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Basic Helix-Loop-Helix Factors in Cortical Development

Review

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Transcription factors with bHLH motifs modulate critical events in the development of the mammalian neocortex. Multipotent cortical progenitors are maintained in a proliferative state by bHLH factors from the Id and Hes families. The transition from proliferation to neurogenesis involves a coordinate increase in the activity of proneural bHLH factors (Mash1, Neurogenin1, and Neurogenin2) and a decrease in the activity of Hes and Id factors. As development proceeds, inhibition of proneural bHLH factors in cortical progenitors promotes the formation of astrocytes. Finally, the formation of oligodendrocytes is triggered by an increase in the activity of bHLH factors Olig1 and Olig2 that may be coupled with a decrease in Id activity. Thus, bHLH factors have key roles in corticogenesis, affecting the timing of differentiation and the specification of cell fate.

Introduction

The neocortex, with its recent evolution and rapid expansion in mammals, is fundamental to everything that makes us human, from abstract thought to consciousness (reviewed in Northcutt and Kaas, 1995). Despite its complexity, the neocortex can be conceptualized as a network of neurons supported by two functionally distinct glial cell types—astrocytes and oligodendrocytes. It is believed that the three principal cell types of the mammalian neocortex are derived from multipotent neural stem cells. How do these cells give rise to the neocortex? What precludes the synchronous formation of neurons and glia (as occurs in *Drosophila*)?

Recent insights into the molecular mechanisms of cortical development show that proliferation, specification, and differentiation of cortical progenitor cells is controlled, to a large degree, by transcription factors with basic helix-loop-helix (bHLH) motifs. The bHLH transcription factors (named in reference to the structural motif that mediates their DNA binding and dimerization functions) (Murre et al., 1989) are a large family with ~125 members encoded in the human genome (Ledent et al., 2002). Several of these factors have been implicated in the formation of the cortex, and this subset of neuro-active bHLH proteins is likely to expand as the roles of novel bHLH factors are analyzed. Here, we focus on a small set of bHLH factors that have been shown to have important roles in cell fate decisions during corticogenesis, which includes members of the NeuroD, Neurogenin, Mash, Olig, Id, and Hes families (Figure 1). We begin this review with a brief introduction to cortical development and cortical progenitors. We then discuss how bHLH transcription factors control four principal stages of cortical development—proliferation of cortical progenitors and the subsequent sequential formation of neurons, astrocytes, and oligodendrocytes.

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Cortical Development

The neocortex is generated from central nervous system (CNS) neural stem cells, a cell type defined by its ability to self-renew and the potential to give rise to neurons, astrocytes, and oligodendrocytes. The neural stem cell is thought of as a tabula rasa with the potential to become any one of the thousands of cell subtypes within the CNS. However, while a cell of this capability exists at some point during development, it is becoming increasingly clear that most proliferating cells in the developing nervous system have already received patterning information that limits their developmental repertoire and proliferative potential (Temple, 2001). Indeed, it is now clear that many progenitor cells are specified some considerable time prior to terminal differentiation. During the greater part of development, therefore, cortical progenitors represent a heterogeneous population whose properties vary depending upon their position within the embryo in space and time. In recognition of this heterogeneity, we have chosen to use the term cortical progenitor in this review, which we define as a cell from the developing cortex that has the capacity to proliferate (it is very difficult to address whether cells meet the rigorous criteria for self-renewal) and the potential to give rise to both neurons and glia.

As a framework within which to consider the regulation of cortical development, we first describe briefly the morphogenic and temporal changes that underlie the formation of this structure (reviewed in Monuki and Walsh, 2001). During the early phases of neural development, the neural plate invaginates to create the neural tube and the neural tube becomes regionalized and subdivided. The anterior-most aspect of the neural tube gives rise to the telencephalon and the dorsal half of the telencephalon gives rise to the cerebral cortices, including the neocortex. As the telencephalon is beginning to form, the majority of progenitors are proliferating rapidly in the ventricular zone. At this time, positional information is helping to specify the cell fate of progenitor cells. Once a progenitor goes through its final round of cell division, it begins to differentiate while simultaneously migrating from the ventricular zone to its final destination. Differentiation of neural precursors into neurons, astrocytes, and oligodendrocytes takes place in overlapping but temporally distinct waves, giving rise

bHLH Factor	Related Factor in Drosophila	Function	DNA Element
NeuroD	Atonal*	activate transcription	E box
Ngn	Tap/Biparous		
Olig	Oli	repressor?	
Mash	Achaete-Scute	activate transcription	
E protein	Daughterless		
Id	EMC	sequester E protein	no DNA binding
Hes	Hairy/(E/Spl)	repress transcription	N box

Figure 1. bHLH Factors Involved in Cortical Development

The phylogenetic tree is based on the analysis of Ledent et al. (2002). Note that branch length in the tree is not proportional to distance of relationship between families. Asterisk: strictly speaking, Atonal is the *Drosophila* homolog of Math1, not NeuroD.

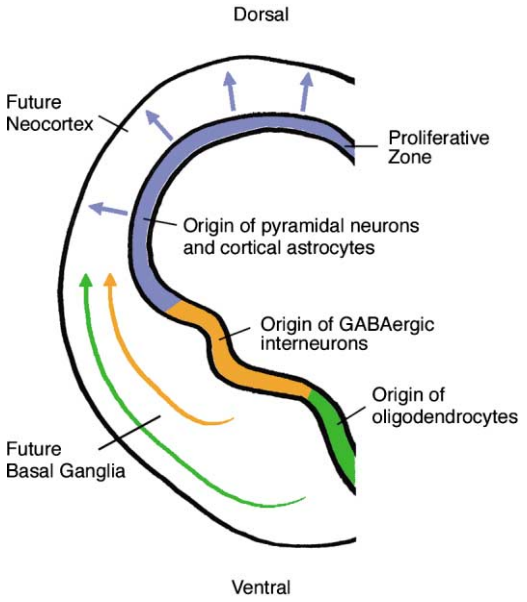

Figure 2. Diagram of the Developing Telencephalon Illustrating the Origin of Neural Cell Types that Make Up the Neocortex

Pyramidal glutamatergic neurons and cortical astrocytes arise from the proliferative layer of the dorsal telencephalon (blue). GABAergic inhibitory interneurons are born in the proliferative zone of the ganglionic eminences (orange). Oligodendrocytes arise from a discrete pool of progenitors in the most ventral region of the telencephalon (green). Not shown are cell types that originate in the ventral telencephalon and remain in the basal ganglia, such as striatal astrocytes and cholinergic neurons.

sequentially to the neurogenic phase, the astroglial phase, and the oligodendroglial phase (Sauvageot and Stiles, 2002). Neuronal differentiation in the mammalian neocortex peaks approximately two-thirds of the way through gestation. Astrocyte differentiation occurs next, peaking at birth in mammals. Oligodendrocyte differentiation follows later still, occurring during the first month of murine life.

Although this review is focused on the regulation of cells that make up the neocortex, to encompass this goal it is necessary to consider a wider region because neocortical cells arise initially from several areas of the telencephalon (reviewed in Marin and Rubenstein, 2001; Parnavelas, 2000). In brief, the telencephalon is subdivided into the ventral telencephalon, which develops into the basal ganglia, and the dorsal telencephalon, which gives rise to the hippocampus, the olfactory cortex, and the neocortex (Figure 2). Pyramidal neurons of the cortex are born locally in the dorsal ventricular zone and migrate outward along radial glia, assembling in an inside-out manner to establish the six laminar layers that characterize the neocortex (Rakic, 1988). Similarly (though later in development), neocortical astrocytes are born in the germinal zone of the neocortex and then migrate locally within this structure. In contrast, two other neocortical cell types, GABAergic interneurons and oligodendrocytes, arise from the germinal zone of the ventral telencephalon and make long journeys, following tangential migratory routes, to their final destinations in the neocortex (Figure 2; Corbin et al., 2001; Parnavelas, 2000; Tekki-Kessaris et al., 2001).

Cortical Progenitors

Cortical progenitors can be isolated from the embryonic cortex and cultured as monolayers on a coated surface or as clusters of floating cells called neurospheres (Johe et al., 1996; Reynolds and Weiss, 1996). In either case, progenitors are selected in a chemically defined medium containing specific growth factors, such as fibroblast growth factor (FGF) or epidermal growth factor (EGF), which maintains the progenitors in a proliferating state. Multipotent cortical progenitors that have the potential to give rise to neurons, astrocytes, and oligodendrocytes are most abundant when cortical tissue is harvested during a time of active neurogenesis, at approximately E14 in the rat (or E13 in the mouse). Treatment of cortical progenitors with platelet-derived growth factor (PDGF) increases the number of neurons that arise from these cultures (Johe et al., 1996; Williams et al., 1997). In addition, neurons are more likely to form if cells are plated at low density, suggesting that a cell-cell contact-mediated signaling pathway inhibits neurogenesis (Tsai and McKay, 2000).

Cortical progenitors isolated from E17 rat cortex are predisposed to form astrocytes and much less likely to undergo neuronal differentiation (Qian et al., 2000). In this way, early and late cortical cell cultures recapitulate the temporal sequence of cortical development wherein neurons are formed before astrocytes. Cytokines such as ciliary neurotrophic factor (CNTF) and leukemia inhibitory factor (LIF) promote the differentiation of astrocytes (Bonni et al., 1997; Johe et al., 1996). Interestingly, depending on the time of development, bone morphogenic protein (BMP) signaling can promote either neuronal or astroglial differentiation of cortical progenitors (Gross et al., 1996; Li et al., 1998).

Cells that differentiate into oligodendrocytes can be isolated from a variety of CNS regions, including the cortex (Williams et al., 1991). However, cultures derived from the cortex would not be expected to contain progenitors that become oligodendrocytes in vivo. Because cortical oligodendrocytes originate in the ventral telencephalon, their antecedents are not represented in cortical cultures, which are derived from the dorsal telencephalon (see Figure 2). The observation that cortical

progenitors form oligodendrocytes in vitro may be explained by the observation that Sonic hedgehog (Shh) can induce the differentiation of oligodendrocytes and that scattered cells within these cultures spontaneously express Shh after 24 hr in serum-free medium (Alberta et al., 2001).

This example of a discrepancy between a cell's destiny in vivo and its potential in vitro illustrates both the advantages and limitations of using cultured cells as a model to understand cortical differentiation. The ease of cell culture systems, in which one can define and manipulate the extracellular environment, facilitates the study of the molecular mechanisms that underlie the programs of neural differentiation. On the other hand, positional information is largely lost in cell culture models. Positional information restricts the potential of a cortical progenitor cell in vivo, whereas an absence of specific patterning cues broadens the range of cell fate potentials that are possible in vitro (Anderson, 2001). As a result, cultured cells can sometimes attain fates in vitro that are excluded from their developmental repertoire in vivo.

Maintenance of Neural Progenitors

Given the striking correlation between cerebral cortical size and intellectual capacity across species, it is clear that regulating cortical cell number is a developmental variable with important consequences. To supply the cortex with the appropriate number of cells, it is critical that cortical progenitors proliferate sufficiently prior to differentiation. What maintains cortical progenitors in a proliferative state and regulates the timing of their differentiation? Two classes of inhibitory bHLH proteins, Hes and Id factors, employ multiple strategies to achieve this goal. Part of the task is accomplished by the ability of these factors to antagonize proneural bHLH factors, whose activity must be quelled to avert neurogenesis. In addition, Ids promote cell cycle progression by interacting with components of the cell-cycle machinery. Thus, cortical progenitors are maintained when the balance of bHLH factors is such that Hes and Id factors predominate.

Hes1 and Hes5 Maintain Cortical Progenitors by Inhibiting Neurogenesis

The important role for Hes factors in neural development was first revealed in *Drosophila* in which the Hes homologs from the Enhancer of split (E(spl)) family were shown to negatively regulate neurogenesis (Knust et al., 1987). Several closely related families make up this class in mammals, including the Hes and the Hey families (also known as Hesr, Herp, HRT, and CHF). While it is likely that many of these genes are involved in the timing of differentiation in a variety of tissues, two of them in particular, Hes1 and Hes5, have been shown to have key roles in telencephalic development (Ishibashi et al., 1995; Ohtsuka et al., 1999). Both Hes1 and Hes5 are expressed in the ventricular zone throughout the telencephalon (Akazawa et al., 1992; Allen and Lobe, 1999; Sasai et al., 1992) where they sustain progenitors in an undifferentiated, proliferative state and inhibit their differentiation into neurons (Ishibashi et al., 1995; Nakamura et al., 2000; Ohtsuka et al., 2001). When, for instance, the Hes1 gene is disrupted, mice show premature neuronal differentiation in vivo, with a 2-fold excess of neurons in the forebrain at E13.5 (Ishibashi et al., 1995; Nakamura et al., 2000). Similarly, suppression of Hes1 expression in ES-derived neural stem cells with antisense oligonucleotides causes neuronal differentiation (Kabos et al., 2002). The role of Hes activity in the maintenance of cortical progenitors is further supported by overexpression studies in the developing cortex, which show that Hes1 blocks neurogenesis and increases the proportion of mitotically active cortical progenitors (Ishibashi et al., 1994).

How do Hes factors work? Studies to date have revealed that Hes proteins inhibit neuronal differentiation through two distinct mechanisms. First, Hes factors form homodimers and heterodimers with closely related family members and bind to DNA elements called N boxes (CACNAG) to repress the expression of target genes, such as Mash1, that are required for neuronal differentiation (Chen et al., 1997; Davis and Turner, 2001) (Figure 3A). Transcriptional repression is mediated by the interaction of Hes proteins with transcriptional corepressors of the Groucho/transducin-like enhancer of split (Gro/TLE) family, via a conserved tetrapeptide motif (WRPW) (Paroush et al., 1994). The histone deacetylase activity associated with these repressor complexes modifies chromatin structure, making DNA inaccessible to transcriptional activation (reviewed in Naar et al., 2001).

In addition, Hes factors interact physically with proneural bHLH proteins, and this interaction functionally antagonizes the activity of proneural bHLH proteins (Alifragis et al., 1997; Sasai et al., 1992). Recent work in *Drosophila* has helped uncover the molecular mechanism underlying this inhibition. E(spl) repressors (*Drosophila* homologs of Hes factors) interact simultaneously with proneural bHLH proteins and with the corepressor Groucho (Giagtzoglou et al., 2003). This allows the recruitment of Groucho to the promoters at which proneural bHLH/E protein dimers are bound, resulting in the repression of proneural bHLH target genes (Figure 3B). Importantly, Hes factors do not require a DNA binding domain to inhibit proneural protein function, but are tethered to DNA through their ability to interact with proneural bHLH proteins.

Much of our understanding of Hes function comes from the appreciation that Hes1 and Hes5 are key target genes that are transactivated in response to Notch signaling (reviewed in Justice and Jan, 2002). Accordingly, disruption of Notch/Hes signaling through loss of Notch, Hes1, or Hes5 results in similar CNS phenotypes, including premature neuronal differentiation in vitro and decreased proliferative ability in vivo (de la Pompa et al., 1997; Ishibashi et al., 1994). Moreover, the ability of Notch to inhibit neuronal differentiation is dependent on Hes1 and Hes5. In cortical progenitors from $Hes1^{-/-};Hes5^{-/-}$ mice, constitutively active Notch signaling is unable to block neurogenesis (Ohtsuka et al., 1999). These findings indicate that Hes1 and Hes5 are critical Notch effectors that act to promote the maintenance of cortical progenitors by blocking their differentiation into neurons.

Notch/Hes signaling may function during cortical development to maintain a balance between the number of developing neurons and the number of remaining

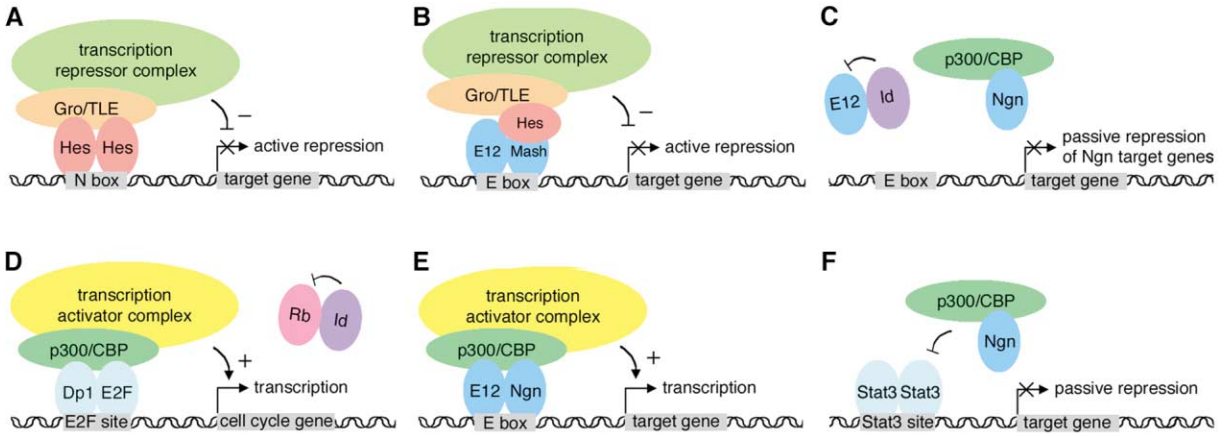

Figure 3. Mechanisms of bHLH Factor Activity

(A) Hes factors form homodimers and heterodimers within their class and bind to DNA elements called N boxes (CACNAG) to repress the expression of target genes. Transcriptional repression is mediated by the interaction of Hes proteins with corepressors, such as Groucho/TLE, which recruit large repressor complexes.

(B) Recent work in *Drosophila* suggests that Hes proteins may inhibit the transcription of genes at which proneural bHLH/E protein dimers are bound through direct interaction with proneural bHLH proteins (Giagtzoglou et al., 2003). Mammalian homologs are depicted here, though further experiments are required to determine whether this mode of repression is conserved in mammals.

(C) Ids inhibit bHLH activity by competing for binding to E proteins, sequestering E proteins away from bHLH factors, such as Ngn, that would otherwise bind to an E box and regulate transcription.

(D) Ids promote proliferation by inhibiting the ability of Rb to interfere with E2F-mediated transactivation of genes that are critical for cell cycle progression.

(E) Several tissue-specific bHLH factors, such as Ngn, dimerize with E proteins, such as E12, and bind to DNA elements called E boxes (CANNTG) to promote the expression of target genes. This transactivation is mediated by the interaction of bHLH dimers with coactivators, such as p300/CBP, which recruit a large complex that includes the basal transcriptional machinery.

(F) Ngn1 blocks astrocyte differentiation by sequestering p300/CBP from Stat3 and thereby inhibiting the transactivation of Stat3 target genes.

progenitors by a phenomenon known as lateral inhibition (reviewed in Beatus and Lendahl, 1998). This process provides a mechanism by which a progenitor gives rise to nonequivalent descendents. Newly formed neurons upregulate the Notch ligand, Delta, as they differentiate, and Delta activates Notch/Hes signaling in adjacent cells to block progenitor differentiation. Lateral inhibition thus allows the differentiation of some cortical progenitors into neurons while preventing simultaneous neurogenesis of all cortical progenitors. As newly differentiating neurons migrate out of the ventricular zone, lateral inhibition in the ventricular zone diminishes, thereby allowing a new set of cortical progenitors to begin neuronal differentiation. Lateral inhibition is likely achieved, in part, by the ability of Hes factors to functionally antagonize the activity of proneural bHLH proteins (Giagtzoglou et al., 2003). In addition, Hes factors directly repress the transcription of the proneural gene Mash1 (Chen et al., 1997; Ishibashi et al., 1995). However, there are likely to be additional target genes that are directly repressed by Hes factors, and it is not yet clear how Hes factors regulate other aspects of the progenitor phenotype, such as their ability to proliferate. Future identification of Hes target genes will help to clarify the roles of this repressor family.

Ids Maintain Cortical Precursors by Inhibiting Differentiation and Promoting Proliferation

Ids are expressed in the ventricular zone of the telencephalon (Jen et al., 1997), a site of active progenitor proliferation, where their expression is required to inhibit precocious differentiation of cortical progenitors. In this regard, Id and Hes factors have similar roles. However, these two families act through different mechanisms to inhibit neuronal differentiation. Whereas Hes factors inhibit gene transcription by recruitment of corepressors, Ids inhibit gene transcription by sequestration. Specifically, Ids form dimers with E proteins and thereby inhibit the activity of bHLH factors that require E proteins for activity (including members of the Mash, Neurogenin, NeuroD, and Olig families; see below) (Norton, 2000). In other words, Ids inhibit bHLH activity by sequestering E proteins away from bHLH factors that, when bound to E proteins, would activate (or repress) transcription (Figure 3C). Although Ids are considered members of the bHLH superfamily, they do not strictly fit into this category because they lack the basic DNA binding region. As a consequence, Id/E heterodimers do not bind DNA.

Interestingly, Ids inhibit the precocious differentiation of cortical progenitors into neurons and oligodendrocytes, but not astrocytes. In mouse models lacking both Id1 and Id3, cortical progenitors exit the cell cycle prematurely and undergo accelerated neuronal differentiation in vivo (Lyden et al., 1999). Similarly, when oligodendrocyte precursors from Id2 knockout mice are cultured in vitro, they undergo precocious growth arrest and differentiation, yielding premature oligodendrocytes (Wang et al., 2001). Gain-of-function studies in which Ids are overexpressed also suggest an inhibitory role for Ids in differentiation. Forced expression of Ids in cell culture blocked both neurogenesis and oligodendrocyte formation (Kondo and Raff, 2000; Nakashima et al., 2001; Toma et al., 2000; Wang et al., 2001). In contrast, astrocyte differentiation is not inhibited by Id activity. It

is likely that astrogliogenesis is different in this respect because the formation of astrocytes, unlike that of neurons and oligodendrocytes, is not promoted by bHLH factors that dimerize with E proteins (such as Ngn or Olig) and hence not inhibited by factors that sequester E proteins.

In addition to their roles as temporally regulated inhibitors of neuronal and oligodendroglial differentiation, Id2 and Id4 also play a direct role in promoting cell proliferation (Figure 3D). Ids stimulate progression through the cell cycle by inhibiting the ability of retinoblastoma (Rb) family members to interfere with E2F-mediated transcription (Iavarone et al., 1994; Norton, 2000). Transcription factors of the E2F family play a critical role in inducing the transcription of genes that mediate cell cycle progression. Members of the Rb family interact physically with E2F transcription factors and block their activity. Id2 and Id4 function in part by releasing E2Fs from inhibition by Rb so that E2Fs can activate genes that promote the proliferation of cortical progenitors. Recent studies have underscored the degree to which the regulation of cell cycle exit is critical for proper cortical development. Mouse mutants that express a stabilized form of β-catenin (a transcriptional regulator of the Wnt signaling pathway) within neural progenitors show a massive increase in the size of the cerebral cortex (Chenn and Walsh, 2002). Analysis of these mice revealed that progenitors expressing constitutive β-catenin are twice as likely to reenter the cell cycle than to undergo neuronal differentiation. Given that expression of the Id2 gene is directly promoted by the Wnt/β-catenin signaling pathway (Rockman et al., 2001), it is possible that the effect of ectopic β-catenin on progenitor proliferation is due, at least in part, to the ability of Id2 to inhibit Rb activity and thereby promote cell cycle progression. Consistent with this interpretation, mice lacking Rb in the telencephalon also show an increase in cortical cell number due to the increased proliferation of cortical progenitors (Ferguson et al., 2002).

Recent experiments suggest that one way in which the activity of Ids may be regulated is through control of Id entry into the nucleus. Though Ids contain the helix-loop-helix domain that mediates dimerization with other bHLH factors, they lack both the basic region, required for DNA binding, and the nuclear localization sequence, required for retention in the nucleus. As a result, the localization of Ids to the nucleus depends upon their interaction with nuclear proteins, such as E proteins. Recently it was found that Id2 relocalizes from the nucleus to the cytoplasm at the onset of oligodendrocyte differentiation (Wang et al., 2001). This observation raises the possibility that dimerization between Id2 and E proteins may be inhibited just prior to the conversion of oligodendrocyte precursors into oligodendrocytes. It is not yet clear what regulates the dimerization of Ids with E proteins, but studies in nonneural systems suggest that Id activity is regulated by phosphorylation. For instance, the ability of Id2 and Id3 to inhibit dimerization between E12 and MyoD, a bHLH factor involved in myogenesis, is regulated by the phosphorylation of these Ids by cyclin-dependent kinase 2 (Deed et al., 1997; Hara et al., 1997). Further studies are required to determine whether similar types of regulation are involved in cortical development.

Formation of Neurons

The initial phases of cortical development involve the rapid proliferation of cortical progenitors through symmetric division in which one cell gives rise to two identical proliferating daughter cells. At the time of neurogenesis, however, a subset of cortical progenitors becomes restricted to a neuronal lineage. It is likely that this restriction involves asymmetric division in which one cell is maintained as a multipotent cortical progenitor, while the other is fated to differentiate into a neuron within a few rounds of cell division.

Recent studies suggest this restriction is due, at least in part, to the activity of proneural bHLH factors. In particular, for the cell that is specified to the neuronal fate, the transition from proliferation to neurogenesis involves a coordinate increase in proneural bHLH activity and a decrease in Hes and Id activity. Because these classes of transcriptional regulators functionally antagonize one another, this concomitant, yet reciprocal, change in their activities initiates an irreversible cascade culminating in terminal neuronal differentiation. Subsequent steps in the differentiation process are mediated by successive waves of neuronal bHLH differentiation genes. Neurogenesis is therefore mediated by two broad categories of bHLH factors, proneural bHLH factors (e.g., Ngns and Mash), which are involved in initiating neurogenesis, and neuronal differentiation bHLH factors (e.g., NeuroD), which are involved in mediating terminal differentiation. The expression of a particular bHLH gene (e.g., Ngn versus Mash), in turn, is part of the mechanism that determines which subtype of neuron will form.

Proneural bHLH factors and neuronal differentiation bHLH factors are transcriptional transactivators. As such, these proteins bind DNA as heterodimeric complexes together with E proteins (E12, E47, E2-2, HEB). The basic domain of these bHLH factors mediates interaction with DNA sequences that contain the core hexanucleotide motif CANNTG, known as an E box (Figure 3E; reviewed in Bertrand et al., 2002). Transactivation is mediated by the interaction of bHLH heterodimers with coactivators, such as p300/CBP and PCAF. These coactivators recruit a large complex that includes the basal transcriptional machinery. In addition, coactivators facilitate transcription by acetylating histones, leading to the unraveling of DNA from a tightly packed structure to one that is accessible to the transcriptional machinery (reviewed in Roth et al., 2001).

Proneural bHLH Factors Initiate Neurogenesis

Three proneural bHLH factors are known to be expressed in the telencephalon, Ngn1, Ngn2, and Mash1 (Guillemot and Joyner, 1993; Lo et al., 1991; Sommer et al., 1996). During development, these bHLH factors are expressed at low levels while neural progenitors are being specified, and a transient increase in their expression results in the initiation of neurogenesis. Consistent with this idea, proneural bHLH factors are expressed in the ventricular zone, where progenitors begin differentiation, but not in the cortical plate, where fully differentiated neurons are situated. Interestingly, Ngns are expressed in the dorsal telencephalon, which gives rise to glutamatergic neurons, whereas Mash1 is predominantly expressed in the ventral telencephlon, which gives rise to GABAergic and cholinergic neurons, hinting at the possibility that different proneural genes are in-

volved in the specification of different neuronal subtypes (Wilson and Rubenstein, 2000).

An important role for proneural bHLH factors in telencephalic development has been revealed by loss-of-function studies. In the Mash1 knockout mouse, the decrease in proneural bHLH activity causes a loss of progenitor cells in the ventral telencephalon, resulting in loss of GABAergic interneurons in the cortex (Casarosa et al., 1999). When both Ngn2 and Mash1 are absent, this phenotype is exacerbated. In this case, a failure of progenitor specification in both the ventral and dorsal telencephalon drastically reduces the number of cortical neurons that develop (Fode et al., 2000). These findings indicate that proneural bHLH activity is required for the specification of precursors in the telencephalon and/or the initiation of neurogenesis. When cortical progenitors from the Ngn2/Mash1 double knockout mouse are cultured in vitro, they produce colonies containing significantly more astrocytes and significantly fewer neurons (Nieto et al., 2001). Collectively, these studies suggest that proneural bHLH genes are involved in the specification of cortical progenitors to a neuronal fate and that in their absence cortical progenitors become astrocytes instead.

A different kind of phenotype is observed in the Ngn2 knockout mouse, perhaps because in this case, loss of Ngn2 results in upregulation of Mash1 in the dorsal telencephalon. A result of Mash1 misexpression in the dorsal telencephalon is that some progenitors in this region differentiate into GABAergic neurons (Fode et al., 2000). Similarly, when the coding sequence of Mash1 is swapped into the Ngn2 locus using a knockin strategy, cortical progenitors originating in the dorsal telencephalon are misdirected in their fate and become GABAergic neurons. In addition, many Ngn2-deficient neurons make the unusual journey from the dorsal to ventral region of the telencephalon, instead of migrating radially within the cortex (Chapouton et al., 2001). Thus, when Mash1 is expressed instead of Ngn2, the normal number of neurons is generated, but their identity is changed, emphasizing the importance of Mash1 in the specification of GABAergic neurons. The observation that Mash1 is sufficient to specify the GABAergic phenotype in cells that ought to be glutamatergic raises the question as to whether the reverse is true: is Ngn2 sufficient to specify the glutamatergic phenotype in cells that ought to be GABAergic? While this might seem an attractive possibility, studies to date argue against it. When Ngn2 replaces Mash1 at the Mash1 locus, neurons of the ventral telencephalon differentiate normally and show no overt change in phenotype (Parras et al., 2002). These findings emphasize that while proneural bHLH factors play a role in neuronal subtype specification in the telencephalon, their activities are dependent upon cellular context.

The idea that proneural bHLH factors initiate a neurogenic program was derived initially from gain-of-function studies in *Drosophila* and *Xenopus*. Similarly, in cultured mammalian cortical progenitors, ectopic expression of Ngn1 results in the differentiation of cortical progenitors into neurons (Sun et al., 2001). Overexpression of Mash1, like Ngn1, can induce the expression of panneuronal markers, such as β-tubulin and neurofilament-M, suggesting that either factor can initiate a program of gene expression that results in a neuronal phenotype (Farah et al., 2000). However, it is likely that there are programs of gene expression elicited by specific proneural bHLH factors that are unique to the proneural gene that is expressed. In *Xenopus*, for instance, while ectopic expression of either XNgnr1 or Xash1 results in neuronal differentiation, only XNgnr1 mediates the expression of XNeuroD and Xath3 (Talikka et al., 2002). Likewise in the mammalian telencephalon, NeuroD, NeuroD2, and Nex are a part of a genetic cascade downstream of Ngns but not Mash1. Indeed, the programs of gene expression that are mediated by Mash1 activity in the ventral telencephalon are completely unknown. Defining the subset of genes that are common to Mash1 and Ngn2 versus those that are distinct to each proneural gene will be of great value in understanding how panneuronal and subtype-specific programs of gene expression work together to mediate neurogenesis.

A second important subject for future studies is the nature of the regulatory mechanisms that initiate the expression of proneural bHLH genes. Whereas members of the Hes family are known to repress the expression of Mash1 (Chen et al., 1997; Ishibashi et al., 1995), transcriptional regulators that upregulate Ngns or Mash have not been identified. Likewise, the signaling pathways and mechanisms that positively regulate the activity of proneural factors are unknown. Ngn1, Ngn2, and Mash1 all have consensus sequences that correspond to glycogen synthase kinase 3 (GSK3) phosphorylation sites, and it has been suggested that this may represent a common mechanism to regulate the activity of these bHLH factors (Moore et al., 2002). Because two families of morphogens, Wnts and FGFs, are known to regulate the activity of GSK3, it is tempting to speculate that bHLH factors couple extrinsic signals and intrinsic pathways through GSK3-mediated phosphorylation.

Finally, a key aspect of the irreversible commitment to undergo neurogenesis is terminal exit from the cell cycle, and proneural bHLH genes appear to be involved in this transition. Ectopic Ngn2 expression decreases the proportion of proliferating progenitors, indicating that Ngn2 can promote cell cycle arrest (Lo et al., 2002). When progenitors from wild-type mice are compared to those from mice lacking both Ngn2 and Mash1, it is clear that the presence of Ngn2 and Mash1 limits the number of cell divisions (Nieto et al., 2001). Progenitors derived from Ngn2/Mash1 double mutant mice give colonies that are three times larger than those derived from wild-type mice. Although a role for proneural bHLH factors in cell cycle withdrawal is suggested by these experiments, it is not clear whether the effects on cell cycle withdrawal are direct or simply a consequence of differentiation. For example, while proneural bHLH factors induce the expression of cyclin-dependent kinase inhibitors, whether they do so directly or through activation of a neuronal differentiation gene, such as NeuroD, is unknown. Distinguishing between these possibilities is further complicated by the fact that these bHLH factors bind to the same or similar DNA elements (E boxes). Defining the direct targets of Ngns and Mash1 using approaches such as chromatin immunoprecipitation will be helpful to assess which transcription factors are bound to which promoters in vivo.

bHLH Differentiation Genes Mediate Terminal Neurogenesis

According to the prevailing model of neurogenesis, the transient expression of proneural bHLH factors induces a second, sustained wave (or waves) of bHLH differentiation genes, which mediate the terminal differentiation of neurons (Kageyama and Nakanishi, 1997; Lee, 1997). These differentiation genes are members of the NeuroD/Nex family and include NeuroD, NeuroD2 (also called NeuroD-related factor, NDRF), and Nex (also called Math2). Like proneural bHLH factors, bHLH differentiation proteins are E box binding transcriptional activators that, when overexpressed, are sufficient to induce cell cycle arrest and neuronal differentiation in culture (Farah et al., 2000). The expression pattern of the NeuroD/Nex family, however, hints at a distinct function in vivo. Whereas proneural bHLH factors are expressed in progenitors that subsequently give rise to both neurons and glia (Nieto et al., 2001), members of the NeuroD/Nex family, at least in the CNS, are specific to cells that are (or will become) neurons. The expression of these bHLH differentiation genes begins in the immature neuron and is maintained during neuronal differentiation (Lee et al., 2000). Thus, these factors are expressed in the cortical plate, but not in the ventricular zone (Schwab et al., 1998).

Although there is strong evidence from studies in knockout mice that the NeuroD/Nex family of genes is required for the differentiation of glutamatergic neurons in the hippocampus and cerebellum, no phenotype has been reported in the neocortex for the single knockouts (NeuroD, NeuroD2, or Nex2) or the NeuroD/Nex1 double knockout (Miyata et al., 1999; Olson et al., 2001; Schwab et al., 1998, 2000). The lack of an observable phenotype in the neocortex may indicate that members of the NeuroD family function in a largely redundant manner. Alternatively, the absence of an obvious phenotype in mice lacking one or more members of the NeuroD family may point to the existence of yet uncharacterized transcription factors that mediate the terminal differentiation of neurons in the neocortex. In this regard, it is noteworthy that bHLHb5, a largely uncharacterized bHLH factor in the Olig family, is expressed in postmitotic neurons of the superficial layer of the neocortex (Xu et al., 2002). However, its function in neuronal differentiation remains to be elucidated.

Formation of Astrocytes

Once the majority of neurons have differentiated and migrated to their final destinations, a second wave of differentiation ensues, the astroglial phase. Astrocytes are critical for CNS function in part because they promote an extracellular environment that sustains neuronal health. In addition, recent studies have revealed a novel role for astrocytes in the regulation of CNS development, where they provide cholesterol, a limiting factor that is critical for neuronal synapse formation (Mauch et al., 2001; Ullian et al., 2001). Interestingly, the appearance of cortical astrocytes and the formation of neuronal synapses occur concurrently. This temporal coupling may be required to safeguard newly formed neurons from damage by delaying the formation of active synapses until a protective environment of astrocytes has been created.

In *Drosophila*, a novel zinc-coordinating transcription factor (Cohen et al., 2002) glial cells missing (GCM) regulates a binary switch from neuronal to glial determination (Hosoya et al., 1995; Jones et al., 1995; Klaes et al., 1994). Two mammalian homologs of GCM have been isolated (Akiyama et al., 1996; Altshuller et al., 1996), and one of them can substitute functionally for its *Drosophila* counterpart (Kim et al., 1998). However, in mammals, the major expression sites of both GCM genes are not in the CNS (Kim et al., 1998), and targeted disruption studies suggest that neither gene plays a role in brain development (Anson-Cartwright et al., 2000; Gunther et al., 2000; Schreiber et al., 2000). Although there is no evidence that precludes the existence of a specific transcription factor in vertebrates that specifies formation of astrocytes, none has been described to date. Rather, recent experiments suggest that astrocytes may arise via a shift in the balance of bHLH factors.

Hes Activity Promotes the Formation of Astrocytes

The concept that Hes activity promotes astroglial formation seems somewhat at odds with the finding that these repressors support the maintenance of cortical progenitors. Nevertheless, accumulating evidence suggests that this is the case. How does Hes activity promote two seemingly opposite fates? While this question remains unanswered, it highlights the important concept that bHLH factors may have more than one function during development. Consequently, an increase in Hes activity prior to the neurogenic phase may maintain cortical precursors in an undifferentiated state, whereas an increase in Hes expression subsequent to the neurogenic phase may support astrocyte differentiation.

Precedence for this idea comes from other CNS models, where a number of recent papers have revealed a role for Hes factors in gliogenesis. In cultured adult hippocampal progenitors, for example, Notch promotes astrocyte differentiation (Tanigaki et al., 2001), suggesting that progenitors of the hippocampus are directed toward an astrocyte fate by Hes activity. In the retina, forced expression of Hes1 or Hes5 promotes the conversion of retinal progenitors into Muller glia (Furukawa et al., 2000; Hojo et al., 2000), whereas disruption of the Hes5 gene causes a decrease in the number of Müller glia that form. Similarly, in progenitors derived from the spinal cord, overexpression of Hes1 causes the differentiation into astrocytes (Wu et al., 2003). Collectively, these findings suggest that regulation of astrocyte formation by Hes factors is a general mechanism throughout the CNS. In the developing cortex, however, there is only circumstantial evidence for this idea at the moment. When cortical progenitors are cultured in vitro, treatment with BMP to induce astrocyte differentiation causes a rapid increase in the expression of Hes5, raising the possibility that an induction of Hes5 is part of the astrocyte differentiation program (Nakashima et al., 2001). Furthermore, cell-cell contact in vitro promotes an astrocyte fate, whereas isolated cells are more likely to become neurons (Tsai and McKay, 2000), consistent with the idea that Notch signaling promotes the formation of astrocytes. Indeed, the intracellular domain of Notch has been shown to directly activate the GFAP promoter (Ge et al., 2002). Thus, Notch may act both

directly and indirectly, via induction of Hes factors, to promote astrocyte formation in the cortex.

Although it is likely that Hes activity in the developing cortex is involved in the differentiation of cortical astrocytes just as it is in other CNS regions, some studies suggest that Hes activity may not be sufficient. When Hes1 or Hes5 was ectopically expressed early in the developing telencephalon, it failed to promote astrocyte differentiation (Ohtsuka et al., 2001). Instead, this misexpression of Hes factors maintained cells in a precursor-like state. It is possible that Hes expression only promotes astrocyte differentiation when active late in telencephalic development. Alternatively, it is possible that Hes activity is not sufficient for the differentiation of neocortical astrocytes, but rather works in combination with other transcription factors to mediate this fate. For example, Stats and Smads have been implicated in astrocyte differentiation (see below) and may work in combination with Hes factors to regulate the formation of astrocytes.

Proneural bHLH Factors Block Astrocyte Differentiation

The expression of proneural factors peaks at the height of neurogenesis and subsides during astrocyte differentiation (Ma et al., 1997; Sommer et al., 1996). Similarly in culture, there is an inverse relationship between the level of Ngn expression and the propensity of the neural progenitors to undergo differentiation into astrocytes, suggesting that proneural bHLH factors might act to suppress the formation of astrocytes (Sun et al., 2001). In support of this idea, overexpression of Ngn1 in neural progenitor cells in vitro almost completely blocks the formation of astrocytes (Sun et al., 2001). Loss-of-function studies likewise emphasize the inhibitory role of proneural bHLH factors in astrocyte differentiation. When the proneural genes Ngn2 and Mash1 are disrupted, there is a dramatic increase in the likelihood that neural progenitors will become astrocytes rather than neurons, indicating that a decrease in proneural bHLH activity leads to an astrocyte fate (Nieto et al., 2001). During development, a decrease in proneural bHLH activity may be initiated by Notch signaling, as this pathway was recently shown to cause ubiquitination and degradation of the human Mash1 homolog (Sriuranpong et al., 2002). Notch signaling may therefore antagonize Mash1 function at several levels, by blocking de novo expression of Mash1 (via Hes), by inhibiting Mash1-mediated transcription (via Hes) and by triggering degradation of preexisting Mash proteins.

Studies using cultured cortical progenitors have helped reveal how proneural bHLH factors inhibit astrocyte differentiation. Treatment of cortical progenitors with cytokines, such as CNTF or LIF, triggers astrocyte differentiation (Bonni et al., 1997; Johe et al., 1996; Park et al., 1999). The effect of these cytokines is dependent on the signal transducer and activator of transcription (Stat) family of transcription factors. Phosphorylation by Janus kinases (JAKs) results in the dimerization and translocation of Stats to the nucleus where they participate in the induction of astrocyte-specific programs of gene expression (Bonni et al., 1997; Nakashima et al., 1999b; Stahl and Yancopoulos, 1994). Treatment with BMPs potentiates astrocyte differentiation by activating Smad transcription factors, which form a complex with Stat3 that is bridged by the p300/CBP coactivators (Nakashima et al., 1999a). A recent study demonstrated that Ngn1 blocks cytokine-induced astrocyte formation by disrupting this Stat3/Smad/CBP transcriptional coactivator complex (Sun et al., 2001). Association of CBP with Ngn1 blocks the interaction of CBP with Stat3, inhibiting Stat3-mediated transcription (Figure 3F). Importantly, this effect is independent of DNA binding and is therefore distinct from the proneural functions of Ngn1. This finding illustrates the idea that one way in which transcription factors can regulate cell fate is by competing for limiting cofactors. Interestingly, Ngn1 appears to employ a second strategy to inhibit JAK/STAT signaling. In particular, Ngn1 acts through an unknown mechanism to block the phosphorylation of Stat3 in response to cytokine treatment (Sun et al., 2001). One possibility is that Ngn1 induces the expression of a gene such as SOCS (suppressors of cytokine signaling), but if so the identity of this gene remains unknown. In any case, it is clear that Ngn1 acts through multiple mechanisms to inhibit gliogenesis. The expression of relatively high levels of proneural bHLH factors may thus delay astrocyte differentiation until the neuronal phase of differentiation is completed and the levels of proneural bHLH factors decline.

Formation of Oligodendrocytes

Once most cortical neurons have migrated to the correct positions, are surrounded by supportive glia, and have formed appropriate synaptic connections, the formation of oligodendrocytes occurs. During this phase, a subset of cells from the ventral region of the telencephalon differentiates into oligodendrocyte progenitors that migrate throughout the telencephalon. These cells are critical for cortical function because they enable saltatory conductivity of the nervous impulse. Interestingly, there are no myelin-producing cells in the nervous systems of invertebrates. It is for this reason in part that the cellular and genetic origins of myelin-producing cells in the vertebrate nervous system have not been well understood until recently. With the discovery of bHLH factors Olig1 and Olig2, it is now apparent that bHLH transcription factors regulate specification of oligodendrocytes (Lu et al., 2000; Takebayashi et al., 2000; Tekki-Kessaris et al., 2001; Zhou et al., 2000). Olig1 and Olig2 are expressed in the ventral region of developing telencephalon, where they are found in presumptive oligodendrocyte precursors (Tekki-Kessaris et al., 2001). The expression of Olig1 and Olig2 persists as oligodendrocyte precursors migrate from the germinal zone into the mantle layer and is maintained in fully differentiated oligodendrocytes (Lu et al., 2000; Zhou et al., 2000).

Compound disruption of Olig1 and Olig2 results in a complete loss of the oligodendrocyte lineage throughout the central nervous system (Zhou and Anderson, 2002). Disruption of Olig2 alone is sufficient to completely inhibit oligodendrocyte development in the spinal cord; however, localized clusters of early oligodendrocyte progenitors form in the ventral forebrain, midbrain, diencephalon, cerebellum, and medulla of Olig2 null mice, suggesting that Olig1 can partially compensate for loss of Olig2 in developing brain (Lu et al., 2002; Takebayashi et al., 2002). This view is supported

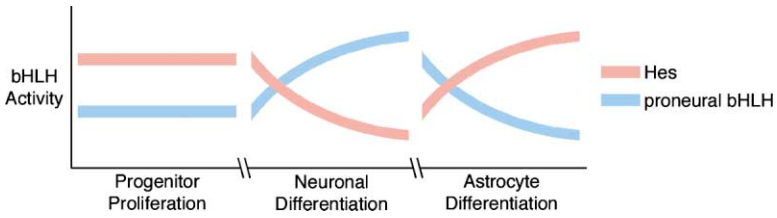

Figure 4. The Timing of Differentiation in the Telencephalon Is Regulated by the Balance of Antagonistic bHLH Factors

Conceptual model based on loss-of-function and gain-of-function studies illustrating the idea that a balance in the activity of proneural bHLH factors and Hes factors is involved in the regulation of progenitor maintenance, neuronal differentiation, and possibly astrocyte differentiation in the neocortex. Oligodendrocyte differentiation may be regulated by an analogous manner by the balance between Ids and Oligs.

by gain-of-function studies showing that overexpression of Olig1 in the developing mouse forebrain results in an increase in the number of progenitors that become oligodendrocytes (Lu et al., 2001). Ectopic expression of Olig1 in cortical progenitors in vitro induces the expression of NG2, a marker of oligodendrocyte precursors, suggesting that the effects of Olig1 in vivo might be cell autonomous (Lu et al., 2000). Other subtle functions of Olig1 on spinal cord patterning and on maturation of oligodendrocytes within the spinal cord have been noted (Lu et al., 2002). However, the apparent confinement of the major Olig1 functions to developing brain is of interest. Orthologs of Olig2 are found from humans to flies. In contrast, exhaustive efforts have thus far failed to find an Olig1 ortholog in chicken (Zhou and Anderson, 2002) or in zebrafish (Lu et al., 2002), suggesting that Olig1 arose from Olig2 by a gene duplication event that occurred coordinately with expansion of the neocortex in mammals. In support of this idea, Olig1 and Olig2 are colocalized to within 40 kb of each other in the mouse and human genomes.

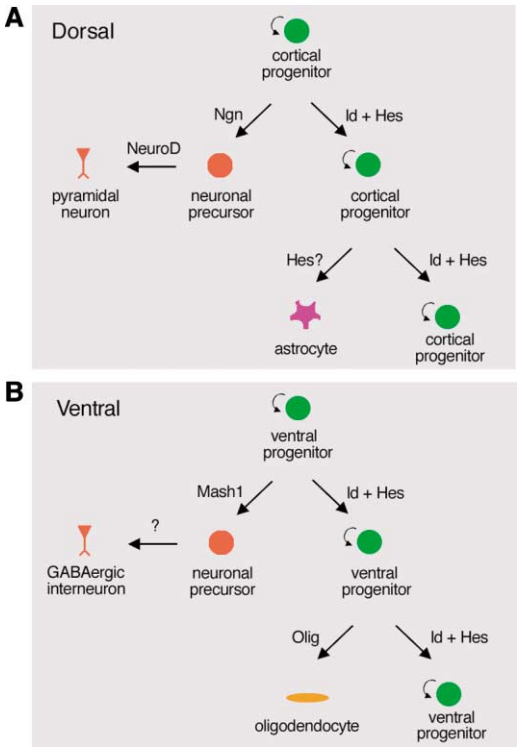

Figure 5. Regulation of Progenitor Specification and Differentiation by bHLH Factors in the Dorsal and Ventral Telencephalon

(A) In the dorsal telencephalon, cortical progenitors are maintained by Id and Hes factors. Members of the Ngn and NeuroD families are involved in early and late phases of neuronal differentiation, respectively, which give rise to pyramidal neurons. Hes factors may also be involved in astrocyte differentiation.

(B) In the ventral telencephalon, Mash1 is involved in the specification of ventral progenitors as GABAergic neurons, whereas members of the Olig family are involved in the specification of oligodendrocytes. To date, no bHLH factors have been identified as mediators of terminal differentiation in Mash-expressing neuronal precursors. Note that while Mash is sufficient for some aspect of the GABAergic phenotype, not all Mash-expressing cells become GABAergic neurons. Also, although all oligodendrocytes in the telencephalon arise from the ventral region, only a subset of ventral progenitors that form glia give rise to oligodendrocytes; many others give rise to astrocytes.

What is the antecedent cell that gives rise to the oligodendrocyte? None of the Olig null mice suffer any defects in formation of astrocytes within the forebrain. Fate mapping studies in the telencephalon show that Olig-expressing cells go on to form neurons and oligodendrocytes but not astrocytes (Lu et al., 2002). Within the forebrain, Olig2 is expressed both earlier and more broadly than expected for a factor whose sole role is to specify oligodendrocyte precursors, raising the possibility that Olig2 has additional uncharacterized roles in telencephalic development (Takebayashi et al., 2000). Collectively, the simplest interpretation of these findings is that the immediate progenitors of oligodendrocytes in the telencephalon are cells that also give rise to neurons. A concrete example of such a lineage relationship is seen within developing spinal cord where the expression of Olig2 occurs in two waves, the first coinciding with the development of motor neurons, the second with the birth of oligodendrocytes (Lu et al., 2000; Takebayashi et al., 2000; Tekki-Kessaris et al., 2001; Zhou et al., 2000). When Olig2 is knocked out, both motor neurons and oligodendrocytes are lost (Lu et al., 2002).

Oligs form heterodimers with E proteins (H. Dong and C.D.S., unpublished observation), as do members of the Ngn, Mash, and NeuroD families. However, Oligs may function as transcriptional repressors, whereas most bHLH factors that dimerize with E proteins are transcriptional activators. For instance, the effects of Olig2 are mimicked when the DNA binding domain of Olig2 is fused to the repressor Engrailed (Novitch et al., 2001; Zhou et al., 2001). Conversely, when Olig2 is fused to the transcriptional activator VP-16, it inhibits endogenous Olig2 function and blocks oligodendrocyte formation (Zhou et al., 2001). Transcriptional reporter assays also support the idea that Olig2 represses gene transcription

(Novitch et al., 2001). Because Oligs dimerize with E proteins, it is likely that Ids, which sequester E proteins, inhibit Olig activity. In support of this idea, overexpression of Id2 blocks the formation of oligodendrocytes (Wang et al., 2001). This finding suggests that oligodendrocyte differentiation may be triggered when the balance of bHLH factors favors Oligs over Ids.

Conclusions

bHLH factors are now appreciated to control both proliferation and differentiation in the most complex region of the mammalian brain, the cortex. Through the analysis of the roles of particular bHLH factors in this developmental process, several fundamental themes have come to light that give insight into the mechanisms that regulate the timing of differentiation and determine the specification of cell fate. It is also becoming increasingly clear that bHLH factors have more than one function during cortical development, and that their function at any given time and place depends on their cellular context.

One recurring theme is that different classes of bHLH factors crossregulate one another to mediate the cell fate choice between a neuron, an astrocyte, or an oligodendrocyte, and that the timing of differentiation in the telencephalon is regulated by the balance of these factors. This idea is illustrated in the model shown in Figure 4, whereby coordinate changes in proneural and Hes activity are shown to regulate the shift in the stages of development from progenitor proliferation to neurogenesis to astrogliogenesis. Initially, when Hes activity predominates, neural progenitors proliferate. A concurrent increase in proneural bHLH activity and decrease in Hes activity triggers the formation of neurons, whereas the converse favors the formation of astrocytes. In an analogous manner, the balance of Olig and Id activity may regulate the timing of oligodendrocyte differentiation. Clearly, further studies are required to substantiate this model, and the molecular mechanisms that shift in balance of bHLH factors in the developing telencephalon need to be identified. Are these transitions due to an intrinsic clock (Qian et al., 2000), or is the developmental clock regulated by extrinsic factors? These questions remain to be addressed.

A second emerging theme is that specific bHLH factors are involved in determining a progenitor's developmental repertoire. Thus, as illustrated in Figure 5, progenitors from different regions of the telencephalon express different bHLH factors, and particular bHLH factors are involved in determining the neuronal and glial subtypes that will form from a given pool of progenitors. In the dorsal telencephalon, for instance, a transcriptional program initiated by Ngns, likely in combination with other transcription factors, results in a cascade of bHLH differentiation genes that mediate the differentiation of pyramidal neurons. In the ventral telencephalon, Mash1 is involved in specification to the GABAergic phenotype, whereas Olig genes are required for oligodendrocyte formation.

A final emerging theme is that bHLH transcription factors play important roles in the formation of the two vertebrate glial lineages—astrocytes and oligodendrocytes. Both astrocytes and oligodendrocytes are more closely related to neurons than hitherto believed and may not arise from glial restricted progenitors. From a teleological perspective, the coupling of neurons and their supportive glia during development may have facilitated the evolution of specialized roles within the CNS.

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