Neuron
Review

bHLH Factors in Self-Renewal, Multipotency, and Fate Choice of Neural Progenitor Cells

Itaru Imayoshi[1,2,3,4,*] and Ryoichiro Kageyama[1,3,5,*]
[1]Institute for Virus Research, Kyoto University, Shogoin-Kawahara, Sakyo-ku, Kyoto 606-8507, Japan
[2]The Hakubi Center, Kyoto University, Kyoto 606-8302, Japan
[3]World Premier International Research Initiative–Institute for Integrated Cell-Material Sciences (WPI-iCeMS), Kyoto University, Kyoto 606-8501, Japan
[4]Japan Science and Technology Agency, Precursory Research for Embryonic Science and Technology (PRESTO)
[5]Japan Science and Technology Agency, Core Research for Evolutional Science and Technology (CREST)
4-1-8 Honcho, Kawaguchi, Saitama 332-0012, Japan
*Correspondence: iimayosh@virus.kyoto-u.ac.jp (I.I.), rkageyam@virus.kyoto-u.ac.jp (R.K.)
http://dx.doi.org/10.1016/j.neuron.2014.03.018

Multipotent neural progenitor cells (NPCs) undergo self-renewal while producing neurons, astrocytes, and oligodendrocytes. These processes are controlled by multiple basic helix-loop-helix (bHLH) fate determination factors, which exhibit different functions by posttranslational modifications. Furthermore, depending on the expression dynamics, each bHLH factor seems to have two contradictory functions, promoting NPC proliferation and cell-cycle exit for differentiation. The oscillatory expression of multiple bHLH factors correlates with the multipotent and proliferative state, whereas sustained expression of a selected single bHLH factor regulates the fate determination. bHLH factors also regulate direct reprogramming of adult somatic cells into neurons and oligodendrocytes. Thus, bHLH factors play key roles in development and regeneration of the nervous system. Here, we review versatile functions of bHLH factors, focusing on telencephalic development.

Introduction

During the course of development, neural progenitor cells (NPCs) are responsible for generating the diverse types of neurons and glial cells that build the nervous system (McConnell, 1995; Okano and Temple, 2009; Breunig et al., 2011). In the developing telencephalon, NPCs of the lateral ventricular wall undergo changes in morphology and property and produce different progeny as brain development proceeds (Fishell and Kriegstein, 2003; Kriegstein and Alvarez-Buylla, 2009). Neuroepithelial cells, the earliest form of NPCs, constitute a single layer of pseudostratified columnar epithelium (Figure 1). Neuroepithelial cells are gradually transformed into elongated radial glial (RG) cells that span the thickness of the brain wall, retaining their cell bodies in the innermost layer, called the ventricular zone (VZ) (Figure 1). RG cells undergo asymmetric cell division: each RG cell divides into two distinct cell types, one RG cell and one immature neuron or an intermediate progenitor (Götz and Huttner, 2005; Miller and Gauthier, 2007). Immature neurons migrate out of the VZ into the outer layers, where they become mature neurons, while intermediate progenitors migrate into the subventricular zone (SVZ), proliferate further, and give rise to more neurons (Figure 1). Thus, the SVZ is a secondary germinal zone where further divisions of intermediate progenitors occur to enlarge the neuronal populations. Neocortical NPCs also give rise to a new type of RG cells, known as outer RG cells, whose cell bodies are located in the outer VZ (Figure 1) (Hansen et al., 2010; Fietz et al., 2010; Wang et al., 2011; Shitamukai et al., 2011). After producing neurons, RG cells finally differentiate into glial cells, but some of them are maintained as NPCs in the postnatal and adult brain.

It has been shown that basic helix-loop-helix (bHLH) factors play key roles in self-renewal of NPCs and fate determination of neurons, oligodendrocytes, and astrocytes (Figure 2) (Bertrand et al., 2002; Ross et al., 2003; Meijer et al., 2012; Namihira and Nakashima, 2013). Repressor bHLH factors like Hes1 regulate the self-renewal of NPCs, whereas proneural bHLH factors, such as Ascl1 (also called Mash1) and Neurog2, promote neuronal differentiation. Other bHLH factors, Olig1 and Olig2, regulate oligodendrocyte differentiation, while Hes1 induces astrocyte formation at later stages. Thus, bHLH factors play key roles in all these steps (Figure 2), but recent studies revealed that the regulation is not that simple. Below, we discuss the recent findings regarding the complex regulations and functions of these bHLH factors.

bHLH Factors in Telencephalic Development

Maintenance of NPCs by Notch-Hes Signaling

To develop the nervous system with the appropriate number of neurons and glia, it is essential that NPCs and intermediate progenitors proliferate sufficiently prior to differentiating (Caviness et al., 1995; Kriegstein and Alvarez-Buylla, 2009). In addition, a sufficient number of NPCs must be maintained until adulthood in the SVZ of the lateral ventricles and the hippocampal dentate gyrus, as they are important for higher brain functions such as learning and memory. Thus, the maintenance of NPCs throughout life is essential for brain morphogenesis and functions. NPCs are maintained in an undifferentiated state by bHLH factors, such as Hes, Hey, and Id family members. *Hes1* and *Hes5* are widely expressed by NPCs in the VZ of the developing telencephalon. In addition, *Hes3* is expressed at early stages in the developing nervous system.

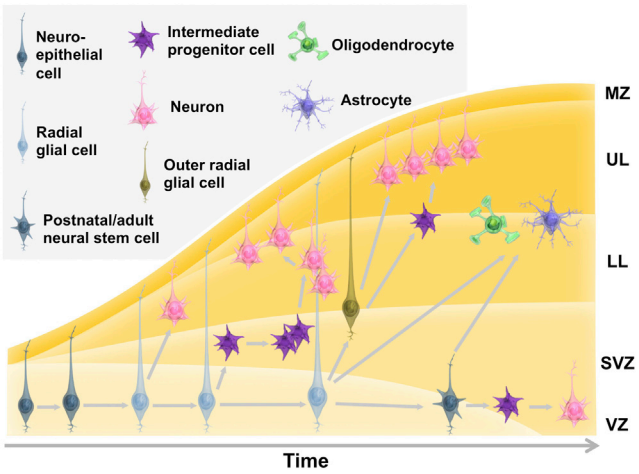

Figure 1. Neural Progenitor Cells in the Developing Telencephalon
MZ, Marginal zone; UL, upper layer; LL, lower layer; SVZ, subventricular zone; VZ, ventricular zone.

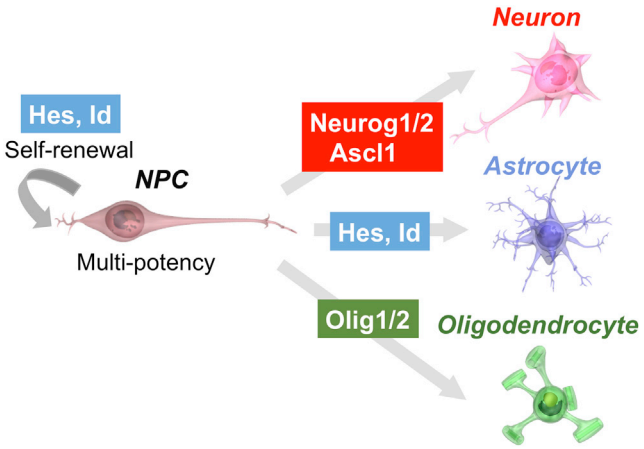

Figure 2. Simplified Roles of bHLH Factors in NPC Self-Renewal and Cell Fate Determination

Hes factors form homodimers and bind to specific DNA elements (i.e., N-box, E-box, or C-site) to repress the expression of target genes (Figure 3Aa) (Sasai et al., 1992). Although there are many downstream targets of Hes factors, proneural genes are the most important targets in the context of neural development. Hes factors directly repress the expression of proneural genes, such as *Ascl1* and *Neurog2*, and in the absence of *Hes* genes, proneural gene expression is upregulated, accelerating neurogenesis (Hatakeyama et al., 2004; Imayoshi et al., 2008). Hes factors can also antagonize the activity of proneural bHLH proteins by physical interaction: the Hes-proneural bHLH complex can bind and repress neuronal target genes, resulting in the inhibition of neurogenesis (Figure 3Ab) (Giagtzoglou et al., 2003). Thus, Hes factors inhibit neurogenesis by antagonizing proneural factors via two independent mechanisms: direct transcriptional repression and physical interaction.

Hes factors cooperatively regulate NPC maintenance and therefore only in compound *Hes* knockout mice do severe defects of NPC maintenance occur. In *Hes1;Hes5* double knockout mice, the expression of proneural factors is upregulated, leading to severe premature neuronal differentiation, rapid depletion of NPCs, and disorganized structures of the developing nervous system (Ohtsuka et al., 1999; Hatakeyama et al., 2004). Even more severe and wider defects of premature neurogenesis and depletion of NPCs occur in the *Hes1;Hes3;Hes5* triple knockout mice (Hatakeyama et al., 2004). However, in the developing telencephalon, NPCs are maintained and proliferate almost normally even in the absence of *Hes1*, *Hes3*, and *Hes5* (Imayoshi et al., 2008), indicating that the requirement for Hes factors is different between the telencephalon and other regions. In the developing telencephalon, *Hey1* is highly expressed, and this expression is upregulated in the absence of *Hes1*, *Hes3*, and *Hes5* (Imayoshi et al., 2008), suggesting that Hey1 may compensate for Hes factors to regulate telencephalic development. Further evidence of the role of Hes factors in the maintenance of NPCs was obtained by overexpression studies, which showed that Hes factors inhibit neurogenesis and increase the proportion of undifferentiated NPCs (Ishibashi et al., 1994; Ohtsuka et al., 2001).

In the developing nervous system, proneural factors, such as the bHLH transcription activators Ascl1, Neurog1, and Neurog2, induce neuronal differentiation (Figure 2, see below) (Bertrand et al., 2002; Wilkinson et al., 2013). These factors also upregulate the expression of ligands for Notch signaling, such as the transmembrane proteins Delta-like1 (Dll1) and Jagged1 (Jag1), which activate the transmembrane protein Notch in neighboring cells (Castro et al., 2006; D'Souza et al., 2008; Henke et al., 2009). Upon activation of Notch, the Notch intracellular domain (NICD) is released from the transmembrane portion and transferred to the nucleus, where it forms a complex with the DNA-binding protein RBPjκ and the coactivator Mastermind-like (Maml) (Kopan and Ilagan, 2009). The NICD-RBPjκ-Maml complex is a transcriptional activator and induces the expression of bHLH transcriptional repressors, such as Hes1 and Hes5, constituting a so-called canonical pathway. By contrast, Hes3 expression is induced by a noncanonical pathway of Notch signaling (Androutsellis-Theotokis et al., 2006). Hes factors then repress the expression of proneural genes and *Dll1*, thereby inhibiting neuronal differentiation and promoting the maintenance of NPCs. Thus, differentiating neurons inhibit neighboring cells from differentiating into the same cell type via Notch signaling, a process called lateral inhibition. This lateral inhibition prevents simultaneous differentiation of all NPCs, thereby achieving prolonged NPC maintenance into later stages of development (Imayoshi et al., 2010). Besides Notch signaling, Hes5 expression is upregulated by Gcm genes (Hitoshi et al., 2011). By contrast, in differentiating neurons, even though Notch signaling is activated, Hes5 expression is suppressed by Bcl6, which excludes the coactivator Maml1 from NICD and recruits the histone deacetylase Sirt1 to the *Hes5* promoter, thereby reinforcing neuronal differentiation (Tiberi et al., 2012). Thus, Hes expression is controlled at multiple levels.

In addition to Hes factors, Id factors are expressed by NPCs in the developing brain. In *Id1;Id3* double knockout mice, NPCs exit the cell cycle prematurely and undergo accelerated neuronal differentiation (Lyden et al., 1999). Thus, Id factors inhibit

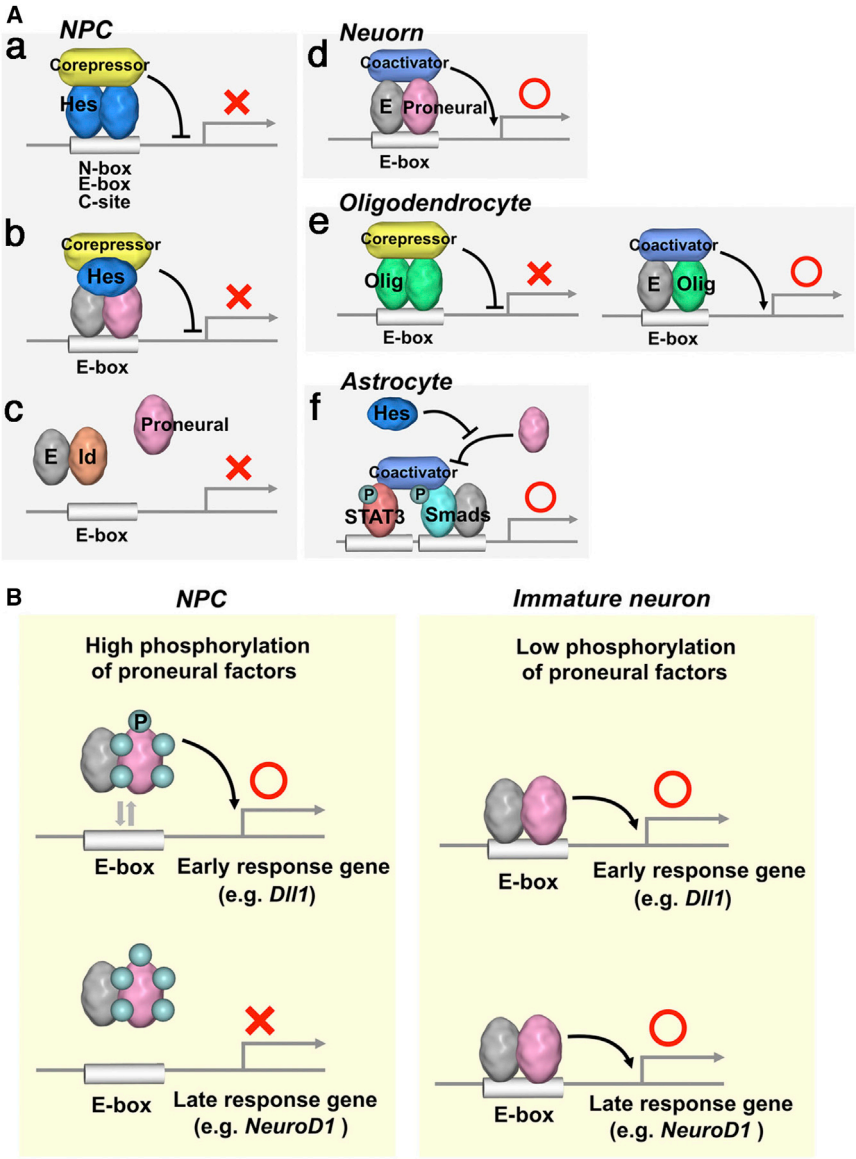

Figure 3. Mechanisms of bHLH Factor Regulation

(A) bHLH factors activate or repress gene expression by physically interacting with each other.

(B) Phosphorylation-dependent modulation of transcriptional activity of proneural factors.

precocious differentiation of NPCs into neurons. Conversely, overexpression of Ids in NPC culture blocks neurogenesis, indicating that Id factors are sufficient for NPC maintenance (Figure 2) (Bai et al., 2007). Id factors form dimers with and sequester E proteins away from proneural bHLH proteins. Because Id proteins lack the basic DNA binding motif, the Id/E heterodimer complex cannot bind to DNA (Figure 3Ac). Therefore, Id factors function as dominant-negative antagonists of proneural bHLH transcription factors (Perk et al., 2005). Unlike Hes1, Id expression is not activated by Notch signaling but strongly regulated by bone morphogenetic protein (BMP) signaling (Nakashima et al., 2001; Mira et al., 2010).

Proneural Factors for Neurogenesis

In the developing and postnatal brain, proneural bHLH factors are key regulators of neurogenesis, coordinating a generic neuronal fate and a specific subtype identity (Bertrand et al., 2002; Wilkinson et al., 2013). These factors are transcriptional activators that bind to target DNA sequences as heterodimer complexes with ubiquitously expressed bHLH E proteins (E12, E47, HEB, or E2-2) (Figure 3Ad). Target genes for proneural factors include those encoding Notch ligands, such as Dll1, which activate Notch signaling in neighboring cells (Castro et al., 2006; Henke et al., 2009). Thus, proneural factors are components of the intercellular regulation of Notch signaling, contributing to NPC maintenance. Proneural factors and Notch ligands are expressed in NPCs, as well as in intermediate progenitors and immature neurons, all of which send Notch signaling inputs to neighboring NPCs in the germinal zone (Nelson et al., 2013).

There is also evidence that Ascl1 directly promotes proliferation of NPCs, in addition to cell-cycle exit and differentiation, highlighting the multiple functions of proneural factors (Castro et al., 2011). Chromatin immunoprecipitation (ChIP)-chip analysis revealed that Ascl1 directly regulates the genes involved in cell-cycle exit, neurotransmitter biosynthesis, and neurite outgrowth, as expected. However, unexpectedly, this analysis also showed that Ascl1 directly regulates the genes involved in cell-cycle progression, including those essential for the G1/S transition and entry into mitosis. Furthermore, when Ascl1 is acutely inactivated, NPCs in the VZ and the SVZ exit the cell cycle prematurely (Castro et al., 2011). These results indicate that Ascl1 promotes the expansion of NPCs as well as their subsequent cell-cycle exit and neuronal differentiation. This contradictory dual activity seems to have been evolutionally conserved, because the *Drosophila* Ascl1 ortholog Asense promotes self-renewal of neuroblasts but inhibits proliferation of neuroblast daughter cells (Wallace et al., 2000; Southall and Brand, 2009).

Dynamic Regulation of Proneural bHLH Factors

The expression of proneural factors, such as Ascl1 and Neurog2, seems to be dynamically controlled at both the mRNA and protein levels. In NPCs, the RNase III Drosha destabilizes *Neurog2* mRNA in a microRNA-independent manner by recognizing the hairpin structures of 3′ UTR of *Neurog2* mRNA. Therefore,

inactivation of Drosha promotes neuronal differentiation by stabilization of *Neurog2* mRNA (Knuckles et al., 2012), suggesting that a reduction in Drosha activity regulates the onset of neuronal differentiation. In addition, Neurog1/2 proteins have short half-lives, less than 30 min, which, along with their proneural activity, are dynamically controlled in NPCs and intermediate progenitors (Ali et al., 2011; Hindley et al., 2012). In the mouse Neurog2 protein, nine serine-proline sites are subjected to phosphorylation by Cyclin-dependent kinases (CDKs). When the overall phosphorylation state is increased, the protein stability, DNA binding to the E-box, and neuronal differentiation potency are diminished, indicating that cell-cycle machinery negatively regulates neurogenesis through the phosphorylation of Neurog2 protein. Indeed, an unphosphorylated form of Neurog2 can more efficiently activate expression of the neuronal differentiation gene *Neurod1* than can the phosphorylated form (Figure 3B, bottom). However, this transcriptional activity is promoter specific, and both phosphorylated and unphosphorylated forms can similarly activate expression of the Notch ligand Dll1 (Figure 3B, top). A recent study showed that glycogen synthase kinase 3 (GSK3) also regulates Neurog2 proneural activity in the developing neocortex (Li et al., 2012). Threonine-proline phosphorylation by GSK3 inhibits the transcriptional activity of Neurog2 by preventing the dimer formation. Interestingly, early stage cortical NPCs have low levels of GSK3 activity, whereas at later embryonic periods GSK3 is progressively activated in cortical NPCs. Indeed, the proneural activity of Neurog2 is stronger in early stage cortical progenitors than in later stages, oppositely correlating with GSK3 activity (Li et al., 2012).

Ascl1 protein also seems to be destabilized in NPCs. It was reported that NICD induces ubiquitin-proteasome-dependent rapid degradation of Ascl1 protein with a half-life of just 14 min in human cells (Sriuranpong et al., 2002). However, Ascl1 is stabilized when NICD is absent (half-life is 50 min) (Sriuranpong et al., 2002), suggesting that this stabilization leads to accumulation of Ascl1 protein, thereby promoting neuronal differentiation. Furthermore, posttranslational modification is important for Ascl1 protein stability. Nonphosphorylated Ascl1 protein is very unstable, whereas CK2-mediated phosphorylation of S152 stabilizes Ascl1 protein (Viñals et al., 2004).

Neurog1 and Neurog2 expression is restricted to the dorsal telencephalon, which gives rise to pyramidal cells (glutamatergic neurons), whereas Ascl1 is predominantly expressed in the ventral telencephalon, which gives rise to cortical interneurons (GABAergic neurons), suggesting that proneural factors also contribute to specification of neuronal subtype identities. Indeed, in the dorsal telencephalon, loss- and gain-of-function studies have revealed that Neurog1 and Neurog2 are necessary and sufficient to specify a glutamatergic neuronal identity (Fode et al., 2000; Parras et al., 2002; Schuurmans et al., 2004; Mattar et al., 2004, 2008; Britz et al., 2006; Kovach et al., 2013). In the ventral telencephalon, similar studies have revealed that Ascl1 is necessary and sufficient to specify a GABAergic interneuron identity (Casarosa et al., 1999; Horton et al., 1999; Berninger et al., 2007; Poitras et al., 2007). Below, we summarize how proneural bHLH factors contribute to the specification of distinct neuronal cell fates.

Glutamatergic Neuronal Specification by Neurogenins

Neurog1 and Neurog2 are expressed in the developing dorsal telencephalon, which includes the anlagen of the neocortex and hippocampus. Essential roles of Neurog1/2 in neocortical development were revealed by loss-of-function studies (Schuurmans and Guillemot, 2002). The formation of cortical projection neurons is severely impaired in *Neurog2* and *Neurog1/2* double-mutant mice. Furthermore, the number of Cajal-Retzius neurons, a layer I cortical population, is diminished in *Neurog2* mutant mice (Imayoshi et al., 2008; Dixit et al., 2014). Defects of fate specification of layer V and VI early-born neurons to glutamatergic neurons are most evident in the absence of *Neurog2* (Schuurmans et al., 2004). The production of hippocampal projection neurons and dentate granule cells is also affected in *Neurog2* mutant mice (Galichet et al., 2008). Conversely, overexpression studies provided evidence for the sufficiency of Neurog1/2 in the fate specification of these glutamatergic neurons (Mattar et al., 2004, 2008; Kovach et al., 2013). Thus, Neurog1/2 are necessary and sufficient to specify glutamatergic neurons throughout telencephalic development. Interestingly, Neurog1/2 repress the differentiation program of GABAergic neurons. In the dorsal telencephalon of *Neurog1/2* double- and even *Neurog2* single-mutant mice, misexpression of Ascl1 and many GABAergic neuronal markers, such as Dlx, Gad1/2, and VGAT, occurs (Schuurmans et al., 2004). Furthermore, in the knockin mice, in which the coding region of the Neurog2 locus was swapped for the Ascl1 sequence, dorsal cortical progenitors are misdirected toward the GABAergic neuronal fate (Fode et al., 2000).

In the dorsal telencephalon, RG cells express the homeodomain transcription factor Pax6, whereas intermediate progenitors express the T-box transcription factor Tbr2. Most cortical pyramidal neurons are generated via Tbr2-positive intermediate progenitors, and these cells typically go through one round of cell division (Götz and Huttner, 2005). This division is believed to increase the neuronal population of the neocortex. It was shown that Neurog2 directly regulates expression of Tbr2 and that Neurog2 is necessary and sufficient to promote the transition of RG cells to intermediate progenitors (Ochiai et al., 2009). Neuronal differentiation is tightly coupled with cell-cycle exit, and it is likely that increased expression of Neurog1/2 leads to gradual accumulation of cell-cycle inhibitors, such as p21 (Cip1), p27 (Kip1), p57 (Kip2), and BM88 (Cend1) (Politis et al., 2007; Lange et al., 2009). Interestingly, p27 also promotes neurogenesis by stabilizing Neurog2 protein through direct interaction (Nguyen et al., 2006).

Neuronal migration is critical for establishing neocortical cell layers, and migration defects can cause neurological and psychiatric diseases. Neurog2 is important for the radial migration of cortical pyramidal neurons by regulating the formation of neuronal polarity and development of a leading process. It was found that RhoA inhibits neuronal migration by tight regulation of F-actin polymerization and microtubule assembly. Neurog2 enhances neuronal migration, independently of its proneural activity, but by activating Rho-GAP expression via C-terminal tyrosine (Y241) phosphorylation that leads to inhibition of RhoA activity (Hand et al., 2005). Furthermore, Neurog2 promotes neuronal migration by upregulating the expression of Rnd2, an

inhibitor of RhoA signaling, independently of Y241 phosphorylation (Heng et al., 2008). Thus, Neurog2 can promote neuronal migration by inhibiting RhoA activity via two independent mechanisms: Y241 phosphorylation and Rnd2 induction.

After glutamatergic neuronal specification by Neurog1/2, terminal differentiation of cortical and hippocampal neurons are regulated by other bHLH factors. Transient expression of Neurog1/2 in NPCs and early-phase intermediate progenitors induces the subsequent expression of bHLH gene cascades, such as those encoding NeuroD1/2/4/6, bHLHb5, Nscl1/2, and Math6 (so-called bHLH differentiation factors) (Mattar et al., 2004, 2008; Kovach et al., 2013). As in the case of proneural bHLH factors, overexpression of bHLH differentiation factors induces neuronal differentiation and cell-cycle exit. Like proneural bHLH factors, bHLH differentiation factors specifically bind to E-box consensus sequences and activate downstream gene expression. These bHLH differentiation factors redundantly regulate various maturation processes of glutamatergic neurons, such as migration, polarization, axon and dendrite maturation, survival, and synaptic formation. Thus, neuronal differentiation and maturation defects are evident only in compound mutants of these factors (Schwab et al., 2000).

GABAergic Neuronal Specification by Ascl1

Ascl1 is predominantly expressed by NPCs in the ventral telencephalon, such as in the medial, lateral, and caudal ganglionic eminences. It is known that GABAergic inhibitory neurons are born in the germinal zone of the ganglionic eminence and tangentially migrate to the neocortex. In *Ascl1* knockout mice, the production of GABAergic neurons is massively attenuated (Casarosa et al., 1999; Horton et al., 1999), whereas overexpression of Ascl1 induces ectopic production of GABAergic neurons from dorsal cortical NPCs (Berninger et al., 2007; Poitras et al., 2007). In *Ascl1* knockout mice, defects in interneuron production are the most pronounced in the medial ganglionic eminence, whereas striatal neuronal formation occurs normally in the lateral ganglionic eminence, suggesting that other factors can substitute for Ascl1 in the generation of striatal neurons. Indeed, striatal development is severely affected in *Ascl1;Gsx2* double knockout mice (Wang et al., 2009). Conversely, transient accumulation of Ascl1 in NPCs and early-phase progenitors induces the subsequent expression of GABAergic differentiation gene cascades, such as those encoding Dlx1/2/5/6 and Lhx6 (Yun et al., 2002; Petryniak et al., 2007; Miyoshi et al., 2010; Bartolini et al., 2013). Overexpression of Neurog2 at high levels by in utero electroporation can induce a cortical projection neuron phenotype in the ventral telencephalon (Mattar et al., 2008). However, loss of Ascl1 does not induce ectopic expression of Neurog2 or other glutamatergic neuronal markers in the ventral telencephalon, and replacement of Ascl1 by Neurog2 by a "knockin" method does not result in the respecification of GABAergic neurons into glutamatergic neurons in the ventral telencephalon (Parras et al., 2002). These results indicate that additional pathways act in parallel to those involving proneural proteins to specify GABAergic neuron identities in the ventral telencephalon, such as those involving the homeodomain transcription factors Gsx1/2.

Although Neurog1/2 and Ascl1 have opposing cell fate specification functions in the telencephalon (glutamatergic versus GABAergic), these factors are coexpressed or sequentially expressed in the same lineage, such as in Cajal-Retzius neurons or in postnatal-born hippocampal dentate granule cells (Kim et al., 2007; Dixit et al., 2011). In the dorsal telencephalon, Ascl1 is also coexpressed with Neurog1/2 by a subset of cortical VZ and SVZ progenitors (Britz et al., 2006), albeit at lower levels than in the ventral telencephalon, and regulates neuronal migration by activating another Rho GTPase, Rnd3 (Pacary et al., 2011).

Olig1/2 for Oligodendrocyte Formation

Olig1/2, two closely related bHLH transcription factors, have been identified as essential factors in the fate choice of oligodendrocytes (Figure 2) (Meijer et al., 2012) and also regulate their subsequent differentiation, maturation, and myelination. *Olig2* single and *Olig1/2* double knockout mice lack oligodendrocyte lineage cells (Lu et al., 2002; Zhou and Anderson, 2002; Takebayashi et al., 2002), whereas forced expression of Olig1/2 in NPCs is sufficient to induce the specification of oligodendrocyte precursor cells (OPCs) (Zhou et al., 2001; Lu et al., 2001). Olig2 functions as a repressor (Figure 3Ae) (Novitch et al., 2001; Zhou et al., 2001) but directly activates the expression of the oligodendrocyte-specific gene *Sox10* by forming a heterodimer with E proteins (Figure 3Ae) (Küspert et al., 2011). Initial studies analyzed the functions of Olig1/2 in the developing spinal cord and hindbrain, where motor neurons and oligodendrocytes are sequentially derived from common NPCs of the pMN domain (Goulding, 2009). It was shown that Olig1 and Olig2 have overlapping functions in oligodendrogenesis, although Olig2 plays a dominant role in patterning the pMN domain. In the absence of *Olig2*, the formation of the pMN domain is severely diminished, and both motoneurons and oligodendrocytes are mostly missing, indicating that Olig2 is essential for the specification of both motoneurons and oligodendrocytes in the developing spinal cord and hindbrain (Lu et al., 2002; Zhou and Anderson, 2002; Takebayashi et al., 2002). In the absence of *Olig1*, maturation of oligodendrocytes is affected (Lu et al., 2002; Xin et al., 2005), suggesting a role for Olig1 in the differentiation process leading to myelinating oligodendrocytes. This feature is more apparent in response to demyelinating injury: *Olig1* knockout mice display a limited ability to repair demyelinated lesions that were induced by gliotoxins (Arnett et al., 2004).

Functions of Olig2 in oligodendrocyte formation in the telencephalon have been intensively analyzed by conditional deletions at various time points during differentiation, from NPCs to OPCs and mature oligodendrocytes, unveiling stage-specific regulatory roles of Olig2 (Yue et al., 2006; Cai et al., 2007; Zhu et al., 2012; Mei et al., 2013). Similar to the results in the spinal cord and the hindbrain, Olig1 and Olig2 have nonoverlapping roles in proliferation and differentiation of NPCs and OPCs. Olig1 promotes the differentiation of committed OPCs, and this function is more apparent in a repairing process than in normal development (Arnett et al., 2004). By contrast, Olig2 functions at earlier stages of development, promoting the fate determination to OPCs and certain types of neurons, such as cholinergic neurons, in the telencephalon (Furusho et al., 2006).

The differentiating functions of Olig2 are regulated by post-transcriptional modifications, especially by phosphorylation (Setoguchi and Kondo, 2004; Meijer et al., 2012). For example,

serine phosphorylation at the ST box of mouse Olig2 protein by CK2 kinase activates the activity for generating OPCs (Huillard et al., 2010). By contrast, serine (S147) phosphorylation in its bHLH domain by PKA is important for motor neuron formation, and its dephosphorylation triggers the transition from motor neuron to oligodendrocyte formation in the pMN domain (Li et al., 2011). This phosphorylation in the bHLH domain promotes homodimer formation of Olig2 protein, whereas dephosphorylation promotes heterodimer formation with Neurog2 protein, whose proneural activity is essential for motoneuron specification. It is likely that Olig2-Neurog2 heterodimer formation sequesters Neurog2 in the pMN domain, thereby preventing motoneuron formation. Indeed, Olig2 mutant mice carrying a serine-to-alanine substitution at position 147, which promoted Olig2-Neurog2 heterodimer formation, impaired motoneuron specification (Li et al., 2011).

At early developmental stages, Olig2 opposes differentiation and promotes proliferation of self-renewing NPCs. This proliferative function of Olig2 is critically regulated by developmentally controlled phosphorylation of a triple serine motif at the N-terminal region. Mouse Olig2 protein is phosphorylated at these residues in NPCs during the early stage of embryos, whereas in the postnatal white matter, the triple serine motif is nonphosphorylated (Sun et al., 2011). When phosphorylated at these positions, the proliferative function of Olig2 becomes dominant over its differentiation functions. Interestingly, this triple serine motif of Olig2 is highly phosphorylated in malignant gliomas. These results indicate that Olig2 has contradictory functions, NPC proliferation and oligodendrocyte formation, depending on the phosphorylated status.

In addition to Olig1/2, it has been reported that Ascl1 also specifies an OPC cell fate in telencephalic NPCs at later developmental stages (Parras et al., 2004, 2007; Nakatani et al., 2013) and can force an oligodendrocyte fate when overexpressed in NPCs of the adult dentate gyrus (Jessberger et al., 2008).

Astrocyte Fate Determination

Currently, a single master bHLH factor for astrocyte fate determination has not been identified, unlike proneural factors in neurons and Olig factors in oligodendrocytes (Ross et al., 2003; Namihira and Nakashima, 2013). Many studies reported the importance of Notch signaling and downstream Hes factors as well as Id factors in astrocyte formation, but these factors are not sufficient for specifying embryonic NPCs into astrocytes (Figure 3Af) (Cai et al., 2000; Ohtsuka et al., 2001; Tanigaki et al., 2001; Wu et al., 2003; Namihira et al., 2009). For example, overexpression of Hes or Id factors in NPCs increased the proportion of astrocytes when analyzed long after the manipulation, or promoted the formation of astrocytes in late-phase or adult NPCs (Cai et al., 2000; Ohtsuka et al., 2001; Tanigaki et al., 2001). However, Hes and Id fail to prematurely induce astrocyte formation, when these factors are overexpressed at early stages in NPCs in the developing telencephalon (Cai et al., 2000; Ohtsuka et al., 2001). Therefore, the increased activities of Notch-Hes and Id factors are not sufficient for astrocyte fate determination and may permissively instruct astrocytic fate by inhibition of the other fates, neurons, and oligodendrocytes. Accumulating evidence indicates that extrinsic cues, such as cytokine signaling through the JAK/STAT3 pathway and BMP signaling, and epigenetic modification of astrocytic genes are critically involved in astrocyte fate determination and differentiation (Namihira and Nakashima, 2013).

The gp130-Janus kinase (JAK) is activated by leukemia inhibitory factor (LIF) or cardiotrophin-1 (CT-1) and phosphorylates the downstream effector STAT3, thereby activating it. Phosphorylated STAT3 can then directly bind to the promoter sequences of many astrocyte genes, such as *Gfap*, and upregulate their expression (Figure 3Af). Treatment of cultured NPCs with another group of cytokines, BMPs, can synergistically promote astrocyte formation via the JAK-STAT pathway by activating downstream Smad transcriptional factors (Nakashima et al., 1999). Activated Smads can form a complex with STAT3, which is mediated by the transcriptional coactivator p300/CBP, and participate in the induction of astrocytic gene expression (Figure 3Af). Interestingly, this process is inhibited by the proneural factor Neurog1, which sequesters the Smads-p300/CBP complex away from STAT3 (Sun et al., 2001). Therefore, competition for limiting cofactors by fate determination factors may be involved in mutually exclusive cell fate choices. Indeed, astrocyte formation is ectopically induced in compound proneural factor mutant mice (i.e., *Neurog2;Ascl1* and *Ascl1;Math3* double knockout mice) (Tomita et al., 2000; Nieto et al., 2001). Thus, proneural factors actively inhibit astrocyte formation, thereby reinforcing the neuronal fate determination. Furthermore, the oligodendrocyte determination factor Olig2 is a negative regulator of astrocytic genes and inhibits astrocyte formation in NPCs and glial-restricted progenitors (Cai et al., 2007).

Astrocyte formation is strongly inhibited at early developmental stages; combinatorial treatment of cultured NPCs with LIF and BMP very efficiently induces astrocyte formation at late stages but fails to do so at early stages (Nakashima et al., 1999; Takizawa et al., 2001). This stage-dependent regulation is achieved by epigenetic modifications, such as DNA and histone methylation. Because the promoter sequences of astrocytic genes, including *Gfap* and *S100β*, are highly methylated in early-stage NPCs, STAT binding to these promoters is hindered (Takizawa et al., 2001). As the development proceeds, DNA sequences of astrocytic genes become demethylated and NPCs then become responsive to astrocyte-inducing cytokines, indicating that the timing of astrocyte formation during brain development is critically regulated by DNA methylation. Various mechanisms for regulating DNA methylation of astrocytic genes have been revealed. For example, nuclear factor 1A/B (NFIA/B), in cooperation with Notch signaling, plays a critical role in the demethylation and initiation of astrocytic gene expression (Deneen et al., 2006; Namihira et al., 2009). Hypoxia-inducible factor (HIF)-1α and orphan receptor Coup-TFI/II also contribute to the demethylation of astrocytic genes during brain development (Naka et al., 2008; Mutoh et al., 2012). In addition to DNA methylation, histone methylation is also involved in astrocyte formation. The H3K9 methyltransferase ESET and high mobility group A (HMGA) proteins are known to repress accelerated astrocyte production (Tan et al., 2012a; Kishi et al., 2012), whereas Polycomb promotes a neurogenic to astrogenic fate transition (Hirabayashi et al., 2009). In summary, the negative bHLH factors Hes and Id promote astrocyte formation by inhibiting neuronal

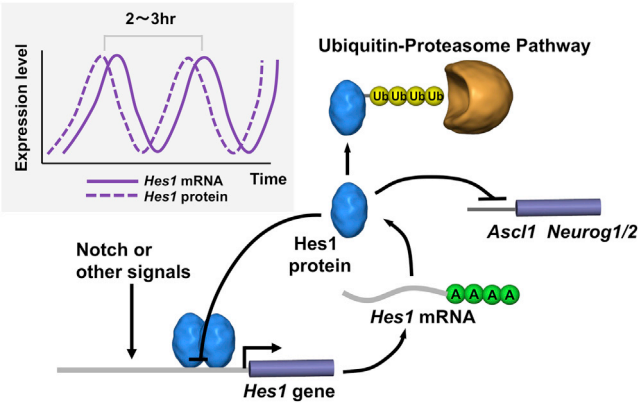

Figure 4. Hes1 Oscillation by Negative Feedback

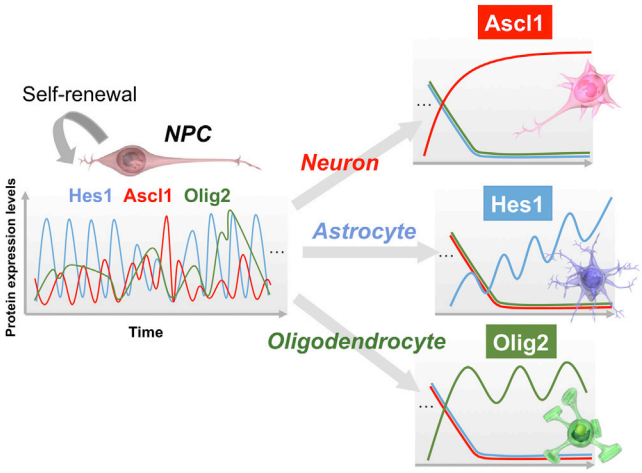

Figure 5. Expression Dynamics of bHLH Factors in Multipotency and Cell Fate Choice

and oligodendrocyte lineage choice with the help of astrocyte-inducing cytokines and epigenetic modifiers.

Expression Dynamics of bHLH Factors in Multipotency and Cell Fate Choice

Oscillatory Expression in Multipotent NPCs

As discussed above, it has been shown that cell fate determination factors such as Ascl1, Hes1, and Olig2 have contradictory functions: promoting NPC proliferation versus cell differentiation (Ascl1 for neurons, Hes1 for astrocytes, and Olig2 for oligodendrocytes). However, the detailed mechanisms by which they display such contradictory functions still remain to be analyzed.

It was previously shown that Hes1 expression oscillates in many cell types (Figure 4) (Hirata et al., 2002; Masamizu et al., 2006; Shimojo et al., 2008; Kobayashi et al., 2009). Activation of the *Hes1* promoter generates *Hes1* mRNA and then Hes1 protein, which can repress its own expression by directly binding to the *Hes1* promoter. Due to this negative feedback, *Hes1* mRNA disappears rapidly, because it is extremely unstable. Hes1 protein is also rapidly degraded by the ubiquitin-proteasome system, allowing the next round of activation of the *Hes1* promoter. In this way, Hes1 expression oscillates autonomously with a period of 2 to several hours (Figure 4). Time-lapse imaging analyses using the *Hes1* promoter-driven destabilized luciferase reporter, which monitors *Hes1* mRNA production, revealed that *Hes1* mRNA expression oscillates with a period of about 2 to 3 hr in NPCs (Shimojo et al., 2008). However, because it is known that transcription and translation can be dissociated in stem cells (Lu et al., 2009), live imaging with new transgenic reporter mice was employed to monitor protein expression. These reporter mice carried a bacterial artificial chromosome (BAC) clone in which luciferase or fluorescent cDNA was inserted into the 5′ region of each factor gene so that a fusion protein was expressed. In these reporter mice, the luciferase or fluorescent activity precisely monitors the endogenous protein expression (Imayoshi et al., 2013). Time-lapse imaging analyses clearly showed that Hes1 and Ascl1 protein expression oscillate with a period of about 2 to 3 hr, while Olig2 protein expression oscillates with a period of about 5 to 8 hr in NPCs (Figure 5). Inactivation of Hes1 does not affect Olig2 oscillation but abolishes Ascl1 oscillation. Thus, it is likely that Hes1 oscillation periodically represses Ascl1 expression, thereby driving Ascl1 oscillation, but that Olig2 expression oscillates independently of Hes1 and Ascl1 oscillation (Imayoshi et al., 2013). Together, these results indicate that three types of cell fate determination factors are expressed in an oscillatory manner by multipotent NPCs (Figure 5).

Sustained Expression during Cell Fate Choice

Time-lapse imaging analyses of individual cells showed that during neuronal fate choice Ascl1 expression occurs in a sustained manner after cell division (Figure 5) and that 6 to 8 hr later expression of the early neuronal marker Doublecortin (DCX) starts. Ascl1 expression continues to be upregulated in many differentiating cells but not in others; more than 20% of differentiating immature neurons soon downregulate Ascl1 expression after DCX is expressed, suggesting that the minimal requirement for neuronal fate determination is accumulation of Ascl1 over 6 to 8 hr during G1 phase (Imayoshi et al., 2013). What causes the transition from oscillatory to accumulative Ascl1 expression in NPCs? During the neuronal fate choice process, the levels of NICD, an active form of Notch signaling, fluctuate in NPCs, which results in unstable Hes1 oscillation and even in disappearance of Hes1 expression, leading to sustained upregulation of Ascl1. When stable levels of NICD expression are induced in NPCs, Hes1 oscillation continues in a stable manner, and Ascl1 expression is never upregulated. These results suggest that fluctuation in NICD levels triggers the transition from oscillatory to sustained Ascl1 expression (Imayoshi et al., 2013).

NPCs frequently undergo asymmetric cell division, in which one daughter cell remains undifferentiated while the other differentiates into a neuron. Before this cell division, Hes1 expression is downregulated, and concomitantly Ascl1 expression is upregulated in a sustained manner. Ascl1 seems to be equally distributed into both daughter cells, and the daughter NPC resumes Hes1 and Ascl1 oscillations, whereas the daughter neuron maintains repressed Hes1 expression and accumulates Ascl1. Thus, transient upregulation of Ascl1 before cell division is the first sign for a bias toward asymmetric cell division with neuronal differentiation (Imayoshi et al., 2013). However, it is not a decisive sign, as many NPCs produce two daughter NPCs even when Ascl1 is

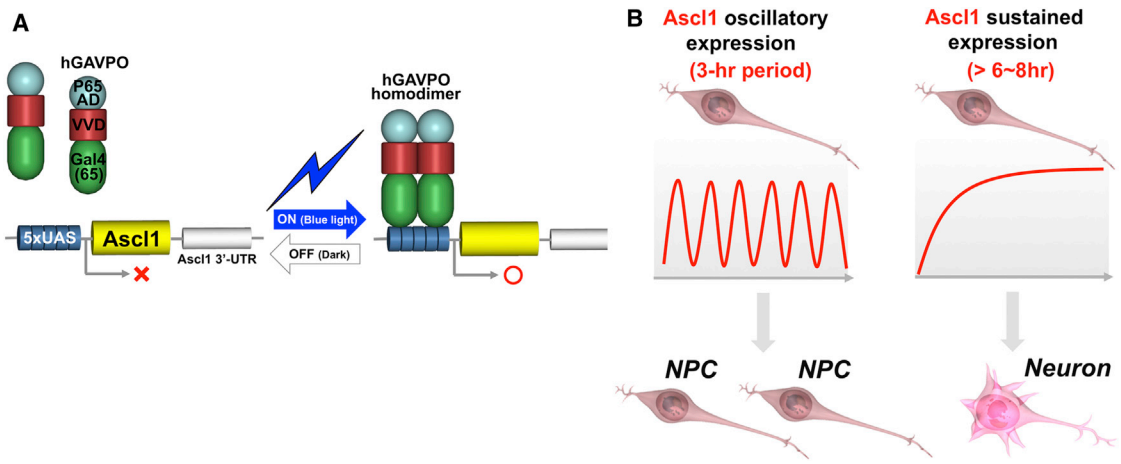

Figure 6. Optogenetic Approach to Control Expression Dynamics
(A) hGAVPO activates gene expression by blue light illumination.
(B) The hGAVPO system shows that oscillatory expression of Ascl1 activates the proliferation of NPCs, whereas sustained expression of Ascl1 promotes neuronal differentiation.

transiently upregulated before cell division. These results indicate that accumulation of Ascl1 during G1 phase is the only decisive sign for neuronal fate determination.

During astrocyte and oligodendrocyte differentiation, the expressions of Hes1 and Olig2, respectively, are upregulated, although they are still oscillatory (Figure 5). However, even during trough phases, both Hes1 and Olig2 levels are higher than they are in NPCs, indicating that Hes1 and Olig2 expressions continue in a sustained manner during astrocyte and oligodendrocyte differentiation (Imayoshi et al., 2013). When Hes1 or Olig2 becomes dominant, the expression of the other two factors is downregulated. These results indicate that Ascl1, Hes1, and Olig2 are expressed in an oscillatory manner in multipotent NPCs and that one of them becomes dominant during cell fate choice. Thus, the multipotent state correlates with oscillatory expression of several fate determination factors, whereas the differentiated state correlates with sustained expression of a selected single factor (Figure 5).

Optogenetic Approach to Control Expression Dynamics

To address whether the correlation between expression dynamics (oscillatory versus sustained) and outcomes (proliferation versus differentiation) has a causative relationship, an optogenetic approach has been employed to control the Ascl1 expression patterns. GAVPO is a light-activatable, hybrid protein consisting of the light-inducible dimerizing protein Vivid (VVD), a Gal4 DNA-binding domain, and a p65 transcriptional activation domain (Figure 6A) (Wang et al., 2012). A dimer form, but not a monomer form, of the Gal4 DNA-binding domain can interact with UAS sequences. Blue light illumination activates VVD, forming a dimer, and a dimer form of the Gal4 DNA-binding domain binds to the UAS sequences. Then, the p65 transcriptional activation domain upregulates the gene expression under the control of the UAS sequences (Figure 6A). Optimizing the codon usage of GAVPO (hGAVPO) and destabilizing the target mRNA enable the control of dynamic gene expression (e.g., oscillatory or sustained expression) by changing the blue light illumination patterns. The significance of gene expression dynamics was examined by introducing this Ascl1-inducible system into *Ascl1* null NPCs. Light-induced sustained Ascl1 expression enhances neuronal differentiation, whereas light-induced oscillatory Ascl1 expression with 3 hr periodicity activates proliferation of NPCs (Figure 6B). When this system is introduced into NPCs in the dorsal telencephalon, sustained expression of Ascl1 increases the number of differentiating neurons that migrate out of the ventricular zone, whereas oscillatory expression of Ascl1 maintains dividing NPCs in the ventricular zone. Thus, distinct (oscillatory versus sustained) expression dynamics of Ascl1 are important for the choice between proliferation and differentiation (Imayoshi et al., 2013). Together, these data suggest that the relationship between the gene expression dynamics of Ascl1 and its functions is not just correlative but also causative and that manipulation of Ascl1 expression can impose a choice favoring NPC proliferation or neuronal differentiation according to whether the expression is oscillatory or sustained.

The 3 hr periodicity of Ascl1 oscillation is important for NPC proliferation, because a 6 hr period does not activate it (Imayoshi et al., 2013). Whether the period of oscillatory expression correlates with that of cell cycle remains to be determined. The expression of bHLH factors might oscillate more slowly or become steady in slowly cycling NPCs, and further analyses are required to understand the significance of oscillation periods and dynamics.

This light-inducible system can precisely change the duration of sustained Ascl1 expression to determine the minimal requirement for the neuronal fate choice. This analysis revealed that only 6 to 8 hr of sustained Ascl1 expression is needed to activate neuronal differentiation (Imayoshi et al., 2013). This agrees well with the above time-lapse imaging results showing that the early neuronal marker expression starts after 6 to 8 hr of sustained Ascl1 expression during the G1 phase. There may be a critical period within the G1 phase for neuronal fate determination, and therefore a period longer than 8 hr of sustained Ascl1 expression shows a higher chance of neuronal fate choice.

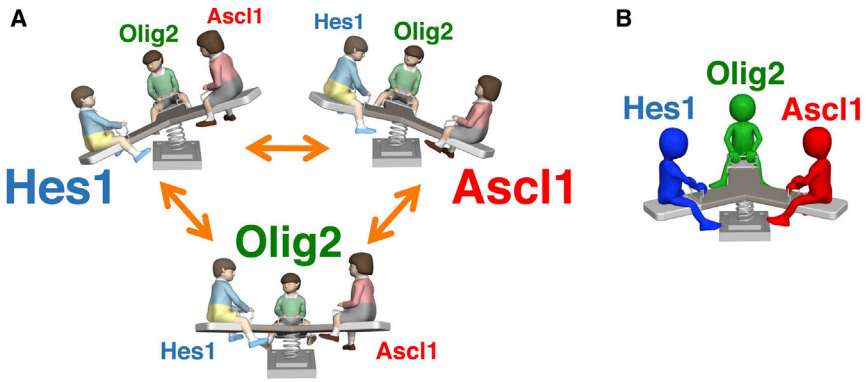

Figure 7. Three-Way Seesaw Models for Multipotency
Oscillatory expression (A) or balanced coexpression (B) of three cell fate determination factors may lead to the multipotent state.

Seesaw Model for Multipotency

The above results indicate that multipotency is a state of oscillatory expression of multiple fate determination factors. In multipotent NPCs, which can generate three different cell lineages, three types of cell fate determination factors, Ascl1, Hes1, and Olig2, oscillate in competition with one another, in a sort of three-way seesaw (Figure 7A). At the peak of Ascl1 oscillation, cells have a higher tendency to differentiate into neurons, but this tendency is not decisive; oscillating Ascl1 just maintains the potency to generate neurons. Producing new proteins but degrading them at once sounds like an enormous waste of energy. Why do stem cells waste such energy? An alternative and more thrifty way to maintain multipotency might be to repress the expression of cell fate determination factors but keep their gene promoters open, so that any cell fate determination factor can be expressed when it is necessary. Such a repressive state can be induced in NPCs at early stages by sustained Hes1 expression, which leads to repression of other cell fate determination factors. These cells do not differentiate into astrocytes but remain undifferentiated, because they are epigenetically resistant to astrocyte differentiation at early stages. However, as described above, cell fate determination factors such as Ascl1 can actually promote cell-cycle progression when they are expressed in an oscillatory manner. Such periodic production of cell fate determination factors seems to be a driving force for cell-cycle progression, although the exact mechanism remains to be determined. Indeed, sustained expression of Hes1 represses proneural gene expression and inhibits proliferation of NPCs (Baek et al., 2006). Furthermore, in the isthmus, roof plate, and floor plate of the developing nervous system, where Hes1 expression is sustained, cells are negative for proneural gene expression, and they are mostly quiescent (Baek et al., 2006). Thus, it is likely that oscillatory expression of multiple fate determination factors leads NPCs to actively divide, whereas sustained Hes1 expression with the concomitant sustained repression of other fate determination factors generates quiescent NPCs. It has been shown that sustained Hes1 expression is required for the quiescent state of fibroblasts and that, without Hes1, cells become senescent, an irreversible dormant state in which cells never enter the cell cycle (Sang et al., 2008).

Another way to maintain multipotency might be sustained and balanced expression of three types of cell fate determination factors, like the original seesaw model proposed for induced pluripotent stem cell (iPSC) formation (Figure 7B) (Shu et al., 2013). Surprisingly, in this model, co-overexpression of mesendodermal specifiers such as Gata6 and ectodermal specifiers such as GMNN can counteract each other for lineage specification, facilitate reprogramming, and synergistically induce pluripotency (Shu et al., 2013). The mesendodermal specifiers inhibit ectodermal specification, and the ectodermal specifiers inhibit mesendodermal specification. When the expressions of these two specifiers are balanced, cells cannot choose a cell fate and are likely to enter the pluripotent state. Thus, it is possible that balanced coexpression of Ascl1, Hes1, and Olig2 in NPCs does not enable them to choose a cell fate and may lead to multipotency, like the original seesaw model. However, such a balance is not robust and seems to be easily broken by intrinsic and extrinsic fluctuating stimulants (such as fluctuating NICD), making it difficult to maintain such a balance or the NPC state. Thus, we speculate that although oscillatory expression of multiple cell fate determination factors may spend enormous energy, it enables a metastable state to maintain proliferative and multipotent conditions with some resistance to fluctuating stimuli or noise.

Regulation and Function of Oscillating Factors

It is surprising that Ascl1 can have opposite functions depending on its expression dynamics, but how the oscillatory and sustained expressions of Ascl1 differentially regulate downstream gene expression is unknown. It was reported that, depending on the phosphorylation status, the proneural factor Neurog2 exhibits different transcriptional activities in NPCs and differentiating neurons (Figure 3B) (Ali et al., 2011; Hindley et al., 2012). Ascl1 may also be differentially phosphorylated in NPCs and neurons, thereby controlling different gene expression, although the posttranslational regulation of Ascl1 functions remains to be analyzed. It was reported that cell-cycle progression genes controlled by Ascl1 have RBPjκ-binding sites as well as Ascl1-binding sites (Castro et al., 2011). Thus, Ascl1 and Notch signaling may cooperatively activate these genes in NPCs, although further studies are required to understand the detailed mechanism.

The expression dynamics of Ascl1 target genes is largely unknown. Time-lapse imaging analyses showed that *Dll1* mRNA expression, which is activated by Ascl1 and repressed by Hes1, oscillates in NPCs but is sustained in neurons, although it remains to be determined whether Dll1 protein expression also oscillates in NPCs (Shimojo et al., 2008). Sustained expression of Hes1 in subsets of NPCs constitutively represses *Dll1* expression, leading to inactivation of Notch signaling in neighboring NPCs. This results in premature neuronal differentiation in the VZ, suggesting that oscillatory expression of Hes1 and

Dll1 is required for proper maintenance of NPCs in the developing nervous system (Shimojo et al., 2008). Other downstream factors may be expressed in different manners. If they are stable, they cannot oscillate, and Hes1 and Ascl1 oscillations may lead to accumulation of downstream factors in a stepwise manner in NPCs. When the expression levels of such downstream factors reach a certain value, new events might occur. In this case, information about the number of pulses of Hes1 and Ascl1 oscillations can possibly be converted into the timing of the next event. Such a model was proposed for microRNA-9 (miR-9), which regulates the stability of *Hes1* mRNA (Bonev et al., 2012; Tan et al., 2012b). Overexpression of miR-9 represses Hes1 expression by destabilizing *Hes1* mRNA. Because Hes1 represses miR-9 precursor expression, Hes1 oscillation drives the oscillatory expression of the miR-9 precursor. However, mature miR-9 is very stable, and therefore the oscillatory expression of the miR-9 precursor leads to accumulation of mature miR-9 over time. It has been proposed that this accumulation of miR-9 terminates Hes1 expression and initiates neuronal differentiation, suggesting that Hes1 oscillation functions as a timer for the switch of neuronal differentiation (Bonev et al., 2012). Further analyses are required to determine whether Hes1 and Ascl1 oscillations contribute to such a cellular clock mechanism.

Direct Reprogramming of Fibroblasts to Neurons by Proneural bHLH Factors

Direct reprogramming of the lineage of human fibroblasts into neurons using defined combinations of transcription factors is a promising approach for human disease modeling and regenerative medicine. It was thought that the lineage identity of differentiated cells is very stable and that the conversion of particular cell types across lineage boundaries is difficult due to strict chromatin configurations, epigenetic DNA modifications, and reinforced transcription factor networks. However, it has been shown that nuclear transfer into oocytes and cell fusion can overcome these epigenetic barriers and induce cell-fate reprogramming to pluripotency (Gurdon, 2006). Reprogramming can also be induced artificially through the introduction of exogenous factors, usually transcription factors. For instance, overexpression of four defined factors, Oct4, Sox2, Klf4, and c-Myc, recapitulates somatic cell nuclear transfer or oocyte-based reprogramming to generate iPSCs (Takahashi and Yamanaka, 2006).

Direct reprogramming of adult somatic cells into alternative cell types has been shown for several lineages, including neurons (Vierbuchen et al., 2010). This approach allows the transformation of easily available somatic cell types (typically fibroblasts) directly into neurons without transition via a pluripotent intermediate. It has been reported that three neuronal transcriptional factors, Ascl1, Brn2, and Myt1l (BAM factors), are sufficient to convert mouse fibroblasts or hepatocytes into functional neurons, termed induced neuronal (iN) cells (Vierbuchen et al., 2010). As mentioned above, the proneural bHLH factor Ascl1 is a crucial regulator of neurogenesis during normal development. Ascl1 is also a central and essential component of direct reprogramming of mouse and human fibroblasts to iN cells (Wapinski et al., 2013). Notably, Ascl1 is sufficient to convert mouse fibroblasts into immature neurons. The addition of Brn2 and Myt1l improves the conversion efficacy and results in fully matured neuronal characteristics. These factors also successfully convert astrocytes into neurons in the mouse striatum in vivo (Torper et al., 2013). Furthermore, overexpression of the BAM factors converts human fibroblasts into functional neurons, albeit with lower efficacy (Pang et al., 2011), and additional introduction of NeuroD1 (Pang et al., 2011) or Zic1 (Qiang et al., 2011) can direct reprogramming of human fibroblasts to functional neurons with enhanced neuronal yield and purity. Although Ascl1 is a proneural bHLH factor regulating GABAergic neurogenesis, most of the iN cells induced by the BAM set of transcription factors show characteristics of glutamatergic neurons, judged by mRNA or marker protein expression profiles and electrophysiological properties (Vierbuchen et al., 2010). Interestingly, the combinatorial use of two proneural bHLH factors, Ascl1 and Neurog2, results in more efficacious reprogramming than the use of the BAM factors, but iN cells induced by Ascl1 and Neurog2 exhibit mixtures of glutamatergic and GABAergic neurons (Ladewig et al., 2012). Another combination of Ascl1 and the SRY-box factor Sox2 more predominantly converts pericytes into GABAergic neurons (Karow et al., 2012). Direct lineage reprogramming approaches have been also applied to other specific neuronal subtypes, such as dopaminergic neurons (Caiazzo et al., 2011; Pfisterer et al., 2011). In the in vivo brain, the same factor can induce different neuronal subtypes, depending on the environmental condition: Neurog2 can induce GABAergic neurons in the striatum and glutamatergic neurons in the neocortex (Grande et al., 2013). Despite these intensive studies, the generation of iN cells by current protocols is relatively inefficacious, and the heterogeneity of the induced cells and limited scalability to obtain postmitotic neurons need to be improved.

Many of the underlying mechanisms by which these reprogramming processes can be induced by just a small number of transcriptional factors, remain obscure. To improve the efficacy and purity of direct reprogramming to neurons, it is essential to fully characterize the mechanisms by which reprogramming factors, including Ascl1, Neurog2, or NeuroD1 bHLH factors, contribute to this process. Recent integrative genomic analysis of fibroblasts-to-iN cell reprogramming using the BAM factors revealed the ''on-target pioneer factor'' activity of Ascl1 (Wapinski et al., 2013). Ascl1 can access nucleosomal DNA and immediately bind to its authentic neurogenic target genes across the fibroblast genome. Ascl1 seems to be able to bind to its target genes and activate their expression irrespective of whether genomic sites are freely available or are nucleosome bound. This study also discovered the specific chromatin configurations that favor access of Ascl1 to its target sites.

In addition to direct reprogramming to neurons, direct conversion to oligodendrocyte precursor cells (OPCs) or NPCs have been reported (Najm et al., 2013; Yang et al., 2013). In OPC reprogramming studies, Olig2 and Sox10, both of which play essential roles in oligodendrocyte formation during normal development, are used as the core components of reprogramming factors. Many methods and protocols of direct reprogramming to NPCs have been also reported, and similar to other lineage reprogramming, transcription factors that are important for achieving and maintaining cell-type-specific identity during normal development function as the core component of reprogramming cocktails. Sox2 is a central and essential component of

direct reprogramming to induced NPCs, and the conversion efficacy is promoted by combinatorial induction of other factors, such as FoxG1, Brn2/4, Klf4, and c-Myc (Yang et al., 2011).

Most protocols for direct cellular reprogramming are currently based on cotransduction of multiple lentiviral vectors having constitutively active promoters. Therefore, reprogramming factors are usually overexpressed throughout all reprogramming processes, although conditional expression using the Tet system has also been tried. As mentioned above, the expression and activity of many transcription factors regulating NPCs are dynamically regulated, and these dynamic regulations are critical for self-renewal, multipotency, and fate choice of NPCs (Imayoshi et al., 2013). For example, the proneural bHLH factors Neurog2 and Ascl1 are transiently expressed in NPCs and immature neurons, but their expression is rapidly downregulated during neuronal maturation. Prolonged expression of these fate-determination factors is inhibitory to maturation and even toxic to differentiated cells. Indeed, one important problem to be overcome is that most reprogrammed neural cells, including iN cells, are phenotypically immature, and the proportion of fully matured cells is very low (Yang et al., 2011). These problems might be resolved by developing reprogramming protocols that manipulate the mode, order, and magnitude of expression, as well as the activity of transcription factors by recapitulating their dynamic regulation during normal development. Furthermore, oscillating expression of bHLH factors, including Hes, Ascl1, Neurog2, and Olig2, in NPCs is important for forming a metastable state that maintains their proliferative and multipotent potentials. Therefore, inducing oscillatory and transiently accumulative expression (e.g., by a light-inducible expression system) may greatly improve the low conversion efficacy of direct cellular reprogramming of neural cells.

Conclusions and Perspectives

It is now clear that bHLH factors have multiple functions that are controlled by posttranslational modifications and expression dynamics. Particularly, the proneural bHLH factor Ascl1 exhibits contradictory functions (NPC proliferation versus neuronal differentiation) when the expression is oscillatory or sustained. It is likely that Hes1 and Olig2 also have such contradictory functions depending on their expression dynamics. One important question here is how genes involved in cell-cycle progression are activated by bHLH factors like Ascl1 when the expression is oscillating but repressed by the same bHLH factors when the expression is sustained. The presence or absence of NICD may be one such mechanism because RBPjκ-binding sites are present in the promoters of genes involved in cell-cycle progression, but further studies are definitely required to understand the precise mechanisms for such differential gene regulations. For the purpose of efficacious reprogramming to generate mature neurons or oligodendrocytes, time-controlled oscillatory and sustained expression of bHLH factors may be important, and recent emerging optogenetic technologies will be advantageous to precisely recapitulate such expression patterns.

Another issue is that NPCs change their competency during neocortical development; lower-layer neurons are generated first, then upper-layer neurons, and lastly glial cells. It is known that the epigenetic status, such as their DNA methylation and histone modifications, changes over time in NPCs, leading to different competencies, but the mechanisms of such time-dependent changes are unknown. Oscillatory bHLH factors might lead to gradual upregulation or downregulation of the expression of their target genes, which might affect their epigenetic status. In this case, oscillatory bHLH factors may function as an internal clock in NPCs, but this possibility remains to be addressed.

Neurogenesis occurs continuously in the adult brain and plays an important role in higher brain functions such as learning and memory. Reduced neurogenesis in the adult brain results in brain dysfunctions such as memory defects and depression. Unlike embryonic NPCs, adult NPCs are slowly dividing or quiescent and only occasionally divide to give rise to new neurons. It was found that the same bHLH factors (i.e., Hes1 and Ascl1) are expressed by NPCs and differentiating neurons, respectively, in the adult brain. An emerging question is how Hes1 and Ascl1 regulate the maintenance of active embryonic NPCs and dormant adult NPCs. One possible mechanism might be their different expression dynamics in embryonic and adult NPCs. Hes1 and Ascl1 expression oscillates in embryonic NPCs but might be nonoscillatory in adult NPCs. Clearly, further studies are required to test this model, and if this is the case, it will be important to test the idea whether forced oscillatory expression of Hes1 and Ascl1 can transform dormant NPCs into active NPCs. Further understanding of developmental mechanisms will be helpful to develop new methods for the effective generation of neurons, which are applicable to regenerative medicine.

ACKNOWLEDGMENTS

We thank H. Takebayashi and G. Miyoshi for their critical reading of this manuscript. This work was supported by Core Research for Evolutional Science and Technology (R.K.), Grant-in-Aid for Scientific Research on Innovative Areas (MEXT 22123002) (R.K.), Scientific Research (A) (JSPS 24240049) (R.K.), and Young Scientists (A) (JSPS 24680035) (I.I.).

REFERENCES

Ali, F., Hindley, C., McDowell, G., Deibler, R., Jones, A., Kirschner, M., Guillemot, F., and Philpott, A. (2011). Cell cycle-regulated multi-site phosphorylation of Neurogenin 2 coordinates cell cycling with differentiation during neurogenesis. Development *138*, 4267–4277.

Androutsellis-Theotokis, A., Leker, R.R., Soldner, F., Hoeppner, D.J., Ravin, R., Poser, S.W., Rueger, M.A., Bae, S.K., Kittappa, R., and McKay, R.D. (2006). Notch signalling regulates stem cell numbers in vitro and in vivo. Nature *442*, 823–826.

Arnett, H.A., Fancy, S.P., Alberta, J.A., Zhao, C., Plant, S.R., Kaing, S., Raine, C.S., Rowitch, D.H., Franklin, R.J., and Stiles, C.D. (2004). bHLH transcription factor Olig1 is required to repair demyelinated lesions in the CNS. Science *306*, 2111–2115.

Baek, J.H., Hatakeyama, J., Sakamoto, S., Ohtsuka, T., and Kageyama, R. (2006). Persistent and high levels of Hes1 expression regulate boundary formation in the developing central nervous system. Development *133*, 2467–2476.

Bai, G., Sheng, N., Xie, Z., Bian, W., Yokota, Y., Benezra, R., Kageyama, R., Guillemot, F., and Jing, N. (2007). Id sustains Hes1 expression to inhibit precocious neurogenesis by releasing negative autoregulation of Hes1. Dev. Cell *13*, 283–297.

Bartolini, G., Ciceri, G., and Marín, O. (2013). Integration of GABAergic interneurons into cortical cell assemblies: lessons from embryos and adults. Neuron *79*, 849–864.

Berninger, B., Guillemot, F., and Götz, M. (2007). Directing neurotransmitter identity of neurones derived from expanded adult neural stem cells. Eur. J. Neurosci. *25*, 2581–2590.

Bertrand, N., Castro, D.S., and Guillemot, F. (2002). Proneural genes and the specification of neural cell types. Nat. Rev. Neurosci. *3*, 517–530.

Bonev, B., Stanley, P., and Papalopulu, N. (2012). MicroRNA-9 modulates Hes1 ultradian oscillations by forming a double-negative feedback loop. Cell Rep. *2*, 10–18.

Breunig, J.J., Haydar, T.F., and Rakic, P. (2011). Neural stem cells: historical perspective and future prospects. Neuron *70*, 614–625.

Britz, O., Mattar, P., Nguyen, L., Langevin, L.M., Zimmer, C., Alam, S., Guillemot, F., and Schuurmans, C. (2006). A role for proneural genes in the maturation of cortical progenitor cells. Cereb. Cortex *16* (*Suppl 1*), i138–i151.

Cai, L., Morrow, E.M., and Cepko, C.L. (2000). Misexpression of basic helix-loop-helix genes in the murine cerebral cortex affects cell fate choices and neuronal survival. Development *127*, 3021–3030.

Cai, J., Chen, Y., Cai, W.H., Hurlock, E.C., Wu, H., Kernie, S.G., Parada, L.F., and Lu, Q.R. (2007). A crucial role for Olig2 in white matter astrocyte development. Development *134*, 1887–1899.

Caiazzo, M., Dell'Anno, M.T., Dvoretskova, E., Lazarevic, D., Taverna, S., Leo, D., Sotnikova, T.D., Menegon, A., Roncaglia, P., Colciago, G., et al. (2011). Direct generation of functional dopaminergic neurons from mouse and human fibroblasts. Nature *476*, 224–227.

Casarosa, S., Fode, C., and Guillemot, F. (1999). Mash1 regulates neurogenesis in the ventral telencephalon. Development *126*, 525–534.

Castro, D.S., Skowronska-Krawczyk, D., Armant, O., Donaldson, I.J., Parras, C., Hunt, C., Critchley, J.A., Nguyen, L., Gossler, A., Göttgens, B., et al. (2006). Proneural bHLH and Brn proteins coregulate a neurogenic program through cooperative binding to a conserved DNA motif. Dev. Cell *11*, 831–844.

Castro, D.S., Martynoga, B., Parras, C., Ramesh, V., Pacary, E., Johnston, C., Drechsel, D., Lebel-Potter, M., Garcia, L.G., Hunt, C., et al. (2011). A novel function of the proneural factor Ascl1 in progenitor proliferation identified by genome-wide characterization of its targets. Genes Dev. *25*, 930–945.

Caviness, V.S., Jr., Takahashi, T., and Nowakowski, R.S. (1995). Numbers, time and neocortical neuronogenesis: a general developmental and evolutionary model. Trends Neurosci. *18*, 379–383.

D'Souza, B., Miyamoto, A., and Weinmaster, G. (2008). The many facets of Notch ligands. Oncogene *27*, 5148–5167.

Deneen, B., Ho, R., Lukaszewicz, A., Hochstim, C.J., Gronostajski, R.M., and Anderson, D.J. (2006). The transcription factor NFIA controls the onset of gliogenesis in the developing spinal cord. Neuron *52*, 953–968.

Dixit, R., Zimmer, C., Waclaw, R.R., Mattar, P., Shaker, T., Kovach, C., Logan, C., Campbell, K., Guillemot, F., and Schuurmans, C. (2011). Ascl1 participates in Cajal-Retzius cell development in the neocortex. Cereb. Cortex *21*, 2599–2611.

Dixit, R., Wilkinson, G., Cancino, G.I., Shaker, T., Adnani, L., Li, S., Dennis, D., Kurrasch, D., Chan, J.A., Olson, E.C., et al. (2014). Neurog1 and Neurog2 control two waves of neuronal differentiation in the piriform cortex. J. Neurosci. *34*, 539–553.

Fietz, S.A., Kelava, I., Vogt, J., Wilsch-Bräuninger, M., Stenzel, D., Fish, J.L., Corbeil, D., Riehn, A., Distler, W., Nitsch, R., and Huttner, W.B. (2010). OSVZ progenitors of human and ferret neocortex are epithelial-like and expand by integrin signaling. Nat. Neurosci. *13*, 690–699.

Fishell, G., and Kriegstein, A.R. (2003). Neurons from radial glia: the consequences of asymmetric inheritance. Curr. Opin. Neurobiol. *13*, 34–41.

Fode, C., Ma, Q., Casarosa, S., Ang, S.L., Anderson, D.J., and Guillemot, F. (2000). A role for neural determination genes in specifying the dorsoventral identity of telencephalic neurons. Genes Dev. *14*, 67–80.

Furusho, M., Ono, K., Takebayashi, H., Masahira, N., Kagawa, T., Ikeda, K., and Ikenaka, K. (2006). Involvement of the Olig2 transcription factor in cholinergic neuron development of the basal forebrain. Dev. Biol. *293*, 348–357.

Galichet, C., Guillemot, F., and Parras, C.M. (2008). Neurogenin 2 has an essential role in development of the dentate gyrus. Development *135*, 2031–2041.

Giagtzoglou, N., Alifragis, P., Koumbanakis, K.A., and Delidakis, C. (2003). Two modes of recruitment of E(spl) repressors onto target genes. Development *130*, 259–270.

Götz, M., and Huttner, W.B. (2005). The cell biology of neurogenesis. Nat. Rev. Mol. Cell Biol. *6*, 777–788.

Goulding, M. (2009). Circuits controlling vertebrate locomotion: moving in a new direction. Nat. Rev. Neurosci. *10*, 507–518.

Grande, A., Sumiyoshi, K., López-Juárez, A., Howard, J., Sakthivel, B., Aronow, B., Campbell, K., and Nakafuku, M. (2013). Environmental impact on direct neuronal reprogramming *in vivo* in the adult brain. Nat. Commun. *4*, 2373.

Gurdon, J.B. (2006). From nuclear transfer to nuclear reprogramming: the reversal of cell differentiation. Annu. Rev. Cell Dev. Biol. *22*, 1–22.

Hand, R., Bortone, D., Mattar, P., Nguyen, L., Heng, J.I., Guerrier, S., Boutt, E., Peters, E., Barnes, A.P., Parras, C., et al. (2005). Phosphorylation of Neurogenin2 specifies the migration properties and the dendritic morphology of pyramidal neurons in the neocortex. Neuron *48*, 45–62.

Hansen, D.V., Lui, J.H., Parker, P.R., and Kriegstein, A.R. (2010). Neurogenic radial glia in the outer subventricular zone of human neocortex. Nature *464*, 554–561.

Hatakeyama, J., Bessho, Y., Katoh, K., Ookawara, S., Fujioka, M., Guillemot, F., and Kageyama, R. (2004). Hes genes regulate size, shape and histogenesis of the nervous system by control of the timing of neural stem cell differentiation. Development *131*, 5539–5550.

Heng, J.I., Nguyen, L., Castro, D.S., Zimmer, C., Wildner, H., Armant, O., Skowronska-Krawczyk, D., Bedogni, F., Matter, J.M., Hevner, R., and Guillemot, F. (2008). Neurogenin 2 controls cortical neuron migration through regulation of Rnd2. Nature *455*, 114–118.

Henke, R.M., Meredith, D.M., Borromeo, M.D., Savage, T.K., and Johnson, J.E. (2009). Ascl1 and Neurog2 form novel complexes and regulate Delta-like3 (Dll3) expression in the neural tube. Dev. Biol. *328*, 529–540.

Hindley, C., Ali, F., McDowell, G., Cheng, K., Jones, A., Guillemot, F., and Philpott, A. (2012). Post-translational modification of Ngn2 differentially affects transcription of distinct targets to regulate the balance between progenitor maintenance and differentiation. Development *139*, 1718–1723.

Hirabayashi, Y., Suzki, N., Tsuboi, M., Endo, T.A., Toyoda, T., Shinga, J., Koseki, H., Vidal, M., and Gotoh, Y. (2009). Polycomb limits the neurogenic competence of neural precursor cells to promote astrogenic fate transition. Neuron *63*, 600–613.

Hirata, H., Yoshiura, S., Ohtsuka, T., Bessho, Y., Harada, T., Yoshikawa, K., and Kageyama, R. (2002). Oscillatory expression of the bHLH factor Hes1 regulated by a negative feedback loop. Science *298*, 840–843.

Hitoshi, S., Ishino, Y., Kumar, A., Jasmine, S., Tanaka, K.F., Kondo, T., Kato, S., Hosoya, T., Hotta, Y., and Ikenaka, K. (2011). Mammalian Gcm genes induce Hes5 expression by active DNA demethylation and induce neural stem cells. Nat. Neurosci. *14*, 957–964.

Horton, S., Meredith, A., Richardson, J.A., and Johnson, J.E. (1999). Correct coordination of neuronal differentiation events in ventral forebrain requires the bHLH factor MASH1. Mol. Cell. Neurosci. *14*, 355–369.

Huillard, E., Ziercher, L., Blond, O., Wong, M., Deloulme, J.C., Souchelnytskyi, S., Baudier, J., Cochet, C., and Buchou, T. (2010). Disruption of CK2beta in embryonic neural stem cells compromises proliferation and oligodendrogenesis in the mouse telencephalon. Mol. Cell. Biol. *30*, 2737–2749.

Imayoshi, I., Shimogori, T., Ohtsuka, T., and Kageyama, R. (2008). Hes genes and neurogenin regulate non-neural versus neural fate specification in the dorsal telencephalic midline. Development *135*, 2531–2541.

Imayoshi, I., Sakamoto, M., Yamaguchi, M., Mori, K., and Kageyama, R. (2010). Essential roles of Notch signaling in maintenance of neural stem cells in developing and adult brains. J. Neurosci. *30*, 3489–3498.

Imayoshi, I., Isomura, A., Harima, Y., Kawaguchi, K., Kori, H., Miyachi, H., Fujiwara, T., Ishidate, F., and Kageyama, R. (2013). Oscillatory control of factors determining multipotency and fate in mouse neural progenitors. Science *342*, 1203–1208.

Ishibashi, M., Moriyoshi, K., Sasai, Y., Shiota, K., Nakanishi, S., and Kageyama, R. (1994). Persistent expression of helix-loop-helix factor HES-1 prevents mammalian neural differentiation in the central nervous system. EMBO J. *13*, 1799–1805.

Jessberger, S., Toni, N., Clemenson, G.D., Jr., Ray, J., and Gage, F.H. (2008). Directed differentiation of hippocampal stem/progenitor cells in the adult brain. Nat. Neurosci. *11*, 888–893.

Karow, M., Sánchez, R., Schichor, C., Masserdotti, G., Ortega, F., Heinrich, C., Gascón, S., Khan, M.A., Lie, D.C., Dellavalle, A., et al. (2012). Reprogramming of pericyte-derived cells of the adult human brain into induced neuronal cells. Cell Stem Cell *11*, 471–476.

Kim, E.J., Leung, C.T., Reed, R.R., and Johnson, J.E. (2007). In vivo analysis of Ascl1 defined progenitors reveals distinct developmental dynamics during adult neurogenesis and gliogenesis. J. Neurosci. *27*, 12764–12774.

Kishi, Y., Fujii, Y., Hirabayashi, Y., and Gotoh, Y. (2012). HMGA regulates the global chromatin state and neurogenic potential in neocortical precursor cells. Nat. Neurosci. *15*, 1127–1133.

Knuckles, P., Vogt, M.A., Lugert, S., Milo, M., Chong, M.M., Hautbergue, G.M., Wilson, S.A., Littman, D.R., and Taylor, V. (2012). Drosha regulates neurogenesis by controlling neurogenin 2 expression independent of microRNAs. Nat. Neurosci. *15*, 962–969.

Kobayashi, T., Mizuno, H., Imayoshi, I., Furusawa, C., Shirahige, K., and Kageyama, R. (2009). The cyclic gene Hes1 contributes to diverse differentiation responses of embryonic stem cells. Genes Dev. *23*, 1870–1875.

Kopan, R., and Ilagan, M.X. (2009). The canonical Notch signaling pathway: unfolding the activation mechanism. Cell *137*, 216–233.

Kovach, C., Dixit, R., Li, S., Mattar, P., Wilkinson, G., Elsen, G.E., Kurrasch, D.M., Hevner, R.F., and Schuurmans, C. (2013). Neurog2 simultaneously activates and represses alternative gene expression programs in the developing neocortex. Cereb. Cortex *23*, 1884–1900.

Kriegstein, A., and Alvarez-Buylla, A. (2009). The glial nature of embryonic and adult neural stem cells. Annu. Rev. Neurosci. *32*, 149–184.

Küspert, M., Hammer, A., Bösl, M.R., and Wegner, M. (2011). Olig2 regulates Sox10 expression in oligodendrocyte precursors through an evolutionary conserved distal enhancer. Nucleic Acids Res. *39*, 1280–1293.

Ladewig, J., Mertens, J., Kesavan, J., Doerr, J., Poppe, D., Glaue, F., Herms, S., Wernet, P., Kögler, G., Müller, F.J., et al. (2012). Small molecules enable highly efficient neuronal conversion of human fibroblasts. Nat. Methods *9*, 575–578.

Lange, C., Huttner, W.B., and Calegari, F. (2009). Cdk4/cyclinD1 overexpression in neural stem cells shortens G1, delays neurogenesis, and promotes the generation and expansion of basal progenitors. Cell Stem Cell *5*, 320–331.

Li, H., de Faria, J.P., Andrew, P., Nitarska, J., and Richardson, W.D. (2011). Phosphorylation regulates OLIG2 cofactor choice and the motor neuron-oligodendrocyte fate switch. Neuron *69*, 918–929.

Li, S., Mattar, P., Zinyk, D., Singh, K., Chaturvedi, C.P., Kovach, C., Dixit, R., Kurrasch, D.M., Ma, Y.C., Chan, J.A., et al. (2012). GSK3 temporally regulates neurogenin 2 proneural activity in the neocortex. J. Neurosci. *32*, 7791–7805.

Lu, Q.R., Cai, L., Rowitch, D., Cepko, C.L., and Stiles, C.D. (2001). Ectopic expression of Olig1 promotes oligodendrocyte formation and reduces neuronal survival in developing mouse cortex. Nat. Neurosci. *4*, 973–974.

Lu, Q.R., Sun, T., Zhu, Z., Ma, N., Garcia, M., Stiles, C.D., and Rowitch, D.H. (2002). Common developmental requirement for Olig function indicates a motor neuron/oligodendrocyte connection. Cell *109*, 75–86.

Lu, R., Markowetz, F., Unwin, R.D., Leek, J.T., Airoldi, E.M., MacArthur, B.D., Lachmann, A., Rozov, R., Ma'ayan, A., Boyer, L.A., et al. (2009). Systems-level dynamic analyses of fate change in murine embryonic stem cells. Nature *462*, 358–362.

Lyden, D., Young, A.Z., Zagzag, D., Yan, W., Gerald, W., O'Reilly, R., Bader, B.L., Hynes, R.O., Zhuang, Y., Manova, K., and Benezra, R. (1999). Id1 and Id3 are required for neurogenesis, angiogenesis and vascularization of tumour xenografts. Nature *401*, 670–677.

Masamizu, Y., Ohtsuka, T., Takashima, Y., Nagahara, H., Takenaka, Y., Yoshikawa, K., Okamura, H., and Kageyama, R. (2006). Real-time imaging of the somite segmentation clock: revelation of unstable oscillators in the individual presomitic mesoderm cells. Proc. Natl. Acad. Sci. USA *103*, 1313–1318.

Mattar, P., Britz, O., Johannes, C., Nieto, M., Ma, L., Rebeyka, A., Klenin, N., Polleux, F., Guillemot, F., and Schuurmans, C. (2004). A screen for downstream effectors of Neurogenin2 in the embryonic neocortex. Dev. Biol. *273*, 373–389.

Mattar, P., Langevin, L.M., Markham, K., Klenin, N., Shivji, S., Zinyk, D., and Schuurmans, C. (2008). Basic helix-loop-helix transcription factors cooperate to specify a cortical projection neuron identity. Mol. Cell. Biol. *28*, 1456–1469.

McConnell, S.K. (1995). Constructing the cerebral cortex: neurogenesis and fate determination. Neuron *15*, 761–768.

Mei, F., Wang, H., Liu, S., Niu, J., Wang, L., He, Y., Etxeberria, A., Chan, J.R., and Xiao, L. (2013). Stage-specific deletion of Olig2 conveys opposing functions on differentiation and maturation of oligodendrocytes. J. Neurosci. *33*, 8454–8462.

Meijer, D.H., Kane, M.F., Mehta, S., Liu, H., Harrington, E., Taylor, C.M., Stiles, C.D., and Rowitch, D.H. (2012). Separated at birth? The functional and molecular divergence of OLIG1 and OLIG2. Nat. Rev. Neurosci. *13*, 819–831.

Miller, F.D., and Gauthier, A.S. (2007). Timing is everything: making neurons versus glia in the developing cortex. Neuron *54*, 357–369.

Mira, H., Andreu, Z., Suh, H., Lie, D.C., Jessberger, S., Consiglio, A., San Emeterio, J., Hortigüela, R., Marqués-Torrejón, M.A., Nakashima, K., et al. (2010). Signaling through BMPR-IA regulates quiescence and long-term activity of neural stem cells in the adult hippocampus. Cell Stem Cell *7*, 78–89.

Miyoshi, G., Hjerling-Leffler, J., Karayannis, T., Sousa, V.H., Butt, S.J., Battiste, J., Johnson, J.E., Machold, R.P., and Fishell, G. (2010). Genetic fate mapping reveals that the caudal ganglionic eminence produces a large and diverse population of superficial cortical interneurons. J. Neurosci. *30*, 1582–1594.

Mutoh, T., Sanosaka, T., Ito, K., and Nakashima, K. (2012). Oxygen levels epigenetically regulate fate switching of neural precursor cells via hypoxia-inducible factor 1alpha-notch signal interaction in the developing brain. Stem Cells *30*, 561–569.

Najm, F.J., Lager, A.M., Zaremba, A., Wyatt, K., Caprariello, A.V., Factor, D.C., Karl, R.T., Maeda, T., Miller, R.H., and Tesar, P.J. (2013). Transcription factor-mediated reprogramming of fibroblasts to expandable, myelinogenic oligodendrocyte progenitor cells. Nat. Biotechnol. *31*, 426–433.

Naka, H., Nakamura, S., Shimazaki, T., and Okano, H. (2008). Requirement for COUP-TFI and II in the temporal specification of neural stem cells in CNS development. Nat. Neurosci. *11*, 1014–1023.

Nakashima, K., Yanagisawa, M., Arakawa, H., Kimura, N., Hisatsune, T., Kawabata, M., Miyazono, K., and Taga, T. (1999). Synergistic signaling in fetal brain by STAT3-Smad1 complex bridged by p300. Science *284*, 479–482.

Nakashima, K., Takizawa, T., Ochiai, W., Yanagisawa, M., Hisatsune, T., Nakafuku, M., Miyazono, K., Kishimoto, T., Kageyama, R., and Taga, T. (2001). BMP2-mediated alteration in the developmental pathway of fetal mouse brain cells from neurogenesis to astrocytogenesis. Proc. Natl. Acad. Sci. USA *98*, 5868–5873.

Nakatani, H., Martin, E., Hassani, H., Clavairoly, A., Maire, C.L., Viadieu, A., Kerninon, C., Delmasure, A., Frah, M., Weber, M., et al. (2013). Ascl1/Mash1 promotes brain oligodendrogenesis during myelination and remyelination. J. Neurosci. *33*, 9752–9768.

Namihira, M., and Nakashima, K. (2013). Mechanisms of astrocytogenesis in the mammalian brain. Curr. Opin. Neurobiol. *23*, 921–927.

Namihira, M., Kohyama, J., Semi, K., Sanosaka, T., Deneen, B., Taga, T., and Nakashima, K. (2009). Committed neuronal precursors confer astrocytic potential on residual neural precursor cells. Dev. Cell *16*, 245–255.

Nelson, B.R., Hodge, R.D., Bedogni, F., and Hevner, R.F. (2013). Dynamic interactions between intermediate neurogenic progenitors and radial glia in embryonic mouse neocortex: potential role in Dll1-Notch signaling. J. Neurosci. *33*, 9122–9139.

Nguyen, L., Besson, A., Heng, J.I., Schuurmans, C., Teboul, L., Parras, C., Philpott, A., Roberts, J.M., and Guillemot, F. (2006). p27kip1 independently promotes neuronal differentiation and migration in the cerebral cortex. Genes Dev. *20*, 1511–1524.

Nieto, M., Schuurmans, C., Britz, O., and Guillemot, F. (2001). Neural bHLH genes control the neuronal versus glial fate decision in cortical progenitors. Neuron *29*, 401–413.

Novitch, B.G., Chen, A.I., and Jessell, T.M. (2001). Coordinate regulation of motor neuron subtype identity and pan-neuronal properties by the bHLH repressor Olig2. Neuron *31*, 773–789.

Ochiai, W., Nakatani, S., Takahara, T., Kainuma, M., Masaoka, M., Minobe, S., Namihira, M., Nakashima, K., Sakakibara, A., Ogawa, M., and Miyata, T. (2009). Periventricular notch activation and asymmetric Ngn2 and Tbr2 expression in pair-generated neocortical daughter cells. Mol. Cell. Neurosci. *40*, 225–233.

Ohtsuka, T., Ishibashi, M., Gradwohl, G., Nakanishi, S., Guillemot, F., and Kageyama, R. (1999). Hes1 and Hes5 as notch effectors in mammalian neuronal differentiation. EMBO J. *18*, 2196–2207.

Ohtsuka, T., Sakamoto, M., Guillemot, F., and Kageyama, R. (2001). Roles of the basic helix-loop-helix genes Hes1 and Hes5 in expansion of neural stem cells of the developing brain. J. Biol. Chem. *276*, 30467–30474.

Okano, H., and Temple, S. (2009). Cell types to order: temporal specification of CNS stem cells. Curr. Opin. Neurobiol. *19*, 112–119.

Pacary, E., Heng, J., Azzarelli, R., Riou, P., Castro, D., Lebel-Potter, M., Parras, C., Bell, D.M., Ridley, A.J., Parsons, M., and Guillemot, F. (2011). Proneural transcription factors regulate different steps of cortical neuron migration through Rnd-mediated inhibition of RhoA signaling. Neuron *69*, 1069–1084.

Pang, Z.P., Yang, N., Vierbuchen, T., Ostermeier, A., Fuentes, D.R., Yang, T.Q., Citri, A., Sebastiano, V., Marro, S., Südhof, T.C., and Wernig, M. (2011). Induction of human neuronal cells by defined transcription factors. Nature *476*, 220–223.

Parras, C.M., Schuurmans, C., Scardigli, R., Kim, J., Anderson, D.J., and Guillemot, F. (2002). Divergent functions of the proneural genes Mash1 and Ngn2 in the specification of neuronal subtype identity. Genes Dev. *16*, 324–338.

Parras, C.M., Galli, R., Britz, O., Soares, S., Galichet, C., Battiste, J., Johnson, J.E., Nakafuku, M., Vescovi, A., and Guillemot, F. (2004). Mash1 specifies neurons and oligodendrocytes in the postnatal brain. EMBO J. *23*, 4495–4505.

Parras, C.M., Hunt, C., Sugimori, M., Nakafuku, M., Rowitch, D., and Guillemot, F. (2007). The proneural gene Mash1 specifies an early population of telencephalic oligodendrocytes. J. Neurosci. *27*, 4233–4242.

Perk, J., Iavarone, A., and Benezra, R. (2005). Id family of helix-loop-helix proteins in cancer. Nat. Rev. Cancer *5*, 603–614.

Petryniak, M.A., Potter, G.B., Rowitch, D.H., and Rubenstein, J.L. (2007). Dlx1 and Dlx2 control neuronal versus oligodendroglial cell fate acquisition in the developing forebrain. Neuron *55*, 417–433.

Pfisterer, U., Kirkeby, A., Torper, O., Wood, J., Nelander, J., Dufour, A., Björklund, A., Lindvall, O., Jakobsson, J., and Parmar, M. (2011). Direct conversion of human fibroblasts to dopaminergic neurons. Proc. Natl. Acad. Sci. USA *108*, 10343–10348.

Poitras, L., Ghanem, N., Hatch, G., and Ekker, M. (2007). The proneural determinant MASH1 regulates forebrain Dlx1/2 expression through the I12b intergenic enhancer. Development *134*, 1755–1765.

Politis, P.K., Makri, G., Thomaidou, D., Geissen, M., Rohrer, H., and Matsas, R. (2007). BM88/CEND1 coordinates cell cycle exit and differentiation of neuronal precursors. Proc. Natl. Acad. Sci. USA *104*, 17861–17866.

Qiang, L., Fujita, R., Yamashita, T., Angulo, S., Rhinn, H., Rhee, D., Doege, C., Chau, L., Aubry, L., Vanti, W.B., et al. (2011). Directed conversion of Alzheimer's disease patient skin fibroblasts into functional neurons. Cell *146*, 359–371.

Ross, S.E., Greenberg, M.E., and Stiles, C.D. (2003). Basic helix-loop-helix factors in cortical development. Neuron *39*, 13–25.

Sang, L., Coller, H.A., and Roberts, J.M. (2008). Control of the reversibility of cellular quiescence by the transcriptional repressor HES1. Science *321*, 1095–1100.

Sasai, Y., Kageyama, R., Tagawa, Y., Shigemoto, R., and Nakanishi, S. (1992). Two mammalian helix-loop-helix factors structurally related to Drosophila hairy and Enhancer of split. Genes Dev. *6* (12B), 2620–2634.

Schuurmans, C., and Guillemot, F. (2002). Molecular mechanisms underlying cell fate specification in the developing telencephalon. Curr. Opin. Neurobiol. *12*, 26–34.

Schuurmans, C., Armant, O., Nieto, M., Stenman, J.M., Britz, O., Klenin, N., Brown, C., Langevin, L.M., Seibt, J., Tang, H., et al. (2004). Sequential phases of cortical specification involve Neurogenin-dependent and -independent pathways. EMBO J. *23*, 2892–2902.

Schwab, M.H., Bartholomae, A., Heimrich, B., Feldmeyer, D., Druffel-Augustin, S., Goebbels, S., Naya, F.J., Zhao, S., Frotscher, M., Tsai, M.J., and Nave, K.A. (2000). Neuronal basic helix-loop-helix proteins (NEX and BETA2/Neuro D) regulate terminal granule cell differentiation in the hippocampus. J. Neurosci. *20*, 3714–3724.

Setoguchi, T., and Kondo, T. (2004). Nuclear export of OLIG2 in neural stem cells is essential for ciliary neurotrophic factor-induced astrocyte differentiation. J. Cell Biol. *166*, 963–968.

Shimojo, H., Ohtsuka, T., and Kageyama, R. (2008). Oscillations in notch signaling regulate maintenance of neural progenitors. Neuron *58*, 52–64.

Shitamukai, A., Konno, D., and Matsuzaki, F. (2011). Oblique radial glial divisions in the developing mouse neocortex induce self-renewing progenitors outside the germinal zone that resemble primate outer subventricular zone progenitors. J. Neurosci. *31*, 3683–3695.

Shu, J., Wu, C., Wu, Y., Li, Z., Shao, S., Zhao, W., Tang, X., Yang, H., Shen, L., Zuo, X., et al. (2013). Induction of pluripotency in mouse somatic cells with lineage specifiers. Cell *153*, 963–975.

Southall, T.D., and Brand, A.H. (2009). Neural stem cell transcriptional networks highlight genes essential for nervous system development. EMBO J. *28*, 3799–3807.

Sriuranpong, V., Borges, M.W., Strock, C.L., Nakakura, E.K., Watkins, D.N., Blaumueller, C.M., Nelkin, B.D., and Ball, D.W. (2002). Notch signaling induces rapid degradation of achaete-scute homolog 1. Mol. Cell. Biol. *22*, 3129–3139.

Sun, Y., Nadal-Vicens, M., Misono, S., Lin, M.Z., Zubiaga, A., Hua, X., Fan, G., and Greenberg, M.E. (2001). Neurogenin promotes neurogenesis and inhibits glial differentiation by independent mechanisms. Cell *104*, 365–376.

Sun, Y., Meijer, D.H., Alberta, J.A., Mehta, S., Kane, M.F., Tien, A.C., Fu, H., Petryniak, M.A., Potter, G.B., Liu, Z., et al. (2011). Phosphorylation state of Olig2 regulates proliferation of neural progenitors. Neuron *69*, 906–917.

Takahashi, K., and Yamanaka, S. (2006). Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell *126*, 663–676.

Takebayashi, H., Nabeshima, Y., Yoshida, S., Chisaka, O., Ikenaka, K., and Nabeshima, Y. (2002). The basic helix-loop-helix factor olig2 is essential for the development of motoneuron and oligodendrocyte lineages. Curr. Biol. *12*, 1157–1163.

Takizawa, T., Nakashima, K., Namihira, M., Ochiai, W., Uemura, A., Yanagisawa, M., Fujita, N., Nakao, M., and Taga, T. (2001). DNA methylation is a critical cell-intrinsic determinant of astrocyte differentiation in the fetal brain. Dev. Cell *1*, 749–758.

Tan, S.L., Nishi, M., Ohtsuka, T., Matsui, T., Takemoto, K., Kamio-Miura, A., Aburatani, H., Shinkai, Y., and Kageyama, R. (2012a). Essential roles of the histone methyltransferase ESET in the epigenetic control of neural progenitor cells during development. Development *139*, 3806–3816.

Tan, S.L., Ohtsuka, T., González, A., and Kageyama, R. (2012b). MicroRNA9 regulates neural stem cell differentiation by controlling Hes1 expression dynamics in the developing brain. Genes Cells *17*, 952–961.

Tanigaki, K., Nogaki, F., Takahashi, J., Tashiro, K., Kurooka, H., and Honjo, T. (2001). Notch1 and Notch3 instructively restrict bFGF-responsive multipotent neural progenitor cells to an astroglial fate. Neuron *29*, 45–55.

Tiberi, L., van den Ameele, J., Dimidschstein, J., Piccirilli, J., Gall, D., Herpoel, A., Bilheu, A., Bonnefont, J., Iacovino, M., Kyba, M., et al. (2012). BCL6 controls neurogenesis through Sirt1-dependent epigenetic repression of selective Notch targets. Nat. Neurosci. *15*, 1627–1635.

Tomita, K., Moriyoshi, K., Nakanishi, S., Guillemot, F., and Kageyama, R. (2000). Mammalian achaete-scute and atonal homologs regulate neuronal versus glial fate determination in the central nervous system. EMBO J. *19*, 5460–5472.

Torper, O., Pfisterer, U., Wolf, D.A., Pereira, M., Lau, S., Jakobsson, J., Björklund, A., Grealish, S., and Parmar, M. (2013). Generation of induced neurons via direct conversion in vivo. Proc. Natl. Acad. Sci. USA *110*, 7038–7043.

Vierbuchen, T., Ostermeier, A., Pang, Z.P., Kokubu, Y., Südhof, T.C., and Wernig, M. (2010). Direct conversion of fibroblasts to functional neurons by defined factors. Nature *463*, 1035–1041.

Viñals, F., Reiriz, J., Ambrosio, S., Bartrons, R., Rosa, J.L., and Ventura, F. (2004). BMP-2 decreases Mash1 stability by increasing Id1 expression. EMBO J. *23*, 3527–3537.

Wallace, K., Liu, T.H., and Vaessin, H. (2000). The pan-neural bHLH proteins DEADPAN and ASENSE regulate mitotic activity and cdk inhibitor dacapo expression in the Drosophila larval optic lobes. Genesis *26*, 77–85.

Wang, B., Waclaw, R.R., Allen, Z.J., 2nd, Guillemot, F., and Campbell, K. (2009). *Ascl1* is a required downstream effector of *Gsx* gene function in the embryonic mouse telencephalon. Neural Dev. *4*, 5.

Wang, X., Tsai, J.W., LaMonica, B., and Kriegstein, A.R. (2011). A new subtype of progenitor cell in the mouse embryonic neocortex. Nat. Neurosci. *14*, 555–561.

Wang, X., Chen, X., and Yang, Y. (2012). Spatiotemporal control of gene expression by a light-switchable transgene system. Nat. Methods *9*, 266–269.

Wapinski, O.L., Vierbuchen, T., Qu, K., Lee, Q.Y., Chanda, S., Fuentes, D.R., Giresi, P.G., Ng, Y.H., Marro, S., Neff, N.F., et al. (2013). Hierarchical mechanisms for direct reprogramming of fibroblasts to neurons. Cell *155*, 621–635.

Wilkinson, G., Dennis, D., and Schuurmans, C. (2013). Proneural genes in neocortical development. Neuroscience *253*, 256–273.

Wu, Y., Liu, Y., Levine, E.M., and Rao, M.S. (2003). Hes1 but not Hes5 regulates an astrocyte versus oligodendrocyte fate choice in glial restricted precursors. Dev. Dyn. *226*, 675–689.

Xin, M., Yue, T., Ma, Z., Wu, F.F., Gow, A., and Lu, Q.R. (2005). Myelinogenesis and axonal recognition by oligodendrocytes in brain are uncoupled in Olig1-null mice. J. Neurosci. *25*, 1354–1365.

Yang, N., Ng, Y.H., Pang, Z.P., Südhof, T.C., and Wernig, M. (2011). Induced neuronal cells: how to make and define a neuron. Cell Stem Cell *9*, 517–525.

Yang, N., Zuchero, J.B., Ahlenius, H., Marro, S., Ng, Y.H., Vierbuchen, T., Hawkins, J.S., Geissler, R., Barres, B.A., and Wernig, M. (2013). Generation of oligodendroglial cells by direct lineage conversion. Nat. Biotechnol. *31*, 434–439.

Yue, T., Xian, K., Hurlock, E., Xin, M., Kernie, S.G., Parada, L.F., and Lu, Q.R. (2006). A critical role for dorsal progenitors in cortical myelination. J. Neurosci. *26*, 1275–1280.

Yun, K., Fischman, S., Johnson, J., Hrabe de Angelis, M., Weinmaster, G., and Rubenstein, J.L. (2002). Modulation of the notch signaling by Mash1 and Dlx1/2 regulates sequential specification and differentiation of progenitor cell types in the subcortical telencephalon. Development *129*, 5029–5040.

Zhou, Q., and Anderson, D.J. (2002). The bHLH transcription factors OLIG2 and OLIG1 couple neuronal and glial subtype specification. Cell *109*, 61–73.

Zhou, Q., Choi, G., and Anderson, D.J. (2001). The bHLH transcription factor Olig2 promotes oligodendrocyte differentiation in collaboration with Nkx2.2. Neuron *31*, 791–807.

Zhu, X., Zuo, H., Maher, B.J., Serwanski, D.R., LoTurco, J.J., Lu, Q.R., and Nishiyama, A. (2012). Olig2-dependent developmental fate switch of NG2 cells. Development *139*, 2299–2307.