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A Turn of the Helix: Preventing the Glial Fate

Minireview

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The development of the nervous system is a carefully orchestrated process resulting in the ordered generation of the mature cellular elements of the central nervous system: neurons, astrocytes, and oligodendrocytes. All of these cell types arise from progenitors that are initially multipotent but gradually become restricted in their potential to either the neuronal or glial lineage (Gage, 2000). Differentiation occurs in a stereotyped sequence whereby neurons are generated first, followed by glial cells, which differentiate after neurogenesis is largely complete (Bayer and Altman, 1991). One of the most fundamental decisions faced by progenitor cells during development is whether to generate neurons or glia. There has been considerable interest recently in understanding how this choice is regulated.

A subset of transcription factors belonging to the basic helix-loop-helix (bHLH) family have long been recognized for their ability to positively regulate the neuronal fate decision in both invertebrates and vertebrates (Lee, 1997). Knockout studies in mice have revealed that bHLH factors are required for the development of distinct subpopulations of neurons. Conversely, overexpression of various proneural bHLH factors in *Xenopus* embryos is sufficient to convert ectodermal cells into neurons. It has been less appreciated to what extent bHLH factors contribute to the glial fate decision. Since most neurons are generated before gliogenesis begins, there must be regulatory mechanisms in place to coordinate the timing of differentiation of these cell populations. Several recent papers highlight a central role for bHLH factors in this process and provide a mechanistic understanding of how bHLH factors required for neurogenesis delay the onset of gliogenesis.

NGN1 Suppresses Glial Development In Vitro

How do bHLH factors influence the fate choices of individual progenitor cells? To determine at what step in the differentiation process bHLH factors are functioning, the fate decisions made by defined progenitor cells during development must be followed. This is difficult to do in vivo and is complicated by the fact that during development progenitor cells are exposed to complex environmental cues that influence cell fate decisions, hampering attempts to tease apart the mechanisms contributing to any given fate choice. Recent advances in neural stem cell research have made it possible to explore the mechanisms regulating progenitor cell differentiation by analyzing these events in vitro. Multipotent progenitor cells in culture can differentiate to give rise to neurons, astrocytes, and oligodendrocytes, similar to what has been observed for multipotent progenitor cells in vivo, and the fate choices made by individual cells or defined cell populations can be followed and manipulated (Rao, 1999). Taking advantage of this, Sun et al. (2001) examined how bHLH factors regulate neurogenesis and gliogenesis in multipotent cortical progenitor cells. Overexpression of the bHLH factor neurogenin1 (ngn1) caused enhanced neuronal differentiation and loss of progenitor cell markers, consistent with a role for ngn1 in positively regulating neurogenesis. Notably, ngn1 also reduced the ability of multipotent progenitor cells to differentiate into astrocytes, even in response to treatment with the cytokine LIF/CNTF, which normally promotes astrocyte differentiation. This suggested that ngn1 expression was able to actively inhibit the ability of progenitors to respond to glial-inducing cues.

Is the inhibition of astrocyte differentiation a direct effect or an indirect consequence of promoting neurogenesis? Sun et al. (2001) found that ngn1 could inhibit the expression of glial-specific genes, but that this was independent of its ability to promote neurogenesis. The authors introduced a promoter for the astrocyte marker glial fibrillary acidic protein (GFAP) coupled to a luciferase reporter into astrocyte-enriched cortical cultures. By monitoring luciferase activity, they found that ngn1 inhibited activation of this reporter construct. However, two observations showed that the inhibitory activity of ngn1 was independent of DNA binding. First, the region of the promoter that responded to ngn1 inhibition did not contain E boxes, which are the binding sequences for bHLH factors, and second, mutations in ngn1 that interfered with DNA binding did not prevent inhibition of the reporter construct. In contrast, the ability of ngn1 to promote neurogenesis did require DNA binding and activation of transcriptional targets such as the bHLH factor NeuroD. These important observations suggest that the proneural activity of ngn1 can be uncoupled from the glial inhibitory activity.

Competition for Limiting Cofactors

If ngn1 does not need to bind DNA to inhibit glial specific gene expression, what is the mechanism underlying this inhibition? A clue was provided through analysis of the GFAP promoter when it was found that one of the most important elements for inhibition by ngn1 contained a STAT binding site. STAT proteins are essential components of the signaling pathway activated by cytokines such as LIF/CNTF, which can promote astrocyte differentiation (Bonni et al., 1997). Binding of CNTF to its receptor activates an associated tyrosine kinase, the Janus kinase (JaK1), which phosphorylates the STAT proteins, resulting in their translocation to the nucleus (Leonard and O'Shea, 1998). In order for STAT proteins to activate expression of target genes, such as GFAP, they must bind to the transcriptional coactivator CBP/p300. Sun et al. (2001) found that Ngn1 also binds to CBP/p300 and can prevent this essential coactivator from associating with the STAT proteins, thus blocking activation of glial-specific genes. The ability of ngn1 to sequester CBP/p300 was independent of its ability to bind DNA, explaining how the glial inhibitory activity of ngn1 could be uncoupled from its ability to promote

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neurogenesis. Ngn1 was also found to inhibit phosphorylation of the STAT proteins through an unknown mechanism, suggesting that multiple mechanisms may be used by ngn1 to inhibit gliogenesis.

Lif/CNTF can also synergize with bone morphogenetic proteins (BMPs) to promote astrocyte differentiation (Nakashima et al., 1999). BMP signaling by receptor binding leads to phosphorylation of Smad proteins, which then translocate to the nucleus. Smad1 binds to CBP/p300, which also interacts with STAT proteins, thus Smad1 is recruited to glial promoters through its indirect association with STAT proteins. In contrast, BMP stimulation of cortical progenitors in which ngn1 is expressed elicits neuronal differentiation. In this case, the CBP/p300-Smad1 complex preferentially interacts with ngn1 to induce neurogenesis at the expense of gliogenesis (Sun et al., 2001). Once again, the ability of ngn1 to sequester CBP/p300 and associated proteins has an inhibitory effect on gliogenesis.

Based upon these results, it is reasonable to propose that CBP/p300 is limiting in cortical progenitor cells, thus precluding neurogenesis and gliogenesis from occurring simultaneously. This model suggests that when ngn1 is expressed in progenitors during normal development, neurogenesis is actively promoted through activation of ngn1 target genes, while gliogenesis is inhibited through sequestration of CBP/p300 and through inhibition of STAT phosphorylation. When ngn1 levels drop later in development, CBP/p300 would become available for recruitment to the promoters of glial-specific genes. To test this idea, Sun et al. (2001) showed that coexpressing CBP with ngn1 in cortical progenitors relieves the inhibition of astrocyte differentiation by ngn1. This suggests that exogenous ngn1 is only inhibitory to gliogenesis when CBP/p300 levels are limiting. Is this also true for endogenous ngn1? Sun et al. (2001) found that endogenous CBP/p300 coimmunoprecipitated with endogenous STAT3 from extracts of P3 cortical subventricular zone, where ngn1 expression is not expressed and glia are being generated. However, in extracts from E14 cortex, when ngn1 expression is high and neurogenesis is taking place, CBP/p300 was bound to ngn1, but not to STAT3, even though STAT3 was expressed. This argues that CBP/p300 is also limiting with respect to endogenous levels of STAT3 and ngn1, although this has not been tested directly.

Loss of bHLH Factors Causes Enhanced Gliogenesis

Together, these findings suggest a model in which ngn1 interferes with glial differentiation by binding to the transcriptional coactivator CBP/p300 and limiting its availability for activation of glial-specific genes (Figure 1). A prediction of this model is that precocious or enhanced glial differentiation should occur when bHLH factor expression is eliminated. This has not been directly examined in ngn1 knockout animals. However, Nieto et al. (2001) analyzed the differentiation of progenitors derived from ngn2 and Mash1 knockout animals to determine whether there were any effects on neuronal or glial differentiation. Ngn2 and Mash1 are both expressed in the germinal layers of the developing cerebral cortex, with ngn2 being expressed at high levels and Mash1 at low levels. When the ngn2 gene is disrupted, there is compensatory upregulation of Mash1 expression, which masks effects on neurogenesis or gliogenesis due to loss of

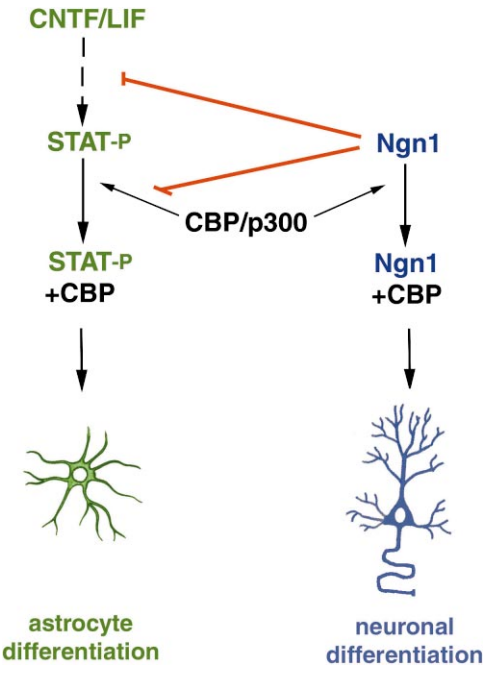

Figure 1. bHLH Factors Controlling Neurogenesis Suppress Gliogenesis

Treatment of cortical progenitors with CNTF promotes astrocyte differentiation. In reponse to CNTF stimulation, STAT proteins become phosphorylated, translocate to the nucleus, and interact with the transcriptional coactivator CBP/p300 to regulate glial-specific gene expression. Expression of the bHLH factor neurogenin 1 (ngn1) in progenitors suppresses glial differentiation in two ways. Ngn1 sequesters CBP/p300 so that it is not available for association with STAT proteins, thus preventing activation of glial-specific genes. In addition, ngn1 can prevent phosphorylation of STAT proteins in response to CNTF through an unknown mechanism.

ngn2 (Fode et al., 2000). Therefore, Nieto et al. (2001) isolated progenitors from Mash1/ngn2 double knockout animals and tested for their ability to differentiate in culture. They found a decrease in the proportion of strictly neuronal clones and an increase in the proportion of mixed clones that contained both neurons and GFAP-positive astrocytes. In general, these clones were larger and consisted of predominantly astrocytes, with only a few neurons. The authors concluded that the loss of these two bHLH factors increased the probability that cortical progenitors would differentiate into glia.

Nieto et al. (2001) also found that cortical progenitors are heterogeneous in their expression of ngn2. Using cells derived from animals heterozygous for a knockin of β-galactosidase into the ngn2 locus, the authors were able to sort progenitors expressing ngn2 from those not expressing ngn2. When this was done in a Mash1 mutant background, it was found that clones derived from progenitors expressing ngn2, but not Mash1, differentiated normally, while those derived from progenitors lacking both ngn2 and Mash1 were much more likely to contain only GFAP-positive astrocytes. Although it has not yet been shown whether ngn2 or Mash1 can sequester CBP/p300 in a manner similar to ngn1, this data suggests that loss of expression of these bHLH factors biases progenitor cells toward adopting a glial fate in vitro,

consistent with the model described above. Other bHLH factors are expressed in the developing central nervous system, so it will be important to test whether these factors can also regulate gliogenesis through interaction with CBP/p300.

The Neuronal/Glial Fate Decision In Vivo

In vivo, the levels of endogenous bHLH factor expression have the potential to regulate the availability of CBP/p300 for gliogenesis, and loss of bHLH factor expression in vivo should cause precocious or enhanced gliogenesis. The effects of loss of bHLH factor function on neurogenesis and gliogenesis in vivo have been analyzed in double knockout animals by Nieto et al. (2001) and Tomita et al. (2000). There were much more dramatic effects on cortical development in ngn2/Mash1 double knockout animals than seen with either single mutant alone (Nieto et al., 2001). There was a dramatic decrease in the numbers of differentiating neurons at E12.5 and E13.5, consistent with a requirement for these bHLH factors in neurogenesis. By E15.5, the ventricular zone in ngn2/Mash1 double mutant animals was disorganized, with BrDU-positive cells no longer restricted to the ventricular surface, and the distributions of ventricular zone and subventricular zone markers disturbed. There were also effects on glial populations in these animals. At E15.5, the radial glia were disorganized and by E18.5 showed morphological signs of premature differentiation along the astrocytic pathway, which normally occurs much later. In addition, glial markers, such as BLBP, tenascinC, and Hes5, were expressed by cells found scattered throughout the cortex. Similarly, in Mash1/Math3 double knockout animals, Tomita et al. (2000) found reduced neurogenesis and increased astrocytic differentiation in the tectum, hindbrain, and retina. However, there was enhanced cell death in the ventricular zone of Mash1/Math3 knockout animals, raising the possibility that neural progenitors were dying and glial progenitor differentiation was increased as a secondary consequence.

Although there was evidence of increased numbers of cells becoming committed to the glial lineage, and early astrocytic markers were expressed, consistent precocious expression of GFAP was not detected in the cortex of Mash1/ngn2 double mutant animals, or in the tectum of Mash1/Math3 double mutant animals, indicating that in these regions complete astrocyte differentiation did not occur (Tomita et al., 2000; Nieto et al., 2001). This suggests that in some parts of the nervous system, loss of bHLH expression is not by itself sufficient to result in the formation of mature astrocytes. It is possible that additional bHLH factors, such as ngn1, continue to be expressed in progenitors in these regions and prevent premature astrocyte differentiation. Alternatively, other signals that are temporally and spatially regulated may be required. For example, signals known to promote astrocyte development, such as ligands for the Notch receptor, are expressed by differentiating neurons. Since the numbers of differentiating neurons are reduced in the mutant animals, appropriate signals for complete astrocyte differentiation may be missing. Alternatively, astrocyte-promoting signals may be temporally regulated, precluding premature differentiation, even in the absence of bHLH factor expression.

Overexpression of bHLH factors in cortical progenitors in vivo does also not appear to be sufficient to inhibit glial differentiation. Cai et al. (2000) overexpressed various bHLH factors, including ngn1, in vivo using retroviral vectors and found that some bHLH factors, including ngn1, ngn2, and Mash1, could cause a modest increase in the proportion of infected cortical progenitors that differentiated into neurons. In addition, there was a corresponding modest inhibition of glial differentiation, but neither astrocyte differentiation nor oligodendrocyte differentiation was completely suppressed in infected cells. Since the levels of Ngn1 protein were not directly examined, it is possible that low levels of exogenous bHLH factor expression limited the effects on gliogenesis. Nevertheless, the findings of Cai et al. (2000) suggest that additional factors may be sufficient to promote gliogenesis in a CBP/p300-independent manner. Several recent studies have found that activation of the Notch pathway can promote glial development (Wang and Barres, 2000). Furthermore, Tanigaki et al. (2001) provide evidence that Notch-dependent activation of GFAP does not depend upon the STAT binding site in the GFAP promoter. Further studies under more defined conditions will be necessary to determine whether these pathways or others can override inhibition of gliogenesis by bHLH factors such as ngn1. Notably, overexpression of bHLH factors is sufficient to block Müller glial differentiation in the retina in vivo (Cai et al., 2000), suggesting that there may be regional differences in the ability of bHLH factors to regulate gliogenesis in the CNS.

Negative Regulation of Oligodendrocyte Development

Oligodendrocytes are also derived from multipotent progenitors, although the effect of proneural bHLH factors on their differentiation has not yet been established. However, recent evidence suggests that a positively acting bHLH factor likely regulates their development, and that the timing of oligodendrocyte differentiation is regulated by inhibiting bHLH activity. Oligodendrocytes differentiate from proliferating oligodendrocyte precursors (OPCs) (Rogister et al., 1999). Using a degenerate PCR approach to examine the profile of HLH factors expressed in purified OPCs, Wang et al. (2001 [this issue of *Neuron*]) found that these cells express high levels of Id2, a HLH protein that lacks the DNA binding basic domain. Id proteins can inhibit the activity of positively acting bHLH factors by heterodimerizing with them and preventing DNA binding. The authors found that overexpressing Id2 in oligodendrocyte precursors efficiently blocked their differentiation. This finding would predict that during normal development in order for OPCs to begin differentiation, endogenous Id2 inhibition must be relieved. Wang et al. (2001) demonstrated that in OPCs stimulated to differentiate in culture, Id2 protein translocated out of the nucleus prior to the onset of differentiation.

If Id2 is functioning to inhibit oligodendrocyte differentiation in OPCs, then OPCs lacking Id2 should differentiate prematurely. Since the Id2 knockout mice die in the early postnatal period, this was assessed using OPCs purified from P5 Id2 mutant mice. OPCs lacking Id2 expression showed a modest increase in the rate of differentiation and a significant decrease in proliferation. The fact that a more dramatic effect on the rate of differentiation was not observed argues that additional mech-

anisms must be in place to regulate the timing of OPC differentiation. Kondo and Raff (2000) provide evidence that Hes5 also plays a role in regulating the timing of OPC differentiation. Although oligodendrocyte-specific bHLH transcription factors such as olg1 and olg2 have been identified recently (Lu et al., 2000; Zhou et al., 2000), it is not yet clear whether Id2 is acting to inhibit their function or the function of other as yet unidentified bHLH factors. Further experiments will be necessary to define the targets of Id2 inhibitory action during oligodendrocyte development.

Remaining Issues

Multipotent progenitor cells undergo progressive restriction in potential to give rise to either a neuronal or glial lineage, but at what step in this process do the bHLH factors function to contribute to the lineage restriction? Nieto et al. (2001) found that ngn2 is not expressed in multipotent progenitors, but surprisingly is expressed in both neural restricted and glial restricted progenitors. Ngn2 may therefore not be regulating the choice of multipotent progenitors to become restricted in their potential to either the neuronal or glial lineage, but may instead act to reinforce this decision. The expression of ngn1 has not been described in this level of detail, so it is not clear whether ngn1 is also acting in separate precursors for neurons and glia. If so, then additional signals may be necessary to initially distinguish the neural lineage from the glial lineage. What would be the function of the neurogenins in the glial-restricted precursors? One possibility is that proneural bHLH factors act in glial-restricted precursors to limit their complete differentiation to mature astrocytes, perhaps by inhibiting the expression of glial-specific genes, such as GFAP. Alternatively, proneural bHLH factors may play an additional role in regulating the astrocyte versus oligodendrocyte fate decision. In OPCs, Wang et al. (2001) found that Mash1 is expressed but that loss of Mash1 expression had no effect on the proliferation or differentiation of these cells. It is possible that other bHLH factors expressed in OPCs, such as olg1 or olg2 (Lu et al., 2000; Zhou et al., 2000), can compensate for the loss of Mash1 expression. At this time the function of proneural bHLH factors in the glial restricted lineage remains to be determined.

Together, the studies highlighted above provide us with unprecedented insight into the molecular mechanisms regulating the neuronal/glial fate choice during development and demonstrate that bHLH factor expression can be an important contributor to this decision. Will these advances enhance our ability to use neural stem cells to therapeutically treat nervous system disease and injury? There is no doubt that defining the molecular mechanisms governing stem cell differentiation will make this much more likely. Sun et al. (2001) have suggested a therapeutic application for their work by stating that manipulation of proneural bHLH factor expression could be used to enhance the probability that neural stem cells differentiate into neurons rather than glia in vivo. However, sustained expression of bHLH factors in differentiated neurons in vivo eventually causes neuronal cell death (Cai et al., 2000), suggesting caution about this approach. In the end, we must still make the leap from controlling the differentiation of these cells under defined conditions in culture, and understanding how these events happen in the complex cellular environment encountered by these cells in vivo. Nevertheless, the recent studies highlighted above are significant for providing an illuminating glimpse into the mechanisms governing the neuronal/glial fate decision during development.

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