Ryoichiro Kageyama · Tetsuo Yamamori
Editors

Cortical Development

Neural Diversity and Neocortical Organization

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Preface

Development of the cerebral cortex, the center for higher brain functions such as cognition, memory, and decision making, is one of the major targets of current research. This book reviews recent progress in cortical development research, focusing on the mechanisms of neural stem cell regulation, neuronal diversity and connectivity formation, and neocortical organization. The cerebral cortex is divided into many areas, including motor, sensory, and visual cortices, each of which consists of six layers containing a variety of neurons with different activities and connections. Such diversity of neuronal types and connections is generated at various levels. First, the competency of neural stem cells changes over time, giving sequential rise to distinct types of neurons and glial cells: initially deep layer neurons, then superficial layer neurons, and lastly astrocytes. The activities and connections of neurons are further modulated via interactions with other brain regions, such as the thalamocortical circuit, and via input from the environment. Extensive studies are gradually elucidating the mechanisms by which the diversity in such neuronal types and connections is formed. To accelerate exchanges of the most recent findings and interactions among leading researchers, we organized a symposium titled "Cortical Development" in Okazaki, Japan, held March 10–13, 2012, which was supported by a Grant-in-Aid for Scientific Research on the Innovative Area "Neural Diversity and Neocortical Organization" from the Ministry of Education, Culture, Sports, Science and Technology (MEXT) of Japan. The symposium was very timely and attracted many young researchers, who were eager to interact with leading researchers and learn about the most recent hot topics. Because the symposium was so successful, we decided to publish a book on cortical development and asked the researchers in this field to contribute chapters. We were happy that many of them responded positively and, although they were very busy, contributed chapters that review hot topics in this field. Many of the topics discussed in the symposium are included in this book.

We are pleased to be able to publish this book, and we would like to thank all the authors who contributed state-of-the-art reviews to it. We also thank our editorial partners, Mr. Kaoru Hashimoto and Ms. Mari Hata at Springer Japan, for their initial suggestion and continued promotion of the project.

Ryoichiro Kageyama
Tetsuo Yamamori

Contents

Chapter 1
Dynamic Notch Signaling in Neural Progenitor Cells

Hiromi Shimojo, Yuki Maeda, Toshiyuki Ohtsuka, and Ryoichiro Kageyama

Abstract Notch signaling plays an essential role in maintenance of neural progenitor cells. Differentiating neurons express Notch ligands such as Delta-like1 (Dll1), which activate Notch signaling in neighboring cells. Activation of Notch signaling induces the expression of Hes1 and Hes5, which repress proneural gene expression, thereby maintaining neural progenitor cells. Thus, differentiating neurons keep their neighbors undifferentiated. Interestingly, Hes1 expression oscillates with a period of 2–3 h by negative feedback, and Hes1 oscillations drive the oscillatory expression of *Dll1* and the proneural gene *Neurogenin2* (*Neurog2*). *Neurog2* oscillation cannot induce neuronal differentiation, and *Dll1* oscillation leads to the mutual activation of Notch signaling between neighboring cells. Thus, neural progenitor cells also keep each other undifferentiated via oscillation in Notch signaling. Not all cells express Hes1 in an oscillatory manner: cells in boundary regions such

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as the isthmus express Hes1 in a sustained manner, and this sustained Hes1 expression seems to be important for the maintenance of boundary regions. Thus, Notch signaling molecules regulate various aspects of neural development by changing the expression dynamics.

Keywords Basal progenitor • Neuroepithelial cell • Oscillatory expression • OSVZ progenitor • Proneural gene • Radial glia

1.1 Introduction

Neuroepithelial cells, which extend from the ventricular surface to the pial surface of the neural tube, repeat symmetric cell division, where each neuroepithelial cell divides into two neuroepithelial cells (Fig. 1.1) (Alvarez-Buylla et al. 2001; Fishell and Kriegstein 2003; Fujita 2003; Götz and Huttner 2005; Miller and Gauthier 2007). As the wall of the neural tube becomes thicker, neuroepithelial cells gradually elongate and become radial glial cells, which have cell bodies in the ventricular zone and radial fibers reaching the pial surface (Fig. 1.1). Radial glial cells undergo asymmetric cell division, where each radial glial cell divides into two distinct cell types, a radial glial cell and an immature neuron or a progenitor (Fig. 1.1) (Malatesta et al. 2000; Miyata et al. 2001; Noctor et al. 2001). Immature neurons migrate

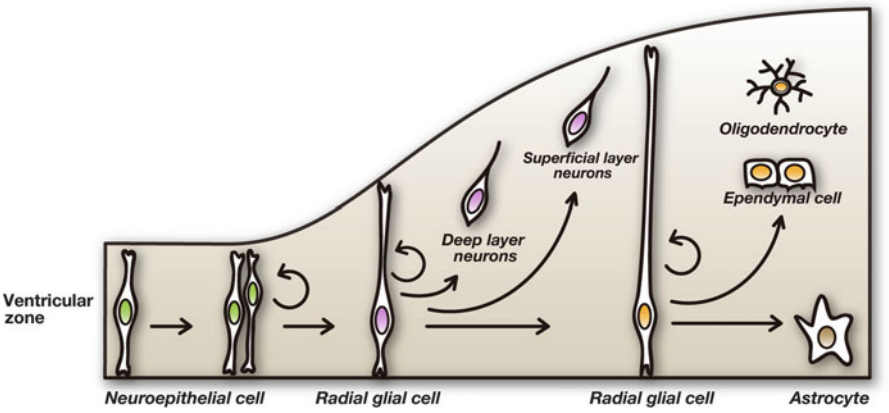

Fig. 1.1 Neural progenitor cells and their differentiation in the embryonic brain. Initially, neuroepithelial cells undergo self-renewal by symmetric division and expand. As development proceeds, neuroepithelial cells elongate to become radial glial cells, which have cell bodies in the inner region (the ventricular zone) of the neural tube and radial fibers that reach the pial surface. Radial glial cells give rise to neurons or basal progenitors. After the production of neurons, some radial glial cells give rise to oligodendrocytes and ependymal cells. Radial glial cells finally differentiate into astrocytes. Both neuroepithelial cells and radial glial cells are considered embryonic neural progenitor cells

outside of the ventricular zone along radial fibers into the cortical plate, where these cells become mature neurons. Progenitors migrate out of the ventricular zone into the subventricular zone (SVZ), proliferate further, and give rise to more neurons, which then migrate into the cortical plate (see Fig. 1.6). Radial glial cells give rise to different types of neurons, initially deep layer neurons and then superficial layer neurons, by repeating asymmetric cell division (Fig. 1.1). Radial glial cells also give rise to oligodendrocytes and ependymal cells and finally differentiate into astrocytes (Fig. 1.1). Both neuroepithelial and radial glial cells are considered neural progenitor cells.

As described above, neural progenitor cells produce a variety of cell types sequentially during development by gradually changing their competency over time. Thus, it is very important to maintain neural progenitor cells until the final point of development in order to generate the proper number of cells and the full diversity of cell types. It has been shown that Notch signaling plays an essential role in the maintenance of neural progenitor cells (Kopan and Ilagan 2009; Fortini 2009; Pierfelice et al. 2011). Here, we review the recent progress on the mechanism and role of Notch signaling in neural development.

1.2 The Core Pathway of Notch Signaling

Notch signaling plays an important role in cell proliferation and differentiation by communication between neighboring cells. Notch ligands such as the transmembrane proteins Delta-like1 (Dll1) and Jagged1 activate Notch receptors such as the transmembrane protein Notch1 in neighboring cells. Notch is cleaved at the S1 site by Furin into two fragments that remain associated to form the functional heterodimer receptor consisting of the Notch extracellular domain and the transmembrane part (Fig. 1.2). Upon Notch ligand binding, Notch receptors undergo successive cleavages: the transmembrane part of Notch proteins is cleaved at the S2 site by TACE and then at the S3 site by γ-secretase, releasing the Notch intracellular domain (NICD) from the transmembrane domain (Fig. 1.2). NICD next moves to the nucleus and forms a complex with the DNA-binding protein Rbpj and the transcriptional co-activator Maml (Fig. 1.2). This ternary complex (NICD-Rbpj-Maml) activates target genes such as the basic helix-loop-helix (bHLH) repressor genes *Hes1* and *Hes5*, mammalian homologues of *Drosophila hairy* and *Enhancer of split* (Jarriault et al. 1995; Fortini 2009; Honjo 1996; Kageyama et al. 2008; Kopan and Ilagan 2009; Pierfelice et al. 2011). Hes factors act as repressors by interacting with the corepressor TLE/Grg, a homologue of Drosophila Groucho, through the C-terminal Trp-Arg-Pro-Trp sequence called the WRPW domain (Akazawa et al. 1992; Sasai et al. 1992; Grbavec and Stifani 1996). Groucho is known to modify the chromatin structure by recruiting the histone deacetylase Rpd3 (Chen et al. 1999). *Hes1* and *Hes5* repress the proneural genes such as the bHLH transcriptional activators *Ascl1* and *Neurogenin2* (*Neurog2*), which induce neuronal differentiation (Bertrand et al. 2002; Ross et al. 2003). As a result, *Hes1* and *Hes5* inhibit neuronal differentiation and maintain neural progenitor cells (Ishibashi et al. 1994; Ohtsuka et al. 1999; Hatakeyama et al. 2004; Kageyama

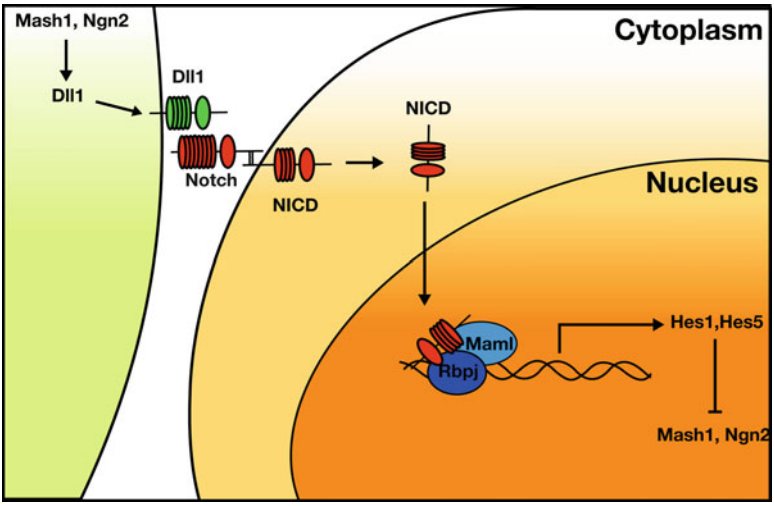

Fig. 1.2 The core pathway of Notch signaling. Proneural genes such as *Ascl1* (also called Mash1) and *Neurog2* (Ngn2) promote neuronal differentiation and induce the expression of Dll1, which in turn activates Notch signaling in neighboring cells. Notch is cleaved at the S1 site by Furin into two fragments that remain associated to form the functional heterodimer receptor consisting of the Notch extracellular domain and the transmembrane part. Upon activation of Notch, the Notch intracellular domain (NICD) is released from the transmembrane domain and transferred to the nucleus, where it forms a complex with the DNA-binding protein Rbpj and the transcriptional co-activator Maml. The NICD-Rbpj-Maml ternary complex induces the expression of transcriptional repressor genes such as *Hes1* and *Hes5*. Hes1 and Hes5 repress the expression of proneural genes and Dll1, thereby leading to the maintenance of neural progenitor cells

et al. 2007). *Hes* genes also repress the expression of Notch ligand genes. Notch ligand expression is induced by proneural genes, and therefore neurons express Notch ligands and inhibit neighboring cells to differentiate into neurons by activating Notch signaling. This process, called lateral inhibition, is essential to maintain neural progenitor cells in the developing nervous system. In the absence of Notch signaling, all cells express proneural genes and initiate neuronal differentiation, resulting in premature depletion of neural progenitor cells without generating later-born cell types (Ishibashi et al. 1995; Hatakeyama et al. 2004; Imayoshi et al. 2010).

While the Notch signaling pathway is important for maintenance of neural progenitor cells, this regulation also suggests that neurons expressing Notch ligands are required to activate the Notch pathway, raising the question as to how neural progenitor cells are maintained during early stages of development before neurons are born.

1.3 Oscillatory Expression of Notch Signaling Genes

In the developing mouse dorsal telencephalon, neural progenitor cells express the proneural gene *Neurog2*, the Notch ligand gene *Dll1*, and *Hes1* in a salt-and-pepper pattern at early stages before neurons are born. It is likely that *Neurog2* induces the

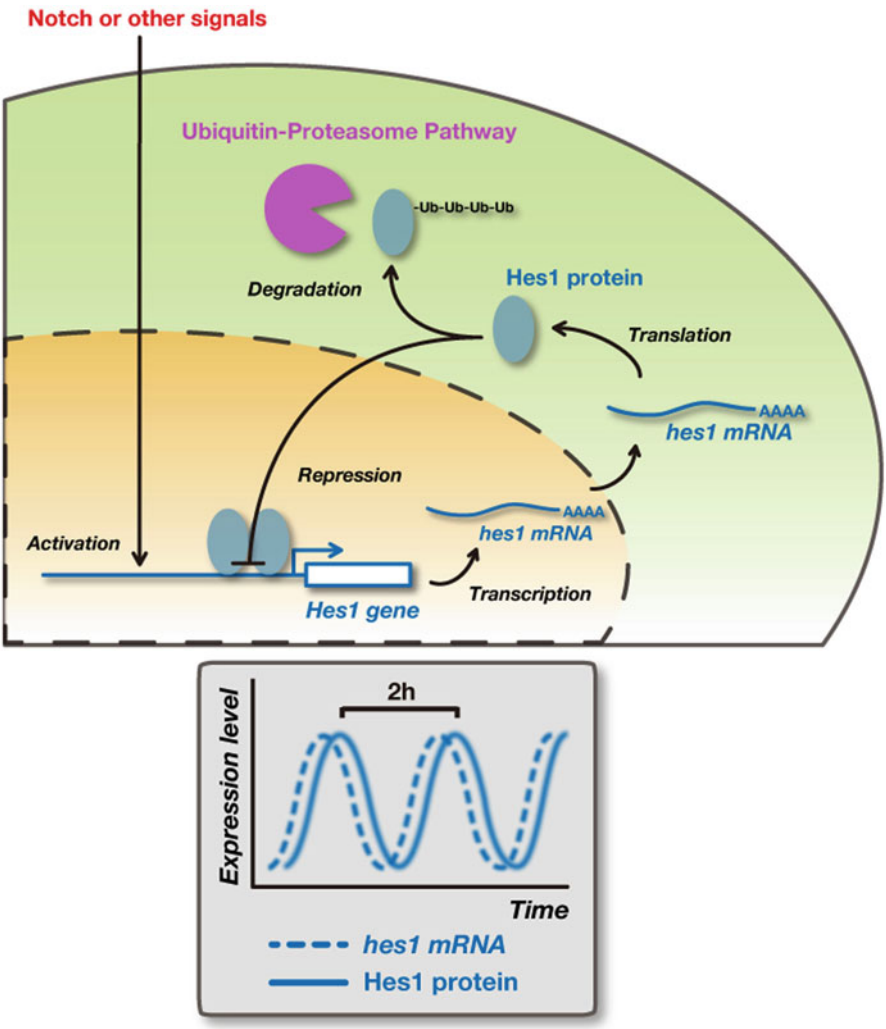

Fig. 1.3 Oscillatory expression of Hes1 by negative feedback. Hes1 expression oscillates with a period of ~2–3 h in many cell types such as neural progenitor cells and fibroblasts. Hes1 represses its own expression by directly binding to its promoter. This negative feedback leads to the disappearance of Hes1 mRNA and protein, because they are extremely unstable, allowing the next round of its expression. In this way, Hes1 autonomously starts oscillatory expression

expression of Dll1, which upregulates Hes1 expression in neighboring cells, suggesting that Notch signaling is active before neurons are born. This observation raises another question: why neurons are not formed during early stages, although the proneural gene *Neurog2* is expressed.

It was previously shown that Hes1 expression oscillates with a period of about 2–3 h in many cell types (Hirata et al. 2002). This oscillatory expression is regulated by negative feedback with a delayed timing (Fig. 1.3) (Hirata et al. 2002).

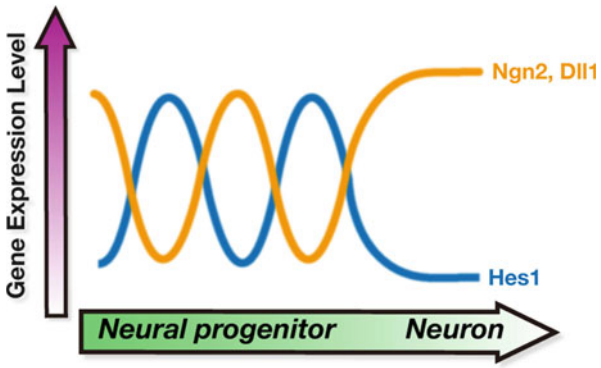

Fig. 1.4 Expression dynamics of *Hes1*, *Neurog2* (Ngn2), and *Dll1* in neural progenitor cells and differentiating neurons. Hes1 expression oscillates with a period of ~2–3 h in neural progenitor cells. In these cells, Hes1 oscillation drives the oscillatory expression of *Neurog2* and *Dll1* by periodic repression. It is likely that Neurog2 cannot induce neuronal differentiation when the expression is oscillatory because many of its downstream genes do not respond to Neurog2 oscillation. In contrast, when Hes1 expression disappears, *Neurog2* expression becomes sustained, promoting neuronal differentiation. Thus, the oscillatory versus sustained expression dynamics of Neurog2 may be important for the choice between neural progenitor cells and neurons

Hes1 represses its own expression by directly binding to multiple N box sequences (CACNAG) of its promoter (Takebayashi et al. 1994). Once the promoter is repressed, Hes1 mRNA and protein disappear rapidly because they are extremely unstable, and the disappearance of Hes1 protein allows the next round of its expression. In this way, Hes1 expression autonomously oscillates with a period of ~2–3 h (Hirata et al. 2002). Because this oscillation is unstable and nonsynchronous, Hes1 expression levels are variable between neighboring cells, suggesting that a salt-and-pepper pattern of Hes1 expression is due to unstable and non-synchronous oscillation. Indeed, time-lapse imaging analysis revealed that *Hes1* expression oscillates in neural progenitor cells (Fig. 1.4) (Masamizu et al. 2006; Shimojo et al. 2008). Hes1 expression exhibits an inverse correlation with Neurog2 protein and *Dll1* mRNA expression in neural progenitor cells, suggesting that Hes1 oscillation induces the oscillatory expression of *Neurog2* and *Dll1* by periodic repression (Shimojo et al. 2008). Time-lapse imaging analysis revealed that *Neurog2* and *Dll1* expression also oscillates in neural progenitor cells, where Hes1 expression oscillates (Fig. 1.4) (Shimojo et al. 2008). However, in differentiating neurons, where Hes1 expression disappears, *Neurog2* and *Dll1* expression becomes sustained (Fig. 1.4) (Shimojo et al. 2008). It is likely that Neurog2 cannot induce neuronal differentiation when its expression oscillates, probably because many of its downstream genes do not respond to *Neurog2* oscillation, and that Neurog2 induces neuronal differentiation only when its expression becomes sustained. When *Neurog2* expression oscillates, only rapidly responding genes such as *Dll1* may be selectively induced, and *Dll1* oscillations may lead to the mutual activation of Notch signaling and the maintenance of neural progenitor cells. Indeed, it was recently demonstrated that Neurog2 is phosphorylated by cyclin-dependent kinases in neural progenitor

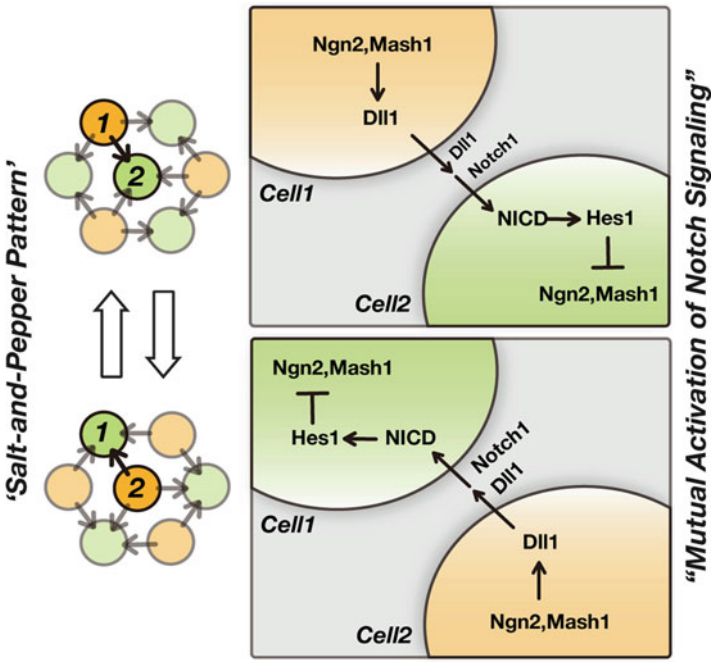

Fig. 1.5 Maintenance of neural progenitor cells by the mutual activation of Notch signaling. When Hes1 expression is low in a subset of cells (Cell 1 in the *upper panel*), Neurog2 (Ngn2) and Dll1 expression becomes high, leading to the activation of Notch signaling and the upregulation of Hes1 in neighboring cells (Cell 2 in the *upper panel*). In the latter cells, high levels of Hes1 repress Neurog2 and Dll1 expression, but due to oscillations, Hes1 expression becomes low after ~1 h, while Neurog2 and Dll1 expression becomes high (Cell 2 in the *lower panel*), leading to the activation of Notch signaling in the former cells (Cell 1 in the *lower panel*). In this way, Dll1 oscillations lead to the mutual activation of Notch signaling between neural progenitor cells without the aid of neurons

cells and that phosphorylated Neurog2 can induce Dll1 expression efficiently but not other gene expression (Ali et al. 2011; Hindley et al. 2012). These results suggest that Neurog2 may lead to two opposite outcomes, depending on its expression dynamics: when its expression oscillates, Neurog2 induces the maintenance of neural progenitor cells, but when its expression is sustained, Neurog2 induces neuronal differentiation.

These observations suggest that salt-and-pepper patterns of *Neurog2*, *Dll1*, and *Hes1* expression during early stages of development are the result of oscillatory expression. It is generally thought that Neurog2- or Dll1-positive cells are selected to become neurons first, while negative cells remain neural progenitor cells. However, time-lapse imaging analyses indicated that positive cells could become negative, while negative cells could become positive a few hours later, suggesting that positive and negative cells may be equivalent to each other. We speculate that *Neurog2* and *Dll1* oscillations enable the maintenance of neural progenitor cells by activation of Notch signaling without the aid of neurons. When Hes1 expression is low in a subset of cells (Cell 1 in the upper panel of Fig. 1.5), Neurog2 and Dll1

expression becomes high, leading to the activation of Notch signaling and the upregulation of Hes1 in neighboring cells (Cell 2 in the upper panel of Fig. 1.5). In the latter cells, high levels of Hes1 repress Neurog2 and Dll1 expression, but due to oscillations, Hes1 expression becomes low after ~1 h, while Neurog2 and Dll1 expression becomes high (Cell 2 in the lower panel of Fig. 1.5), leading to the activation of Notch signaling in the former cells (Cell 1 in the lower panel of Fig. 1.5). In this way, Dll1 oscillations lead to the mutual activation of Notch signaling between neural progenitor cells (Shimojo et al. 2008). Thus, oscillatory expression is advantageous for maintaining a group of cells undifferentiated without any input from neurons. In agreement with this notion, when sustained Hes1 expression is induced in neural progenitor cells, their neighboring cells prematurely initiate neuronal differentiation (Shimojo et al. 2008). This is probably because sustained Hes1 expression represses Neurog2 and Dll1 expression continuously, resulting in inactivation of Notch signaling in the neighboring cells. These observations also suggest that Notch signaling is not a one-way mechanism (neuron to neural progenitor cell), but functions by reciprocal transmission (neural progenitor cell to neural progenitor cell).

At later stages of development, many differentiating neurons express Dll1 in a sustained manner and activate Notch signaling in neural progenitor cells. Thus, Neurog2 and Dll1 expression mostly occurs in neurons but not in neural progenitor cells, although Hes1 expression in neural progenitor cells oscillates even at later stages (Shimojo et al. 2008).

1.4 The Mechanism of Oscillatory Expression: Lessons from the Segmentation Clock

The negative feedback loop is important but not sufficient for oscillatory expression. Both the instability of gene products and negative feedback with delayed timing are required for sustained oscillations, which was initially predicted by mathematical modeling (Lewis 2003; Monk 2003; Jensen et al. 2003; Kageyama et al. 2012). The detailed mechanism for oscillatory expression has been analyzed in the somite segmentation clock. Somites are transient metameric structures, which later give rise to the vertebral column, ribs, skeletal muscles, and subcutaneous tissues. A bilateral pair of somites is formed by segmentation of the anterior parts of the presomitic mesoderm (PSM), which is located in the caudal part of embryos. In mouse embryos, each pair of somites is formed every 2 h, and this process is controlled by *Hes7*, a member of *Hes* gene family (Bessho et al. 2001). Like *Hes1*, *Hes7* is expressed in an oscillatory manner in the PSM. Both loss of expression and sustained expression of Hes7 lead to severe somite fusion, suggesting that oscillatory expression of Hes7 is important for periodic somite segmentation. Hes7 oscillations drive cyclic expression of many downstream genes such as genes in Notch signaling and Fgf signaling (Bessho et al. 2001; Niwa et al. 2007, 2011). Hes7 oscillation is

also regulated by negative feedback: Hes7 protein directly binds to its own promoter and represses its expression (Bessho et al. 2003).

Hes7 protein is very unstable: the half-life is only ~22 min, and further analyses revealed that this instability is very important for Hes7 oscillations. Introduction of a K14R point mutation (the 14th lysine residue is mutated to arginine) stabilizes Hes7 protein (the half-life is ~30 min) without changing the transcriptional repressor activity. This mutation was found to dampen the Hes7 oscillation rapidly in mouse embryos, resulting in steady (non-oscillatory) Hes7 expression and disorganized somite segmentation after a few normal cycles, which agreed well with the prediction by mathematical modeling (Hirata et al. 2004). Another feature required for sustained Hes7 oscillation is the intronic delays, which include transcription and splicing of intron sequences. Hes7 gene has three introns, and intronic delays for Hes7 expression were found to be about 19 min (Takashima et al. 2011). It was shown that Hes7 oscillations were abolished by deletion of all three introns, as predicted by mathematical modeling, indicating that intronic delays are essential for Hes7 oscillation (Takashima et al. 2011). Removal of two introns shortens the intronic delays by about 5 min, and according to the mathematical modeling, this shortened delays would dampen but accelerate the oscillation. Indeed, deletion of two introns accelerates Hes7 oscillation and somite segmentation, increasing the number of somites and vertebrae in the cervical and upper thoracic region (Harima et al. 2013).

Hes1 protein is also very unstable (about ~20 min half-life), and the gene has three introns. Thus, point mutations that change the stability of Hes1 protein and deletions of introns would affect the dynamics of Hes1 oscillations, as observed with Hes7 oscillations. It would be interesting to see what effects on neural progenitors are caused by such mutations in the *Hes1* gene.

1.5 Basal Progenitors and Outer Subventricular Zone (OSVZ) Progenitors

As stated above, radial glial cells not only generate neurons but also produce progenitors, which migrate into the subventricular zone (SVZ). There are two types of progenitors, basal progenitors and outer SVZ (OSVZ) progenitors. Basal progenitors formed by Tbr2 migrate into the SVZ, retract their apical and basal processes, and generally divide only once to generate two neurons (Sessa et al. 2008). Thus, basal progenitors have a limited proliferation ability. In these cells, *Hes1* and *Hes5* expression is downregulated, suggesting that Notch signaling is not active (Fig. 1.6) (Mizutani et al. 2007; Kawaguchi et al. 2008). By contrast, OSVZ progenitors divide multiple times in the OSVZ and generate a large number of neurons (Hansen et al. 2010; Fietz et al. 2010). These cells have radial glia-like morphology extending radial fibers to the pial surface but lack apical processes and therefore are not in contact with the ventricular surface (Fig. 1.6). OSVZ progenitors undergo asymmetric cell division, where each cell divides into a daughter cell that inherits

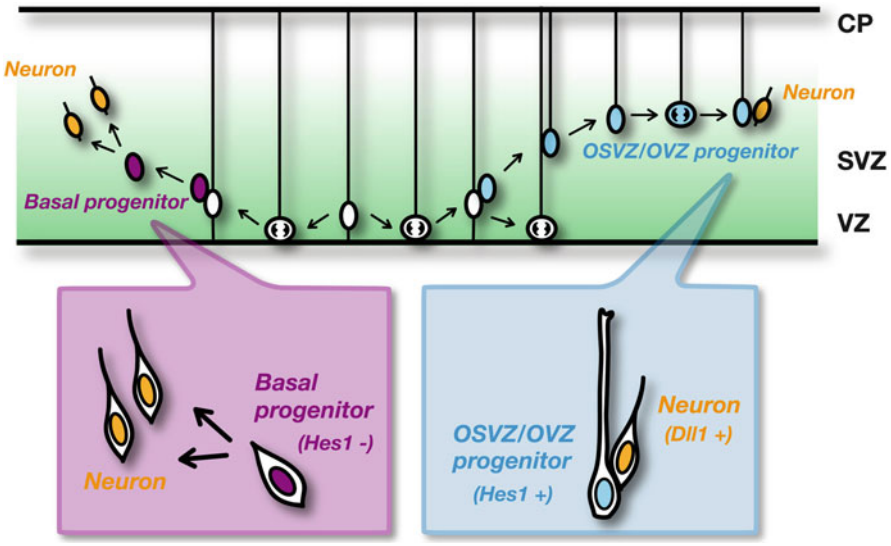

Fig. 1.6 Basal progenitors and OSVZ progenitors. Basal progenitors retract apical and basal processes and generally divide only once to generate two neurons. In these cells, *Hes1* expression is downregulated, suggesting that Notch signaling is not active. OSVZ progenitors have radial glia-like morphology extending radial fibers to the pial surface but lack apical processes. These cells repeatedly undergo asymmetric cell division, each dividing into a daughter cell that inherits the radial fiber (OSVZ progenitor) and the other that does not (neuron). Neurons express Notch ligands and activate Notch signaling in their sibling OSVZ progenitors

the radial fiber (OSVZ progenitor) and the other that does not. The one that inherits the radial fiber seems to repeat asymmetric cell division multiple times, while the other differentiates into postmitotic neurons. The former cells (OSVZ progenitors) express Hes1, and inhibition of Notch signaling by treatment with a γ-secretase inhibitor induces OSVZ progenitors to differentiate into neurons or $Tbr2^{+}$ basal progenitors (Hansen et al. 2010), suggesting that Notch signaling is required for maintenance of OSVZ progenitors (Fig. 1.6). Interestingly, these daughter cells (OSVZ progenitor and neuron) maintain contact with each other for several hours, and neurons express Notch ligands and activate Notch signaling in their sibling OSVZ progenitors (Fig. 1.6) (Shitamukai et al. 2011). These observations suggest that asymmetric cell division is required to activate Notch signaling in OSVZ progenitors by their sibling neurons.

OSVZ progenitors seem to play a major role in the increase of the neuronal number in the cortex, and indeed it was shown that the developing human neocortex has an expanded outer region in the SVZ, suggesting that OSVZ progenitors are responsible of the expansion of the cortex. It is possible that the cells that migrate into the SVZ may become OSVZ progenitors when Notch signaling is active, whereas they may become basal progenitors when Notch signaling is inactive. It remains to be determined how Notch signaling is regulated in the SVZ and whether Hes1 expression oscillates in OSVZ progenitors, as observed in radial glial cells.

1.6 Sustained Hes1 Expression in Boundary Cells

The developing nervous system is partitioned into many compartments by boundaries such as the isthmus and the zona limitans intrathalamica (Fig. 1.7). The nervous system is also partitioned into the right and left halves by the roof plate and the floor plate (Fig. 1.7). These boundaries function as the signaling centers by expressing signaling molecules such as Fgf8, Shh, and Wnt and regulate specification of neural progenitor cells and neurons in neighboring compartments (Kiecker and Lumsden 2005). Cells in these boundaries do not proliferate actively or usually do not give rise to neurons. Thus, the proliferation and differentiation characteristics are different between boundary cells and neural progenitor cells.

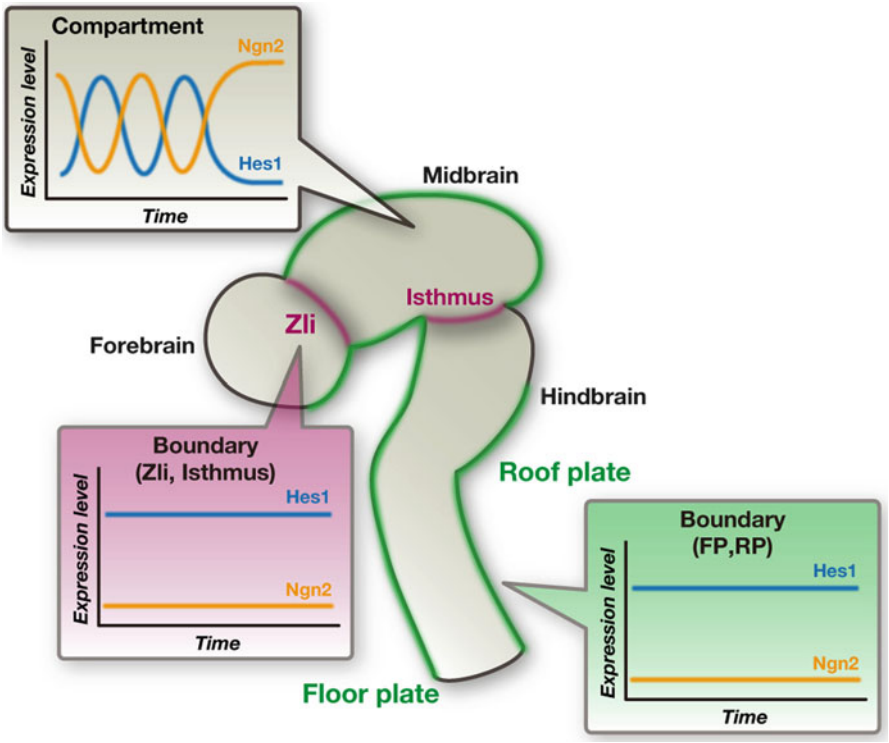

Fig. 1.7 Different expression dynamics of Hes1 in the developing nervous system. The developing nervous system is partitioned into many compartments by boundaries such as the isthmus and the zona limitans intrathalamica (Zli). The nervous system is also partitioned into the right and left halves by the roof plate and the floor plate. Cells in boundary regions are mostly dormant with regard to proliferation and differentiation, in contrast to neural progenitor cells present in compartments. Boundary cells express Hes1 in a sustained manner, while neural progenitor cells present in compartments express Hes1 in an oscillatory manner

Cells in boundary regions express Hes1 in a sustained manner, which suppresses proneural gene expression (Fig. 1.7) (Baek et al. 2006). It was found that the retrovirus-mediated introduction of sustained Hes1 expression into neural progenitor cells inhibits their proliferation and neuronal differentiation (Baek et al. 2006). Sustained Hes1 expression downregulates not only Notch ligand and proneural genes but also cell cycle regulators such as cyclin D1 and cyclin E2 (Shimojo et al. 2008). By contrast, when *Hes* genes are inactivated, boundaries are not properly maintained, and cells in the boundary regions can express proneural genes and differentiate into neurons (Hirata et al. 2001; Baek et al. 2006). These results suggest that cells with sustained Hes1 expression are rather dormant with regard to proliferation and differentiation and that oscillatory expression of Hes1 may be important for proliferation and differentiation of neural progenitor cells. This feature is similar to the one observed in fibroblasts, where sustained Hes1 overexpression leads to reversible quiescence (Sang et al. 2008).

The mechanism by which oscillatory versus sustained Hes1 expression is regulated remains to be determined. In fibroblasts, Jak-Stat signaling is involved in Hes1 oscillations. Jak2 activates Stat3 by phosphorylation, and phosphorylated Stat3 (pStat3) induces Socs3 expression, which in turn inhibits Jak2. Due to this negative feedback, pStat3 and Socs3 levels oscillate in fibroblasts (Yoshiura et al. 2007). Interestingly, blockade of this pathway with a Jak inhibitor inhibits Hes1 oscillations by stabilizing the Hes1 protein, and Hes1 expression becomes steady in fibroblasts (Yoshiura et al. 2007). Similarly, treatment with a Jak inhibitor inhibits Hes1 oscillations in neural progenitor cells, suggesting that Jak-Stat signaling is also involved in the regulation of Hes1 oscillations in these cells (Shimojo et al. 2008).

It was shown that the Id-mediated regulation is involved in sustained expression of Hes1. Id proteins, HLH factors without a basic region, form heterodimers with Hes1 through their HLH domains and inhibit Hes1 from binding to the N box in the Hes1 promoter (Bai et al. 2007), suggesting that Id factors prevent Hes1 from negative autoregulation. Thus, Id proteins could inhibit the oscillatory expression of Hes1, although it remains to be determined whether Id factors lead to steady Hes1 expression in boundaries.

Another possible mechanism is microRNA-9 (miR-9), which interacts with the 3′-untranslated region (UTR) sequence of *Hes1* mRNA. MiR-9 is important for the short half-life of *Hes1* mRNA and downregulation of Hes1 protein expression. Interestingly, knockdown of miR-9 inhibits the oscillatory expression of Hes1 (Bonev et al. 2012; Tan et al. 2012b). Because it was previously shown that the instability of gene products is essential for continuous oscillation of Hes7, another member of the Hes family (Hirata et al. 2004), it is likely that miR-9-induced short half-life of *Hes1* mRNA may be required for Hes1 oscillation. Expression analyses showed that miR-9 is highly expressed in the ventricular zone, where neural progenitor cells reside, whereas it is absent in boundary regions (Tan et al. 2012b), suggesting that the lack of miR-9 expression leads to sustained expression of Hes1 in boundaries.

1.7 Downstream Events of Hes1 and Neurog2 Oscillations

Some downstream genes such as *Dll1* are expressed in an oscillatory manner via periodic repression by Hes1 and periodic activation by Neurog2. Other downstream genes could be gradually up- or downregulated over time in response to Hes1 and Neurog2 oscillations, which could lead to changes in competency of neural progenitor cells (Fig. 1.8). It was previously shown that sustained overexpression of Hes1 or Hes5 accelerates astrocyte formation (Ohtsuka et al. 2001), raising the possibility that compared to Hes1 oscillation, sustained Hes1 expression accelerates the transition from neurogenesis to astrogenesis. Identification of downstream genes for Hes1 will be required to understand the mechanism of how such transition is controlled.

One candidate gene involved in the transition from neurogenesis to astrogenesis is *ESET/Setdb1/KMT1E*, a histone H3 Lys-9 (H3K9) methyltransferase gene,

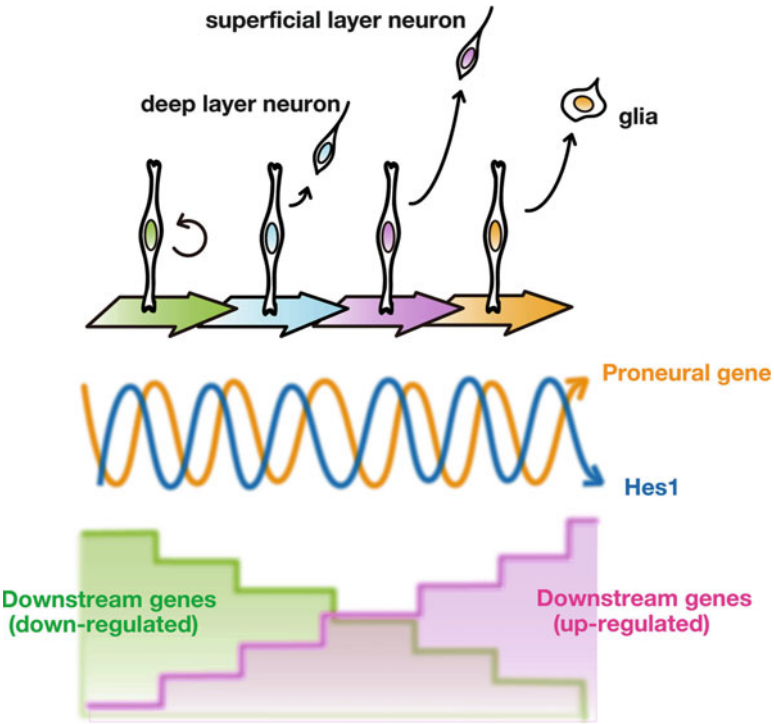

Fig. 1.8 Possible downstream events of Hes1 and Neurog2 oscillations. Some downstream genes could be gradually up- or downregulated over time in response to Hes1 and Neurog2 oscillations, which could lead to changes in competency of neural progenitor cells during development. *ESET* expression in neural progenitor cells is gradually downregulated, and this gene expression could be regulated by Hes1 and Neurog2 oscillations

because this gene has multiple Hes1-binding sites in the promoter, although it remains to be determined whether Hes1 regulates *ESET* expression. ESET is highly expressed by neural progenitor cells at early stages of development, but the expression is downregulated over time and becomes significantly low or almost absent at later stages when the transition from neurogenesis to astrogenesis occurs (Tan et al. 2012a). Inactivation of *ESET* in the forebrain derepresses the expression of endogenous retrotransposons and their neighboring genes as well as non-neural gene expression and leads to impairment of formation of early-born (deep layer) neurons and enhancement of astrocyte formation. Formation of late-born (superficial layer) neurons is also accelerated because of impairment of early neurogenesis, but because astrocyte formation is also accelerated, the final number of late-born neurons is mostly normal (Tan et al. 2012a). Conversely, overexpression of ESET decreases the astrocyte differentiation. These results suggest that decreasing expression of ESET during development may be one of the internal clock mechanisms that regulate the timing of cell fate switches of neural progenitor cells, from deep layer neurogenesis to superficial layer neurogenesis and finally to astrogenesis. Further analyses will be required to determine whether Hes1 oscillation is involved in gradual downregulation of ESET.

1.8 Conclusions

Oscillatory versus sustained Hes1 expression leads to different outcomes in neural progenitor cells. When its expression oscillates, neural progenitor cells proliferate actively and differentiate into mature cells. By contrast, when its expression is sustained, neural progenitor cells become dormant. Adult neural stem cells are known to be mostly dormant, suggesting that Hes1 expression is non-oscillatory. If this is the case, it would be interesting to induce Hes1 oscillation in these cells to see whether adult neural stem cells are activated in proliferation. Similarly, oscillatory versus sustained Neurog2 expression leads to different outcomes. When its expression is sustained, neural progenitor cells differentiate into neurons. By contrast, when its expression oscillates, neural progenitor cells remain undifferentiated. Thus, not just the expression but the dynamics of these genes are very important for the outcomes. Oscillatory versus sustained Hes1 expression is regulated by Jak-Stat signaling, Id, and miR-9, although the exact mechanism remains to be determined. Oscillatory versus sustained Neurog2 expression is regulated by Hes1. However, the dynamics of downstream genes for Hes1 and Neurog2 oscillations are mostly unknown. The expression of some genes may be gradually up- or downregulated over time in response to Hes1 and Neurog2 oscillations, which could be responsible for changes in differentiation competency of neural progenitor cells. Further analysis of the downstream events will be required to understand the complex regulatory mechanism of neural development.

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Chapter 2
Proneural Proteins and the Development of the Cerebral Cortex

Julian Heng and François Guillemot

Abstract Proneural transcription factors are key regulators of neurogenesis. This chapter focuses on the proneural proteins Ascl1, Neurog1 and Neurog2 and their multiple roles in development of the mammalian cerebral cortex. The first part of the chapter considers the different aspects of telencephalic development that are regulated by proneural proteins, including the neuronal versus glial fate decision, the specification of glutamatergic and GABAergic neuronal phenotypes, and the radial migration, dendritic morphogenesis and axonal projection patterning of cortical neurons. The second part turns to the molecular mechanisms through which proneural proteins exert their activities and discusses the regulation of their expression and activity, the identification of the many genes they regulate and finally the nature of the transcription factors and cofactors that they interact with to regulate gene expression. Together, this chapter illustrates how studies focused on the functions and modes of action of a small group of proteins have greatly improved our general understanding of cortical development.

2.1 Introduction

Proneural proteins comprise a small group of transcription factors that have unique and crucial functions in neurogenesis throughout the animal kingdom. They belong to the vast class of basic-helix-loop-helix (bHLH) proteins, which are characterized by a short stretch of basic amino acids conferring sequence-specific DNA-binding

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activity and an adjacent helix-loop-helix region involved in dimerization with other bHLH proteins (Massari and Murre 2000). Many bHLH transcription factors have important functions in the generation and differentiation of tissues in both animal and plant organisms.

There are two clearly distinct groups of bHLH proteins that are specifically expressed within the developing nervous system, proneural factors and neuronal differentiation factors, which differ in both expression patterns and loss-of-function phenotypes. Proneural factors are expressed almost exclusively in progenitor cells and they specify neuronal fates and initiate differentiation programmes. Neuronal differentiation factors are expressed in postmitotic neurons and in some cases in committed but still mitotic neuronal progenitors and are involved in the execution of the differentiation programmes. It is worth noting that these two categories of proteins cannot be distinguished by their gain-of-function phenotypes as both can induce the formation of new neurons when ectopically expressed in competent cells (e.g. Lee et al. 1995; Farah et al. 2000). In mammals, proneural bHLH factors expressed in the telencephalon comprise just three proteins, the Achaete-Scute-like factor Ascl1/Mash1 and the two Neurogenin factors Neurog1 and Neurog2. On the other hand, the neuronal differentiation bHLH factors of the telencephalon comprise six factors, including four Neurod proteins, Neurod1, Neurod2, Neurod4 and Neurod6, and two Nscl proteins, Nscl1 and Nscl2.

This chapter focuses on the role of the proneural proteins Ascl1, Neurog1 and Neurog2 in the development of the mammalian cerebral cortex. Within the embryonic telencephalon, Neurog1 and Neurog2 are specifically expressed in the dorsal division that produces all cortical projection neurons, while Ascl1 is expressed mostly in the ventral division, which produces cortical interneurons as well as all basal ganglia neurons. We will also discuss a few studies performed in other regions of the nervous system, which provide important information on proneural proteins, relevant to their role in cortical development. Not included here is a discussion of the role of proneural factors in direct reprogramming, such as the conversion of non-neural cells including fibroblasts and hepatocytes into neurons by forced expression of Ascl1 (Vierbuchen et al. 2010; Marro et al. 2011; Pang et al. 2011). These studies have obviously important medical implications, e.g. by providing a source of human neurons for modelling disease processes, but how relevant the reprogramming process is to the normal function of proneural proteins remains an open question.

In the first part of this chapter, we will discuss the main cellular functions that have been ascribed to proneural proteins during telencephalic development, namely, the regulation of the neuronal versus glial fate decision (Sect. 2.2.1), the specification of glutamatergic and GABAergic neuronal phenotypes (Sects. 2.2.2 and 2.2.3) and the regulation of the radial migration, dendritic morphology and axonal projection pattern of cortical projection neurons (Sects. 2.2.4 and 2.2.5). In the second part, we will turn to the molecular mechanisms through which proneural proteins exert their activities and account for their functional diversity, including the mechanisms that regulate the expression and activity of proneural proteins spatially and temporally (Sect. 2.3.1), the nature of the transcriptional targets of proneural proteins (Sect. 2.3.2) and finally the nature of the transcription factors

and cofactors that proneural proteins interact with to regulate gene expression (Sects. 2.3.3 and 2.3.4). We hope that this chapter will provide an accurate and up-to-date overview of the central role that these fascinating molecules have in the development of the cerebral cortex.

2.2 Cellular Functions of Proneural Genes in Telencephalic Development

2.2.1 Proneural Genes and the Neuronal Versus Glial Fate Decision

The main cell type expressing proneural proteins in the developing telencephalon is radial glial stem cells. Radial glial cells divide asymmetrically and self-renew while generating postmitotic neurons or mitotic neuronal precursors. They convert into astroglial precursors at the end of the neurogenic period. Proneural proteins have redundant roles in controlling the neuronal specification of radial glial progenitors (Fig. 2.1). As a result, mice carrying mutations in a single proneural gene have relatively mild phenotypes compared with mice with mutations in two proneural genes. Embryos mutant for *Ascl1* alone lack radial glial cells in a defined region of the ventral telencephalon, the medial ganglionic eminence, and lack also neuronal populations derived from the missing progenitors (Casarosa et al. 1999). *Neurog2* single mutant embryos have lost many neurons in the subplate and layers 6 and 5 of the cortical plate. These defects are exacerbated in *Neurog1, Neurog2* double mutants, while *Neurog1* single mutant mice do not present overt defects (Fode et al. 2000; Nieto et al. 2001). Embryos that are mutant for both *Ascl1* and *Neurod4* present a severe reduction of neurogenesis as well as ectopic astrogliogenesis in the midbrain and hindbrain, while single mutants present only subtle defects in these structures (Tomita et al. 2000). Similarly, loss of both *Neurog2* and *Ascl1* results in a profound deficit in neuronal production coupled with premature initiation of astroglial generation in the embryonic cortex (Nieto et al. 2001). Importantly, analysis of clonal cultures of mutant cortical progenitors demonstrated that either Neurog2 or Ascl1 is required in radial glial cells of the dorsal telencephalon to maintain their neurogenic potential and prevent activation of the gliogenic programme (Nieto et al. 2001).

Analysis of the molecular mechanisms underlying Neurog1 activity in cortical progenitors demonstrated that this factor induces neurogenesis and inhibits glial differentiation via two distinct mechanisms (Sun et al. 2001). While induction of neurogenesis involves a classical mode of transcriptional regulation requiring DNA binding of Neurog1, the suppression of glial differentiation did not require DNA binding but the sequestration of a complex formed by the transcription factor Smad1 and the cofactor CREB-Binding Protein (CBP) to prevent association of this complex with STAT transcription factors for activation of the promoters of the astrocyte-specific genes S100β and GFAP (Sun et al. 2001). Therefore, the function

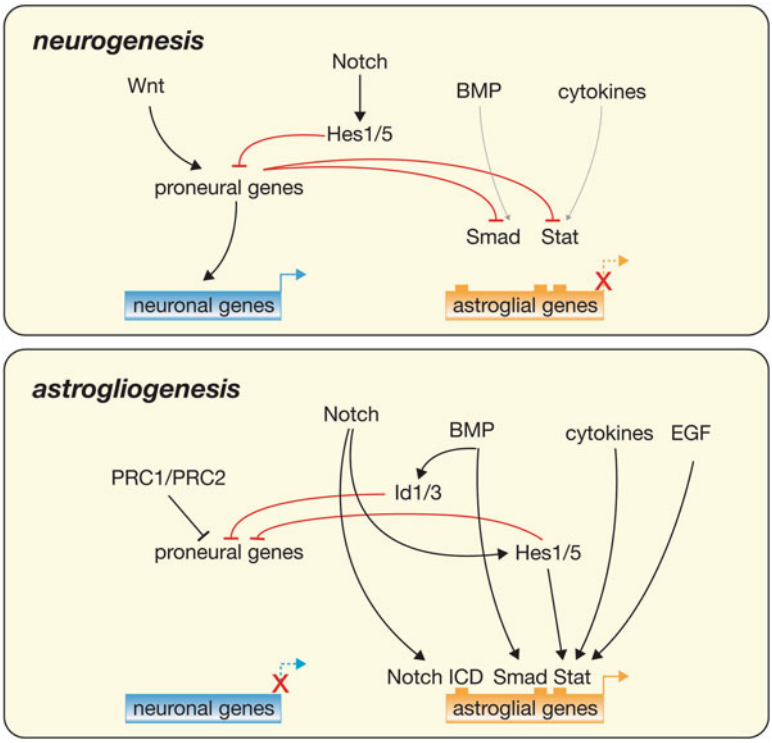

Fig. 2.1 Proneural genes regulate the neuronal versus glial fate decision. *Top panel*: Proneural genes/proteins interact with signalling pathways to promote neurogenesis during telencephalic development. Neurog1 expression is induced by Wnt signalling and expression of all proneural genes is repressed by Notch/Hes signalling in a process of lateral inhibition. Neurog1 induces expression of neuronal genes while suppressing transcription of the astroglial genes GFAP and S100β through sequestration of a Smad1/CBP complex. *Bottom panel*: Signalling pathways suppress proneural protein expression and activity and promote the switch to astrogliogenesis at the end of the neurogenic period of cortical development. Expression of all proneural genes is repressed by Hes1/5 downstream of Notch signalling and that of Neurog1 is specifically repressed by the Polycomb complexes PRC1 and PRC2, and the activity of proneural proteins is inhibited by Id proteins downstream of BMP signalling. Astroglial gene transcription is activated by recruitment of Notch intracellular domain (Notch ICD), Smad proteins downstream of BMP signalling and STAT proteins downstream of cytokine and EGF signalling. STATs act synergistically with Hes and Smads to transactivate astroglial promoters (Sun et al. 2001; Hirabayashi et al. 2004; Hirabayashi et al. 2009; Martynoga et al. 2012)

of Neurog1 in radial glial stem cells involves the simultaneous induction of neuronal differentiation genes and inhibition of the gliogenic JAK-STAT signalling pathway. The transition from neurogenesis to gliogenesis, which takes place around embryonic day 18 in the mouse cerebral cortex, requires the active repression of proneural gene expression and/or the suppression of their activity. The BMP and Notch pathways promote gliogenesis in part by inducing transcriptional repressors of the Id and Hes families, which in turn act by inactivating proneural protein and repressing

proneural genes, respectively (Nakashima et al. 2001; Louvi and Artavanis-Tsakonas 2006). Transcription of Neurog1 is also repressed at the end of the neurogenic period by the Polycomb Repressive Complexes PRC1 and PRC2 (Hirabayashi et al. 2009), as discussed more extensively in Sect. 2.3.1.

In contrast with its inhibitory role in astrogliogenesis, Ascl1 has a positive role in the development of oligodendrocytes, as it is required for the specification of oligodendrocyte progenitors in the embryonic ventral telencephalon and in the postnatal brain (Parras et al. 2004; Parras et al. 2007). The switch from the neurogenic to the oligodendrogenic function of Ascl1 may involve a cooperation with the transcription factor Olig2 as well as a Notch-mediated repression of the neurogenic factors Dlx1/2 (Parras et al. 2007; Petryniak et al. 2007). Ascl1 has also been shown to regulate later steps of oligodendrocyte maturation in the spinal cord (Sugimori et al. 2008) and may have a similar role in the telencephalon as well.

2.2.2 *Neurogenin Proteins and the Specification of Telencephalic Projection Neurons*

When it was first observed, the spatially restricted expression patterns of proneural genes in the developing nervous system suggested that these genes might be important for the specification of neuronal identities. In the telencephalon, *Ascl1* is expressed at its highest level by progenitors in the ganglionic eminences of the ventral telencephalon (Guillemot and Joyner 1993; Casarosa et al. 1999), suggesting a role in the specification of ventrally born basal ganglia neurons and cortical interneurons. In contrast, *Neurog1* and *Neurog2* are expressed exclusively in the dorsal telencephalon, suggestive of important roles in the specification of dorsally born cortical projection neurons (Fig. 2.2). *Ascl1* and *Neurog* genes are also expressed in complementary progenitor populations in several other regions of the nervous system, including the spinal cord and peripheral nervous system, suggesting an extensive role for these proneural genes in neuronal fate specification. It should be noted, however, that *Ascl1* and *Neurog* genes are not always expressed in nonoverlapping progenitor populations. In several regions of the embryonic central nervous system, including the dorsal telencephalon, and in adult neurogenic regions, Ascl1-expressing stem cells produce Neurog2-expressing intermediate progenitors or postmitotic neurons, with transient co-expression of the two factors (Britz et al. 2006).

Analysis of mutant mice has demonstrated that Neurog2 is indeed critical for the specification of excitatory cortical projection neurons, while Ascl1 is required for the specification and production of cortical interneurons as well as interneurons and projection neurons of the basal ganglia (Casarosa et al. 1999; Fode et al. 2000; Marin et al. 2000). Loss of *Neurog2* and, to a further extent, loss of both *Neurog2* and *Neurog1* result in the absence of a subset of glutamatergic projection neurons in the deep layers (layers 6 and 5) of the cortex, which are generated during the first half of corticogenesis. The generation of neurons in the upper cortical layers, which are generated during the second half of corticogenesis, is in contrast unaffected in

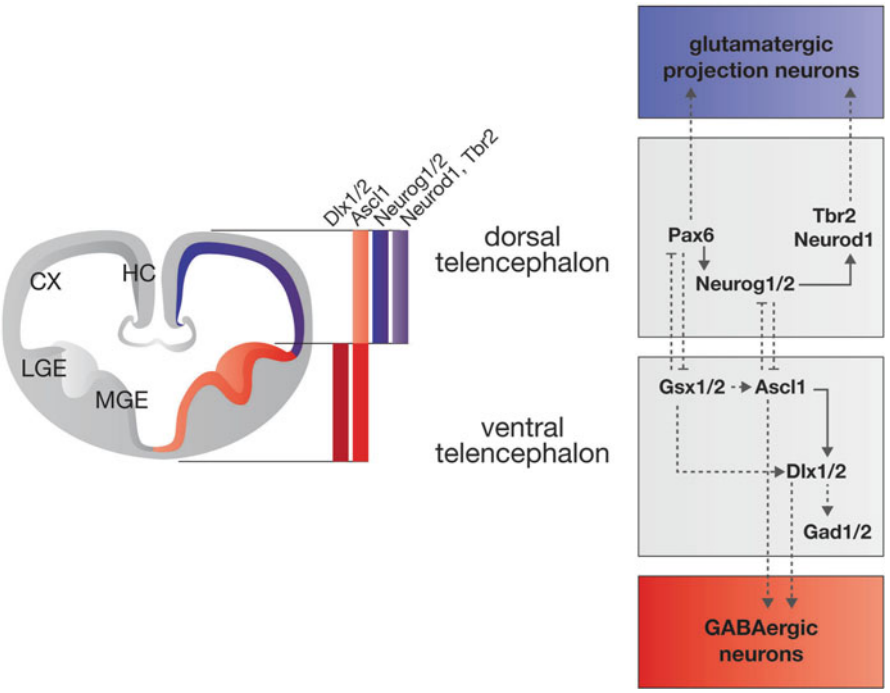

Fig. 2.2 Proneural proteins play a central role in the specification of glutamatergic and GABAergic neuronal fates in the embryonic telencephalon. *Left panel*: Expression of Neurog1 and Neurog2 and expression of their targets Neurod1 and Tbr2 are restricted to the dorsal telencephalon, while Ascl1 is expressed at a high level in the ventral telencephalon and at a lower level dorsally, and expression of its targets Dlx1/2 is restricted to the ventral telencephalon. *Right panel*: Interactions that take place between transcription factors in telencephalic progenitors result in the generation of glutamatergic neurons from dorsal progenitors and GABAergic neurons from ventral progenitors. Neurog2 expression is induced by Pax6 in the dorsolateral telencephalon, and Neurog2 induces Neurog1 and suppresses Ascl1 expression (most likely indirectly) in progenitors throughout the dorsal telencephalon. Neurog2 controls the specification of dorsal telencephalic progenitors into glutamatergic neurons by activating a programme that includes direct transcriptional activation of the transcription factors Neurod1 and Tbr2. In the ventral telencephalon, Gsx1 promotes Ascl1 expression (through direct or indirect regulation). Ascl1 controls the specification of GABAergic neurons in part through direct activation of the Dlx1/2 genes, which in turn induce the GABA biosynthetic enzymes Gad1 and Gad2. Gsx1 and Gsx2 can promote the expression of Dlx1/2 and differentiation of GABAergic neurons in the ventrolateral telencephalon (lateral ganglionic eminence) even in the absence of Ascl1 (Fode et al. 2000; Scardigli et al. 2003; Schuurmans et al. 2004; Poitras et al. 2007; Wang et al. 2009; Martynoga et al. 2012). *Solid lines* represent direct transcriptional regulation, while *dotted lines* represent direct or indirect regulation. *HC* hippocampus, *CX* cortex, *LGE* lateral ganglionic eminence, *MGE* medial ganglionic eminence

Neurog2 null mutant mice. Upper layer neurons appear to depend instead for their generation on other transcription factors, including Pax6 and Tlx (Schuurmans et al. 2004). Identification of the genes that are downregulated in the cortex of *Neurog1*; *Neurog2* mutant embryos by microarray transcript profiling has shown that Neurog

proteins promote the expression of a large number of transcription factors specifically expressed in the glutamatergic cortical projection neuron lineage, including Neurod1, Neurod2, Neurod6 and Tbr2, and for the expression of the vesicular glutamate transporters vGlut1 and vGlut2 (Schuurmans et al. 2004; Kovach et al. 2012) (see also Sect. 2.3.2). Thus, the Neurog proteins have a central role in initiating the glutamatergic neuronal differentiation programme in cortical progenitors.

Neurog2 is not only required but also sufficient for the specification of telencephalic neurons with a glutamatergic projection neuron phenotype. Forced expression of Neurog2 in neural stem cell cultures or in cortical astrocyte cultures generates neurons with molecular and electrophysiological characteristics of glutamatergic cortical neurons, thus demonstrating that Neurog2 has an instructive role in establishing this neurotransmission phenotype (Berninger et al. 2007; Heinrich et al. 2010). Moreover, expression of Neurog2 in cortical progenitor cultures promotes the differentiation of neurons with one major dendrite, a morphological characteristic of pyramidal cortical neurons, while neurons that do not express Neurog2 in such cultures adopt mostly a multipolar morphology, i.e. they grow multiple thin dendrites from the cell body (Hand et al. 2005). This result suggests that Neurog2 specifies not only the glutamatergic neurotransmission of cortical projection neurons but also their pyramidal morphology. This conclusion is supported by in vivo experiments in which silencing of *Neurog2* expression by RNAi or expression of mutant forms of the protein results in a fraction of neurons adopting a multipolar instead of a pyramidal morphology (Hand et al. 2005; Hand and Polleux 2011). Along the same lines, and as discussed further in the next section of the chapter, Neurog2 also promotes the radial migration of newborn neurons from the ventricular zone of the dorsal telencephalon to the cortical plate and controls the callosal axonal trajectory of upper layer projection neurons. Therefore, Neurog2 coordinately specifies all the major features defining the identity of cortical projection neurons, including their neurotransmission phenotype, mode of migration, axonal projection pattern and pyramidal morphology.

It is worth noting that Neurog2 is also expressed in adult neurogenic progenitors in the dentate gyrus of the hippocampus and in the dorsal part of the subependymal zone. Neurog2-expressing adult progenitors produce Tbr2-expressing and Neurod1-expressing precursors and glutamatergic neurons (granule cells in the hippocampus and juxtaglomerular neurons in the olfactory bulb). This suggests that the crucial role of Neurog2 in specifying glutamatergic neuronal phenotypes is not restricted to the embryonic cortex but extends to the adult brain (Brill et al. 2009; Roybon et al. 2009a; Roybon et al. 2009b; Winpenny et al. 2011).

2.2.3 Ascl1 Protein and the Specification of Telencephalic GABAergic Neurons

An important feature of *Neurog* mutant embryos besides the loss of cortical projection neurons is an overexpression of Ascl1 by dorsal telencephalic progenitors as well as

their subsequent ectopic expression of ventral markers, including Dlx1, Dlx2, Dlx5 and the GABA biosynthetic enzymes Gad1 and Gad2 (Fode et al. 2000). Therefore, *Neurog* genes have a dual role of inducing a cortical-specific neuronal differentiation programme while suppressing an alternative basal ganglia differentiation programme (Schuurmans et al. 2004; Kovach et al. 2012). When *Ascl1* is also deleted in *Neurog2*; *Ascl1* double mutant embryos, these ventral markers are absent from the cortex, demonstrating that their ectopic expression in the *Neurog2-deficient* cortex results from the overexpression of *Ascl1* and therefore that Ascl1 can instruct a GABAergic ventral telencephalic identity in the embryonic cortex (Schuurmans et al. 2004). A similar conclusion was reached by analysing mice in which *Ascl1* is expressed instead of *Neurog2* by cortical progenitors, as a result of the "knock-in" of the *Ascl1* coding sequence in the *Neurog2* locus (Fode et al. 2000). Moreover, forced expression of Ascl1 in neural stem cell cultures or cortical astrocyte cultures produces neurons with molecular and electrophysiological characteristics of GABAergic neurons, thus confirming the instructive role of Ascl1 in the specification of GABAergic telencephalic neurons (Berninger et al. 2007; Heinrich et al. 2010).

Loss of *Ascl1* in the mouse telencephalon results in a depletion of progenitors that is restricted to the medial ganglionic eminence and a loss of neurons generated in this region, namely, interneurons that will populate the cortex as well as different populations of basal ganglia neurons (Casarosa et al. 1999; Marin et al. 2000). In contrast, the lateral ganglionic eminence and the striatal projection neurons that derive from it remain unaffected in *Ascl1* null mutant mice. However, the striatum is severely reduced in mice double mutant for *Ascl1* and *Gsx2* (while it is only mildly affected in *Gsx2* single mutants), suggesting that the homeodomain transcription factor Gsx2, or a target gene of Gsx2, can substitute for Ascl1 in the generation of striatal neurons (Wang et al. 2009). Loss of *Ascl1* does not result in the derepression of *Neurog2* or other components of the glutamatergic cortical differentiation programme in the ventral telencephalon, and replacement of Ascl1 by Neurog2 in ventral telencephalic progenitors, by "knock-in" of *Neurog2* in the *Ascl1* locus, does not result in the respecification of ventral telencephalic neurons into glutamatergic cortical neurons (Parras et al. 2002). However, Neurog2 can induce a cortical projection neuron phenotype, including induction of *Neurod* genes and vGlut2, when ectopically expressed at a high and sustained level in the ventral telencephalon by in utero electroporation (Mattar et al. 2008). Therefore, progenitor cells in both ventral and dorsal divisions of the telencephalon can produce either GABAergic or glutamatergic neurons. However, additional pathways acting in parallel to proneural proteins to specify telencephalic neuron identities appear to have a more important role in the specification of GABAergic neurons ventrally than in the specification of glutamatergic neurons dorsally.

Together, the studies discussed in this section demonstrate unequivocally that proneural proteins are critical for the specification of the identity of telencephalic neurons. However, the important subtype specification function of proneural proteins must be qualified for the following reasons: (1) Proneural proteins do not appear to be involved in the further specification of neuronal identities within the GABAergic and glutamatergic telencephalic neuron populations, e.g. factors other

than Neurog2 control the decision of cortical progenitors to produce layer 6 versus layer 5 neurons, and factors other than Ascl1 control the decision of ventral telencephalic progenitors to generate specific subtypes of interneurons or pallidal or striatal projection neurons; (2) Neurogs and Ascl1 are not the only factors that can initiate glutamatergic and GABAergic differentiation programmes in telencephalic progenitors, since only subsets of neurons lose their identities in *Neurog1*; *Neurog2* and in *Ascl1* mutant mice. The roles of Pax6 and Tlx in the cortex and of Gsx2 in the striatum have already been mentioned but other genes are certainly also involved (e.g. Rouaux and Arlotta (2010)); (3) the function of Neurog2 and Ascl1 in the specification of glutamatergic and GABAergic neurons is specific to the telencephalon. Proneural genes contribute to the specification of neuronal identities throughout the peripheral and central nervous system, but their particular roles differ in different parts of the nervous system (Bertrand et al. 2002; Parras et al. 2002).

2.2.4 *Proneural Proteins and the Regulation of Neuronal Migration*

Proneural proteins are present in dividing progenitors and early postmitotic neurons of the dorsal telencephalon at the time when these cells initiate their migration towards the cortical plate (Hand et al. 2005; Britz et al. 2006; Ochiai et al. 2009; Hand and Polleux 2011), suggesting that these factors could contribute to the migratory process. Manipulations of the expression of Ascl1 and Neurogs in cortical cells, either in vivo or in tissue explants, have indeed demonstrated their role in the regulation of neuronal migration. Forced expression of either *Neurog2* or *Ascl1* induces the migration of embryonic cortical neurons in an in vitro cell aggregate migration assay (Ge et al. 2006). Conversely, loss of *Neurog2*, and to a lesser extent loss of *Ascl1*, results in distinct radial migration defects. Abnormal neuronal migration has been observed in the whole cortex of null mutant embryos and in individual neurons in which proneural protein expression has been acutely eliminated by shRNA-mediated silencing or electroporation of the recombinase Cre in conditional mutant embryos (Hand et al. 2005; Heng et al. 2008; Hand and Polleux 2011; Pacary et al. 2011). In the case of *Neurog2*, histological examination of the cortex of newborn *Neurog2* mutant mice has revealed hallmarks of a neuronal migration disorder, in particular the presence of heterotopic clusters of cells located deep within the cortical parenchyma (Hand et al. 2005).

At a mechanistic level, both Neurog2 and Ascl1 act primarily by downregulating RhoA signalling, since the migration defects of cortical neurons that have lost the expression of either of these transcription factors can be restored by downregulating RhoA signalling (Hand et al. 2005; Pacary et al. 2011). The molecular link between proneural proteins and the RhoA pathway is established by Rnd genes, which belong to a small family of atypical small GTP-binding molecules (Riou et al. 2010). Rnd2 is a direct target of Neurog2 while Rnd3 is a direct target of Ascl1, and both Rnd2 and Rnd3 promote radial migration of newborn cortical neurons by controlling

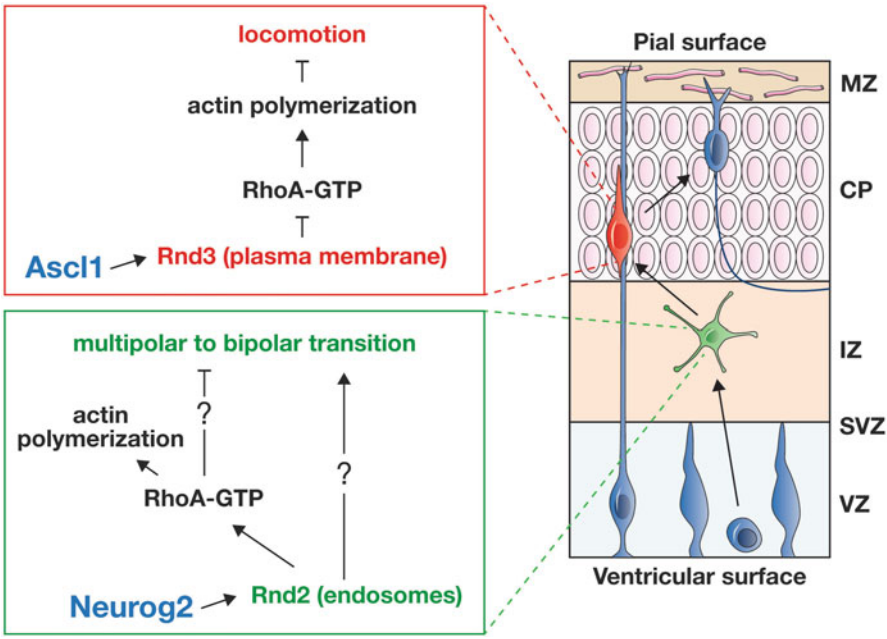

Fig. 2.3 Neurog2 and Ascl1 control different steps of radial migration of cortical neurons through induction of different Rnd proteins. *Top left panel*: Ascl1 induces the expression of plasma membrane-localized Rnd3, which inhibits RhoA and induces filamentous actin depolymerization, which in turn promotes the locomotion of cortical neurons along radial glial fibres in the cortical plate. *Bottom left panel*: Neurog2 induces the expression of endosome-associated Rnd2, which promotes neuronal polymerization and extension of the leading process at the multipolar to bipolar transition in the intermediate zone. Rnd2 acts in part through inhibition of RhoA, but filamentous actin depolymerization is not necessary for Rnd2 activity (Heng et al. 2008; Pacary et al. 2011). *VZ* ventricular zone, *SVZ* subventricular zone, *IZ* intermediate zone, *CP* cortical plate, *MZ* marginal zone

actin cytoskeleton dynamics through suppression of RhoA signalling (Heng et al. 2008; Pacary et al. 2011) (Fig. 2.3). Interestingly, the two Rnd genes regulate distinct steps of migration in the embryonic cortex. In particular, Rnd2-deficient neurons fail to transit from the multipolar to the bipolar stage as they migrate in the intermediate zone, while Rnd3-deficient neurons progress successfully through this transition but present specific locomotion defects as they migrate in the cortical plate (Heng et al. 2008; Pacary et al. 2011). Swapping experiments involving silencing one Rnd gene and overexpressing the other have demonstrated that the divergent functions of Rnd2 and Rnd3 in radial migration reflect both different intrinsic properties and distinct timing of expression in migrating neurons (Pacary et al. 2011). An important difference between the two molecules is that they regulate RhoA signalling in distinct subcellular compartments, with only Rnd3 being associated with the cell membrane (Pacary et al. 2011). The processes regulated by the Rnd-RhoA pathways at the cell membrane and inside the cell have not yet been identified.

Rnd genes are expressed by neurons not only when they initiate their migration in the ventricular and subventricular zone but also as they migrate through the intermediate zone and cortical plate. In contrast, Neurog2 and Ascl1 are expressed mostly by progenitor and early born neurons in the ventricular and subventricular zones, and only very transiently in the intermediate zone for Neurog2 (Hand et al. 2005; Britz et al. 2006; Ochiai et al. 2009; Hand and Polleux 2011). Therefore, proneural genes must induce the expression of Rnd genes in young neurons but not maintain their expression throughout the migratory process. Interestingly, Neurog2 induces the expression of neuronal differentiation genes of the NeuroD family and of the T-box protein Tbr2, which have been shown to be able to activate the same *Rnd2* enhancer element as Neurog2, suggesting that Neurog2 and downstream transcription factors induce and maintain *Rnd2* transcription, respectively, via a relay mechanism (Heng et al. 2008). Factors acting downstream of Ascl1 may similarly maintain Rnd3 expression in migrating cortical neurons but the identity of such factors remains unknown.

Interneurons are largely missing from the cortex of Ascl1 mutant embryos, suggesting that, in addition to its role in their specification discussed earlier, Ascl1 may also be required for the tangential migration of cortical interneurons (Casarosa et al. 1999; Marin et al. 2000). *Rnd3* expression is severely reduced in the ventral telencephalon of Ascl1 mutant embryos, where cortical interneurons are born and initiate their long journey into the cortex (Pacary et al. 2011). However, whether Rnd3 or other targets of Ascl1 are implicated in the tangential migration of these neurons has not yet been investigated.

2.2.5 Neurog2 and the Regulation of Axon Projections

Cortical neuron populations display very specific patterns of axonal projections. Most neurons in deep cortical layers project laterally towards subcortical regions, while neurons in superficial layers project towards the midline to innervate other cortical areas. In keeping with the important role of Neurog2 in specification of multiple aspects of the phenotype of cortical projection neurons, and with its transient expression in early postmitotic cortical neurons when axon growth is initiated (Hand et al. 2005; Ochiai et al. 2009), Neurog2 has been shown to determine axon projection patterns in the cortex. Silencing Neurog2 when neurons that will populate superficial layers (layers 2–4) are generated, causes many of these neurons to project their axons laterally towards the ipsilateral cortex and the thalamus instead of projecting medially towards the contralateral cortex (Seibt et al. 2003; Hand and Polleux 2011). Moreover, *Neurog2* null mutant embryos present a reduced and abnormal corpus callosum (Hand and Polleux 2011). These results demonstrate that Neurog2 specifies the callosal projection pattern of late-born cortical neurons, therefore suggesting that it plays an important role in an early step of cortical circuit formation.

2.3 Molecular Mechanisms Underlying Proneural Gene Activity

2.3.1 Regulation of Proneural Gene Expression and Activity

The genetic manipulations that we have discussed above have shown that proneural proteins are potent molecules whose overexpression or silencing results in dramatic cell fate changes. It is therefore logical that their expression and activity are tightly controlled by a multitude of regulatory mechanisms acting on multiple levels, including transcription, transcript stability, protein stability and cofactor interactions. The expression of proneural genes in broad dorsal and ventral domains in the embryonic telencephalon is likely controlled by the same pathways that establish the dorsal and ventral subdivisions of the telencephalon, but the information available is only fragmentary. Expression of Neurog2 in the dorsolateral cortex is controlled by the regional determinant Pax6, which has been shown to bind and activate an enhancer element in the Neurog2 gene (Scardigli et al. 2001; Scardigli et al. 2003). The cortical expression of Neurog1 is induced by Neurog2 (Fode et al. 2000) as well as by Wnt signalling through direct binding of the β-catenin/TCF transcriptional complex to its promoter (Hirabayashi et al. 2004). The telencephalic expression pattern of Ascl1 is more complex than that of the Neurog genes as it includes both ventral progenitors at a high level and cortical progenitors at a lower level. The regulatory elements that direct Ascl1 transcription in these territories have not been identified, but mouse mutant analysis indicates that Ascl1 expression in the lateral ganglionic eminence of the ventral telencephalon is controlled by the homeodomain protein Gsx1 (Wang et al. 2009) (Fig. 2.2).

Within these regional domains, the expression of proneural genes is strictly regulated temporally and restricted to proliferating progenitors and newborn neurons of the ventricular and subventricular zones. Furthermore, they are only expressed by subsets of ventricular zone progenitors in a salt-and-pepper manner. This is due to a process of lateral inhibition mediated by Notch signalling, whereby cells expressing a proneural gene induce the expression of the Notch ligand Delta1, which activates Notch signalling and represses proneural gene expression in neighbouring cells (Louvi and Artavanis-Tsakonas 2006). However, Ryoichiro Kageyama and colleagues made the important discovery that the suppression of Neurog2 expression by Notch signalling in cortical ventricular zone cells is in fact a dynamic process, with the Notch effector Hes1 oscillating with a period of 2–3 h and Neurog2 and its target Delta1 oscillating in opposite phase (Kageyama et al. 2008; Shimojo et al. 2008). Interestingly, Neurog2 not only oscillates but is also phosphorylated by cyclin-dependent kinases (cdks) on multiple serine-protein sites in proliferating progenitors, which results in a reduced ability to bind regulatory elements (Ali et al. 2011). Therefore, the transition from proliferating progenitors to differentiating neurons involves both the downregulation of Notch signalling and stabilization of *Neurog2* transcription, and a reduction in cdk kinase activity and the ensuing dephosphorylation of Neurog2. Delta1 is the only target of Neurog2 known to be

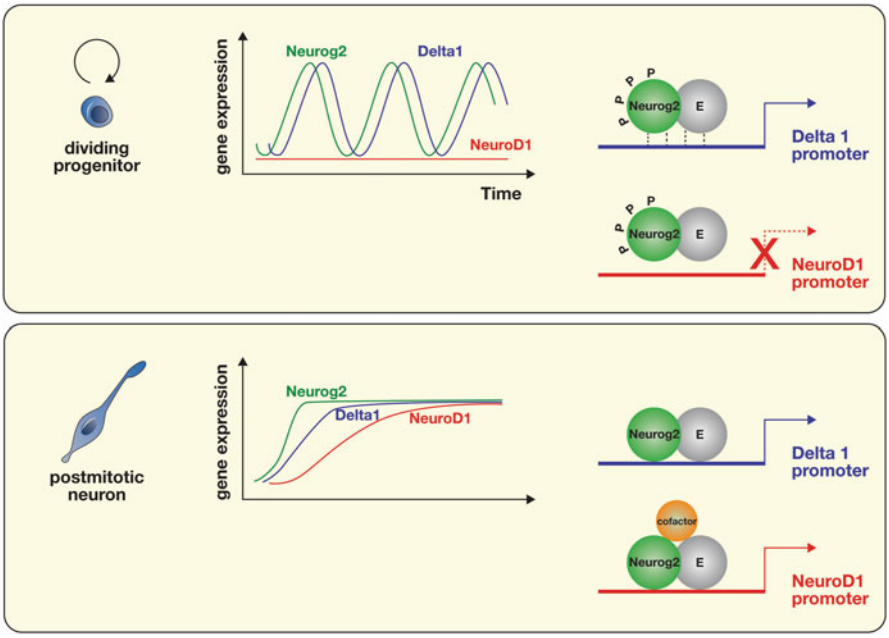

Fig. 2.4 Neurog2 activates the Delta1 promoter in both proliferating progenitors and postmitotic neurons but activates the Neurod1 promoter only in postmitotic neurons. *Top panel*: In proliferating cortical progenitors, Neurog2 and Delta1 transcript and protein levels oscillate and Neurog2 protein is phosphorylated at multiple serine residues by cyclin-dependent kinases. Short bursts of phosphorylated Neurog2 can activate the Delta1 promoter but not the Neurod1 promoter. *Bottom panel*: In postmitotic neurons, Neurog2 promoter activity is stabilized and Neurog2 protein is dephosphorylated, resulting in stable transcription of Delta1 and, with a slower kinetic, of Neurod1 (Kageyama et al. 2008; Shimojo et al. 2008; Ali et al. 2011; Hindley et al. 2012)

transcribed in proliferating progenitors, because the Delta1 promoter has a unique ability to be activated by Neurog2 even when it is phosphorylated and transiently available during oscillations (Hindley et al. 2012). In contrast, transcription of other targets including the neuronal differentiation gene *Neurod1* requires dephosphorylation as well as a more stable expression of Neurog2 and is therefore initiated only when cells exit the cell cycle (Hindley et al. 2012) (Fig. 2.4). The mechanisms that underlie the differential sensitivity of target promoters to the duration and strength of Neurog2 binding are not known and clearly deserve further investigation.

The fine-tuning of Neurog2 expression that is required to achieve a proper balance between progenitor self-renewal and differentiation involves not only transcriptional regulation of the *Neurog2* gene by Notch signalling but also regulation of the stability of both Neurog2 transcript and protein. Neurog2 transcripts are actively degraded by Drosha, a component of the microRNA microprocessor that has RNAse activity. Silencing of Drosha with shRNAs results in stabilization of *Neurog2* transcripts, in particular in the subventricular and intermediate zones of the

cortex, leading to premature neuronal differentiation of cortical progenitors (Knuckles et al. 2012). Neurog proteins are also rapidly degraded by ubiquitin-mediated proteolysis (Vosper et al. 2009), and the stabilization of Neurog2 in progenitors that exit the cell cycle and begin to differentiate requires an interaction with the cdk inhibitor $p27^{Kip1}$ (Nguyen et al. 2006). By independently suppressing cdk kinase activity and inhibiting Neurog2 protein degradation, $p27^{Kip1}$ mechanistically links several crucial events in the development of the cortical neuron lineage, including the cell cycle exit of progenitors and the increase in Neurog2 activity by stabilization of this protein.

While multiple mechanisms stimulate Neurog expression during cortical neurogenesis, new mechanisms appear at the end of this period to downregulate their expression and allow the switch to gliogenesis to take place. Repression of Neurog1 at the end of cortical neurogenesis has been shown to involve the Polycomb Repressive Complexes PRC1 and PRC2. Elimination from the embryonic cortex of the PRC1 component Ring1B or of the PRC2 component Ezh2 results in delayed downregulation of Neurog1 expression while Neurog2 remains unaffected. This is sufficient to extend the duration of the neurogenic period and to delay the onset of astrogenesis (Hirabayashi et al. 2009). Therefore, Polycomb complexes play an important role in regulating the timing of the switch from neurogenesis to gliogenesis, but other mechanisms are likely to be involved as well, e.g. for the downregulation of Neurog2 (Fig. 2.1).

Neurog2 function in cortical development is controlled not only at the level of its expression but also by regulation of its activity, and phosphorylation appears to be the main mechanism used to control the activity of the Neurog2 protein. Phosphorylation of a tyrosine residue, Y241, is essential for the ability of Neurog2 to specify the dendritic morphology of pyramidal cortical projection neurons and for the initiation of radial migration, since forced expression of a mutant form of Neurog2 that cannot be phosphorylated at position 241 results in migration defects and abnormal morphologies of cortical neurons (Hand et al. 2005). In contrast, Y241 phosphorylation is dispensable for the proneural functions of Neurog2 such as activation of the Neurod1 promoter, suggesting that phosphorylation of Y241 regulates the interaction of Neurog2 with a co-regulator that is specifically required for activation of a subset of target genes controlling neuronal migration and dendritic polarity (Hand et al. 2005). While this hypothetical co-regulator and the kinase phosphorylating Neurog2 at tyrosine 241 have not yet been identified, phosphorylation of two other residues, serines S231 and S234, by the kinase GSK3 has been shown to regulate the interaction of Neurog2 with a well-characterized transcriptional complex involved in spinal motor neuron induction (Ma et al. 2008), as discussed in more detail in Sect. 2.3.3. Phosphorylation of Neurog2 by GSK3 has also been implicated in the decline of Neurog2 activity in late cortical neurogenesis. GSK3 has been shown to influence the choice of Neurog2 dimerization partner, favouring the formation of Neurog2-E47 heterodimers at the expense of more active Neurog2-Neurog2 homodimers (Li et al. 2012). Finally, and as already discussed above, phosphorylation of Neurog2 by cyclin-dependent kinases reduces the ability of Neurog2 to bind DNA and activate its neuronal differentiation targets in

proliferating progenitors. Thus, Neurog2 is the target of multiple phosphorylation events that regulate its activity in various ways, including modulation of its interaction with cofactors, with dimerization partners and with target gene promoters.

2.3.2 Transcriptional Targets of Proneural Genes

Since proneural proteins are transcriptional regulators, a detailed understanding of their functions in neurogenesis would be greatly enhanced by the characterization of their direct targets, i.e. the genes whose transcription they regulate through binding to their promoters and/or enhancer elements. The first attempts at identifying target genes of Neurogs and Ascl1 were made using subtractive hybridization screens or transcriptome analysis of neural cell lines and mouse embryos lacking or overexpressing these proneural genes (Hu et al. 2004; Mattar et al. 2004; Gohlke et al. 2008). Although the genes identified were clearly part of the differentiation programmes regulated by proneural proteins, it was however not clear whether they were direct targets or regulated further down the regulatory cascade. Progress was made with the identification of genes that are induced in Xenopus embryonic explants by a *Neurog* gene in the presence of the protein synthesis inhibitor cycloheximide and which are therefore likely direct transcriptional targets (Seo et al. 2007). This approach identified a large number of Neurog targets and, in particular, multiple transcription factors such as Neurod1, Neurod4 and Myt1. These factors are also regulated by Neurog2 in the mouse embryonic cerebral cortex (Gohlke et al. 2008), suggesting that Neurog proteins activate neuronal differentiation by inducing a common battery of transcription factors in different regions of the nervous system and in different organisms. In contrast, other transcription factors directly regulated by Neurog2 are expressed in a region-specific manner and contribute to the subtype specification of neurons, such as Tbr2 for the specification of glutamatergic neurons in the embryonic cerebral cortex (Ochiai et al. 2009; Kovach et al. 2012) (Fig. 2.5).

Improvements to the chromatin immunoprecipitation technique have now made it possible to directly probe proneural protein binding to regulatory elements and therefore, when combined with gene expression studies, to rigorously identify their direct targets. The identification of Ascl1 direct targets in the ventral telencephalon has shown that this factor directly controls all the major steps of neurogenesis through regulation of large number of genes that control, for example, lateral inhibition, cell fate specification, axon morphogenesis and neuronal differentiation (Castro et al. 2006; Poitras et al. 2007; Henke et al. 2009; Castro et al. 2011) (Fig. 2.5). Interestingly, two different classes of target genes with opposite roles in cell cycle control were also identified, including core cell cycle regulators (e.g. Cdk1, Cdk3, E2f1) as well as genes implicated in cell cycle arrest (e.g. Btg2, Gadd45g, Ccng2), thus suggesting that Ascl1 has a dual role in cell cycle control during neurogenesis, promoting sequentially cell cycle progression of neural progenitors and subsequently their cell cycle exit (Castro et al. 2011).

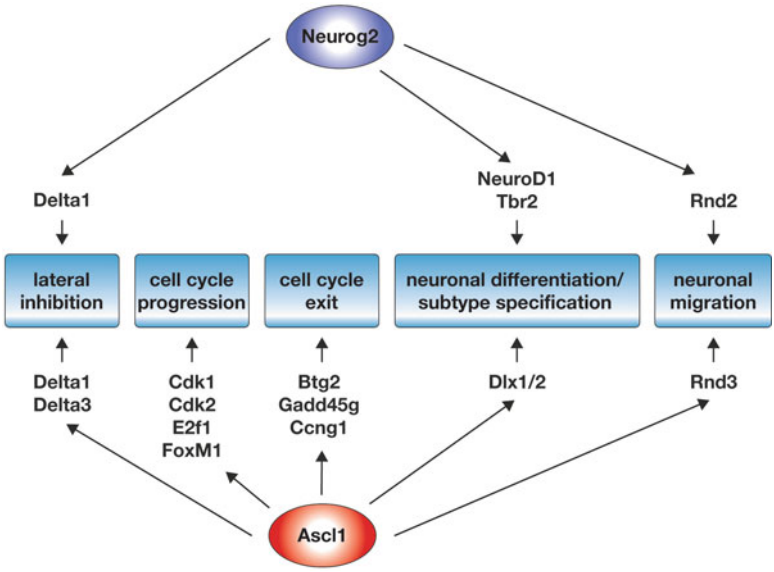

Fig. 2.5 Neurog2 and Ascl1 control the major steps of neurogenesis through direct regulation of multiple target genes. *Top panel*: Only relatively few of the direct target genes of Neurog2 in the developing telencephalon are known since no genome-wide chromatin immunoprecipitation analysis has been published yet. Separate studies have reported the direct regulation by Neurog2 of the Notch ligand Delta1 involved in lateral inhibition, of the transcription factor Neurod1 and Tbr2 involved in glutamatergic neuron differentiation and of Rnd2 involved in radial migration. *Bottom panel*: A genome-wide analysis of the direct transcriptional targets of Ascl1 in the telencephalon has demonstrated that this factor directly controls the major steps of neurogenesis through regulation of a large number of targets. This analysis also revealed that Ascl1 promotes sequentially the cell cycle progression and the cell cycle exit of telencephalic progenitors (Castro et al. 2006; Ochiai et al. 2009; Castro et al. 2011; Hindley et al. 2012)

The identification of large numbers of target genes involved in the different steps of neurogenesis raises the question of whether some of these targets are more important than others in mediating the function of proneural genes. This question has been addressed for the control of cortical neuron migration by proneural proteins. As discussed in Sect. 2.2.4, the genes coding for the small GTP-binding proteins Rnd2 and Rnd3 are directly regulated by Neurog2 and Ascl1, respectively, and they promote the radial migration of cortical projection neurons (Heng et al. 2008; Pacary et al. 2011). Remarkably, overexpression of Rnd2 in *Neurog2*-deficient neurons restores efficiently their migratory behaviour (Heng et al. 2008). Similarly, Rnd3 overexpression rescues the migratory defect of Ascl1-deficient neurons (Pacary et al. 2011). Thus, for this particular step of neurogenesis at least, a single target gene mediates the regulation of a complex cellular process by a proneural protein. These findings raise hope that it will be feasible, using similar rescue experiments, to identify the different molecular pathways through which proneural proteins control the major steps of neurogenesis in the mammalian brain.

2.3.3 *Interaction of Proneural Proteins with Other Transcription Factors*

Proneural factors regulate the expression of target genes as part of large transcriptional complexes. Such multi-protein complexes can include other sequence-specific DNA-binding factors which bind DNA in combination with proneural proteins and therefore restrict the number of sites in the genome where the protein complex can bind. Transcriptional complexes also contain non-DNA-binding cofactors that are recruited to the DNA by transcription factors and promote or suppress transcription by modifying the organization of the chromatin at the bound loci. The diversity of targets of proneural proteins and in particular their ability to specify different neuronal identities and initiate distinct differentiation programmes in different regions of the nervous system suggest that their activity and choice of targets are modulated by interactions with other factors. Indeed, examples have emerged of both DNA-binding transcription factors and chromatin-remodelling cofactors that interact with proneural proteins and modulate their activity. However, the few known interactors of proneural proteins very likely represent the tip of the iceberg and much remains to be learned in this area.

The best-documented example of an interaction between a proneural protein and other transcription factors has been obtained in the context of Neurog2 involvement in the generation of motor neurons in the ventral spinal cord (Lee and Pfaff 2003), but since similar interactions are likely to regulate Neurog2 activity in the developing cerebral cortex, this study is being discussed here. The LIM homeodomain proteins Lhx3 and Isl1 have been shown to specify motor neuron identity by binding to a well-characterized enhancer element in the motor neuron determination gene Hb9 (Thaler et al. 2002). Overexpression of combinations of transcription factors in the chick spinal cord has demonstrated that Lhx3 and Isl1 must cooperate with Neurog2 and the downstream factor Neurod4 to induce motor neurons (Lee and Pfaff 2003). Importantly, this interaction is specific for Neurog2 and Neurod4 as Ascl1 fails to induce motor neurons when co-expressed with Lhx3 and Isl1. This result is in keeping with the study of mice in which the sequences coding for Neurog2 and Ascl1 had been swapped, which showed that replacing Neurog2 by Ascl1 in motor neuron progenitors results in a deficit in motor neuron production and ectopic differentiation of ventral spinal cord interneurons (Parras et al. 2002).

Interestingly, the interaction between Neurog2 and Lhx3/Isl1 requires phosphorylation of Neurog2 by the kinase GSK3 at serine residues S231 and S234, as mentioned above. Mutation of these residues to Alanine prevents Neurog2 from cooperating with Lhx3/Isl1 to induce motor neurons (Ma et al. 2008). Biochemical experiments suggest a model whereby Neurog2 directly interacts via the phosphorylated serine residues with an adaptor protein, NLI (also known as Ldb, CLIM or Chip), which acts as a bridge between Neurog2 and the LIM homeodomain proteins (Ma et al. 2008). An antibody specific for the serine-phosphorylated form of Neurog2 detects a signal in nuclear extracts from embryonic telencephalon, suggesting that Neurog2 is involved in similar molecular interactions in the developing

cortex. Indeed, Soo-Kyung Lee and colleagues have recently shown that Neurog2 interacts with NLI and the non-DNA-binding LIM only protein Lmo4 in the embryonic cortex, where they act as coactivators of Neurog2 (Asprer et al. 2011). Overexpression of NLI/Lmo4 enhances the neuronal differentiation activity of Neurog2 in cellular assays and its pro-migratory activity in vivo. More importantly, loss of Lmo4 in mice results in a moderate but significant reduction in the expression of Neurog2 targets including *Tbr2* and *Rnd2* and in a deficit in cortical neurogenesis (Asprer et al. 2011). LIM proteins therefore play important roles in regulating the activity of Neurog proteins through direct and phosphorylation-dependent protein-protein interactions. Whether they differentially regulate the diverse functions of Neurogs (e.g. promoting subtype specification over pan-neuronal differentiation) and whether they also regulate the activity of Ascl1 remains to be investigated.

In motor neuron precursors, Neurog2 binds to the Hb9 enhancer where it interacts not only with the NLI/Lhx3/Isl1 complex but also with retinoic acid receptor (Lee et al. 2009). Interestingly, the Neurog2-retinoic acid receptor interaction is independent of the presence of retinoic acid. However, the recruitment of the coactivator p300/CBP to this complex requires the presence of retinoic acid, which as a result synergizes with Neurog2, Isl1 and Lhx3 for the upregulation of Hb9 and the induction of motor neuron differentiation (Lee et al. 2009). Motor neuron specification thus provides a very compelling model of how interaction with other transcription factors and with a signalling pathway controls the neurogenic activity of Neurog2. Future studies will determine whether similar mechanisms operate during cortical development, which seems likely.

Transcription factors from another family, the POU domain proteins Brn1 and Brn2, have been shown to cooperate with Ascl1 in the regulation of a subset of its target genes (Castro et al. 2006). The gene coding for the Notch ligand *Delta1*/*Dll1* is one of the best characterized direct transcriptional targets of both Ascl1 and Neurog2, and analysis of an enhancer element in the *Delta1* gene identified an evolutionarily conserved 15 bp motif that is essential for activation of this enhancer by Ascl1. Interestingly, this motif contains an E-box (the bHLH protein consensus-binding sequence) separated by 1 bp from a consensus-binding motif for POU proteins. Biochemical and mutagenesis experiments showed that Ascl1 binds cooperatively with the POU proteins Brn1 and Brn2 on the *Delta1* enhancer, and functional experiments showed that the Ascl1-Brn1/2 interaction is required for induction of Delta1 expression in vivo. Interestingly, an almost identical evolutionary conserved 15 bp motif is found in the vicinity of 21 other genes, including five other components of the Notch signalling pathway as well as genes involved in other steps of neurogenesis (Castro et al. 2006), suggesting that Ascl1 interacts with Brn proteins to regulate a subset of the neurogenic programme. Important questions remain as to how many other transcription factors must interact with proneural proteins in order to activate their differentiation programmes and whether interactions with different factors control different aspects of these programmes. To this end, a bioinformatics search for motif enrichment near putative Neurog2 and Ascl1 binding sites in proneural protein target genes has identified a significant number of

potential co-regulators, largely distinct for Ascl1 and Neurog2 (Gohlke et al. 2008). Biochemical and functional studies are now required to determine which of these candidates are bona fide proneural protein interactors and to elucidate their contributions to the different steps of neurogenesis.

2.3.4 Cofactors of Proneural Proteins

Proneural proteins appear to exert their functions mostly by dimerizing with E proteins such as E12 and E47 (encoded by the gene Tcf3), E2-2/Tcf4 and HEB/Tcf12. A few studies have reported that the activity of the proneural protein-E protein heterodimer is potentiated by recruitment of non-DNA-binding cofactors with chromatin-remodelling activity. For example, Neurog1 has been shown to recruit Brg1, the catalytic subunit of the ATP-dependent SWI/SNF chromatin-remodelling complex, and Brg1 function is essential for Neurog1 activity in Xenopus embryos and in the pluripotent cell line P19 (Seo et al. 2005). Neurog1 and Neurog2 also recruit the coactivators p300/CBP and PCAF, which stimulate transcriptional activity through their histone acetyltransferase activity. In motor neuron precursors, p300/CBP is recruited by the Neurog2-retinoic acid receptor complex in the presence of retinoic acid, and this results in marked elevation of histone H3 and histone H4 acetylation at the Neurog2-bound Hb9 enhancer (Lee et al. 2009). Recruitment of p300/CBP has also been shown to be required for the transcriptional activity of Neurog factors in Xenopus embryos, in mammalian neuronal precursors in culture and in motor neuron and cortical neuron precursors in vivo (Koyano-Nakagawa et al. 1999; Sun et al. 2001; Lee et al. 2009). An interesting question to address in the future is whether proneural protein targets are differentially dependent on cofactor activity for their transcription, which may result in different kinetics of activation during neurogenesis.

2.4 Conclusion

Proneural proteins regulate an amazing array of cellular processes during cortical development. While the nature of the functions of proneural proteins has become clearer in recent years, how they execute each of these activities has just begun to be addressed. Future work will identify the different target genes that proneural proteins must regulate to promote each step of neurogenesis. It will also be important to characterise the different transcriptional regulators and chromatin modifiers that interact with proneural proteins in order to better understand how different categories of targets are regulated in a precise temporal manner by such a small group of transcription factors. Finally, we still know very little of how the expression and activity of proneural proteins is controlled at each step of cortical development. Elucidating how extracellular signalling pathways converge with proneural-driven transcriptional programmes is another exciting research direction for the future.

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Chapter 3
The Role of the Transcription Factor Pax6 in Brain Development and Evolution: Evidence and Hypothesis

Noriko Osumi and Takako Kikkawa

Abstract During mammalian corticogenesis, the dorsal telencephalon is patterned through secreted molecules and transcription factors. Expression of the transcription factor Pax6 demarcates the dorsal telencephalon, thereby patterning the future cortical primordium. Pax6 is also crucial in neurogenesis in the developing cortex through its role in balancing proliferation and differentiation of neural progenitor cells (NPCs). In this chapter, we address the role of Pax6 and its downstream molecules in cortical development and evolution. We also note the possible involvement of Pax6 in the onset of neurodevelopmental diseases.

3.1 Pax6 is a Highly Conserved Transcription Factor

Historically, the *Pax6* gene has been identified as the gene responsible for the *Small eye* mouse (*Sey*) (Hill et al. 1991). In addition, the human *PAX6/AN1* gene is cloned from the 11p13 chromosomal region and has been identified as the gene responsible for congenital aniridia through loci deletions in cases of WAGR syndrome showing Wilms' tumor, aniridia, genital ridge defects, and mental retardation (Ton et al. 1991). *Pax6/PAX6* encodes a transcription factor that has two DNA-binding domains: a paired domain and paired-type homeodomain (Walther and Gruss 1991). Phylogenetically, Pax6 is well conserved in both vertebrates and invertebrates and critical in eye development [see below; (Hanson and Van Heyningen 1995; Gehring 1996)].

Analyses of Pax6 expression in vertebrate embryos have revealed that Pax6 also plays crucial roles in the nervous system (Osumi 2001; Manuel and Price 2005).

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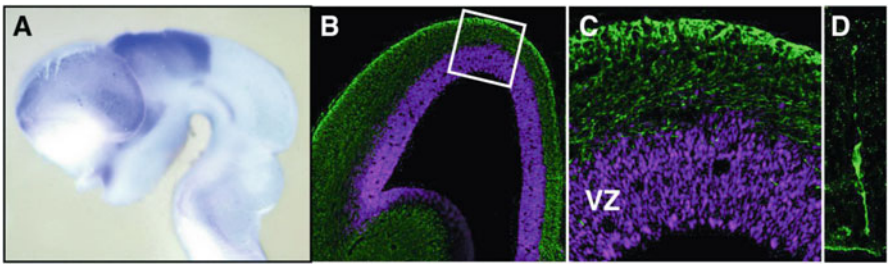

Fig. 3.1 Expression of Pax6 in the developing cortex. Whole-mount in situ hybridization of an E13.5 rat neural tube (**a**) and immunohistochemistry of E14.5 mouse brain sections (**b**–**d**). (**a**) *Pax6* mRNA (*blue*) is specifically expressed in the dorsal telencephalon (i.e., the primordium of the neocortex) and part of the diencephalon. Pax6 is also expressed in the hindbrain (i.e., the primordia of the cerebellum and brain stem). (**b**) Pax6 protein expression (*magenta*) is restricted to the ventricular zone (VZ) where neural progenitor cells exist. (**c**) Higher magnification of the *square* shown in (**b**). (**d**) A radial progenitor labeled with *green fluorescent protein* showing a hyperpolarized morphology with long and thin apical and basal processes stretching from the VZ (*bottom*) to the pial surface (*upper*)

Expression of Pax6 begins with the initial development of the central nervous system (CNS) in the mouse (Inoue et al. 2000). Pax6 expression in the neural tube is quite regionally specific; it is expressed first in the forebrain, hindbrain, and spinal cord, but not in the midbrain (Stoykova and Gruss 1994; Osumi et al. 1997). That is, Pax6 is regionalized to the forebrain and midbrain. Within the forebrain, Pax6 expression becomes restricted to the dorsal telencephalon and to specific regions of the diencephalon [Fig. 3.1a; (Mastick and Andrews 2001; Hirata et al. 2002)]. These expression patterns up to this stage of brain patterning have been most carefully analyzed in rodents but are similar in other vertebrates and even in lampreys (Macdonald et al. 1994; Pera and Kessel 1997; Murakami et al. 2001). Because brain patterning guides subsequent neuronal migration and axonal extension, Pax6 also has essential roles in these contexts (Hirata et al. 2002; Mastick et al. 1997; Talamillo et al. 2003; Nomura and Osumi 2004; Nural and Mastick 2004; Nomura et al. 2006). The dorsal telencephalon gives rise to the neocortex in mammals. In later embryonic stages, the expression of Pax6 in the dorsal telencephalon shows graded patterns, i.e., a rostrally high and caudally low gradient and a laterally high and medially low gradient. These gradients thereby pattern the developing neocortex in collaboration with other transcription factors (O'Leary and Sahara 2008; Bishop et al. 2002).

Pax6 further contributes to the development of glial cells. Pax6 is weakly expressed in astrocytes, and the loss of Pax6 perturbs astrocytic maturation and increases astrocytic proliferation (Sakurai and Osumi 2008). Consistent with this, it seems that reduced Pax6 expression may be related to the formation of glioma (Zhou et al. 2003, 2005; Pinto et al. 2007; Maekawa et al. 2010). A small population of oligodendrocyte precursor cells, another type of glia, tested positive for Pax6 (Kimura and Osumi unpublished). These results suggest that Pax6 plays multiple

roles in promoting cell proliferation and cell differentiation in NPCs, neurons, and glial cells in highly context-dependent manners.

3.2 Pax6 in Embryonic Neurogenesis

During the period of cortical neurogenesis, Pax6 is expressed by NPCs in the ventricular zone (VZ), and for this reason, Pax6 is widely used as a marker for NPCs [Fig. 3.1b, c; (Osumi et al. 2008)]. NPCs initially divide symmetrically to proliferate and subsequently divide asymmetrically to produce one NPC daughter and another daughter fated to become a neuron or a basal progenitor cell (BP) (Noctor et al. 2007; Pontious et al. 2008). BPs do not have radial processes and divide symmetrically a few times to produce neurons. Cell division of the Pax6+ NPCs occurs at the apical surface of the VZ (Tamai et al. 2007); thus, these NPCs are also called apical progenitors (APs). The APs become longer and longer during neurogenesis, stretching from the apical side to the basal (pial) side of the cortical primordium. Therefore, these cells are also called "radial progenitors" or "radial glia (RG)," although they are not really glial cells (Hevner et al. 2006; Fish et al. 2008). Pax6 expression shuts off in daughter cells that are fated to differentiate into neurons or BPs (Fig. 3.1b, c).

Although Pax6 is not expressed in cortical excitatory neurons derived from the Pax6+ NPCs of the dorsal telencephalon, intense expression of Pax6 is observed in neurons of the amygdala, thalamus, and cerebellum (Stoykova and Gruss 1994) (also see Genepaint.org). Thus, it is expected that Pax6 is important in the production of neurons. Indeed, overexpression of Pax6 in developing cortical NPCs inhibits its proliferation and promotes neurogenesis (Heins et al. 2002). Therefore, Pax6 was once believed to be a strong inducer of neuronal differentiation (Gotz et al. 1998). However, both mice and rats with spontaneous mutations in the *Pax6* gene exhibit an intriguingly dual phenotype; not only the cortical plate (containing neurons) but also the VZ (containing NPCs) becomes thinner (Warren et al. 1999; Fukuda et al. 2000). Detailed analysis of the cell cycle has revealed that Pax6 is indeed crucial in the maintenance of proliferative NPCs in the cortex (Estivill-Torrus et al. 2002). That is, the level of Pax6 expression is essential for controlling the balance between the proliferation and neuronal differentiation of neuronal progenitors in the cerebral cortex; intense expression of Pax6 inevitably induces neuronal differentiation in NPCs and even in astrocytes (Gomez-Lopez et al. 2011; Heins et al. 2002; Sansom et al. 2009).

It is evident that Pax6 is important in the cell division of APs (Quinn et al. 2007). One of the roles of Pax6 in cell division seems to be the regulation of the orientation of the cleavage plane (Asami et al. 2011). APs often divide asymmetrically in the developing cortex of *Sey/Sey* embryos. The same is true in acute depletion of Pax6 in *Pax6-floxed* mice transfected with *Cre-IRES-GFP* vector. This can be as a result of an unequal inheritance of the apical membrane domain and adherens junctions in the absence of Pax6 function. A related phenotype seen in *Pax6* mutant rat homozygotes

(*rSey²*/*rSey²*) is impairment in interkinetic nuclear movement (Tamai et al. 2007). Another possibility for altered cell division in the loss-of-Pax6 condition could be abnormal integrin signaling from the basement membrane or from the marginal zone [(Costa et al. 2007; Radakovits et al. 2009); see below].

3.3 Pax6 in Postnatal Neurogenesis

Pax6 also plays a crucial role in postnatal neurogenesis. Pax6 is expressed at medium levels in embryonic NPCs, but continues to be expressed in the adult NPCs located in the hippocampal dentate gyrus and in the SVZ of the lateral ventricle (Maekawa et al. 2005; Nacher et al. 2005; Englund et al. 2005; Kohwi et al. 2005). Transcriptional regulation of NPCs is similar in both embryonic and postnatal neurogenesis, and the following is a common transition of transcription factors: Pax6 ->Tbr2 ->NeuroD ->Tbr1 from NPCs to neurons (Hevner et al. 2006; Englund et al. 2005).

The precise mechanisms by which Pax6 regulates postnatal neurogenesis remain rather fragmentary at the molecular level. However, haploinsufficiency of *Pax6* causes reduced neurogenesis in the dentate gyrus by depleting the NPC pool and accelerating precocious differentiation (Maekawa et al. 2005). Similarly, reduced and mis-specified interneurons are formed in the olfactory bulb of *Sey*/+ mice (Kohwi et al. 2005; Hack et al. 2005; Vergano-Vera et al. 2006; Haba et al. 2009). Pax6-deficient embryonic stem cells show reduced neurogenic capacity in culture (Quinn et al. 2010). In neurosphere assays using NPCs derived from the cortical primordium of *rSey²*/+ rat embryos, the proliferation of NPCs is decreased and astrocytic differentiation is conversely increased (Sakayori et al. 2012). Therefore, it is safe to say that the importance of Pax6 in the maintenance of NPCs and production of neurons is well conserved both in embryonic and postnatal neurogenesis.

The activation of neurogenesis is sometimes observed in non-conventional neurogenic regions. For example, brain injuries such as stab wounds and focal ischemia can induce neurogenesis in the murine neocortex (Buffo et al. 2005). More recently, focal laser lesions were found to promote neurogenesis in the visual cortex of juvenile rats (Sirko et al. 2009). Pax6 expression is generally induced in the hippocampus and SVZ after injury (Nakatomi et al. 2002; Wei et al. 2011; Zhang et al. 2011). This phenomenon may be a recapitulation that reflects embryonic neurogenesis.

3.4 Pax6 Expression in the Developing Primate Neocortex

The mammalian brain is characterized by a proportionally large neocortex, as the cortical primordium has become larger and more complex throughout mammalian evolution. To make a bigger cortex, it is obvious that more NPCs must be recruited. In primates, there appears to be, in addition to the VZ, another germinal layer in the

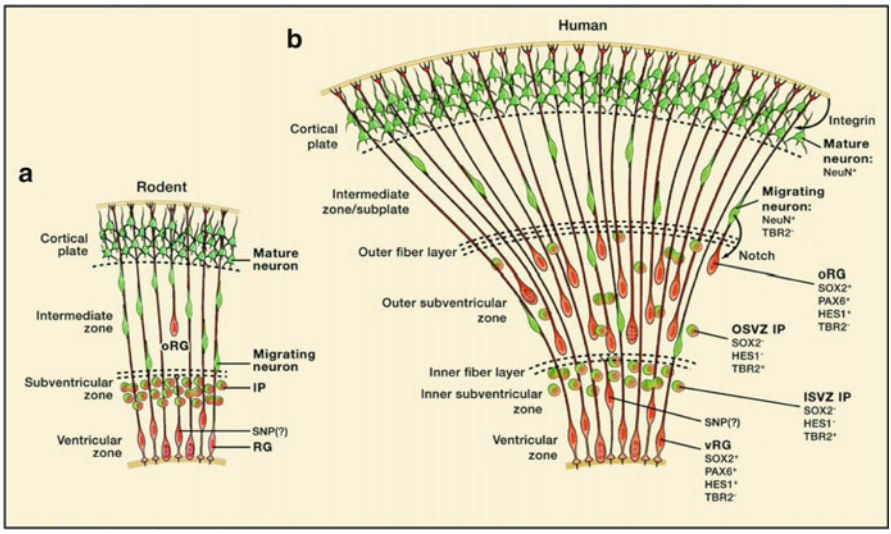

Fig. 3.2 Importance of Pax6 and radial processes in neocortical development and evolution. (**a**) In rodent, radial glial (RG) cells most often generate intermediate progenitor (IP) cells that divide to produce pairs of neurons. These neurons use RG fibers to basally migrate to the cortical plate. Although it is not fully acknowledged, it seems that small numbers of outer subventricular zone radial glia-like (oRG) cells exist in the mouse. (**b**) In human, outer subventricular zone (OSVZ) is well developed and contains oRG cells, IP cells, and migrating neurons (*red to green*). It is speculated that the number of produced neurons is significantly increased with the addition of oRG cells over ventricular RG (vRG) cells. Importantly, oRG cells have basal processes and receive integrin signaling via the pial membrane that is rich in extracellular matrix (Illustration is slightly modified from Liu et al., 2011)

outer subventricular zone (OSVZ) of the developing cortex [Fig. 3.2; (Hansen et al. 2010; Fietz et al. 2010)]. The NPCs in the OSVZ divide several times and greatly contribute to the production of the larger cortex of primates. Owing to the existence of the OSVZ and an inside out pattern of neuronal migration, the primate neocortex can dramatically expand in radial directions.

Unlike the BPs in the SVZ, NPCs in the OSVZ have long and thin basal processes, but no apical processes that stretch toward the pial surface (Hansen et al. 2010; Fietz et al. 2010). That is, NPCs in the OSVZ exhibit radial morphology common to RG in the VZ. The NPCs in the OSVZ are thus called outer radial glia (oRG) (Fig. 3.2; see review in Lui et al. (2011) and LaMonica et al. (2012)).

Interestingly, Pax6 expression is also expressed in oRG [Fig. 3.2; (Fietz et al. 2010; LaMonica et al. 2012; Reillo et al. 2011)]. Extrapolating from evidence in rodents, Pax6 might function to maintain the morphology and proliferative character in the oRG. Thus, it would be interesting to see how Pax6 contributes to the formation of primate neocortices, eventually of the gigantic human neocortex.

3.5 Molecules Downstream of Pax6

Multiple functions of Pax6 are mediated by the transcriptional regulation of different target genes. In corticogenesis, Pax6 balances cell proliferation (Fukuda et al. 2000; Estivill-Torrus et al. 2002) and neuronal differentiation (Heins et al. 2002). For example, we have shown that a gene encoding brain-type fatty acid protein (Fabp7/BLBP), the expression of which is regulated by Pax6, is required for the maintenance of proliferating NPCs in the developing cortex (Arai et al. 2005). CD15 is another NPC marker (Lewis X/SSEA-1) that is synthesized by fucosyltransferase 9 (Fut9) and is downregulated in *rSey*^{2}/*rSey*^{2} rats (Shimoda et al. 2002). Pax6 also directly regulates the expression of *Sox2* and controls the proliferation of the NPC's cortical SVZ (Wen et al. 2008). Neuronal differentiation, however, is promoted by so-called proneural molecules. One of the proneural genes, *Neurogenin2* (*Neurog2*), is directly regulated by Pax6 in the telencephalon and hindbrain [(Scardigli et al. 2001, 2003); also see below]. These lines of evidence suggest that Pax6 coordinates the proliferation and differentiation of NPCs by regulating various genes in a nested manner.

In addition to functions of self-renewal and neurogenesis, Pax6 controls genes involved in cell adhesion. *Cadherin 4* (*Cdh4*, *R-cadherin*), a gene encoding a hemophilic adhesion molecule, is expressed in the cortical region, and this expression is decreased in *Sey*/*Sey* mice (Stoykova et al. 1997). Cdh4 promotes the outgrowth of the tract of the preoptic commissure (TPOC) and rescues the defect of TPOC axon growth in *Sey*/*Sey* mice (Andrews and Mastick 2003). Microarray analyses using *Sey*/*Sey* mouse forebrains and an electrophoretic mobility shift assay identified *catenin delta 2* (*Ctnnd2, δ-catenin*) as a direct target of Pax6 (Duparc et al. 2006). Ctnnd2 colocalizes and interacts with adhesive junction proteins in the CNS (Lu et al. 1999). Moreover, tenascin C (Tnc), an extracellular matrix (ECM) glycoprotein, is lost in the *Sey*/*Sey* cortical primordium (Gotz et al. 1998). Pax6 acts as a modulator of alternative splicing and regulates the expression of various Tnc isoforms in NPCs in vitro (von Holst et al. 2007). Therefore, Pax6 may have an important role in the maintenance of RG cells via cell adhesion and production of ECM molecules [reviewed in Marthiens et al. (2010)].

Pax6 also regulates the expression of transcription factors. As previously mentioned, the proneural transcription factor Neurog2 is downstream of Pax6 (Scardigli et al. 2001, 2003). We have identified another transcription factor downstream of Pax6 called *double-sex* and a *mab-3*-related transcription factor-like family A1 (*Dmrta1*) (Fukuzaki and Osumi 2007) (Kikkawa et al. unpublished data). We have shown that Dmrta1 is markedly downregulated in the telencephalon of *rSey*^{2}/*rSey*^{2} rats and that Dmrta1 regulates expression of proneural genes (Kikkawa et al. unpublished data). Er81, a transcription factor in the ETS family, is expressed in layer 5 neurons of the neocortex (Weimann et al. 1999). Pax6 binds to the putative *Er81* promoter (Tuoc and Stoykova 2008), suggesting that a direct genetic interaction between Pax6 and Er81 may be involved in the neuronal subtype identity of layer 5 neurons. In silico and ChIP experiments have shown that FoxP2 is a novel direct

target of Pax6 (Coutinho et al. 2011). *FoxP2* expression is severely reduced in *Sey*/*Sey* mice and *Pax6*-knockdowned zebra fish (Coutinho et al. 2011). FoxP2 regulates the transition of NPCs to intermediate progenitors and neurons during corticogenesis, and the introduction of humanized *FOXP2* in mice enhances neurogenesis (Tsui et al. 2013). *FOXP2* has also received attention as a "language gene" in humans (Fisher and Scharff 2009) in the field of evolution (see below). These transcription factors that are regulated by Pax6 harmonize to control expression of other genes, which results in the formation of complicated structures of the mammalian cortex.

Recently, the emergence of ChIP-chip technology enabled us to perform a genome-wide mapping of transcription factor-binding sites in various organisms. A novel target of Pax6 is the *fragile X mental retardation 1* (*Fmr1*) gene, which encodes a protein called FMRP (Sansom et al. 2009). FMRP is an RNA-binding protein and functions to transport mRNA of synaptic molecules to synapses at a long distance (Ashley et al. 1993). It has been shown that FMRP is expressed in the RG cells during neocortical development and suppresses the transition from RG cells to intermediate progenitors by regulating actin organization (Saffary and Xie 2011). Another direct target of Pax6 is the *sperm-associated antigen 5* (*Spag5*) gene, which encodes a microtubule-associated protein (Sansom et al. 2009). Knocking down *Spag5* phenocopies the impaired orientation of cell division observed in *Sey*/*Sey* mice (Asami et al. 2011). Therefore, these findings highlight the importance of exploring Pax6 target genes to better understand the molecular networks of cortical development.

In a wider point of view, various genes appear to be Pax6 target candidates in different cell types and tissues. A most recent ChIP-chip study using newborn mouse lenses, E15 forebrains, and adult pancreas β-cells showed that nearly 20 % of genes/promoters are accessible to Pax6 in multiple tissues (Xie et al. 2013). The authors of this study identified novel common targets of Pax6 in the eye and forebrain: *kinesin family member 1B* (*Kif1b*) and *α-synuclein* (*Scna*). Kif1B, a kinesin motor protein, functions in the axonal transport of mitochondria and synaptic vesicles (Nangaku et al. 1994). Scna also regulates dopamine release and transport (Chandra et al. 2004; Tong and Shen 2009), and a mutation in human *SNCA* causes Parkinson's disease and Lewy body disease (Winslow et al. 2010). These findings represent a new aspect of the molecular networks regulated by Pax6 in regard to neurological and psychiatric diseases (see below).

3.6 Importance of Basal Processes and Possible Involvement of Pax6

As mentioned above, both RG and oRG cells have long basal processes. At the basal extreme, these processes attach to the basement membrane (the pia mater) where ECM molecules and growth factors are accumulated (Fietz et al. 2012; Radner et al. 2013). The basement membrane ECM molecules function in maintaining RG cells

via integrin signaling in mice (Radakovits et al. 2009; Loulier et al. 2009; Halfter et al. 2002; Haubst et al. 2006). Near the pia matter, there is a specific type of early born neuron called Cajal-Retzius cells that produce reelin and other morphogenic molecules (Ogawa et al. 1995; Griveau et al. 2010). Therefore, as proposed more than a decade ago by Tamamaki (Tamamaki 2002), RG have another important function in which they maintain their progenitor states in order to produce a large number of cortical neurons; this is in addition to their classical role as a scaffold for their radial migration.

The same situation might be true for primate oRG cells that also have long and thin basal processes attached to the basal membrane. Reelin-producing Cajal-Retzius cells also exist in the developing primate cortex (Meyer and Goffinet 1998). Furthermore, the oRG cells of human fetuses express various ECM molecules at higher levels than mice (Fietz et al. 2012). Thus, it is assumed that oRG cells receive more integrin signaling from the ECM at their pial surfaces, which enables the enormous proliferation that expands the progenitor pool and eventually forms a bigger brain.

Another important aspect of radial glial basal processes is their inheritance at mitosis. A cell-cycle-promoting molecule, Cyclin D2, accumulates in the most basal tip (called the basal end foot) of RG cells in the developing mouse cortex (Tsunekawa et al. 2012). During the cell division of RG cells, one of the daughters inherits the basal process and the other does not. Eventually, Cyclin D2 at the basal end foot is sequestered from the daughter that does not inherit the basal process. Through this process, the daughter cell that inherits the basal end foot eventually obtains Cyclin D2 and remains a progenitor cell, while the other cell that does not inherit the basal end foot is fated to be a neuron or basal/intermediate progenitor. A gain or loss of function of Cyclin D2 in mouse cortical primordium increases or decreases the number of apical progenitors, respectively, indicating that Cyclin D2 can actually promote cell proliferation (Tsunekawa et al. 2012). That is, Cyclin D2 works during neurogenesis as a negative determinant of neuronal fate (see Fig. 3.3 in Tsunekawa and Osumi (2012)). In the human fetal cortex, a similar distribution of Cyclin D2 is observed (Tsunekawa et al. 2012); thus, Cyclin D2 may also be important in the maintenance of oRG cells through a mechanism similar to that observed in the mouse.

What is the role of Pax6 in the basal processes of RG (and possibly oRG)? In the developing cortex of the *Sey/Sey* mouse, alignment of radial fibers is disrupted (Gotz et al. 1998). Thus, Pax6 may function to maintain the radial morphology of the RG cells. As discussed above, through functional analyses of Fabp7 (a molecule downstream of Pax6) in rodent corticogenesis, we found that loss of function of Fabp7 abolishes the radial morphology of the RG in addition to decreasing cell proliferation and promoting precocious neurogenesis (Arai et al. 2005). The disruption of radial morphology induced by Fabp7 knockdown is quite reasonable because the Fabp protein is, in general, a shuttle that transfers fatty acids toward endoplasmic reticulum to provide fatty acids to the cell membrane. The cell membrane is definitely indispensable to the formation of the long and thin radial fibers of the RG cells. Therefore, the Pax6-Fabp7 network is crucial for maintaining radial morphology (Fig. 3.3).

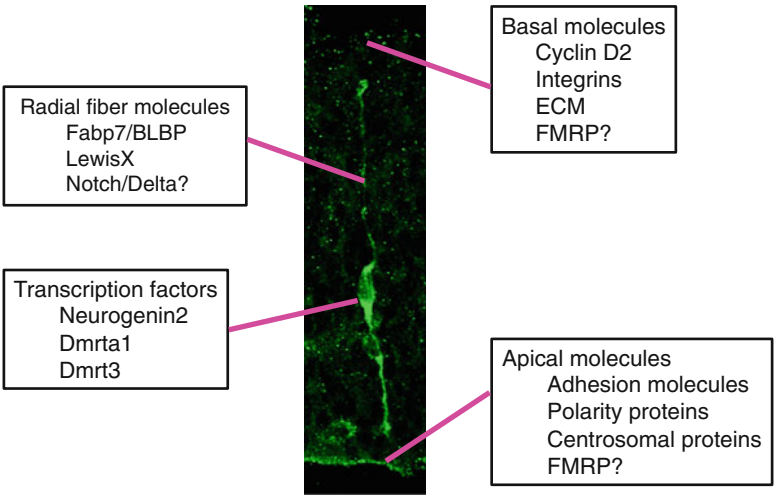

Fig. 3.3 Possible Pax6-downstream molecules categorized according to their localization within the radial glial (RG) cell (illustrated schematically in *magenta*). The expression of RG-specific molecules is likely harmonized by Pax6

ChIP-chip assays have suggested the possibility that Pax6 regulates the expression of the *Fmr1* gene encoding FMRP (Sansom et al. 2009). If so, this may be another interesting scenario. FMRP is an RNA-binding protein and transfers various synaptic molecules over long distances within neurons (Bagni and Greenough 2005; Bassell and Warren 2008; Bhakar et al. 2012). In the RG cells, FMRP protein is localized both in the apical and basal end feet (Saffary and Xie 2011). We have shown that Cyclin D2 mRNA is transported toward the basal end foot via its cis element that is located in 3′-UTR region (Tsunekawa et al. 2012). Thus, we can assume that FMRP might transfer the mRNA of various basal molecules, including Cyclin D2, integrins, and secreted ECM molecules, toward the basal end feet of the RG cells. Considering the accumulation of FMRP at the apical side of RG cells, the same might be true for apical molecules such as centrosomal proteins, polarity molecules, and adhesion molecules. Indeed, we have noticed that localization of one centrosomal protein, ninein, and another apical protein, Fez1 (the function of which is unknown), is impaired in the *rSey*^{2}/*rSey*^{2} rat cortical primordium (Shinohara and Osumi unpublished). Thus, Pax6 may coordinate and maintain the characteristics of RG cells by orchestrating the subcellular distribution of polarized molecules (Fig. 3.3).

3.7 The Role of Pax6 in Brain Evolution: A Hypothesis

Pax6 is a highly conserved molecule throughout evolution. A homolog of Pax6 exists in the sea urchin, which has no eyes or no brain, and the expression of Pax6 is seen in the sensory foot (Czerny and Busslinger 1995; Agca et al. 2011).

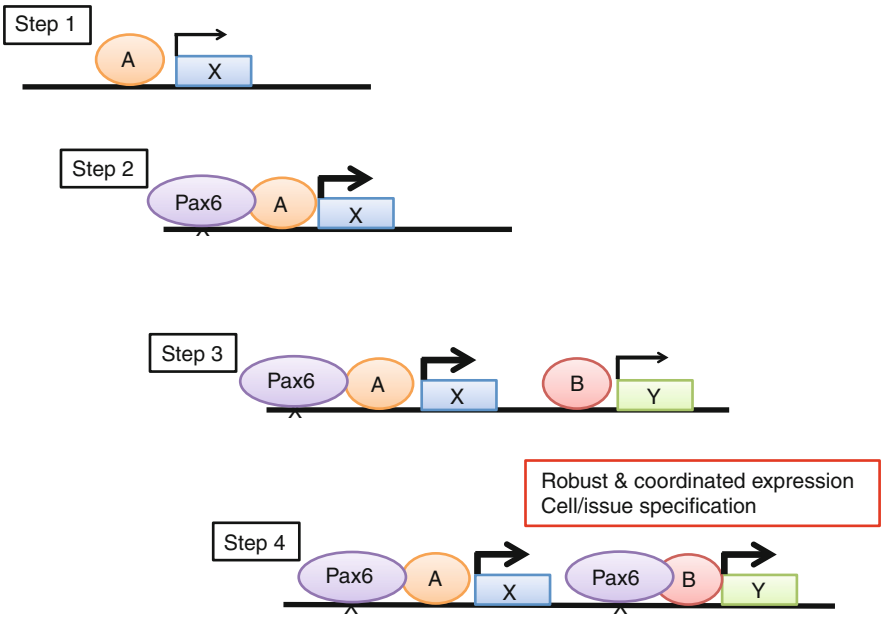

Fig. 3.4 Possible evolutionary scenario detailing how the Pax6 transcription factor could come to coordinately regulate the expression of its target genes X and Y in collaboration with the other transcription factors *A* and *B*. For details, see the text

Although structures are dramatically different, Pax6 is expressed in the various eye primordia of different species (Gehring 2002; Kozmik 2005; Erclik et al. 2009). Within rodent tissues, Pax6 also contributes to the formation of the pineal gland, pituitary organ, and pancreas (Bentley et al. 1999; Kioussi et al. 1999; Pichaud and Desplan 2002; Gosmain et al. 2011). Thus, the role of Pax6 in various tissues and cell types across wide phylogenies is intriguing.

The molecular structure of Pax6 is well conserved, and a function of Pax6 is interchangeable across species; the fly *eyeless*, mouse *Pax6,* and human *PAX6* genes can all induce compound eyes in the imaginal discs of the fly (Halder et al. 1995). However, putative Pax6-binding sites on target genes vary dramatically (Xie et al. 2013) compared to other transcription factor-binding sites such as E-box. Therefore, let us imagine that the acquisition of Pax6-binding sites may contribute to robust expression of target genes.

A putative scenario is the following (Fig. 3.4): In an area forming a certain tissue, a gene X is transcribed by a transcription factor A (step 1). A mutation has occurred near the binding site of X that creates a Pax6-binding site (step 2). Pax6, together with the transcription factor A, binds to induce strong expression of the gene X (step 3). Another gene, Y, is also recruited after a mutation has occurred in its regulatory region (step 4). In this manner, genes X and Y together can place a certain tissue under the control of Pax6 (step 5). We propose that such an evolutionary change has

occurred in the regulatory regions of cortex-specific genes and that Pax6 harmonizes their expression. These cortex-specific genes may involve *Fabp7*, *FucT9*, *Cdh4, Ctnnd2*, and *Fmr1* in the context of NPC maintenance and *Dmrta1* and *Neurog2* in the context of neuronal differentiation (Fig. 3.3).

We should also consider evolutionary changes in the regulation of the expression of Pax6 itself. It would be especially interesting to elucidate the regulatory elements of the *Pax6* gene that govern its expression within oRG. These regulatory elements might possibly be key to the processes by which evolution created primate cortices from non-primate ones.

3.8 Pax6: Possible Involvement in Neurodevelopmental Diseases?

As mentioned earlier, *PAX6* was originally identified within loci deleted in patients with WAGR syndrome (Ton et al. 1991). Considering the enormous contribution of Pax6 to cortical development, it is unsurprising that WAGR patients exhibit mental retardation (Xu et al. 2008). Autism is one of the neurodevelopmental diseases that have heterogeneous symptoms with core and other comorbid features. The three core features of autism are impairments in social interaction, impairments in verbal and nonverbal communication, and stereotyped patterns of behavior. Autistic phenotypes are also seen in patients with WAGR syndrome, and patients with *PAX6* mutations often show aggressive and autistic behaviors in addition to mental retardation and aniridia (see the meta-analyses of Davis et al. (2008)). There is reproducible genetic evidence showing the association of the 11p13 locus with autism (The Autism Genome Project Consortium 2007; (Duvall et al. 2007; Abrahams and Geschwind 2008).

We have previously reported 15 mutations in the *PAX6* gene that were unique to 285 autistic patients and not seen in 2,120 normal or non-autistic subjects (Maekawa et al. 2009). We have also demonstrated autistic phenotypes in *Pax6* mutant rats; these rats exhibit impairments of social interaction, sensorimotor gating, and vocal communication (Umeda et al. 2010). The defects in ultrasonic vocalization seen in the *Pax6* mutant rats are reproducibly observed in *Sey* mouse pups (Kimura and Osumi unpublished). In brain imaging studies, anatomic changes in various brain regions have been reported in autistic patients [see the review of (Amaral et al. 2008)]. These areas are related to the core symptoms. For example, the orbitofrontal cortex is related to social impairment and repetitive behaviors, the amygdala is related to social impairment, the cerebellum and pontine nuclei are related to communication deficits, and the thalamus is related to communication deficits and repetitive behaviors. Interestingly, Pax6/PAX6 is highly expressed in the NPCs or neurons of all of the above-mentioned brain areas. In addition, autistic patients often show comorbid mild impairments in motor skills, and Pax6 is actually involved in motor neuron development (Osumi et al. 1997; Ericson et al. 1997). As discussed above, the molecules downstream of Pax6

include several autistic genes such as FMRP, Foxp2, cadherins, Ctnnd2, and PTEN (Sansom et al. 2009; Stoykova et al. 1997; Coutinho et al. 2011; Rubenstein 2010). Thus, we speculate that many symptoms of autism may be explained by malfunctions of one gene, *PAX6,* which is expressed in various autism-related brain regions and regulates various autism-related target genes.

Mutations in the *Pax6/PAX6* gene occur both in coding and noncoding regions. In the latter case, it may be possible that eye development is normal but brain development is impaired because the expression of PAX6 can be reduced in a tissue-specific manner through the control of differential usage of its enhancers (Kammandel et al. 1999; Dimanlig et al. 2001; Zhang et al. 2003; Kleinjan et al. 2004, 2008; McBride et al. 2011). There are many CpG islands in the mouse *Pax6* gene (Kleinjan et al. 2004), and the CpG islands in the human *PAX6* gene are frequently hypermethylated in astrocytoma and other cancers (Cross et al. 1999; Bender et al. 1999; Markl et al. 2001; Wu et al. 2010). Therefore, the *PAX6* gene may be a good candidate for de novo mutations that might cause neurodevelopmental disorders such as autism.

3.9 Future Perspectives

From an evolutionary perspective, it is interesting that a greater number of genes have come to be under the control of Pax6, which may secure the robust transcription of target genes. However, it should be suspected that such an evolutionarily new innovation to genetic networks would be fragile. Pax6-regulatory programs in brain development and maintenance may be a good example of this fragility that illustrates why molecules downstream of Pax6 are vulnerable in terms of mental diseases. More in-depth analyses involving sequencing of the whole genome and searches for hypermethylated sites in patient brains would comprehensively reveal the roles of PAX6 in neurodevelopmental diseases.

Regarding the identification of the pathogenesis of neurodevelopmental diseases, the contribution of inhibitory interneurons should be interesting. In cortical development, Pax6 defines the fate of neurons generated in the dorsal telencephalon as excitatory projection neurons. However, Pax6 is also expressed in the NPCs of a part of the caudal ganglionic eminence that produces cortical interneurons that later intermingle with projection neurons within the cortex (Tang et al. 2012). Therefore, exploration of the involvement of Pax6 in the development of inhibitory interneurons would be of interest.

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Chapter 4
Regulatory Mechanisms Underlying the Neurogenesis-to-Gliogenesis Switch by Neural Stem Cells

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Abstract During development of the vertebrate central nervous system (CNS), neural stem cells (NSCs) first generate neurons, followed by glia. This sequential production of specific cell types is advantageous for the organism, since glia play pivotal roles in the maintenance and function of neurons and also, under some conditions, in the inhibition of axonal growth. The latter may be related to the conservation of the newly established neuronal circuitry. The temporal regulation of stem cell differentiation is captivating, given that the loss of stem cell plasticity is often part of the standard mammalian aging process. The reduced plasticity of adult stem cells, including NSCs, directly affects the capacity of the metazoan to regenerate lost or damaged neural tissue and seems to have occurred over the course of evolution. Indeed, the injured adult mammalian brain is scarcely capable of regeneration, not only due to the limited number of adult NSCs but also because of their low neurogenic capacity, except for in certain restricted CNS regions. By contrast, some lower vertebrates (e.g., red-spotted newts) show high regenerative capacity in the brain, with the efficient induction of neurogenesis after injury. Therefore, addressing the regulatory mechanisms underlying the neurogenesis-to-gliogenesis switch by NSCs during development is critical to understanding the restricted plasticity of the adult mammalian CNS. Accordingly, this chapter will review the recent progress in the field of NSC biology, especially regarding the temporal regulation of neurogenesis and gliogenesis.

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4.1 Introduction

The concept of central nervous system (CNS) stem cells dates back to the end of the nineteenth century, with the microscopic observation of dividing cells in the developing chick neural tube. Wilhelm His suggested that the proliferating cells in the ventricular zone (VZ) of the chick embryo could be separated into two morphologically distinct groups, one capable of generating neurons and the other capable of generating glia (for a historical perspective, see Jacobson 1991). By contrast, Schaper (1897) proposed that the two VZ cell types previously categorised by His instead represent a single-cell population that can give rise to both neurons and glia. Later, Sauer (1935) convincingly demonstrated that the two morphologically distinct VZ cell types are indeed members of the same population, but at different phases of the cell cycle.

Despite recent technological advances for the analysis of CNS development, the complexity of the VZ continues to preclude elucidation of how neurons and glia originate in vertebrates. Early work with [^{3}H]-thymidine labelling of proliferating cells in the VZ, including multiple birthdating analyses, originally suggested that this region is composed of a single-cell population that sequentially produces neurons and glia (Bayer and Altman 1991). On the other hand, precise investigation of the cell cycle length of VZ cells in the developing mouse telencephalon intimates that the VZ comprises at least two distinct cell populations, with different generation times (Takahashi et al. 1993, 1995). Moreover, the expression of glial fibrillary acid protein (GFAP, a marker of astrocytes) in a subpopulation of proliferating VZ cells termed radial glial cells (RGCs) during the peak period of neurogenesis in the embryonic primate brain supports the hypothesis that neuronal and glial precursor cells coexist during early stages of CNS development (Levitt and Rakic 1980; Levitt et al. 1981, 1983).

Finally, clonal lineage analyses of progenitor cells by live cell imaging in vitro and by viral vector-mediated genetic labelling and tracer injection techniques in vivo suggest that the CNS harbours neuronal, neuroglial and glial progenitors during early development, depending on the brain region in question and the developmental stage (Soula et al. 1993; Qian et al. 1998, 2000; Noctor et al. 2008; see also reviews by Costa et al. 2009 and Kriegstein and Alvarez-Buylla 2009). However, it is still unclear whether these progenitors are derived from a single stem cell population or are born independently in the early neuroepithelium, as there is no reliable marker for the prospective identification of neural stem cells (NSCs). NSCs can only be retrospectively identified in the developing CNS via limited in vitro culture systems that permit assessment of stem cell self-renewal and differentiation (Reynolds and Weiss 1992; Reynolds et al. 1992; Louis et al. 2008; see also review by Conti and Cattaneo 2010).

In contrast to vertebrates, the origins of neurons and glia in the developing *Drosophila melanogaster* (fruit fly) CNS are well clarified. This organism is extremely accessible to both genetic and molecular analyses, and definitive markers are available to distinguish between NSCs and neuronal and glial restricted

progenitors (NRPs and GRPs, respectively). *Drosophila* NSCs delaminate from the ectoderm, whereas GRPs (glioblasts) delaminate from the neuroectoderm and are an exceptional population in the fruit fly (Jones 2001). While lineage relationship patterns (i.e., the occurrence of NSCs, NRPs, and GRPs) are common to *Drosophila* and vertebrates, conclusions from the *Drosophila* studies may not lend themselves to hypotheses regarding the origins of neurons and glia in vertebrates. In particular, many environmental, developmental, and molecular mechanisms of glial differentiation are not evolutionally conserved between fruit flies and vertebrates. For instance, the functions of vertebrate counterparts of mutant *Drosophila* glial cells missing *gcm*, the gene encoding the primary gliogenic transcription factor in *Drosophila*, are controversial. No defects in gliogenesis are observed in mutant mice lacking either of the two mammalian *Gcm* homologues, *Gcm1* or *Gcm2*, whereas forced expression of Gcm1 in the developing mouse and chick CNS promotes astrocyte differentiation and neurogenesis, respectively (Iwasaki et al. 2003; Soustelle et al. 2007; Mao et al. 2012). Confounding the issue, glial phylogenetics supports the repeated appearance and disappearance of glia and the emergence of new glial functions throughout evolution (Hartline 2011).

This chapter will focus on the mechanisms underlying the birth of neurons and glia (neurogenesis and gliogenesis) from NSCs during vertebrate CNS development. Emphasis will be placed on knowledge that is now commonly accepted, as well as on issues that remain to be clarified.

4.2 Stem Cell Development and Progenitor Heterogeneity

According to in vitro studies in mammals, NSCs that can give rise to various types of neurons and glia in spatially and temporally regulated patterns (Temple 2001) may exist in the ventricular neuroaxis throughout the life of vertebrates. NSCs (defined by their ability to self-renew and differentiate into neurons and glia in culture) can be first detected during the period of neural induction (Tropepe et al. 1999). The cells in the neural plate then multiply, which leads to closure of the neural groove to form the neural tube comprising the neuroepithelium, a layer of rapidly proliferating progenitor cells that include neuroepithelial progenitors (NEPs). During this period, the regional identity of NSCs can be determined by their position along the dorsal-ventral and rostral-caudal axes and through their response to assorted inductive signals to generate regionally specific neuronal phenotypes (Altmann and Brivanlou 2001; O'Leary and Nakagawa 2002).

Experimental evidence suggests that NEPs initially divide symmetrically to expand the progenitor population in the VZ. On the other hand, a possible specification for GRPs among NEPs in the forebrain has been suggested by retrospective cell fate analyses (McCarthy et al. 2001; Delaunay et al. 2008). NEPs then divide asymmetrically to initiate the generation of neurons and undergo transformation to RGCs. The RGCs elongate their processes (radial fibres) to the pial surface and express the glutamate/aspartate transporter (GLAST), brain lipid-binding protein

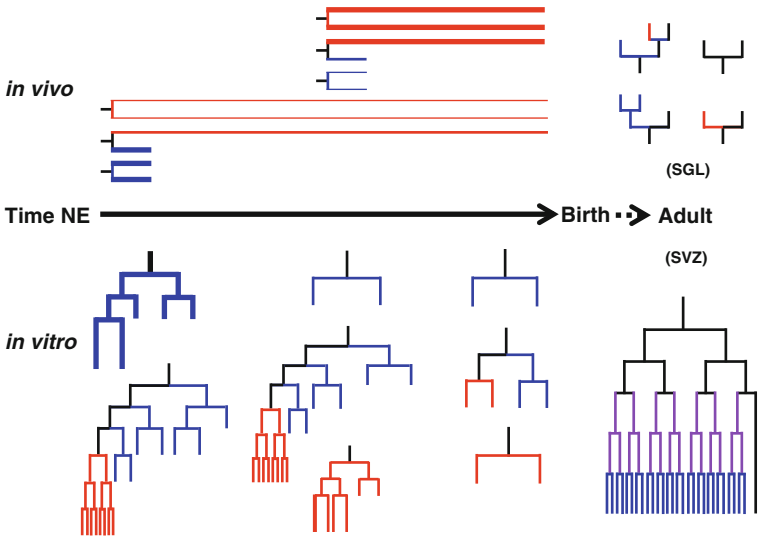

Fig. 4.1 Progenitor heterogeneity in the developing CNS. Results of in vivo clonal lineage analyses and in vitro time-lapse clonal lineage analyses in the developing and adult vertebrate CNS from several research groups are summarised and depicted as lineage trees. *Blue branches* represent neuronal lineages, and *red branches* represent glial lineages. *Purple branches* in the adult SVZ represent transit-amplifying cells that can differentiate into both neuronal and glial lineages. The thickness of the branches indicates the relative frequency of each cell type in vivo and each clone type in vitro for clones born during the same time period. The frequency of GRCs increases during development at the expense of NRCs. The expandability of progenitors in vitro decreases at the late embryonic stage

(BLBP, also known as fatty acid-binding protein 7, FABP7), and GFAP. The latter is only expressed in primate and human RGCs. GLAST, BLBP, and GFAP are also expressed in astrocytes. Although RGCs, like NEPs, divide symmetrically for their expansion and generally behave like multipotent stem cells in vitro, they divide asymmetrically during the peak period of neurogenesis to generate neurons. Neurogenesis from RGCs terminates at the appropriate time point depending on the CNS region. RGCs finally differentiate into astrocytes or ependymal cells or remain as NSCs throughout the life of the organism (Kriegstein and Alvarez-Buylla 2009).

In vivo and in vitro clonal lineage tracing studies in vertebrates suggest that progenitor cells in the VZ are characterised by heterogeneity and developmental changes in specific progenitor populations and differentiation potential (Soula et al. 1993; Qian et al. 1998, 2000; Noctor et al. 2008; see also reviews by Costa et al. 2009 and Kriegstein and Alvarez-Buylla 2009) (Fig. 4.1). Fates of NEPs appear to be mostly neuronal or neuroglial. In particular, progenitors fated to only become neurons represent the largest population of NEPs, while neuroglial progenitors fated to sequentially generate neurons and glia are in the minority. However, retroviral vector-mediated genetic labelling of NEPs in the embryonic day (E) 9.5 mouse forebrain revealed a significant number (18.8 %) of putative glial-specific progenitors, primarily in the

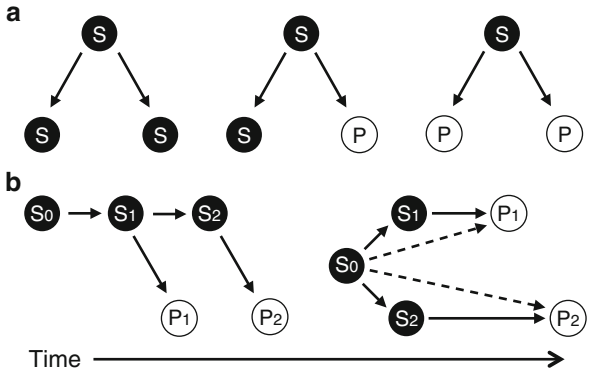

Fig. 4.2 Patterns of stem cell division. (**a**) *Left to right*: symmetric self-renewing division to double stem cells; asymmetric division to self-renew and generate a differentiating progeny cell; and differentiative division to generate two differentiating progeny cells. The fate of each daughter cell may be determined stochastically, by intrinsic programs in ancestor stem cells, and/or by local environmental signals after birth. (**b**) Alternatively, temporally regulated generation of distinct types of progeny cells may be caused by deterministic division of a single stem cell lineage (*left*) or by asymmetric division of a primitive stem cell fated to generate two different types of stem cells or progeny cells that terminally differentiate at varying times (*right*). *S* stem cell, *P* differentiating progeny cell

ventral telencephalon (McCarthy et al. 2001). At mid-gestational stages, GRP-like cells become the major population of progenitor cells in vivo, but they are still scarce in vitro. The GRP-like progenitor population increases in the brain over time, while the proliferative capacity of all types of progenitors and the neurogenic capacity of neuroglial progenitors decrease. Thus, the developmental change in the composition of progenitor subtypes, as retrospectively defined by their differentiation fates and proliferative and differentiation potential in vitro, is the major factor determining the basic pattern of cytogenesis in the developing CNS: the initial generation of a large number of neurons, followed by glia.

The lack of definitive stem cell markers makes it problematic to ascertain how the heterogeneity and differentiation of progenitors in the VZ are controlled in vertebrates. It is difficult to know whether the final fate of each progenitor cell depends on its own original differentiation potential or instead results from intrinsically and/or extrinsically regulated patterns of differentiation of ancestor stem cells. As shown in Fig. 4.2a, basic patterns of stem cell division can be divided into three types, each with its own implications for progenitor differentiation: symmetric self-renewing division to double stem cells, asymmetric division to self-renew and generate a differentiating progeny cell, and differentiative division to generate two differentiating progeny cells. To date, no means are available to determine whether the lineage commitment of mammalian embryonic NSCs occurs stochastically following symmetric self-renewal division, by asymmetric division, or via both processes because stem cells and committed intermediate progenitor cells (IPCs) are mostly indistinguishable in the VZ.

In fact, there may be no clear-cut criteria to differentiate between the stem cell and IPC stages for individual VZ progenitors. Rather, some intrinsic bias may exist in each ancestor stem cell. Candidate biases include fate determinants such as Numb, which inhibits Notch signalling and is localised at the apical border of dividing VZ cells, as well as tripartite motif-containing protein 32 (TRIM32), which ubiquitinates and degrades the transcription factor c-Myc, and also binds argonaute-1 and thereby increases the activity of specific microRNAs such as let-7. TRIM32 is enriched at the basal pole of the cell body of RGCs and appears to be preferentially inherited by differentiating daughter neuronal precursors in the developing mouse cortex (Shen et al. 2002; Schwamborn et al. 2009).

The partitioning defective protein (Par) complex is another fate determinant that is essential for specifying the polarity of neuroblasts and ensuring their asymmetric cell division during CNS development in *Drosophila*. The Par complex is concentrated at the luminal surface of the VZ, particularly in the ventricular end feet of interphase RGCs. This complex specifies the polarity of dividing RGCs to control daughter cell fate specification and differentiation by modulating the signalling activity of Notch, a key regulator of stem cell vs neuronal fate determination (Costa et al. 2008; Bultje et al. 2009). In the developing zebra fish brain, the Par complex promotes Notch signalling by controlling the asymmetric localisation of the E3 ubiquitin ligase Mindbomb. Mindbomb promotes Notch signalling by modulating the endocytosis of Notch ligands and is essential during cell cleavage for the proper neurogenic asymmetric division of VZ progenitors (Dong et al. 2012). Finally, asymmetric inheritance of cyclin D2, located at the tip of the basal processes of basally positioned daughter cells, preferentially results in the acquisition of a self-renewing stem cell phenotype (Tsunekawa et al. 2012). However, no such intrinsic bias for the specification of glial fate has been found.

In contrast to the situation in the VZ, the combinatorial use of several markers permits a certain amount of discrimination between NSCs and IPCs in the subgranular layer (SGL) of the hippocampal dentate gyrus and the subventricular zone (SVZ) of the lateral ventricle, both of which are neurogenic regions in the adult mammalian brain (Ming and Song 2011). The ability to distinguish between progenitor stages in the SGL and SVZ greatly increases accessibility to precise mechanisms of stem cell differentiation. For instance, an initial study of CNS development of *Pten* mutant mice revealed the involvement of Pten in the proliferation of VZ progenitors and the self-renewal of stem cells in vitro (Groszer et al. 2001, 2006). In addition, precise clonal analyses of the fate of stem cells having selective loss of Pten in the SGL indicated that this protein is involved in regulating stem cell quiescence and differentiation (Bonaguidi et al. 2011).

4.3 Stochastic Differentiation

Multipotent stem cells are characterised by two primary patterns of differentiation, stochastic differentiation (Fig. 4.2a) and deterministic differentiation (Fig. 4.2b). A classical view of NSC differentiation in vertebrates is stochastic differentiation

into either neurons or glial cells, irrespective of whether the NSC undergoes cell division. Identification of numerous environmental factors that direct or bias the differentiation fate of stem cells supports the stochastic differentiation model.

One such example is the directed differentiation of cultured neural progenitors (NPs) and stem cells toward GFAP-expressing astrocytes at the expense of neurons and oligodendrocytes by exposure to bone morphogenetic protein (BMP) 2/4 and interleukin (IL)-6 family cytokines (e.g., ciliary neurotrophic factor (CNTF), leukaemia inhibitory factor (LIF) and cardiotrophin-1 (CT-1)) (Miller and Gauthier 2007). BMPs bind to a tetrameric complex of type I and type II serine/threonine kinase receptors that phosphorylate and activate Smad (mothers against decapentaplegic homologue) transcription factors (Mueller and Nickel 2012), while IL-6 family cytokines bind to receptors that share a common co-receptor (glycoprotein 130, gp130), thereby triggering activation of the Janus kinase (JAK) family of non-receptor tyrosine kinases. The JAK family in turn activates members of the signal transducer and activator of transcription (STAT) family of transcription factors, STAT1 and STAT3 (Heinrich et al. 2003).

Nakashima et al. (1999) proposed that BMP signals and gp130-mediated cytokine signals synergistically facilitate astrocyte differentiation via the cooperative activation of Smad1 and STAT3. Activated Smad1 and STAT3 then move into the nucleus, recruiting and non-competitively binding to p300, a member of the p300/CREB-binding protein (CBP) coactivator family with histone acetylase activity. The net result is the activation of astrocytic genes such as *Gfap*. Consistent with these results, Sun et al. (2001) showed that the basic helix-loop-helix (bHLH) transcription factor neurogenin 1 (Neurog1) inhibits astrocyte differentiation by inhibiting the activation of STATs and sequestering the CBP-Smad1 transcription complex away from astrocyte differentiation genes. At the same time, Neurog1 directly activates neuronal differentiation genes such as NeuroD1, an additional neurogenic bHLH transcription factor, likely through association with the CBP-Smad1 complex.

Pituitary adenylate cyclase-activating polypeptide (PACAP) also promotes the differentiation of NPs into astrocytes via activation of a cAMP-dependent pathway. However, PACAP apparently does not affect the differentiation of NPs into neuronal or oligodendroglial lineage cells, unlike BMP and gp130-mediated cytokine signalling (Vallejo 2009). Nonetheless, it is unclear whether BMP and gp130 signals induce the specification of astrocytes from NSCs, as opposed to exclusively promoting the maturation of astrocytes from committed precursors. In this regard, BMP/gp130 studies focused solely on the expression of GFAP, which is expressed not only in mature astrocytes but also in postnatal NPs (Imura et al. 2003; Morshead et al. 2003) and RGCs in primates (Levitt and Rakic 1980; Levitt et al. 1981, 1983). Indeed, conditional deletion of gp130 in late RGCs does not influence the number or distribution of astrocytes in adult mice (Drögemüller et al. 2008). On the other hand, Kohyama et al. (2010) showed that BMP2 stimulation of REST (induction of RE1 silencer of transcription)/NRSF (neuron-restrictive silencer factor) facilitates astrocyte differentiation from NPs derived from the mouse embryonic cortex, as assessed by the expression of glutamine synthase and S100β, while suppressing neuronal differentiation.

The generation of oligodendrocyte progenitors (OLPs) also supports, at least in part, the stochastic differentiation model. Although OLPs are initially derived from

several specific regions of the VZ in the developing CNS (Kessaris et al. 2008), it is unknown whether these cells can be generated stochastically from stem cells, deterministically from GRPs, or both. The mechanism of OLP specification is still enigmatic. In vivo, sonic hedgehog (Shh) is initially secreted from the ventral portions of the neural tube (including the floor plate in the spinal cord and notochord), where it is required for the dorsal-ventral patterning of the progenitor domain, including sites of OLP origin. Hence, Shh is apparently a major inductive signal for the first acquisition of OLPs in the VZ, although the detailed molecular mechanisms by which Shh actions are transduced remain to be determined (Kessaris et al. 2008).

Nonetheless, analysis of mice lacking Shh or Smoothened (an essential component of all hedgehog (Hh) signalling pathways) demonstrated the existence of a Shh-independent pathway for OLP specification (Chandran et al. 2003; Cai et al. 2005). Moreover, in vitro cultures of NPs from the early embryonic cortex and dorsal spinal cord generated oligodendrocytes, although no OLPs as defined by the expression of Olig1/2 (bHLH transcription factors essential for OLP specification) were detected. In addition, fibroblast growth factor (FGF)-2 but not Hh signalling was required for oligodendrocyte differentiation in these cultures (Chandran et al. 2003; Gabay et al. 2003; Kessaris et al. 2004), suggesting that FGF-2 might also be an inducer of OLPs. However, these results may simply reflect temporal changes in the differentiation potential of NSCs during in vitro culture, as will be discussed later.

In contrast to Shh and FGF-2, BMPs are negative regulators of OLP specification. Exposure of cultured NPs derived from the embryonic forebrain or spinal cord to BMPs inhibited oligodendrocyte differentiation, even in the presence of Shh or FGF-2, whereas exposure to Noggin (a BMP antagonist) increased oligodendrocyte differentiation both in vitro and in vivo (Gross et al. 1996; Mekki-Dauriac et al. 2002; Yung et al. 2002; Vallstedt et al. 2005). In all of these studies, the number of astrocytes was always inversely correlated with the number of oligodendrocytes.

Unlike Shh signalling, the mechanisms underlying the actions of BMP signalling on glial lineage specification have been convincingly elucidated. Samanta and Kessler (2004) showed that BMP4 induces the expression of *Id* (inhibitors of differentiation) family genes to increase the expression levels of Id proteins in cultured NPs. Id proteins are bHLH proteins related to Olig1/2 that, when overexpressed in vivo or in vitro, inhibit neuronal differentiation while promoting cell proliferation and astrogenesis (Cai et al. 2000; Jung et al. 2010). Samanta and Kessler (2004) also showed that Id4 and Id2 inhibit oligodendrocyte differentiation from NPs by complexing with Olig1/2 and their potential cofactors E12 and E47 and that Olig proteins co-localised with Id2 and Id4 are retained in the cytoplasm of differentiating NPs in the presence of BMP4. Thus, Id proteins apparently mediate BMP signalling to inhibit OLP specification by blocking the transcriptional actions of Olig proteins. Taken together, the results from these studies suggest that BMP signalling regulates the stochastic differentiation of NSCs/NPs and/or GRPs into astrocytes rather than into oligodendrocytes.

Retinoic acid (RA) plays pleiotropic roles in the differentiation of NPs. Exposure of cultured NPs to RA promotes both neurogenesis and astrocyte differentiation

(Wohl and Weiss 1998; Asano et al. 2009). In the developing mouse cortex, RA secreted from the meninges enveloping the cortex is probably required for the switch from symmetric to asymmetric neurogenic division of RGCs (Siegenthaler et al. 2009). Analysis of mice lacking a critical RA synthesising enzyme, retinaldehyde dehydrogenase 2, revealed that RA is involved in the maintenance of NSCs by sustaining high levels of FGF and Notch signalling, as well as in the promotion of neuronal differentiation in the developing spinal cord (Paschaki et al. 2012).

4.4 Deterministic Differentiation

Time-lapse in vitro fate analyses of individual progenitors isolated from the rodent embryonic cortex in the early neurogenic phase suggest the existence of deterministic differentiation of NSCs (Qian et al. 1998, 2000). Deterministic differentiation is defined as the neurogenic-to-gliogenic switch of cytogenesis by single stem cells. Timed generation of early- to late-born neurons by single progenitors has also been demonstrated (Shen et al. 2006). This temporal regulation of cytogenesis can be observed in cultured NEPs derived from both mammalian embryos and embryonic stem cells (ESCs) (Conti and Cattaneo 2010). Thus, the neurogenesis-to-gliogenesis transition in the developing CNS seems to largely depend on the temporal regulation of cytogenesis by NSCs. However, it is still possible that some GRPs, if not all, are specified stochastically early in the lifespan of NEPs and initiate expansion and differentiation afterwards (Delaunay et al. 2008). Indeed, an example of a time lag between specification and differentiation of NPs has been shown by Franco et al. (2012), who reported that a subset of cortical progenitors is specified to generate late-born, upper-layer neurons early on, but actually produces these upper-layer neurons predominantly later than their early-born, lower-layer counterparts.

Temporal changes in the responsiveness of NPs to environmental regulatory factors support the existence of intrinsic timer mechanism(s) for the neurogenic-to-gliogenic switch during NSC development. For instance, exposure of NPs including NEPs in the early neurogenic phase to BMPs and IL-6 family cytokines does not induce astrocyte differentiation (Mehler et al. 2000; Molne et al. 2000; Takizawa et al. 2001; He et al. 2005; Naka et al. 2008). Instead, BMPs facilitate neurogenesis from NPs at this developmental phase (Li et al. 1998; Yung et al. 2002). Conversely, activation of canonical Wnt signalling, which directs neuronal differentiation (Hirabayashi et al. 2004; Israsena et al. 2004), does not enhance neurogenesis from NPs at the late gliogenic phase (Hirabayashi et al. 2009).

Epigenetic regulation of gliogenic- and neurogenic-specific genes seems to be involved in the neurogenic-to-gliogenic switch. For example, the epigenetic status of the STAT3-binding site in the *Gfap* promoter is responsible for the JAK-STAT pathway-dependent expression of GFAP. Takizawa et al. (2001) showed that the CpG dinucleotide within the STAT3-binding site in the murine promoter is highly methylated in early neurogenic NPs but is gradually demethylated during development. Furthermore, a genome-wide DNA methylation profiling of mouse cortical

progenitor cells between E11.5 and E14.5 revealed that many astrocytic genes are demethylated in late-stage NPs.

Song and Ghosh (2004) demonstrated a time-dependent change in the methylation status of histone H3 at the STAT-binding site (i.e., promotion of Lys4 methylation and suppression of Lys9 methylation), with a change in the responsiveness of cultured cortical progenitors derived from E18 rat embryos to CNTF regarding upregulation of GFAP in the presence of FGF-2. Furthermore, Hirabayashi et al. (2009) showed that Polycomb group proteins epigenetically suppress the *Neurog1* locus and restrict responsiveness to neurogenic Wnt signals in cortical NPs during the gliogenic phase. One problem with these studies is, again, a lack of reliable stem cell markers, meaning the causal relationship between changes in the epigenetic status of progenitors and lineage commitments is unclear. For example, the conditional deletion of the maintenance DNA methyltransferase I (*Dnmt1*) gene in mouse NPs results in DNA hypomethylation of the *Gfap* and *Stat1* promoters, as well as precocious astroglial differentiation, as assessed by GFAP and S100β expression. However, the deletion of this gene does not consistently induce precocious differentiation of NPs into astrocytes during the early neurogenic period (Fan et al. 2005). Accordingly, only the maturation of astroglial lineages might be enhanced by the absence of *Dnmt1*.

The responsiveness of NSCs to Notch signalling, one of the key factors controlling cell fate decisions in metazoans, also changes during development. In canonical Notch signalling, binding of ligands such as Delta and Jagged to Notch receptors at the cell surface leads to nuclear translocation of the Notch intercellular domain (NICD) after proteolytic cleavage of Notch. NICD subsequently associates with the coactivator Mastermind (Mam) and the DNA-binding protein RBPjk/CSL to form a transcriptional complex to activate Notch target genes, such as the *hairy and enhancer of split* (*Hes*) family genes of transcription factors in *Drosophila* (Guruharsha et al. 2012). Forced expression of constitutively active NICD in NEPs promotes the maintenance of NPs and the inhibition of neurogenesis early in development, whereas deletion or inhibition of components of the Notch signalling pathway consistently results in the depletion of NPs and premature neurogenesis (reviewed in Yoon and Gaiano 2005). By contrast, introduction of NICD at later stages into NPs, including adult hippocampus-derived multipotent progenitors, promotes astrogliogenesis (Chambers et al. 2001; Tanigaki et al. 2001; Grandbarbe et al. 2003). Similar results have been obtained in gain- and loss-of-function studies of *Hes* family genes (reviewed in Kageyama et al. 2008).

Moreover, the Notch pathway effector RBPjk/CSL directly binds to and modulates the activity of the *Gfap* promoter in cooperation with the JAK-STAT pathway (Ge et al. 2002). If the JAK-STAT pathway is not activated, RBPjk/CSL associates with a repressive transcriptional co-repressor, nuclear receptor co-repressor (N-CoR), instead of NICD and Mam, and astrogliogenesis does not occur. Stimulation of the CNTF/JAK-STAT pathway in wild-type embryonic cortical NPs leads to the translocation of N-CoR to the cytoplasm. On the other hand, embryonic cortical progenitors derived from *N-CoR*-null mutant mice display impaired self-renewal and spontaneous premature differentiation into GFAP-expressing astroglia-like cells (Hermanson et al. 2002).

However, NPs derived from *RBPjk/CSL*-null mutant mouse embryos can differentiate into GFAP + astrocytes at a normal rate, but the differentiation is delayed relative to that of wild-type cells (Ge et al. 2002).

The function of Notch signalling in oligodendrogliogenesis is controversial. Overexpression of NICD or Hes1, but not Hes5, in GRPs derived from the embryonic rat spinal cord promotes an astrocyte fate at the expense of an oligodendrocyte fate (Wu et al. 2003). By contrast, Notch signalling appears to be essential for the development of the oligodendrocyte lineage during zebra fish CNS development (Kim et al. 2008). Taken together, one can conclude that Notch signalling is required for the maintenance of NSCs and the maturation of astrocytes, but may not be essential for the commitment of NSCs to an astrocyte lineage.

4.5 Acquisition of Gliogenic Competence

To elucidate the regulatory mechanisms underlying the deterministic differentiation of NSCs that yields the neurogenesis-to-gliogenesis switch, it is critical to understand how stem cells acquire competence to respond to extrinsic neurogenic and gliogenic signals. Several transcription factors are reportedly involved in the acquisition of gliogenic competence by NSCs, as discussed below.

The nuclear factor I (NFI) family of transcription factors is composed of four family members in vertebrates, NFIA, NFIB, NFIC and NFIX. NFI factors bind to the promoters of many genes and regulate the expression of certain radial glia and astrocyte markers (e.g., BLBP and GFAP) (Mason et al. 2008). In particular, NFIA and NFIB are involved in the initiation of gliogenesis and the differentiation of astrocytes. *Nfia*- and *Nfib*-deficient mice fail to form midline glial populations located dorsally and ventrally to the corpus callosum. The number of GFAP + cells in the developing spinal cords of these mutant mice is reduced (Mason et al. 2008). Overexpression of *Nfia* or *Nfib* in the developing chick spinal cord during the neurogenic phase causes precocious expression of GLAST in the VZ and subsequent precocious migration of GLAST-positive cells from the VZ, while knockdown of *Nfia* causes a loss of markers for progenitors with gliogenic potential (i.e., GLAST, FGF receptor 3 (FGFR3), and Olig2) in the VZ (Deneen et al. 2006). Moreover, NFIA is required for the maintenance of NPs to provide GRPs at later gliogenic stages and functions in this context by inducing the expression of Hes5 in the developing chick spinal cord (Deneen et al. 2006).

Interestingly, Notch signalling induces the expression of NFIA in mouse cortical NPs at mid-gestation, probably through the direct binding of the RBPjk/CSL complex to the *Nfia* promoter, resulting in the demethylation of the *Gfap* promoter (Namihira et al. 2009). Moreover, a NFI-binding site in the *Gfap* promoter is essential for its full activation in response to PACAP and CNTF (Cebolla and Vallejo 2006). Thus, NFIs apparently play a major role in the acquisition of gliogenic competence by NSCs such that they can respond to Notch, PACAP and gp130-mediated signalling.

Sox9 ((sex-determining region Y)-box 9), a member of the SOX family of high mobility group (HMG) transcription factors, is also essential for the acquisition of gliogenic competence upstream of NFIA. *Sox9*-deficient mice exhibit a prolonged period of motoneurogenesis in the developing spinal cord, coupled with a delay in the onset of oligodendrogliogenesis (Stolt et al. 2003). Conditional gain-of-function and loss-of-function studies in chick and mouse revealed that Sox9 is required for the initiation and maintenance of multipotent NP populations, as well as gliogenesis in the embryonic and adult CNS (Cheng et al. 2009; Scott et al. 2010). Shh signalling probably controls Sox9 expression (Scott et al. 2010). In addition, Kang et al. (2012) provided evidence that Sox9 induces NFIA to initiate gliogenesis and then cooperatively controls the induction of adenomatosis polyposis coli downregulated 1 and myotonic muscular dystrophy type 2, which promote astroglial precursor migration and energy metabolism, respectively.

Naka et al. (2008) showed that the orphan nuclear receptors Coup-TFI/II (chicken ovalbumin upstream promoter transcription factors I and II) also play crucial roles in the ability of NSCs to acquire gliogenic competence. Coup-TFI/II expression is transiently upregulated in NPs during the early neurogenic period and markedly decreases before the onset of gliogenesis in the developing mouse CNS. Coup-TFI/II expression is similarly downregulated in NPs derived from cultured ESCs, which normally exhibit a neurogenesis-to-gliogenesis switch when serially passaged over an extended period of time. Furthermore, double knockdown of *Coup-tfi/ii* results in prolonged neurogenesis at the expense of gliogenesis in the developing mouse forebrain, as well as in ESC-derived NPs. *Coup-tfi/ii* double knockdown also results in prolonged epigenetic silencing of the *Gfap* promoter in ESC-derived NPs and their loss of responsiveness to BMP2 and LIF, which otherwise promote astrocyte differentiation. However, because *Coup-tfi/ii* double knockdown does not induce significant changes in the expression levels of Sox9, NFIA or NFIB, Coup-TFI/II may act in parallel with these factors in wild-type NSCs to regulate the acquisition of gliogenic competence. In support of this hypothesis, the overexpression of Coup-TFI/II alone in the early neurogenic period does not induce precocious gliogenesis.

Nagao et al. (2008) proposed an association between the self-renewal capacity of NSCs and the neurogenesis-to-gliogenesis switch mediated by the Myc family of transcription factors and the $p19^{ARF}$-p53 pathway. Loss-of-function and gain-of-function studies in rat and mouse suggested that the opposing actions of Myc and $p19^{ARF}$ coordinate the extent of self-renewal and the timing of the production of neurons and glia during CNS development. At early neurogenic stages, a Myc-dominant status (high expression of Myc and low expression of $p19^{ARF}$) links a high self-renewal capacity in NPs with a high neurogenic propensity. A time-dependent increase in $p19^{ARF}$ expression attenuates self-renewal and neurogenesis, while facilitating gliogenesis via the actions of p53. The upregulation of $p19^{ARF}$ also occurs in cultured NPs following multiple passages in the presence of high concentrations of epidermal growth factor (EGF) and FGF-2. This model is well adapted to the screw model of stem cell self-renewal and differentiation proposed by Loeffler and Potten (1997) (Fig. 4.3a), as discussed below.

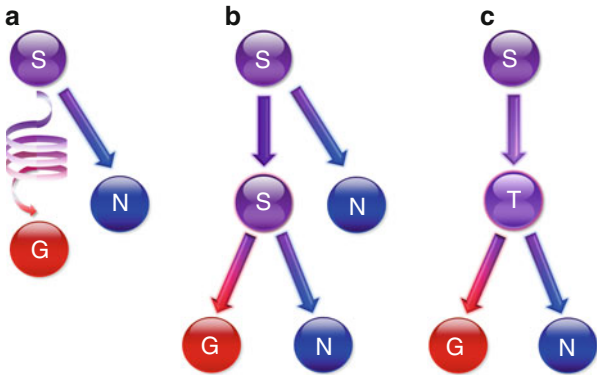

Fig. 4.3 Possible patterns of temporal regulation of neurogenesis and gliogenesis by stem cells. (**a**) The screw model of NSC differentiation into glia. A neurogenic stem cell lineage "spirals down" to a glial lineage over time. (**b**) A neurogenic stem cell lineage acquires gliogenic competence to generate glia stochastically and/or in response to environmental signals. The screw model can be adapted to the glial differentiation of the original neurogenic stem cell lineage. (**c**) A stem cell divides asymmetrically to generate a transit-amplifying progenitor called a type C cell, which can self-renew to some extent and differentiate into neurons or glia stochastically and/or in response to environmental signals in the adult SVZ. The differentiation of type C cells into glia may also possibly result from a spiral down from the stem cell-like state. *S* stem cell, *N* neuron. *G* glia, *T* transit-amplifying progenitor

In the screw model, self-renewing stem cells gradually "spiral down" to a differentiated state like corkscrew through a process that includes a transit-amplifying progenitor cell population that retains some self-maintenance ability. Myc may block the spiral down of stem cells into the glial lineage, whereas the $p19^{ARF}$-p53 pathway promotes it. However, this is probably not the case for adult NSCs, which remain highly neurogenic while also giving rise to glia (Menn et al. 2006; Bonaguidi et al. 2011). Interestingly, a subpopulation of highly neurogenic EGF receptor (EGFR) + adult SVZ stem cells and their type C cell progeny (Fig. 4.1) behave as gliogenic multipotent NPs following exposure to EGF or FGF-2 in vitro (Gritti et al. 1996; Doetsch et al. 1999, 2002), and perhaps in vivo as well. On the other hand, quiescent SVZ stem cells, unlike activated SVZ stem cells, have no detectable expression of EGFR (Doetsch et al. 2002), which is acquired in most VZ cells (including NPs) at mid-gestation (Burrows et al. 1997; Represa et al. 2001). Adult SVZ stem cells may thus retain a juvenile neurogenic state throughout life, but not to the same extent as early NEPs, which are highly neurogenic even in the presence of elevated growth factor levels (Conti and Cattaneo 2010).

As noted above, activated adult EGFR + SVZ stem cells are likely to become multipotent transit-amplifying, NP-like C cells (Gonzalez-Perez and Alvarez-Buylla 2011) (Fig. 4.3c). EGF signalling facilitates gliogenesis by embryonic NPs, as well as by adult NPs (Burrows et al. 1997; Gonzalez-Perez et al. 2009). It is unclear whether EGFR + stem cells in adult neurogenic regions such as the SVZ are direct descendants of highly neurogenic NEPs that acquire gliogenic competence

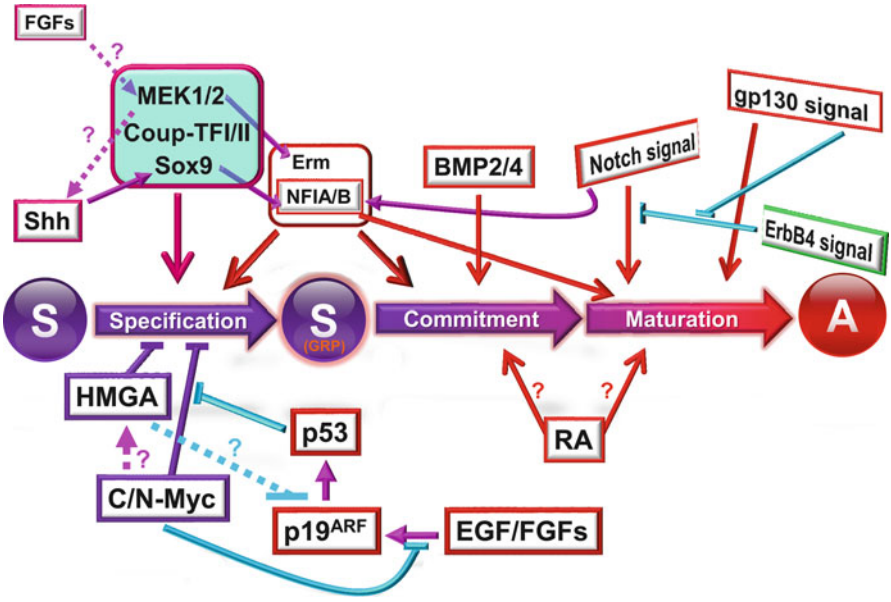

Fig. 4.4 A hypothetical model for the mechanism of temporal specification and differentiation of NSCs into astrocytes. Many intrinsic and extrinsic factors are involved in the differentiation process from neurogenic stem cells toward astrocytes. Early NSCs (*S*) that initially differentiate exclusively into neurons are specified to become GRPs or to acquire gliogenic competence in response to astrogliogenic signals such as BMPs, Notch and/or gp130. *Arrows* and *T-bars* show stimulation and inhibition by the regulators, respectively. *A* astrocytes

with the maturation of the organism or originate independently in the neuroepithelium early on in development.

Candidate effectors of Myc function in the neurogenesis-to-gliogenesis switch may include HMGA proteins (Fig. 4.4). The expression levels of HMGA1 and HMGA2 are elevated in the early neurogenic VZ and decrease with age (Nishino et al. 2008; Sanosaka et al. 2008; Kishi et al. 2012). Furthermore, these proteins are regulated by Myc in non-neural cell types, and loss- and gain-of-function studies of HMGA proteins exhibit similar phenotypes to that of Myc (Nagao et al. 2008; Kishi et al. 2012). HMGAs also seem to regulate the global chromatin state of developing NPs, such that the chromatin gradually becomes more condensed as NPs lose their neurogenic capacity (Kishi et al. 2012). On the other hand, overexpression of HMGA2 does not alter the time-dependent change in the DNA methylation status of the *Gfap* promoter (Sanosaka et al. 2008). However, HMGA2 represses the transcription of the *Ink4* locus encoding $p19^{arf}$ and $p16^{ink4a}$ via the suppression of JunB transcription in adult mouse SVZ NPs (Nishino et al. 2008), increasing their capacity for self-renewal, but not in early-stage cortical NPs (Kishi et al. 2012). These observations again suggest that the nature of adult SVZ stem cells is quite different from that of the NSCs that eventually differentiate into glia during development.

Other than transcriptional regulators, intracellular signalling systems may regulate gliogenic competence. Conditional deletion in NPs of both *Mek1* and *Mek2*, which encode MAPK (mitogen-activated protein kinase)/ERK (extracellular signal-regulated kinase) kinases (MEK) 1 and 2, respectively, leads to attenuated gliogenesis and prolonged neurogenesis, whereas forced expression of constitutively active MEK1 (caMEK1) robustly increases the number of astrocytes at the expense of neurons in the mouse developing cortex (Li et al. 2012). In addition, *Mek1/2*-deleted NPs cannot respond to the astrogliogenic signal CNTF. In these NPs, gp130 expression and phosphorylated STAT3 levels are profoundly reduced. Moreover, loss-of-function mutations of the gene that encodes neurofibromin 1 (a RAS GTPase that converts the active guanosine triphosphate (GTP)-bound form of RAS proteins to the inactive guanosine diphosphate (GDP)-bound form) lead to hyperactivation of the RAF/MEK/ERK pathway (Cichowski and Jacks 2001), as well as NP fate specification defects that are quite similar to those observed in caMEK1-expressing mice (Hegedus et al. 2007; Wang et al. 2012). Thus, the MEK/ERK signalling pathway may play a major role in the acquisition of gliogenic competence in NSCs during development (Fig. 4.4). In this context, Li et al. (2012) demonstrated that Erm, a member of the Ets family of transcription factors, is a downstream effector of the MEK/ERK pathway. However, the upstream effectors for MEK activation remain to be determined.

Potential upstream effectors include FGF and Shh (Fig. 4.4). The Shh and FGF/MAPK signalling pathways cooperatively induce cortical NPs to express Olig2, which is required for oligodendrogliogenesis, as well as astrogliogenesis and OLP generation (Kessaris et al. 2004). Shh signalling is additionally required for the induction of Sox9, as mentioned above (Scott et al. 2010), and basal levels of MEK1/2 and/or ERK activity are probably required for the activation of Shh signalling (Kessaris et al. 2004). It is noteworthy that FGF signalling through ERK synergises with Shh to promote the activity of the GLI1 transcription factor (a mediator of Hh signalling that acts through the MEK1-responsive GLI NH2-terminal domain in NIH3T3 cells (Riobo et al. 2006)), providing additional evidence for a possible functional synergy between Shh and FGF signalling via MEK/ERK to promote the neurogenesis-to-gliogenesis switch. Moreover, Shh signalling may synergise with EGF signalling in the developing cortex through its regulation of EGFR expression to support the proliferation of NPs, and possibly gliogenesis as well (Palma and Ruiz i Altaba 2004).

Contrary to the above discussion, several lines of evidence indicate that the MEK/ERK pathway also promotes neurogenesis (Miller and Gauthier 2007). Differences in experimental approaches and/or cellular targets for the functional analyses may be responsible for this discrepancy. For instance, the reduction in neurogenesis from cortical progenitors by the forced expression of dominant-negative MEK, as shown by Menard et al. (2002), might be caused by platelet-derived growth factor (PDGF)-mediated suppression of NRP proliferation (Erlandsson et al. 2001). Moreover, ERK5 was initially shown to direct cortical progenitors toward a neuronal fate but was later found to potentiate the transcriptional activity of Neurog1 (Liu et al. 2006; Cundiff et al. 2009). Neurog1 is exclusively expressed in NRPs (Kawaguchi et al. 2008;

Namihira et al. 2009) and is induced by Wnt-β-catenin signalling to facilitate the neuronal differentiation of NRPs (Hirabayashi et al. 2004; Munji et al. 2011). Thus, the MEK/ERK pathway may facilitate the proliferation and differentiation of NRPs and/or neuronally biased progenitors, as well as gliogenesis from NSCs.

4.6 Timing of Differentiation

Normally, the timing of astrocyte differentiation, as defined by the expression of known astrocyte-specific markers in vivo, occurs later than the timing of acquisition of gliogenic competence by stem cells during the neurogenic period (Takizawa et al. 2001). Unlike the generation of Olig1/2+ OLPs, the acquisition of gliogenic competence can only be assessed in vitro due to the lack of markers that distinguish multipotent NPs from astrocyte precursors. The temporal regulation of terminal astrocyte differentiation in vivo is most likely mediated by extrinsic signals. In the developing mouse cortex, CT-1 is secreted by newly born neurons; thereafter, its local concentration gradually increases during development (Barnabe´-Heider et al. 2005). The early postnatal cortex of *ct-1*-deficient mice relative to wild-type mice exhibits a 50–70 % reduction in the expression of GFAP and CD44, another marker of astrocytes and GRPs (Barnabe´-Heider et al. 2005). Thus, maximal activation of the JAK-STAT pathway to induce the expression of astrocyte markers may occur after the end of neurogenesis. Similarly, the activation of Notch signalling within radial glial may increase along with an increase in the number of neurons that express high levels of Notch ligands, such as Delta (Namihira et al. 2009). Moreover, Delta expression in neuronal lineage cells is positively regulated by the JAK-STAT pathway (Yoshimatsu et al. 2006). Accordingly, a non-cell-autonomous positive feedback loop may facilitate cooperation of Notch signalling and the JAK-STAT pathways to induce astrocytic genes.

Conversely, neuregulin-1 can suppress astrogliogenesis via binding to its ErbB2 and ErbB4 receptors during the neurogenic period in the developing mouse cortex (Miller and Gauthier 2007). Ligand activation results in the cleavage of the ErbB4 receptor and the release of its intracellular receptor domain, which then forms a complex with the adaptor proteins transforming growth factor-β-activated kinase (TAK)-binding protein 2 (TAB2) and N-CoR (Sardi et al. 2006). This complex translocates to the nucleus to repress the transcription of *Gfap* and *S100β*. Loss of function of ErbB2 or ErbB4 results in the premature expression of GFAP and S100β after mid-gestation (Schmid et al. 2003; Sardi et al. 2006). Hence, ErbB2/ErbB4 activation in RGCs during neurogenic periods may lead to the formation of an ErbB4/TAB2/N-CoR/RBPjk/CSL complex that represses the expression of gliogenic genes. By contrast, increased levels of astrogliogenic cytokines (e.g., CT-1) may facilitate JAK-STAT signalling during gliogenesis, leading to the translocation of N-CoR to the cytoplasm and the derepression of RBPjk/CSL to overcome the anti-astrogliogenic actions of neuregulin-1. Therefore, the timing of differentiation and/or maturation of astrocytes in vivo may be determined by a balance between the JAK-STAT pathway and ErbB4-mediated signalling.

4.7 Concluding Remarks

Numerous studies have attempted to determine how neurons and glia are generated by stem cells during vertebrate development. The absence of definitive stem cell markers has undoubtedly resulted in divergent interpretations of experimental results. In particular, the gliogenic nature of stem cells, as exemplified by the expression of glial markers from the late embryonic stage to the adult phase, is poorly understood. For instance, the role of gp130-mediated signalling in the regulation of stem cell differentiation remains controversial (Deverman and Patterson 2009). The activation of gp130 promotes the maintenance of NSCs but still facilitates the induction of astrocytic genes and the differentiation of NPs into astrocytes. However, gp130-mediated signalling induces GFAP expression in NPs but not their irreversible differentiation into astrocytes, whereas BMP signalling instructs GFAP + late-stage NPs to terminally differentiate into mature astrocytes by forcing cell cycle exit (Bonaguidi et al. 2005). Intriguingly, OLPs can dedifferentiate and revert to multipotent stem-like cells, depending on the culture conditions, engendering the necessity for careful interpretation of results achieved from experiments using cultured NPs (Kondo and Raff 2000). Unfortunately, in vitro self-renewal and differentiation assays are currently the only available methods to elucidate the developmental potential and fate specifications of single progenitors. Thus, it is uncertain how and when irreversible differentiation of NPs into glia occurs, although it is quite difficult to force dedifferentiation of parenchymal astrocytes in mammals (Imura et al. 2006).

The acquisition of gliogenic competence is also open to argument. Stem cells may not really acquire gliogenic competence; rather, they may undergo a "corkscrew"-like, time-dependent process to differentiate into glia, particularly in CNS regions that are not characterised by adult neurogenesis (see the screw model of NSC differentiation, Fig. 4.3a). Time-lapse fate analyses of single cortical progenitors support this hypothesis (Qian et al. 1998, 2000; Costa et al. 2009) (Fig. 4.1). Retrospectively identified multipotent clones always show a deterministic sequential differentiation pattern in vitro; that is, neurons are generated first, followed by glia. Moreover, the number of GRPs increases as development progresses. Therefore, the capacity of mammalian NPs to respond to gliogenic signals at mid-gestation may largely reflect the appearance of non-terminally differentiated GRPs, although these GRPs might retain the plasticity to revert to multipotent progenitors under certain conditions (Kondo and Raff 2000). In this case, dormant stem and/or progenitor cells in non-neurogenic regions after birth, including the GFAP + NPs defined by in vitro culture, might possibly represent a minor population that have exited the neurogenic stage but are still somewhat plastic. Indeed, the self-renewal capacity of NPs derived from the rodent embryonic forebrain gradually decreases with age (Nagao et al. 2008).

The capacity of NSCs to differentiate into GRPs may be regulated by both intrinsic and extrinsic factors, such as $p19^{ARF}$ and EGF signals, respectively. Alternatively, many stem cells, if not all, may acquire competence to differentiate into glia stochastically and/or in response to environmental factors during development (Fig. 4.3b). However, a definitive demonstration of gliogenic competence does not

preclude the idea that glial differentiation of stem cells may transpire, at least in part, by a corkscrew-like process. Finally, I propose a hypothetical model for the mechanism of temporal specification and/or differentiation of NSCs toward astrocytes, based on current knowledge in the literature.

Future studies in the field of NSC fate specification should include the advancement of methods for the prospective identification of stem cells as well as GRPs to clarify the precise mechanisms by which stem cells are fated toward glial lineages during development. Even if no definitive markers for stem cells are identified, the combinatorial use of several progenitor markers should enable this goal, at least in part, as has been the case for adult stem cells (Ming and Song 2011). For instance, Gsx2 (GS homeobox 2) is a transcription factor that is specifically expressed in the VZ of the lateral ganglionic eminence (LGE), where a gradient of VZ precursor differentiation is defined by Gsx2, Ascl1 (achaete-scute homologue 1), and Dlx (Distal-less) 12 expression (Gregg and Weiss 2005). Gsx2 may thus be a good candidate NSC marker in the LGE because Gsx2-positive, Ascl1-negative, Dlx1/2-negative cells are likely to be the most primitive cell population within the VZ. Moreover, the combinatorial use of Gsx2 and markers expressed in RGCs committed to an astrocyte lineage (e.g., GLAST, FGFR3, and GFAP) may be a worthwhile approach toward understanding the neurogenic-to-gliogenic switch.

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Chapter 5
Specification of GABAergic Neocortical Interneurons

Goichi Miyoshi, Robert P. Machold, and Gord Fishell

Abstract Inhibitory GABAergic interneurons within the cerebral cortex are critical for fine-tuning the activity of cortical circuits and thus are thought to be involved in generating the distinct oscillatory patterns that underlie higher brain functions such as consciousness and memory. Understanding how cortical interneurons are specified during development is important not just from the standpoint of basic research but also is likely to provide key insights into how cognitive disorders emerge. Although interneurons only consist of around 20 % of the neurons within the neocortex, they are extremely diverse with regard to their morphologies, molecular expression profiles, intrinsic electrophysiological properties, and synaptic connections. In rodents, most neocortical interneurons originate during embryogenesis from ventrally located structures, primarily the ganglionic eminences, and therefore must migrate over long distances following discrete pathways to reach the appropriate cortical areas. Thus, proper coordination of the distinct migration programs followed by pyramidal cells and interneuron precursors during development is crucial for the assembly of functional microcircuits within the cerebral cortex. Here, we review and discuss emerging views of how cortical GABAergic interneuron specification, migration, and integration occur from embryonic to early postnatal stages.

5.1 GABAergic Interneurons of the Cerebral Cortex

The mammalian cerebral cortex is composed of a sophisticated neuronal network that processes higher order information such as perception, consciousness, and memory. While glutamatergic pyramidal neurons represent the fundamental

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excitatory component of cortical circuits and integrate information between distinct brain regions, GABAergic interneurons are considered to be essential for local information processing via feedforward and feedback inhibition (Callaway 2004; Connors and Long 2004; Isaacson and Scanziani 2011; Lawrence and McBain 2003; Morishita and Hensch 2008) and are critically involved in the generation of frequency-specific rhythmic activity across neuronal ensembles (Bartos et al. 2007; Buzsáki and Draguhn 2004; Buzsáki and Wang 2012; Klausberger and Somogyi 2008; Mann and Paulsen 2007; Moore et al. 2010; Whittington and Traub 2003). Although GABAergic interneurons are greatly outnumbered by pyramidal neurons and only represent around 20 % of the total neurons within the neocortex, an impressive diversity has long been appreciated within this population (Fig. 5.1, right). The morphological heterogeneity of interneuron populations was first recognized by Ramon y Cajal over a century ago through his application of the Golgi staining method. More recently, the extent of interneuron diversity has been further explored through analyses of their molecular expression profiles, electrophysiological intrinsic properties, and their specific axonal projection patterns (DeFelipe et al. 2013; Fino et al. 2012; Krook-Magnuson et al. 2012; Kubota et al. 1994; Markram et al. 2004; Thomson and Lamy 2007) (further reviewed in Chap. 8).

Beyond the considerable interest from the standpoint of basic research, GABAergic interneuron dysfunction has been implicated in neurodevelopmental disorders such as autism and schizophrenia (Buonanno 2010; Chao et al. 2010; Inan et al. 2013; Lewis et al. 2012; Marín 2012; Nakazawa et al. 2012; Rossignol 2011; Rubenstein and Merzenich 2003), and thus understanding how these cells are specified and integrated within the cortex will likely provide key insights into the development of novel cognitive therapies.

5.2 Neocortical Interneurons Originate from the Ventral Telencephalon

Initially, both pyramidal cells and interneurons were thought to arise from within the neocortical primordium, either sequentially or in parallel, during the course of development. One of the reasons for this thinking was the observed similarity in layering between early to late-born pyramidal neurons and GABAergic interneurons, both of which are established in the cortex in an inside-out manner during development (Fairen et al. 1986; Miller 1985). The birthdating of specific interneuron classes was further analyzed by combining the use of DNA analogs and molecular markers (Cavanagh and Parnavelas 1988, 1989, 1990), but at the time, the embryonic neocortex was still considered to be the origin of these populations. This view was radically revised in 1997, when Stewart Anderson, John Rubenstein, and their colleagues demonstrated that cortical interneurons in mice are not generated from progenitors within the neocortex but are actually derived from a distantly located set of embryonic ventral structures known as the ganglionic eminences (GE, Fig. 5.2, left), a group of highly proliferative germinal zones located adjacent to the lateral ventricle (Anderson et al. 1997). To demonstrate

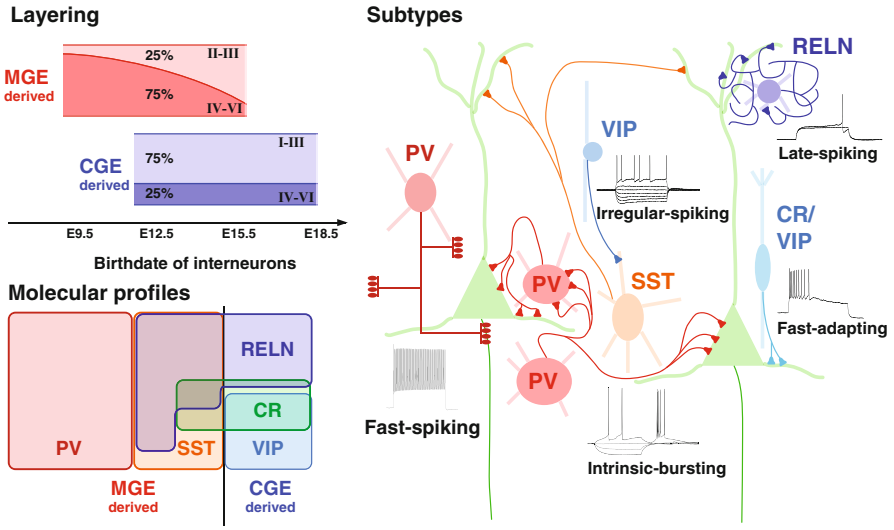

Fig. 5.1 Developmental origins of MGE- and CGE-derived interneuron subtypes. Note that MGE- and CGE-derived interneuron subtypes are colored in *red* and *blue*, respectively. *Layering*: Correlation between the birthdate and final laminar locations are indicated for MGE- and CGE-derived interneuron populations. MGE-derived interneurons populate the neocortical layers of II to VI, roughly in an inside-out manner. CGE-derived interneurons occupy superficial layers irrespective of their birthdate, with around 75 % of cells occupying layers I to III. The initiation and peak generation (not shown) of cortical interneurons is delayed in the CGE compared to the MGE. *Molecular profiles*: The four molecular markers of PV (Parvalbumin), SST (Somatostatin), RELN (Reelin), and VIP (Vasoactive intestinal polypeptide) cover over 95 % of neocortical GABAergic interneurons. While MGE-derived interneurons mainly express PV or SST, CGE-derived cells express RELN or VIP. Note that RELN is also expressed in around 70 % of SST-expressing cells. CR (Calretinin) is expressed in both MGE- and CGE-derived lineages and SST/CR and CR/VIP co-expression mark physiologically distinct subtypes within each group. *Subtypes*: A schematic correlating the morphology, molecular expression profile, electrophysiological properties, and the connectivity of GABAergic neocortical interneuron subtypes. Note that only a subset of information is included in this simplified diagram. The *dark red cell* on the *left* represents the chandelier cell type that mostly targets axon-initial segments, and the *two red round* somas in the *middle* of the diagram represent basket cells that preferentially synapse onto cell bodies. All of these cells show fast-spiking behavior when challenged with suprathreshold current injections. The *orange cell* at the *center* represents the Martinotti cell type that mainly targets apical dendritic shafts and tufts of pyramidal neurons (depicted in *green*). *Three blue-colored cells* on the *top right* represent the primary subtypes derived from the CGE. RELN-expressing cells show dense plexus or neurogliaform morphologies, primarily target dendrites, and show characteristic late-spiking behavior at near spike threshold. Many of the VIP- and CR/VIP-expressing subtypes are bipolar or tufted. VIP-single populations are highly enriched at the dorsal border of layers II/III

this, they labeled GE cells with DiI (a fluorescent dye) and directly showed that these cells migrate tangentially up into the neocortex. Furthermore, they demonstrated that cells mutant for the homeodomain transcription factors *Dlx1* and *Dlx2* could no longer invade the cortex and instead remained within the ventral telencephalon. Although it has been suggested that at least some primate interneurons may arise from cortical

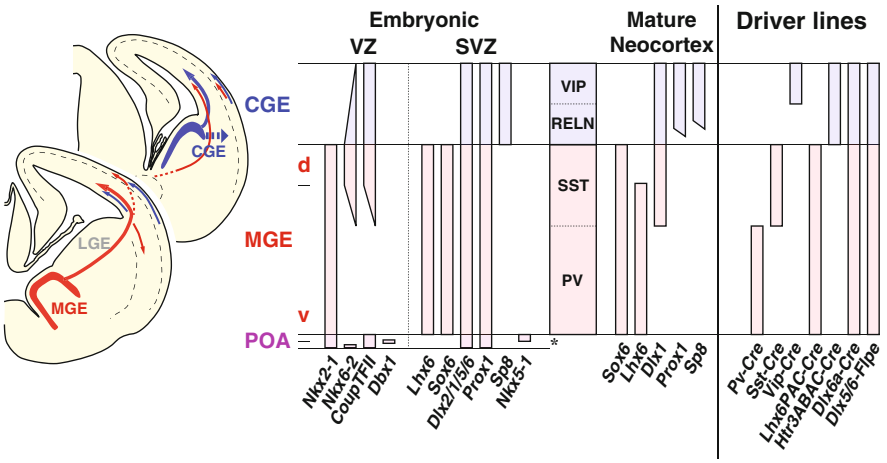

Fig. 5.2 Lineage specific transcription factor expression and driver lines to target distinct subtypes of cortical interneurons. *Left*: A coronal schematic of the E14.5 telencephalon with the MGE (*red*), CGE (*blue*), and LGE domains indicated. Preoptic area (POA) is not depicted here as it is located ventral to the MGE and is in between these two coronal planes. *Arrows* indicate the migration routes of interneurons from the MGE and CGE at this stage and also at later time points. *Embryonic*: Transcription factors expressed in the VZ (ventricular zone) or SVZ (subventricular zone) are indicated as *bar graphs*. Dorsal (d) and ventral (v) territories of the MGE are indicated. POA domain 1 (above) and 2 (below) are also considered to be distinct areas (Gelman et al. 2011). *Mature*: The expression patterns of selected transcription factors in the four basic interneuron subgroups of PV, SST, RELN (without SST), and VIP at maturity are indicated, illustrating the dynamic changes in gene expression that take place during development. For example, while *Dlx1* is widely expressed in most interneuron precursors at embryonic stages, its expression becomes restricted to specific subsets of interneurons at adult stages. *Asterisk* indicates that POA-derived interneurons give rise to diverse subtypes. *Driver lines*: While *Cre* driver lines generated based on mature interneuron markers are useful to target each subgroup (Hippenmeyer et al. 2005; Taniguchi et al. 2011), genes expressed during development within specific lineages (Fogarty et al. 2007) can be utilized to target specific interneuron populations as well. By using the transgenic driver lines of *Dlx6a-Cre* (Monory et al. 2006), *Dlxl1/2b-Cre* (Potter et al. 2009), *Dlx5/6-Cre-IRES-EGFP* (Stenman et al. 2003), or *Dlx5/6-Flpe* (Miyoshi et al. 2010), all of which utilize *Dlx* enhancer elements, a large majority of GABAergic neocortical interneurons can be targeted

progenitors (Letinic et al. 2002), in this review, we will mostly focus on the development of rodent GABAergic cortical interneurons, of which we have accumulated the most knowledge to date largely as a result of the powerful genetic tools available in this model organism (Miyoshi and Fishell 2006).

5.3 Spatial and Temporal Origins of Interneuron Subtypes

The discovery that GABAergic neocortical interneurons are not locally generated but instead are derived from the ventral telencephalon opened up exciting new avenues of research. From which specific germinal zones of the ventral telencephalon are the distinct subtypes of interneurons actually generated? Is a specific interneuron

subtype derived from a particular region or embryonic stage? How do interneurons migrate tangentially to their appropriate neocortical destination, and are there other genes besides *Dlx1* and *Dlx2* that regulate this process? Answers to many of these questions have begun to emerge in recent years.

As alluded to above, the embryonic GE is comprised of three morphologically distinct bulges: the medial-, lateral-, and caudal-ganglionic eminences (MGE, LGE, and CGE, Fig. 5.2 left). They are only transiently distinguishable during embryonic stages; for example, the prominent sulcus between the MGE and LGE/CGE at embryonic day 12.5 (E12.5) disappears after E15.5 in mice (Fig. 5.5). Although these morphological distinctions provide convenient landmarks, a more accurate map of the distinct germinal territories within the eminences is provided by gene expression profiles (Flames et al. 2007). The earliest effort to determine the embryonic origins of different interneuron subtypes was carried out by combining classical transplantation methods with ultrasound backscatter microscopy (UBM), a visualization method similar to that widely used for monitoring the growth of the human fetus, albeit with considerably higher resolution. In this study, genetically labeled E13.5 MGE or LGE donor cells were transplanted back into the host MGE or LGE of the same age, and their embryonic migration pattern and subsequent fates in the mature cortex were analyzed (Wichterle et al. 2001). The conclusions from both of these studies as well as a subsequent study testing the lineages derived from the CGE (Nery et al. 2002) were that (1) the MGE and CGE but not the LGE produce neocortical interneurons at E13.5 and (2) the MGE and CGE seem to have intrinsically determined fates for their interneuron subtypes. The second point was demonstrated by heterotopically transplanting the donor MGE or CGE cells into the host CGE or MGE and later confirming that the donor identity largely predicts the mature phenotype of the transplanted cells. Furthermore, *in vitro* cell cultures from the MGE and CGE have demonstrated that they give rise to populations with distinct marker profiles (Xu et al. 2004). Later studies expanded upon these findings by carrying out electrophysiological analyses and by demonstrating that some MGE cells migrate through the CGE (Butt et al. 2005), and thus the lineages arising from the MGE and CGE are quite distinct. Similarly, the LGE also produces a distinct set of neuronal populations and while it does not appear to generate substantial numbers of cortical interneurons, it does give rise to striatal cells as well as interneurons that migrate to the olfactory bulb (Wichterle et al. 2001).

Following these transplantation studies, advances in mouse genetic techniques opened up new and exciting approaches to fate map embryonic populations based on gene expression rather than by anatomical location alone. Specific cell lineages defined by gene expression could be labeled with a reporter protein (e.g., β-galactosidase/*LacZ*, alkaline phosphatase, or fluorescent proteins) *in vivo* through the use of gene targeting or transgenesis. These reporter proteins can be expressed under the direct regulation of a particular gene (e.g., *Dlx2-LacZ*) (Nery et al. 2003; Stuhmer et al. 2002) or can be driven from a ubiquitous promoter (e.g., *CAG* promoter, *ROSA26* locus) in a conditional manner that relies on the activity of a site-specific recombinase (e.g., Cre, CreER, Flpe, PhiC31) selectively expressed in the desired genetic lineage (e.g., *Dlx-Cre*) (Branda and Dymecki 2004; Dymecki and Kim 2007; Imayoshi et al. 2012; Joyner and Zervas 2006). In this review, we

will focus on the observations from genetic fate-mapping studies that mostly use fluorescent reporters, but it is worth mentioning that these same genetic techniques can be applied towards manipulating the activity or gene expression within specific cell populations in order to study both developmental processes and the mature functions of interneurons (Luo et al. 2008).

5.3.1 The Four Major Classes of Neocortical Interneurons

Although a large diversity of GABAergic interneuron subtypes are found in the neocortex, we propose that they can be classified into four major subgroups based on their developmental and genetic origins, molecular expression profiles, morphologies, and intrinsic electrophysiological properties (as obtained from slice whole-cell patch recordings) (Fig. 5.1, right). By itself, the combinatorial expression of four major markers, PV (*Pvalb*, Parvalbumin), SST (*Sst*, Somatostatin), RELN (*Reln*, Reelin), and VIP (*Vip*, Vasoactive intestinal polypeptide), is generally sufficient to identify the majority of neocortical interneuron subtypes. The MGE gives rise to two broad subtypes: PV-expressing, basket/chandelier morphology interneurons with fast-spiking properties (roughly 40 % of total interneurons), and SST-expressing, Martinotti morphology cells often with intrinsic-bursting characteristics (~30 %). The CGE-derived interneurons also have two distinct groups: RELN-expressing, neurogliaform/dense plexus morphology cells with a late-spiking character (~15 %), and VIP-expressing, bipolar/bitufted morphology interneurons with diverse firing properties (e.g., fast-adapting, irregular spiking, burst followed with nonadapting, ~15 %). When Calretinin (CR) expression is also considered (Fig. 5.1 bottom left, green), interneurons have six groups and consistent with this; both SST/CR- and CR/VIP-co-expressing cells are electrophysiologically distinct from the SST- and VIP-single interneurons. It is interesting that both of the MGE- and CGE-derived classes contain a subtype that is highly multipolar with many dendritic processes directly protruding from the soma (PV- and RELN-expressing subtypes, Fig. 5.1 right). The remaining populations in each of the MGE- and CGE-derived interneuron classes have relatively stereotypic projection patterns of axons with a large majority of SST- and VIP-expressing cells sending their axons towards superficial and deep directions, respectively. It is worth mentioning that SST-expressing cells in layer IV are exceptional and they have their axons confined largely to layer IV (Xu et al. 2013). We will discuss a few exceptions below, but basically, these four major subtypes comprise over 95 % of the entire GABAergic interneuron population within the mouse neocortex (Miyoshi et al. 2010).

5.3.2 MGE-Derived Interneurons

Figure 5.2 summarizes the recent findings correlating gene expression in the ventral telencephalon (progenitor domains) with mature interneuron fates and available

genetic tools for targeting these populations. In the developing forebrain, the expression of the homeodomain transcription factor *Nkx2-1* in progenitors is largely coincident with the MGE, although the dorsal limit of this gene does not extend to the sulcus between the MGE and LGE/CGE but is located slightly more ventrally (Miyoshi et al. 2007). In order to characterize the interneuron subtypes derived from the *Nkx2-1*-expressing progenitor domain, BAC (bacterial artificial chromosome, typically 200–300 kb) transgenic *Cre* driver lines were generated (Fogarty et al. 2007; Xu et al. 2008). Interestingly, although these two distinct groups generated their transgenic lines independently, in both cases Cre expression was not observed in the most dorsal part of the *Nkx2-1*-expressing domain. Nevertheless, these tools were sufficient to demonstrate that the majority of PV- and SST-expressing interneurons are derived from the MGE. These observations were further validated by fate mapping the forebrain *Lhx6* lineages by the use of an *Lhx6PAC-Cre* driver line. *Lhx6* is a downstream target of *Nkx2-1* (Du et al. 2008), and in this study nearly all PV and SST populations were shown to originate from the MGE (Fogarty et al. 2007).

While it has been demonstrated that almost all of the PV- and SST-expressing interneurons are derived from the MGE, considerable questions concerning the generation of diversity remain. Is there any regional bias in the production of PV- and SST-expressing interneurons within the MGE? Is there a temporal sequence in generating these populations? Do these two populations arise from a common progenitor lineage, and if their lineages are distinct, at what point do they diverge? As shown in Fig. 5.2, there are genes specifically expressed within the dorsal domains of the MGE (*Nkx6-2* and *CoupTFII*), suggesting that there is patterning within the MGE that might direct interneuron subtype specification. This in fact turned out to be the case and has been demonstrated by (1) transplantation of dorsal versus ventral MGE and (2) genetic fate mapping with *Cre* drivers under the control of a dorsal-MGE-biased gene. The transplantation studies that compared the fates of dorsal- versus ventral-MGE progenitors have demonstrated that there is dorsal-SST, ventral-PV bias in the generation of interneurons from the MGE (Flames et al. 2007; Wonders et al. 2008). Similarly, genetic fate-mapping studies on *Nkx6-2* lineages (using both *Cre* and *CreER*) demonstrated that these dorsal-MGE progenitors give rise primarily to SST- but very few PV-expressing interneurons (Fogarty et al. 2007; Sousa et al. 2009). More recently, through the use of a *Shh-Cre* driver line, in which recombination is mostly confined to a population of cells within the ventral MGE and dorsal preoptic area, it has been shown that this lineage gives rise to only a small fraction (~5 %) of PV-expressing neocortical interneurons (Flandin et al. 2010), underscoring the complexity of populations arising from this anatomical region.

Is the generation of PV- and SST-expressing interneuron populations temporally regulated within the MGE? *CreER*-mediated inducible genetic fate mapping demonstrated that MGE-derived interneurons are generated in an inside-out manner (Fig. 5.1 top left, Miyoshi et al. 2007), consistent with previous findings from the use of DNA-analog-based birthdating studies (Fairen et al. 1986; Miller 1985; Rymar and Sadikot 2007). Furthermore, in a manner very similar to pyramidal neurons, MGE-derived interneurons rarely occupied layer I. In this study, PV-expressing fast-spiking interneurons with basket morphologies were found to originate from the MGE consistently over time (E9.5 to E15.5). In contrast, SST-expressing

interneurons were mostly produced from the early MGE (E9.5 to E12.5), and their generation declined dramatically at later time points (E15.5). Furthermore, SST-expressing interneurons with distinct intrinsic electrophysiological profiles had different temporal origins in the MGE. Notably, the SST/Calretinin co-expressing interneurons were mostly produced from the later MGE (E15.5). Since in rats, the calcium-binding protein Calretinin (CR) is not co-expressed in SST-positive interneurons (Kawaguchi and Kubota 1997; Kubota et al. 1994), CR had been generally considered as a non-MGE lineage marker, and this has lead to confusion when comparing results from mice and rats. As shown in this fate-mapping study, SST/CR co-expressing interneurons are late MGE-derived interneurons with distinct morphological and physiological characteristics (Xu et al. 2006) than the SST-single populations described previously (Ma et al. 2006).

Recently, experiments to simultaneously address the spatial and temporal origin of MGE-derived interneurons have been carried out (Inan et al. 2012). In this study, the E13.5 and E15.5 MGE was dissected into three divisions (dorsal, medial, ventral) and transplanted into the early postnatal cortex, and subsequently the fates of the grafted cells were characterized. While the dorsal-SST, ventral-PV bias described above was confirmed in this study, the authors also found that the ventral-E15.5 MGE was biased for the production of chandelier neurons (~25 %), the fast-spiking PV-expressing interneuron class that selectively targets axon-initial segments (Inan et al. 2012). Temporal fate-mapping experiments performed with a *Nkx2-1-CreER* driver line demonstrated that at E17.5, the late MGE mostly gave rise to chandelier cells (70 % in the somatosensory cortex), whereas SST-expressing interneurons were not found to be produced at this stage at all (Taniguchi et al. 2013).

In summary, the MGE produces neocortical interneurons roughly in the following manner: early (by E13), PV-basket and SST-Martinotti cells; mid (around E15), PV-basket and SST/CR-Martinotti cells; and late (around E17), PV-basket and PV-chandelier cells.

5.3.3 CGE-Derived Interneurons

Studies on the developmental origins of CGE-derived interneurons have lagged behind the analyses carried out on the populations derived from the MGE. This is not just due to relatively smaller population of CGE-derived interneurons compared to that originating from the MGE but also because of the absence of genes identified to date that is selectively expressed within the CGE. Our own study made inroads in revealing the developmental process of CGE-derived interneuron subtypes (Miyoshi et al. 2010) by utilizing a BAC transgenic *Mash1BAC-CreER* driver line (Battiste et al. 2007) that efficiently targeted the cells born from the LGE and CGE but not the ones derived from the MGE domain. This study also reported the discovery that expression of RELN, a secreted glycoprotein critical for cortical development (Ogawa et al. 1995; Tissir and Goffinet 2003), allows us to define a previously unidentifiable population of CGE-derived interneurons. We labeled almost all

cortical interneurons with EGFP by combining *Dlx5/6-Flpe* driver and *RCE:FRT* reporter lines and found that a large proportion of cells that were not labeled with PV-, SST- or VIP-expressed RELN. In fact, over 40 % of interneurons fate mapped by the *Mash1BAC-CreER* driver were consistently labeled with RELN. It is important to note that in addition to the CGE-derived populations, many (~70 %) SST-expressing interneurons, which are derived from the MGE, also co-express RELN (Fig. 5.1 bottom left). With these findings, we now can molecularly distinguish over 95 % of the entire population of GABAergic neocortical interneurons based on four markers: PV, SST, RELN, and VIP. Experiments carried out in the Kessaris lab complement this study in that the authors used "genetic algebra" to negatively subtract out the MGE-derived interneurons from the entire population of GABAergic cells and reported similar conclusions (Rubin et al. 2010). However, in these two studies, both CGE and LGE lineages were labeled, and hence the best evidence to date that only the CGE generates interneurons is the finding that the transplanted E13.5 and E15.5 CGE cells but not E13.5 LGE cells give rise to cortical interneurons (Nery et al. 2002; Wichterle et al. 2001; Butt et al. 2005). In fact, cellular migration from the LGE into the cortex has been reported in the context of *in vitro* brain slice cultures (Anderson et al. 2001), and many genes expressed in the CGE show continuous expression across the LGE subventricular zone, such as *Sp8* (Ma et al. 2012) and *CoupTFII* (Kanatani et al. 2008). Generation and analysis of a *Cre* driver line that selectively targets either the LGE or CGE progenitors will be required to resolve this fundamental and important distinction.

Interneuron generation from the CGE was found to be delayed compared to the MGE for both the initial (E12.5) and peak (E16.5) generation by about 2 days (Fig. 5.1, top left). In addition, while early to late-born MGE-derived interneurons established themselves in the cortex in an inside-out manner, respectively (Fig. 5.1, top left), CGE-derived interneurons primarily inhabited superficial layers (~75 %) regardless of birthdate. Consistent with this finding, the CGE produced relatively similar populations of interneurons expressing RELN or VIP over time, with only a few exceptions. RELN-expressing (SST-negative) interneurons possess neurogliaform or dense plexus morphologies with characteristic late-spiking electrophysiological properties. RELN-positive interneurons at near threshold current injections show a single spike after a long ramp (late-spiking, Fig. 5.1, right) and at above threshold current injections, show regular spiking. VIP-expressing interneurons have diverse intrinsic firing properties but can be divided into two populations based on whether they co-express CR or not. Through the use of BrdU injections to birthdate cells, the CR/VIP co-expressing population was found to be a relatively early-born CGE-derived population (E12.5-E14.5) and is barely generated after E16.5 (Xu et al. 2004). This implies that while SST/CR cells are an intermediately generated MGE-derived population, CR/VIP cells are an early CGE-derived population, with both of their peak birthdates being around E14.5. Taken together, these two complementary *in vivo* genetic inducible fate-mapping studies on MGE- and CGE-derived interneuron populations (Miyoshi et al. 2007; Miyoshi et al. 2010) and the recent late (E17.5) MGE fate-mapping study (Taniguchi et al. 2013) have defined the distinct spatiotemporal origins of the vast majority of cortical interneuron subtypes.

While the vast majority of cortical interneurons arise from the MGE or CGE, are there other ventral structures that generate subpopulations of interneurons? Recently, existence of a preoptic area (POA)-derived population of interneurons was discovered (Gelman et al. 2009; Gelman et al. 2011). The preoptic area is located ventral to the MGE, and interestingly, this domain also expresses *Nkx2-1* (Fig. 5.2) and gives rise to at least two separate lineages of cortical interneurons. One interneuron population originating from the POA is selectively labeled with an *Nkx5-1BAC-Cre* transgenic driver, and around one third of these cells express Neuropeptide-Y (NPY) exclusively (Gelman et al. 2009). The other POA-derived population of interneurons can be fate mapped with a *Dbx1-Cre* driver (Fig. 5.2) and consists of highly diverse subtypes that express PV, SST, RELN, and VIP (Gelman et al. 2011). Other potential sources of GABAergic neocortical interneurons might be uncovered in additional *Dlx*-expressing domains such as the embryonic septum, which is located in the ventromedial walls anterior to hippocampus. However, a fate map of the septum using the transgenic *Zic4BAC-Cre* driver line did not reveal any labeling of neocortical interneurons (Rubin et al. 2010).

5.3.4 Interneuron Markers Beyond PV, SST, RELN, and VIP

In addition to the four primary molecular markers of PV, SST, RELN, and VIP, we would like to briefly discuss some of the other molecules that have been widely used in studies of neocortical interneuron development and function. CR (*Calb2*, Calretinin), as we have already described above, is expressed in electrophysiologically distinct subpopulations of SST- and VIP-expressing interneurons (Fig. 5.1, bottom left). In addition to these two interneuron classes of SST/CR and CR/VIP cells, CR expression can be found in a small fraction of the RELN-expressing late spikers (CGE-derived, non-SST) and also in some of the interneurons located primarily in layer I that do not express the four major markers (Fig. 5.1, bottom left). It is also important to mention that CR is transiently expressed in a subpopulation of pyramidal neurons at early postnatal stages (Schierle et al. 1997), in addition to the embryonic subplate and Cajal–Retzius cells (Del Rio et al. 1995; Fonseca et al. 1995).

NPY (*Npy*, Neuropeptide Y) is expressed broadly in all four major classes of neocortical interneuron subtypes (Karagiannis et al. 2009), and its expression is dynamically regulated by the activity state of the neocortical network (Baraban et al. 1997). nNOS (*Nos1*, Neuronal nitric oxide synthase 1) is found to be expressed at high levels in about 2 % of the neocortical SST-expressing interneurons (Jaglin et al. 2012; Magno et al. 2012) and also at low levels in an overlapping manner with the four major interneuron markers (Type II cells) (Perrenoud et al. 2012). CB (*Calb1*, Calbindin) expression within neocortical interneurons occurs exclusively in MGE-derived lineages (Fogarty et al. 2007). Similar to the other calcium-binding protein CR, CB, at least in rats, is weakly expressed in early postnatal pyramidal neurons (Alcantara et al. 1996). Because of its early onset, CB has been frequently used as a marker for migrating interneuron precursors during the embryonic period

(Anderson et al. 1997; Colombo et al. 2007). CCK (*Cck*, Cholecystokinin)- and PV-expressing interneurons are the two major basket cell types within the hippocampus (Freund 2003). However, in the mouse neocortex, CCK is only rarely expressed in interneurons (Xu et al. 2010b), and consistent with what has been found in the rat (Kawaguchi and Kubota 1997), around 75 % of CCK-positive interneurons co-express VIP (G.M. unpublished observations). Htr3A (*Htr3A*, 5-Hydroxytryptamine/serotonin receptor 3A) is expressed in neocortical interneurons (Morales and Bloom 1997) but interestingly is restricted to the CGE-derived populations (Lee et al. 2010; Vucurovic et al. 2010). During the embryonic and early postnatal periods, Htr3A is also expressed in the subplate and Cajal–Retzius cells. Alpha actinin 2 (*Actn2*, Actinin alpha 2) is uniformly expressed in rat late-spiking neocortical interneurons (Kubota et al. 2011), which are equivalent to the mouse RELN-expressing (SST-negative) populations. Similarly, there are additional markers such as cortistatin (De Lecea et al. 1997), CRF (corticotropin-releasing factor), and substance P receptor (Kubota et al. 2011) that have been identified to date whose role in interneuron classification and function has yet to be fully explored.

5.4 Interneuron Specification by Transcription Factors

Here, we will focus on the cell-autonomous role of transcription factors expressed within interneurons and their progenitors, with an emphasis on loss-of-function studies. Most transcription factors that have been identified as being necessary for proper interneuron development are broadly expressed in other ventral telencephalic lineages or are required at early patterning stages to specify the eminences in general. For example, early (by E9) dorsal–ventral and anterior–posterior patterning of the telencephalon by sonic hedgehog (Shh) and fibroblast growth factor (Fgf) signaling-induced transcriptional pathways are crucial for the proper specification of the MGE, LGE, and CGE (Campbell 2003; Guillemot and Zimmer 2011; Hébert and Fishell 2008; Hoch et al. 2009; Lupo et al. 2006; Rallu et al. 2002; Sousa and Fishell 2010). It is interesting and worth mentioning that continuous action of *Smo*-mediated hedgehog signaling is required to maintain MGE identity, otherwise interneuron subtypes generated from this domain become mis-fated to fewer PV- and more SST- and CR-expressing bipolar (CGE-derived subtype) populations (Xu et al. 2005; Xu et al. 2010a).

5.4.1 Telencephalic GABAergic Cell Fate Specification

We will begin by discussing the regulation of GABAergic neuron differentiation in general, and later will delve into the mechanisms involved in specifying interneuron subtypes. GABAergic neurons exist throughout the entire central nervous system but within the telencephalon of rodents, they are exclusively produced ventrally.

By focusing on the two organizing centers of the zona limitans intrathalamica (often abbreviated as ZLI) and the isthmus, both of which are a source of morphogens such as Shh and Fgf8, the anterior–posterior axis of the central nervous system are thought to be divided into three distinct regions (Puelles and Rubenstein 2003; Rubenstein et al. 1994). The telencephalon, hypothalamus, and prethalamus (the latter two are considered as compartments within the anterior diencephalon) are located anterior to the ZLI, and thalamus and pretectum (considered posterior compartments of the diencephalon) and mesencephalon are located in between the ZLI and isthmus. Most caudally from the isthmus are the rhombencephalon (cerebellum/hindbrain) and the spinal cord. Interestingly, distinct transcriptional programs control GABAergic neurogenesis within each anterior–posterior compartment. In particular, GABAergic cell specification anterior to the ZLI requires *Dlx* (Anderson et al. 1997), between ZLI and isthmus, *Helt* (Guimera et al. 2006; Miyoshi et al. 2004; Nakatani et al. 2007), and posterior to isthmus, *Ptf1a* (Glasgow et al. 2005; Hoshino et al. 2005).

5.4.2 GABAergic Cell Fate Specification Within the Ventricular and Subventricular Zones

Several proteins involved in GABAergic specification within the ventricular and subventricular zones (VZ/SVZ) have been identified to date. *Vax1* (ventral anterior homeobox-containing gene 1) is expressed in the VZ/SVZ of the ventral telencephalon and regulates cell proliferation (Taglialatela et al. 2004). Very interestingly, loss-of-function studies of *CyclinD2*, which is selectively expressed within the SVZ but not in the VZ of the MGE have suggested that while SST-expressing interneurons are produced directly from the VZ, PV-expressing subtypes are produced from the SVZ, a domain where VZ-originated progenitors undergo additional mitoses prior to becoming postmitotic interneuron precursors (Glickstein et al. 2007). While it is possible that this *CyclinD2* mutant phenotype was simply due to the loss of later-born subtypes caused by progenitor depletion, it is consistent with recent observations from a clonal study on MGE-derived lineages (Brown et al. 2011). In this study, RCAS retrovirus was specifically transfected into the *Nkx2-1*-expressing progenitor pool using a conditional genetic approach, and the resulting populations of labeled cells were carefully studied across serial brain sections. Very interestingly, clonally related interneurons preferentially formed vertical or horizontal clusters within the neocortex, and each cluster contained either or both PV- or SST-expressing subtypes (Brown et al. 2011).

Another interesting set of genes that could participate in GABAergic subtype specification at VZ/SVZ stages are proneural transcription factors that are broadly required throughout the CNS for neurogenesis (Bertrand et al. 2002). In particular, *Mash1* (*Ascl1*) is strongly expressed in the VZ/SVZ of the ventral eminences, in a salt and pepper pattern (Casarosa et al. 1999; Torii et al. 1999). *Mash1* promotes neural differentiation in part by stimulating the expression of the Notch ligand Dll1

(*Delta-like 1*) (Castro et al. 2006). In addition to the ventral telencephalon, a low level of Mash1 expression is observed in pyramidal neuron precursors (Britz et al. 2006; Pacary et al. 2011) and also in migrating interneuron precursors primarily arising from the CGE (Miyoshi et al. 2010). While Mash1 function is required for GABAergic neurogenesis in the ventral telencephalon, *Neurog2* (*Neurogenin2*), which is another proneural gene normally expressed exclusively in cortical (pallial) progenitors (Mattar et al. 2008; Miyata et al. 2004; Shimojo et al. 2008), can substitute for Mash1 when expressed from the *Mash1* locus, at least with regard to numbers of migrating GABAergic neuronal precursors and *Dlx1* expression (Fode et al. 2000; Parras et al. 2002). Interestingly though, GABAergic neurogenesis can be induced in cortical progenitors by *Mash1* overexpression (Fode et al. 2000), notwithstanding the fact that *Mash1* is normally present at low levels within the cortical VZ (Britz et al. 2006). Thus, while the proneural gene *Mash1* appears to regulate GABAergic neurogenesis through its role in Delta/Notch signaling, it is also capable of stimulating GABAergic transcriptional programs ectopically in the cortical VZ.

5.4.3 Postmitotic Regulation of Interneuron Development

As we have described above in Sect. 5.2, when both *Dlx1* and *Dlx2* genes are mutated, the vast majority of cortical GABAergic interneuron precursors fail to migrate tangentially up into the cortex and instead remain in the ventral telencephalon (Anderson et al. 1997). This is in part due to the requirement for *Dlx* function in the suppression of neurite growth by Pak3 kinase-dependent mechanisms (Cobos et al. 2007) (Fig. 5.3). It has been shown that Necdin associates with Dlx proteins to promote the differentiation of GABAergic neurons (Kuwajima et al. 2006). Furthermore, Dlx activity is necessary to restrict oligodendroglial fate from a common progenitor pool in the SVZ (Petryniak et al. 2007). The X-linked transcription factor *Arx* acts downstream of *Dlx* (Cobos et al. 2005a; Colasante et al. 2008) and regulates the migration of interneurons (Colombo et al. 2007; Friocourt and Parnavelas 2011; Kitamura et al. 2002) through the expression of a number of genes including Pak3 and Cxcr4/7 (Colasante et al. 2009; Friocourt and Parnavelas 2011; Fulp et al. 2008) (Fig. 5.3). Very interestingly, sustained *Dlx1* expression (Fig. 5.2, mature) plays an important role during postnatal development specifically in ~90 % of SST-expressing and all of the CR-positive (includes the ones co-expressing SST or VIP; see Fig. 5.1) neocortical interneurons (Cobos et al. 2005b). *Dlx5* and *Dlx6* are induced downstream of *Dlx2* and *Dlx1* (Long et al. 2009) and found expressed in developing and mature cortical interneurons (Cobos et al. 2006; Panganiban and Rubenstein 2002). In the *Dlx5* and *Dlx6* double mutants, while embryonically interneuron precursors show a severe reduction in Cxcr4 expression and attenuated tangential migration, within the postnatal neocortex, PV- but not SST-expressing interneuron populations are selectively reduced (Wang et al. 2010).

CoupTF1 (*Nr2f1*, nuclear receptor subfamily 2 group F member 1, or chicken ovalbumin upstream protein-transcription factor 1) is expressed in over 80 % of

neocortical interneurons at P8, but by P21 is highly enriched in bipolar CR-expressing interneurons (Lodato et al. 2011b) that presumably co-express VIP, and is almost absent from PV-expressing cells. In the conditional *CoupTF1* loss-of-function mutants, within the neocortex, the VIP-expressing population is reduced by 30 %, while the numbers of PV-expressing interneurons were 30 % increased (Lodato et al. 2011b). Thus, *CoupTF1* plays a pivotal role in regulating the balance between MGE- versus CGE-derived interneuron subtypes within the neocortex. The paralog of *CoupTFI*, *CoupTFII,* is expressed in the dorsal MGE and CGE (Kanatani et al. 2008) (Fig. 5.2), and consistent with this pattern, it is maintained in migrating interneuron precursors derived from both the MGE and CGE (Miyoshi et al. 2010). Although CoupTFII is expressed in the dorsal MGE, within the mature cortex, it is absent from SST/CR co-expressing interneurons (Cai et al. 2013) but is present in SST-single and CGE-derived interneuron populations (Ma et al. 2012). Recently, *CoupTFII* mutants were reported to have no obvious phenotype in the development of cortical interneurons, at least with regard to their marker expression profiles (Tang et al. 2012), suggesting that *CoupTF1* may compensate for loss of *CoupTFII* in this context. The transcription factor *Npas1* (Neuronal PAS domain 1) appears to play a role in interneuron specification and/or maturation, although its expression profile in interneuron subclasses has not been fully characterized to date. In single or double *Npas1* and *Npas3* mutants, GAD67, PV, and CR expression are not affected, but RELN expression is severely reduced (Erbel-Sieler et al. 2004). Considering that there are substantial numbers of Npas1-expressing cells within layer I (Cobos et al. 2006), *Npas1* likely regulates the development of SST/RELN- and RELN-expressing interneuron subtypes derived from the MGE and CGE, respectively (Fig. 5.1, bottom left).

5.4.4 Transcriptional Cascades for Specific Interneuron Subtypes

Beyond the basic specification of GABAergic neuron identity, several transcription factor cascades have been identified that impart a clear lineage bias in cortical interneuron precursors. For MGE-derived subtypes of interneurons, the *Nkx2-1–Lhx6–Sox6/SatB1* transcriptional cascade has been demonstrated to be critical for specification and migration of these populations. Although the appropriate rescue experiments have not yet been performed for each downstream gene(s), the hierarchical order of this cascade is well established (Fig. 5.3). Loss of *Nkx2-1* function invariably results in the loss of *Lhx6* expression (Butt et al. 2008; Du et al. 2008; Sussel et al. 1999). While the total loss of *Nkx2-1* in null embryos results in severe mis-patterning of the MGE domain (Sussel et al. 1999), conditional removal of this gene at mid-gestation stages redirects interneuron precursors towards the fate of CGE-derived subtypes (Butt et al. 2008). Analysis of *Lhx6* loss-of-function animals (Liodis et al. 2007; Zhao et al. 2008) demonstrates that this gene, which is expressed within the SVZ of the MGE in an overlapping manner with Nkx2-1 and is subsequently maintained in migrating interneuron precursors, plays an important role in

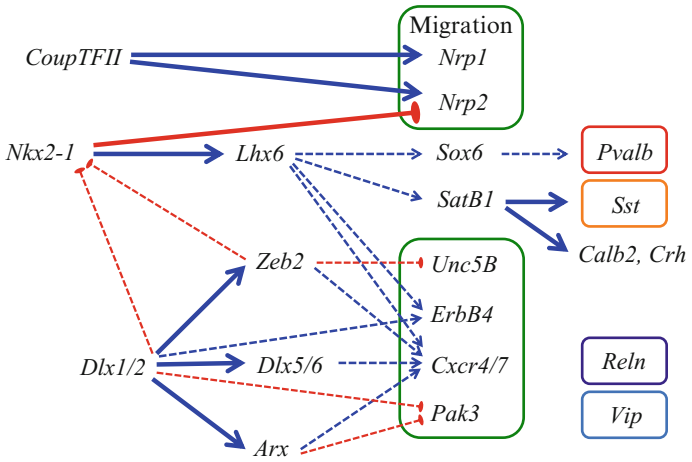

Fig. 5.3 Gene networks regulating the development of neocortical interneurons (partial view). *Blue arrows* and *red bars* indicate activation and repression, respectively, within the cascade of genes expressed during cortical interneuron development. In the cases where direct transcriptional regulation has been demonstrated, the *lines* are shown in *bold*; otherwise the relationship is depicted with a *dotted line*. References: (Azim et al. 2009; Balamotis et al. 2012; Batista-Brito et al. 2009; Cobos et al. 2005a, b; Cobos et al. 2007; Colasante et al. 2008; Colasante et al. 2009; Denaxa et al. 2012; Du et al. 2008; Long et al. 2009; McKinsey et al. 2013; Nobrega-Pereira et al. 2008; Tang et al. 2012; van den Berghe et al. 2013; Wang et al. 2010; Zhao et al. 2008)

the specification of MGE-derived interneurons of both PV- and SST-expressing classes. In fact, *Nkx2-1* directly binds to the promoter region of *Lhx6* in vivo and induces its transcription (Du et al. 2008). In addition, Lhx6 expression can rescue many aspects of the *Nkx2-1* null phenotype in cortical interneurons. Among the transcriptional targets of *Lhx6* are the transcription factors *Sox6* and *SatB1*, both of which are severely reduced within *Lhx6* mutant cortical interneurons (Batista-Brito et al. 2009; Denaxa et al. 2012). In *Lhx6* mutants, MGE-derived interneuron precursors migrate abnormally, and by the second postnatal week, *Lhx6* mutant cells are misplaced in locations above (layer I) and below (white matter) the cortical plate with no obvious reduction in total cell numbers (Liodis et al. 2007). Although this is the case, the expression of PV and SST is almost completely eliminated, indicating that *Lhx6* is required for the specification and/or maintenance of these interneuron subtypes in addition to its role in regulating migration.

The loss of function of *Sox6*, which acts downstream of *Lhx6* (Fig. 5.3), phenocopies the laminar distribution defects observed in the *Lhx6* mutants. Interestingly, in this mutant, interneurons of the PV-expressing lineages are particularly affected and the expression of PV is dramatically reduced (Azim et al. 2009; Batista-Brito et al. 2009). In addition, the proportion of cells expressing the potassium channels normally found in this population (Kv3.1b and Kv3.2) is reduced by 50 %, and accordingly, these cells show deficits in their fast-spiking properties (Batista-Brito et al. 2009). In contrast to the severe phenotype in SST-expressing cells caused by the loss of *Lhx6*, in the *Sox6* mutants, SST-expressing interneurons were only mildly

affected (Batista-Brito et al. 2009). Subsequent work has found that the transcriptional regulator *SatB1* is the critical downstream gene target of *Lhx6* for the specification of the SST-expressing Martinotti subpopulation of MGE-derived interneurons. Furthermore, *SatB1* is sufficient to induce SST expression in *Lhx6* deficient cells (Denaxa et al. 2012) through its ability to bind directly to the cis-regulatory elements of the *Sst* gene (Balamotis et al. 2012). *SatB1* expression is initiated in MGE-derived interneuron precursors at the time they are about to complete their tangential migration, and may regulate the onset of their post-migratory maturation (Close et al. 2012; Denaxa et al. 2012). In *SatB1* mutants, while PV-expressing interneurons are only moderately affected, SST-expressing cells are severely depleted (Balamotis et al. 2012; Close et al. 2012; Denaxa et al. 2012; Narboux-Neme et al. 2012) through apoptosis during the postnatal developmental period (Close et al. 2012).

For CGE-derived neocortical interneurons, *Sp8* (zinc finger type) is the only known transcription factor specifically expressed within this lineage (Fig. 5.2) and is present in about half of the RELN-expressing and most of the VIP-expressing interneurons within the adult neocortex (Ma et al. 2012). During the embryonic period, Sp8 is expressed in the subventricular zones of both the LGE and CGE and is maintained in the migrating cortical interneuron precursors derived from the CGE but not MGE (Ma et al. 2012). Although the *Sp8* loss of function severely affects the LGE-derived olfactory bulb granule cells and interneurons (Li et al. 2011; Waclaw et al. 2006), no obvious defects were found in CGE-derived neocortical interneurons, at least within the molecular expression profiles examined (Ma et al. 2012). We have recently carried out a microarray expression screen comparing the transcriptomes of MGE- versus CGE-derived interneuron precursors and found that the homeodomain factor *Prox1* is highly enriched in CGE-derived lineages. Preliminary studies on the conditional removal of *Prox1* indicate that this gene is specifically required for the proper development of CGE-derived neocortical interneurons (G.M., unpublished observations).

5.5 Interneuron Migration and Integration into the Cerebral Cortex

The process by which interneurons migrate and integrate into the neocortex can be divided into roughly three phases based on developmental transitions that are shared by all interneuron subtypes. Initially, as soon as interneuron precursors become postmitotic within the ventricular or subventricular zone of the ventral telencephalon, it is necessary for them to choose an appropriate route for their tangential migration into the developing neocortex (Fig. 5.2, left, red and blue routes). After reaching the neocortex, a large majority of interneuron precursors tangentially migrate through the intermediate zone (IZ), which is located below the cortical plate and above the cortical ventricular/subventricular zones (VZ/SVZ) (Fig. 5.4, embryonic) in order to reach the desired neocortical area (e.g., visual, auditory, barrel, motor, prefrontal). After reaching the appropriate area, interneuron precursors change their migration

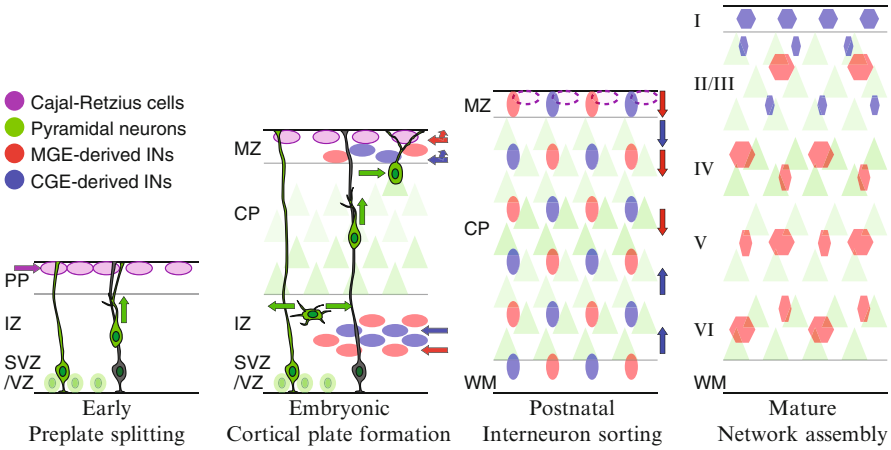

Fig. 5.4 Coordinated migration of excitatory and inhibitory neurons within the developing neocortex. Throughout development, distinct neuronal lineages show characteristic migration behaviors during the formation of a functional neocortical network. At very early stages (by E11.5), the cortical plate emerges from the invasion of newborn pyramidal cells in between the Cajal–Retzius (*purple*) and subplate (not shown) cells to split the preplate (PP). During embryonic stages, while pyramidal neuron precursors are locally generated in the subventricular and ventricular zones (SVZ/VZ) and migrate radially into the cortical plate (CP), they transiently become multipolar in the intermediate zone (IZ) and disperse in tangential directions. While a large majority of MGE (*red*)- and CGE (*blue*)-derived interneuron precursors tangentially migrate through the lower IZ in the lateral to medial direction, the ones that have reached the marginal zone (MZ) also migrate towards caudal directions. Note that the multipolar pyramidal cell precursors in the IZ, Cajal–Retzius cells in the MZ, and meninges above the MZ (not shown) secrete the ligand Cxcl12, in order to guide the tangential migration of interneuron precursors in two discrete streams. Within the postnatal cortex, interneurons undergo a highly dynamic radial migration phase to reach their proper laminar destinations. At this time, most of Cajal–Retzius cells disappear from the MZ. After the age of P7, the cell bodies of both pyramidal and interneurons are no longer migrating, allowing assembly of the neuronal network to proceed

mode from tangential to radial (Fig. 5.4, postnatal) to populate the appropriate neocortical layers and to begin their integration into the emerging neuronal network (Fig. 5.4, mature). Interestingly, recently it has been shown that the ratio between pyramidal neurons and interneurons is relatively constant at 4:1 over the course of mid-embryonic throughout postnatal developmental periods (Sahara et al. 2012), suggesting that integration of glutamatergic and GABAergic populations occurs in a synchronized manner during the assembly of the neocortex.

5.5.1 Initiation of Tangential Migration Towards the Developing Neocortex

Through the use of genetic tools to label GABAergic interneuron precursors (e.g., fate mapping with *Cre* driver lines for *Dlx*, *Nkx2-1*, *Lhx6*) or just by simply

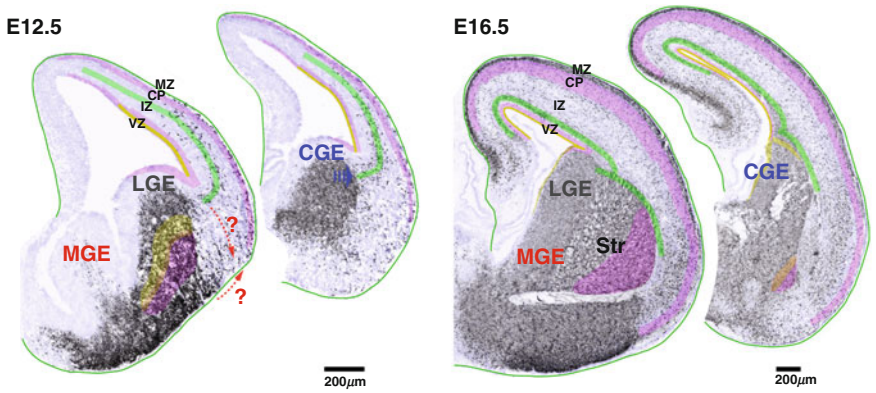

Fig. 5.5 Guidance cues for GABAergic neocortical interneuron migration. By combining the *Dlx5/6-Flp*e driver and *RCE:FRT* reporter lines, the vast majority of GABAergic neuronal precursors are labeled with EGFP (*pseudo-colored in black*) in coronal sections of the E12.5 and E16.5 telencephalon. Nuclear counter staining with DAPI is also presented *pseudo-colored in blue*. *E12.5*: Since the earliest cohort of cortical interneurons derived from the CGE is born at around this time, all of the interneuron precursors visualized in the cortex at this stage are derived from the MGE. Two clear pathways of cell migration can be observed in the intermediate (IZ) and marginal (MZ) zones of the cortex. It is still not known whether there is a direct migration from the ventral MZ to the cortical MZ (shown in *red dotted arrows*). Cxcl12 is expressed in multipolar pyramidal neuron precursors (*thick green lines*), Cajal–Retzius cells, and the meninges (together, *thin green lines*) and guides interneurons expressing Cxcr4/Cxcr7. Sema3A/Sema3F (*purple*) are expressed in the striatal mantle and cortical plate cells and prevent migrating interneuron precursors expressing Nrp2 (possibly Nrp1 as well) from entering these domains. Nrg1 is expressed in between the SVZ of the LGE and the striatal mantle (*yellow*) and the cortical VZ/SVZ (*yellow*). In addition, Nrg3 is expressed in the cortical plate (*purple*). The extent of overlap between the Nrg1 and Sema3A/3F (*yellow* and *purple*) in the striatal mantle is unconfirmed. Both Nrg1 and Nrg3 regulate cell migration through ErbB4 receptor expressed in interneuron precursors. *E16.5*: At this time, the cortical plate and the IZ in the cortex is proportionally thicker compared to E12.5. While at this time a large number of interneuron precursors are located in the MZ, the lower IZ stream is still highly visible. Nrg1 expression in the striatum (Str) is more restricted at E16.5 (Shimogori et al. 2010) than at E12.5. Earlier to this stage at around E14.5, additional populations of Cxcl12-expressing cells appear as a band of cells (*thick green lines*) extending from the corticostriatal boundary alongside the lateral edge of striatum. Notably, from this stage onwards, streams of interneuron precursors are observed in this Cxcl12-expressed domain, suggesting that Cxcl12 expression in this area guides interneuron migration in a similar manner to the cortical IZ and MZ streams

analyzing the patterns of genes selectively expressed in migrating interneuron precursors (e.g., *Dlx1*, *Dlx2*, *Lhx6*, *Gad1*), two streams of cells from the ventral eminences towards the cortex can be visualized as early at E12.5 (Fig. 5.5, left). One stream is located medially to the LGE and MGE, reaching the intermediate zone (IZ) of the neocortex, and the other curves laterally around the LGE towards the marginal zone (MZ) of the ventral eminences (Fig. 5.5, *Dlx* fate mapping with EGFP). Cell migration through the medial path has been well documented, and one of the earliest observations of this route was in coronal brain slices subsequent to dye labeling within the MGE (Anderson et al. 2001). However, to our knowledge,

direct evidence demonstrating that interneuron precursors migrate directly from the ventral MZ into the neocortical MZ has not yet been obtained (Fig. 5.5, left, dotted arrows). In several in vitro brain slice culture studies, labeled interneuron precursors migrating through the ventral MZ do not seem to enter the cortex directly through MZ, but instead dispersed at the IZ of the cortex (Marín et al. 2001; Zimmer et al. 2011). In fact, cell migration from the MGE to the ventral MZ is not generally observed in in vitro coronal slice culture experiments, possibly because the radial glial fibers that extend from the MGE ventricular zone to the pial surface (Malatesta et al. 2003; Remedios et al. 2007) are disrupted in slice preparations.

How does the medial migration stream in the ventral telencephalon form, and through which mechanisms are cells guided into this path? One of the key molecules is ErbB4, which is a receptor for Nrg (*Neuregulin*) ligands (Buonanno 2010; Rico and Marín 2011) and is robustly expressed in MGE-derived interneuron precursors (Flames et al. 2004; Yau et al. 2003). The membrane-bound form of Nrg1 (TypeIII) is expressed specifically in between the VZ/SVZ of the LGE and striatal mantle, forming a corridor-like structure and acting as a permissive factor for migrating MGE-derived interneuron precursors (Fig. 5.5, yellow). The other isoforms of Nrg1 (Type I and II) are secreted variants expressed in the cortical VZ/SVZ domains (Fig. 5.5, yellow), acting as chemoattractants for MGE-derived interneuron precursors (Flames et al. 2004). Consistent with the attractive role of Nrg1 signaling, loss of the *ErbB4* receptor decreases the migration of interneuron precursors from the MGE, resulting in a reduction of total interneuron numbers within the mature neocortex (Flames et al. 2004). Recently, it has been suggested that Nrg1 and Nrg3 can also act as repellents for ErbB4-expressing interneurons (Li et al. 2012). While Nrg1 is mainly expressed in the striatal mantle and the cortical VZ (Fig. 5.5, yellow), Nrg3 is expressed in the cortical plate, and both Nrg1 and Nrg3 were observed to inhibit ErbB4-expressing interneuron precursors from entering the Nrg-rich domains (Li et al. 2012).

In addition to attractant signaling cues, repellents play equally important roles in guiding the migration of interneuron precursors into the developing neocortex. Migrating MGE-derived interneuron precursors express Nrp2 (*Neuropilin 2*), which is a receptor for Sema3A and Sema3F (*Semaphorin 3A* and *3F*) ligands. Both Sema3A and Sema3F are expressed in the mantle of the striatum (Fig. 5.5, purple), and both act as repellents to block Nrp2-expressing MGE-derived interneuron precursors from migrating into the developing striatum (Marín et al. 2001). This has been shown through *Nrp2* loss of function or by mis-expressing a dominant-negative construct for *Nrp2* in MGE-derived interneuron precursors, and in both cases, manipulated cells do not migrate properly into the neocortex and instead populate the striatum. Moreover, while postmitotic maintenance of Nkx2-1 expression is important for MGE-derived striatal interneurons (e.g., cholinergic interneurons) to migrate into the mantle of LGE, it is crucial for tangentially migrating cortical interneuron precursors to downregulate Nkx2-1, which directly represses Nrp2 expression, in order to migrate into the neocortex (Nobrega-Pereira et al. 2008). Recently, CoupTFII has been shown to directly induce the expression of both *Nrp1* and *Nrp2*, thereby regulating the migration of cells from the CGE into the amygdala (Tang et al. 2012).

This raises the interesting possibility that Nkx2-1 and CoupTFII counteract each other in repressing and inducing *Nrp* expression (Fig. 5.3), respectively, for guiding the dorsal-MGE- and preoptic area-derived cells into the neocortex (Fig. 5.2).

How is Nkx2-1 expression differentially regulated in cortical versus striatal interneurons arising from the MGE? Recently, the transcription factor *Zeb2* (zinc finger E-box binding homeobox 2, also known as *Sip1* or *Zfhx1B*), was identified as a direct target of *Dlx1* and *Dlx2* (McKinsey et al. 2013) and was found to critically regulate dorsal migration of MGE-derived interneuron precursors into the neocortex (McKinsey et al. 2013; van den Berghe et al. 2013). Interestingly, *Nkx2-1* downregulation in postmitotic MGE-derived cortical interneurons does not take place in the absence of *Zeb2*, in a similar manner to *Dlx1/2* double mutants, and this leads to the accumulation of mutant cells within the striatum (McKinsey et al. 2013; van den Berghe et al. 2013). As noted above, downregulation of Nkx2-1 in postmitotic cortical interneurons is critically required for these cells to derepress *Nrp2* (Fig. 5.3), which then facilitates tangential migration towards the neocortex by repelling cells from the striatum (Marín et al. 2001; Nobrega-Pereira et al. 2008). In the *Zeb2* mutant, many marker genes that are expressed in migrating neocortical but not striatal interneurons, such as *Cxcr7*, are diminished, further reinforcing the idea that *Zeb2* regulates a number of signaling pathways that affect the responsiveness of cortical interneurons to migratory cues (McKinsey et al. 2013). Interestingly, *Zeb2* was found to repress the expression of the receptor *Unc5B*, whose ectopic expression in MGE-derived interneuron precursors decreased the proportion of cells entering the neocortex and whose knockdown could partially rescue the migration defects observed in the *Zeb2* mutant (van den Berghe et al. 2013).

In contrast to the interneuron precursors arising from the MGE, much less is known about the migration guidance mechanisms for CGE-derived interneuron subtypes. When we fate mapped temporally distinct cohorts of interneuron precursors derived from the CGE, we always observed cells initially migrating through the IZ of the caudal but not rostral part of the neocortex (Miyoshi et al. 2010). Consistent with this observation, it has been demonstrated that CGE cells are intrinsically predisposed to preferentially migrate in a caudal direction (Yozu et al. 2005). When labeled MGE or CGE cells are placed into the CGE, MGE cells were still able to disperse into all rostro-caudal directions, and not surprisingly, CGE cells were able to normally migrate in a caudal direction. However, when cells from the MGE or CGE are placed into the MGE, MGE-derived cells exhibit normal migration behavior, whereas cells originating from the CGE were not able to migrate out of the MGE (Yozu et al. 2005). Subsequently, this same group demonstrated that the intrinsic mechanisms that direct CGE cells to migrate caudally are at least partially controlled by the transcription factor *CoupTFII* (*Nr2f2*) (Kanatani et al. 2008). While knockdown of *CoupTFII* reduced the ability of CGE-derived cells to migrate caudally, transduction of MGE cells with *CoupTFII* allowed them to preferentially migrate in caudal directions when transplanted into the CGE (Kanatani et al. 2008). Interestingly, *CoupTFII*-expressing cells arising from the preoptic area (POA) (Cai et al. 2013) located ventrally to the MGE migrated in caudal directions in a similar manner to CGE-derived cells (Kanatani et al. 2008). Consistent with this

observation, it has been demonstrated that the MGE and POA, both of which are contained within the Nkx2-1-expressing territory of the ventral telencephalon, produce interneurons that migrate into the neocortex via distinct pathways (Zimmer et al. 2011). When MGE and POA cells are placed in the POA of an E14.5 brain slice, while MGE cells migrated medially through the LGE into the cortex, POA cells traveled via the pial surface and dispersed into the cortex at the corticostriatal boundary. It seems that the segregation of migration streams for MGE- and POA-derived interneuron precursors is at least partially mediated by bidirectional EphrinB3 and EphA4 signaling (Zimmer et al. 2011).

5.5.2 Tangential Migration Within the Neocortex

During their tangential migration within the embryonic neocortex, at E12.5, the most prominent migratory stream of interneuron precursors is observed within the lower part of the intermediate zone (IZ) right above the subventricular/ventricular zones (SVZ and VZ, Fig. 5.5). Many interneuron precursors are also observed in the marginal zone (MZ) after E12.5, and by E16.5 this route outnumbers the IZ stream (Fig. 5.5), indicating that there is either a lower IZ to MZ migration or a separate cohort of cells that reach the MZ directly in a delayed manner.

What signaling cues guide cortical interneuron precursors during their tangential migration? Interneurons express Cxcr4 and Cxcr7 (Tiveron et al. 2010), receptors for the secreted ligand Cxcl12 (*Cxcl12*, chemokine ligand 12, also called *SDF1*) that is expressed in three distinct non-GABAergic cell populations (Fig. 5.5, green lines). One population is the pyramidal neuron precursors that transiently express Cxcl12 in the lower IZ during their radial migration towards the cortical plate (Fig. 5.5, thick green lines). Interestingly, at this stage pyramidal neuron precursors can transiently adopt a multipolar morphology and disperse in a tangential direction (Noctor et al. 2004; Tabata and Nakajima 2003), and it has been shown that the proper regulation of this multipolar phase is critical for pyramidal neuron migration and development (Miyoshi and Fishell 2012; Ohshima et al. 2007; Pinheiro et al. 2011; Torii et al. 2009). It has been shown that Cxcl12 expression induced in multipolar pyramidal neuron precursors is critical for the guidance of GABAergic interneuron precursors (Li et al. 2008; Lopez-Bendito et al. 2008; Tiveron et al. 2006; Lysko et al. 2011). Interestingly, the induction of Cxcl12 expression depends on the activity of the transcription factor *Tbr2* (Sessa et al. 2010). Thus, within the lower IZ, two separate lineages of cells are migrating or dispersing in a tangential manner, with locally derived pyramidal neuron precursors actively providing guidance cues to migratory interneuron cohorts. The other two sources of Cxcl12 in the dorsal telencephalon are the meninges, located above and adjacent to the MZ external to the neocortex, and Cajal–Retzius cells in the MZ (Fig. 5.5, together shown in thin green lines). These Cxcl12 sources are important for retaining interneuron precursors in the MZ (Li et al. 2008; Lopez-Bendito et al. 2008; Lupo et al. 2006; Tanaka et al. 2009). In migrating interneuron precursors, Cxcr7 is necessary to stabilize Cxcr4

protein levels (Sanchez-Alcaniz et al. 2011), and thus loss of function of either gene results in similar defects in interneuron positioning in the mature neocortex (Li et al. 2008; Lopez-Bendito et al. 2008; Sanchez-Alcaniz et al. 2011; Wang et al. 2011).

Based on the general role of Cxcl12-mediated signaling in guiding interneuron precursor migration within the neocortex, we would like to propose the following hypothesis. Although at early stages (E12.5) Cxcl12 expression is mainly observed in the lower IZ of the neocortex, meninges and Cajal–Retzius cells, at around E14.5, a distinct population of cells expressing Cxcl12 appears between the striatum and lateral cortical areas, and interestingly, this population only mildly depends on the function of *Tbr2* (Sessa et al. 2010) (Fig. 5.5, green in E16.5). Coincident with this, a stream of interneuron precursors expressing such genes as *Cxcr7*, *Lhx6,* and *Dlx2* is observed in the ventrolateral region in a pattern similar to Cxcl12. Thus, it seems likely that this lateral population of Cxcl12-expressing cells, in a similar manner to the dorsal neocortical lower IZ, is providing guidance cues to attract GABAergic interneurons into the neocortex, which is expanding laterally and caudally as embryogenesis proceeds. Furthermore, the emergence of lateral Cxcl12-expressing cells at E14.5 coincides well with the appearance of CGE-derived interneuron precursors within the neocortex. Thus, we speculate that this lateral Cxcl12 expression facilitates CGE-derived interneuron precursors to enter into the neocortex, since they primarily migrate caudally (Yozu et al. 2005) and are not capable of migrating through the MGE SVZ.

As a repellent cue, in a similar manner to its role in deterring interneuron precursors from entering into the striatum (Marín et al. 2001), Sema3F expressed in pyramidal neurons (Fig. 5.5, purple) prevents tangentially migrating cells from entering into the cortical plate (Tamamaki et al. 2003). This is most likely the reason that cells splitting off from the Cxcl12-expressing lower IZ stream (Fig. 5.5, thick green lines) form another minor cell stream right beneath the cortical plate in the upper IZ (Fig. 5.5) (Polleux et al. 2002).

In summary, the neocortical lower IZ provides a major highway for interneuron precursors to tangentially migrate over long distances from the ventral MGE, CGE, and POA to their final destinations. While cells are continuously entering the dorsal neocortex and hippocampal area through this lower IZ path, many cells then disperse into the MZ where they can also move in rostro-caudal directions (Ang et al. 2003; Tanaka et al. 2009; Yokota et al. 2007) in addition to the lateral to medial route (Fig. 5.4, embryonic). By late embryonic and early postnatal stages, interneuron precursors have gathered in the MZ and the lower IZ and VZ/SVZ (Nadarajah et al. 2002) waiting for the right time to enter into the developing cortical plate by radial migration.

5.5.3 Radial Migration During Laminar Fate Determination

During the early postnatal period, interneuron precursors within the neocortex undergo a highly dynamic radial migration process in order to become allocated within specific layers (Hevner et al. 2004; Miyoshi and Fishell 2011; Yamasaki

et al. 2010; Baudoin et al. 2012; Higginbotham et al. 2012). It is well established that pyramidal neuron layers are generated in an inside-out manner, with later-born cells migrating radially through the earlier-born layers to populate more superficial layers (Franco and Muller 2013; Kwan et al. 2012; Leone et al. 2008; Lui et al. 2011; Martynoga et al. 2012; Molyneaux et al. 2007; Monuki and Walsh 2001; Nguyen et al. 2006; Rakic 2009). As we described above in Sect. 5.3, while MGE-derived interneurons generally populate the cortex in an inside-out manner similar to pyramidal cells, interneuron subtypes originating from the CGE target superficial layers irrespective of their birthdate. How does each interneuron subtype recognize its proper laminar location and through which migration paths do they achieve this positioning?

Evidence that interneuron positioning is determined based on the laminar identity of pyramidal neurons comes from studies on the Reelin pathway, in which mutations of either ligand (*Reln*) or receptor/adaptor (*Lrp8*, *Vldlr,* and *Dab1*) molecules result in the inversion of pyramidal neuron layers together with a failure in preplate splitting (Cooper 2008; Franco et al. 2011; Olson and Walsh 2002; Sanada et al. 2004; Tissir and Goffinet 2003). In this inverted neocortex, where early-born pyramidal neurons are located superficial to later-born ones, interneuron migration largely followed the layer inversion (Hammond et al. 2006; Hevner et al. 2004; Pla et al. 2006). Interestingly, while wild-type and *Dab1* mutant MGE cells showed similar layer distributions when transplanted into a wild-type cortex, in the *Dab1* mutant cortex, transplanted wild-type MGE cells followed the inversion of pyramidal neuron layers. Thus, the cortex seems to be providing critical migratory cues to interneuron precursors to guide their laminar selection. This idea is supported by experiments where the identity of specific pyramidal neuron layers was altered through manipulation of a laminar-specific transcription factor *Fezf2*, which is required for the specification of layer V cortico-fugal pyramidal neurons (Chen et al. 2005; Molyneaux et al. 2005). Loss of *Fezf2* results in the conversion of layer V pyramidal neurons into layer II/III-like cells, and consequently, MGE-derived interneurons, which preferentially target deep cortical layers, were dispersed towards superficial layers in the *Fezf2* mutant. Furthermore, ectopic relocation of layer II/III- or layer V-like cell aggregates in the white matter preferentially attracted CGE- or MGE-derived interneurons, respectively (Lodato et al. 2011a). Thus, layer-specific gene programs within pyramidal neurons appear to be providing critical cues that guide the migration of MGE- and CGE-derived interneurons into their correct laminar destinations.

How plastic is the laminar fate of newly born interneuron precursors? When MGE cells from early (E12.5) and late (E15.5) embryonic stages were transplanted into the late MGE, the donor cells from the two distinct time points were able to maintain their respective intrinsic laminar fates. However, when early and late MGE cells were transplanted into the early MGE, their respective laminar fates were now predominantly restricted to deep layers, with late-born MGE interneurons now behaving in a similar manner to early-born MGE cells (Pla et al. 2006). It is currently not known how the intrinsic laminar fate of late-born MGE cells is re-specified in the early MGE environment. However, this observation is very interesting since this temporal fate restriction of MGE-derived interneurons is the opposite to

what has been observed in pyramidal neuron precursors, where the laminar fates of later-born cortical neurons are already restricted and not affected by heterochronic transplantation, but early-born donor cells are able to adopt a later-born laminar fate (Desai and Mcconnell 2000; Frantz and Mcconnell 1996).

How segregated are the migration routes for MGE- and/or CGE-derived interneuron precursors that are destined to occupy distinct cortical layers? Intriguingly, although early (E12.5) and late (E15.5) MGE donor cells transplanted into the late MGE maintained their intrinsic laminar preferences and ultimately migrated to deep and superficial layers, respectively, they were both similarly distributed within the cortex two days after the transplantation, suggesting that their segregation into distinct layers occurs at later stages (Pla et al. 2006). We addressed this question by examining interneuron precursors born at the same time (E12.5) but arising from separate lineages (MGE versus CGE) that are destined to occupy distinct layers (deep versus superficial, Fig. 5.1, top left) (Miyoshi and Fishell 2011). We found that age-matched E12.5 MGE- and CGE-derived interneuron precursors reached the cortical IZ after 2 days, showed similar distributions in migration routes within E16.5 cortex, and surprisingly, occupied all cortical layers in a comparable manner at P1. Only after this time did we begin to observe distinct laminar distributions of the E12.5 MGE- and CGE-derived interneurons, suggesting that layer preferences for each lineage manifest themselves during radial sorting within the cortical layers at postnatal stages (Fig. 5.4, postnatal). Moreover, to our surprise, later-born E16.5 CGE-derived interneurons showed a similar sorting pattern to that observed for the earlier-born E12.5 population. This finding indicated that CGE-derived interneuron precursors occupy similar layers irrespective of birthdate as a result of a synchronized radial sorting process that occurs within the cortical plate during early postnatal development (Miyoshi and Fishell 2011).

Once interneuron precursors have reached their correct laminar location, what kind of mechanisms are involved in the termination of radial migration? When stimulated by ambient GABA, the GABA receptors expressed on the cell surface of interneuron precursors open up and chloride ion (Cl^-) flows inward or outward, dependent on the internal Cl^- concentration, leading to inhibition or excitation of the cell, respectively (Ben-Ari 2002; Owens and Kriegstein 2002). It is known that at later developmental stages, interneuron precursors initiate Kcc2 (K/Cl transporter, *Slc12a5*) expression to decrease the intracellular Cl^- concentration and thus become inhibited by GABA. Prior to synaptogenesis, Kcc2 upregulation is necessary and sufficient to reduce the motility of MGE-derived interneuron precursors (Bortone and Polleux 2009). Moreover, the reason that interneuron motility is almost abolished by the end of the first postnatal week seems to be due to Kcc2 upregulation (Inamura et al. 2012). Conversely, within the marginal zone, the random walk behavior exhibited by interneuron precursors (Tanaka et al. 2009) is disrupted by Kcc2 expression (Inada et al. 2011).

So, the switch in GABA signaling from excitatory to inhibitory plays a pivotal role in the cessation of interneuron precursor motility. Consistent with this observation, when cell excitability is decreased by the expression of the inward rectifying channel Kir2.1 (Cancedda et al. 2007; Yu et al. 2004) before the age of P3,

CGE-derived interneuron precursors ended up in ectopic laminar locations (De Marco Garcia et al. 2011). More interestingly, within the three classes of CGE-derived interneuron subtypes, only RELN-expressing and CR/VIP co-expressing but not VIP (without CR) single populations were affected by this manipulation. Thus, the motility of VIP (without CR)-expressing interneurons is likely regulated in an activity-independent manner, and, in fact, only this population lacked the expression of the Rac activator protein, Elmo1 (De Marco Garcia et al. 2011). Within the early postnatal neocortex, distinct patterns of network activity sequentially take place over development: initial calcium spikes (E20, in rat), followed by synchronous plateau assemblies (SPA) mediated through gap junctions (P0), early network oscillations (ENO) (P3), and synapse-driven giant depolarizing potentials (GDP) (P7) (Allene et al. 2008). In order to achieve these sequential waves of distinct network activities in the hippocampus, some of the earliest-born GABAergic interneurons that possess widespread axonal arbors already at early postnatal stages play pivotal roles as "hub" cells for coordinating network activity (Bonifazi et al. 2009; Picardo et al. 2011). It is intriguing to speculate that, in a similar manner to the hippocampus, there is an early-born interneuron population with widespread axonal arbors within the neocortex that is regulating local activity patterns in a manner that guides the radial migration of later-born counterparts.

5.5.4 Migration of Hippocampal Interneuron Precursors Through the Neocortex

Consistent with what has been observed in the neocortex, hippocampal interneurons were almost completely missing in *Dlx1/2* double mutants and were severely reduced in *Nkx2-1* mutants (Pleasure et al. 2000), suggesting that they are generated from the MGE and CGE. In fact, while in vivo cell transplantation studies (Nery et al. 2002; Wichterle et al. 2001) have indicated that hippocampal interneurons are derived from both the MGE and CGE, studies utilizing in vitro brain slice culture demonstrated that MGE-derived interneurons reach the hippocampal area by migrating through the neocortex (Pleasure et al. 2000; Polleux et al. 2002). While this is the case for MGE-derived populations, since CGE cells have a propensity to migrate in caudal directions, they can directly enter and populate the hippocampus without passing through the neocortex (Yozu et al. 2005). Supporting this notion, while interneurons are first observed reaching the neocortex at E12.5 and E14.5 for each MGE- and CGE-derived population, respectively, they both simultaneously appear in the hippocampus at E15.5 (Tricoire et al. 2011). Recently, the origins of hippocampal interneurons have been reexamined by utilizing the novel genetic fate-mapping tools described above, and very interestingly, the proportion of interneuron subtypes within each lineage (MGE and CGE) between the neocortex and hippocampus was found to be quite distinct (Tricoire et al. 2010; Tricoire et al. 2011). Briefly, the MGE and CGE provided proportionally larger numbers of nNOS- and CCK-expressing interneurons, respectively, into the hippocampus

compared to the neocortex. These populations could have been specifically increased in the hippocampus by direct migration from their origins and by avoiding the entrance into the neocortex. In a similar manner to the subpopulation of CGE-derived interneurons (Yozu et al. 2005), some MGE-derived populations may directly migrate into the hippocampus via a caudal route, similar to the preoptic area-derived populations, which are known to exhibit caudal migration (Kanatani et al. 2008). In fact, substantial numbers of hippocampal interneurons appear to originate from the preoptic area (Gelman et al. 2009). Conversely, this further indicates that in order to precisely understand the integration process of interneurons into the neocortex, it will be necessary to develop a strategy to distinguish hippocampal versus cortical interneuron precursors while they both are tangentially migrating through the developing cortex.

5.6 Novel Concepts in Neocortical Interneuron Development

Several conceptual advancements for understanding the developmental processes of neocortical interneurons have been made recently. In a similar manner to olfactory bulb GABAergic interneurons and granule cells, a population of neocortical interneurons labeled by the *Htr3ABAC-EGFP* reporter was observed to arise from the subventricular zone during early postnatal stages (Inta et al. 2008). A report that a few ventrally derived interneuron precursors proliferate while tangentially migrating through the neocortex (Wu et al. 2011) suggests that this mechanism, previously observed in primates (Letinic et al. 2002), similarly exists in rodents. Very surprisingly, the group of Alvarez-Buylla has found that around 40 % of neocortical interneurons are eliminated during postnatal stages after P5 (Southwell et al. 2012). This elimination process was through *Bax* (Bcl-2-associated X)-dependent apoptosis and the timing for death was intrinsically regulated and did not depend on the environment. Clearly, further studies are needed to characterize the role of programmed cell death in cortical interneuron development as well as in other cell populations that only transiently exist during development, such as Cajal–Retzius (Caronia-Brown and Grove 2011) and subplate cells.

A tremendous amount of diversity is now well appreciated in neocortical GABAergic interneurons, and in fact, one of the most interesting questions at present is how subtype-specific synapse formation takes place onto discrete cellular subdomains, such as soma, dendritic tufts, or axon-initial segments (Fig. 5.1, right). Considering their extensive migration history, it is remarkable how precisely interneuron precursors are able to polarize in the proper orientation and then form the appropriate connections to integrate into the neuronal network. With the latest sets of genetic tools such as the mGRASP method to visualize the formation of specific synapses (Kim et al. 2012; Yamagata and Sanes 2012), Syp (*Synaptophysin*) fusion indicator proteins for visualizing presynaptic termini and cell polarization (Li et al. 2010) and transsynaptic viruses for circuit tracing (Beier et al. 2011; Wickersham et al. 2007), we are increasingly able to tackle this fundamentally

important question that will provide deep insights into the biological significance of interneuron diversity.

Finally, we would like to recommend a number of earlier reviews on cortical interneuron biology for additional reading (Batista-Brito and Fishell 2009; Corbin and Butt 2011; Cossart 2011; Gelman et al. 2012; Hernandez-Miranda et al. 2010: Huang et al. 2007; Lehmann et al. 2012: Marín 2012; Marín and Rubenstein 2003; Marín et al. 2010; Metin et al. 2006; Tanaka and Nakajima 2012; Wonders and Anderson 2006).

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Chapter 6
Regulation of Cortical Circuit Formation

Fernanda M. Rodríguez-Tornos, Beatriz Cubelos, and Marta Nieto

Abstract The complex functions of the mammalian neocortex depend on the formation of precise networks and subnetworks among its many neuron types during development. These networks are formed in a stereotyped manner that creates a reproducible human cortex and facilitates common human behavior. The accuracy and complexity of cortical circuitry predicts that the developmental mechanisms that direct each of these neurons to connect with its siblings must be precise. In recent years, remarkable advances have been made in our understanding of the several developmental mechanisms that direct cortical connectivity, but we still know only a fraction of the coordinated events and molecular elements involved. An additional difficulty is that the intricate connectivity and physiology of these circuits is far from being definitively untangled. Much of the knowledge comes from relatively simple animal models, such as rodents, ferrets, and cats. Relevant information is also derived from the study of human genetic conditions that affect intellectual capabilities. This chapter briefly describes the connectivity of excitatory neurons of the cerebral cortex, which integrate and transmit information among neocortex regions and to other regions of the brain. We will try to give an extended overview of the mechanisms that shape this connectivity during development, with special emphasis on implications in humans.

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6.1 Regulation of Cortical Circuit Formation

The mammalian neocortex is a complex, highly organized structure that contains hundreds of different neuronal cell types and diverse types of glial cells (Guillemot 2007; Molyneaux et al. 2007). It is the most anterior part of the telencephalon and is responsible for sensory perception, high cognitive functions, and consciousness; as such, it has undergone pronounced expansion during evolution, with maximal representation in the human cortex (Selzer 1990). The complex cortical functions rely on the formation of precise networks and subnetworks among the many neuron types during development. These networks form in a stereotyped manner able to create a reproducible human cortex and to facilitate common human behavior. Certain cortical circuits are also preserved among species throughout evolution, while new circuits and functions have been added to the more primitive existing structures (Innocenti 2011; Molnar 2011).

The accuracy and complexity of cortical circuitry predicts that the developmental mechanisms that direct each of these neurons to connect with its siblings must necessarily be precise. Several processes are conserved during evolution, and certain mechanisms are added or modified to create new networks that expand the cognitive capabilities of the cortex. Remarkable advances have been made in recent years in our understanding of these mechanisms and their spatial and temporal coordination, but we still know only a fraction of them. An additional difficulty is that the intricate connectivity and physiology of these circuits is far from being definitively untangled. Much of the knowledge comes from relatively simple animal models, including mice, which have a lissencephalic (smooth) cortical surface, whereas the close resemblance and evolutionary distance of the gyrencephalic brain of ferrets and cats provide excellent tools for deciphering processes exclusive to higher mammals. Relevant information is also derived from the study of human genetic conditions that affect cognitive capabilities, such as schizophrenia, autism, micro- and macroencephaly, and other syndromic and non-syndromic forms of mental retardation (Clowry et al. 2010; Manzini and Walsh 2011). In the near future, the field will benefit from the use of these approaches combined with new technologies and computer modeling to make a decisive step forward.

Neurons of the cerebral cortex can be classified into two broad classes, excitatory and inhibitory neurons. Inhibitory GABAergic, locally connecting neurons are born in the basal telencephalon and have modulatory functions. Excitatory neurons are of dorsal origin and are pyramidal neurons (most abundant) and spiny stellate excitatory interneurons of layer IV. Pyramidal neurons are projecting neurons; some extend their axons to distant subcortical and subcerebral targets, and others project to local and distant intracortical targets (Selzer 1990). This chapter will focus mainly on the connectivity of excitatory neurons, which integrate and transmit information between different neocortex regions and to other regions of the brain (subcortical targets).

6.2 General Structure of Cortical Connectivity

The cerebral cortex is a laminated structure, and each lamina or layer contains neurons with similar morphologies, connectivity patterns (Selzer 1990), and molecular identities (Molyneaux et al. 2007) that originate sequentially during development from radial precursors (Caviness et al. 1996; Takahashi et al. 1996; Heins et al. 2002; Malatesta et al. 2003; Hansen et al. 2010). The number of layers, their thickness, cell composition, and architecture varies throughout the tangential surface of the cortex and among the different functionally specialized areas. The neocortex, and by extension most of the cortex, is composed of six layers, numbered I to VI, which show further expansion and subdivisions in human. Most sensory information is routed to the cerebral cortex from the thalamus (Selzer 1990) and is conveyed to extracortical targets via corticofugal projections (Fig. 6.1). Nevertheless, the vast majority of cortical neuron connections are from one cortex region to another (intracortical) rather than to subcortical targets, allowing complex processing and integration (Fig. 6.2).

Cortical connectivity can be visualized in a simple scheme that reflects its hierarchical organization and the mechanism of origin during development. Radial interlaminar connectivity establishes the most essential intracortical circuit, the so-called cortical columns (Fig. 6.1). These columns, composed of neurons from different

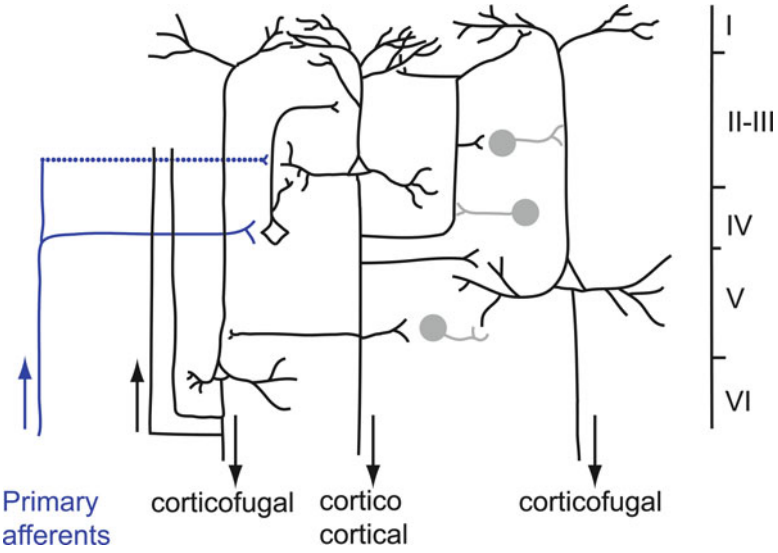

Fig. 6.1 The cortical column. Scheme showing the connectivity of a column in the somatosensory cortex. The precise connectivity of columns shows some variations on this general pattern among functional areas. *Circular grey cells* represent inhibitory interneurons; *diamonds* indicate excitatory interneurons in layer IV

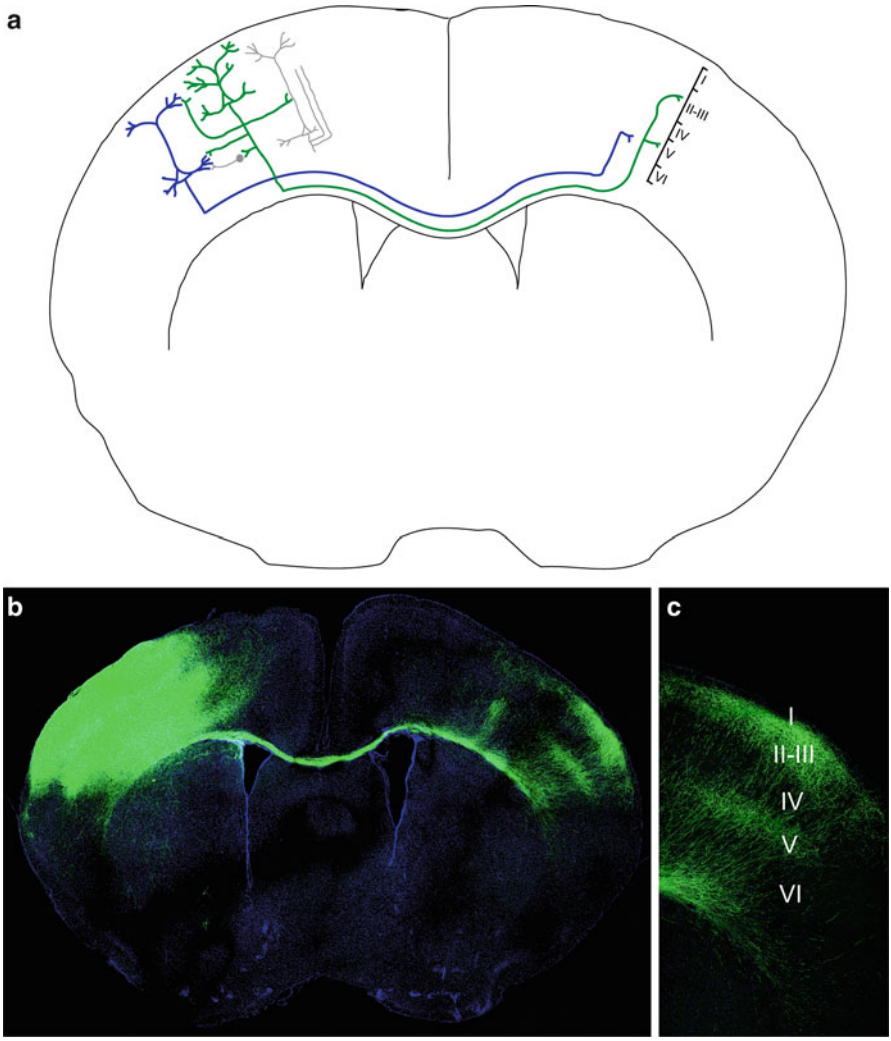

Fig. 6.2 The corpus callosum. (**a**) Myelinated axons of the CC project from neurons in layers II and III (~80 % in mouse) and in layer V (~20 %), and a very small population from cells in layer VI (not shown). (**b**) Confocal micrographs showing somas and CC axons of GFP-expressing layer II–III neurons in the P21 cortex. Neuronal morphology was analyzed at P21 after in utero electroporation at E15.5. Axons of CC neurons projecting from layers II–III invade the cortical plate at homotypic areas (six layered cortex), where they branch and profusely innervate layers II–III and V (**a** and **b**). (**c**) Layer specific pattern of innervation in the contralateral site. Magnification from **b**

layers, were described from early electrophysiological recordings in the sensory cortex demonstrating that neurons inside the column respond with similar activity to precise stimuli. The thalamic input is preferentially distributed vertically in columns to superficial and deep layers, rather than horizontally (Mountcastle et al. 1957). In the sensory cortex, neurons in a cortical column all process sensory

information from the same peripheral location and submodality (Feldmeyer et al. 2013). These studies were extended in the visual cortex by Hubel and Wiesel, who showed among other things that innervation of the visual cortex from the two eyes is also organized in columns (ocular dominance) and discovered orientation columns (Hubel and Wiesel 1962, 1963). Columnar organization is also the result of the common precursor foundation and the migration mode of cortical pyramidal neurons during development (Rakic 1988; Heins et al. 2002; Malatesta et al. 2003; Torii et al. 2009; Jones and Rakic 2010).

Columns communicate tangentially through laminar connectivity, essentially through layers II–III and V, to form the functionally specialized areas of the cortex generally classified as sensory, motor, and association areas (Rakic 1988). In the adult, the transition from one neocortical area to another can be defined by differences in cytoarchitecture, gene expression patterns, input projections, and by the specific mode of connections between neurons of the column. These properties determine the physiology and connectivity of specific circuits to allow the functional specializations that distinguish areas. For example, the somatosensory area in the mouse is defined by a thicker layer IV, expression of markers such as Rorβ, input from the whiskers and barrel formation. Finally, areas are interconnected, facilitating integration and complex behavior. Interhemispheric commissural axons permit information exchange between the cerebral hemispheres, whereas other axons that do not cross the midline, but run along the anterior posterior axis, connect areas from the same hemisphere.

In essence, the mechanisms that control cortical circuit formation during development select axon pathways and influence formation of dendritic structures and synapses, as will be discussed below. Studies in recent years have shown remarkable coordination between intrinsic molecular programs that specify neuronal cell identity and those regulating their connectivity. In the last two decades, numerous studies have reported examples of transcription factors (TF) expressed only by selected neuronal subtypes that regulate discrete aspects of connectivity (Hevner et al. 2001; Molnar et al. 2003; Jacobs et al. 2007). The pattern of overlapping functions of these TF creates cell diversity and acts as a genetic code that encrypts the rules that govern cortical networks. These intrinsic programs regulate fundamental aspects such as neurotransmitter expression, cell morphology, and the ability to respond selectively to external cues, including soluble factors and membrane-bound molecules. These mechanisms are discussed separately in this chapter. Finally, during postnatal stages, experience- and activity-mediated mechanisms involved in plasticity ultimately shape the circuits and give rise to the final stereotypical networks (Metin et al. 1997; Molnar and Cordery 1999).

6.3 Corticofugal Neurons

Projection neurons extend their axons to distant subcortical targets to transmit information to other brain regions. They are located mainly in the deeper layers of the cerebral cortex and are generally referred to as extracortical projection neurons or

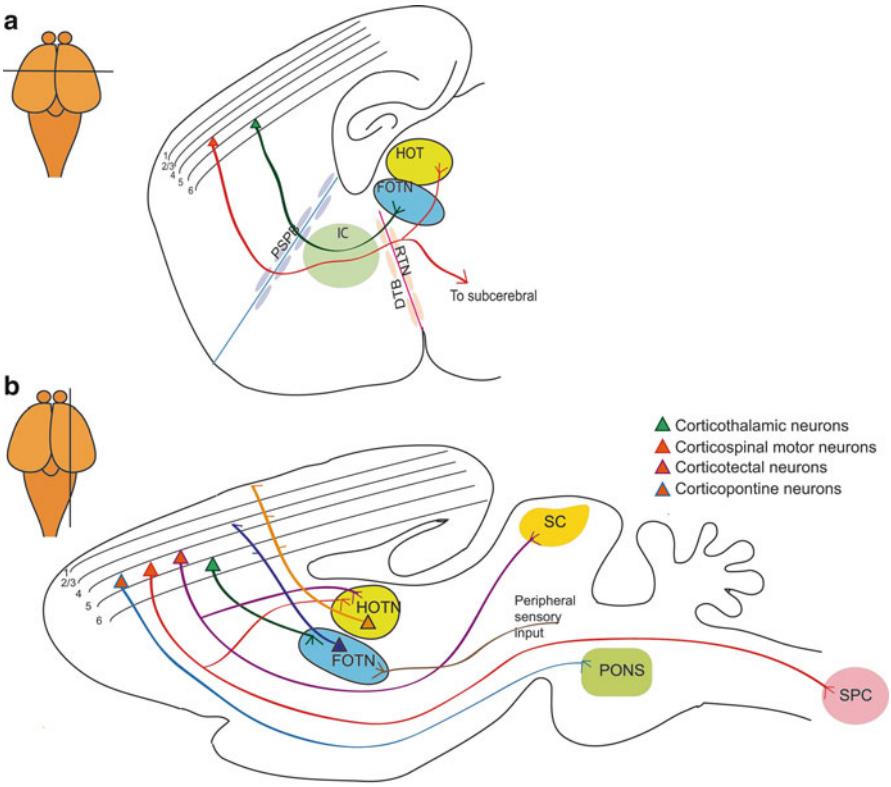

Fig. 6.3 The development of corticofugal axons. Scheme of the axonal pathway of corticofugal neurons of cortex layers V and VI. The different anatomic and genetic regions these axons encounter are depicted. Coronal (**a**) and sagittal views (**b**). *DTB* dorsal telencephalic boundary, *FOTN* first order thalamic nuclei, *HOTN* high-order thalamic nuclei, *PSPB* pallial-subpallial boundary, *RTN* reticular thalamic nuclei, *SC* superior colicullum, *SPC* spinal cord

corticofugal neurons, and are subdivided in *subcerebral* and *corticothalamic*. *Subcerebral* projection neurons reside mostly in layer V and innervate different parts of the brain stem and cerebellum, as well as the higher-order thalamic nuclei (HOTN) through secondary collaterals (Fig. 6.3). The neurons of the HOTN relay cortico-cortical information by projecting excitatory fibers to layers I, IV, and VI of a cortical area distinct from that from which they receive input. Subcerebral projecting layer V neurons can be subdivided into three major subpopulations, *corticotectal*, *corticospinal*, and *corticopontine. Corticotectal* neurons are located in the visual cortex; they send their primary axon to the superior colliculus and secondary collaterals to the rostral pons. *Corticospinal* motor neurons reside in the sensorimotor area of the cortex; they send primary projections to the spinal cord and secondary collaterals to the striatum red nucleus, caudal pons and medulla. Finally, *corticopontine* neurons are in charge of transmitting information to the pons (Molyneaux et al. 2007) (Fig. 6.3).

Corticothalamic neurons are located in layer VI and enable cortical processing of peripheral data. They project axons to and receive input from the first-order thalamic nuclei (FOTN) (Fig. 6.3). These nuclei receive peripheral sensory input and relay it to layer IV and VI neurons. Corticothalamic primary axons generate numerous small synapses with thalamic neurons, providing signals for peripheral information. Corticothalamic neurons projecting from layer VI primary visual cortex (V1) send axons to the dorsal lateral geniculate nucleus (dLGN); those in the auditory cortex (A1) project to the medial geniculate nucleus (MGN) and those in the primary somatosensory cortex (S1), to the ventrobasal nucleus (VB). The axon collaterals of these neurons innervate the reticular thalamic nucleus (RTN).

6.3.1 Development of Corticofugal Tracts

Development of corticofugal tracts follows a complex process by which distinct neuron subpopulations innervate specific extracortical regions in a temporal pattern with characteristic axon outgrowth kinetics. The subset of TF expressed by each neuron confers a unique identity, essential for its connectivity pattern and behavior. This identity would nonetheless be worthless in the absence of long- and short-range guidance cues that follow spatiotemporal dynamics. The development of corticofugal tracts is also closely associated with thalamocortical tract formation, since axons that form both tracts establish the physical association necessary to guide each other and to complete their development. Considerable controversy nonetheless remains regarding the relative importance of this interaction and of other intrinsic and extrinsic mechanisms. This will not be discussed here in detail, as the reader can find many complete reviews (Cang et al. 2005; Torii and Levitt 2005; Rash and Grove 2006; Rubenstein 2011).

The preplate contains the first subsets of cortical differentiated neurons and gives rise to Cajal-Retzius and to subplate cells. The latter are the first cortical neurons to extend their axons into the internal capsule, the natural path to extracortical territories. These initial projections act as a scaffold for subsequent corticofugal axons; the majority will disappear in the early postnatal period, correlating with a wave of cell death that eliminates their somas (Hevner et al. 2001; Jacobs et al. 2007). Neurons that form permanent connections between the cortex and extracortical regions will begin to extend their neurites at around embryonic day (E)10. Depending on their location and identity, their axons take a lateral, medial, rostral, or caudal trajectory, and grow until they reach the region adjacent to the lateral internal capsule. The distinct populations arrive at this zone at slightly different times between E13 and E15.5, depending on the position of their somas; the lateral fibers are the first to arrive and the dorsally derived fibers, the last (Fig. 6.3a). At this point, temporal synchronization requires axons to align in order to continue their journey together. The first incoming axons await the arrival of the others before continuing growth; this is termed the first waiting period. All the axons then cross the pallial-subpallial boundary (PSPB) and enter the internal capsule. The PSPB is a major boundary that

expresses a very specific subset of TF (high Pax6, null Emx1, Dlx1). This territory has modulatory potential, making early corticofugal projections turn sharply from their original ventrolateral to a medial trajectory, to cross the subpallium. The internal capsule is the site at which early corticofugal axons emitted from the subplate and thalamocortical projections first meet and establish a close interaction that will be maintained throughout the intermediate zone, PSPB, and the lateral sector of the internal capsule; this interaction is needed for guidance (Hevner et al. 2001, 2002; Lopez-Bendito et al. 2007; Chen et al. 2012; Grant et al. 2012) (23).

Once the axons exit the internal capsule, they arrive at the diencephalon-telencephalon boundary (DTB), where they enter the prethalamus and encounter the cells of the perireticular nuclei (PRN) and RTN at E16 (Fig. 6.3a). The extension will undergo a second pause that lasts until E17.5 (second waiting period). At this time, corticofugal projections continue through different pathways (Fig. 6.3a, b). Layer V primary axons continue to grow and cross the cerebral peduncle to the brainstem and spinal cord. Layer VI primary axons and layer V collaterals change direction to enter the thalamus, a process that takes several days and results in postnatal innervation of most thalamic nuclei. In higher mammals, this correlates with the functional establishment of behaviors associated with the relevant sensory systems; somatosensory and motor functions mature before visual and auditory functions. For example, in mice, somatosensory ventrobasal and motor ventrolateral nuclei are innervated earlier (E18.5 and P0.5) than auditory MGN and visual dLGN, which are not fully innervated until postnatal day (P)8 (O'Leary and Koester 1993; Metin et al. 1997; Molnar and Cordery 1999; Molnar et al. 2003; Jacobs et al. 2007; Grant et al. 2012; Lickiss et al. 2012).

One of the most fascinating characteristics of layer V and VI axons is therefore that they must navigate through several distinct territories until they reach their target. This requires dynamic recognition of territory-specific signals and modulation of axon responses. It has become apparent that several neuron populations and their axons, such as the thalamic afferents discussed above, provide structural support essential for crossing these anatomic regions and their boundaries. Pioneer axons are those of neurons (in this case, subplate neurons) that, thanks to their intrinsic electrical activity, can navigate without the help of preexisting axons and pave the way for follower axons. Voltage-gated ion channels, which in subplate neurons are voltage-gated K+3.4 (Kv3.4), are responsible for the intrinsic electrical activity patterns of neurons, and are thus necessary for corticofugal development (Huang et al. 2012). The corridor cells, a population derived from the lateral ganglionic eminence, also illustrate these cooperative interactions. These cells are needed to generate a permissive substrate for cortical axon growth across the medial ganglionic eminence (MGE). The axon guidance functions of corridor cells overlap with the guidance and sorting functions of PRN neurons, thought to have a role in directional change in the internal capsule (Lopez-Bendito et al. 2006; Grant et al. 2012).

Following spatiotemporal dynamics, axons respond differently to distinct sets of cues in the environment they traverse. These specific behaviors enable correct navigation and innervation of their targets. These guiding factors include intrinsic factors at the neuron that emits the axon (e.g., cell surface receptors or molecules that

influence intracellular signaling) as well as extrinsic factors (membrane-bound and soluble factors presented or secreted by intermediate or final targets); the latter act at short and long range, and affect growth cone extension as well as orientation by generating repulsive or attractive responses. Soluble molecules often establish concentration gradients critical for precise axon guidance of corticofugal neurons.

6.3.2 Guidance Factors and Receptors that Direct Corticofugal Axons

Although gaps remain in our knowledge, several families of guidance molecules are known to determine the trajectory of corticofugal axons. We summarize a series of illustrative examples. The semaphorin family provides early context-dependent cues. Pioneer explant experiments showed that Sema3A expression in the most superficial cortical plate, the marginal zone (MZ), is responsible both for repelling axons toward the VZ (Polleux et al. 1998) and for attracting apical dendrites (Polleux et al. 2000). Further complementary studies demonstrated that combinations of Sema3 molecules have a specific effect on the corticofugal axon pathway. For example, in addition to the superficial cortical plate, Sema3A is expressed throughout the ventricular zone and lower subventricular zone, and Sema3C is expressed in the intermediate and the subventricular zones. Although cortical axons are exposed to Sema3A and Sema3C concurrently, Sema3A has a repulsive effect that overrides Sema3C attraction, even at very low concentrations. As a result, corticofugal axons grow over the corridor generated at the intermediate zone and the upper SVZ, where Sema3C is expressed alone (Ruediger et al. 2012). Likewise, Sema5B is expressed in many regions of the corticofugal pathway, including the ventricular zone and the ventrolateral cortices, and inhibits axon entry into these territories (Bagnard et al. 2001; Lett et al. 2009). Sema molecules bind to neuropilins, whose expression and differential association with plexins also critically modulate cortifugal axon responses and dynamics (Pasterkamp 2012). Several pathways involving Sema signaling alone can thus explain many of the corticofugal axon turns and trajectories.

Netrin-1 is expressed in the internal capsule and mediates long-range attraction of corticothalamic axons at E12.5–13.5. The attractive effects of netrin-1 can induce axon turning and thus appears to be responsible for corticofugal growth cone reorientation toward the ventral telencephalon. Slit1 and 2 have a major role in corticothalamic and thalamocortical axon guidance within the ventral telencephalon and diencephalon, mainly through binding to Robo1 and Robo2 receptors, which appear to have partially redundant functions. In Robo mutant mice, and more markedly in Robo1 and Robo2 double mutants, corticothalamic axons do not grow through the internal capsule but are aberrantly directed to cross the midline. In addition, Robo1 (but not Slit) appears to act as a slowing signal, since both corticothalamic and thalamocortical axons grow faster in Robo1 knockouts (ko) than in WT mice. This deceleration might be relevant in the developmental control of the temporal

dynamics of these tracts, specifically in the regulation of the two waiting periods (Andrews et al. 2006; Lopez-Bendito et al. 2007; Grant et al. 2012).

Finally, the EphA family of tyrosine kinase receptors and their ligands are essential for the initial establishment of corticothalamic targeting. Neocortical neurons express an EphA7 gradient that controls the topography of corticothalamic projections, through local interactions within individual thalamic nuclei. Other EphA proteins, such as EphA5, also have a role in the correct patterning of corticothalamic and thalamocortical wiring (Sestan et al. 2001; Cang et al. 2005; Torii and Levitt 2005; Torii et al. 2013).

Further studies are needed to better delineate the elements that determine corticofugal connectivity. As these neurons are characterized by their long-distance journeys, the challenge is not only to understand what these signals are and how they are transduced, but also the nature of the spatiotemporal mechanisms that regulate them.

6.4 The Formation of Intracortical Circuits

6.4.1 The Development of Callosal Projecting Neurons

Interhemispheric connections are essential components of intracortical circuits and contribute to the integration ability and high associative function of the mammalian brain. The corpus callosum (CC) and the anterior commissure formed by axons of layer V are the main commissures that connect the hemispheres. The CC is the major commissural track of the mammalian brain. Partial or total CC agenesis is associated with many human developmental syndromes that affect the brain (Fame et al. 2011). Most myelinated axons of the CC project from neurons in layers II and III (~80 % in the mouse) and in layer V (~20 %), and a very minor population from cells in layer VI. A number of callosal neurons also send axonal collaterals to the same hemisphere (ipsilateral) and communicate cortical areas. There are also dual connections to the contra- and ipsilateral striatum. Axon guidance cues and synaptic maturation mechanisms that target callosal neurons and their projections are critical in the development of this important cortical circuitry.

In the several steps of axon routing involved in CC formation, different glial and neuronal cells act as intermediate guideposts and present secreted and membrane-bound navigation signals. Defects in hemisphere fusion cause partial or total CC agenesis; fusion occurs simultaneously as callosal neurons are born, just before they extend their axons, and is necessary for axons to cross the midline. Early studies showed that CC axons are guided across the cerebral midline by a glial population, then termed sling-like glial and now known as the glial sling. These astroglial populations form a bridge-like structure at the midline between the two lateral ventricles (Hankin et al. 1988; Silver et al. 1993). It was shown early on, that in acallosal mice midline crossing could be restored postnatally when this glial scaffold was

presented artificially (Silver and Ogawa 1983). More recent observations in mice and humans nonetheless show that many neurons are also present within the glial slings (Shu et al. 2003a; Ren et al. 2006). Semaphorin 3C expression in one of these transient neuronal populations helps to attract callosal axons to and through the midline (Niquille et al. 2009). Additional glial structures in the CC are considered relevant for axon navigation, including radial glial cells in the glial wedge (GW) and astrocytes in the indusium griseum (IG) (Shu and Richards 2001; Shu et al. 2003b). In the developing CC, GW-expressed Slit2 guides callosal axons to the corticoseptal boundary (Bagri et al. 2002; Shu et al. 2003c). Robo receptors bind to Slit proteins; callosal axons express Robo1, and mice deficient in this protein (*Robo1*$^{-/-}$) have defects in CC formation (Shu and Richards 2001; Andrews et al. 2006; Lopez-Bendito et al. 2007). Once axons cross the midline, the same signal repels them from this boundary (Bagri et al. 2002; Shu et al. 2003c). Other long-range molecules such as Wnt are necessary for the guidance of callosal axons. Wnt5a is expressed by the GW and the IG cells, and stimulates both outgrowth and repulsion of developing callosal axons via Ryk receptors (Keeble et al. 2006; Li et al. 2010). Other signals such as ephrins and their receptors (EphA5, EphB1 and EphrinB3) act at a shorter range and are essential not only for callosal formation, but also have a broader effect on other commissures (Mendes et al. 2006; Lindwall et al. 2007).

CC formation is also highly dependent on the earlier extensions emitted by a population of pioneer callosal neurons. This is the earliest neuron population to extend axons across the midline, at around E17 in the mouse. The cell bodies of these neurons are located in the most medial part of the cortical plate and the cingulate cortex, and their axons appear to guide the neocortical callosal projections (Koester and O'Leary 1994; Rash and Richards 2001; Fame et al. 2011). Short-range signals such as neuropilin 1 (Nrp1) regulate crossing of these early axons (Hatanaka et al. 2009; Piper et al. 2009).

Callosal axons initiate their journey guided by this plethora of signals. After midline crossing, they travel along the CC; they make a sudden turn in their trajectory and invade the contralateral cortical plate at homotypic areas. Little is known about the mechanisms that trigger this turn, but it might imply changes in axon capacity to respond to cortical cues, similar to those that occur when they cross the midline. Recognition of the correct contralateral territories might also imply recognition of lateral gradients at the cortical plate, although these mechanisms remain unclear.

Axons are able to branch and extend many synapses along their length, which allows neurons to send information to various cells simultaneously. Callosal axons branch at several points during their trajectory; most branches profusely innervate layers II–III and V in the ipsilateral and contralateral columns (Fig. 6.2), although some neurons (termed dual projecting) also send collaterals to other areas and regions. Despite their probable importance in human cognition, the patterns of these branched connections are not fully resolved, although they are likely to be responsible for certain associative properties of the cortex. For example, an undetermined number of callosal projecting neurons from the sensory cortex simultaneously extend exuberant projections to the contralateral homotypic cortex and to both

contralateral and ipsilateral areas of the motor cortex. Laterally located superficial neurons can also extend dual axons toward the midline and the internal capsule, although in the latter case, they apparently retract at P11 (Garcez et al. 2007). Similar schemes of dual projections are found in certain callosal neurons of the motor cortex, which send dual axonal projections to sensory areas (Mitchell and Macklis 2005). In mice, these dual projections show maximum numbers at P8; they are refined until approximately P21, probably through activity-dependent mechanisms, but many persist into adulthood (Innocenti and Price 2005; Mitchell and Macklis 2005). Little is currently known of the molecular control of these double connections.

6.4.2 Factors that Regulate Selectivity of the Synapse: From Intra-Columnar and Intra-Laminar Connectivity to Microcircuits

Based on the work discussed above, it is clear that scientists have successfully identified several crucial regulatory mechanisms responsible for delivering axons to the vicinity of their targets. After this arduous journey, however, only half the job is done. Axons do not establish synapses without a pattern. The nervous system shows considerable specificity at this level, and connections are made only with certain neurons; there is even selection of specific cell compartments. This is extreme in the case of cortical circuits, which implicate hierarchical organization in layers: axons selectively establish connections with certain layers, certain cells within the layers, and even choose between apical or basal dendrites. The cellular and genetic mechanisms responsible for the assembly of specific connections in the nervous system are the subject of intense study. These mechanisms involve coordinated expression of homophilic adhesion molecules by both pre- and postsynaptic partners, including the diverse cadherins and immunoglobulin superfamily (IgSF) proteins. Repulsive signals also prevent abnormal innervation (Shen and Scheiffele 2010; de Wit et al. 2011).

Few of the mechanisms known to select synaptic targets in other parts of the nervous system have been reported or tested in the cortex; there is an intriguing relative lack of knowledge about the elements that implement the beautiful patterns of cortical laminar connectivity. Barrels, which are prominent sensory units in the rodent somatosensory cortex, have been examined in detail. Data suggest that the initial gross formation of the barrel map relies on molecular cues, while refinement of its topography depends on neuronal activity. Temporal and cell-specific expression of cadherins contributes to the barrel-like distribution of thalamic axonal inputs into layer IV (Huntley and Benson 1999; Inan and Crair 2007). The development of excitatory synapses between axons emitted from layer II–III neurons with dendrites in layers II–III and V, but not those in layers IV and VI, is another perfect paradigm of layer-specific synaptic organization. Activity has a role in determining the relative innervation of layers II–III and V by contralateral CC afferent connections. Reduced firing results in increased innervation of superficial layers at the expense

of layer V innervation (Mizuno et al. 2007). Recent work identified an unexpected molecular regulators of innervation of layers II–III and V in Shh, a secreted molecule known mainly for its patterning and axon guidance effects, and in its high-affinity receptor Brother of CDO (Boc) (Okada et al. 2006). The restricted Shh expression in layer V promotes synaptic formation with Boc-bearing axons; these axons are precisely those of neurons in layers II–III. Genetic manipulation of mice showed that conditional Shh deletion in the dorsal telencephalon mimics Boc ko phenotypes of layer V neurons. Boc-depleted layer V neurons show reduced dendritic complexity, spine density and synaptic strength as a result of decreased innervation from layer II–III callosal projecting neurons (Harwell et al. 2012). Although alteration of activity or the Shh-Boc pathway did not result in layers being ectopically innervated, these studies open the path to understand layer-specific connections and the possible implications of other patterning molecules in cortical wiring, perhaps in conjunction with activity.

These studies of synaptic specificity mechanisms are also extremely important when considering the existence and formation of microcircuits and subnetworks embedded within cortical circuits. There is cellular and molecular heterogeneity not only between layers and cortical areas, but also within the neurons of the same layer (Fame et al. 2011); this results in the expression of different membrane and secreted proteins that might contribute to generating networks in the cortex. In layers II–III, microcircuits have been described functionally by the characterization of neuron firing patterns (Burgalossi et al. 2011). They have also been identified genetically, through visualization of GFP-labeled neurons that express high c-fos levels, and are highly interconnected, as shown by electrophysiology studies (Yassin et al. 2010). Common neuronal birth origin might be implicated in the formation of these microcircuits and in columnar formation. A common progenitor increases the probability of synapse between neurons, the probability to form strong electrical coupling with each other rather than with adjacent non-sister excitatory neurons, and the likelihood of producing similar excitatory responses (Yu et al. 2009, 2012; Li et al. 2012).

6.4.3 The Regulation of Dendritic Structures

Another facet of the regulation of cortical circuitry is the modulation of postsynaptic structures: dendrites, spines, and synapses. Dendritic branching specifies connectivity with selected axonal input and determines neuron morphology (Shen and Scheiffele 2010). Morphology in turn influences the way information is processed, amplifies or silences presynaptic input depolarization signals (Mainen and Sejnowski 1996), and even affects plasticity (Feldman 2012). Spine density and spine morphology determine the number, strength, and stability of synaptic contacts (Tada and Sheng 2006; Edbauer et al. 2010; Shen and Scheiffele 2010).

Developmental mechanisms that target regulation of postsynaptic structures and compartments have considerable importance in cortical function and circuit modulation, and are critical for the acquisition of higher intellectual abilities. Alterations

in dendritic morphology and in spine number and structure are defects that often correlate with cognitive disorders and mental retardation (Tada and Sheng 2006; Bourgeron 2009; Jan and Jan 2010; Kulkarni and Firestein 2012). Many of the mechanisms involved in the control of dendritic structures and synapses were thus identified during the study of human intellectual disabilities, including autism and fragile X syndrome, the most frequent cause of mental retardation. Analysis of human mutations linked to autism often shows alterations in genes that regulate the cytoskeleton and synaptic scaffold (Segal 2001); this is the case of Shank proteins (Bourgeron 2009), kalirin (Penzes and Remmers 2012), and mutations that affect the Ras/Epac2 pathway (Srivastava et al. 2012). Autism-related genes also appear to target postnatal mechanisms of plasticity and synaptic refinement. Human mutations linked to fragile X syndrome (Zhang et al. 2001) affect *FMR1*, a gene that encodes the RNA-binding protein FMRP (fragile mental retardation protein), which regulates transport and local translation to axons and dendrites (Tada and Sheng 2006; Napoli et al. 2008; Boda et al. 2010; Darnell et al. 2011; Penzes et al. 2011; van Bokhoven 2011; De Rubeis et al. 2012).

Human and mouse genes that encode TF also control dendrite and synapse development. In mice, *Mef2a* controls activity-dependent dendritogenesis (Fiore et al. 2009) as well as activity-dependent spine deletion (Flavell et al. 2006), which involves downstream use of FMRP (Pfeiffer et al. 2010). *Neurog2* regulates early neuritogenesis and alters neuron migration via phosporylation of the small GTPase Rnd2 (Hand et al. 2005), and by forming a DNA-binding complex with the LIM-only protein LMO4 (Asprer et al. 2011). Calcium signals and calcium-binding TF such as CREB are also involved in migration and dendritogenesis in the cortex (Redmond et al. 2002; Redmond and Ghosh 2005). Of the several cortical layer-specific TF described so far, the expression in mice of *Fezf2/Zfp312* in layer V neurons (Chen et al. 2005) and of *Cux1* and *Cux2* in layers II-IV regulate dendrite formation, and also synaptogenesis in the case of Cux proteins (Chen et al. 2005; Cubelos et al. 2010). *Cux* TF functions might be linked to evolution; the number of superficial layers in mammals expands together with brain volume and is maximal in humans (Hill and Walsh 2005). This correlates with the fact that upper layer neurons participate in highly associative circuits and tasks, and show an extreme degree of interconnectivity. It is thus possible that *Cux* optimize these neurons to increase their connectivity and their capacity to integrate information.

In a similar conceptual line, the two human-specific duplications of *SRGAP2* are proposed to be a delay mechanism for synaptic maturation which expands the temporal window of neonatal plasticity in humans. Mice bear one copy of the SRGAP2 gene, while humans have three alleles (A, B, and C). In the mouse neocortex, *SRGAP2* promotes spine maturation and limits spine density. The human *SRGAP2B* and *SRGAP2C* duplications are partial and encode truncated forms that dimerize with the ancestral SRGAP2 (SRGAP2A) protein. Surprisingly, this dimerization inhibits normal SRGAP2 function. Thus, experiments in mice show that ectopic expression of hSRGAP2C phenocopies SRGAP2 deficiency; in both cases, mice have abundant, immature long spines. These findings suggest that inhibition of SRGAP2 function by its human-specific paralogs has contributed to evolution of the

human neocortex (Charrier et al. 2012). In sum, these studies suggest that specific mechanisms that target dendritic structures and synapses contribute to the evolution of cerebral cortical circuits and the definition of human intellectual capacity.

6.5 Molecular Identity of Cortical Neurons: Layer and Area Identity as Determinants of Connectivity

Molecular identity is broadly defined by the subset of genes expressed by each neuron. Subtype-specific TF ultimately determine the molecular identity of neurons by initiating and maintaining specific genetic programs. Expression of these TF is often interconnected through gene expression cascades (Molyneaux et al. 2007; Leone et al. 2008; Fame et al. 2011). Neuron identity programs are initiated early in dividing cells by progenitor-specific TF and passed on to neuronal progeny through expression of the same or other subtype-specific TF (Molyneaux et al. 2007; Leone et al. 2008; Fame et al. 2011). Because laminar organization of the cortex coincides with the segregation of neuron subpopulations, many of the TF that specify neuronal identity have been identified as layer specific (Fig. 6.4). In the last two decades, genetic studies in mice have shown how several of these layer-specific TF modulate

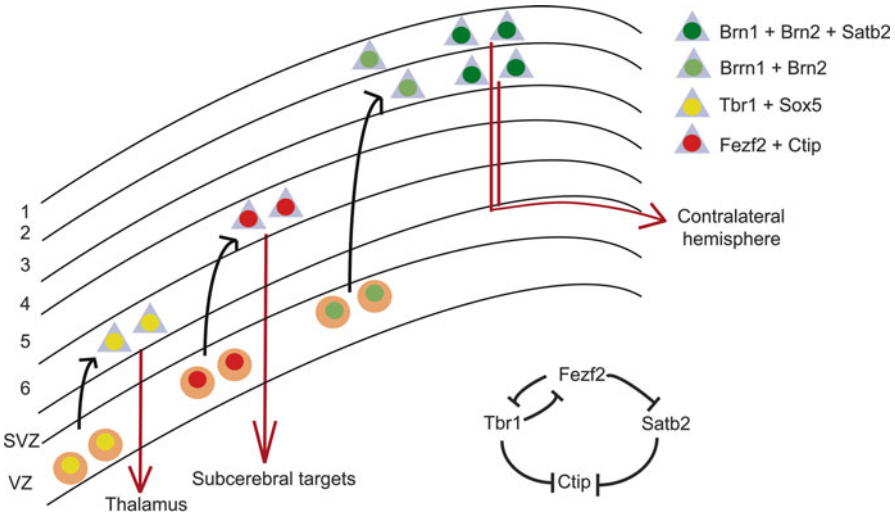

Fig. 6.4 The molecular identity of cortical neurons. Molecular identity is defined by the subset of TF expressed by each neuron. Many of the TF that specify neuronal identity have been identified as layer-specific factors. Neuron identity programs are initiated early in dividing cells by progenitor-specific TF and passed on to neuronal progeny through expression of the same or other subtype-specific TF. This identity determines the connectivity pattern of these neurons. Schematic representation of reported molecular and genetic interactions that inter-regulate the expression of subclass-specific TF

different aspects of connectivity during development and indicate that they are related to almost every process the neurons undergo. This is a fascinating and dynamic field, as indicated by the ongoing identification of genes essential for determination of each neuron's fate and behavior and, thus, its connectivity. The most recent studies clearly established that there is even further molecular diversity within the layers, which could explain the instructive signals that direct formation of cortical circuits and microcircuits.

It is increasingly apparent that there are many TF genes common to all projection neurons, which would explain the common pattern of initial development. A smaller group of TF defines closely related subtypes of projection neurons and an even smaller group is characteristic of each neuron population (Arlotta et al. 2005; Molyneaux et al. 2007; Leone et al. 2008). Most studies analyze the phenotypes of neurons with loss and gain of function of specific genes. More research is needed to fully understand the specification of all these neuron subtypes and the molecular mechanisms underlying their integration into selected circuitries. We can nonetheless begin to define some mechanisms that are quite illustrative of the extreme importance of the TF selective mode of control.

6.5.1 Transcription Factors in Lower Layers

Sox5, *Ctip2* (*COUP-TF*-interacting protein 2), and *Tbr1* expression patterns selectively mark distinct subtypes of corticofugal populations (Fig. 6.4). Subplate neurons express an intermediate level of *Sox5*, high *Tbr1*, and low *Ctip2* levels; corticothalamic neurons in layer VI express *Sox5* and *Tbr1* strongly and little *Ctip2*, and subcerebral projection neurons in layer V show high *Ctip2* levels, intermediate *Sox5*, and little *Tbr1*. These expression patterns prompt the hypothesis that these proteins form a coregulatory network that governs the adoption of neuronal fates (Fig. 6.4) (Arlotta et al. 2005; Molyneaux et al. 2007).

Tbr1, a T-box family TF gene, is expressed soon after cortical progenitors begin to differentiate (Fig. 6.4). It is found at high levels in early-born neurons of the preplate and layer VI and is necessary for their correct differentiation, as it is for cortical laminar organization and guidance of cortical afferent and efferent axons (Bulfone et al. 1995; Hevner et al. 2001). Several studies suggest that its functions overlap partially with those of *Sox5*, although defects in *Tbr1* ko mouse cortex are more severe. In the absence of *Tbr1*, the corticothalamic tract disappears and there is greater upregulation of neuronal markers than in *Sox5* ko mice. Chromatin immunoprecipitation and luciferase assays demonstrated that Tbr1 binds to and inhibits *Fezf2* promoter (McKenna et al. 2011).

Studies of *Sox5* ko mice and of its overexpression demonstrate that *Sox5* is critical for generation of diversity in extra-cortical projecting neurons, as it regulates and coordinates timing of sequential emergence of the different corticofugal neuron types (subplate, corticothalamic, and subcerebral) during early corticogenesis.

Sox5 expression is essential for correct differentiation of corticothalamic and subplate neurons, and blocks premature emergence of subcerebral neurons. When *Sox5* is absent, subplate and corticothalamic neurons locate to more superficial areas, while subcerebral neurons accumulate within layer VI and the white matter. This is interpreted as an anomalous overlap in the generation of the three principal corticofugal neuron subtypes. In addition, in the *Sox5* ko mouse cortex, subplate neurons aberrantly express molecular hallmarks and connectivity patterns of subcerebral projection neurons, resulting in the appearance of additional subcerebral projection tracts. Differentiation of corticothalamic neurons is imprecise, and that of subcerebral projection neurons is accelerated. In contrast, *Sox5* gain of function at later stages of corticogenesis causes reemergence of neurons with corticofugal features (Lai et al. 2008).

Ctip2 is one of the molecular targets of *Sox5* that is upregulated in the subplate of Sox5 ko mice (Lai et al. 2008). *Ctip2* is also a major downstream effector of *Fezf2*; it is expressed at high levels in layer V corticospinal and corticotectal neurons, and at much lower levels in layer VI corticothalamic neurons. *Ctip2* expression begins once neurons reach the cortical plate and is not implicated in early specification of cortical precursors (Arlotta et al. 2005). *Ctip2* participates in directing the extension, fasciculation, and refinement of subcerebral axonal projections, particularly the ability of corticospinal neurons to extend projections to the spinal cord during formation of the corticospinal tract. Thus, *Ctip2* ko axons fail to extend past the pons to reach the spinal cord (Arlotta et al. 2005; Lickiss et al. 2012).

Fezf2 represses callosal neuron identity, is sufficient for specification of layer V subcortical projection neurons, and is needed for layer VI neuron maturation (Rouaux and Arlotta 2010). *Ctip2*- and *Fezf2*-null mice have very similar phenotypes. In *Fezf2* ko mice, the corticospinal tract disappears; corticotectal and pontine projections are also greatly reduced; inappropriate new projections appear instead (Chen et al. 2005; Molyneaux et al. 2005). In *Fezf2* ko mice, *Ctip2* expression is absent, whereas forced expression of *Fezf2* by in utero electroporation induces upregulation of *Ctip2* in neurons that would not normally express it (Chen et al. 2005, 2008). This suggests that these two genes might act in a common pathway and that *Fezf2* is a key upstream regulator of corticospinal projection neuron differentiation.

Although the genetic regulatory pathways of the TF described above are relatively well characterized, there are many other TF that define lower layer identities or are involved in axon extension and pathfinding. *Otx1* is expressed in 40–50 % of subcerebral neurons, primarily those of the visual cortex, as well as by a number of cells in layer VI; it is essential for development of the corticotectal projection neurons and controls the refinement and pruning of their axon collaterals (Weimann et al. 1999). *Opn3* is a marker of layer V and *Foxp2* of layer VI. *Er81* is expressed in layer V cortico-cortical and subcerebral projection neurons; *Nfh* and *Pou3f1* are expressed primarily in layer V subcerebral projection neurons (Frantz et al. 1994; Ferland et al. 2003; Hevner et al. 2003; Voelker et al. 2004; Arlotta et al. 2005).

6.5.2 Transcription Factors in Superficial Layers

Several TF define the molecular identity of the superficial layers. We will mention some that exemplify distinct roles in neuron differentiation. *Brn1* and *Brn2* are two POU domain transcriptional regulators expressed in superficial cortical neurons and are necessary for correct migration and cortical lamination (McEvilly et al. 2002; Sugitani et al. 2002). Genetic loss of *Brn1* and *Brn2* in mice thus abrogates the appearance of late-born superficial neurons (Sugitani et al. 2002). Other TF directly implement programs that regulate connectivity. *Satb2* (AT-rich sequence-binding protein 2) is a chromatin-remodeling TF expressed in a broad subset of layer II–III neurons and in a smaller subpopulation of layer V neurons. Loss of *Satb2* expression in mice results in agenesis of the corpus callosum and reorientation of axons toward subcortical targets through the internal capsule. This abnormal wiring scenario is explained by the observation that *Satb2* represses expression of *Ctip2*, which regulates corticofugal identities; *Satb2*-deficient neurons also have other molecular features of corticofugal projecting neurons (Alcamo et al. 2008; Britanova et al. 2008). An epigenetic regulator, the proto-oncogene *Ski*, cooperates with *Satb2* for callosal axon guidance (Baranek et al. 2012).

Cut-like homeobox proteins *Cux1* and *Cux2* also mark layers II–III and IV specifically (Nieto et al. 2004; Zimmer et al. 2004). As mentioned above, in cortical layers II–III, both genes regulate dendritogenesis, spine formation, and synaptogenesis in a non-redundant manner and act in combination to specify the final dendritic tree and the synapses of these neurons (Cubelos et al. 2010). *CUX2* also defines the upper layers of the human cerebral cortex (Arion et al. 2007), and a possible association of *CUX1* polymorphisms with failure of antidepressant response is reported (Sasayama et al. 2012). Additional TF, including Id2, act as markers of the molecular identity of superficial layers. The functions of *Bhlhb5*, which marks superficial layers but is also found in layer V, are described below.

6.5.3 Area-Specific TF

Neocortical areas are characterized by unique molecular profiles and cyto-architecture, which ultimately reflect specific modes of axonal and dendritic connectivity. A strong deterministic function of TF expressed in the progenitor pools was demonstrated in relation to cortical area formation. Four murine TF, *Coup-TFI* (Armentano et al. 2007; Faedo et al. 2008), *Emx2, Pax6* (Bishop et al. 2000; Mallamaci et al. 2000), and *Sp8* (Sahara et al. 2007), all of which are expressed in gradients across the embryonic cortical axis, determine cortical area sizes and positions by specifying or repressing area identities within cortical progenitors. Early expression of area-specific progenitor TF is modulated by morphogens and signaling molecules secreted by patterning centers that are positioned at the perimeter of the dorsal telencephalon. These centers generate graded TF expression in cortical progenitors. Two major patterning centers are the commissural plate, which expresses *Fgf8* and

Fgf17, and the cortical hem, which expresses *Bmps* and *Wnts* (O'Leary and Nakagawa 2002). Progenitor area-specific TF also interact genetically, thus modifying the expression of one another; for example, *Pax6* and *Emx2* are mutually exclusive (Bishop et al. 2000; Mallamaci et al. 2000). There is interplay between intrinsic genetic mechanisms and extrinsic information conveyed by thalamocortical input to the cortex, especially to layer IV. The relative contribution of each of these early mechanisms to area formation is still debated, and has been reviewed extensively (O'Leary et al. 2007; O'Leary and Sahara 2008).

Expression of progenitor area-specific TF can be downmodulated (*Emx2*, *Pax6*) or maintained in postmitotic neurons (*Coup-TFI*). Area-specific TF generally inhibit or promote expression of other area-specific genes including *Cadherin8, Eph* receptors and other layer-specific TF such as *Satb2, Rorβ*, and *Id2* (O'Leary et al. 2007; O'Leary and Sahara 2008). *Coup-TFI* is expressed as a gradient and, during corticogenesis, is needed to maintain the balance between frontal/motor and sensory areas (Armentano et al. 2007). This factor temporally inhibits generation of corticospinal motor neurons, which in large numbers characterize motor areas (Tomassy et al. 2010), and regulates axon outgrowth as well as the formation of the CC and other brain commissures (Armentano et al. 2006), and governs neuronal migration (Alfano et al. 2011).

Arealization is closely linked to the identity of the postmitotic neurons. Moreover, certain layer-specific TF have a role in this process. *Bhlhb5* is selectively expressed in layers II–IV and V and regulates area identity; during embryonic development, it shows a transient high caudomedial to low rostrolateral gradient. It is gradually downmodulated in the postnatal brain to produce a sharp boundary between sensory and caudal motor cortices around P4, and practically disappears at P14. *Bhlhb5*-null mice show aberrant expression of layer-specific markers and disorganization of vibrissal barrels, and those layer V corticospinal motor neurons of the motor cortex that normally express this TF also show aberrant development (Joshi et al. 2008).

Our picture of arealization mechanisms is still incomplete. Fortunately, considerable research is ongoing to further our understanding of this process. These studies include the contribution of other TF expressed in postmitotic neurons to area specification and how they might coordinate with the action of thalamocortical input, as well as with activity and experience. Unraveling circuit formation in the cerebral cortex will help us to comprehend the precise modes of connections in the cortex and that are altered in many human conditions that affect cognition, from mental retardation to neurodegeneration.

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Chapter 7
Neocortical Neurogenesis and Circuit Assembly

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Abstract The mammalian brain is an extraordinarily complex organ. The outermost part of the brain is the cerebral cortex, which plays a key role in higher-order brain functions, such as perception, language, and decision-making. Since the invention of Golgi staining, which allowed for visualization of individual neurons, defining neural circuits underlying various brain functions has been a field of intense study for over a century. In this chapter, we will discuss the formation of neocortical circuits, emphasizing on how individual components are generated and assembled during development and how early developmental processes, including neurogenesis and neuronal migration, may guide precise circuit assembly.

The mammalian cerebral cortex is composed of the archicortex (hippocampal region), the paleocortex (olfactory cortex), and the neocortex. As the evolutionarily newest addition, the neocortex is the site of higher brain function. It contains two primary types of neurons: glutamatergic neurons (70–80 %) and GABA (γ-aminobutyric acid)-ergic neurons (20–30 %). Glutamatergic neurons release glutamate as neurotransmitter, which elicits excitation in the postsynaptic neuron, and are the principle neurons in the neocortex responsible for generating circuit output. GABAergic neurons, on the other hand, release GABA as neurotransmitter, which usually triggers inhibition in the postsynaptic neuron, and are critical for shaping circuit output.

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7.1 Excitatory Neuron Production, Migration, and Laminar Organization

Glutamatergic excitatory neurons are generated by progenitor cells residing in the proliferative zone of the dorsal telencephalon during embryonic development (Fig. 7.1a). As the precursor to the central nervous system, the neural tube is composed of a single layer of neuroepithelial cells (NE) proliferating via symmetric divisions to expand the progenitor pool (Breunig et al. 2011). This leads to thickening of the wall of neural tube and the formation of pseudostratified neuroepithelium that exhibits interkinetic nuclear oscillation during cell cycle (Sauer 1934). A small fraction of NEs undergo asymmetric division to produce the first wave of postmitotic neurons that migrate out radially to form a transient structure called the preplate (Marin-padilla 1970; Marin-Padilla 1971, 1978; Del Río et al. 2000). As development proceeds, NEs transform into a more fate-restricted progenitor type referred to as the radial glial cells (RGC). RGCs display a characteristic bipolar morphology with a short apical process that reaches the luminal surface of the ventricular zone (VZ) (i.e., the ventricular end foot) and an elongated process that extends basally to the pial surface (i.e., the radial glial fiber). With their unique long radial processes, RGCs were initially described to be the scaffold supporting neuronal migration in the developing cortex (Rakic 1971, 1972). In the past decade, extensive genetic and imaging studies have demonstrated that these cells represent a major population of neural progenitors, giving rise to nearly all excitatory neurons in the cortex, directly or indirectly (Anthony et al. 2004; Fishell and Kriegstein 2003; Malatesta et al. 2000, 2003; Miyata et al. 2001; Noctor et al. 2001, 2004). It is generally believed that RGCs first undergo symmetric division to expand the progenitor pool and then switch to asymmetric division where they self-renew and simultaneously produce daughter cells that are either postmitotic neurons or intermediate progenitor cells (IPC). IPCs then undergo additional rounds of symmetric division in the subventricular zone (SVZ) to produce neurons (Kowalczyk et al. 2009; Noctor et al. 2004). Toward the end of neurogenesis, RGCs either undergo terminal symmetric neurogenic division or switch to gliogenesis to generate astrocytes or oligodendrocytes (Fishell and Kriegstein 2003; Götz and Huttner 2005; Magavi et al. 2012).

Recently two additional types of neuronal progenitor cells were described in the developing cortex: short neural precursors (SNP) and outer subventricular zone radial glial progenitor (oRGs). Morphologically, SNPs maintain their ventricular end feet, but their basal processes are of variable length (Gal et al. 2006), and unlike RGCs, they generate neurons directly in the VZ instead of going through IPCs (Stancik et al. 2010). On the other hand, oRGs maintain the basal processes but lack the apical processes and are capable of undergoing asymmetric division in the outer subventricular zone. They were initially discovered in human and later found in primate, ferret, mouse, and other species (Fietz et al. 2010; Hansen et al. 2010; Kelava et al. 2012; Shitamukai et al. 2011; Wang et al. 2011). Notably, the abundance of oRG population has been suggested to underlie the evolutionary expansion of the cortex from mouse to human (Lui et al. 2011).

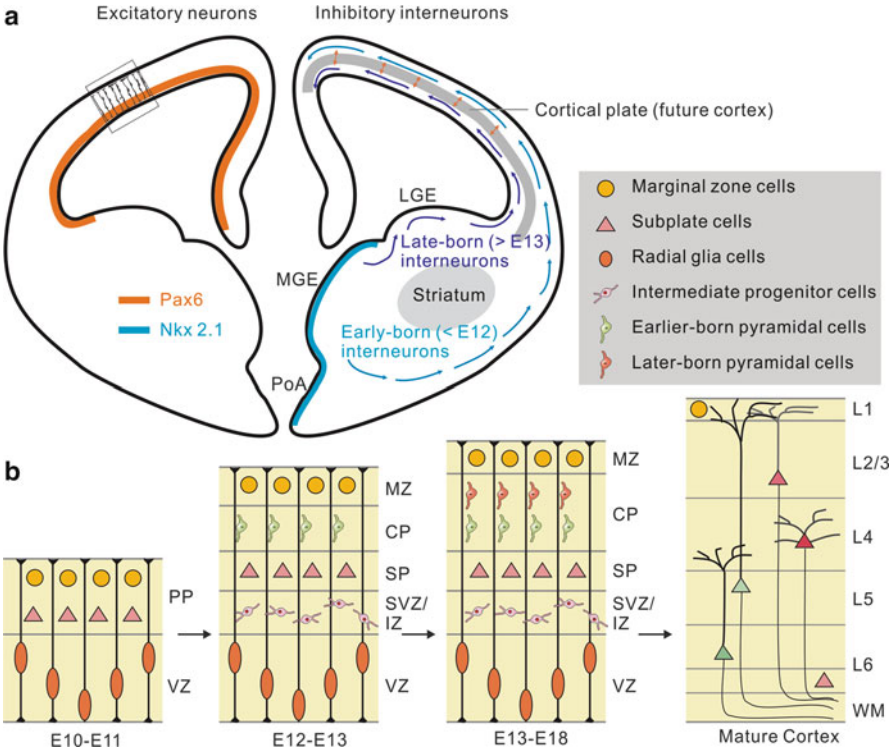

Fig. 7.1 Generation and migration of neocortical excitatory and inhibitory neurons. (**a**) Cortical excitatory neurons are generated from progenitor cells (Pax6+) residing in the VZ of the dorsal telencephalon. Newborn excitatory neurons undergo radial glial fiber-guided radial migration and settle into the developing CP. Cortical inhibitory interneurons are mainly generated from progenitor cells (Nkx2.1+) located in the proliferative zone of the ventral telencephalon, mainly MGE and CGE. A small population of cortical inhibitory interneurons is produced from PoA. Newborn inhibitory interneurons follow two tangentially oriented migratory streams to enter the cortex: a superficially migrating early cohort (before E12 in the mouse) mainly migrates through the MZ (*light blue arrows*); a deeply migrating second and more prominent cohort after E13 migrates predominantly through the lower IZ/SVZ (*dark blue arrows*). Upon reaching the cortex, they switch to radial migration and settle into their final laminar position in the CP (*orange arrows*). (**b**) Inside-out fashion of cortical layer formation. In early stages of neurulation (E10–E11), the neural tube is composed of a single layer of NEs. A small fraction of NEs undergo asymmetric division to generate the first wave of postmitotic neurons that migrate out radially and form the PP. As development proceeds, newborn excitatory neurons split the existing PP into a superficial MZ and a deeper SP (E12–E13). Successive waves of newly generated excitatory neurons migrate past the existing neurons to occupy more superficial region in the CP (E13–E18), creating the adult pattern of a six-layered cortex. *VZ* ventricular zone, *CP* cortical plate, *MGE* medial ganglionic eminence, *LGE* lateral ganglionic eminence, *PoA* preoptic area, *IZ* intermediate zone, *SVZ* subventricular zone, *NE* neuroepithelial cells, *PP* preplate, *MZ* marginal zone, *SP* subplate, *WM* white matter

Following the preplate formation around embryonic day (E) 10–11 in mouse, the continuous production and radial migration of neurons cause the split of the existing preplate into a superficial marginal zone (MZ) and a deeper subplate (SP) and the emergence of a new region called the cortical plate (CP, the future cortex) that

harbors newly arrived neurons. Between E12 and E18, successive waves of neurons undergo radial glial fiber-guided radial migration and settle in the CP, creating cortical layers (L) 2–6. Remarkably, the time of birth of a given neuron is encoded in its final laminar position: birth-dating experiments revealed that the lamination of the cortex occurs in an "inside-out" fashion, that is, early-born neurons reside in the deeper layers, whereas late-born neurons migrate past the early-born neurons and occupy more superficial layers (Angevine and Sidman 1961) (Fig. 7.1b). Many molecules have been implicated in regulating excitatory neuron migration, such as Reelin (D'Arcangelo et al. 1995; Ogawa et al. 1995), Dab1 (Howell et al. 1997), cyclin-dependent kinase 5 (Cdk5) (Ohshima et al. 1996), and doublecortin (Des Portes et al. 1998; Gleeson et al. 1998). In addition, it has been shown that the adhesive function of gap junctions is necessary for radial migration in the neocortex (Elias et al. 2007).

Compared to inhibitory interneurons (discussed below), excitatory neurons have long been considered a more homogenous population; however, this notion has been challenged with the discovery of distinct neuron subtypes based on the morphology, projection pattern, and gene expression profile (Molyneaux et al. 2007). In the mature neocortex, L1 is a cell-sparse zone that lacks excitatory neurons. It is predominantly comprised of the distal dendritic tufts of excitatory neurons, axon terminations, Cajal-Retzius cells, and GABAergic interneurons (Douglas and Martin 2004). L2/3 consists of predominantly commissural neurons, which, in addition to participating in local circuits, project their axon collaterals across the corpus callosum (CC) and mediate the communication between the two cerebral hemispheres. L4 contains two main types of morphologically distinct excitatory neurons: classic and star pyramids. In primary sensory areas, L4 has an additional cell type: glutamatergic spiny stellate cells, which comprise an important cell population receiving thalamic innervation. Several transcription factors have been identified to express specifically in L2–4, including Cux1, Cux2, and Lhx2 (Molyneaux et al. 2007). L5 contains two main types of pyramidal neurons: thin-tufted and thick-tufted pyramidal neurons. "Thin-tufted" pyramidal neurons with a relatively thin apical dendrite and a small tuft in L1 are mainly found in upper L5; they are callosal neurons and project intracortically to other regions as well as to the contralateral cortex. "Thick-tufted" pyramidal neurons with a thick apical dendrite and a large tuft in L1 are mainly found in lower L5; they are corticofugal neurons that project to subcortical regions as well as the pons and spinal cord. Interestingly, L5 neurons that project to different target areas seem to have distinct electrophysiological characteristics, such as intrinsic membrane properties and firing properties, suggesting that they could transmit different types of cortical activity to their long-range targets (Hattox and Nelson 2007). Besides general L5-specific transcription factors, such as Opn3, several genes have been found to have restricted expression in different subtypes of projection neurons in L5. For example, *Ctip2* is highly expressed in subcerebral projection neurons, whereas *Lmo4* is expressed in callosal neurons (Molyneaux et al. 2007). L6 has the greatest diversity of excitatory pyramidal neurons, with at least four types based on projection patterns: corticocortical, cortico-thalamic, cortico-callosal, and cortico-claustral. Most of the apical dendrites of

these cells terminate in the middle layers, with only a small fraction reaching L1 (Markram 2010; Svoboda et al. 2010). L6-specific genes include Foxp2 and *Igh6* (Molyneaux et al. 2007).

7.2 Inhibitory Interneuron Production, Migration, and Laminar Distribution

Unlike excitatory neurons, GABA-containing inhibitory interneurons do not arise in the dorsal VZ. Instead, they are born in a transient region in the embryonic ventral telencephalon referred to as the ganglionic eminence (GE) (Anderson et al. 2002; Butt et al. 2005; Valcanis and Tan 2003; Wichterle et al. 1999, 2001; Xu et al. 2004, 2006, 2008). This region is subdivided into three zones: lateral (LGE), medial (MGE), and caudal (CGE) ganglionic eminence. The GE first appears around E11 in mouse as a sloping swelling at the telo-diencephalic junction and protrudes into the lateral and third ventricles. By E12, a second sloping swelling appears, and the two are called MGE and LGE, respectively. The CGE is defined as the more posterior region where the LGE and MGE fuse together. Around E14–15, the sulcus between MGE and LGE begins to disappear, and by the end of embryonic development, it has completely receded into the wall of the lateral ventricle. Fate-mapping studies have demonstrated that whereas LGE primarily gives rise to the striatal projection neurons and interneurons of the olfactory bulb (Anderson et al. 1997), MGE and CGE are the predominant sources of cortical interneurons (Nery et al. 2002; Xu et al. 2008). More specifically, the MGE produces approximately 70 % of neocortical interneurons (Lavdas et al. 1999; Xu et al. 2008) and the CGE gives rise to the remaining 30 % of cortical interneurons (Butt et al. 2005; Miyoshi et al. 2010; Nery et al. 2002). In addition, a small subpopulation (less than 3 %) of cortical interneurons is derived from the embryonic preoptic area (PoA), located in the telencephalic stalk, close to the pallidal domain (Gelman et al. 2009) (Fig. 7.1a).

The MGE is characterized by the expression of the homeobox transcription factor, Nkx2.1 (Marin et al. 2000; Sussel et al. 1999) (Fig. 7.1a), which is critical for the specification of MGE-derived cortical interneurons as well as suppression of surrounding cell fate programs; loss of Nkx2.1 results in the absence of cortical parvalbumin (PV)- and somatostatin (SST)-expressing interneurons with a concomitant increase in CGE-derived cortical interneurons as well as an increase in LGE-derived medium spiny neurons in the striatum (Butt et al. 2005, 2008). Notably, it has been suggested that in humans and nonhuman primates, besides the GEs, the VZ/SVZ of the dorsal telencephalon also produces a significant population of cortical GABAergic neurons (Fertuzinhos et al. 2009; Jakovcevski et al. 2011; Letinic et al. 2002; Petanjek et al. 2009). However, additional studies are needed to further explore this (Hansen et al. 2010). Nonetheless, the origin and migration of cortical interneurons may be more complex in humans and higher mammals, which could contribute to their increased diversity and allow for the formation of more elaborate cortical circuits.

One of the most distinct features of interneurons in the adult neocortex is their incredibly rich diversity in the morphology, biochemical expression, electrophysiological properties, and synaptic connectivity patterns. The distinct subcellular targeting and firing patterns of each subtype allows populations of interneurons to exert their inhibitory influence on surrounding neurons in numerous ways, thereby shape circuit output dynamically and allow for a wide range of neuronal computations. Two important determinants of subtype specification of cortical interneurons are the place and time of birth.

The spatial bias of progenitors in subtype specification is apparent when comparing progenitors in MGE versus CGE, which give rise to largely nonoverlapping subtypes of cortical interneurons. Progenitors in the MGE give rise to the majority of cortical interneurons that consist of PV-positive, fast-spiking (FS) basket and chandelier cells and SST-positive, burst-spiking cells which include Martinotti cells. The CGE, on the other hand, is the source of the remaining cortical interneurons that are more heterogenous (Lee et al. 2010; Miyoshi et al. 2010). These include the reelin-positive multipolar population and the vasointestinal-peptide (VIP)-positive (calretinin, CR-positive) bitufted, irregular spiking as well as VIP-positive (CR-negative), bipolar population that displays a fast-adapting firing pattern (Miyoshi et al. 2010; Nery et al. 2002). A comprehensive molecular map of the VZ in the developing mouse GE revealed that there may be as many as 18 progenitor domains in this region as marked by the combinatorial expression patterns of several transcription factors (Flames et al. 2007). Within the MGE, the dorsal-most region expresses Nkx6.2 and is thought to specifically generate SST-expressing cortical interneurons, whereas PV-expressing cortical interneurons are preferentially derived from more ventrally located Nkx2.1-positive progenitors (Flames et al. 2007; Fogarty et al. 2007; Sousa et al. 2009; Wonders et al. 2008). Anatomically defined subpallial regions can, therefore, be further divided into molecular subdomains that give rise to functionally distinct interneuron subtypes.

In addition to the presence of spatially distinct progenitor domains, a temporal bias in neurogenesis may contribute to interneuron subtype specification. Fate mapping of MGE progenitors showed that they undergo temporal changes that progress from generating mainly SST into mainly PV interneurons (Miyoshi et al. 2007). Recent transplantation and lineage-tracing experiments elegantly demonstrated that chandelier cells in the mouse neocortex are selectively born in the MGE at late stages of embryonic development (Inan et al. 2012; Taniguchi et al. 2012).

Although spatiotemporal dynamics of neurogenesis evidently contribute to diversification of neocortical interneurons, it remains unclear if interneuron subtype specification occurs at the population or single progenitor level. Using mouse genetics in combination with in utero retroviral labeling, Brown et al. were the first to conduct a clonal analysis of the MGE at single progenitor level to show neocortical interneurons are produced as spatially organized clonal units in the MGE and are subsequently organized into spatially isolated clusters in the adult neocortex. Individual RGCs within the MGE are able to generate clones of cortical interneurons that share the same neurochemical markers as well as clones that contain interneurons expressing different neurochemical markers (Brown et al. 2011).

More extensive morphological and physiological characterization of interneuron subtypes within clonal clusters will help elucidate how many subtypes a single progenitor can generate and in what combinations and the early developmental principles that generate interneuron diversity.

In contrast to excitatory cells, which migrate radially into the developing CP, interneurons must undergo a long and tortuous journey from their subpallial origins to reach the cortex. *En route* to the cortex, interneurons avoid the developing striatum and hence migrate superficial or deep relative to the striatal mantle (Marín et al. 2001). Tracing studies have identified two main migratory streams of interneurons that follow tangentially oriented paths to enter the cortex: an early cohort (before E12) migrates superficially through the MZ of the cortex (Lavdas et al. 1999) and a second and more prominent cohort (after E13) migrates deeply, predominantly through the lower intermediate zone (IZ) and SVZ (Wichterle et al. 2001). Upon reaching the cortex, interneurons adopt a radial trajectory to settle into their final laminar position within the cortex (Ang et al. 2003; Hevner et al. 2004; Polleux et al. 2002; Tanaka et al. 2003).

Many transcriptions factors (e.g., Dlx1/2, Lhx6), motogenic factors (e.g., HGF), neurotrophic factors (e.g., BDNF, NT-4), guidance cues (e.g., netrin/Dcc, Slit/Robo, semaphorin/neuropilin), and other molecules have been demonstrated to play important roles in guiding the migration of interneurons from their ventral origins to the cortex (Oscar Marín and Rubenstein 2003). The general ventral to dorsal direction of interneuron migration is established by the synergistic actions of chemoattractive and chemorepulsive factors produced by the cortex and embryonic PoA, respectively (Marín et al. 2003; Wichterle et al. 2003). In order to avoid the striatum, interneurons destined for the cortex selectively express neuropilin 1/2, thereby responding to the chemorepulsion produced by the semaphorin-expressing striatal mantle (Marín et al. 2001). Upon reaching the cortex, interneurons adopt a radial trajectory to settle into their final laminar position within the CP (Ang et al. 2003; Hevner et al. 2004; Polleux et al. 2002; Tanaka et al. 2003). Interestingly, gap-junction-mediated adhesion is crucial for the radial but not tangential migration of interneurons (Elias et al. 2010). In contrast to disrupted migration of excitatory neurons which typically results in severe malformation of the cortex (Ross and Walsh 2001), perturbations in interneuron migration often lead to subtle morphological defects that are nevertheless associated with severe physiological alterations in cortical activity (Powell et al. 2003). Identification of new genes underlying tangential migration is, therefore, an important area of research to better understand the etiology of complex neurological disorders.

Similar to excitatory neurons, interneurons are distributed in the cortex in a laminar fashion. Classic birth-dating studies as well as recent transplantation experiments have demonstrated that interneurons born at different times in the MGE or CGE populate specific layers of the neocortex in an inside-out order (Nery et al. 2002; Valcanis and Tan 2003). This is also apparent in the case of CGE-derived interneurons that are born relatively late during embryonic neurogenesis and tend to occupy more superficial layers of the cortex in comparison to most MGE-derived interneurons (Miyoshi et al. 2010). Hence, the acquisition of laminar identity by

cortical interneurons appears to correlate with their time of birth. Interestingly, cortical interneurons and projection neurons born at roughly the same time appear to occupy the same cortical layer (Valcanis and Tan 2003).

7.3 Neocortical Circuits

Underlying the rich diversity of neural computations in the neocortex are incredibly complex cortical circuits. We are beginning to understand the logic and principles of how neocortical circuits operate. Here we summarize several most prominent and well-studied features of neocortical circuits.

7.3.1 Functional Columns

The concept of neocortical column was first introduced by Mountcastle in 1957 and is invaluable in understanding the functional organization of the cortex. In recording the somatosensory cortex of cats, he made a key observation that neurons sharing common functional properties, such as peripheral receptors, receptive fields, and firing latencies, were located in a radial column extending from pial surface to white matter (Mountcastle 1957). He later extended this finding to the monkey cortex, where the dimensions of columns (~0.5 mm) were similar. Working alongside Mountcastle, his colleague Powell suggested that the vertical palisades of cells in stained sections of somatosensory cortex were the elementary structural units underlying the functional columns revealed by electrophysiology (Powell and Mountcastle 1959).

The seminal work by Hubel and Wiesel in the 1960s and 1970s brought tremendous interest and enthusiasm in studying neocortical column (Douglas and Martin 2007). Echoing Mountcastle's observation in the somatosensory cortex, they found that cells with similar orientation selectivity were located in a single radial penetration from pial surface to white matter (*orientation columns*) (Hubel and Wiesel 1962; Wiesel 1963). Later, aided by hints from kittens with artificially induced divergent squint, they discovered that two eyes differentially activated cortical neurons: cells with similar eye preference were grouped together into columns (*ocular dominance columns*) and left and right eye dominant columns alternated across the cortex (Wiesel and Hubel 1963). Subsequently, similar functional columns were discovered in the cat primary auditory cortex (Abeles and Goldstein 1970) and many other cortical areas (Mountcastle 1997). These discoveries prompted a deep thought that *"the cells behave as though they shared certain connections among themselves, but not with cells of neighboring columns, and in this sense a single group of cells is looked upon as a more or less autonomous functional unit…The machinery may be roughly uniform over the whole striate cortex, the difference being in the inputs…*

It may be that there is a great developmental advantage in designing such machinery once only, and repeating it over and over monotonously, like a crystal" (Hubel and Wiesel 1974a).

Despite the long history of successful electrophysiological recordings of functional columns, the structural basis of these functional columns has remained elusive. Minicolumns, which are chains of neurons (typically contain 80–120 neurons) derived from the same proliferative units, spanning across cortical layers whose cell bodies are vertically aligned within a diameter of 40–50 μm, have been proposed to be the basic unit of the neocortex. About 50–80 minicolumns link together and form the structural basis of functional columns (Mountcastle 2003). Another candidate is a closely related but not identical structure referred to as bundles, which are mainly comprised of closely associated apical dendrites of pyramidal cells whose cell bodies are located in different layers (Peters and Kara 1987; Peters and Sethares 1991, 1996; Peters and Walsh 1972; Peters et al. 1997). However, both views have met ample criticism (Rockland and Ichinohe 2004). Whether there is a structural correlate of functional columns at all, therefore, remains controversial. One obvious challenge is that the definition of functional columns is based on the functional properties of neurons, which may not be simply reflected anatomically. A more effective search for the structural correlate of functional columns requires a precise characterization of the function properties of individual neurons. Recent advance of in vivo Ca^{2+} imaging provides a powerful route to bridge the gap between structure and function (Bock et al. 2011; Chen et al. 2011; Ko et al. 2011; Li et al. 2012; Ohki and Reid 2007; Ohki et al. 2005).

7.3.2 Canonical Neocortical Circuit

Incorporating the essence of columnar organization of the neocortex, that is, a fundamental computational unit that repeats itself, Douglas and Martin developed the concept of "canonical cortical circuit" based on electrophysiological and modeling studies in the cat visual cortex (Douglas and Martin 1991; Douglas et al. 1989). Instead of focusing on identifying the distinct cellular and anatomical module underlying functional columns, the canonical cortical circuit concept focuses on the rules that govern the synaptic connections between different neuronal types in the neocortex, including recurrent excitation and inhibition, amplification of weak inputs into the cortex, and balance of excitation/inhibition (Rodney J. Douglas and Martin 2007; da Costa and Martin 2010). This has greatly advanced our understanding of the wiring principle of cortical circuits.

In the canonical circuit of the neocortex, thalamic relay cells provide input into the cortex and mainly target L4, although they also form synapses with neurons in other layers. This thalamic input is relatively weak and is amplified by recurrent excitation of L4 excitatory neurons. Recurrent excitation can be potentially harmful in leading to hyperexcitability of the circuit; inhibition is therefore needed to modulate

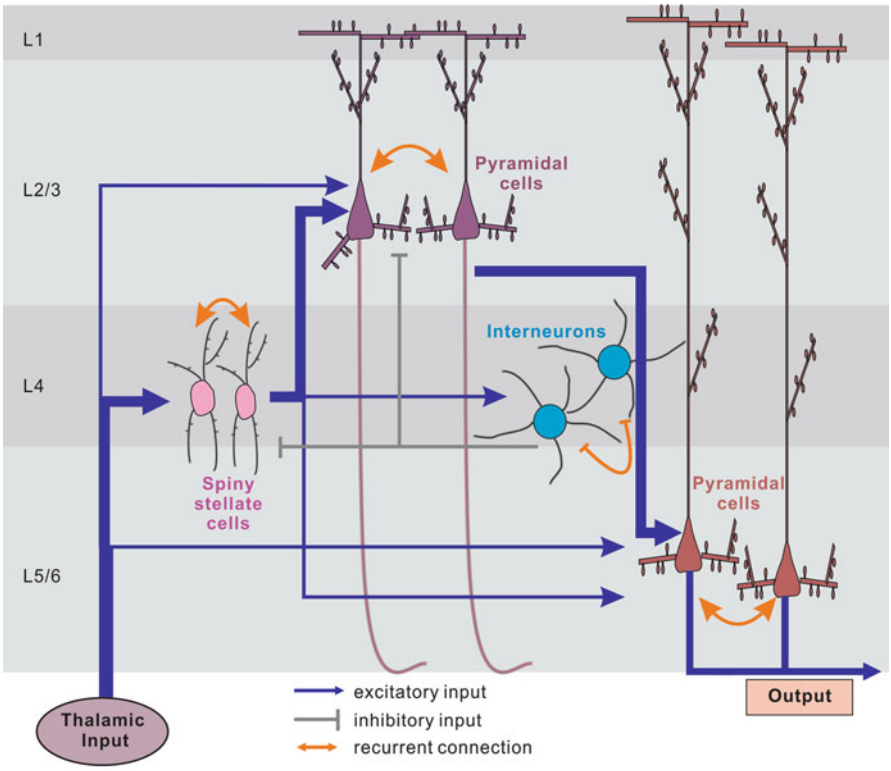

Fig. 7.2 Canonical neocortical circuit. In the canonical circuit, thalamic relay cells provide input to the cortex and mainly target L4, although they also target neurons in other layers. This thalamic input is relatively weak and is amplified by recurrent excitation of L4 excitatory neurons. Within all layers, excitatory and inhibitory neurons form recurrent connections. Between cortical layers, information flow has a strong directional tendency: from L4 up to L2/3 and down to L5/6. There is also a weaker projection from L4 directly down to L5/6. Deep layer excitatory neurons provide major cortical output to subcortical regions as well as the pons and spinal cord. *Note*: inhibitory interneurons are present in all layers, but for simplicity, only a pair in L4 is shown

this potentially strong excitation. Within all layers, excitatory and inhibitory neurons form recurrent connections. Between cortical layers, information flow has a strong directional tendency: from L4 up to L2/3 and down to L5/6. There is also a weaker projection from L4 directly down to L5/6 (Fig. 7.2). The principles of canonical circuit can be applied to other cortical areas, such as the motor cortex, suggesting that they may reflect the underlying organization of the entire cortex (Douglas and Martin 2007). Notably, recent studies also suggested that there might be variations in circuit organization in certain cortical areas, e.g., the somatosensory cortex (Bruno and Sakmann 2006; Meyer et al. 2010; Oberlaender et al. 2012; Wimmer et al. 2010).

7.4 Neocortical Circuit Assembly

Connectivity in the neocortex can be broadly divided into local connections (referred to as "microcircuit") and long-range connections. Excitatory neurons participate in both modes of connections, whereas inhibitory interneurons predominantly exhibit local connectivity. Below we discuss how these connections are constructed during development. We will also briefly discuss how neuronal activity shapes and refines neocortical circuits.

7.4.1 Microcircuit Construction

The logic of neocortical microcircuit assembly has long been debated. Some believe that the connections between neurons are not specific, but are statistical, semi-random, or random (Hansel and van Vreeswijk 2012; Hill et al. 2012). However, recent evidence suggests that even in rodents, which lack the obviously identifiable functional columns, cortical synaptic connections are highly nonrandom (Song et al. 2005) and exhibit fine-scale specificity (Yoshimura and Callaway 2005; Yoshimura et al. 2005).

7.4.1.1 Projection-Dependent Specificity

Within a single layer, excitatory neurons can be categorized into subgroups based on their input and output patterns. These subgroups may differ in their local connection properties, thus forming subnetworks that process information in relatively independent or even parallel ways (Fig. 7.3a) (Krook-Magnuson et al. 2012). Indeed, input- and output-dependent neuronal networks have been discovered in rodent neocortex, revealing a level of fine-scale specificity based on projection pattern.

Hebbian learning rule states that *"any two cells or systems of cells that are repeatedly active at the same time will tend to become 'associated,' so that activity in one facilitates activity in the other."* One possibility of achieving simultaneous activation is by sharing excitatory input. Indeed, using photo-stimulation and cross-correlation analysis, this concept was demonstrated in rat visual cortex: when adjacent L2/3 pyramidal neurons are synaptically connected to each other, they share common input from L4 and within L2/3; on the contrary, when they are not connected, they share little common input from L4 and L2/3. Thus, depending on the input from L4 and within L2/3, excitatory neurons in L2/3 form selectively interconnected fine-scale subnetworks that allow different information to be processed in relatively independent ways (Yoshimura et al. 2005). Interestingly, a recent study demonstrated that in L2/3 of mouse visual cortex, neurons that share similar functional properties are more likely to be synaptically connected (Ko et al. 2011). It will be interesting to test if this preferential connectivity results from shared visual input conveyed by L4 neurons.

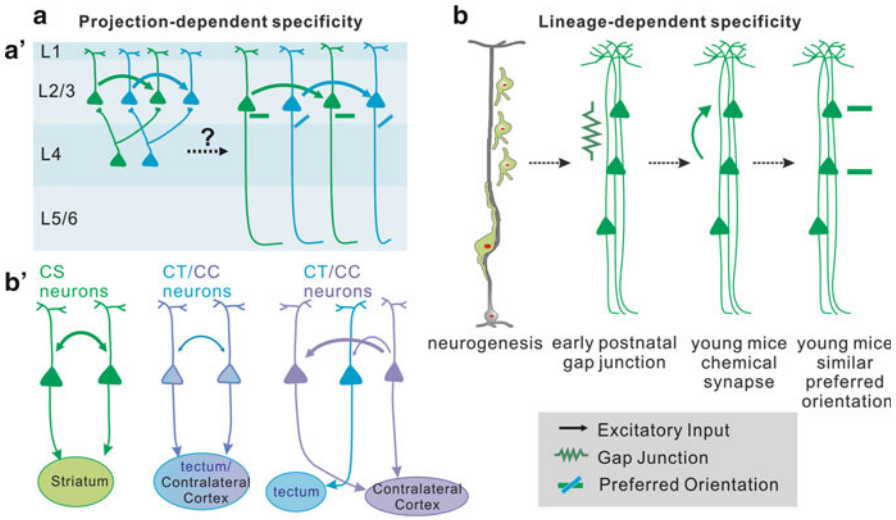

Fig. 7.3 Precise microcircuit assembly in the neocortex. (**a**) Projection-dependent specificity. (*A′*) In rat visual cortex, when adjacent L2/3 pyramidal neurons are synaptically connected to each other, they share common input from L4 and within L2/3; when they are not connected, they share little common input from L4 and L2/3 (Yoshimura et al. 2005). Interestingly, in L2/3 of mouse visual cortex, neurons that share functional properties are more likely to be synaptically connected (Ko et al. 2011). It will be interesting to test if this preferential connectivity results from shared input from L4. (*A″*) Connectivity within a subgroup of excitatory neurons depends on their long-range target, e.g., connection probability between a pair of CS neurons (~30–40 %) is much higher than a pair of CT or CC neurons (~10–15 %) (Brown and Hestrin 2009). In certain circuits, excitatory neurons preferentially connect to those that have different long-range targets, e.g., CC neurons preferentially connect to CT neurons rather than other CC neurons (Brown and Hestrin 2009). (**b**) Lineage-dependent specificity. Sister neurons derived from the same RGCs migrate along the radial glial fiber and form ontogenetic columns. Sister neurons are preferentially coupled through electric synapses (gap junctions) in the first postnatal week (Yu et al. 2009). In the second postnatal week, sister neurons develop preferential chemical synapses with each other (Yu et al. 2012), and in L2/3 of mouse visual cortex, sister neurons have similar orientation tuning response properties (Yu et al. 2012). *CS* corticostriatal, *CT* corticotectal, *CC* corticocortical

L5 excitatory neurons provide a good starting point for studying output-dependent specificity because of their diverse long-range projection patterns. *First*, although generally cortical excitatory neurons are sparsely connected (connection probability 1–12 %), the local connection probability between two neurons could differ significantly depending on their long-range projection targets. For example, in mouse visual cortex, the probability of identifying monosynaptic connections among corticostriatal (CS) neurons (~30–40 %) is significantly higher than those among corticocortical (CC) or corticotectal (CT) neurons (~10–15 %) (Brown and Hestrin 2009). *Second*, in certain areas, excitatory neurons may preferentially connect to other excitatory neurons with the same long-range projection targets. One example is in rat frontal cortex, where two types of corticostriatal pyramidal neurons are present in L5: corticopontine (CPn) cells that project to the pons and

ipsilateral striatum and crossed corticostriatal (CCS) cells that project to both ipsilateral and contralateral striatum but not to the pons. Interestingly, CPn cells form strong reciprocal connection with other CPn cells, but rarely connect with CCS cells (Morishima and Kawaguchi 2006; Morishima et al. 2011). *Third*, in some other areas, excitatory neurons may selectively connect to other excitatory neurons that have different long-range projection targets rather than those that share the same targets. One example of this is in L5 of mouse visual cortex, where CC neurons provide selective feed-forward monosynaptic connections to CT neurons, rather than connect to other CC neurons (Brown and Hestrin 2009). These results collectively suggest that distinct subnetworks of pyramidal neurons specified by their long-range projection patterns are embedded within local circuits of L5, which could contribute to the generation of diverse cortical outputs.

7.4.1.2 Lineage-Dependent Specificity

In 1988, Rakic proposed the "radial unit hypothesis." According to this hypothesis, neurons derived from the same proliferative unit in the VZ migrate along the radial glial fiber and form "ontogenetic/embryonic" columns, which are the building blocks for the cerebral cortex (Rakic 1988). Based on the similarity of a vertical organization of neurons, it was postulated that ontogenetic columns might relate to functional columns. However, this hypothesis has not been experimentally tested until recently. Yu et al. injected EGFP-expressing retroviruses intraventricularly into the developing mouse embryos at E12–E13 to label individual asymmetrically dividing RGCs, which give rise to ontogenetic columns composed of 4–5 vertically aligned sister excitatory neurons spanning different cortical layers. Multiple-electrode whole-cell patch clamp recordings at postnatal stages (P10–P21) revealed that sister neurons are preferentially connected compared to nearby non-sister neurons. Interestingly, the direction of interlaminar connectivity among sister neurons in an ontogenetic column resembles those observed in the mature cortex, suggesting that these ontogenetic columns could lead to the formation of functional columns in the cortex (Yu et al. 2009). Tracing this back to even earlier developmental stages, sister excitatory neurons preferentially form transient electrical synapses with each other (peak at P1~P2, largely disappear after P6), which allow for selective electrical communication and promote action potential generation/synchronous firing. Although these gap junctions largely disappear before functional chemical synapses can be detected, they are necessary for the formation of specific chemical synapses between sister neurons (Yu et al. 2012). This line of studies not only demonstrates a new principle of circuit specificity that depends on the lineage relationship of neurons, but also suggests that ontogenetic columns may contribute to the emergence of the functional columns in the neocortex.

To directly test this, Li et al. used the same retrovirus labeling technique to label sister excitatory neurons derived from the same RGCs at E15–E17 in mouse visual cortex and performed in vivo two-photon Ca^{2+} imaging to measure their orientation tuning response properties at P12–P17. They found that sister neurons have similar

orientation preferences compared to nearby non-sister neurons. Interestingly, in line with the findings of Yu et al., blockade of electrical coupling between sister neurons abolished the functional similarity between sister neurons, highlighting the role of early gap-junction-mediated electrical communication in establishing specific connections between sister neurons and their shared functional properties (Li et al. 2012) (Fig. 7.3b).

In a similar study, Ohtsuki et al. utilized a different approach to label lineage-related neurons. They used a transgenic mouse Cre-driver line in which Cre is express sparsely in progenitor cells early in forebrain development, generating individual clones containing 600–800 fluorescence-labeled neurons derived from the same progenitors. They then used in vivo two-photon Ca^{2+} imaging to examine the orientation tuning response properties of clonally related neurons and nearby non-clonally related neurons. Interestingly, orientation preferences among clonally related neurons were still more similar than those among unrelated neurons. However, they also pointed out that there was considerable diversity within the large clones, such that nearly half of all neuronal pairs have preferred orientations with a difference greater than 30° and a quarter of them with a difference greater than 60° (Ohtsuki et al. 2012).

Together, these studies suggest that, at least in the mouse, lineage plays a crucial role in guiding precise neocortical microcircuit construction. Ontogenetic columns formed by clonally related neurons could be the basic structural and functional unit that constitutes the neocortex. One important question is whether these lineage-related specific microcircuits also exist in other mammalian species, especially higher mammals such as cat and monkey. Recent studies revealed a tremendous interspecies difference in the organization of orientation preference maps (Ohki and Reid 2007; Ohki et al. 2005), which could be due to the differences in patterns of neurogenesis and the layout of ontogenetic columns. It was proposed that extensive proliferation capacity in the SVZ may underlie cortical expansion from rodents to higher mammals including ferret, monkey, and human (Kriegstein et al. 2006), which presumably will give rise to ontogenetic columns that are much bigger in size and with many more horizontal features. The identification of oRGs in the SVZ with increased proliferation capability supports this hypothesis (Lui et al. 2011). Interestingly, computational modeling based on wire length minimization principle predicts that strong horizontal connection pattern would lead to smooth varying maps, as those discovered in cats and monkeys (Blasdel 1992; Bonhoeffer and Grinvald 1991; Hubel and Wiesel 1974b, 1977; Wolf and Geisel 1998). In comparison, the proliferation potential of IPCs in the SVZ of rodents is much more limited, and lack of specific horizontal connections is predicted to produce apparent salt-and-pepper organization of maps (Koulakov and Chklovskii 2001). In this regard, it will be interesting to test if ontogenetic columns are the long-awaited structural basis of functional columns in higher mammals. It is important to note that the cellular organization of the thalamus appears different between rodents and monkeys, and this difference may also fundamentally influence the functional organization of the cortex.

7.4.1.3 Interneuron Synaptic Targeting Specificity

Within the laminar and columnar architecture of the neocortex, dendritic and axonal arbors of excitatory neurons are extensively intermingled with those of inhibitory interneurons. Despite this, connections between these two types of cortical neurons are highly specific such that axons terminating in any given region selectively connect to some cell types while avoiding others. For instance, FS cells in L2/3 of the rat visual cortex were shown to preferentially target pyramidal cells that provided them with reciprocal excitatory connections (Yoshimura and Callaway 2005). In addition, reciprocally connected FS and pyramidal cells shared more common excitatory input from L4 than those that were not (Yoshimura and Callaway 2005). Pairs of SST-positive interneurons and pyramidal cells, on the other hand, shared little or no common input, regardless of their direct connectivity (Yoshimura and Callaway 2005). In the rat frontal cortex, however, L2/3 pyramidal cells preferentially innervate connected pairs of SST-positive interneurons and pyramidal cells in L5 while avoiding connected pairs of PV-expressing interneurons and pyramidal cells (Otsuka and Kawaguchi 2009). Formation of specific subnetworks may, therefore, depend not only on the interneuron subtype involved, but also on the cortical areas and layers considered. Connectivity from interneurons to pyramidal neurons varies from 20 % to 60 % (Thomson and Lamy 2007). This seems to be highly dependent on distance, such that interneurons (SST- and PV-positive) and pyramidal cells within 200 μm are up to 70 % as likely to be connected (Fino and Yuste 2011; Packer et al. 2012).

Axons of different subtypes of inhibitory interneurons are also highly selective in the postsynaptic neuronal compartment (i.e., soma, dendritic tree, or axon initial segment) they target (Fig. 7.4a). Perisomatic inhibition controls the output of the postsynaptic neuron and is primarily mediated by PV-containing basket cells. Dendritic inhibition sculpts the local input of the postsynaptic neuron and is mainly mediated by SST-expressing interneurons, predominantly Martinotti cells (McGarry et al. 2010). PV-positive chandelier (also referred to as axo-axonic) cells specifically target the axon initial segment of pyramidal cells and are capable of abolishing the excitatory output of the postsynaptic neuron (Pouille et al. 2009).

7.4.1.4 Interneuron Electrical-Coupling Specificity

Interneurons in the mature cortex not only provide inhibition to local circuits, but also play a critical role in generating network oscillations, which in turn modify the response of local circuits to incoming signals. This rhythmic electrophysiological activity of neural ensembles forms the basis of brain rhythms essential for information processing. In attempting to understand the cellular origin and subtype specificity in rhythm induction, the strongest case made so far is the role of PV-positive FS interneurons in the induction of gamma oscillations (Cardin et al. 2009; Traub et al. 1996). FS interneurons have a high incidence of electrical coupling amongst each

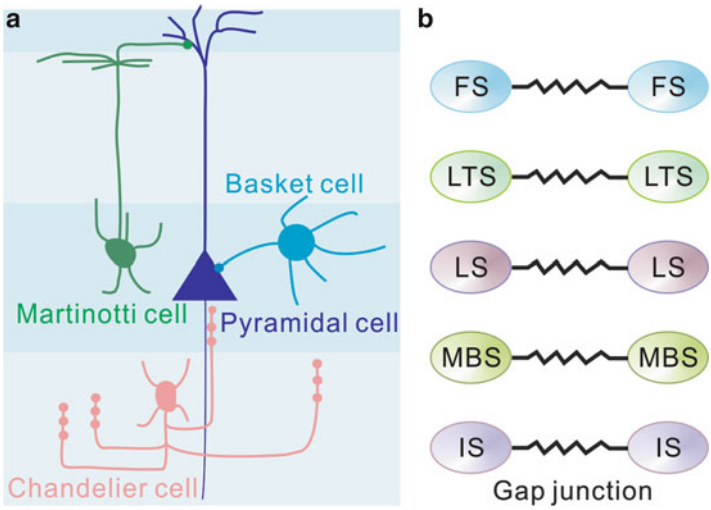

Fig. 7.4 Subcellular targeting specificity of inhibitory interneurons and electrically coupled networks of interneurons. (**a**) PV-containing basket cells form perisomatic synapses on pyramidal cells; chandelier cells specifically target the pyramidal cell axon initial segment; SST-containing Martinotti cells mainly innervate the pyramidal cell dendrites in L1. (**b**) Gap junctions are found almost exclusively on the interneurons of the same subtype. *Note*: one exception is LS neurogliaform cells, which can also be involved in heterologous electrical coupling. *FS* fast spiking, *LTS* low-threshold spiking, *LS* late spiking, *MBS* multipolar burst spiking, *IS* irregular spiking

other via gap junctions (Galarreta and Hestrin 1999; Galarreta and Hestrin 2002; H Meyer et al. 2002); they provide large, synchronous inhibition to local excitatory neurons that is sufficient to induce 20–80 Hz oscillations (Börgers et al. 2005; Hasenstaub et al. 2005; Wang and Buzsáki 1996). In fact, gap-junction-mediated electrical coupling is a unique feature of GABAergic interneurons that is critical for cortical function. Interestingly, gap junctions are found almost exclusively between GABAergic interneurons of the same subtype (Fig. 7.4b) (Galarreta and Hestrin 1999; Gibson et al. 1999). In addition to FS basket and chandelier cells, pair-wise electrophysiological recordings have identified electrically coupled networks of low-threshold spiking (LTS) Martinotti cells (Beierlein et al. 2000; Deans et al. 2001; Galarreta and Hestrin 2001; Gibson et al. 1999, 2005; Venance et al. 2000), late-spiking (LS) neurogliaform cells (Chu et al. 2003), multipolar bursting (MB) cells (Blatow et al. 2003), as well as a population of irregular spiking (IS), large basket cells expressing cannabinoid receptors (Galarreta et al. 2004). An exception to the rule of homologous electrical coupling is the case of LS neurogliaform cells, which, in addition to participating in homologous electrical coupling, are also involved in heterologous coupling with other types of interneurons with diverse morphology and firing patterns (Simon et al. 2005).

7.4.2 Long-Range Connection Establishment

In addition to encompassing rich and dynamic local microcircuits, the neocortex also receives long-range input from and provides long-range output to different brain areas. The most prominent long-range input into the neocortex is from the thalamus, which carries peripheral sensory information; the two main outputs from the neocortex are mediated by corticofugal projection neurons and callosal projection neurons. We discuss below how these long-range connections are formed during development. One emerging theme is that the establishment of overall topography of cortical afferents and efferents depends heavily on genetic information, especially axon guidance mechanisms (Polleux 2005).

7.4.2.1 Thalamocortical Projections

The thalamus plays a key role in mediating sensory responses. Almost all peripheral sensory information (except for olfaction) is processed in distinct nuclei in the thalamus and projected topographically onto corresponding cortical areas (Sherman and Guillery 2002). During development, thalamocortical axons (TCA) have to navigate an impressively long distance, across the thalamic eminence, corridor, and ventral telencephalon to reach their target cortical areas (Garel and Rubenstein 2004). Extensive studies have been carried out to investigate the cellular and molecular mechanisms that guide TCA pathfinding. Briefly, after exiting the thalamic eminence, TCAs avoid the hypothalamus and turn toward the direction of the diencephalic-telencephalic border through a combination of Slit-mediated repulsion from the hypothalamus (Braisted et al. 2009; López-Bendito et al. 2007) and the attractive activity exerted by the mantle region of the ventral telencephalon (Braisted et al. 1999, 2000; Métin and Godement 1996). As they enter the ventral telencephalon, TCAs navigate through a narrow corridor located between the proliferative zone of MGE and globus pallidus (GP). This corridor is composed of GABAergic interneurons derived from LGE (expressing *Islet-1*) and migrating into MGE. These cells express a membrane-bound isoform of neuregulin-1 and create a permissive domain for TCA pathfinding (López-Bendito et al. 2006). Inside the ventral telencephalon, which constitutes a main intermediate target for TCA projections, TCAs from different nuclei go through a sorting process along the rostrocaudal axis. In this stage, TCAs express different axon guidance cue receptors and respond to gradients of attractant and repellent cues (e.g., ephrinA5, netrin-1, Sema3A, Sema3F, and Slit) (Dufour et al. 2003; Polleux 2005). After crossing the ventral telencephalon and past the pallial-subpallial boundaries, TCAs wait in the subplate region for considerable time periods before growing into the cortex. Within the neocortex, additional molecular cues (e.g., FGF8, Pax6, COUP-TFI, Emx2, and Sp8) and activity-dependent mechanisms promote the final synaptic targeting of TCAs (*for more details, please refer to a review by* Molnár et al. 2012).

7.4.2.2 Corticofugal and Callosal Projections

Corticofugal projection neurons respect the midline and project subcerebrally to the thalamus, brainstem, and spinal cord. These neurons are mainly located in the deeper layers of the cortex. In adult, these cortical efferents are specific to cortical regions, for example, only neurons in L5 of the sensorimotor cortex project to the spinal cord and only neurons in the visual cortex and auditory cortex project to the tectum. In rodents, this area-specific projection is accomplished through selective elimination of unwanted axon branches. For example, initially L5 neurons from all cortical areas project toward the spinal cord and then send collateral axon branches to invade the midbrain. As development proceeds, neurons from the sensorimotor cortex selectively remove their collaterals from the tectum, while neurons from the visual cortex retract their axon branch from the spinal cord (O'Leary and Koester 1993). Recent studies have begun to uncover the molecules underlying these processes, such as Otx1 (Weimann et al. 1999), Fezl (Chen et al. 2005), Ctip2 (Chen et al. 2008), and plexin (Low et al. 2008).

Callosal projection neurons project across the corpus callosum and mediate the interaction between two hemispheres; their axons do not leave the telencephalon. Callosal projection neurons are mainly located in L2/3 (80 %) and, to a lesser extent, in L5 (20 %) (Fame et al. 2011). As these neurons are born, two cerebral hemispheres begin to fuse. Glia and local neurons form a transient bridge-like subcallosal sling across the midline (Niquille et al. 2009; Shu et al. 2003a). The axons of callosal projection neurons first descend toward the IZ and then turn toward the midline to cross the corticoseptal boundary; when they encounter the contralateral glial wedge, these axons turn dorsally and ascend into the cortex toward homotopic targets (Fame et al. 2011). A number of molecules have been implicated in midline crossing, including guidance molecules such as Slit/Robo (Bagri et al. 2002; López-Bendito et al. 2007; Shu et al. 2003b), Wnt (Keeble et al. 2006; Li et al. 2010), and the Netrin family (Serafini et al. 1996).

7.4.3 Activity-Dependent Modification of Neocortical Circuits

Whereas genetically specified molecular cues are thought to underlie early events in cortical development by providing the structural framework for a stereotyped neural circuit (Goodman and Shatz 1993; Kaschube et al. 2002; Tessier-Lavigne and Goodman 1996), a later phase of neural activity is required to refine early synaptic connections in order to generate specific patterns of connectivity characteristic of the mature brain (Katz and Shatz 1996). Neural activity consists of an experience-independent (spontaneously driven) phase observed during the prenatal/early neonatal stages of development, followed by an experience-dependent (sensory-evoked) phase during early postnatal development.

Hubel and Wiesel's seminal work shed light on the importance of early visual experience during the critical period for plasticity of visual circuits (Hubel and

Wiesel 1965). Studies since then have revealed that neurons in the developing visual system are active long before the critical period. In fact, work in the newborn mouse visual cortex has shown that spontaneous retina waves are present and propagate throughout the entire visual system before eye opening (Ackman et al. 2012), and the development of precise cortical maps requires spontaneous patterned activity in the retina (Cang et al. 2005; Huberman et al. 2006; Xu et al. 2011). During early postnatal development (i.e., during the critical period), sensory-evoked activity further modifies the circuit in an experience-dependent manner based on Hebbian learning rules so that unequal levels of activity result in the dominance of connections from the more active eye at the expense of the less active one. This competitive Hebbian learning is thought to be accomplished at the cellular level through spike-timing-dependent synaptic plasticity, so that inputs that arrive at the postsynaptic neuron a few milliseconds before postsynaptic spikes lead to strengthening of the synapse (long-term potentiation, LTP), whereas those that arrive after postsynaptic spikes lead to weakening of the synapse (long-term depression, LTD) (Caporale and Dan 2008).

LTP and LTD are NMDA receptor-dependent synaptic modifications that are crucial for circuit plasticity during the critical period (Daw et al. 1999; Di Cristo et al. 2001; Heynen et al. 2003; Rittenhouse et al. 1999; Roberts et al. 1998). Interestingly, sensory experience is associated with maturation of inhibitory innervations in visual and somatosensory cortices (Chattopadhyaya et al. 2007; Jiao et al. 2006; Morales et al. 2002). In fact, it has been suggested that GABAergic inhibition is central to the regulation of the critical period of plasticity (Hensch 2005; Huang et al. 1999; Morales et al. 2002).

7.5 Concluding Remarks

In 1937, Cajal made an extraordinary far-reaching statement that the neocortex, albeit the highest center of the brain, was still built from stereotypic circuit elements, similar to those discovered in the retina, cerebellum, hippocampus, and spinal cord (Cajal 1937; Douglas and Martin 2007). This belief motivated generations of neuroscientists to embark on the journey of deciphering the basic circuit with which the neocortex operates. Over the last century, and especially within the past two decades, we have gained significant insight into the basic principles of neocortical circuit organization and function. Nevertheless, many questions remain to be answered: for example, what is the fundamental structural and functional unit that constitutes the neocortex? What are the mechanisms that ensure the correct assembly of neocortical circuits during development? How is relevant information about the external world processed and integrated in the neocortex, allowing the organism to comprehend and act upon? Recent advent of technology has endowed neuroscientists with an unprecedented edge to approach these questions. We are optimistic that many mysteries about neocortex development and function will be unraveled in the near future.

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Chapter 8
Hierarchical Organization of Neocortical Neuron Types

Yasuo Kawaguchi

Abstract The neocortex consists of many diverse neuron populations distributed across cortical layers having specialized connectivity and projection patterns. Glutamatergic pyramidal cells, which are cortical projection neurons, reside in all layers except layer 1, while GABAergic nonpyramidal cells are ubiquitous throughout all cortical layers. These broad classes of excitatory and inhibitory neurons comprise specialized neuron subtypes that have specific morphological, physiological, and chemical properties. However, while much is now known about the types in the cortex, less is known regarding the rules governing their selective connectivity into cortical and extracortical circuits. In layer 5 of the rat frontal cortex, several distinct populations of pyramidal cells are identifiable based on their distinct extracortical projections, firing characteristics, morphologies, and positions within layer 5. We have characterized highly selective synaptic connectivity among and between these pyramidal cell populations, which likely contributes to their establishing and maintaining functional loops between the frontal cortex, basal ganglia, and thalamus. However, less is known about how GABAergic neuron subpopulations are selectively incorporated into cortical circuits or how they might differentially regulate cortical output to subcortical targets.

8.1 Introduction

Corresponding to its involvement in higher-level cognitive functions, the neocortex has developed highly complicated neural circuits, but its operating characteristics remain to be investigated. To understand the function of the neocortex, we must

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reveal the structural basis of its circuitry. For this purpose, the first step is to identify the functional classes of neocortical neurons from various aspects.

Neocortical neurons are morphologically divided into pyramidal and nonpyramidal cells (Peters and Jones 1984). Pyramidal cells comprise approximately 80 % of neocortical neurons and use glutamate as an excitatory transmitter (DeFelipe and Fariñas 1992). They project to various subcortical structures as well as to diverse cortical areas (Jones 1984). In addition to extracortical projections, they emit axon collaterals locally, thereby also acting as local circuit neurons (Markram et al. 1997; Thomson and Bannister 2003). Typical pyramidal cells extend an apical dendrite toward the cortical surface and issue basal dendrites around the soma. In contrast, nonpyramidal cells are morphologically diverse in both axons and dendrites (Karube et al. 2004; Kawaguchi et al. 2006; Kubota et al. 2011a). Glutamatergic nonpyramidal cells called spiny stellate cells are found in layer 4 of the primary sensory areas, but most other nonpyramidal cells are GABAergic.

In this chapter, I will introduce the following concepts: (1) pyramidal and GABAergic cells are both composed of several independent groups that are identified by their morphological, physiological, and molecular aspects; (2) excitatory local subnetworks are formed by selective connections among these pyramidal cell subtypes projecting to different extracortical targets; (3) pyramidal cells in different sublayers of L5 may differently participate in reinforcement learning executed in the basal ganglia; and (4) diverse GABAergic cells may differentially regulate multiple excitatory subnetworks in unique ways.

8.2 Pyramidal Cell Organization

The neocortex is composed of several layers that vary in neuron density and somatic size. Extracortical targets are different between the layers, and even in the same layers, pyramidal cells project to diverse extracortical targets (Gabbott et al. 2005). I would like to introduce neocortical laminar structure and pyramidal cell diversity according to axonal projection, dendritic morphology, and firing pattern in the context of the rodent frontal cortex that we are currently investigating.

8.2.1 Extracortical Target Differences Between Layers

Layer I (L1) contains mostly GABA cells, but the other layers contain both pyramidal and GABA cells (Fig. 8.1). In the rat frontal cortex, layer 2/3 (L2/3) has predominantly cortico-cortical cells that connect other ipsilateral cortical regions (association cells) and the contralateral cortex through the corpus callosum [commissural (COM) cells]. Amygdala-projecting cells also exist in L2/3 (Hirai et al. 2012).

Layer 5 (L5) contains both COM cells and pyramidal cells projecting to the pontine nuclei, namely, corticopontine (CPn) cells. Some CPn cells also innervate

Fig. 8.1 Dendritic morphology of pyramidal cells in the rat frontal cortex. Pyramidal cells in the superficial layers project to the contralateral cortex as well as other areas in the ipsilateral cortex. Deep-layer pyramidal cells project to various subcortical structures in addition to other cortical regions

the thalamus [corticothalamic (CTh) cells] and spinal cord (Kita and Kita 2012). Some L5 pyramidal cells project to the ipsilateral cortical areas (Rockland 1997; Veinante and Deschênes 2003). The relationship between L5 association cells and CPn and COM cells remains to be investigated.

Some layer 6 (L6) output cells are modified pyramidal cells that lack typical apical dendrites toward L1 (Thomson 2010). This layer projects to the thalamus (CTh cells) and the contralateral cortex (COM cells). L6 also contains association cells that project to other ipsilateral areas.

8.2.2 Selective Cholinergic Modulation of Superficial and Deep Pyramidal Cells

Superficial and deep-layer pyramidal cells are different in their projection targets. Their outputs may be differentially regulated by extracortical inputs. Acetylcholine is involved in arousal and cognitive functions, and indeed, acetylcholine actions differ between L2/3 and L5 pyramidal cells.

Acetylcholine is assumed to diffuse from the release site and exert its action nonselectively (Descarries et al. 1997). The increased extracellular concentration of ambient acetylcholine increases pyramidal cell excitability. However, some cholinergic terminals make differentiated synaptic structures and may exert synaptic actions selectively for the postsynaptic neuron type and surface domain.

Tonic application of acetylcholine induces depolarization and suppresses hyperpolarizations following the spike discharges in pyramidal cells from each layer. However, phasic acetylcholine application produces an initial transient hyperpolarization in some pyramidal cells, followed by depolarization and

increased excitability (Gulledge and Stuart 2005). The initial hyperpolarization was previously assumed to be caused by inhibitory potentials induced by cholinergic excitation of GABA cells. However, this hyperpolarization is due to direct cholinergic action on deep-layer pyramidal cells. The phasic hyperpolarization easily desensitizes, whereas the later depolarization is less desensitized. In L2/3 pyramidal cells, slow depolarizations are observed. Only deep-layer pyramidal cells show phasic cholinergic inhibition (Gulledge et al. 2007). Hyperpolarization is induced by activation of type 1 muscarinic receptors around the cell body, followed by intracellular calcium increase and opening of SK-type potassium channels.

Thus, direct cholinergic inhibition of pyramidal cells is layer specific. In hippocampal formation, that belongs to the archicortex, cholinergic actions are different between the intrahippocampal regions (Gulledge and Kawaguchi 2007). Hippocampal formation is primarily composed of CA1 and CA3 regions. Tonic application of acetylcholine increases pyramidal cell excitation in both regions. However, initial phasic inhibition is observed only in CA1, but not CA3, pyramidal cells.

In the neocortex, L2/3 to L5 projections are strong excitatory feed-forward connections, whereas in hippocampal formation, they are from the CA3 to CA1 in a major unidirectional excitatory connection. As mentioned earlier, neocortical pyramidal cells in L5, but not L2/3, project to diverse subcortical targets. Similarly, the CA1 projects to diverse areas, including the thalamus, but the CA3 does not (Cenquizca and Swanson 2006; Cenquizca and Swanson 2007). Thus, CA3 and CA1 may correspond to L2/3 and L5, respectively, considering analogies of the direction-selective local connection, cholinergic action specificity, and extracortical projection pattern. L2/3 and CA3 pyramidal cells have higher connection hierarchy than L5 and CA1 cells. Therefore, acetylcholine may phasically inhibit pyramidal cells in lower hierarchical structures, sending diverse outputs from the neocortex and hippocampus.

8.3 Organization of L5 Pyramidal Cells Innervating the Striatum and Pontine Nuclei

Deep-layer pyramidal cells send axons to the ipsi- and contralateral cortices, like superficial ones, as well as to various subcerebral structures. As mentioned above, L5 receives feed-forward excitation from the upstream L2/3 and sends outputs to diverse subcortical targets. Here, I discuss the physiological and morphological diversity of L5 pyramidal cells in the rat frontal cortex. Followed by projection subtype identification, their local connection selectivity is examined. Based on these observations, the functional relationship between pyramidal cell projection subtypes and the local circuitry of target structures is discussed.

8.3.1 L5 Pyramidal Cell Heterogeneity

8.3.1.1 L5 Sublaminar Structures in the Frontal Cortex

L5 is discriminated from L2/3 by larger size of cell bodies and from L6 by their larger size as well as lower density. Type 2 vesicular glutamate transporters (VGluT2) are predominantly expressed in the axon terminals of thalamocortical cells, but not in those of pyramidal cells. L5 is further divided into two sublayers with different VGluT2 immunoreactivity: upper L5 (L5a) has weaker VGluT2 immunoreactivity and lower L5 (L5b) has stronger immunoreactivity (Morishima et al. 2011). Thus, L5b receives more thalamocortical inputs than L5a. Pyramidal cells in the two sublaminae differ in external projections, dendritic morphology, and connection pattern, as described below.

8.3.1.2 Firing Pattern Diversity Among L5 Pyramidal Cells

It is known that L5 pyramidal cells are physiologically heterogeneous (Kasper et al. 1994; Hattox and Nelson 2007). In the rat frontal cortex, two major firing patterns are found in L5 pyramidal cells (Fig. 8.2) (Morishima and Kawaguchi 2006; Otsuka and Kawaguchi 2008). Some pyramidal cells reduce firing frequencies during the application of constant suprathreshold depolarizing current pulses (firing frequency adaptation). The pyramidal cells with stronger adaptations are called fast adapting (FA). Other pyramidal cells show less adaptation of firing frequencies in response to depolarization, and are termed the slowly adapting (SA) type. Among SA pyramidal cells, some fire initially with a higher frequency (the first spike interval > 100 Hz; doublet firing).

8.3.1.3 L5 COM Cell Heterogeneity

The contralateral cortex and pontine nuclei are the two major targets of L5 pyramidal cells in the frontal cortex. Furthermore, L5 COM cells are heterogeneous in firing pattern and striatal projection pattern.

Both FA and SA cells are found among COM cells. Regarding remote projection, COM cells project to the striatum and other ipsilateral cortical regions. Some COM cells project only to the ipsilateral striatum (ipsilateral corticostriatal COM cells, type I COM cells), whereas others project to both sides of the striatum [crossed corticostriatal (CCS) cells, type II COM cells]. CCS COM cells (type II) are mostly the FA type, but ipsilateral corticostriatal COM cells (type I) are predominantly the SA type (Otsuka and Kawaguchi 2011). Morphologically, the apical dendritic tufts are more developed in SA COM cells than in FA COM cells. Thus, COM cells in the same layers are not uniform, but rather are heterogeneous in firing, dendritic morphology, and external projections, suggesting that COM communication is diversified.

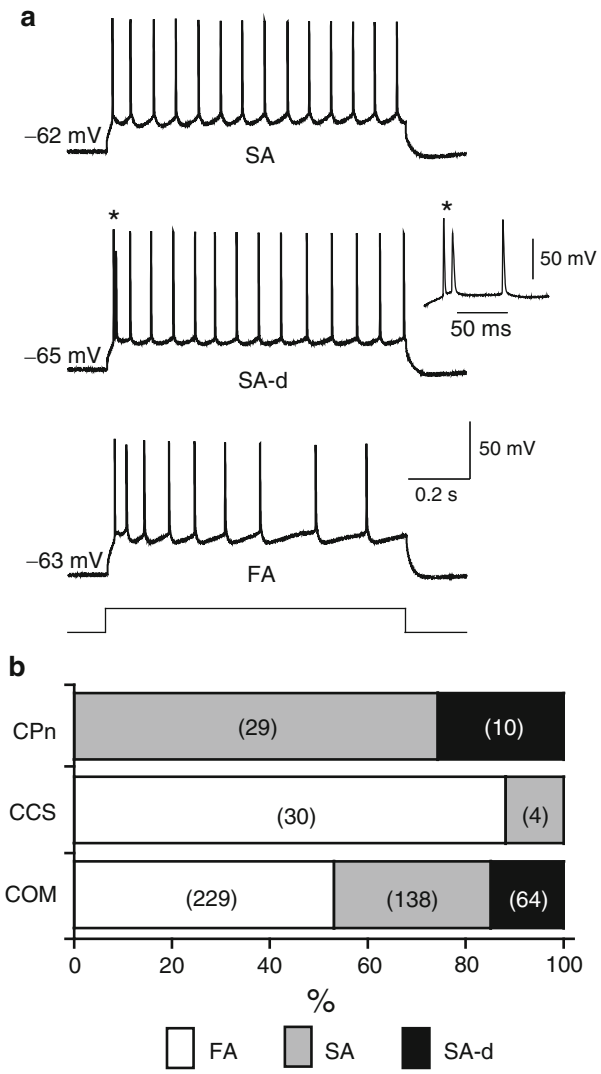

Fig. 8.2 Pyramidal cell firing types and correlation with extracortical projections. (**a**) Three firing patterns of L5 pyramidal cells in response to current pulse injection (500 pA, 1 s). *Inset* in SA-d, initial doublet (*) at an expanded time scale. FA type, fast spike frequency adaptation type; SA type, slow spike frequency adaptation type; SA-d type, slow spike frequency adaptation with initial doublet spikes type. (**b**) Percentage of firing subtypes in L5 CPn, CCS, and COM cells. Reproduced with permission from Otsuka and Kawaguchi (2011)

8.3.1.4 L5 CPn Cell Heterogeneity

Corticothalamic cells distribute mostly in L6 and upper L5 (L5a) (Hirai et al. 2012). L5 and L6 corticothalamic cells are assumed to innervate different thalamic cells (Jones 2001). L5a CTh cells overlap with CPn cells. In contrast,

corticospinal cells are found primarily in L5b, but not L5a (Ueta et al. 2013). Thus, CPn cells are further differentiated according to the L5 sublaminar structure and subcortical targets.

8.3.2 Excitatory Interlaminar Connections from L2/3 to L5

In intracortical wiring, the prominent excitatory interlaminar connection is from L2/3 to L5 pyramidal cells. This feed-forward connection is organized according to postsynaptic L5 pyramidal cell subtype (Otsuka and Kawaguchi 2008). A pair of L5 pyramidal cells of the same firing type receive more common inputs from individual L2/3 pyramidal cells than those with different firing type. Furthermore, a connected pair of pyramidal cells with the same firing type share excitation from L2/3 cells more frequently than an unconnected pair with the same firing type.

L5 COM cells are broadly divided into FA and SA firing types, whereas CPn cells are mostly the SA type. A pair of L5 COM cells of the same firing type is more connected than a pair from different firing types (Otsuka and Kawaguchi 2011). Furthermore, the former shares excitatory inputs from L2/3 more frequently than the latter. Interestingly, L5 CPn cells share L2/3 inputs with SA COM cells more frequently than with FA COM cells. These results suggest that the feed-forward interlaminar connections are partially segregated into functionally different channels according to output structure in the hierarchically lower layer and that some COM cells are more linked with the subcerebral projection. Thus, L5 SA and FA COM cells may transmit distinct information to the contralateral cortex.

8.3.3 Pyramidal Cell Subtypes in the Cortico-Subcortical Loop

L5 pyramidal cells in the frontal cortex participate in two important cortico-subcortical loops: the cortico-ponto-cerebello-thalamocortical and cortico-basal ganglia-thalamocortical loops. Because dysfunctions of the two loop structures are distinct, they are considered to have different functional roles (Doya 1999; Houk 2010). Both are thought to participate in procedural learning, but the basal ganglia loop is more involved in reinforcement learning and the cerebellar one is involved in supervised learning.

Internal basal ganglia and cerebellum structures have been well investigated, but it remains to be clarified how the local circuitry of the frontal cortex is organized according to these two distinct loops. CCS cells belong to the COM cell group and are a unique class of L5 pyramidal cells because they are of the FA type and have innervations to both sides of the striatum, as well as to the contralateral cortex. CCS cells are involved exclusively in the basal ganglia loop, but CPn cells are involved in the basal ganglia and cerebellar loops because CPn cells in the frontal cortex issue axon collaterals to the ipsilateral striatum. CCS and CPn cell morphological and connectional aspects are compared in the following sections (Fig. 8.3).

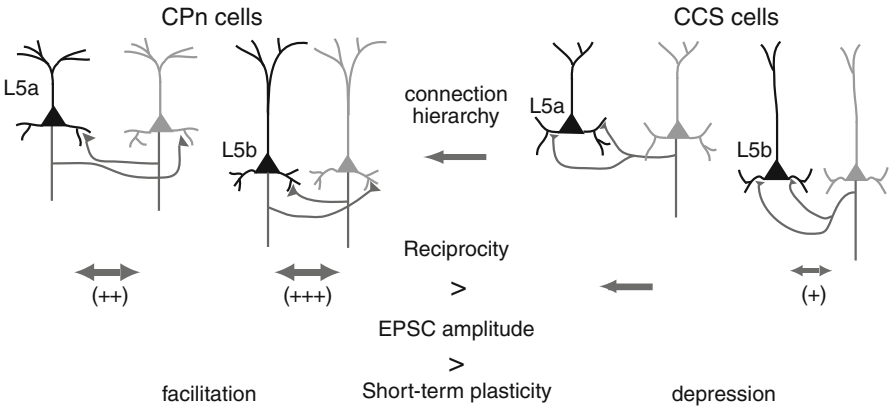

Fig. 8.3 CCS and CPn cell morphology and connectivity. Compared with CCS cells, the apical tufts of CPn neurons are more elaborate in L1. CPn pairs are more reciprocally connected than CCS cells. Reciprocal connections are only found in L5b among CCS pairs. The EPSC amplitude is larger in CPn than in CCS connections. In particular, one direction of reciprocally connected pairs sometimes exhibits large EPSCs (>100 pA). CPn pairs are facilitatory in short-term plasticity, whereas CCS pairs depressive. Reproduced with permission from Morishima et al. (2011)

8.3.3.1 Morphologies of CCS and CPn Cells

The dendritic morphology of L5 pyramidal cells depends on the projection subtype as well as the somatic depth (sublayer-dependent). The basal dendrites have more branch points in CPn cells than in CCS cells. The apical dendritic tufts in L1 are more developed in CPn cells than in CCS cells. L5a neurons have more tuft branches than L5b neurons in both the CCS and CPn cell groups. These suggest different extracortical input patterns between CCS and CPn cells in L1 and L5.

8.3.3.2 Hierarchical Connection from CCS to CPn Cells

We investigated the connection patterns between CCS and CP cells in vitro by using paired recordings from retrogradely labeled cells from the contralateral striatum and the ipsilateral striatum: spike induction in one cell from depolarizing current pulses and postsynaptic current recordings from another (Morishima and Kawaguchi 2006). Connections are found from CCS to CPn cells, but rarely from CPn to CCS cells, suggesting hierarchy from the CCS to CPn cell group. The short-term plasticity of CCS to CPn cell connection is predominantly the depression type, as revealed by paired-pulse stimulation. Axodendritic contact sites have been identified in morphologically reconstructed pairs by using light microscopy. The negative correlation of contact site number with the failure rate and positive correlation with the EPSC amplitude suggests the intimate relationship of the contact site with the release site.

CPn cells rarely induce EPSCs in CCS cells. Two possibilities may be considered for the connection deficits: (1) if CPn cell axons do not spatially come within the range of CCS cell dendrites, synaptic formation would be impossible (axon territory segregation), or (2) CPn cell axons approach CCS cell dendrites (within potential distance for synapse formation) but avoid making synaptic contacts (local contact avoidance). We discriminated between these two cases by reconstructing the axodendritic contact sites as well as the approaching points where the presynaptic candidate neuron axons approach within the spine length. In CCS/CPn cell pairs with a connection from the former to the latter, we only observed contact sites in dendrites of CPn cells but not in CCS cells but found some approach points in both directions. Approximately 20 % of approach points from CCS cell axons to CPn cell dendrites made contact. These data suggest that the unidirectional connection between different L5 projection subtypes is selectively formed, and the reverse connection is actively avoided.

8.3.3.3 Connection Characteristics Within the CCS and CPn Cell Groups

Connection patterns between pyramidal cells of the same projection subtype, such as CCS/CCS and CPn/CPn pairs, have been investigated (Morishima and Kawaguchi 2006). Both types of connections are found at similar probabilities, but reciprocal connections (bidirectional excitations) are found three times more frequently in CPn/CPn pairs than in CCS/CCS pairs. EPSC amplitudes are larger in CPn/CPn pairs than CCS/CCS pairs. In particular, reciprocally connected CPn/CPn pairs exhibit the largest EPSCs.

Temporal characteristics of synaptic transmissions have been investigated using paired-pulse stimulation. CPn/CPn pairs are more facilitatory in short-term plasticity, whereas CCS/CCS pairs are more depressive. CPn/CPn pair facilitation accompanies decrease of coefficient variation (CV) from the first EPSCs to the second EPSCs, but CCS/CCS and CCS/CPn pair depression accompanies its increase. These findings suggest that facilitation and depression in these pairs are caused by changes in presynaptic transmitter release.

It is generally assumed that synaptic transmission with lower release probability is more facilitatory than transmissions with higher ones. Therefore, axon terminals of CPn cells might have a lower release probability than those of CCS cells. This could not be directly tested, but we have examined the relationship between short-term plasticity and release probability in these synapses in two alternative ways, assuming different transmission models.

In pairs with morphological reconstruction, including axodendritic contact sites, the binominal model has been applied (Markram et al. 1997). When the contact site number and CV are given, we can obtain the release probability using the binomial model, further assuming individual contacts with a single release site. In all CPn/CPn, CCS/CCS, and CCS/CPn pairs, the release probabilities are variable. When compared at similar release probabilities, the paired-pulse ratio of CPn/CPn pairs was larger than those of either the CCS/CCS or CCS/CPn pairs.

In addition, the phenomenological approach has been applied for transmission analyses (Markram et al. 1998). A series of synaptic currents induced by a repetitive presynaptic cell firing at short intervals, followed by a longer interval, were obtained. These currents were fitted to the phenomenological model, including the available resource and utilization parameters. The utilization parameter at rest (*U*) corresponds to the release probability in the binominal model. Compared at similar rest utilization parameters (*U*), the paired-pulse ratio of CPn/CPn pairs was larger than that of CCS/CCS pairs. These data suggest that CPn/CPn transmissions are generally more facilitatory than CCS/CCS and CCS/CPn transmissions.

8.3.4 Pyramidal Cell Subtypes and Local Reverberating Circuit

When the cortex retains information temporarily, a given cortical neuron would fire tonically for that period. This tonic mnemonic activity could be executed locally by an intrinsic property of cortical neurons themselves to generate persistent depolarization or excitatory reciprocal connections between them. It is known that there are very few cells firing tonically without external depolarizing currents in vitro. Therefore, reciprocal connections between excitatory neurons would play an important role to support local continuous firing (Wang 2001).

There are more spontaneously firing cells in L5 than in L2/3 (Barth and Poulet 2012). As described above, L5 pyramidal cells are heterogeneous in external projections and firing characteristics. Among them, CCS and CPn cells, two projection subtypes that innervate the ipsilateral striatum, are distinct in synaptic connection characteristics as well as morphology. CPn cells show less firing adaptation during constant depolarization than CCS cells. CPn/CPn pairs are higher in connection reciprocity, more facilitatory in short-term plasticity, and larger in synaptic current amplitude than CCS/CCS pairs. These observations suggest that connected CPn cells could make excitatory reverberations to produce persistent activity. However, the CCS cell group may be transiently activated and represent phasic information.

Importantly, CCS cells unidirectionally connect with CPn cells. Thus, transient excitation of a sufficient number of CCS cells would excite CPn cells enough to induce autonomous firing by their reciprocal connection. The CPn group could amplify their spiking activities using the temporal properties of facilitation, higher reciprocal connectivity, and intrinsic firing properties.

Persistent firing during a given task in the frontal cortex is thought to underlie working memory (Wang 2001). The synaptic properties and interconnections of CPn pairs suggest these neurons may provide a suitable substrate for working memory representation, receiving inputs from CCS cells unidirectionally. Because both CPn and CCS cells project to the ipsilateral striatum, it would be beneficial to consider the interaction of these two types with basal ganglia function.

8.3.5 Relationship Between Corticostriatal Projection Diversity and Basal Ganglia Internal Structure

8.3.5.1 Corticostriatal Input Preferences for Individual Basal Ganglia Pathways

The striatum receives cortical inputs and sends outputs to other nuclei in the basal ganglia. Striatal projection neurons are GABAergic medium-sized spiny neurons (MSN) and can be divided into two major subtypes: (1) neurons that exclusively innervate the external segment of the globus pallidus (GPe; indirect pathway), called iMSN (indirect-pathway MSN) neurons, and (2) neurons that project to basal ganglia output nuclei, such as the substantia nigra pars reticulata (SNr; direct pathway), and issue axon collaterals to the GPe, called dMSN (direct-pathway MSN) neurons (Gerfen and Surmeier 2011). Thus, dMSN neurons directly inhibit SNr cells, but iMSN cells indirectly disinhibit SNr cells because GPe cells are GABAergic.

Importantly, CCS and CPn cells in the frontal cortex do not innervate the dMSN and iMSN uniformly but rather show postsynaptic target preferences (Lei et al. 2004; Reiner et al. 2010). It has been revealed using electron microscopy that dMSN and iMSN are identified by expression differences in dopamine receptor types combined with selective labeling of CCS and CPn cell axons by using tracers in rat. CCS cell axons innervate the dMSN more than iMSN, whereas CPn cells prefer the iMSN. Thus, although both cortical cell subtypes project to both striatal projection subtypes, there exists combinatory preferences between the postsynaptic and presynaptic cell subtypes. Furthermore, CPn terminals are larger than CCS terminals. In primates, by comparing the size of corticostriatal terminals on the dMSN and iMSN, similar corticostriatal connection preferences have been suggested (Reiner et al. 2010). These results indicate that CCS and CPn cells share the striatum as an extracortical target, but synapses are differentially formed there. That is, the local circuit in the frontal cortex differentiates according to the internal organization of the projected areas.

8.3.5.2 Thalamocortical Input Heterogeneity to the Frontal Cortex

GABAergic SNr neurons innervate some thalamic nuclei. The thalamus consists of two major compartments, called the core and matrix (Jones 2001). The thalamic core cells mainly predominantly innervate the cortical middle layer, but the matrix cells prefer L1. The latter often express calbindin, a type of calcium-binding protein. The thalamic cells receiving SNr cell inhibition belong to the matrix system, and thus innervate L1 of the frontal cortex (Kuramoto et al. 2009; Rubio-Garrido et al. 2009).

As discussed above, compared to CCS cells, CPn cells have more apical dendritic branches in L1. Therefore, CPn cells are assumed to receive more input from basal ganglia-related thalamic nuclei. CPn cells would be excited in response to thalamic cell excitation via suppression of SNr cell discharges induced by dMSN firing.

Thus, CPn cells make closed loops with the basal ganglia, whereas the CCS cells make open loops. Therefore, the indirect pathway could excite SNr cells and thereby decrease the activity of the corresponding thalamic cells, and eventually reduce CPn cell activity. These data suggest that the indirect pathway could participate in feedback inhibition of CPn cells via the closed cortico-basal ganglia loop.

8.3.5.3 Mechanism of Induction for Selected Actions Via the Cortico-Basal Ganglia-Thalamic Loop

The frontal cortico-basal ganglia system is thought to participate in the evaluation of performed actions, in addition to action selection. Considering the cortico-basal ganglia circuits, we made the following hypothesis regarding the initiation and termination of selected actions (Morita et al. 2012).

When one action is selected, a group of CCS cells representing that action start to fire and drive dMSN cells representing that action value. These CCS cells also activate a group of CPn cells. Direct pathway activation would excite the corresponding thalamic cells projecting to the frontal cortex. In response to simultaneous excitation by direct intracortical input from the CCS cells to the basal dendrites and the thalamic input via the basal ganglia to the apical tuft, CPn cells then start to fire. When another action is selected and a corresponding group of CCS cells start to fire, the CPn cells excited by the former CCS cell group would lose the excitatory drive from the thalamus via the basal ganglia direct pathway, and, in addition, would be suppressed by feedback inhibition through the basal ganglia indirect pathway, thus terminating that action.

8.3.5.4 Evaluation of Performed Actions Through the Cortico-Striato-Nigro-Striatal Pathway

Dopaminergic neurons in the substantia nigra pars compacta (SNc) are assumed to represent reward prediction error (RPE) signals that change corticostriatal synaptic strength (Schultz et al. 1997; Glimcher 2011), which would be the basis for reinforcement learning. RPE is obtained by subtracting the value of a selected action from the action to be selected next and addition of the reward amount. According to the above model, the direct pathway would represent a value of the action that is selected next, with the indirect pathway representing the value of an already selected action. The subtraction value may be performed at SNr, upon convergence of the direct and indirect pathways, although the sign is opposite. Dopaminergic SNc cells receive convergent inhibition from SNr cells and may receive the reward amount signal, for example, the pedunculopontine nucleus. Therefore, RPE could be calculated at the SNc (Morita et al. 2012). Phasic firing changes in dopaminergic SNc cells, representing the RPE, would transiently modify the dopamine concentration in the striatum and the synaptic strength of corticostriatal terminals involved in that action.

8.4 Pyramidal Cells Projecting to the Parahippocampal Area

Pyramidal cells participate in external projection to subcortical targets as well as to other cortical regions. The frontal cortex reciprocally connects with the perirhinal cortex (PRC), a parahippocampal area, which is involved in the formation and retrieval of declarative memories (Eichenbaum 2006). We have identified distinct subtypes of pyramidal cells projecting from the frontal to perirhinal cortex (PRC-projecting cells) (Hirai et al. 2012).

8.4.1 Multiple Projection Channels from the Frontal Cortex to Perirhinal Cortex

PRC-projecting cells distribute in L2/3, L5, and L6, predominantly found in the upper L2/3 (L2/3a) and upper L5 (L5a) (Fig. 8.4). The perirhinal cortex consists of dorsal area 36 and ventral area 35. L2/3a PRC-projecting cells mainly innervate area 35 as well as the basolateral nucleus of the amygdala. However, L5a PRC-projecting cells send axons mainly to area 36, and partially overlap with CCS cells but are independent of CPn and corticothalamic (CTh) cells. As expected, L5a PRC-projecting and CTh cells are different in dendritic morphologies and electrophysiological properties.

L2/3a PRC-projecting cells innervate both L5a PRC-projecting and CTh cells. In the reverse direction, EPSCs have been detected in L2/3a PRC-projecting cells from L5a PRC-projecting cells, but not from individual CTh cells. Intracortical ascending axons of L5a PRC-projecting cells distribute mainly in lower layer 1 (L1b) and upper layer 2/3 (L2/3a), whereas L5a CTh cell axons preferentially target upper layer 1 (L1a).

The axon collaterals in L1a would innervate the apical tufts. Individual excitatory synapses on pyramidal tuft dendrites in L1a could not effectively generate somatic depolarization on their own. For transmission to the soma, large local events should be induced by joint activation of several inputs at the apical tuft. Interlaminar reciprocal connections are functionally formed between a pair of L2/3a and L5a PRC-projecting cells. However, excitatory transmission from L5a CTh to L2/3a PRC-projecting cells would need collective activation of the former cells.

8.4.2 Local Circuit Specification is Dependent on Procedural and Declarative Memory Systems

Recently, the striatum was shown to participate in declarative memory retrieval as well as reinforcement learning of procedural memories (Scimeca and Badre 2012). L5a CCS cells project to both the striatum and perirhinal cortex area 36. Thus, these

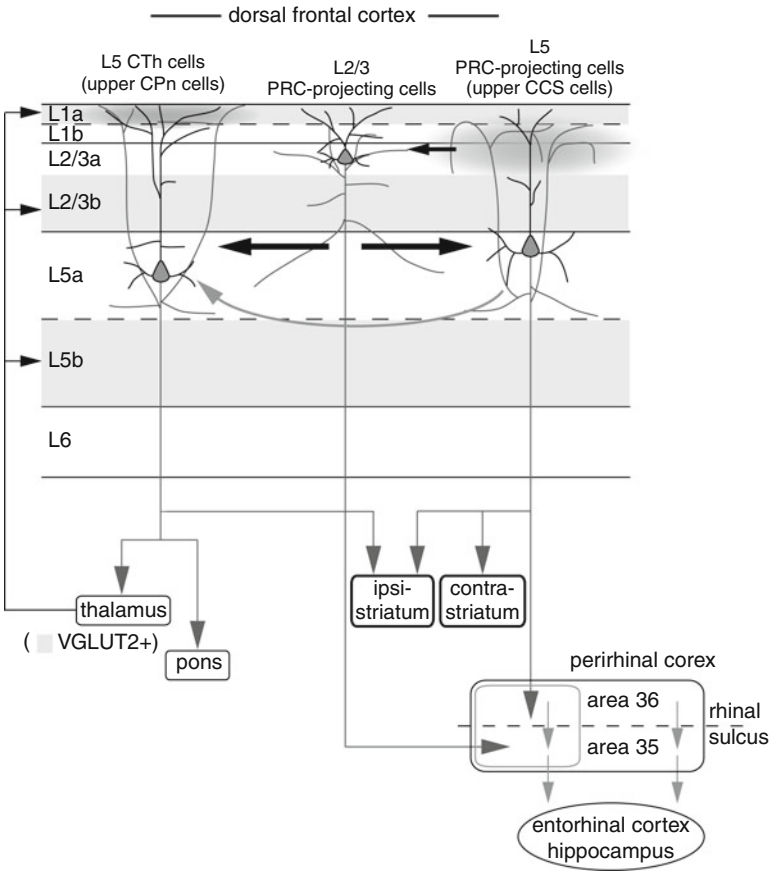

Fig. 8.4 Schematic representation of local/distal circuits and layer distributions of pyramidal cell subtypes projecting to the perirhinal cortex. The fronto-perirhinal projection incorporates two pathways originating from L2/3 cells innervating the rostral part of perirhinal area 35 (L2/3 PRC-projecting cells) and L5 cells innervating the rostral part of perirhinal area 36 (L5 PRC-projecting cells). L5 PRC-projecting cells also innervate both sides of the striatum (CCS cells) and are distinct from CTh cells, which send axons to the pons (CPn cells). Within the frontal cortex, ascending axon branches of L5 PRC-projecting cells mainly innervate L1b and L2/3a, whereas CTh cells innervate L1a. The L2/3 PRC-projecting group sends feed-forward excitation to both L5 PRC-projecting and CTh cells, but the backward connection is from individual L5 PRC-projecting cells. L5 PRC-projecting cells are assumed to send excitation unidirectionally to CTh cells. The perirhinal cortex is composed of excitatory forward connections from area 36 to 35 (Burwell and Amaral 1998). Note that L2/3 PRC-projecting cells not only make forward connections to L5 PRC-projecting cells but also innervate the downstream site of perirhinal cortex, area 35. Reproduced with permission from Hirai et al. (2012)

cells could convey frontal cortex activity simultaneously to the striatum and perirhinal cortex. Concurrent projection to the perirhinal cortex and striatum suggests an intimate relationship between declarative memory retrieval/formation and reinforcement learning (Hirai et al. 2012).

The corticostriatal synapses denote the state or action values used for calculation of the temporal difference error. According to the tentative reinforcement learning hypotheses, CCS cells represent the current state or action to be selected, whereas CPn cells represent actions being executed or those that have already been executed. Thus, some CPn cells should code the state/action that occurred just prior, with others coding those in the distant past (Clark et al. 2012).

CPn cell extracortical targets differ between L5a and L5b. L5b CPn cells, activated by L5b CCS cells, include corticospinal cells that directly participate in action execution and may represent the immediately preceding state/action. L5a CPn cells receive excitation from L5a CCS cells. L5a FA cells, including CCS cells, receive more excitation from L2/3a (Otsuka and Kawaguchi 2008; Hirai et al. 2012). Importantly, both L2/3a pyramidal and L5a CCS cells project to the perirhinal cortex, which is involved in retrieval of declarative memories (Miyashita 2004). Thus, two interlaminar subnetworks from L2/3 to L5 are embedded in the frontal cortex. The connection between L2/3a and L5a could be used for evaluation and selection of actions executed in the distant past, whereas the connection between L2/3b and L5b would be used for actions being executed in series.

8.5 GABAergic Cell Organization

Neocortical GABAergic cells have highly diverse morphology, but their independent subtypes have been identified by combined analyses of molecular expression, electrophysiological, and morphological characteristics (Kawaguchi and Karube 2008). It is important how diverse GABAergic cells regulate pyramidal cell subnetworks dependent on diverse extracortical targets.

8.5.1 Expression Specificity of Molecular Markers in Neocortical GABAergic Cells

In addition to GABA and its synthetic enzymes, nonpyramidal cell somata express several peptides including somatostatin, neuropeptide Y (NPY), cholecystokinin (CCK), vasoactive intestinal polypeptide (VIP), corticotropin-releasing factor (CRF), calcium-binding proteins (such as parvalbumin (PV) and calretinin), and an actinin-binding protein, α-actinin-2 (AAC) (Fig. 8.5). Expression of these markers is not uniform among GABA cells, but is restricted to a fraction of cells. Although the functional significance remains unknown, their specific expression has been very useful for GABA cell subtype identification.

Upon immunohistochemical examination, PV, somatostatin, VIP, and AAC are expressed in different neocortical neurons. Neocortical GABA cells are

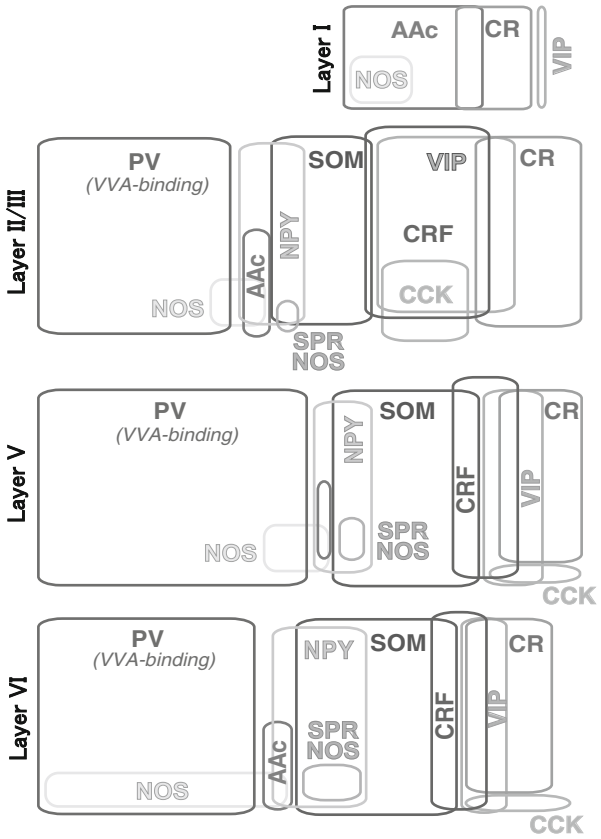

Fig. 8.5 Shared chemical composition of GABA cells in the frontal cortex. The relative number of immunoreactive cells for a particular substance is proportional to the size of the box in each layer. *AAc* alpha-actinin-2, *CCK* cholecystokinin, *CR* calretinin, *CRF* corticotropin-releasing factor, *NOS* nitric oxide synthase, *NPY* neuropeptide Y, *PV* parvalbumin, *SOM* somatostatin, *SPR* substance P receptor, *VIP* vasoactive intestinal peptide. Reproduced with permission from Kubota et al. (2011b)

broadly divided into five major classes in the rat frontal cortex (Uematsu et al. 2008; Kubota et al. 2011b): (a) PV cells; (b) somatostatin cells; (c) VIP cells, calretinin cells, VIP/CRF cells, and VIP/CCK cells; (d) CCK cells; and (e) AAC cells (Fig. 8.5).

The proportion of each subtype among GABA cells varies between layers, but PV cells are the largest group in individual layers. A similar kind of molecular organization has been found in other cortical regions. Therefore, this scheme seems common over the entire neocortex.

8.5.2 Firing Pattern Diversity

The firing characteristics in response to suprathreshold depolarizing current pulses broadly divide nonpyramidal cells into three major categories: fast-spiking (FS), late-spiking (LS), and other non-FS cells.

1. FS cells are more hyperpolarized in resting potential and lower in input resistance, and start to fire at approximately 40 Hz in response to threshold currents.
2. LS cells discharge spikes following slowly developing ramp depolarizations at the threshold strength.
3. Non-FS cells: I have tentatively grouped cells other than FS/LS cells into this category. Therefore, their electrophysiological properties are diverse. However, low-threshold spike (LTS) cells are distinct, generating two to five spikes on LTS induced by transient depolarization from the hyperpolarized potential.

8.5.3 Morphological Diversity

Neocortical GABA cell morphology is highly diverse and their diverse subtypes have been identified previously. The following classification depends on the axonal ramification pattern:

1. Basket cells form multiple axonal boutons on other soma, with somatic boutons greater than 15 % of total boutons.
2. Chandelier cells form vertical arrays of multiple boutons like candle chandeliers and make synapses on initial axon segments of pyramidal cells.
3. Martinotti cells with ascending axons to layer 1 and a modest number of spines along the dendrite. They innervate the dendritic shafts and spines of pyramidal cells.
4. Double bouquet cells with bipolar or multipolar somata and descending axon collaterals, innervating dendritic shafts and spines.
5. Neurogliaform cells with dense axonal innervation around the soma, also called spider web cells, innervate various pyramidal cell domains.

8.5.4 Correlation of Molecular Expression with Physiological and Morphological Characteristics

Thus far, neurochemical, electrophysiological, and morphological diversity have been shown. How are these features relevant to each other in neocortical GABAergic cells? By investigating physiological and anatomical features of molecularly and chemically defined cells, the neuronal organization of GABA cells has gradually

been revealed. The five major chemical groups, as mentioned above, show distinct firing and morphological patterns.

1. PV cells are predominantly FS cells. They are frequently basket cells, but chandelier cells are also found in FS cells.
2. Somatostatin cells are non-FS cells, and are often Martinotti cells. LTS Martinotti cells are frequently found in L5.
3. VIP and calretinin cells are non-FS cells, and include various morphological forms such as double bouquet, small basket, descending basket, and arcade cells.
4. CCK cells with larger somata are often non-FS large basket cells. Thus, large basket cells are composed of two major subtypes: PV and CCK cells.
5. AAC cells are often LS cells and morphologically are neurogliaform cells. They are also chemically positive for NPY, except L1 AAC cells. L1 AAC cells extend axons horizontally, confined to L1, whereas L2/3 AAC neurogliaform cells innervate both L1 and L2/3. AAC cells in L1 are likely distinct from those in other layers and are morphologically different from neurogliaform cells.

Thus, an intimate correlation between molecular, physiological, and morphological classifications has been established.

8.5.5 Selective Cholinergic Modulation of GABA Cell Subtypes

The individual subtypes described above show unique morphological characteristics and axonal selectivity for postsynaptic domains. Furthermore, the transmitter response pattern correlates with this classification (Kawaguchi and Kondo 2002). The cholinergic actions on GABA cell subtypes varied among previous studies. Therefore, we compared phasic responses induced by transient focal application of acetylcholine (Gulledge et al. 2007).

Direct hyperpolarization and depolarization are induced in GABA cells by acetylcholine application. Large CCK basket cells are transiently hyperpolarized by acetylcholine. The responsible receptor is the type 2 muscarinic receptor, which differs from the hyperpolarization induced in L5 pyramidal cells described above.

Slow tonic muscarinic depolarizations are found in somatostatin, VIP, and CCK cells, but not in PV and AAC cells. Phasic depolarization via nicotinic receptors is induced selectively in VIP cells and AAC neurogliaform cells.

Thus, as described above, acetylcholine transiently inhibits L5 pyramidal cells projecting to the subcortical structures. In contrast, GABAergic cells are regulated phasically and tonically by acetylcholine in a diverse fashion: nicotinic depolarization, muscarinic hyperpolarization, and slow depolarization. Because the innervation preference for the pyramidal cell surface domain is dependent on the GABA cell subtype, GABAergic inhibition of circuit activity would dynamically shift according to

temporal changes in local acetylcholine concentration. Further correlation of GABA cell subtypes as defined by the molecular expression, firing, dendritic arborization, and axon target preference with modulator response pattern supports the functional validity of the above classification.

8.6 Synaptic Interactions Between Excitatory and Inhibitory Subnetworks

To understand the functional differentiation of diverse GABA cells, we must reveal their synapse formation rules. As introduced above, pyramidal cells are composed of diverse output groups and form excitatory subnetworks dependent on the extracortical projection pattern. Therefore, the local inhibitory system is assumed to diversify by responding to the diverse excitatory subnetworks.

Most excitatory inputs to pyramidal cells make synapses on the dendritic spine. Using electron microscopic observation, it has been determined that some spines have symmetrical GABAergic synapses together with asymmetrical excitatory synapses (Kubota et al. 2007). The postsynaptic receptors on the spines contain GABA A type. Several GABA cell subtypes, including PV FS and somatostatin Martinotti cells, innervate the spine. This dual input on the same spine would be suitable for inhibiting specific inputs, without affecting other inputs to a given pyramidal cell. By selectively marking of the excitatory inputs from the thalamus and the cortex, some spines innervated by thalamic inputs have been found to receive GABAergic inhibition. Therefore, several GABA cell subtypes inhibit thalamic excitation selectively at the initial input spine stage.

L5 pyramidal cells receive feed-forward excitation from L2/3 pyramidal cells composed of pathways differentiated according to layer 5 pyramidal cell subtypes and their connection patterns (Otsuka and Kawaguchi 2011). PV FS cells frequently make reciprocal connections with nearby pyramidal cells. Inhibitory and excitatory postsynaptic currents are larger in reciprocally connected pairs than in unidirectionally connected ones (Otsuka and Kawaguchi 2009). Sharing the excitatory inputs from individual L2/3 pyramidal cells is similar between connected and unconnected pairs of L5 pyramidal and FS cells. However, common excitatory input probabilities from L2/3 are higher in connected pairs of L5 pyramidal and non-FS cells than unconnected ones. Thus, L5 GABA cells form distinct interlaminar subnetworks with pyramidal cells, depending on inhibitory cell subtypes.

Recently, it has been revealed that neocortical GABA cells inhibit nearby pyramidal cells nonselectively (Fino et al. 2012). Considering the selective inhibition of thalamocortical inputs and the specific inhibitory manner on the abovementioned interlaminar excitatory pathways, however, some connection specificity would exist between multiple GABA cell subtypes and diverse excitatory subnetworks. Elucidation of synaptic interactions among excitatory and inhibitory cell subtypes will promote our understanding of neocortical circuit organization.

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Chapter 9
Emerging Roles of Heparan Sulfate in Axon Guidance Signaling

Masayuki Masu

Abstract Accurate wiring of the neural network is a fundamental for higher brain functions. In the developing brain, growing axons are navigated towards their targets by the concerted actions of chemoattractants and chemorepellents. Recent studies have revealed that heparan sulfate, a glycosaminoglycan sugar chain attached to core proteins in proteoglycans, plays pivotal roles in regulating axon guidance signaling. Here some of the topics related to heparan sulfate in axon guidance are reviewed with emphasis on its structure and activity in relation to its synthesizing and modifying enzymes.

Abbreviations

BMP	Bone morphogenetic protein
DCC	Deleted in colorectal cancer
FGF	Fibroblast growth factor
GDNF	Glial cell line-derived neurotrophic factor
GlcA	Glucuronic acid
GlcNAc	*N*-acetylglucosamine
HB-EGF	Heparin-binding epidermal growth factor
HGF	Hepatocyte growth factor
HS	Heparan sulfate
HSPG	Heparan sulfate proteoglycans
IdoA	Iduronic acid
Shh	Sonic hedgehog
TGFβ	Transforming growth factor-β
VEGF	Vascular endothelial growth factor

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9.1 Axon Guidance Molecules

In embryonic brains, neural networks are formed through neurogenesis, differentiation, axon guidance, synaptogenesis, and activity-dependent refinement of immature synapses. Each of these processes is regulated by the concerted actions of growth factors, transcription factors, axon guidance molecules, and synapse organizers. Genetic defects or environmental perturbation of this harmonized signaling may cause severe brain diseases including congenital brain malformation, mental retardation, and autism spectrum disorders. Therefore, from the medical viewpoint, it is important to elucidate the molecular mechanisms that control brain development.

Genetic, biochemical, and molecular studies over three decades have identified axon guidance molecules that attract or repel specific axons to establish functional neural networks (Yu and Bargmann 2001; Dickson and Zou 2010; Kolodkin and Tessier-Lavigne 2011). These molecules are evolutionarily conserved among diverse species and contribute to the formation of distinct neural networks in different organisms such as nematodes, fruit flies, zebrafish, mice, and humans. Major axon guidance molecules are classified into four families: netrins, semaphorins, slits, and ephrins (Fig. 9.1). Netrins were initially identified as secreted proteins that promoted outgrowth and induced turning of spinal commissural axons in collagen gel culture. Biochemical purification and molecular cloning of netrins led to the discovery that netrins are homologs of the *C. elegans UNC6* gene, which was identified as the mutant gene responsible for the defects in circumferential migration of axons. Both netrins and UNC6 act as chemoattractants for some axons and as chemorepellents for others. Semaphorin 3A (previously termed collapsin 1) was identified as a secreted protein that induced the collapse of the growth cones of cultured dorsal ganglion cells. Subsequently, several homologous molecules were found in invertebrates and vertebrates and classified into seven classes (Sema1-7) (Fig. 9.1). All of them share the Sema domain, while each class has different structural motifs, including an immunoglobulin globular domain, a transmembrane segment, thrombospondin domains, and a glycosylphosphatidylinositol (GPI) anchor domain. Class 2 and 3 semaphorins are secreted proteins, while other semaphorins are associated with the cell surface. Slit was originally described as a mutation in *Drosophila* that resulted in the collapse of the midline structure and axon fascicles in the embryonic nerve cord. Subsequent studies have shown that slits function as chemorepellents that prevent the midline crossing of non-crossing axons. Ephrins were initially identified as ligands for orphan Eph receptor-type tyrosine kinases. Subsequently, ephrins were shown to play roles in regulating axon guidance, migration, and topographic map formation. Ephrins are classified into ephrin As (GPI-anchored molecules) and ephrin Bs (transmembrane molecules) (Fig. 9.1). In general, netrins are bifunctional molecules that mediate attraction or repulsion, while slits, semaphorins, and ephrins act primarily as repellents but can act as attractants in some cases.

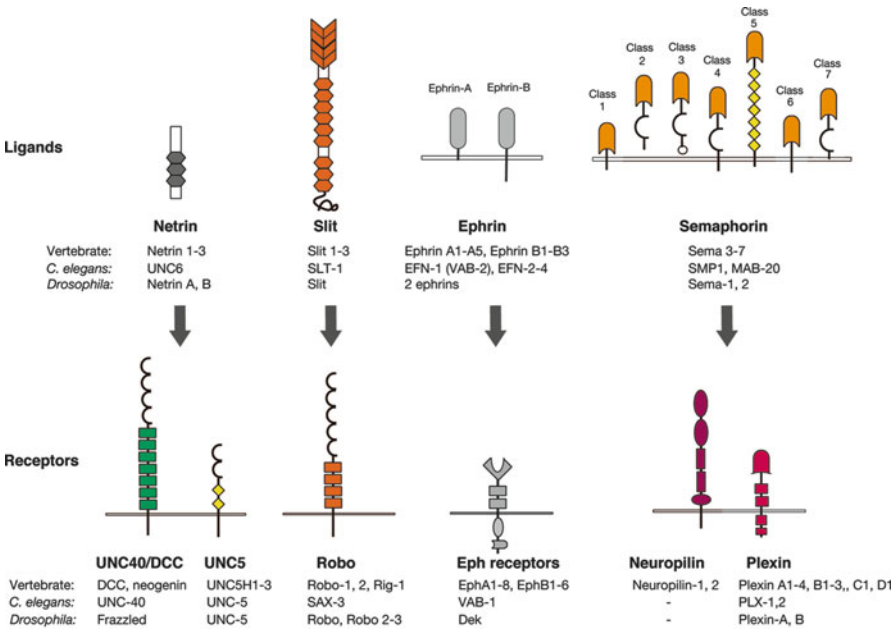

Fig. 9.1 Axon guidance molecules and their receptors. Major axon guidance proteins are classified into four families: netrins, slits, ephrins, and semaphorins. Each family consists of several members. The axon guidance signals are mediated by specific transmembrane receptors. Axon guidance proteins and receptors are evolutionarily conserved from *C. elegans* and *Drosophila* to humans (Modified from Yu and Bargmann 2001)

9.2 Axon Guidance Receptors

The receptors that mediate specific signaling of axon guidance molecules were identified by genetic, biochemical, and molecular approaches (Yu and Bargmann 2001; Dickson and Zou 2010; Kolodkin and Tessier-Lavigne 2011). Netrins activate two classes of receptors, DCC (deleted in colorectal cancer)/UNC40 and Unc5H (Unc5 homologs)/UNC5, both of which are immunoglobulin superfamily molecules with one transmembrane segment (Fig. 9.1). DCC and UNC5 mediate netrin-dependent axonal attraction and repulsion, respectively. In addition, UNC5 converts DCC-mediated attraction to repulsion by silencing DCC responses. Semaphorins have two types of receptors, neuropilins and plexins. Neuropilins are found in vertebrates but not in invertebrates and mediate the repulsion by class 3 semaphorins, while plexins are versatile and involved in all semaphorin-dependent signaling (Fig. 9.1). Roundabouts (Robos) are the receptors for slits and are immunoglobulin superfamily molecules with different domain organization from DCC/UNC40 (Fig. 9.1). Slit-Robo repulsion regulates the midline crossing decision: axons expressing Robo do not cross the midline owing to repulsion by Slit protein present in the midline, whereas axons that lack Robo expression do cross the midline. In fruit flies, a transmembrane protein, commissureless (comm), sorts Robo proteins into endosomes

and lysosomes intracellularly, thereby downregulating Robo activity and making axons insensitive to Slit during midline crossing. In vertebrates, a specific splice variant of Robo3, Robo3.1, inhibits Slit-Robo repulsion, thereby helping commissural axons to cross the midline. Eph receptors are transmembrane-type tyrosine kinases and subdivided into two classes: EphA and EphB. Generally, ephrin As and ephrin Bs activate EphA and EphB receptors, respectively.

9.3 Axon Guidance by Classical Morphogens

More recently, molecules that were originally known as morphogens, such as wingless/Wnt, hedgehog/sonic hedgehog (Shh), and transforming growth factor-β (TGFβ)/bone morphogenetic protein (BMP), turned out to have roles in axon guidance (Charron and Tessier-Lavigne 2005). The first evidence for the guidance role of Wnt came from a *Drosophila* study: in a mutant lacking Derailed (Drl) receptor tyrosine kinase, commissural axons cross the midline only in the posterior commissure and avoid the anterior commissure. Subsequently, Wnt5, one member of the Wnt family, was shown to be a Drl ligand expressed in the posterior commissures that act as a repellent for the Drl-expressing axons that normally cross the midline in the anterior commissure owing to the repulsive effect from the posterior commissures. Interestingly, in the rat spinal cord, Wnt4 attracts post-crossing axons in a rostral direction along the anteroposterior axis in a concentration-dependent manner. Shh functions as a floor plate-derived chemoattractant for spinal commissural neurons through the Shh signaling mediator Smoothened (Smo) and Bidirectional Cdon-binding protein (Boc). In addition, Shh also guides commissural axons in a rostral direction along the longitudinal axis by repelling them after they have crossed the midline. BMP7 contributes to the chemorepellent activity of the roof plate and helps commissural axons to extend ventrally into the spinal cord in collaboration with netrins and Shh. Therefore, these morphogens control axon guidance by using their graded positional information.

9.4 Heparan Sulfate: Its Structural and Functional Diversity

Heparan sulfate proteoglycans (HSPGs) regulate cell growth, differentiation, morphogenesis, migration, axon guidance, synapse formation, and synaptic plasticity (Bülow and Hobert 2006; Esko and Selleck 2002; Lindahl et al. 1998; Perrimon and Bernfield 2000). These diverse functions are mediated by the interaction between heparan sulfate (HS) attached to core proteins in proteoglycans and various signaling molecules, including growth factors, morphogens, axon guidance proteins, chemokines, extracellular matrix components, receptors, and cell adhesion molecules. In the developing brain, for example, HSPGs in the extracellular matrix bind morphogens, contributing to formation of a morphogen gradient. Cell surface HSPGs act

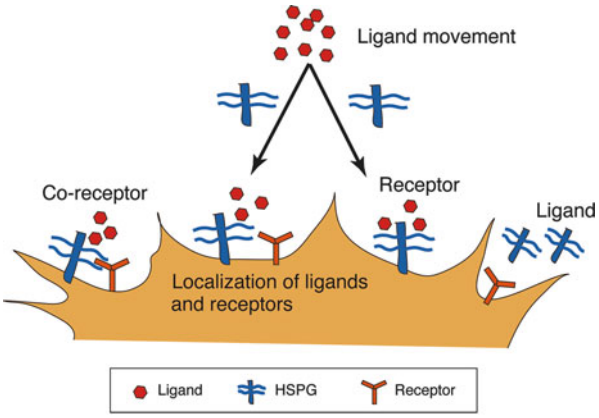

Fig. 9.2 Functions of HSPG in neural development. HSPGs in the extracellular matrix regulate ligand movement or act as the ligand itself. Cell surface HSPGs act as receptors or co-receptors or regulate the localization of ligands and receptors (Modified from Lee and Chien 2004)

as receptors or co-receptors for growth factors and morphogens and also regulate localization of ligands and receptors (Fig. 9.2) (Lee and Chien 2004).

HS is a linear sugar chain composed of repeating disaccharides each of which consists of uronic acid (glucuronic acid [GlcA] or iduronic acid [IdoA]) and *N*-acetylglucosamine (GlcNAc) (Fig. 9.3) (Bülow and Hobert 2006; Esko and Selleck 2002; Lindahl et al. 1998; Perrimon and Bernfield 2000). Biochemical studies have shown that sulfation residues in the sugar chain are important for the interaction between HS and many bioactive molecules. Sulfation can occur at the 2-*O*-position of GlcA or IdoA and the 6-*O*-position, the *N*-position, and, rarely, the 3-*O*-position of glucosamines. Because each of these potential sulfation sites is independently modified and the distribution of sulfation residues is not homogenous, enormous structural heterogeneities are formed in the sugar chain. HS chains contain highly sulfated regions (called the S domain or the NS domain) and poorly sulfated regions (called the NA domain). Generally, S domains in HS offer the sites of biochemical interaction between HS and signaling molecules. Chondroitin sulfate, dermatan sulfate, and keratan sulfate have similar structures but differ in disaccharide compositions. These molecules are collectively called glycosaminoglycans.

9.5 HS Biosynthetic Pathway

HS is synthesized by a series of enzymatic reactions (Fig. 9.4) (Bülow and Hobert 2006; Esko and Selleck 2002; Lindahl et al. 1998; Lee and Chien 2004; Perrimon and Bernfield 2000). First, the tetrasaccharide linkage region is added to the specific serine or threonine residues in core proteins in proteoglycans. Next, the HS sugar backbone is synthesized by HS polymerases, which consist of several

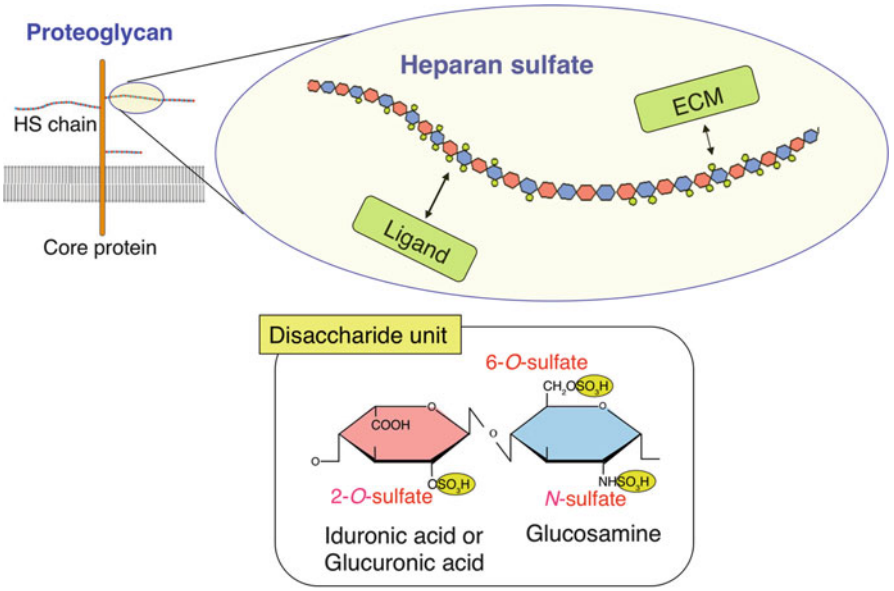

Fig. 9.3 Structure of HSPG. HSPG consists of a core protein and covalently attached HS chains. HS is composed of repeating disaccharides each of which consists of glucuronic acid or iduronic acid and glucosamine. Highly sulfated domains in the HS sugar chain (called the S domain) interact with ligands and extracellular matrix (ECM) molecules, thus regulating their activity

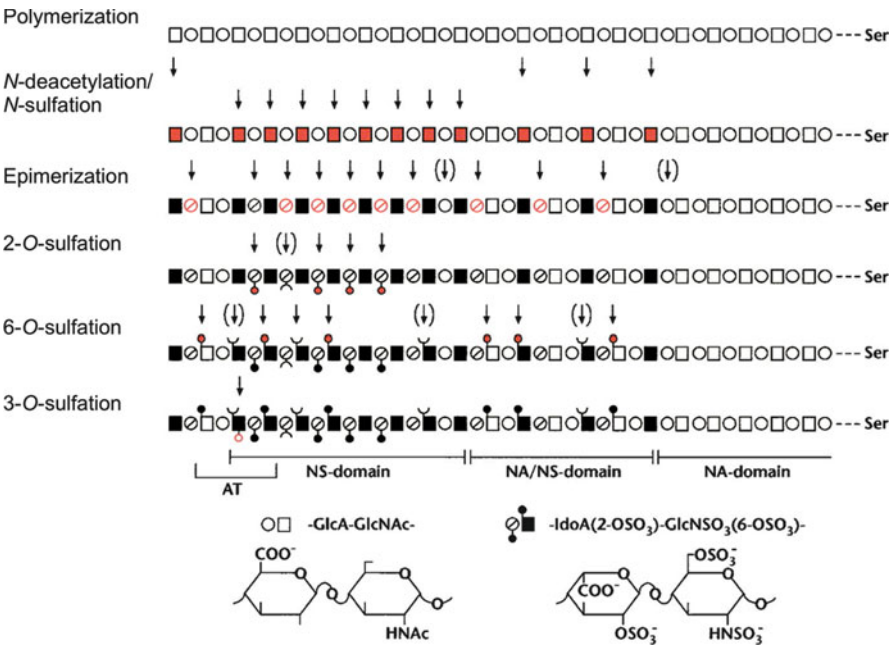

Fig. 9.4 Biosynthesis of the HS chain. HS is synthesized by a series of enzymatic reactions, including polymerization, *N*-deacetylation/*N*-sulfation, epimerization, and sulfation. Each reaction does not proceed to completion, resulting in the formation of structural heterogeneity (Modified from Lindahl et al. 1998)

glycosyltransferases of the Exostosin family. The first member of the Exostosin family, *EXT1*, was identified as a human gene responsible for hereditary multiple exostoses. Mutation in this gene leads to development of benign bone tumors in the long bones, which is caused by overgrowth of cartilages and bones, although the exact mechanisms for this pathophysiology remain unknown. EXT1 and other related molecules (EXT2-3, EXTL1-3) catalyze polymerization of the HS sugar chain by alternately adding GlcA and GlcNAc. Next, some *N*-acetyl moieties in GlcNAc are deacetylated and sulfated by the action of *N*-deacetylase/*N*-sulfotransferase (NDST1-4), and some GlcAs are converted to IdoAs by D-glucuronyl C5-epimerase. Finally the HS chain is modified by a dozen sulfotransferases that specifically add sulfate groups to the 2-*O*-position of GlcA or IdoA and the 6-*O*-position and 3-*O*-position of glucosamines. Differential expression of these HS-synthesizing enzymes in different cells gives rise to distinct HS microstructure, which confers cell-specific responses to signaling molecules. It is generally thought that sulfation patterns, which are critical determinants for ligand binding specificity and affinity, are generated by the combinatorial expression of sulfotransferases expressed in particular cells.

9.6 Roles of HS in Brain Development

Given the importance of HSPG in many signaling pathways, it is easy to imagine that HS plays critical roles in brain development. However, several studies using knockout mice have revealed that disruption of HSPG core proteins did not lead to conspicuous abnormalities, probably owing to HSPG genetic redundancy. In contrast, complete removal of HS by disruption of the *Ext1* gene in mice resulted in growth arrest at the gastrulation stage. This early embryonic lethality of *Ext1* knockout mice indicates essential roles of HS in cell growth and differentiation but hampered the analysis of the roles of HS in brain development. This problem was overcome by the generation of brain-specific *Ext1* knockout mice. Yamaguchi and colleagues generated *Nestin-Cre*;*Ext1*$^{flox/flox}$ mice (*Ext1* cKO mice) and performed morphological analysis of embryonic brains (Holt and Dickson 2005; Inatani et al. 2003; Van Vactor et al. 2006). *Ext1* cKO mice survived until birth and died within the first day of life. The most notable brain phenotype of the *Ext1* cKO mice was the patterning defects in the midbrain-hindbrain region, characterized by the absence of the cerebellum and inferior colliculus. This phenotype was similar to those of mice lacking fibroblast growth factor 8 (FGF8), the key organizer of the cerebellum. The concentrated band of FGF8 protein in the midbrain-hindbrain boundary, which is typically seen in wild-type embryos, was not formed in *Ext1* cKO mice, thus leading to the defects in cerebellar development. The mutant mice showed hypoplasia of the cerebral cortex and the absence of the olfactory bulb. The *Ext1* cKO mice also displayed severe defects in the formation of commissural axon tracts (Inatani et al. 2003). In the forebrain, three major commissural fibers—the

corpus callosum, hippocampal commissure, and anterior commissure—were not formed in the cKO mice. In addition, guidance defects in the axons of retinal ganglion cells were observed. In wild-type mice, most of the retinal axons cross the midline at the chiasm and project into the contralateral tectum. In contrast, in the cKO mice, the retinal axons crossed the midline and then projected ectopically into the contralateral optic nerve instead of extending into the brain. This phenotype was reminiscent of the axonal abnormalities found in the *Slit1/2* double-knockout mice. Slits are HS-binding axonal repellents that are expressed in the optic chiasm and cooperatively guide retinal axons towards the contralateral brain. Genetic interaction between *Ext1* and *Slit1/2* was demonstrated, suggesting that HS is required for the Slit signaling in retinal axon guidance. Similarly, HS requirement in netrin signaling in commissure formation was demonstrated in mice (Matsumoto et al. 2007).

Essential roles of HS in axon guidance have also been shown using zebrafish genetics (Holt and Dickson 2005; Lee et al. 2004; Lee and Chien 2004; Van Vactor et al. 2006). A large-scale screening for retinotectal projection defects in zebrafish embryos led to isolation of several groups of mutants showing different guidance defects. One group, including the mutants *boxer* (*box*), *dackel* (*dak*), and *pinscher* (*pic*), showed defects in optic tract sorting (Karlstrom et al. 1996). In these mutants, the retinotectal projection is generally normal except that a subset of dorsal retinal axons is missorted in the optic nerve. These misrouted axons terminate at their normal topographic positions in the tectum, and the ventral retinal axons project normally. Interestingly, all of three mutants also showed defects in fin and branchial arch development, suggesting that they act in a common developmental pathway. Subsequent studies revealed that *dackel* and *boxer* have mutations in *Ext2* and *Extl3*, respectively (Lee et al. 2004). In the mutant fish, HS levels were severely reduced, indicating Ext2 and Extl3 are required for HS biosynthesis. In addition, the double mutant *dackel/boxer* showed a severer phenotype, which was essentially the same as those observed in *astray* mutants possessing a mutation in a Slit receptor Robo2. These results suggest that HS plays important roles in Slit-Robo-dependent retinal axon guidance in zebrafish.

Critical roles of HSPGs are evolutionarily conserved. In *Drosophila*, a null mutant lacking syndecan (*sdc*), a transmembrane-type HSPG, showed partially penetrant midline crossing defects. Although $sdc^{+/-}$, $slit^{+/-}$, or $robo^{+/-}$ heterozygotes are almost normal, $sdc^{+/-}$;$slit^{+/-}$ or $sdc^{+/-}$;$robo^{+/-}$ transheterozygotes show significantly enhanced defects in midline axonal crossing. The genetic interaction between *sdc* and *slit* or *robo* suggests that HSPG is required for Slit-Robo signaling in *Drosophila* (Lee and Chien 2004; Van Vactor et al. 2006). In *C. elegans*, mutants lacking HS epimerase (*hse-5*), HS 6-*O*-sulfotransferase (*hst-6*), or HS 2-*O*-sulfotransferase (*hst-2*) showed defects in axon guidance that are also observed in Slit (*slt-1*) and robo (*sax-3*) mutants (Bülow and Hobert 2004, 2006; Van Vactor et al. 2006). In conclusion, HS is a versatile regulator of axon guidance signaling beyond species differences.

9.7 Extracellular Sulfatases that Modify HS Structures

Sulfatases are a family of enzyme that hydrolyze sulfate ester bonds in many biologic substrates (Parenti et al. 1997). Most of the sulfatases with known functions degrade glycosaminoglycans in lysosomes. Thus, the genetic defects of these enzymes lead to mucopolysaccharidosis, a pathologic state in which undegraded glycosaminoglycans accumulate abnormally in lysosomes (Parenti et al. 1997). Lysosomal sulfatases show exosulfatase activity at acidic pH. Recently, a novel class of sulfatases, called sulfatase 1 (sulf1) and sulfatase 2 (sulf2), have been identified in human, rats, mice, quails, chicken, and zebrafish (Ai et al. 2003; Dhoot et al. 2001; Morimoto-Tomita et al. 2002; Nagamine et al. 2005; Ohto et al. 2002; Wang et al. 2004). The homologs are also found in *Drosophila* and *C. elegans*. Sulf1 and Sulf2 have many characteristics that distinguish them from lysosomal sulfatases. They have a large hydrophilic domain besides the basic motifs commonly found in sulfatases. They are secreted into an extracellular space via the Golgi apparatus. They have optimal enzymatic activity at neutral to alkaline pH. More importantly, they show specific endosulfatase activity towards HS and heparin. Namely, they can degrade 6-*O*-sulfate in glucosamine residues in intact HS/heparin, especially from the trisulfated disaccharides that are commonly found in the S domain of HS (Fig. 9.5) (Morimoto-Tomita et al. 2002; Lamanna et al. 2006). This trimming of 6-*O*-sulfate in highly sulfated domains of HS has great impacts on cellular signaling (Lamanna et al. 2007). Overexpression of Sulf1 or Sulf2 can activate Wnt signaling by releasing Wnt ligands that are tethered to extracellular matrix molecules through strong interaction with 6-*O*-sulfated HS (Ai et al. 2003). Likewise, Sulfs were shown to mobilize and activate the signaling by Noggin, vascular endothelial growth factor (VEGF),

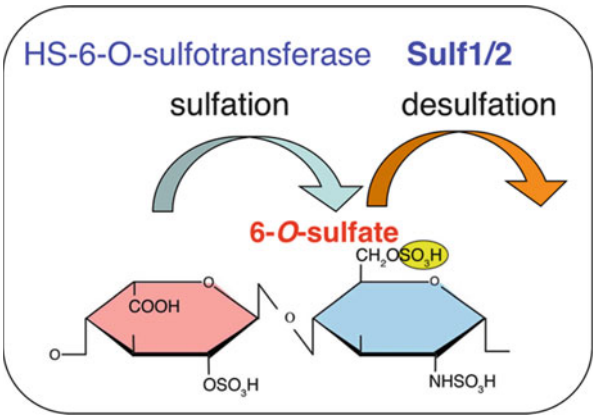

Fig. 9.5 Function of HS endosulfatases. HS-6-*O*-sulfotransferases add sulfate residues to the 6-*O*-position of glucosamines in HS chains, whereas HS endosulfatases, Sulf1 and Sulf2, remove 6-*O*-sulfate especially in the trisulfated disaccharide units in HS chains

FGF, and chemokines (Uchimura et al. 2006; Viviano et al. 2004). Conversely, Sulfs can act as negative regulators for cellular signaling. It is well documented that a ternary complex of FGF, FGF receptor and HS, is required for activation of FGF signaling. In Sulf-expressing cells, 6-*O*-desulfation leads to reduction of FGF receptor dimerization owing to the loss of efficient ternary complex formation (Lamanna et al. 2007). Similarly, Sulfs attenuate the signaling by hepatocyte growth factor (HGF), heparin-binding epidermal growth factor (HB-EGF), glial cell line-derived neurotrophic factor (GDNF), and VEGF. Taken together, Sulfs play roles in fine-tuning HS-dependent cellular signaling.

9.8 Physiologic Roles of Extracellular Sulfatases in the Nervous System

To examine the physiologic roles of *Sulf* genes, several research groups, including ours, generated knockout mice and performed biochemical, developmental, and morphological analyses. *Sulf1* or *Sulf2* single-knockout mice appeared to be almost normal, whereas double-knockout mice died soon after birth or showed growth retardation. In these mice, as predicted from the enzymatic activities of Sulf1 and Sulf2 measured in vitro, the percentages of trisulfated disaccharides in HS were increased when compared with those in wild-type controls (Nagamine et al. 2012). The changes were greater in the double-knockout mice than in the single-knockout mice, indicating genetic redundancy of *Sulf1* and *Sulf2* (Nagamine et al. 2012). The double-knockout mice showed diminished esophageal innervation, subtle skeletal and renal defects, and delayed myogenic differentiation after injury (Holst et al. 2007; Lamanna et al. 2007; Langsdorf et al. 2007; Ratzka et al. 2008). The brain phenotypes, however, remained unknown. Given the importance of HS and 6-*O*-sulfation in neural development (Bülow and Hobert 2006; Lin 2004; Kantor et al. 2004; Pratt et al. 2006), we examined brain phenotypes of *Sulf1/2* double-knockout mice. We found that the double-knockout embryos showed axon guidance defects owing to abnormal localization of axon guidance proteins (Takuya Okada, Kazuko Keino-Masu, and Masayuki Masu unpublished results). These data indicate the critical roles of HS endosulfatases in neural network formation. Further studies are required to elucidate the relationship between Sulf-mediated HS remodeling and axon guidance signaling. Because HS regulates a broad spectrum of cellular signaling in neural cell differentiation, migration, axon guidance, synaptogenesis, and synaptic function and plasticity, it is also necessary to examine the roles of fine-tuning of HS functions by Sulfs in other aspects of the developing and mature nervous system.

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Chapter 10
The Roles of RECK, a Membrane-Anchored Regulator of Pericellular Proteolysis, in Neural Development

Makoto Noda

Abstract The brain is unique in its extracellular matrix composition. Although some extracellular proteases, such as tissue plasminogen activator and matrix metalloproteinases, have been implicated in tissue destruction after brain ischemia, the roles of pericellular proteolysis and its regulation in brain development, functions, and homeostasis remain largely unknown. RECK, a membrane-anchored regulator of extracellular metalloproteases, was initially isolated as a candidate tumor suppressor; subsequent studies revealed its importance in mammalian embryogenesis, especially in the mid-gestation development of vascular and central nervous systems. Emerging evidence in mouse models now suggests its roles in corticogenesis as well as post-ischemic tissue protection and repair in the brain.

Abbreviations

ECM	Extracellular matrix
GPI	Glycosylphosphatidylinositol
MMP	Matrix metalloproteinase
NPC	Neural precursor cells
RECK	Reversion-inducing cysteine-rich protein with Kazal motifs

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10.1 Introduction

Extracellular matrix (ECM) has been implicated in animal development and adult homeostasis. It serves not only as scaffolds or guideposts for resident or migrating cells but also by providing instructive signals or storages for regulated release of diffusible factors (Vu and Werb 2000; Nelson and Bissell 2006). The adult brain ECM is unusual in that (1) it lacks well-defined stromal space and (2) common ECM components, such as collagen and fibronectin, are virtually absent, while some other ECM proteins (e.g., laminin, tenascin, and reelin), proteoglycans (PTGs), and glycosaminoglycans (GAGs) are abundant components filling the intercellular spaces between neurons and glia (Bonneh-Barkay and Wiley 2009). Two major systems that modify the adult brain ECM, especially after brain ischemia, are tissue plasminogen activator (tPA) and matrix metalloproteinases (MMPs) (Adibhatla and Hatcher 2008). Some other proteases (e.g., ADAMTS family members), protease inhibitors (e.g., TIMPs and PAI-1), and carbohydrate-modifying enzymes (e.g., hyaluronidase, heparanase, and chondroitinase) have also been implicated in the regulation of ECM remodeling (Bonneh-Barkay and Wiley 2009). The relevance of ECM and its remodeling to some human conditions such as stroke (Lo et al. 2003), neurodegeneration (Bonneh-Barkay and Wiley 2009), and mental illnesses (Berretta 2012) has also been recognized.

Studies on the roles of ECM regulators in vivo using genetically engineered animals, however, often encounter difficulty due to the multiplicity of related genes in their genomes. For instance, the MMP family consists of more than 20 members with overlapping substrate specificity (Sternlicht and Werb 2001) and redundant functions in vivo (Oh et al. 2004), the TIMP family 4 members (Rivera et al. 2010), and ADAMTS family 19 members with relatively recent gene duplication in mammalian genomes (Jones and Riley 2005).

Reversion-inducing cysteine-rich protein with Kazal motifs (RECK) is a latecomer in matrix biology and has been studied mainly in the context of carcinogenesis due to the historical reason (Noda et al. 2010). RECK, however, is also of interest in the contexts of animal development and evolution, for mice cannot be born without Reck (Oh et al. 2001), and its orthologues are well conserved from fly to man as single genes. Being a regulator of a broad spectrum of matrix metalloproteases (see below), Reck-deficiency provides a unique opportunity for studying the roles of ECM (or excessive ECM turnover) in various physiological and pathological events in vivo. In particular, the central nervous system is a site of abundant Reck expression in normal mouse embryos and a site of most conspicuous defects in Reck-deficient mice (Muraguchi et al. 2007). Hence, the study of Reck in neural development, functions, and homeostasis is expected to shed some new lights on the roles and regulations of pericellular molecules (e.g., cell surface proteins and ECM components) and their turnover in mammalian brain. This chapter summarizes our current knowledge on RECK and its roles in neural development.

10.2 Discovery of *RECK* as a Transformation Suppressor

RECK was first isolated as a transformation suppressor by cDNA expression cloning; *RECK* induces "flat reversion" in NIH3T3 (mouse embryo fibroblast derived) cells transformed by *v-K-ras* oncogene (Takahashi et al. 1998). Later studies have shown that RECK is downregulated in various types of tumors as compared to the normal counterparts and that the levels of residual RECK correlate with better prognoses in such tumors (Noda and Takahashi 2007). Furthermore, activities of RECK to suppress tumor angiogenesis, invasion, and metastasis have been documented in tumor xenograft models (Oh et al. 2001). Thus, the evidence supporting the causal involvement of RECK in human malignancies is accumulating.

10.3 Properties of RECK Protein

The *RECK* gene is conserved from fly to man as a single gene. Mammalian *RECK* encodes a protein of 971 amino acid residues with hydrophobic domains on both termini, five cysteine-rich modules in the N-terminal region, five N-glycosylation sites, a newly identified Frizzled cysteine-rich domain (FZ-CRD) (Pei and Grishin 2012), two EGF-like domains, and three Kazal (serine-protease inhibitor) motifs (Takahashi et al. 1998) (Fig. 10.1). Early studies using cultured cells indicated that the *RECK* gene product is a glycoprotein of about 125 kDa tethered to the membrane through the glycosylphosphatidylinositol (GPI) anchor (Takahashi et al. 1998). When expressed in fibrosarcoma cells, RECK suppresses secretion (in the case of MMP-9) or activation (in the case of MMP-2) of gelatinases, two members of the MMP family (Oh et al. 2001; Takagi et al. 2009). Studies using partially purified proteins have also demonstrated the activity of RECK to bind and inhibit several MMPs in the test tube (Oh et al. 2001; Takahashi et al. 1998; Miki et al. 2007), despite that its primary sequence (i.e., Kazal motifs) predicted serine-protease inhibition. Studies using more purified proteins indicated that RECK forms dimer of a unique cowbell-like shape (Fig. 10.2) and inhibits MMP-7-catalyzed cleavage

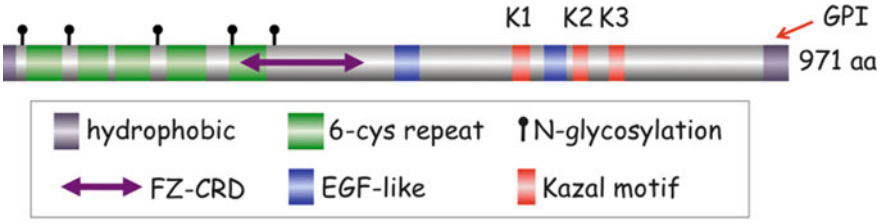

Fig. 10.1 Domain structure of the RECK protein

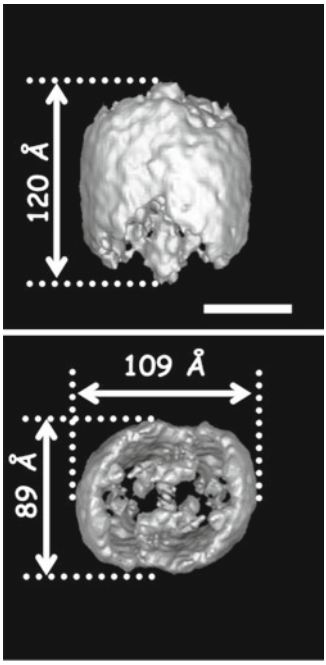

Fig. 10.2 RECK forms cowbell-shaped dimer

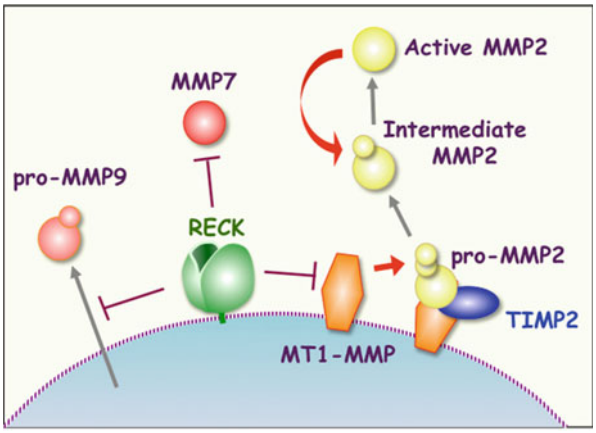

Fig. 10.3 RECK regulates multiple MMPs

of fibronectin (Omura et al. 2009). MMP-7 is a "minimal domain MMP" consisting only of pre-, pro-, and catalytic domains (Sternlicht and Werb 2001), suggesting that RECK interacts directly with the catalytic domain of MMPs. This explains the RECK's ability to regulate relatively broad spectrum of metalloproteinases (Fig. 10.3).

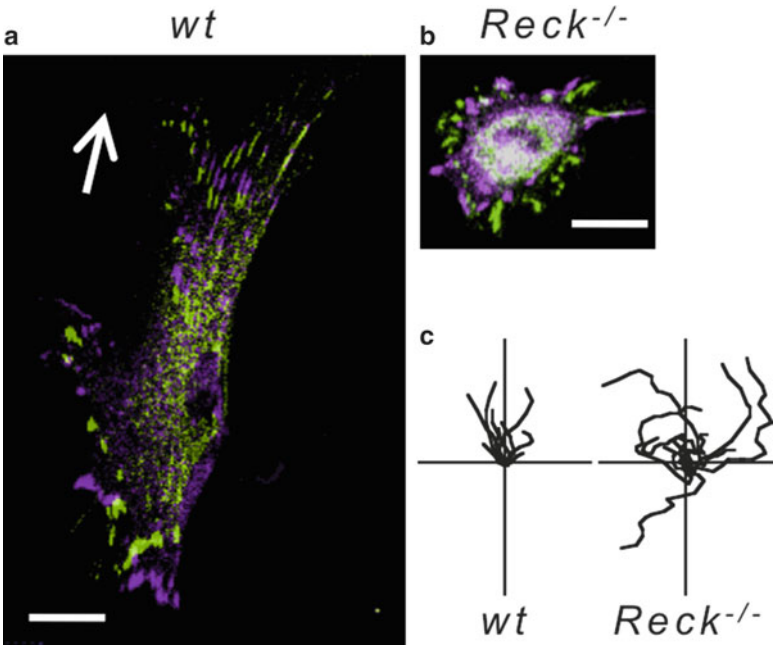

Fig. 10.4 Unstable focal adhesions in a *Reck*-deficient mouse embryo fibroblasts. Focal adhesions as visualized using GFP-vinculin were recorded at time 0 (*purple*) and 30 min (*green*), and two images were superimposed. *Arrow*: direction of migration. Scale bars: 20 μm. (**a**) In normal cells, the discrete, compact focal adhesions (*in purple*) near the leading edge (*top*) subsequently elongated as they moved centripetally in parallel with each other (green signals), while the signals near the retracting end (*bottom*) were more diffuse and less organized. (**b**) In Reck-deficient cells, diffuse, process-shaped, green and purple signals were observed radiating from the cell margin in a disorganized fashion. (**c**) Migration trucks show rapid but less directionally persistent migration of Reck-deficient cells (*right panel*) as compared to the wild-type cells (*left panel*). *Y*-axis indicates the orientation of chemoattractant (PDGF) gradient (top corresponds to higher concentration and the origin the initial positions of the cells)

10.4 RECK in Cell Behaviors

Fibroblasts derived from E10.5 mouse embryos show abnormal behaviors when *Reck* is absent. The cells show reduced spreading, increased speed, and decreased directional persistence in migration and amorphous, unstable focal adhesions (Morioka et al. 2009) (Fig. 10.4). These phenotypes are largely suppressed when plated on fibronectin-coated dishes (Morioka et al. 2009), supporting the idea that fibronectin is an important substrate to be protected by Reck.

In fibrosarcoma cells, on the other hand, Reck may be involved in the endocytic degradation of MT1-MMP (Miki et al. 2007).

10.5 RECK in Development

Global Reck knockout mice die in utero around E10.5 with multiple defects (Oh et al. 2001). The reason for the death is likely to be the defects in vascular development, since the vascular network of mutant embryos is apparently arrested at the primary capillary plexus stage (Oh et al. 2001; Risau 1997) and the timing of death coincides with the stage where the embryos become heavily dependent on their own circulation. In the wild-type embryos and the uteri of pregnant female mice, Reck is abundantly expressed in the cells associated with blood vessels undergoing angiogenesis or remodeling (Chandana et al. 2010). Some of the Reck-positive vessels show morphological features consistent with non-sprouting angiogenesis, such as intussusception (Burri et al. 2004) and pruning (Risau 1997). Experiments using a small hairpin RNAs (shRNAs) in the uterine system indicated that disruption of Reck resulted in the formation of blood vessels with large continuous lumen, rather than the compact, round lumen found in the normal tissues (Chandana et al. 2010), implicating Reck in vascular remodeling, possibly through non-sprouting angiogenesis, in both maternal and embryonic tissues.

Although Reck-deficient mice die around E10.5, wild-type embryos at later stages show interesting patterns of Reck expression; the expression is particularly prominent in developing skeletal muscles and cartilage at around E14.5. Experiments using cultured cells suggested somewhat similar, dual effects in both cell types: suppression of differentiation (myoblast fusion) or morphogenesis (cartilaginous nodule formation) in early stages followed by promotion of tissue maturation (basement membrane sheath formation or bone model consolidation) in later stages (Echizenya et al. 2005; Kondo et al. 2007). The former effect can be explained by the Reck's activity to promote cell-substrate adhesion and suppress cell migration, while the latter by its activity to promote ECM accumulation. In more mature muscles, however, Reck expression declines and becomes confined to the neuromuscular junction (NMJ) (Kawashima et al. 2008). This may confer mechanical strength or some other undefined properties to the NMJ.

The embryonic lethality of the global *Reck* knockout mice, however, hindered our direct testing of these hypotheses using these mice, although expression of shRNA in wild-type mice has been useful in some particular systems. To circumvent this obstacle, we developed two alternative systems: (1) conditional Reck knockout mice and (2) hypomorphic Reck mutant mice exhibiting phenotypes milder than those of the global knockouts. Interesting phenotypes were found in the hypomorphic *Reck* mutant line named $Reck^{Low/-}$. These mice show cutaneous horns (hyperkeratosis) in the dorsal side of all extremity and the right-dominant, forelimb-specific defects in the postaxial skeletal elements. Tissue-specific inactivation of *Reck* indicated that the loss of Reck expression in the early mesenchyme, rather than chondrocytes, is responsible for the skeletal phenotype (Yamamoto et al. 2012). Interestingly, this phenotype is reminiscent of the phenotype of Wnt7a-deficient mice (Parr and McMahon 1995), and the source of Wnt7a is known to be the dorsal ectoderm (DE) in the limb bud (Johnson and Tabin 1997). Since the hypomorphic

Reck mutant mice show severe disruption in the DE tissue at around E11.5 and since a previous report indicates that removal of DE in the limb bud results in the loss of postaxial skeletal elements in chicken (Yang and Niswander 1995), we speculate that Reck in the mesenchyme somehow supports the intactness of DE producing Wnt7a. Although the exact mechanism, or mediator, is still unknown, these findings suggest the role for Reck in mesenchymal-epithelial interactions.

10.6 RECK in Neurogenesis

One of the prominent morphological abnormalities found in the global *Reck-deficient* embryos at E10.5 was their thin and fragile neuroepithelium (Fig. 10.5). Histological studies revealed Reck expression in Nestin-positive neural precursor cells (NPCs) in wild-type embryos (Fig. 10.6a) and reduction in Nestin/BrdU-positive cells in the mutant embryos at E10.5 (Muraguchi et al. 2007). The distribution of TuJ1-positive cells (differentiated neurons) in the mutant embryos (Fig. 10.6b) was reminiscent of that in Hes1/Hes5-deficeint mice (Ohtsuka et al. 1999) and suggests precocious neuronal differentiation. Several lines of evidence obtained using immunohistochemistry, cultured NPCs, shRNA expression in vitro and in vivo, and purified proteins in vitro supported the model that Reck regulates the Adam10-catalyzed shedding of Notch ligand, thereby augmenting Notch signaling, suppressing neuronal differentiation, and promoting NPC proliferation (Fig. 10.7). This model nicely explains why the mice deficient in a metalloproteinase regulator show thin neuroepithelium and precocious neuronal differentiation.

Sockanathan and colleagues identified a retinoid-inducible gene, Gde2 (glycerophosphodiester phosphodiesterase 2), encoding a six-transmembrane protein that is necessary and sufficient to drive spinal motor neuron differentiation in vivo (Rao and Sockanathan 2005). Subsequent study revealed that Gde2 regulates

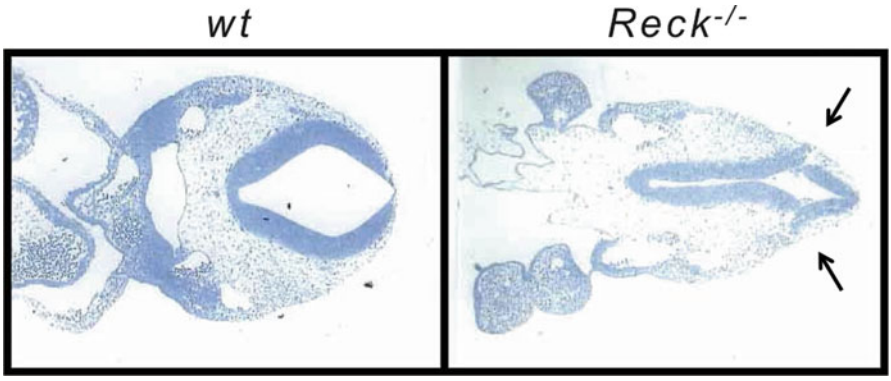

Fig. 10.5 Thin and fragile neuroepithelium in Reck-deficient mice. *Arrow*: breakage frequently found in the Reck-null embryos at E10.5

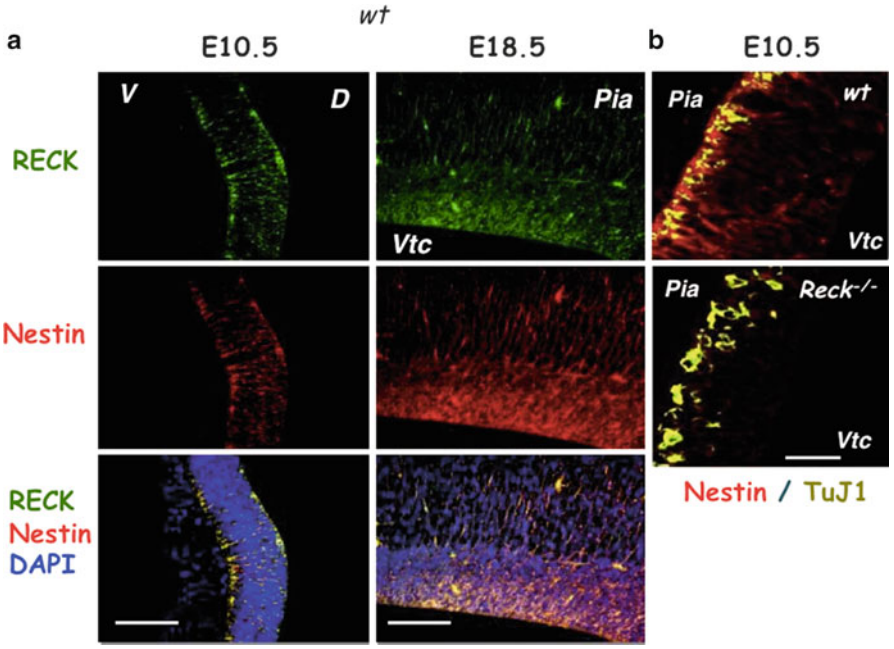

Fig. 10.6 Expression of Reck and phenotype of Reck-deficient mice in the neuroepithelium. (**a**) Expression of RECK and Nestin in neuroepithelium during embryonic development of wild-type mice. *V*, ventral; *D*, dorsal; *Vtc*, ventricle; DAPI, nuclear staining. Scale bars: 200 μm. (**b**) Distribution of Nestin- and TuJ1-positive cells in the wild-type (*top*) and Reck-deficient (*bottom*) embryos at E10.5. Scale bar: 50 μm

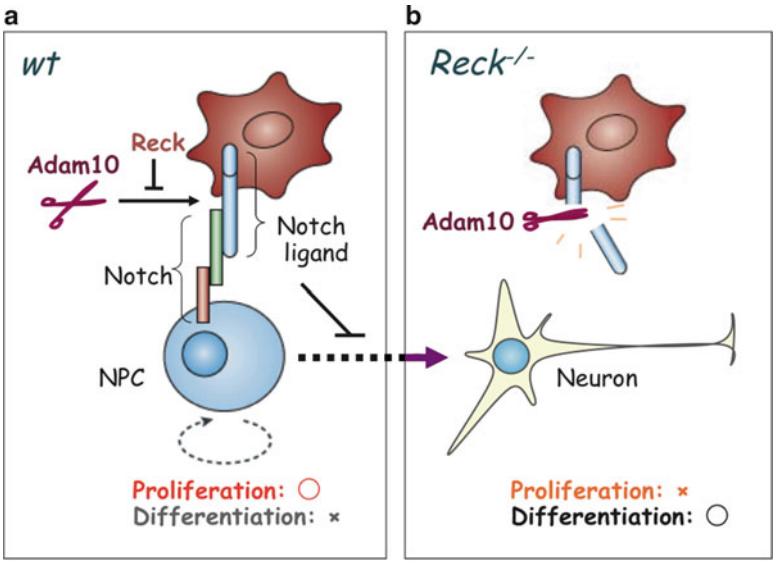

Fig. 10.7 A model to explain the neural phenotype of Reck-deficient mice. (**a**) Reck supports the activity of neighboring cell (*top*) to stimulate Notch signaling in the NPC (*bottom*) by inhibiting Adam10-mediated Notch-ligand shedding. (**b**) In the absence of Reck, Notch ligand is shed, resulting in fewer NPCs and their precocious differentiation

generation of subtype-specific motor neuron through inhibition of Notch signaling (Sabharwal et al. 2011). Their new study indicates that Gde2 promotes neurogenesis by glycosylphosphatidylinositol-anchor cleavage of Reck (Park et al. 2013). Using forward genetics in zebrafish, on the other hand, Prendergast et al. identified RECK as the gene required for the formation of dorsal root ganglia (Prendergast et al. 2012). These studies demonstrate the pivotal role for Reck in spinal cord development.

10.7 RECK in Corticogenesis

What about the role of Reck in brain development? In light of the severe defects found in the neural tube of global *Reck* knockout mice, it was surprising to learn that selective knockout of Reck in Nestin-positive cells (*Reck$^{fl/fl}$;Nestin-Cre*) results in viable animals. These mice, however, do have altered cytoarchitecture in their cerebral cortex (unpublished). More detailed studies on their anatomy as well as behavior are currently under way. The unexpectedly mild phenotype might suggest the consequence of developmental compensation or feedback regulation. To test this possibility, inducible and tissue-specific Reck knockout mice are being developed.

10.8 RECK in Brain Functions

In contrast to its abundance in developing central nervous system, Reck expression is modest in adult brain. We found, however, that after transient cerebral ischemia, NPC-like cells strongly positive for Reck emerge in some areas, such as in the CA2 region of the hippocampus, which then proliferate and differentiate into neurons (Wang et al. 2010) (Fig. 10.8). Although the mice heterozygous for the global *Reck* knockout allele (*Reck$^{+/-}$*) look normal and fertile, they show increased sensitivity to cerebral ischemia, as revealed by increased infarct sizes (Fig. 10.9b, c) and lethality, as compared to the wild-type counterpart. After a milder form of ischemia, recovery of neural function takes more time in the *Reck$^{+/-}$* mice as compared to the wild-type mice (Wang et al. 2010) (Fig. 10.8d). Hence, Reck seems to have at least two functions in postischemic adult brain: (1) protection from tissue damage and (2) functional recovery, which is probably dependent on neurogenesis.

10.9 Future Directions

Our knowledge on the roles of ECM and its remodeling in brain development, functions, and homeostasis is still limited and fragmentary. This is partly due to the inherent difficulty in solubilizing ECM components, which makes their biochemical investigation extremely difficult. An alternative approach would be to use genetics or transgenic animals, together with modern technologies in bio-imaging,

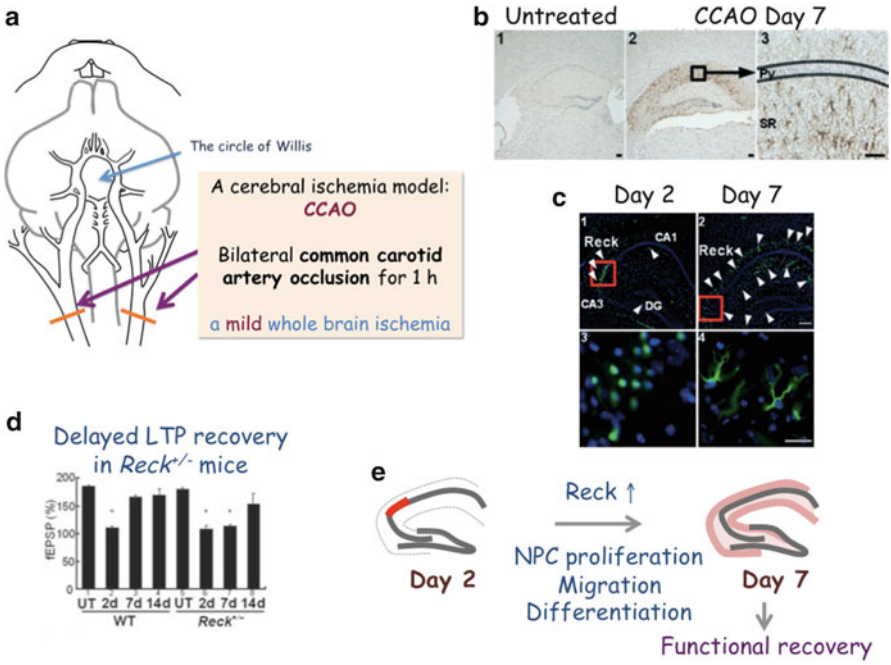

Fig. 10.8 Induction of Reck expression in the hippocampus after a mild form of transient brain ischemia. (**a**) Positions of vascular occlusion in the CCAO model. (**b**) Reck immunoreactivity in the hippocampus from an untreated mouse (UT; panel 1) or a mouse at day 7 after CCAO (Is 7d; panels 2 and 3). *Py*, pyramidal cell layer; *SR*, stratum radiatum. Scale bar: 50 **μ**m. (**c**) Immunofluorescent staining of the hippocampus for Reck (*green*) followed by nuclear counterstaining with DAPI (*blue*). Magnified views of the areas indicated by *red squares* in panels 1 and 2 are shown in panels 3 and 4, respectively. Scale bar: 200 **μ**m (panels 1, 2), 20 **μ**m (panels 3, 4). Some of the Reck-positive cells are indicated by *arrowheads*. The round Reck-positive cells are found in the CA2/CA3 region of the hippocampus at day 2 (panels 1, 3), whereas the Reck-positive process-extending cells are found more widely and abundantly in the hippocampus at day 7 (panels 2, 4). (**d**) Delayed LTP recovery in the hippocampal slices from Reck-null heterozygous mice. See Wang et al. (2010) for more detail. (**e**) Fate of function of Reck-positive cells in the hippocampus after CCAO

omics, etc. Mutations in ECM components are found in human diseases and developmental abnormalities, and such cases are as informative as various animal models for identifying their roles and their functional domains.

As mentioned in Introduction, RECK provides a unique opportunity in learning the roles of cell surface proteins and pericellular proteolysis, since the RECK gene is conserved among various species as a single gene and its loss may lead to deregulation of multiple proteases leading to degradation of multiple pericellular components. We therefore suspect that the roles of Reck in vivo can be as numerous as the number of ECM components or their proteases and that we have a lot more to learn from this pivotal molecule.

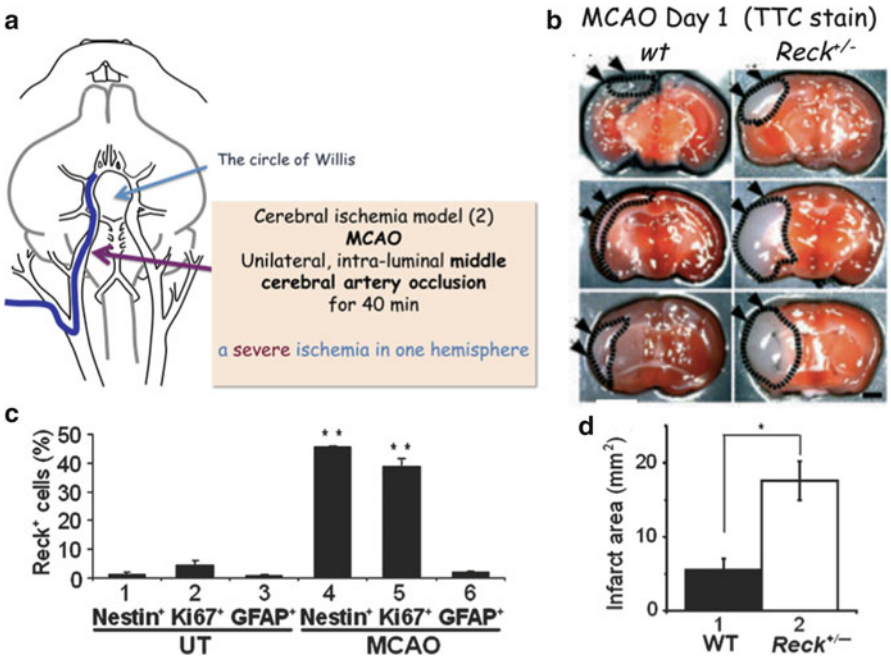

Fig. 10.9 Suppression of brain infarction after a severe form of transient ischemia. (**a**) Method of vascular occlusion in the MCAO model. (**b**) Reduced infarct sizes after MCAO in Reck-null heterozygous mice. Three consecutive coronal brain sections from WT or Reck+/− mice at 24 h after MCAO were stained with TTC. Note the larger infarct lesions (*white areas*) in Reck+/− mice (*arrowheads*). Scale bar: 1 mm. (**c**) The area of infarction was determined from slice images as shown in (**a**). *Bar* represents mean ± SEM ($n = 16$ sections from four animals). (**d**) Quantification of Reck immunoreactivity among Nestin (bars 1, 4)-, Ki67 (bars 2, 5)-, and GFAP (bars 3, 6)-positive cells. Bar represents mean ± SEM ($n = 40$–50 slices from four animals). $^{**}p < 0.01$ vs. UT (Student's *t*-test). $^{*}p < 0.05$

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Chapter 11
Synapse Formation in the Brain

Masayoshi Mishina, Tomoyuki Yoshida, Misato Yasumura, and Takeshi Uemura

Abstract Precise synaptic connections between nerve cells in the brain provide the basis of perception, learning, memory, and cognition. Synapse formation is the key step in the development of neuronal networks and requires the coordinate assembly of large numbers of protein complexes. *Trans*-synaptic cell adhesion molecules are thought to mediate target recognition and induction of pre- and postsynaptic specializations. Despite the wealth of information on the molecular mechanisms of glutamatergic synaptogenesis proposed by in vitro studies using neuronal cell culture models, evidence for their relevance to synaptogenesis in vivo has been lacking. Thus, fundamental questions about how glutamatergic synapses are formed in the mammalian brain have remained unanswered. On the other hand, there is clear in vivo evidence that GluR**δ**2, a member of the **δ**-type glutamate receptor (GluR), plays an essential role in cerebellar Purkinje cell (PC) synapse formation. We found that a significant number of PC spines lack synaptic contacts with parallel fiber (PF) terminals and some of residual PF-PC synapses show mismatching between pre- and postsynaptic specializations in conventional and conditional GluR**δ**2 knockout mice. Recently, we have shown that the *trans*-synaptic interaction of postsynaptic GluR**δ**2 and presynaptic neurexins (NRXNs) through Cbln1 mediates PF-PC synapse formation. The assembly stoichiometry of the synaptogenic GluR**δ**2-Cbln1-NRXN**1β** triad provides the molecular insight into the mechanism of PF-PC synapse formation in the cerebellum. IL1-receptor accessory protein-like 1 (IL1RAPL1) is

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responsible for nonsyndromic mental retardation and autism. We have found that postsynaptic IL1RAPL1 mediates excitatory synapse formation of cortical neurons through *trans*-synaptic interaction with specific variants of presynaptic protein tyrosine phosphatase-**δ**. These results imply the impaired synapse formation as a common pathogenic pathway shared by mental retardation and autism.

11.1 Introduction

Neuronal circuits are formed by a series of developmental events including cell fate determination, cell migration, axon guidance, synapse formation, and synapse maturation. Precise synaptic connections between nerve cells in the brain provide the basis of perception, learning, memory, and cognition. Thus, the elucidation of molecular mechanisms that regulate the formation and maturation of central synapses is essential for the understanding of neural wiring, brain functions, and mental disorders. Excitatory synapse formation in the brain requires the coordinate assembly of large numbers of protein complexes and specialized membrane domains required for synaptic transmission (Scheiffele 2003; Kim and Sheng 2004; Waites et al. 2005; Dalva et al. 2007; McAllister 2007). Using cultured neurons, a number of *trans*-synaptic cell adhesion molecules are shown to induce synapse formation in vitro (Scheiffele et al. 2000; Dean et al. 2003; Graf et al. 2004; Waites et al. 2005; Dalva et al. 2007; McAllister 2007; Südhof 2008; Shen and Scheiffele 2010; Williams et al. 2010; Siddiqui and Craig 2011). However, phenotypic analyses of representative *trans*-synaptic cell adhesion molecules, neuroligin (NLGN) 1, NLGN2, and NLGN3 triple knockout mice, suggest that these molecules are important for synaptic function but are dispensable for synapse formation in vivo (Varoqueaux et al. 2006; Südhof 2008). On the other hand, there is clear evidence that glutamate receptor (GluR)-δ2 plays an essential role in vivo in cerebellar Purkinje cell (PC) synapse formation (Kashiwabuchi et al. 1995; Kurihara et al. 1997; Takeuchi et al. 2005). Recently, we have revealed the molecular mechanism of GluRδ2-mediated cerebellar synapse formation (Uemura et al. 2010; Lee et al. 2012; Mishina et al. 2012).

Mental retardation (MR) and autism are highly heterogeneous neurodevelopmental disorders. MR, defined as a failure to develop cognitive abilities, is the most frequent cause of serious handicap in children and young adults (Chelly and Mandel 2001). A number of genes associated with X-linked MR have been identified by positional-cloning strategies (Chelly et al. 2006; Ropers 2006; Chiurazzi et al. 2008). IL1-receptor accessory protein-like 1 (IL1RAPL1) was identified as the product of an X-linked gene responsible for a nonsyndromic form of MR (Carrié et al. 1999). Nonsyndromic MR is characterized by reduced cognitive function without any other clinical features, thus providing the most direct approach to specifically study the neurobiology of cognition and pathogenesis of MR. Mutations in the gene encoding IL1RAPL1 are associated also with autism (Piton et al. 2008). Autism is comprised of a clinically heterogeneous group of disorders, collectively termed “autism spectrum disorders (ASDs),” that are characterized by severe

deficits in socialization, impaired communication, and limited range of interests and behavior (Abrahams and Geschwind 2008; Levy et al. 2009). Cognitive impairment is common in autism, and ~70 % of autistic individuals suffer from MR (Fombonne 1999). Autism is highly heritable and rare variants in candidate genes have been reported (Pinto et al. 2010; Gilman et al. 2011; Levy et al. 2011; Sanders et al. 2011; Voineagu et al. 2011). Although the underlying causes of these mental disorders are extremely heterogeneous, molecular alterations in monogenic disorders may identify common pathogenic pathways shared by MR or autism or both (Bill and Geschwind 2009). Thus, the elucidation of functional roles of IL1RAPL1 will contribute to our understanding of the pathogenesis of MR and autism. Recently, we found that IL1RAPL1 mediates synapse formation of cortical neurons through *trans*-synaptic interaction with presynaptic protein tyrosine phosphatase (PTP)-δ (Yoshida et al. 2011).

In this chapter, we review the recent progress in the molecular mechanisms of synapse formation in the brain by focusing on GluRδ2 and IL1RAPL1.

11.2 Molecular Mechanism of Synapse Formation in the Cerebellum

The cerebellum receives two excitatory afferents, the climbing fiber (CF) and the mossy fiber-parallel fiber (PF) pathway, both converging onto PCs that are the sole neurons sending outputs from the cerebellar cortex. GluRδ2 is expressed selectively in cerebellar PCs and localized exclusively at the PF-PC synapses (Araki et al. 1993; Lomeli et al. 1993; Takayama et al. 1996; Landsend et al. 1997). In GluRδ2 knockout mice, most of PC spines lost their synaptic contacts with PFs (Kashiwabuchi et al. 1995; Kurihara et al. 1997). Conditional ablation of GluRδ2 in the adult brain also resulted in the appearance of free spines without synaptic contacts (Takeuchi et al. 2005). Thus, GluRδ2 is essential for the formation and maintenance of PF-PC synapses in vivo. Furthermore, we found the appearance of a significant number of mismatching of presynaptic active zones and postsynaptic densities (PSDs). At normal wild-type synapses, the active zone and PSD are well matched to ensure the efficient synaptic transmission. Some of mutant synapses showed that the size of the active zone is smaller than that of the PSD, resulting in the pre- and postsynapse mismatching. Quantitative measurements revealed a strong correlation between the amount of GluRδ2 proteins and the size of active zones. Thus, the maintenance of presynaptic active zones is critically dependent on postsynaptic GluRδ2 proteins. We proposed that GluRδ2 makes a physical linkage between the active zone and PSD by direct or indirect interaction with an active zone component (Takeuchi et al. 2005). Indirect interaction through PSD proteins appears to be less likely since the C-terminal truncation of GluRδ2 has little effect on PF-PC synapse formation, while the mutation impairs cerebellar long-term depression and motor learning (Uemura et al. 2007). We then examined the direct interaction between GluRδ2 and presynaptic terminals by coculture assay (Uemura and Mishina 2008).

GluRδ2 when expressed in HEK293T cells induced the accumulation of presynaptic marker proteins of cultured cerebellar granule cells (GCs). The extracellular N-terminal domain (NTD) of GluRδ2 coated on beads successfully induced the accumulation of presynaptic specializations. These results suggest that GluRδ2 triggers synapse formation by direct interaction with presynaptic component(s) through the NTD (Uemura and Mishina 2008).

After induction of presynaptic differentiation of cultured GCs by GluRδ2-NTD-coated magnetic beads, we cross-linked surface proteins of cerebellar GC axons using non-permeable cross-linker 3,3′-dithiobis (sulfosuccinimidylpropionate). Comparative analysis of the isolated proteins by liquid chromatography-tandem mass spectrometry identified four membrane proteins—neurexin (NRXN) 1, NRXN2, FAT2, and PTPσ—and soluble cerebellin 1 precursor protein (Cbln1) as possible GluRδ2-interacting proteins (Uemura et al. 2010). However, no binding signals for GluRδ2-NTD were detectable on the surface of HEK293T cells transfected with these membrane proteins. When recombinant Cbln1 was added to the culture, we detected significant immunofluorescent signals for GluRδ2-NTD on the surface of HEK293T cells transfected with NRXN1β or NRXN2β. There are large numbers of splice variants of presynaptic NRXNs. We thus examined whether the splice segment of NRXNs affects the interaction with GluRδ2. HEK293T cells transfected with NRXN1β showed robust signals for GluRδ2-NTD-Fc in the presence of HA-Cbln1; however, signals for GluRδ2-NTD-Fc were hardly detectable on the surface of HEK293T cells transfected with a splice variant lacking S4. Thus, GluRδ2 selectively interacts with NRXN variants containing S4.

Direct binding experiments showed that Cbln1 interacts with both GluRδ2 and NRXNs. Since Cbln1 is a ligand for both GluRδ2 and NRXNs, we propose that postsynaptic GluRδ2 interacts with presynaptic NRXN through Cbln1. This ternary interaction provides a physical linkage between PSD and active zone. The synaptogenic activity of GluRδ2 was abolished by knockout of Cbln1 and was hindered by small interference RNA (siRNA)-mediated knockdown of NRXNs. Furthermore, the synaptogenic activity of Cbln1 in cerebellar primary cultures and in vivo was suppressed by the addition of the NTD of GluRδ2 and the extracellular domain (ECD) of NRXN1β. Thus, the *trans*-synaptic interaction of postsynaptic GluRδ2 and presynaptic NRXNs through Cbln1 mediates PF-PC synapse formation in the cerebellum (Uemura et al. 2010). This model well explains previous observations that the size of the presynaptic active zone shrank progressively concomitant with the decrease of postsynaptic GluRδ2 proteins upon inducible Cre-mediated GluRδ2 ablation (Takeuchi et al. 2005) and that Cbln1 knockout mice phenotypically mimic GluRδ2 knockout mice (Hirai et al. 2005).

We then asked how the *trans*-synaptic triad induces synapse formation. In blue native PAGE, the size of native GluRδ2 protein prepared from the membrane fraction corresponded to that of the tetramer. GluRδ2 band collapsed into monomeric and dimeric intermediates by the treatment of 1 % SDS. Thus, GluRδ2 exists as a tetramer in the membrane. On the other hand, GluRδ2-NTD assembled into a stable homodimer. In cultured cerebellar GCs, the majority of varicosities containing presynaptic proteins are not apposed to definite postsynaptic structures (Marxen et al. 1999; Urakubo et al. 2003). Dimeric GluRδ2-NTD exerted little effect on the intensities

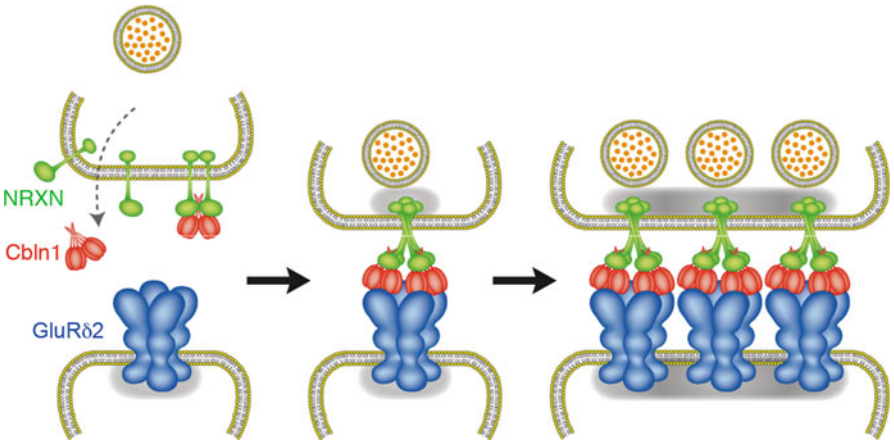

Fig. 11.1 Mechanism of GluR**δ**2-mediated synapse formation in the cerebellum. Before PF-PC synapse formation, Cbln1 secreted from cerebellar GCs may interact with presynaptic NRXNs. However, Cbln1-induced NRXN dimerization is not sufficient to trigger presynaptic differentiation. When the contact between the PF terminal and PC spine takes place, GluR**δ**2 triggers synapse formation by clustering four NRXNs through triad formation. Tetramerization of NRXNs will stimulate the clustering of presynaptic scaffold proteins, leading to the organization of transmitter release machineries. Further pre- and postsynaptic interactions will facilitate the development of synapses (Mishina et al. 2012)

of punctate immunostaining signals for Bassoon and vesicular glutamate transporter 1 (VGluT1) in cultured cerebellar GCs. In contrast, tetrameric GluRδ2-NTD enhanced the accumulation of the active zone and synaptic vesicle proteins in axons of cultured GCs. These results suggest that tetrameric assembly is essential for GluRδ2 to induce presynaptic differentiation (Lee et al. 2012).

We examined the stoichiometry of the GluRδ2-Cbln1-NRXN1β complex. Fast protein liquid chromatography gel-filtration assay and isothermal titration calorimetry analysis consistently showed that dimeric GluRδ2-NTD and hexameric Cbln1 assembled in the molar ratio of one to one and that hexameric Cbln1 and monomeric NRXN1β laminin-neurexin-sex hormone-binding globulin (LNS) domain assembled in the molar ratio of one to two. We thus suggest that the synaptogenic triad is composed of one molecule of tetrameric GluRδ2, two molecules of hexameric Cbln1, and four molecules of monomeric NRXN (Lee et al. 2012). The assembly stoichiometry of the GluRδ2-Cbln1-NRXN1β triad provides the molecular insight into the mechanism of PF-PC synapse formation in the cerebellum. Cbln1 is a high-affinity ligand for NRXNs (Uemura et al. 2010; Joo et al. 2011) and is secreted from cerebellar GCs (Bao et al. 2005), suggesting that the interaction between secreted Cbln1 and presynaptic NRXNs takes place before PF-PC synapse formation. However, Cbln1-induced NRXN dimerization is not sufficient to trigger presynaptic differentiation. When PF terminals contact to PC spines, GluRδ2 induces the clustering of four NRXNs through triad formation and triggers synapse formation (Lee et al. 2012; Mishina et al. 2012) (Fig. 11.1). Tetramerization of NRXNs may stimulate the clustering of scaffold proteins synaptotagmin, CASK, Mint, and

syntenin (Hata et al. 1993, 1996; Butz et al. 1998; Biederer and Südhof 2000; Grootjans et al. 2000) and the organization of transmitter release machineries (Butz et al. 1998; Maximov et al. 1999; Biederer and Südhof 2000, 2001). Further pre- and postsynaptic interaction will stimulate the development of the PF-PC synapse in the cerebellum.

11.3 IL1RAPL1 Mediates Synapse Formation In Vivo of Olfactory Sensory Neurons in Zebrafish

MR and ASD are highly heritable neurodevelopmental disorders characterized by marked genetic heterogeneity. IL1RAPL1 is responsible for MR (Carrié et al. 1999) and is also associated with ASD (Piton et al. 2008). Interestingly, some genetic loci are also overlapping in susceptibility to these disorders (Jamain et al. 2003; Laumonnier et al. 2004; Durand et al. 2007; Kim et al. 2008; Berkel et al. 2010; Laumonnier et al. 2010). Thus, the elucidation of functional roles of IL1RAPL1 will provide an important clue to identify common pathogenic pathways shared by MR and autism (Bill and Geschwind 2009). We found that presynaptic IL1RAPL1 plays a role in presynaptic differentiation of zebrafish olfactory sensory neurons in vivo by controlling both synaptic vesicle accumulation and morphological remodeling of axon terminals, which are mediated by IP_3-dependent Ca^{2+}/calmodulin-protein kinase A signaling and neuronal activity-dependent Ca^{2+}/calmodulin signaling, respectively (Yoshida and Mishina 2005, 2008; Yoshida et al. 2009). Axons of zebrafish olfactory sensory neurons begin to extend toward the olfactory bulb around 22 hours postfertilization (hpf), reach the target sites, and stop the extension at about 50 hpf (Dynes and Ngai 1998; Yoshida et al. 2002). Then a stereotyped pattern of glomerular arrangement in the olfactory bulb—the anatomical basis for an olfactory sensory map—is manifested between 48 and 84 hpf (Wilson et al. 1990; Dynes and Ngai 1998). The contacts between olfactory neuron axons and dendrites of postsynaptic cells in the olfactory bulb are detectable from ~50 hpf, and synaptic vesicles visualized with vesicle-associated membrane protein 2 (VAMP2)-EGFP markedly increase in the axon terminal by 60 hpf (Yoshida and Mishina 2005). Consistently, odor responses in the olfactory bulb become detectable at 60–72 hpf (Li et al. 2005). On the other hand, the morphological remodeling of axon terminals from large and complex shapes to small and simple ones proceeds between 60 and 84 hpf (Yoshida and Mishina 2005). Moreover, the stretch of axon terminals decreases to the size comparable to the diameter of glomeruli during the remodeling, suggesting the refinement of synaptic connections of olfactory sensory neurons with postsynaptic neurons in the olfactory bulb (Yoshida and Mishina 2005). Thus, the early stage of synapse formation between olfactory neuron axons and dendrites of postsynaptic cells in the olfactory bulb is characterized by synaptic vesicle accumulation in the axon terminals and the late stage by axon terminal remodeling. We showed that Ca^{2+}/calmodulin is required for both of the characteristic developmental changes of the axon terminals during synapse formation

(Yoshida et al. 2009). However, PLC inhibitor or olfactory sensory neuron-specific expression of IP_3 5-phosphatase suppressed synaptic vesicle accumulation, but not morphological remodeling. On the contrary, voltage-gated Ca^{2+} channel blocker or olfactory sensory neuron-specific expression of Kir2.1 hardly affected the synaptic vesicle accumulation at 60 hpf but severely inhibited the axon terminal remodeling. Thus, the marked increase in VAMP2-EGFP punctate area in the axon terminal at the early stage of synaptogenesis depends on IP_3 signaling rather than neuronal activities. On the other hand, the morphological remodeling of axon terminals at the late stage of synapse formation requires neural activity and is independent of IP_3 signaling (Yoshida et al. 2009).

Since our previous results show that PKA signaling regulates synaptic vesicle accumulation in the axon terminals of zebrafish olfactory sensory neurons, an intriguing question arises whether IP_3 and PKA signaling work in the same signaling pathway or in parallel. Expression of IP_3 5-phosphatase, dominant negative PKA, or both suppressed VAMP2-EGFP puncta formation to similar degree. Suppression of VAMP2-EGFP puncta formation by IP_3 5-phosphatase was rescued by expression of constitutively active PKA. These results suggest that IP_3 signaling and PKA signaling act sequentially to control synaptic vesicle accumulation and PKA regulates synaptic vesicle accumulation in the downstream of IP_3-mediated Ca^{2+} release from ER (Yoshida et al. 2009). Since type 3 adenylyl cyclase is enriched in rodent olfactory neurons (Bakalyar and Reed 1990; Xia and Storm 1997; Mons et al. 1999), Ca^{2+}/calmodulin-stimulated adenylyl cyclases may transduce IP_3-mediated Ca^{2+} signal to PKA signal.

Thus, Ca^{2+} signaling plays a key role in the regulation of presynaptic differentiation of zebrafish olfactory sensory neurons. However, distinct Ca^{2+} sources are required for the synaptic vesicle accumulation and axon terminal remodeling. At the early stage of synapse formation, IP_3-mediated Ca^{2+}/calmodulin signaling induces synaptic vesicle accumulation in the axon terminals. At the late stage of synapse formation, activity-dependent Ca^{2+}/calmodulin signaling stimulates morphological remodeling of the axon terminals. We demonstrated that zebrafish orthologue of human IL1RAPL1 plays a role in both synaptic vesicle accumulation and the morphological remodeling during presynaptic differentiation of olfactory sensory neurons (Yoshida and Mishina 2008). An intriguing possibility is that IL1RAPL1 may mediate upstream signals to induce axon terminal differentiation during synapse formation (Fig. 11.2).

11.4 IL1RAPL1 is a Synapse Organizer of Mouse Cortical Neurons

We thus examined possible role of IL1RAPL1 in synapse formation of mouse cortical neurons. We expressed IL1RAPL1 in cultured cortical neurons at 12 days in vitro. We detected numerous punctate staining signals for Bassoon along the dendrites of cortical neurons expressing IL1RAPL1. The expression of IL1RAPL1 also

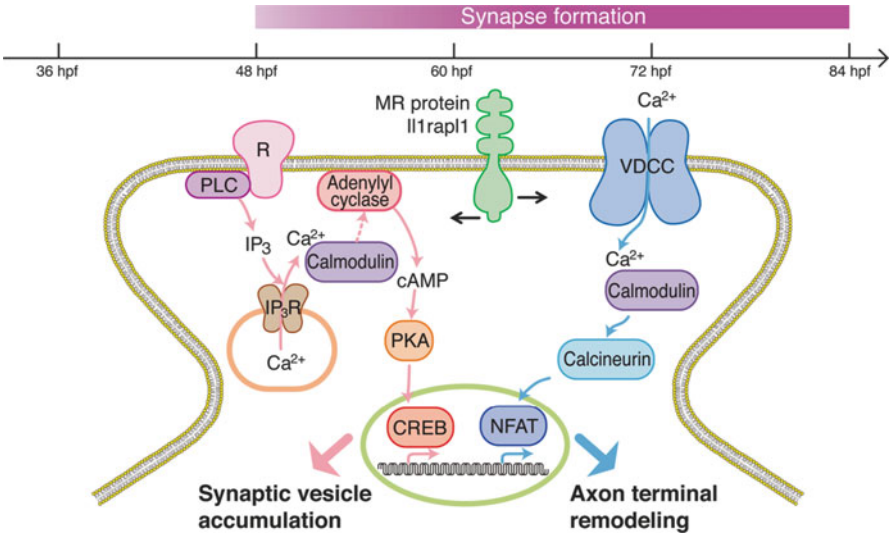

Fig. 11.2 Zebrafish IL1RAPL1 coordinates the presynaptic differentiation of olfactory sensory neurons. During the period of synapse formation between olfactory sensory neurons and postsynaptic cells in the olfactory bulb, distinct Ca^{2+}/calmodulin signaling regulates the presynaptic differentiation. IP_3-mediated Ca^{2+}/calmodulin signaling upstream of PKA and CREB induces synaptic vesicle accumulation in the early stage of synapse formation (*pink arrows*), while neuronal activity-dependent Ca^{2+}/calmodulin signaling upstream of calcineurin and NFAT stimulates morphological remodeling of axon terminal in the late stage of synapse formation (*blue arrows*). IL1RAPL1 controls both synaptic vesicle accumulation and morphological remodeling of axon terminal (modified from Yoshida et al. 2009)

increased the number of protrusions from the dendrites of cortical neurons. Consistently, knockdown of endogenous IL1RAPL1 by siRNAs significantly reduced punctate staining signals for Bassoon along the dendrites and the number of dendritic protrusions. These results suggest that IL1RAPL1 has a synaptogenic activity in cultured cortical neurons (Yoshida et al. 2011) (Fig. 11.3). Furthermore, Sindbis virus-mediated expression of IL1RAPL1-EYFP protein in vivo in cortical layer 2/3 pyramidal neurons of wild-type mouse strongly enhanced VGluT1 staining signals around the basal dendrites of infected neurons.

In coculture assay, IL1RAPL1 expressed in HEK293T cells showed a strong activity to induce the accumulation of presynaptic Bassoon puncta and VGluT1 puncta. However, staining signals for vesicular GABA transporter (VGAT) on the surface of HEK293T cells expressing IL1RAPL1 were as marginal as those of control cells. NLGN induces both excitatory and inhibitory presynaptic differentiation, while cell adhesion molecule N-cadherin shows no synaptogenic activity. These results suggest that IL1RAPL1 triggers excitatory presynaptic differentiation in vitro (Yoshida et al. 2011).

Most likely, IL1RAPL1 exerts its synaptogenic activity by interacting with presynaptic protein(s) since its ECD is required and sufficient to induce presynaptic

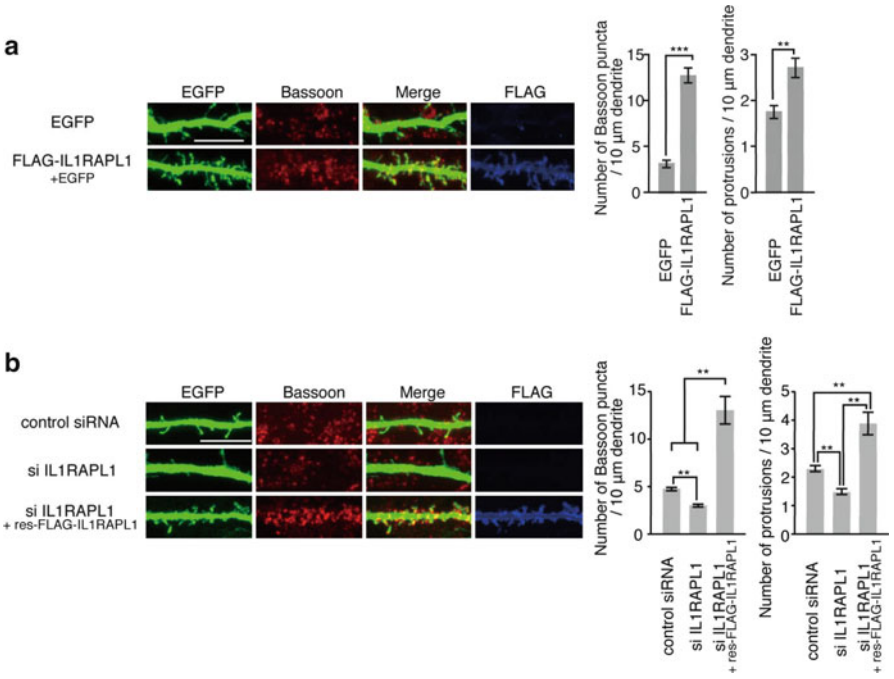

Fig. 11.3 Mouse IL1RAPL1 stimulates Bassoon accumulation and dendritic protrusions in cultured cortical neurons. (**a**) Increases of Bassoon puncta and dendritic protrusions of cultured cortical neurons by FLAG-tagged IL1RAPL1. (**b**) Reduction of numbers of Bassoon puncta and dendritic protrusions of cultured cortical neurons by siRNAs against *Il1rapl1* and rescue by transfection of an siRNA-resistant form of IL1RAPL1 (res-FLAG-IL1RAPL1). Quantitative measurements of numbers of Bassoon puncta and dendritic protrusions are on the right. All values represent mean ± SEM. ** and ***, $p < 0.01$ and 0.001, respectively; Tukey's test. Scale bars represent 10 μm (modified from Yoshida et al. 2011)

differentiation. To examine the issue, we isolated IL1RAPL1-ECD binding proteins by affinity chromatography using IL1RAPL1-ECD protein immobilized on Protein A-Sepharose beads. We obtained two bands of approximately 80 and 90 kDa as IL1RAPL1-ECD binding proteins. These proteins were digested with trypsin, subjected to LC-MS/MS, and were identified as phosphatase subunit (P-subunit) and extracellular subunit (E-subunit) of PTPδ. We confirmed the interaction between IL1RAPL1 and PTPδ by cell surface binding assay, cell aggregation assay, and surface plasmon resonance binding assay. Analysis of the steady-state kinetics of the sensorgrams showed a K_D of 0.3 μM, indicating a high-affinity interaction between IL1RAPL1 and PTPδ.

The developing mouse brain expressed at least six variants of PTPδ derived from alternative splicing at three small exons encoding mini-exon (me) A and meB peptides. Analysis of binding activities of all possible variants revealed that meA and meB peptides in the Ig-like domains of PTPδ were critical for the interaction with

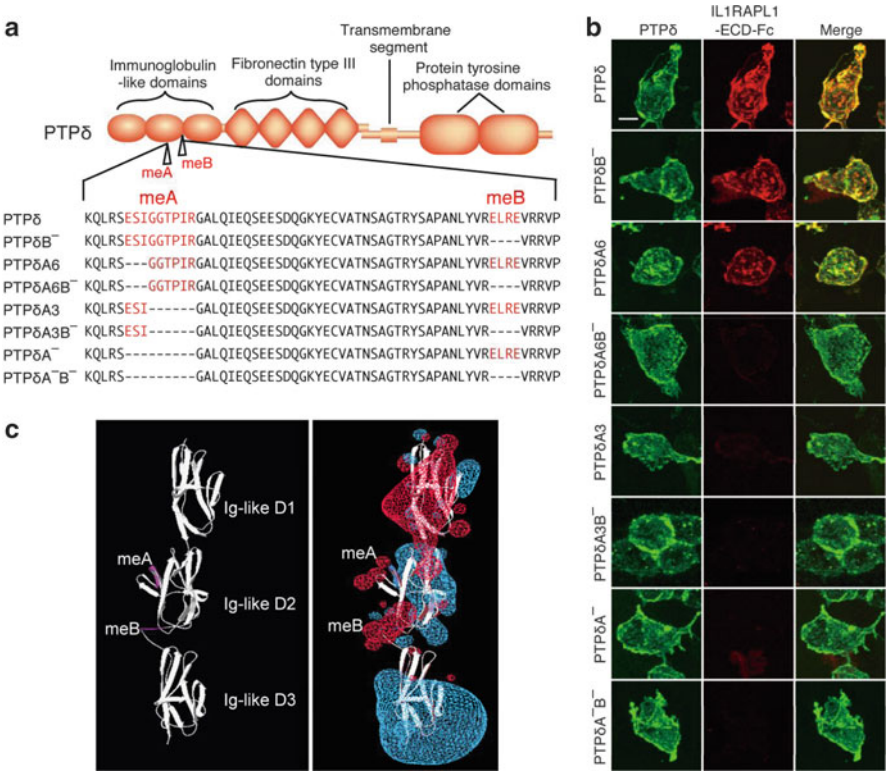

Fig. 11.4 Selective interaction of IL1RAPL1 with variants of PTP**δ**. (**a**) Schematic structure of PTP**δ**. Mini-exon A and B insertion sites are indicated with *arrowheads*. (**b**) Cell surface binding assay. HEK293T cells transfected with splice variants of PTP**δ** were incubated with IL1RAPL1-ECD-Fc. The transfected cells were immunostained with anti-Fc (*red*) and anti-PTP**δ** (*green*) antibodies. Scale bar, 10 **μ**m. (**c**) Structure models of Ig-like domains of PTP**δ**. meA and meB peptides are indicated in *magenta* in *ribbon diagram* (*left*). In the model with electrostatic potential (*right*) (*red*, positive potential; *blue*, negative potential), vicinities of the meA and meB peptides are electrostatically positive (modified from Yoshida et al. 2011)

IL1RAPL1. Interestingly, the meA and meB peptides appear to form positively charged prominences from the domains in a structure model of Ig-like domains of PTPδ according to Titin. PTPδ shares similar domain structures with PTPσ and LAR, constituting 2A-type receptor-like PTPs (RPTPs) (Tonks 2006). PTPσ and LAR expressed in the brain were lacking meA or meB peptide and failed to show any significant binding activity for IL1RAPL1. These results suggest that IL1RAPL1 selectively interacts with specific variants of PTPδ among 2A-type RPTPs, and the mini-exon peptides of PTPδ represent protein codes for the specificity of synapse formation through *trans*-synaptic interaction between IL1RAPL1 and PTPδ (Yoshida et al. 2011) (Fig. 11.4).

Then, we examined the role of IL1RAPL1-PTPδ interaction in synapse formation. IL1RAPL1 expressed in HEK293T cells induced the accumulation of Bassoon

puncta of cortical neurons from wild-type mice. However, these punctate signals were hardly detectable for cortical neurons prepared from PTPδ knockout mice. These results suggest that IL1RAPL1 requires PTPδ for the induction of presynaptic differentiation. In cultured cortical neurons, expression of IL1RAPL1 significantly increased the number of dendritic protrusions and Shank2 staining signals of the transfected neurons when cocultured with cortical neurons from wild-type mice. However, IL1RAPL1 failed to increase dendritic protrusions and Shank2 staining signals in the coculture with cortical neurons from PTPδ knockout mice. These results suggest that postsynaptic IL1RAPL1 requires presynaptic PTPδ for the induction of dendritic protrusions and postsynaptic differentiation. Sindbis virus-mediated expression of IL1RAPL1 protein in vivo in cortical layer 2/3 pyramidal neurons of wild-type mouse strongly enhanced VGluT1 staining signals around the basal dendrites of infected neurons. However, the in vivo synaptogenic activity of IL1RAPL1 was abolished in PTPδ knockout mice. The injection of soluble IL1RAPL1-ECD protein into the cerebral cortex of wild-type mice significantly reduced the spine density of basal dendrites of cortical layer 2/3 pyramidal neurons proximal to the injection site. Notably, the extent of the decrease in the spine density by the injection of IL1RAPL1-ECD was comparable to that observed in PTPδ knockout mice. Furthermore, the suppressive effect of IL1RAPL1-ECD on synapse formation in vivo was abolished in PTPδ knockout mice.

These results suggest that *trans*-synaptic interaction between IL1RAPL1 and PTPδ bidirectionally controls synapse formation of cortical pyramidal neurons (Yoshida et al. 2011). The synapse-organizing role of IL1RAPL1 through the interaction with PTPδ well explains the reduction of the hippocampal dendritic spine density in IL1RAPL1 knockout mice (Pavlowsky et al. 2010).

11.5 Interleukin-1 Receptor Accessory Protein (IL-1RAcP) Organizes Neuronal Synaptogenesis as a Cell Adhesion Molecule

Interleukin-1 (IL-1) family cytokines play important roles in innate and adaptive immune responses (Dinarello 2009; Sims and Smith 2010). Each IL-1 family cytokine binds to its primary receptor subunit, and their interaction recruits a second receptor subunit, IL-1R accessory protein (IL-1RAcP), to induce the response. IL1RAPL1 has no activity to mediate immune signals as a component of the IL-1 family receptors (Born et al. 2000; Sims and Smith 2010) but structurally belongs to the IL-1 receptor family. We thus examined IL-1R family proteins for their abilities to induce presynaptic differentiation by fibroblast-neuron mixed culture assay (Scheiffele et al. 2000). Little staining signals for Bassoon, VGluT1, and VGAT were detectable on the axons crossing the surface of HEK293T cells expressing ligand-binding subunits of the IL-1R family proteins. On the other hand, we detected robust staining signals for Bassoon on the axons in contact with HEK293T cells expressing IL-1RAcP. The intensities of Bassoon signals were comparable between

IL-1RAcP and NLGN1. The synaptogenic activity of IL-1RAcP is preferential for excitatory synapses. The synaptogenic activity is unique for IL-1RAcP among IL-1 receptor family members mediating immune signals (Yoshida et al. 2012).

IL-1RAcP is expressed in numerous tissues including brain, while IL-1RAcPb is an isoform selectively expressed in the central nervous system (Greenfeder et al. 1995; Smith et al. 2009). Knockdown of IL-1RAcP isoforms in cortical neurons suppressed synapse formation as indicated by the reduction of staining signals for presynaptic Bassoon and by the decrease of number of dendritic protrusions. Both phenotypes were rescued by the neuronal isoform IL-1RAcPb, while only presynaptic Bassoon signals were recovered by IL-1RAcP. IL-1RAcP isoforms showed the ability to induce presynaptic differentiation, whereas the abilities to increase the number of dendritic protrusions and to induce Shank2 accumulation were specific for IL-1RAcPb. Because IL-1RAcPb differs from IL-1RAcP only in the C-terminal region (Lu et al. 2008; Smith et al. 2009), it is likely that the C-terminal 240 amino acid sequence specific for IL-1RAcPb is responsible for the spinogenic activity and the activity to induce postsynaptic differentiation. Thus, IL-1RAcPb bidirectionally regulates synapse formation of cortical neurons, while IL-1RAcP induces unidirectionally presynaptic differentiation. The ECD of IL-1RAcPb that is identical to that of IL-1RAcP was sufficient to induce presynaptic differentiation. We identified PTPδ as a ligand for IL-1RAcP isoforms. In addition, LAR and PTPσ, other members of 2A-type RPTP proteins, showed weak binding activities to IL-1RAcP isoforms. The presynaptic differentiation induced by IL-1RAcP isoforms was strongly suppressed in the cultured cortical neurons prepared from PTPδ knockout mice. These results suggest that IL-1RAcP isoforms induce presynaptic differentiation by interacting with presynaptic PTPδ (Yoshida et al. 2012). The cell adherent interaction between IL-1RAcP and PTPδ required neither IL-1 family cytokines nor the ligand-recognition receptors for the IL-1 family cytokines, although IL-1RAcP is the essential component of the signaling-competent receptor complexes for IL-1 family cytokines (Greenfeder et al. 1995; Palmer et al. 2008). Thus, our results revealed the role of IL-1RAcP as a synaptic cell adhesion molecule.

The developing mouse brain expressed at least six variants of PTPδ derived from alternative splicing at three small exons encoding meA and meB peptides as described above. The meB peptide of PTPδ is essential for binding to IL-1RAcP. On the other hand, the meA peptide of PTPδ was critical for the interaction with IL1RAPL1. Thus, the interactions with PTPδ variants are differential between IL-1RAcP and IL1RAPL1. The mini-exon peptides of PTPδ represent protein codes for the specificity of synapse formation through *trans*-synaptic interaction with IL-1RAcP and IL1RAPL1 (Yoshida et al. 2011, 2012).

11.6 Synapse Organizers in the Brain

IL1RAPL1 and IL-1RAcP interact with PTPδ to induce excitatory synapse formation (Yoshida et al. 2011, 2012). Interestingly, neurotrophin receptor tyrosine kinase (Trk) C selectively interacts with PTPσ among 2A-type RPTPs and induces

excitatory synapse formation bidirectionally (Takahashi et al. 2011). Furthermore, PTPσ regulates the synapse number of zebrafish olfactory sensory neurons (Chen et al. 2011). On the other hand, netrin-G ligand-3 (NGL-3) interacts with all members of 2A-type RPTPs and induces presynaptic differentiation of cultured rat hippocampal neurons (Woo et al. 2009; Kwon et al. 2010). The synaptogenic activity of NGL-3 is considerably smaller than that of IL1RAPL1 and appears to be independent of PTPδ since the activity is unaltered in cortical neurons from PTPδ knockout mice (Yoshida et al. 2012). Interestingly, the interaction of TrkC and PTPσ was partly affected by the insertion of meB peptide into the Ig-like domains of PTPσ (Takahashi et al. 2011). On the other hand, the first two fibronectin-3-like domains of LAR and PTPσ were responsible for the interaction with NGL-3 (Kwon et al. 2010). These studies together with our finding suggest that diverse forms of 2A-type RPTPs may provide presynaptic molecular codes for synapse formation by interacting with diverse postsynaptic cell adhesion molecules in a subtype- and variant-specific manner (Fig. 11.5).

We showed that postsynaptic GluRδ2 mediates PF-PC synapse formation in vivo in the cerebellum by *trans*-synaptic interaction with presynaptic NRXNs through Cbln1 (Uemura et al. 2010). GluRδ2 triggers synapse formation by clustering four NRXNs through *trans*-synaptic triad formation (Lee et al. 2012; Mishina et al. 2012). Presynaptic NRXNs interact with postsynaptic NLGNs and leucine-rich repeat transmembrane 2 (LRRTM2) in addition to GluRδ2 in a variant-specific manner (Südhof 2008; de Wit et al. 2009; Ko et al. 2009; Uemura et al. 2010). The selective formation of the huge numbers of synapses in the brain would require specific synaptogenic codes. NRXNs and 2A-type RPTPs may provide presynaptic molecular codes for synapse formation.

There is growing evidence that the functional loss or dysregulation of single synaptic molecules is associated with various neurological diseases. Mutations in the genes encoding IL1RAPL1, NRXN1, NLGN3, NLGN4X, SHANK2, and SHANK3 are associated with ASD and MR (Carrié et al. 1999; Jamain et al. 2003; Laumonnier et al. 2004; Durand et al. 2007; Kim et al. 2008; Piton et al. 2008; Bourgeron 2009; Berkel et al. 2010). These studies suggest the overlap of genetic loci in susceptibility to ASD and MR. In fact, about half of individuals presenting with ASD have intelligence quotients less than 70, and there is a well-documented increased risk for ASD among individuals with some Mendelian forms of MR (Fombonne 1999). NLGNs are involved in proper synapse maturation and function though dispensable for the initial formation of synaptic contacts (Südhof 2008). NRXNs interact with NLGNs as *trans*-synaptic cell adhesion molecules (Südhof 2008). The *trans*-synaptic interaction between NRXNs and postsynaptic GluRδ2 through Cbln1 mediates synapse formation in the cerebellum (Uemura et al. 2010). Shank proteins are scaffolding proteins in excitatory synapses (Sheng and Kim 2000) and promote the maturation of dendritic spines (Sala et al. 2001). Thus, we suggest that synapse formation and following maturation processes represent one of common pathogenic pathways shared by MR and ASD. A better understanding of the complex mechanistic regulations of central synapse formation will be required to provide molecular targets for drug intervention and treatment of human neurological disorders.

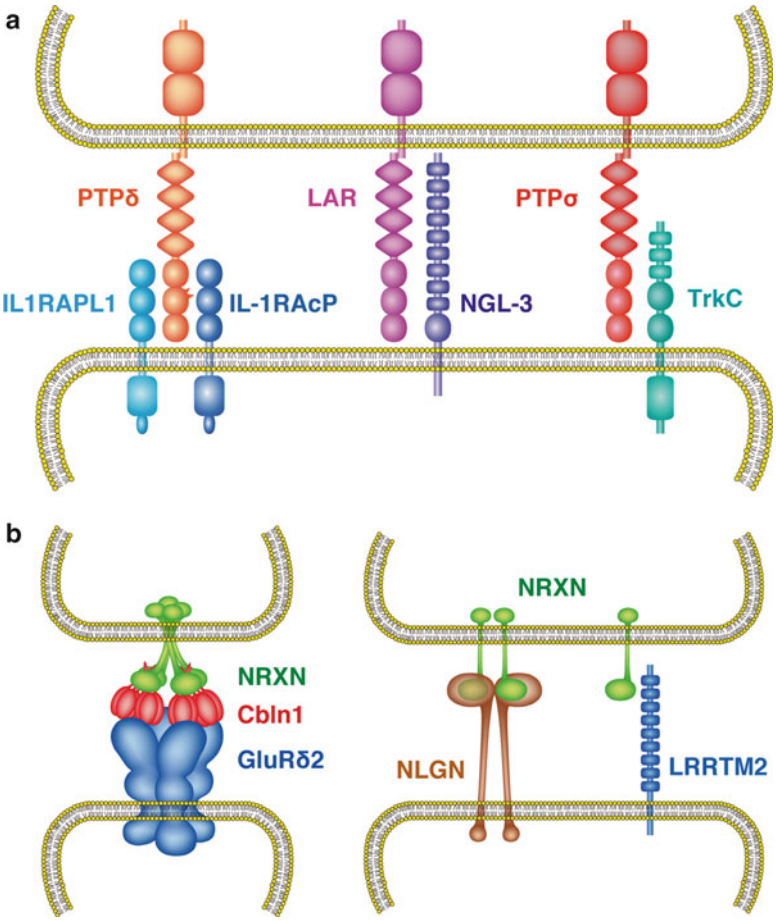

Fig. 11.5 Synapse organizers in the brain. (**a**) In the brain, multiple synapse organizers may mediate synapse formation. Postsynaptic IL1RAPL1 mediates excitatory synapse formation of cortical neurons through *trans*-synaptic interaction with presynaptic PTP**δ**. Other members of 2A-type RPTPs, LAR and PTP**σ**, also stimulate the pre- and postsynaptic differentiation of cultured hippocampal neurons by interacting with NGL-3 and TrkC, respectively. (**b**) Postsynaptic GluR**δ**2 triggers PF-PC synapse formation by clustering presynaptic NRXNs through *trans*-synaptic triad formation in the cerebellum. NRXNs interact with NLGNs and LRRTM2 in addition to GluR**δ**2. Mutations in the genes encoding IL1RAPL1, NRXN1, NLGN3, and NLGN4 are associated with autism and mental retardation. Thus, the impairment of synapse formation and following maturation processes may be a common pathogenic pathway shared by autism and mental retardation

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Chapter 12
Genomic Imprinting in the Mammalian Brain

Wei-Chao Huang and Christopher Gregg

Abstract Genomic imprinting has been primarily studied in the context of embryonic growth and development. However, over the past several years new insights into the roles of imprinted genes in the brain have emerged. Using a novel approach based on next-generation sequencing we recently uncovered hundreds of genes exhibiting complex imprinting effects in the brain, including imprinting effects that are brain region specific, developmental stage specific, and sex specific. Here, we provide a historical perspective on genomic imprinting to introduce this exciting area to the neuroscience field. Further, we comment on emerging concepts related to imprinting in the brain revealed by next-generation sequencing. This work suggests a major frontier exists to understand the functional roles of imprinted genes in the regulation of brain development, function, and behavior.

12.1 Discovery of Imprinting and Roles for Imprinted Genes in Brain Function and Behavior

In a classical Mendelian genetic view, genetic inheritance involves equal contributions from the mother and the father to diploid offspring. This general model is applicable to many conditions, but many complex traits remain unexplained (Fradin et al. 2006). Advances in the field of molecular genetics have revealed many factors that contribute to complex non-Mendelian patterns, including epigenetic effects (Mohtat and Susztak 2010), genetic-environment interactions (Dempfle et al. 2008), and parent-of-origin effects (Wolf et al. 2008). Parent-of-origin effects have been recognized to influence the phenotype and behavior of offspring for centuries (Thomas et al. 1970).

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Parent-of-origin effects may arise from a number of mechanisms that include maternally inherited mitochondrial DNA (mtDNA) (Giles et al. 1980), small ncRNAs differentially transmitted by sperm versus egg (Bourc'his and Voinnet 2010), uterine environment during development (Wolff et al. 1998), and genomic imprinting (Guilmatre and Sharp 2012; Abramowitz and Bartolomei 2012). Genomic imprinting is a process that causes genes to be asymmetrically expressed in offspring depending on their parental origin. The phenomenon has been observed at both the chromosomal level and at the level of individual genes. The first use of "imprinting" to describe epigenetic parent-of-origin effects was in the context of the elimination of paternal chromosomes during spermatogenesis in sciarid flies (Crouse 1960). *Sciara* male and female embryos selectively eliminate a paternally inherited X chromosome, and Crouse proposed that chromosomal imprints are established through the germline in order to functionally distinguish maternal and paternal X chromosomes in the embryo.

The first example of imprinting at the level of a single gene was described in plants 40 years ago (Kermicle 1970). Through experiments focused on the inheritance of maize kernel coloration, Kermicle recognized parent-of-origin effects influencing alleles affecting the red color (anthocyanin pigmentation) of the endosperm's outer layer, the aleurone. Strains were uncovered with red color (*R*) alleles that gave full pigmentation when maternally inherited in a cross with a colorless strain (*r/r*) but mottled pigmentation when paternally inherited. In vertebrates, genomic imprinting was initially uncovered at the chromosomal level for the X chromosome. Imprinting of the X chromosome occurs in female marsupials, such that the paternally inherited X chromosome (Xp) is preferentially silenced in both embryonic and extraembryonic tissues (Cooper et al. 1971). In mice, the Xp is silenced specifically in extraembryonic tissues, but in the embryo proper, both the maternally inherited X (Xm) and the Xp undergo random inactivation (Takagi and Sasaki 1975; West et al. 1977). The first individual imprinted autosomal genes in mammals would not be discovered until 1991 in mice. *Igf2r* (insulin-like growth factor type 2) was mapped to mouse chromosome 17 and identified as a maternally expressed gene (Barlow et al. 1991). The Igf2 gene (insulin-like growth factor type 2) was revealed as a paternally expressed imprinted gene (DeChiara et al. 1991). Finally, the H19 gene (fetal hepatic cDNA clone 19), which is a long noncoding RNA, was demonstrated as a maternal expressed imprinted gene closely located to the Igf2 locus (Bartolomei et al. 1991).

Nuclear transplantation experiments in mice first revealed that maternal and paternal genomic complements are not equivalent in mammals and that both maternally and paternally inherited chromosomes are essential for development. It was discovered by Barton et al. and McGrath and Solter (Surani and Barton 1983, 1984; McGrath and Solter 1984; Barton et al. 1984) that parthenogenetic (PG) and androgenetic (AG) (with a diploid maternally or paternally derived genome, respectively) embryos exhibit early embryonic lethality. The early experimental work also revealed major differences between AG and PG embryos. AG embryos showed reduced fetal growth and excessive extraembryonic growth, whereas PG embryos showed more advanced fetal development with relatively poor extraembryonic growth. The discrepancy in the phenotype between PG and AG embryos implied that paternally expressed genes

(PEGs) have functionally distinct roles compared to maternally expressed genes (MEGs) in offspring and that these effects are tissue specific.

Studies of imprinting in the brain using PG and AG chimeric mice suggested distinct roles for PEGs and MEGs in the regulation of cortical versus hypothalamic brain regions. To overcome the developmental lethality of AG and PG embryos, chimeric mice were generated with wild-type cells that could survive to adulthood (Allen et al. 1995). With regard to brain development, PG chimeras have small bodies with relatively enlarged brains compared to controls, whilst AG chimeras have large bodies, but relatively small brains (Allen et al. 1995). To determine where the PG/AG cells were located in the brain, a lacZ reporter was utilized to label PG and AG cells. Cells with a maternal genome (PG) preferentially contributed to cortical and limbic regions, but were selectively eliminated from hypothalamic regions. In contrast, AG cells contributed preferentially to the hypothalamus, septum, and the preoptic area of the stria terminalis (Keverne et al. 1996). These pioneering studies suggested that alleles that come from mothers and fathers have potentially distinct roles in the development and functions of cortical versus hypothalamic brain regions, respectively (Keverne 1997).

Subsequently, mice with altered dosage of individual or multiple imprinted genes have provided insights into the functional roles of imprinted genes. Many studies have indicated that imprinted genes are involved in fetal growth, postnatal energy homeostasis, organ development, and in several behaviors (Charalambous et al. 2007). Insights into the roles of imprinted genes in humans have largely come from congenital disorders, such as Prader–Willi syndrome (PWS), Angelman syndrome (AS) (Buiting 2010), Beckwith–Wiedemann syndrome (Choufani et al. 2010), and Silver–Russell syndrome (Abu-Amero et al. 2008). Further, recent studies have revealed roles for imprinted genes in complex diseases. A study assessed the relationship between parental origin and disease risk in Iceland and found that a number of alleles within known imprinted loci significantly influenced the risk of breast cancer, basal-cell carcinoma, and type II diabetes (Kong et al. 2009). Major neuropsychiatric disorders have also been associated with imprinted loci, including autism (Arking et al. 2008; Lamb et al. 2005), schizophrenia (DeLisi et al. 2002; Francks et al. 2003), alcoholism (Liu et al. 2005; Wyszynski and Panhuysen 1999), and bipolar affective disorder (Pinto et al. 2011). In addition to clinical studies, bioinformatic approaches suggest that imprinted genes are associated with psychosis, obesity/diabetes, and autism (Sandhu 2010).

Maternal and paternal imprinting effects on human behavior and neurodevelopment have been most extensively explored in PWS and AS. PWS is characterized by mental handicap, severe hypotonia, hypogonadism, poor temperature regulation, and obesity (Cassidy and Driscoll 2009). Infants with PWS show poor suckling reflexes following birth and often show failure to thrive in early infancy, followed by the emergence of hyperphagia and obesity in early childhood. A distinctive behavioral character with temper tantrums, obsessive-compulsive characteristics, and psychiatric disturbance are common findings. The clinical features of AS include mental retardation, microcephaly, gait ataxia, seizures, and repetitive, uncoordinated, but symmetrical movements. Affected individuals with AS usually

exhibit inappropriate laughter and excitability (Williams et al. 2006). These two disorders were the first examples of a human imprinting disease (Buiting 2010). Previous studies reported that the prevalence rate of these two disorders is 1 per 15,000–25,000 live births (Burd et al. 1990; Butler 1996). A deletion in the same chromosome region, 15q11–q13, was identified in patients with PWS and AS (Knoll et al. 1989; Ledbetter et al. 1981; Nicholls et al. 1989). It was initially unclear how two phenotypically distinct syndromes arose from the same genetic mutation.

The chromosomal region 15q11–q13 is the location of a cluster of imprinted genes, expressed from either the paternally or maternally inherited allele in the brain. Paternally expressed transcripts from the relatively centromeric part of this locus, including *NDN* (Jay et al. 1997), *SNRPN* (Leff et al. 1992), and its associated noncoding small nucleolar RNAs (snoRNA) gene *SNORD* (Sahoo et al. 2008), are not expressed in PWS and are implicated in this syndrome. In contrast, AS is caused by loss of the UBE3A transcript, which is maternally expressed (Matsuura et al. 1997). How loss or aberrant dosage of these genes affects neuronal function and causes the phenotype of these disorders is a major area of research and has been recently reviewed (Cassidy et al. 2012; Mabb et al. 2011). Several studies have provided enticing mechanistic insights. For example, *Ndn* knockout mice show hypothalamic deficits, including a reduction in oxytocin-producing and luteinizing hormone-releasing hormone-producing neurons, which are similar with the general phenotype of hypothalamic deficit in PWS (Muscatelli et al. 2000). Further, a role for snoRNAs encoded in the PWS locus and pre-mRNA splicing that is essential for neurodevelopmental processes and serotonin signaling has been suggested (Yin et al. 2012; Kishore and Stamm 2006). In terms of etiology of AS, transgenic ablation of maternal Ube3A leads to behavioral deficits that are associated with abnormal dopamine signaling (Riday et al. 2012).

12.2 Regulation of Imprinting

Like the PWS/AS locus mentioned above, greater than 80 % of known imprinted genes are clustered into 16 genomic regions that contain two or more imprinted genes (Edwards and Ferguson-Smith 2007). The fact that imprinting often occurs in clusters implies that the imprinting mechanism is often not exerted in a gene-specific manner. Indeed, for several known imprinted loci, a long-range cis-acting control element called an imprint control element (ICE) or imprint control region (ICR) has been identified (Barlow 2011). Although not all imprinted gene clusters follow the same rules of regulation, in general imprinting involves a differentially methylated ICE region and the expression of a long noncoding RNA with a regulatory role in the maintenance of allele-specific expression.

A well-studied imprinted gene locus regulated by a paternally methylated ICR is the *H19/Igf2* imprinting locus. *H19* and *Igf2* compete for two enhancers located downstream of *H19* (Webber et al. 1998; Bartolomei et al. 1991, 1993; DeChiara et al. 1991). The ICR has 4 binding sites for the insulator protein, CTCF, which binds to the unmethylated maternal allele (Bell and Felsenfeld 2000; Hark et al. 2000).

CTCF binding blocks the enhancer region from interacting with the *Igf2* promoter, leading to exclusive expression of *H19* from the maternal allele. Methylation at the ICR on the paternal allele prevents CTCF from binding and allows the *Igf2* promoter to interact with the enhancers, resulting in paternal Igf2 expression. Maternal *H19* expression is directly involved in regulating the paternal expression of *Igf2,* such that loss of *H19* results in activation of the maternal *Igf2* allele (Leighton et al. 1995). In the developing brain, some differences exist in the regulation of imprinting at this locus that are poorly understood, *H19* is maternally expressed (Hemberger et al. 1998), but *Igf2* expression has been reported to be biallelic (Leighton et al. 1995; Hemberger et al. 1998).

A well-studied example of imprinting involving a maternally methylated ICR is the *Igf2r/Airn* locus. The *Igf2r* gene is maternally expressed (Barlow et al. 1991), and *Airn* gene, which is a long noncoding RNA, is expressed from the paternal allele (Wutz et al. 1997). In this case, the ICR lies within an intron of *Igf2r* (Stoger et al. 1993) and involves a differentially methylated promoter site, for which the maternal allele is methylated. *Airn* is transcribed from the unmethylated paternal allele in an antisense direction to *Igf2r* and represses the expression of cis-linked genes from the paternal allele (Lyle et al. 2000; Wutz et al. 1997), including *Igf2r*, *Slc22a2,* and *Slc22a3* (Sleutels et al. 2002). Conversely, *Airn* expression is repressed on the maternal allele due to methylation at the ICR, allowing expression of cis-linked genes. It is suggested that lncRNAs regulated by differentially methylated ICRs represent a general mechanism that controls gene expression at several imprinted gene clusters. For example, this mechanism also occurs at the *Kcnq1* cluster (Fitzpatrick et al. 2002), *Snrpn* cluster (Horsthemke and Wagstaff 2008), and *Gnas* cluster (Williamson et al. 2011).

Imprinting can occur in a cell-type-specific or developmental-stage-specific manner for many genes. A recent study of the *Dlk1* locus provides some important mechanistic insights into cell-type-specific imprinting in the brain. *Dlk1* is exclusively expressed from the paternal allele during embryogenesis. However, Ferron et al. have demonstrated in the neurogenic niche of the developing and adult brain that *Dlk1* selectively loses imprinting in both NSCs and niche astrocytes resulting in expression of both alleles (Ferron et al. 2011). The underlying mechanism is associated with postnatally acquired hypermethylation at the intergenic DMR that regulates *Dlk1* imprinting. This study reveals that epigenetic mechanisms can dynamically control imprinted gene expression in specific cell types of the brain.

12.3 Next-Generation Sequencing and the Analysis of Imprinting in the Brain

Microarray technology revolutionized our ability to profile and compare levels of gene expression in specific tissues or under different treatment conditions. However, a genome-wide approach to study expression from maternally versus paternally inherited chromosomes has been lacking. To address this problem, Gregg and colleagues developed an approach to compare imprinting in different brain regions

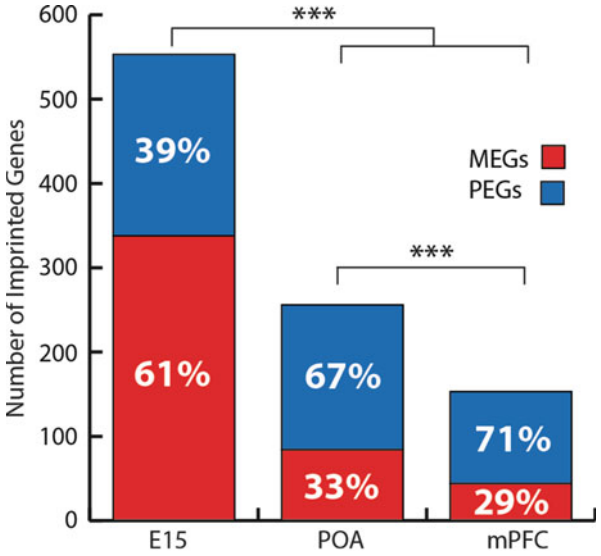

Fig. 12.1 Numbers of maternally and paternally expressed imprinted genes discovered by next-generation sequencing in the embryonic day 15 (E15) brain and preoptic area (POA) and medial prefrontal cortex (mPFC) of the adult brain. The data reveals that the largest numbers of imprinted genes were uncovered in the developing brain and, in the adult brain, the POA had significantly more imprinted genes that the mPFC. In addition, the majority of imprinted genes in the adult brain exhibited a paternal expression bias, while the majority of genes in the developing brain exhibited a maternal bias

and tissues using high-throughput sequencing (Gregg et al. 2010a, b). In this approach, RNA is harvested from microdissected brain regions of F1 hybrid mice generated from reciprocal crosses of the distantly related C57BL/6J (C57)×CAST/EiJ (Cast) mouse strains. Single-nucleotide polymorphisms (SNPs) in the RNA-Seq data are used to distinguish expression from maternally versus paternally inherited alleles. The authors used this high-resolution approach to ask many questions for the very first time. Imprinting was analyzed in the cortex versus the hypothalamus to test the idea that biased maternal control exists in cortical regions and biased paternal control in hypothalamic regions, as discussed above. The authors further tested whether imprinting differs in the adult brain compared to the developing brain and whether sex-based differences in imprinting effects might exist.

The study revealed 256 imprinted genes expressed in the adult preoptic area of the hypothalamus, compared to 153 in the medial prefrontal cortex and 553 in the embryonic brain. Only 47 of the ~100 previously known murine imprinted genes were found to be expressed and imprinted in the brain. Thus, the findings suggested profound differences in imprinting developmentally and between brain regions. Additionally, differences were uncovered between males and females. Remarkably, these results further revealed that a substantial *paternal bias* exists among autosomal imprinted genes expressed in the adult brain, but a maternal bias exists in the developing brain (Fig. 12.1). In both cortical and hypothalamic regions of the brain,

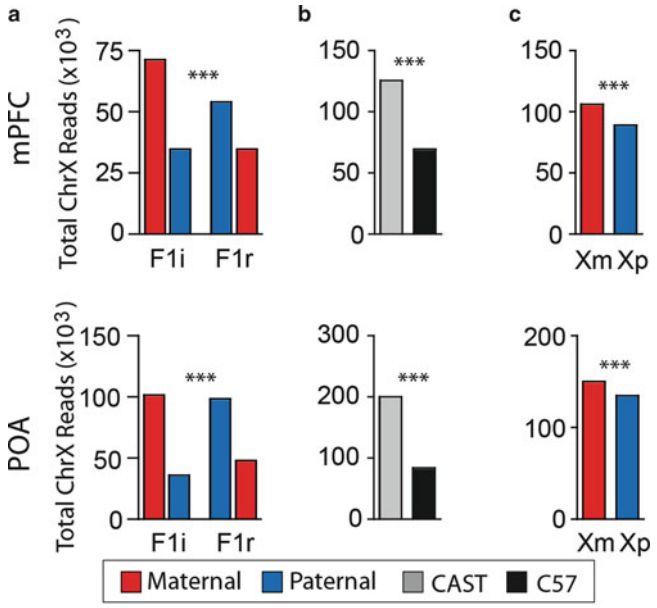

Fig. 12.2 A significant bias to express the maternally inherited X chromosome was uncovered in the mPFC and POA of the adult female brain by next-generation sequencing as revealed using a fisher's exact test (**a**) or a chi-square test on all Xm versus all Xp reads (**c**). Preferential expression of the CAST/EiJ (Cast) X chromosome was also uncovered (**b**, chi-square test)

~70 % of the imprinted genes identified exhibited a paternal expression bias. Thus, the study did not find evidence for maternally biased control over cortical regions and paternally biased control over hypothalamic regions in terms of total numbers of MEGs or PEGs.

These studies also revealed the surprising insight that the maternal X chromosome (Xm) is preferentially expressed in the adult female brain (Fig. 12.2). This observation opposes the long-held assumption that X-inactivation leads to a random mosaic of Xm- and Xp-expressing cells in females. Interestingly, the authors propose that the X chromosome may represent a nexus of maternal influence over gene expression in the adult brain. This proposal is partly inspired by the fact that the X chromosome is enriched for genes that regulate brain function (Nguyen and Disteche 2006; Zechner et al. 2001), that males only inherit a maternal X chromosome, and that the X is postulated to be preferentially influenced by selection effects that act in maternal interests (Haig 2006).

Sexually dimorphic imprinting effects were also uncovered on the autosomes and involved an estimated 347 candidate genes imprinted specifically in males or females. The majority of sex-specific imprinting effects were observed in the preoptic area of the female brain. *Mrpl48* and *Il18* are examples of genes that exhibit sex-specific imprinting in the brain (Fig. 12.3). Interestingly, *Il18* is linked to inflammation and autoimmune diseases, such as multiple sclerosis, which are highly

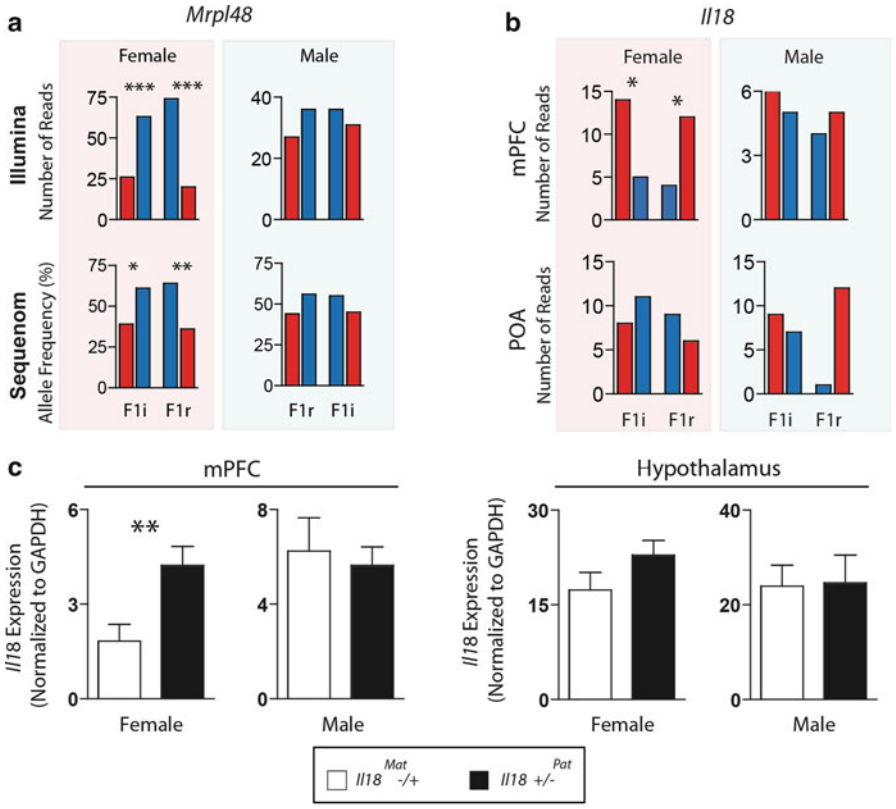

Fig. 12.3 Sex-specific imprinting effects were uncovered for *Mrpl48* and *Il18*. (**a**) The paternal allele is preferentially expressed for the gene *Mrpl48* in the female POA and the effect was independently validated by sequenom. (**b**) *Il18* is maternally expressed in the mPFC of the female brain. The maternal effects were confirmed using *Il18* mutant mice and qPCR. (**c**) *Il18* expression was higher in the mPFC of *Il18* −/+ females compared to *Il18* +/− females, consistent with a maternal expression bias. This effect was not observed in males *Sources*: All figures were previously published in Science by the authors. These can be reproduced in book contributions by the original authors without permission

sexually dimorphic diseases. These new insights into sex-specific imprinting effects may help understand the underlying genetic and epigenetic architecture of these diseases.

Interestingly, none of the new imprinted genes uncovered in these studies exhibited the complete allele-specific silencing that is often associated with canonical imprinting. It was found that the vast majority of novel imprinting effects involve biases in allele-specific gene expression, and future studies are needed to understand the functional significance of these allele-specific biases. One untested explanation is that the biases emerge due to cell-type-specific imprinting effects. Finally, the studies by Gregg et al. offer additional insights and directions for future studies of imprinting in different regions of the brain. The authors mapped the expression pattern of 45 known imprinted genes in 118 different adult mice brain regions to determine whether particular brain regions are relatively enriched for imprinted gene expression

(Gregg et al. 2010b). They found 26 brain regions that exhibited enrichments for the expression of known imprinted genes, and most of these were monoaminergic and hypothalamic nuclei in the brain, such as the dorsal raphe nucleus, the arcuate nucleus, and the preoptic area (Gregg et al. 2010a). Future studies of imprinting in the brain might initially focus on these neural systems.

12.4 Future Directions

Next-generation sequencing allows us to observe genetic imprinting effects from a new prospective. In the same way that microarray technology contributed to the emergence of system-level analyses of gene expression, we anticipate that next-generation sequencing studies of allele-specific gene expression will similarly contribute to allele-specific gene network-level analyses that elucidate maternal versus paternal influences over gene expression in specific regions of the developing and adult brain. Currently, these studies of imprinting are limited to mice and new approaches will need to be devised to uncover imprinting effects in the primate brain. Further, improved RNA-Seq technologies will permit the study of imprinting in specific cell populations of the brain to elucidate maternal versus paternal influences over the function of molecularly defined circuits in the brain. A major question that is largely unaddressed with the exception of a few pioneering studies is whether imprinting can change in response to environmental factors or physiological states. The use of next-generation sequencing to profile imprinting will be fundamental to address these different issues. Importantly, extensive genetic and behavioral studies are required to reveal the function(s) of these complex imprinting effects and how they may contribute to disease susceptibility. We anticipate that insights into the roles of imprinted genes in the brain will provide insights into the evolutionary pressures that shaped the development and function of the brain and of the behavior of different species. Further, given the complex, polygenic nature of neuropsychiatric diseases and disorders, uncovering maternal and paternal epigenetic influences over gene expression in the brain may provide new insights into the biological basis of some of these disorders. Roles for imprinted genes in autism and schizophrenia have already been clearly established through studies of PWS and AS (Wilkinson et al. 2007). In summary, the application of next-generation sequencing to the study of genomic imprinting in the brain has opened an exciting new frontier with many avenues for study.

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Chapter 13
Genes Selectively Expressed in the Visual Cortex of the Old World Monkey

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Abstract The primate neocortex consists of much more evolved areas than those in other mammals. To understand the molecular basis and physiological significance specific to the primate neocortex, we have been searching and characterizing the genes that are selectively and highly expressed in the macaque neocortex. Such gene can be classified into three groups: the primary visual area (V1)-selective genes, the association-area-selective genes, and the motor-area-selective genes (Yamamori, Progress Neurobiol 94:201–222, 2011). The V1-selective genes (*OCC1/FSTL1*, *HTR1B*, and *HTR2A*) may play roles in keeping visual homeostasis in primates to adjust to large changes in light amount in the natural environment. In this article, we report on *SEMA7A* as another V1-selective gene in macaque monkeys. The expression pattern of *SEMA7A* differs from those of the V1-selective genes we previously reported in that it is already expressed in the mid-embryonic stage (embryonic day 83) when the thalamocortical fibers are about to project. In addition, *SEMA7A*

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only shows weak activity-dependent expression upon monocular inhibition by TTX injection into one eye compared with other V1-selective genes. These findings suggest that there are at least two subgroups of genes that show high and V1-selective expression. The significance of these findings is discussed.

13.1 Introduction

The neocortex, which is the most complex and recently evolved structure in mammals, parceled into so-called areas. The notion of areas is best known by the definition of Brodmann (1909). However the delineation of areas has been variable. For example, Oskar Vogt defined more areas and Alfred Campbell defined more conservative areas than that of Brodmann's areas (see Haymaker 1951; Rosa and Tweedale 2005). It took many centuries to determine the brain as the body part that is responsible for mental functions. At the time when the cortical parcellation was proposed, the functional significance was not clear at all. Since then, however, our understanding of the functional localization of areas has been greatly advanced (see, e.g., Gross 1998). Furthermore, recent studies of neocortical formation, some of which are presented in this special issue, revealed principal mechanisms that control layer formation, anteroposterial and lateromedial gradients, and regionalization of the primary sensory areas in the rodent neocortex (O'Leary and Sahara 2008). Nonetheless, regarding area formation, there are many questions that need to be answered.

One important issue is the formation of boundaries between areas. The neocortical areas defined by the difference in cytoarchitectonic structure within different brain areas, which are designated numbers by Brodmann, for example, may have given an impression that there are distinct boundaries between areas. As a matter of fact, the transition from one area to another is usually gradual, and it is not easy to find well-defined borders except for a few areas, most typically the primary visual cortex (V1) in primates (Lukaszewicz, et al. 2006). Earlier studies in rodents suggested that there are no genes that show distinct area-selective expression in the neocortex, although some genes clearly show regional expression patterns (see the review by O'Leary and Nakagawa 2002). Because the boundaries between cytoarchitectonic areas are less clear in the areas that appear in progressively later stages of development, or that evolved more recently (Rosa and Tweedale 2005; Collins et al. 2010), we attempted to identify the genes selectively expressed in representative areas in macaques by the differential display method(Tochitani et al. 2001; Watakabe et al. 2009; Komatsu et al. 2005)and restriction landmark cDNA scanning (RLCS) methods (Takaji et al. 2009; Takahata et al. 2009; Sasaki et al. 2010).

Our study using the above-mentioned methods has revealed two groups of genes that are selectively and highly expressed in particular areas of the macaque neocortex (Yamamori 2011). The first group of genes includes *OCC1/FSTL1*, *HTR1B*, and *HTR2A*, which are expressed in the primary visual cortex. The second group of genes includes *RBP4*, *PNMA5*, and *SLIT1*, which are selectively and highly expressed in the association areas. We have reported that *HTR1B* plays roles in the increase in signal-to-noise (S/N) ratio and *HTR2A* plays in a gain controller to

compensate for the loss and gain effected by the former receptors that are presynaptically localized (Watakabe et al. 2009). *FSTL1* has been reported to play a role in the control of sensory afferent-evoked activity in the rodent dorsal root ganglion cells (Li et al. 2011). Although the functional analysis of *OCC1/FSTL1* in the primate has not been reported yet, we speculate that it plays a similar role in primate V1. The expressions of V1-selective genes in primates show a more or less activity-dependent expression pattern (Takahata et al 2006). The V1-sepcific genes are abundantly expressed in an activity-dependent manner in layer 4 of V1, which receives projections from the lateral geniculate nucleus (LGN), which transmits visual information from the retina (Tochitani et al. 2001). These findings suggest that one of the functions of selectively V1-expressed genes is to adjust visual homeostasis to dramatic changes in light amount in the natural environment on the order of 10^7 (Yamamori 2011).

However, it is likely that there are other types of genes that show V1-selective expression besides those we identified, as described above. To identify and characterize more genes that are expressed in an area-selective manner, we performed a detailed histological study and identified another V1-selective gene of semaphorin 7A (*SEMA7A)*. *SEMA7A* is a membrane-anchored member of the semaphorin family of guidance proteins and immunomodulatory effects and known for its roles in promoting axon outgrowth in rodents (Pasterkamp et al. 2003). In macaque cortex, *SEMA7A* was abundantly expressed in primary sensory areas, particularly the primary visual area (V1), and showed a lower expression level in association areas. Although *SEMA7A* was expressed throughout all the layers, the prominent expressions in layer IV of V1 and the middle layer in other sensory areas seem to be the common characteristic features of the expression that are shown among other V1-selective genes. However, the activity dependence of *SEMA7A* was weak and not as apparent as that shown by other genes selectively expressed in V1. In addition, *SEMA7A* is already expressed during corticogenesis, and its expression level is comparable to those in layer IV of V1 postembryonic and adult levels, whereas the expression of other V1-selective genes in layer IV of V1 undergoes maturation during postnatal development (Tochitani et al. 2003a, b). These findings suggest that a variety of V1-selective gene expression properties may correspond to the unique features of primate V1 and may therefore provide important and useful clues to solving the characteristics of V1-selective genes at the molecular level.

13.2 Genes that are Selectively Expressed in V1

By searching for genes selectively expressed in the cortical area using the RLCS method, we found 28 genes that were expressed differentially among four areas [area 46, primary motor area (M1), temporal association area (TE), and primary visual area (V1)] in African green monkeys (data not shown). The findings were reproduced by semiquantitative PCR analysis in two macaque monkeys. Although the average ratio of maximum expression level to minimum expression level varied among genes, only three expression patterns were observed in terms of

difference in the rank order of expression levels between cortical areas. The first pattern is that the gene expression levels are highest in V1 and lowest in the higher-order association areas 46 and TE. In contrast, the second pattern showed the highest expression levels in the higher-order association areas and the lowest in V1. The third pattern of gene expression showed the highest levels in M1, but this pattern did not have the complementary relationships observed between the gene group expressed in V1 and that expressed in higher-order association areas. Among the gene groups classified on the basis of their expression patterns, V1-selective genes show the most characteristic expression pattern in terms of their expression pattern by in situ hybridization (ISH) (Tochitani et al. 2001; Takahata et al. 2006; Watakabe et al. 2009; Yamamori 2011). Accordingly, V1-selective genes show higher expression levels in primary sensory areas than in other sensory areas. These genes are abundantly expressed in the middle layers among sensory areas, and the expression level in layer IVC in V1 is highest throughout cortical areas. The expressed genes generally reach maturity during postnatal development. In particular, the expression in V1 depends on retinal activity. We wanted to determine whether these properties are common among V1-selective genes. To this end, here, we newly investigate *SEMA7A* screened as a selectively V1-expressed gene by RLCS.

13.3 *SEMA7A* Expression in Macaque Cortical Areas

ISH for macaque cortices demonstrated that *SEMA7A* was expressed at a certain level throughout the cortical areas, but the laminar distribution and the abundance of the *SEMA7A* level in each cell were markedly different among cortical areas. The *SEMA7A* expression level was highest in V1, as shown by RLCS (Fig. 13.1a, b). The border between the primary sensory and adjacent areas was distinct in the visual, auditory, and somatosensory cortices (Fig.13.1a). In V1, *SEMA7A* was expressed in all layers except layer I. In particular, ISH signals were most intense in layer IVC (Fig. 13.1b). In V2, intense signals were observed among the neuronal populations deep in layer III. Similarly in visual cortices, *SEMA7A* also showed high expression levels in the middle layers of other sensory areas, i.e., the auditory and somatosensory-related areas (Fig. 13.1a). Outside the sensory areas, e.g., area 46 and M1, *SEMA7A* showed a lower expression level, and the level of *SEMA7A* expression in one neuron seemed to be low from layers II to VI except for a subpopulation of large pyramidal neurons in layer V in M1, which showed a high level of *SEMA7A* expression.

13.4 Characteristics of *SEMA7A* Expression in Subcortical Regions of Macaque Brain

SEMA7A expression was observed in most of the thalamic nuclei including the sensory relay nuclei, lateral geniculate nucleus (LGN), and medial geniculate nucleus (MGN). The expression was observed in all layers of LGN with higher expression

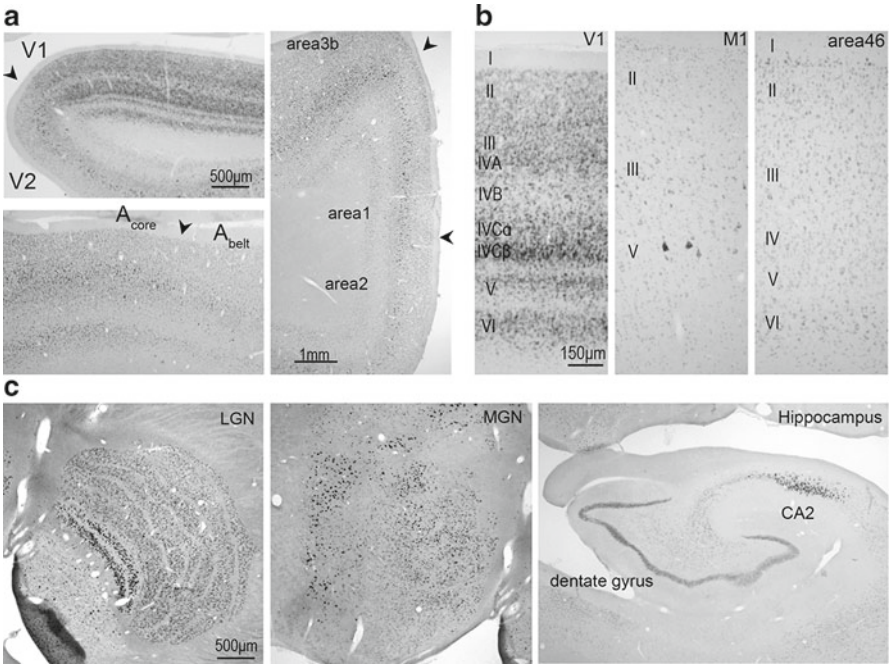

Fig. 13.1 *SEMA7A* expression in sensory areas in *Macaca fuscata* brain. (**a**) Three primary sensory areas, namely, primary visual area (V1), primary auditory area (Acore), and primary somatosensory area (area3b), show higher expression levels than adjacent secondary areas, namely, secondary visual area (V2), secondary auditory area (Abelt), and secondary somatosensory area (area 1). *Arrowhead(s)* indicates the border between the primary and secondary sensory areas. (**b**) Differential laminar distribution among three cortical areas (V1, M1, prefrontal area 46). V1 shows high expression levels from layers II to VI, especially layer IVC**β**. The primary motor cortex (M1) and area 46 show a moderately high expression level except for large pyramidal neurons in layer V of M1. (**c**) *SEMA7A* expression in subcortical regions. The sensory relay nuclei, lateral geniculate nucleus (LGN), and medial geniculate nucleus (MGN) show significantly high signal intensities. In the hippocampus, significantly high signal intensities are observed in CA2 and dentate gyrus. *SEMA7A* signals were detected by ISH (in situ hybridization) as described in our previous reports

levels in magnocellular (M) layers than that of parvocellular (P) layers (Fig. 13.1c). The neurons in M layers project to layer IVA in V1, whereas those in P layers project to layer IVC, in which *SEMA7A* was also highly expressed. Thus, the role of *SEMA7A* may be different between LGN and V1.

Interestingly, we observed that *SEMA7A* was selectively expressed in CA2 in the macaque hippocampus, where other V1-selective genes, *OCC1/FSTL1* (Tochitani et al. 2003a) and *HTR1B* (data not shown), are also expressed. Although the role of CA2 was not clear for many years after its first identification by Lorente de Nó in 1934, a recent study has demonstrated that CA2 neurons are strongly activated by their more proximal dendritic inputs from the entorhinal cortex (EC). CA2 neurons in turn form strong excitatory connections with CA1 neurons. The EC-CA2

synapses are subjected to a strong synaptic plasticity in the form of LTP (Chevaleyre and Siegelbaum 2010). CA2 neurons are the hippocampal target of the supramammillary nucleus (which also targets the dentate gyrus), a structure involved in controlling the frequency of the theta rhythm (Pan and McNaughton 2002). It is also involved in the epileptic spread in the hippocampus (Saji et al. 2000). These findings suggest the modulatory and synchronizing roles of CA2 neurons in the hippocampal network.

In the rodent hippocampus, however, *OCC1/FSTL1* and *HTR1B* do not show CA2-selective expression, but are widely expressed throughout the hippocampus. This pattern of expression may be analogous to that in the visual cortex, where V1-selective expressions of *OCC1/FSTL1 and HTR1B* are only observed in the primate neocortex (Takahata et al. 2008). In this regard, despite the different features of expression pattern of *SEMA7A* and other V1-selective genes thus far characterized by our group, they still share the same characteristics as a group, and *SEMA7A* may play a critical role in formation of a structure of V1 because it is already expressed at prospective V1 during corticogenesis.

13.5 Response of *SEMA7A* Expression to Monocular Inhibition (MI)

To examine the retinal activity-dependent expression of *SEMA*7A in V1, retinal activity was monocularly inhibited with TTX for 6 h, 1 day, 5 days, 14 days, and 21 days, as previously conducted for *OCC1/FSTL1* (Tochitani et al. 2001; Takahata et al. 2008). Compared with *OCC1/FSTL1* expression, *SEMA7A* expression was weak (Fig. 13.2). This is different from other selectively V1-expressed genes, such as *OCC1/FSTL1* and *HTR1B* (Fig. 13.2 and data not shown), which showed a constant and strong reduction of expression even level during the 21-day monocularly inhibited period (Tochitani et al. 2001). The functional meaning of this weak activity dependency of *SEMA7A* is not clear at this moment and needs further investigation.

13.6 *SEMA7A* Expression in Visual Cortex During Development

SEMA7A expression was already observed during the early cortical development (Fig. 13.3). At E83, when the cortex is still undergoing division and the projection of thalamocortical fibers is about to start (Donoghue and Rakic 1999a, b), *SEMA7A* expression was already observed in the occipital region (Fig. 13.3a). The laminar distribution seemed to resemble that of *Ephrin A5* (Donoghue and Rakic 1999b). After this stage, the increase in expression level in the upper layer of V1 seemed to occur until E90, and then, the border between V1 and V2 became well defined (Fig. 13.3b).

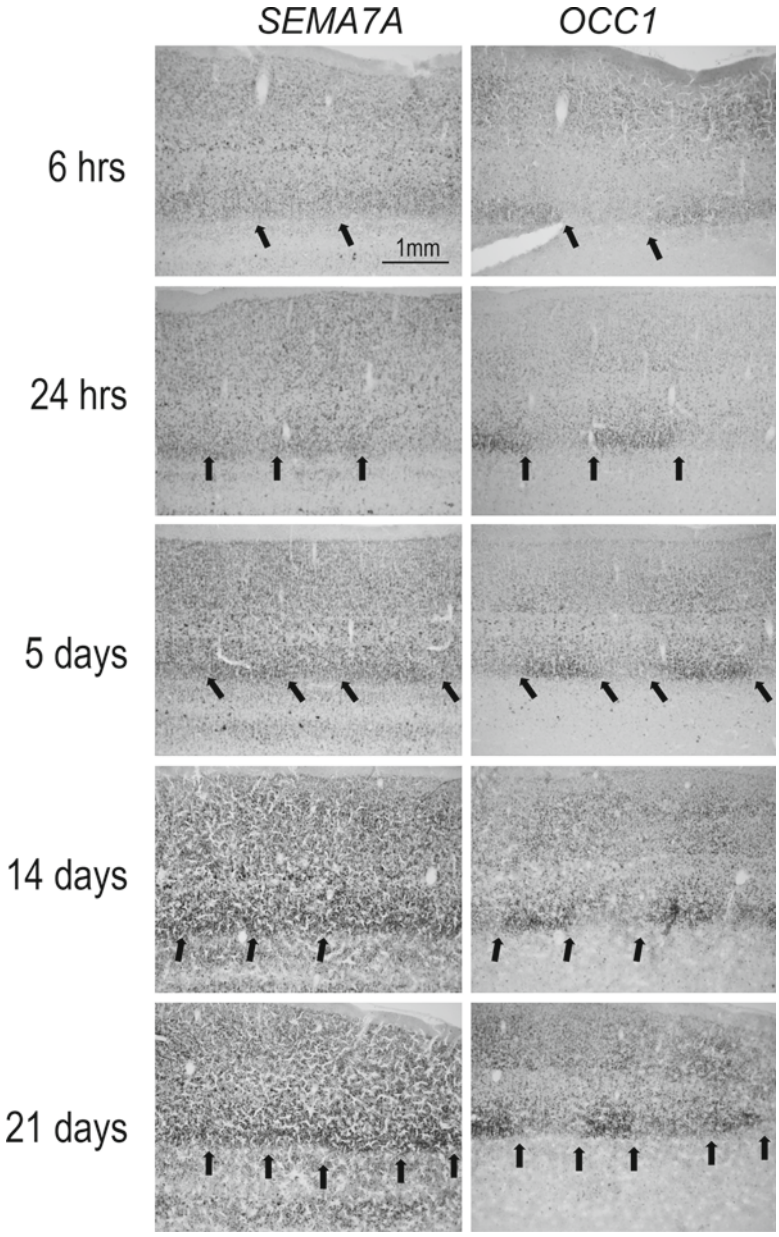

Fig. 13.2 Effect of monocular inhibition on *SEMA7A* expression. Ocular dominance columns in V1 were observed to consistently show decreased *OCC1/FSTL1* signal intensities 6 h, 24 h, 5 days, 14 days, and 21 days after TTX injection into one eye (*Macaca fascicularis* and *Macaca fuscata*, the same samples used in Tochitani et al. 2001). In contrast, the *SEMA7A* expression level seems to be less reduced in the deprived columns at all periods. *Arrows* indicate the borders between the normal and monocularly inhibited ocular columns

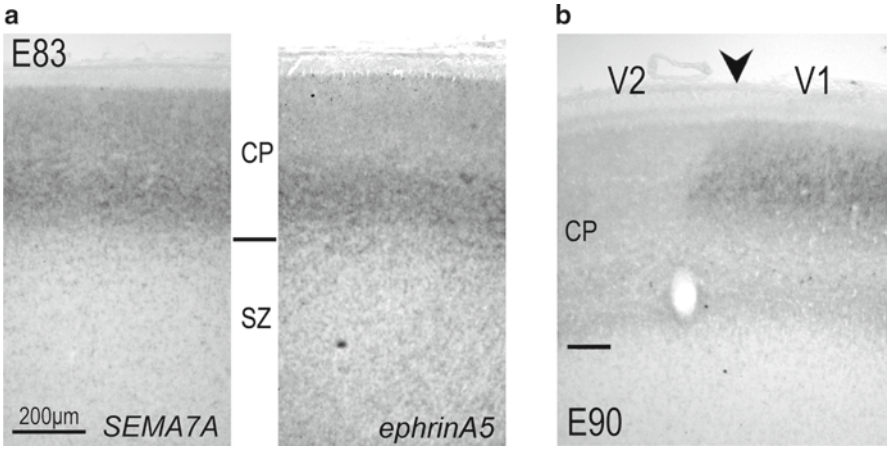

Fig. 13.3 *SEMA7A* expression in early cortical development. (**a**) *SEMA7A* expression was observed throughout the cortical plate (CP) at embryonic day (E) 83 (*Macaca fascicularis*). More intense signals were observed in the deeper part of the cortical plate, corresponding to the region where *ephrinA5* was expressed. Both signals were not clearly observed in the subventricular zone (SZ). (**b**) *SEMA7A* expression at E90. The *SEMA7A* signal clearly visualized the border between area 17 and area 18 and prospective V1 and V2. The expression signal was more intense in upper part of CP

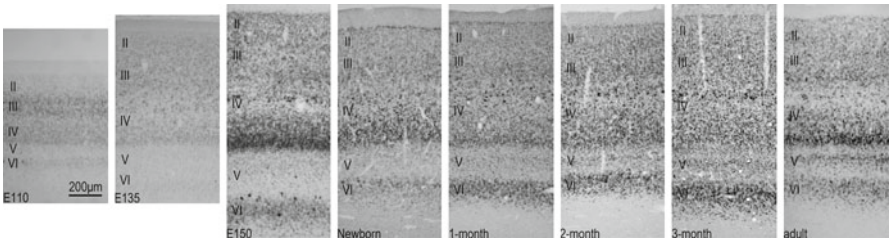

Fig. 13.4 *SEMA7A* expression in prenatal and postnatal development in V1 (*Macaca fascicularis*). SEMA7A signals at eight different developmental stages (E110, E135, E150, newborn, 1 month of age, 2 months of age, 3 months of age, adult) are shown. Layers are shown on the left side of each panel

The prospective V2 is already projected by thalamic fibers, which can be visualized by acetylcholineesterase at this stage (data not shown). However, a similar increase in the level of *SEMA7A* expression was not observed in the cortical plate corresponding to the prospective V2. Thus, these findings suggest that the area selectivity of *SEMA7A* expression is not only dependent on the thalamocortical projection.

From E110 to E150, *SEMA7A* expression was constantly observed in the cortex (Fig. 13.4), and the expression clearly revealed the prospective V1-V2 border (data not shown), whereas the laminar distribution was changed during this period (Fig. 13.4). Notably, the high expression level in layer IV of V1 at E150 was already

comparable to or relatively even higher than that in adult V1. This expression pattern indicates that *SEMA7A* expression in layer IV of V1 has nearly reached maturity before birth. Interestingly, this maturation pattern is different from that of other V1-selective genes; *OCC1/FSTL1* and *HTR1B* expressions undergo maturation by increasing the layer IV expression level in V1 during the early postnatal development (Tochitani et al. 2003a, b; Watakabe et al. 2009).

In contrast to *OCC1* and *HTR1B*, *SEMA7A* was expressed in layers V and VI. The *SEMA7A* expression in layer VI became apparent at E150, and the layer V was enhanced after 1 month of age (Fig. 13.4). We need further investigation to clarify the relationship between the maturation difference of *SMEA7A* expression among V1 layers and the construction of the neuronal circuit in each layer.

13.7 Classification of Selectively V1-Expressed Genes

It is considered that a developing primary visual cortex should be formed such that it can receive a large amount of visual information needed for the primate life in the form of different information streams (channels), while it remains to be studied further what kind of molecules contribute to the development and maintenance of the primate visual cortex. Our studies thus far have found the genes that are selectively expressed in V1 in macaque monkeys, whose expressions are highest at layer IVC (Tochitani et al. 2001; Watakabe et al. 2009). The gene expressions in V1 are strongly affected by the deprivation of neuronal activity from the retina via LGN within 5–6 h, and the reduction in the expression level continues during the monocularly inhibited period up to a maximum of three weeks, the longest length thus far we have examined. However, the characteristic expression pattern of *SEMA7A* revealed in this study has further deepened our knowledge on the property of V1-selective genes. *SEMA7A* expression in V1 was less sensitive to the activity from thalamic inputs even though *SEMA7A* was also expressed abundantly in the receptive layers in V1 with area distributions in the adult cortex similar to those of other selectively V1-expressed genes. These different characteristics among selectively V1-expressed genes may be related to the functional property of the gene products.

We previously reported and discussed the possibility that selective V1 expression is related to the modulatory role of the functional connection between V1 and LGN (Takahata et al. 2010; Yamamori 2011). Actually, those gene expressions mature after birth. On the other hand, it has been recently reported that *Sema7A* plays roles in axon branching and/or presynaptic punctate formation in the mouse thalamocortical projections (Fukunishi et al. 2011). Consistent with this report, we observed that *SEMA7A* expression was already observed during the invasion of thalamocortical fibers into prospective V1 in this study. We expect that a similar function of *SEMA7A* will be observed in developing and adult visual circuits in the Old World monkey brain.

13.8 Expression Patterns of Area-Selective Gene Expressions in Macaque Monkeys and Their Implications

As shown in Fig. 13.5, the genes selectively and highly expressed in certain areas in the macaque neocortex can be categorized into three groups. One group of genes is selectively expressed in the primary sensory areas, particularly in the visual cortex. Another group of genes is selectively expressed in the association areas. The third group of genes is selectively expressed in motor areas. Although the expression patterns of genes within one group in each area are similar, for example, the expressions of *RBP* and *PNMA5* highly overlap in the macaque neocortex (Takaji et al. 2009), the genes selectively expressed in association areas show different types of developmental patterns of expression in the late embryonic stage (Komatsu, Sasaki and Yamamori, unpublished observation). These findings suggest that the regulations of area-selective genes are complex and may be variable. The observation that the

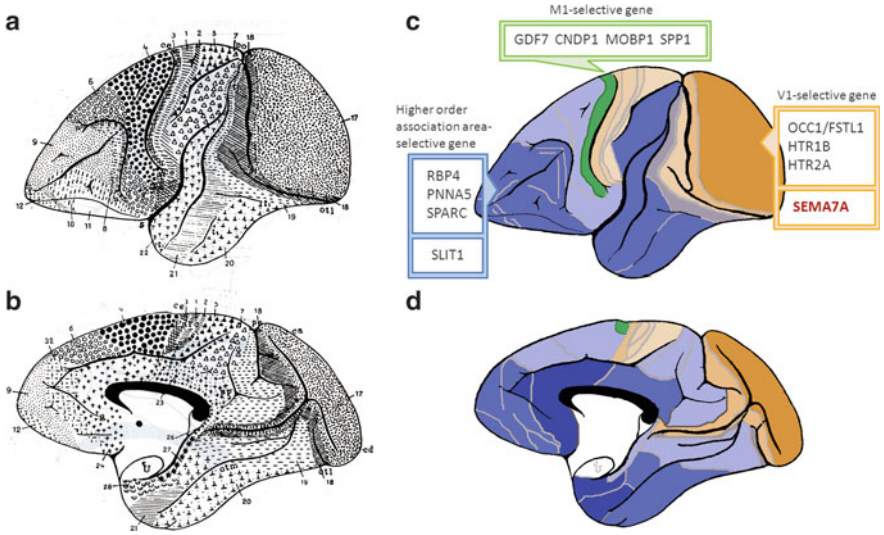

Fig. 13.5 Expression patterns of primate neocortical area-selective genes. (**a** and **b**) Cytoarchitectonic cortical areas in the guenon monkey, as distinguished by Brodmann, from the lateral (**a**) and medial (**b**) views. (**c** and **d**) Illustration of area-selective gene expression. Expression of three area-selective genes is illustrated on the basis of the following data in macaques. *OCC1/FSTL1*, *HTR1B*, *HTR2A*, and *SEMA7A* (*brown*) are expressed in the primary sensory areas, particularly in the visual cortex (Tochitani et al. 2001; Watakabe et al. 2009; this volume). *RBP*, *PNMA5*, *SPARC*, and *SLIT1* (*blue*) are expressed in association areas (Komatsu et al. 2005; Takahata et al. 2009; Sasaki et al. 2010). *GDF7*, *CNDP1*, *MOBP1*, and *SSP1* (*green*) are expressed in the motor area (Watakabe et al. 2001; Sato et al. 2007). *Shaded dark*, *light*, and *pale colors* indicate strong, moderate, and weak expressions of each gene, respectively. *Mixed color* areas of *pale blue* and *brown* indicate where both *RBP* and *OCC1/FSTL1* are expressed in different layers (see Figs. 13.2 and 13.3). The original figure is published in Yamamori and Rockland 2006 and cited with permission

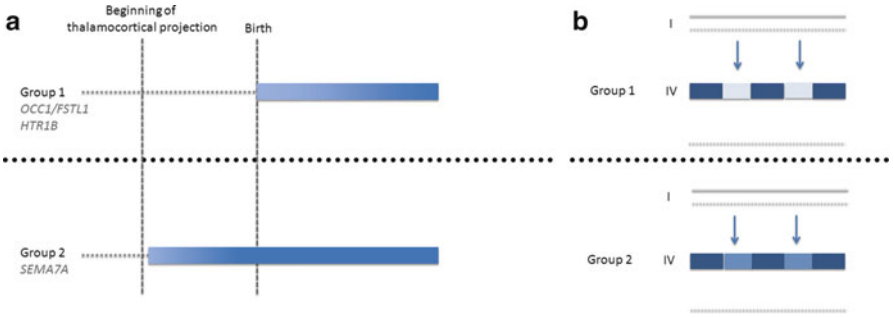

Fig. 13.6 Summary of expression profiles of selectively V1-expressed gene groups in layer IV of V1. (**a**) Gradation of *blue lines* in each group indicates change in expression level throughout the developmental period. *Dotted lines* indicate that gene expression profile remains to be investigated. (**b**) *Arrows* indicate the layer IV deprived of ocular dominance columns

expression patterns become very similar among genes within one group (e.g., *RBP4* and *PNMA5*) in adults suggests that there is a coordinated mechanism within a subgroup. In addition, although *SLIT1* is selectively expressed in association areas, most strongly in the prefrontal cortex, the highest expression level is observed at layer IV where *RBP4*, *PNMA5*, and *SPARC* are expressed at low levels (Sasaki et al. 2010). Therefore, there may be at least two types of expression pattern of association-area-selective genes in the adult neocortex in macaque monkeys.

We summarize the expression pattern of V1-selective genes thus far characterized by our group including that of *SEMA7A* in Fig. 13.6. The difference in expression pattern between *SEMA7A* and the other V1-selective genes is that *SEMA7A* was already expressed even before the start of thalamocortical projections into the neocortex (Fig. 13.3a). Although *SEMA7A* expression varies in pattern to some extent during the late development, it continues until adulthood. On the other hand, the expression levels of other V1-selective genes are low before birth and markedly increase postnatally. In addition, *SEMA7A* shows only weak activity-dependent expression in adult V1, whereas the other V1-selective genes show strong neuronal activity-dependent expression. These findings suggest that there are different regulatory mechanisms between the subgroups of V1-selective genes. However, the overall expression patterns of *SEMA7A* and the other selective V1-expressed genes are similar in the brain including their selective expression in CA2 of the macaque hippocampus. How the similarities and differences between the two groups of V1-selective genes are controlled at the molecular level remains to be elucidated in a future study. The expression patterns of a set of 26 genes were studied in neonate marmoset cortices (Mashiko et al. 2012). Although most of the genes show conserved expression patterns in the mouse and the marmoset, certain genes show very different expressions patterns between the two species. Some of the genes, such as the BTB (POZ) domain containing 3 (*BTBD3*) and connective tissue growth factor (*CTGF*) genes, are highly and selectively expressed in neonatal marmoset V1. Therefore, it is interesting to determine whether genes that show similar expression

patterns to *SEMA7A* exist and, if any, to identify the population of this class of genes in the macaque whole genome.

Bernard et al. (2012) carried out a large-scale analysis of the primate neocortex (*Macaca mulatta*) using laser microdissection and microarray analysis. Their analysis demonstrated that the primary transcriptome-based relationships are determined by spatial proximity, being strongest between neighboring cortical areas and between proximal layers. This principal profile probably reflects the "geometrical relationships" during the course of corticogenesis, which mechanisms are one of the main topics of this special issue. In addition to this neighboring rule, they reported that macaque V1 displays the most distinctive gene expression pattern among the cortical regions. In this article, we focus on the genes that are highly and selectively expressed in V1 among four representative neocortical areas, which generally show more than a fivefold difference between V1 and the area with the least expression level, as determined by semiquantitative RT-PCR analysis. This class of genes plays roles in maintaining visual homeostasis in primates as discussed above. Because the level of V1 selectivity varies among the V1-selective genes, as revealed by their microarray and laser microdissection analysis, namely, from highly selective genes, which we focused on in this research, to V1-selective genes whose significance of expression level is only detectable by statistical analysis, it is necessary to further analyze the functional classification of these V1-selective genes.

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