

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 [³H]-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

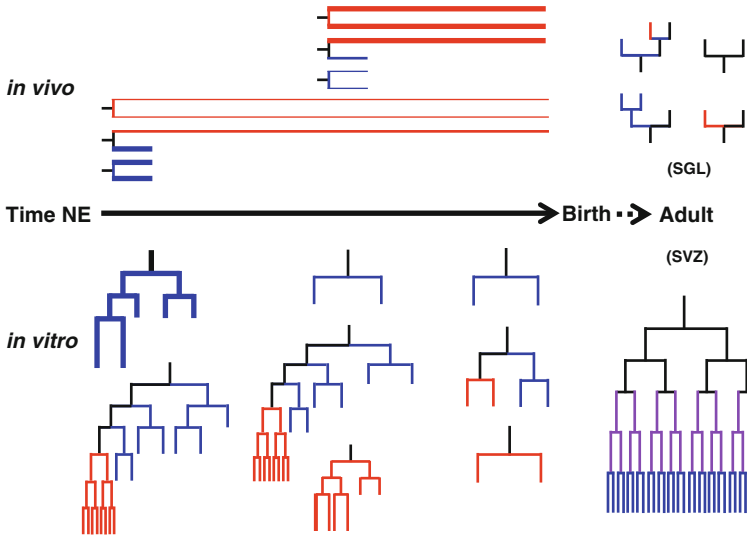


Fig. 4.1 Progenitor heterogeneity in the developing CNS. Results of *in vivo* clonal lineage analyses and *in vitro* time-lapse clonal lineage analyses in the developing and adult vertebrate CNS from several research groups are summarised and depicted as lineage trees. *Blue branches* represent neuronal 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

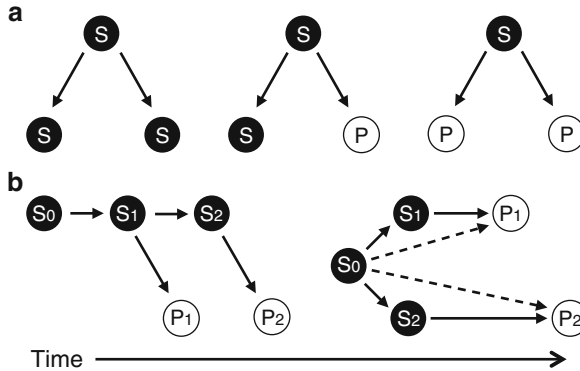


Fig. 4.2 Patterns of stem cell division. **(a)** *Left to right*: symmetric self-renewing division to double stem cells; asymmetric division to self-renew and generate a differentiating progeny cell; and differentiative division to generate two differentiating progeny cells. The fate of each daughter 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 signaling (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 S100 β , 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 (Kessarlis 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 (Kessarlis et al. 2008).

Nonetheless, analysis of mice lacking Shh or Smoothed (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; Kessarlis 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 S100 β 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 astroglialogenesis (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 astroglialogenesis 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 oligodendroglialogenesis 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 oligodendroglialogenesis (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 signaling 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 adenomatous 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.

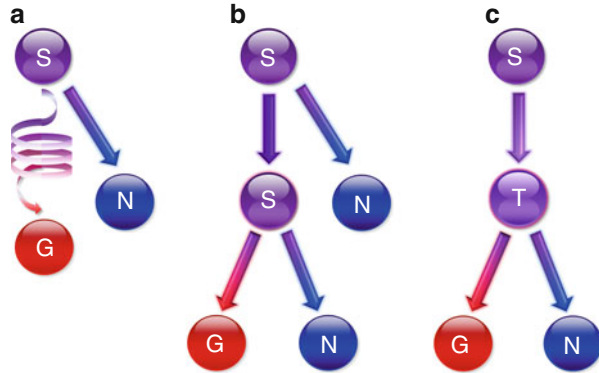


Fig. 4.3 Possible patterns of temporal regulation of neurogenesis and gliogenesis by stem cells. (a) The screw model of NSC differentiation into glia. A neurogenic stem cell lineage “spirals down” to a glial lineage over time. (b) A neurogenic stem cell lineage acquires gliogenic competence to generate 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 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

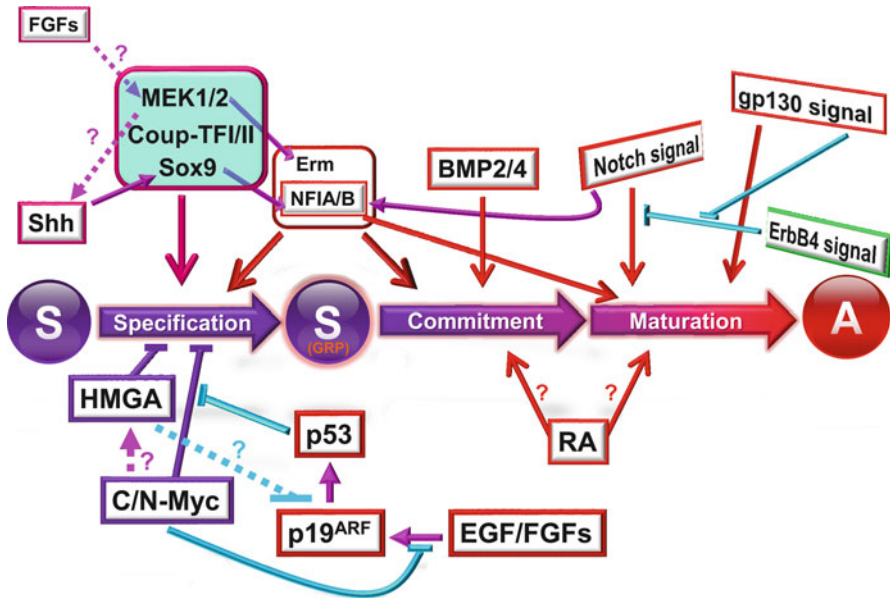


Fig. 4.4 A hypothetical model for the mechanism of temporal specification and differentiation of NSCs into astrocytes. Many intrinsic and extrinsic factors are involved in the differentiation process from neurogenic stem cells toward astrocytes. Early NSCs (S) that initially differentiate exclusively into neurons are specified to become GRPs or to acquire gliogenic competence in response to astroglial 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 HMGAs proteins (Fig. 4.4). The expression levels of HMGAs1 and HMGAs2 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 HMGAs 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 HMGAs2 does not alter the time-dependent change in the DNA methylation status of the *Gfap* promoter (Sanosaka et al. 2008). However, HMGAs2 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 astroglial 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 oligodendroglialogenesis, as well as astroglialogenesis 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 (Barnabé-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 (Barnabé-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 astroglialogenesis 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 *S100 β* . Loss of function of ErbB2 or ErbB4 results in the premature expression of GFAP and S100 β 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 astroglialogenic 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-astroglialogenic 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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