Cell, Vol. 104, 365–376, February 9, 2001, Copyright ©2001 by Cell Press

Neurogenin Promotes Neurogenesis and Inhibits Glial Differentiation by Independent Mechanisms

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Summary

The mechanisms by which neural stem cells give rise to neurons, astrocytes, or oligodendrocytes are beginning to be elucidated. However, it is not known how the specification of one cell lineage results in the suppression of alternative fates. We find that in addition to inducing neurogenesis, the bHLH transcription factor neurogenin (Ngn1) inhibits the differentiation of neural stem cells into astrocytes. While Ngn1 promotes neurogenesis by functioning as a transcriptional activator, Ngn1 inhibits astrocyte differentiation by sequestering the CBP-Smad1 transcription complex away from astrocyte differentiation genes, and by inhibiting the activation of STAT transcription factors that are necessary for gliogenesis. Thus, two distinct mechanisms are involved in the activation and suppression of gene expression during cell-fate specification by neurogenin.

Introduction

The mammalian cerebral cortex originates from a single layer of proliferating neuroepithelial cells. These progenitor cells line the ventricular cavities and sequentially give rise to the three major cell types of the brain: neurons, astrocytes, and oligodendrocytes. Neurogenesis precedes gliogenesis throughout the nervous system (Bayer and Altman, 1991), and retroviral labeling techniques have further shown that a single progenitor can give rise to both neurons and astrocytes (Turner and Cepko, 1987; Luskin et al., 1988; Price and Thurlow, 1988). It thus appears that a common cortical progenitor cell gives rise first to a variety of layer-specific neurons and then switches to producing astrocytes, and ultimately oligodendrocytes. The molecular mechanisms that orchestrate these sequential events during development are unclear.

In culture, cortical progenitor cells isolated at different embryonic stages behave in a manner that mimics the normal process of development (Burrows et al., 1997; Qian et al., 2000). Progenitors from rat embryonic day 14 (E14) cortex (at the peak of neurogenesis) primarily give rise to neurons and to dividing precursor cells (Ghosh and Greenberg, 1995). In E14 cultures, astrocytes are only generated after several days in vitro. By contrast, E17 progenitors give rise to astrocytes immediately (Bonni et al., 1997).

Multipotent neural stem cells can be isolated and expanded from primary cortical cultures after serial passaging in the presence of mitogenic growth factors (Gage et al., 1995). Although neural stem cell cultures are more homogenous than primary cortical cultures, both types of cultures have been used successfully to identify extracellular factors that specifically promote differentiation of stem cells into neurons, astrocytes, or oligodendrocytes.

Neuronal differentiation is promoted by both platelet-derived growth factor (PDGF) and by neurotrophin-3 (NT3). The cytokines leukemia inhibitory factor (LIF) and ciliary neurotrophic factor (CNTF) are potent inducers of astrocyte production, and thyroid hormone induces oligodendrocyte differentiation (Greenberg, 1995; Johe et al., 1996; Bonni et al., 1997; Ghosh and Williams et al., 1997).

LIF and CNTF exert their effects primarily via the JaK/STAT signaling pathway (Bonni et al., 1997; Rajan and McKay, 1998). LIF and CNTF bind to related receptors, which activate a receptor-associated tyrosine kinase, the Janus kinase (JaK1) (Stahl and Yancopoulos, 1994). Activated JaK1 phosphorylates two cytoplasmic proteins, the signal transducers and activators of transcription 1 and 3 (STAT1 and STAT3). This leads to STAT dimerization and translocation to the nucleus where the STATs activate cell type and stimulus-specific programs of gene expression (Bonni et al., 1993).

Other factors, such as bone morphogenetic protein (BMP), can enhance both neuronal and astrocyte differentiation, depending on the age of the stimulated cortical progenitors (Gross et al., 1996; Li et al., 1998). BMP-induced astrocyte differentiation appears to be mediated by the downstream Smad signaling proteins (Nakashima et al., 1999; M. N. V. and M. E. G., unpublished results). BMPs bind a multimeric receptor, which in turn results in the direct phosphorylation of Smad1 (Hoodless et al., 1996). This permits Smad1 to dimerize with Smad4 and to translocate to the nucleus (Massague, 1998), where these factors cooperate with STATs to activate glial-specific programs of gene expression (Nakashima et al., 1999).

The cooperation between Smads and STATs on glial promoters such as the glial fibrillary acidic protein (GFAP) promoter appears to be facilitated by a family of coactivator proteins termed p300/CBP. CBP (CREB binding protein) and p300 are ubiquitously expressed and are involved in the transcriptional coactivation of many different transcription factors (Goodman and Smolik, 2000). STATs and Smads bind to different domains of CBP/p300 (Nakashima et al., 1999), and the

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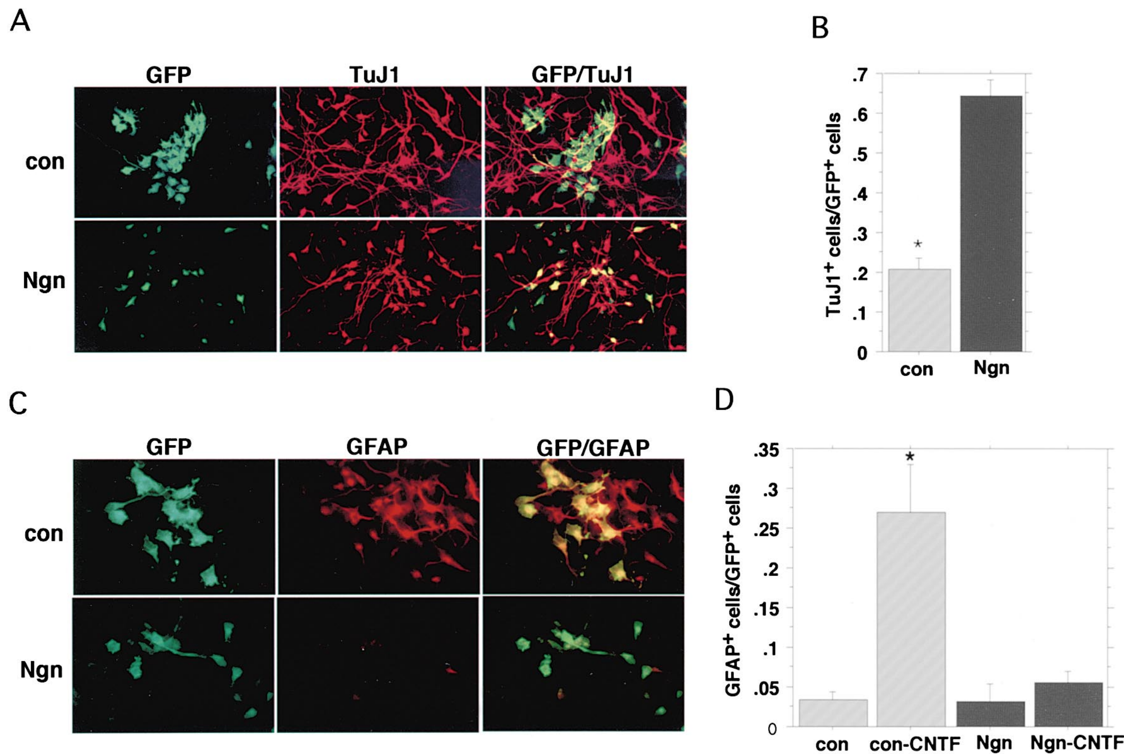


Figure 1. Ngn1 Promotes Neurogenesis and Inhibits Astrocyte Differentiation in E14 Rat Cortical Progenitor Cells

Primary rat E14 cortical cultures were infected with *Ngn1* or *Ngn1(nm)*/control (con) retroviruses. Four days after plating/infection, cells were fixed and subjected to immunostaining for neuronal (TuJ1) and astrocytic (GFAP) differentiation markers. Infected cells were positive for GFP (A and C). In (C), but not in (A), cells were treated with CNTF for four days to promote astrocyte differentiation. The quantitative analyses of the experiments are presented in (B) and (D) (*; $p < 0.001$).

STAT/p300/Smad complex, acting at the STAT binding element in the astrocyte-specific GFAP promoter, is particularly effective at inducing astrocyte differentiation in neural stem cells (Nakashima et al., 1999).

The signaling mechanisms by which neuronal or oligodendrocytic cell fates are specified are less well understood. However, several basic helix-loop-helix (bHLH) transcription factors have been implicated as mediators of neuronal or oligodendrocyte differentiation in the developing CNS. These bHLH factors include oligo1 and oligo2 for oligodendrocyte specification (Lu et al., 2000; Zhou et al., 2000), and neurogenin1 and 2 (Ngn1 and Ngn2), Mash1, and Math1 for neuronal differentiation (Guillemot et al., 1993; Ma et al., 1996; Ben-Arie et al., 1997; Fode et al., 1998).

In the developing mammalian cerebral cortex, two closely related bHLH factors, Ngn1 and Ngn2, are expressed exclusively in the cortical ventricular zone, where neuroepithelial precursor cells reside, and only during the limited period of time when neurogenesis is taking place (Gradwohl et al., 1996; Sommer et al., 1996; Ma et al., 1997). Both Ngn1 and Ngn2 dimerize with ubiquitous bHLH proteins, such as E12 or E47. These heterodimers then bind via their positively charged basic domains to DNA sequences that contain the E box consensus motif, CANNTG (Gradwohl et al., 1996). E box binding has been found to be critical for bHLH proteins to activate tissue-specific gene expression that promotes neuronal differentiation (Cau et al., 1997). The role for neurogenic bHLH proteins in the developing CNS has been substantiated by several recent knockout studies (Ben-Arie et al., 1997; Fode et al., 2000; Tomita et al., 2000).

Despite recent progress, there is much that is still not known about how different cell fates are determined in the developing cortex. A critical issue is how alternative cell fates are suppressed. Inducers of neuronal fate must somehow suppress glial differentiation, and likewise, glial differentiation can only proceed if the neuronal fate is blocked. In this report, we demonstrate that Ngn1 effectively inhibits astrocyte differentiation. We have identified two mechanisms by which Ngn1 represses glial-specific gene transcription, (1) by sequestering the CBP/p300/Smad1 complex away from glial promoters, and (2) by suppressing the JaK/STAT signaling pathway. As neurogenin levels are high during cortical neurogenesis and low during gliogenesis, neurogenin's ability to suppress glial differentiation may explain why astrocytes fail to develop during the period of neurogenesis even in the presence of glial-inducing cues.

Results

Ngn1 Not Only Promotes Neurogenesis but Also Inhibits Astrocyte Differentiation

In the cerebral cortex, *Ngn1* and *Ngn2* mRNAs are detected exclusively in the ventricular zone, where the precursor cells reside, and only during the period of

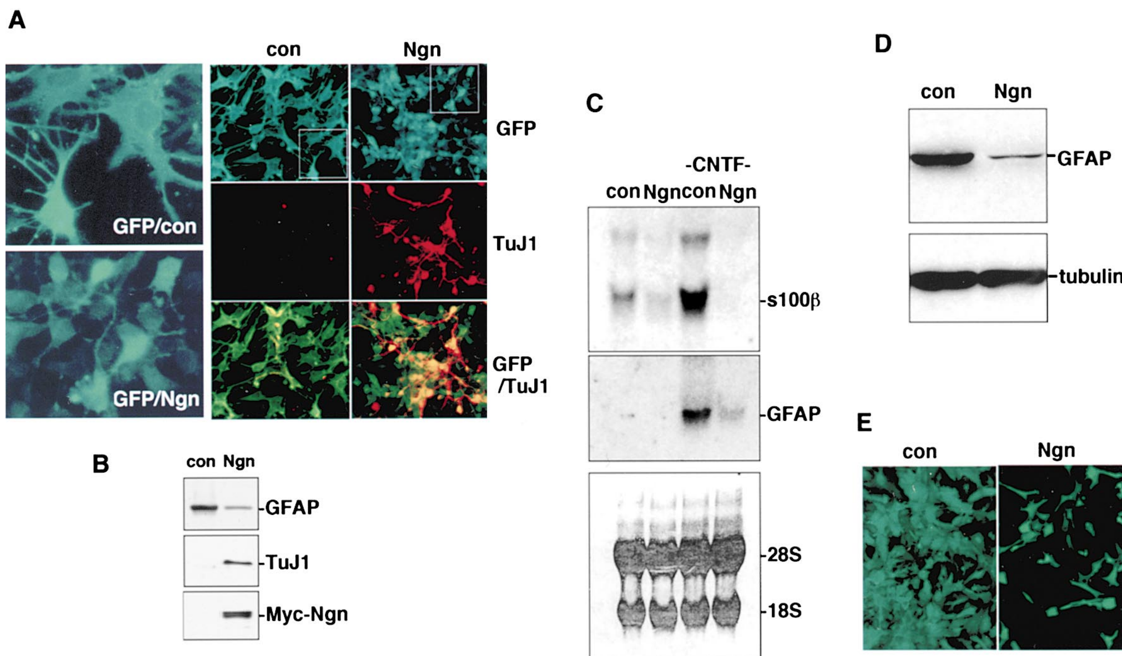


Figure 2. Ngn1 Inhibits Various Aspects of the Astrocytic Phenotype in CNS Stem Cells and Mature Astrocytes

CNS stem cells were infected with *Ngn1* or *Ngn1(nm)*/control (con) adenovirus. GFP was used to mark infected cells. Two days after infection and treatment with CNTF, cells were fixed or lysed for immunocytochemistry (A), Western (B), or Northern (C) analysis and the levels of cell type–specific markers were determined. In astrocyte-enriched cultures, virally expressed Ngn1 significantly reduced the protein levels of one of the astrocytic markers, GFAP (D). Sample loading was normalized by assaying the levels of tubulin expression (see Western blot, D). Mature astrocyte cultures were infected with the GFP/Ngn1 adenoviruses, and Ngn1 expression dramatically altered the astrocytic morphology and cell adhesion (E).

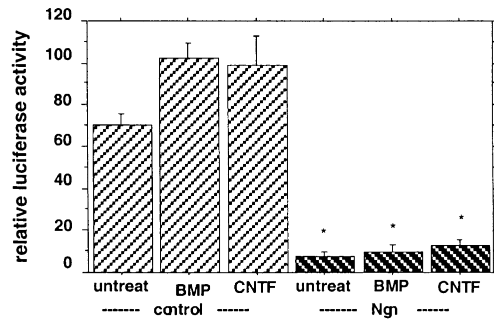
cortical neurogenesis (Gradwohl et al., 1996; Sommer et al., 1996; Ma et al., 1997). To examine whether Ngn1 can influence cell fate commitment, a retroviral vector was generated to introduce exogenous *Ngn1* into dividing cortical precursor cells cultured from rat E14 cortices. For this analysis, we also used a control virus that expresses a mutant *Ngn1* gene, *Ngn1(nm)*, with two stop codons inserted after the initiator methionine codon, so that no Ngn1 protein is produced. When cortical precursor cells were infected with the *Ngn1* or *Ngn1(nm)*/control viruses, we found that exogenous Ngn1 expression led to a significant increase in the number of precursors that become neurons, as compared with the control. Neurons were identified by cell morphology, and by the expression of neuronal-specific markers such as the β-tubulin/TuJ1 antigen (Figures 1A and 1B) and the microtubule-associated protein MAP2 (data not shown). In addition, the expression of Ngn1 led to downregulation of the expression of the neuroepithelial precursor marker Nestin. This finding suggests that ectopic Ngn1 effectively induces the process of neuronal differentiation, rather than simply inducing the expression of selected neuronal-specific markers in progenitor cells.

The induction of neurogenesis by Ngn1 could be due to enhanced neuronal survival or increased proliferation of committed neuroblasts rather than neuronal differentiation per se. However, the exogenous Ngn1-expressing cells do not show increased cell survival or proliferation rates as compared with nonexpressing cells, ruling out either of these alternative explanations (data not shown).

The possibility that Ngn1 instructively promotes neuronal cell fate determination, as opposed to merely promoting neuronal differentiation, implies that Ngn1 induces neurogenesis at the expense of gliogenesis. During cortical development, neurogenesis precedes glial differentiation. One possibility is that glial-inducing cues are only expressed after neuronal differentiation is complete. Alternatively, Ngn1 might actively inhibit gliogenesis even in the presence of glial-inducing cues, ensuring that the process of neurogenesis is completed before gliogenesis can begin.

To explore whether Ngn1 can still induce neurogenesis in the presence of glial-inducing factors, we examined the effect of Ngn1 on neuronal and glial differentiation in cells treated with LIF/CNTF. Ectopically expressed Ngn1, but not Ngn1(nm)/control, was able to inhibit cytokine-induced astrocyte differentiation in both cortical precursor and neural stem cell cultures. Astrocytes were identified by their morphology and by the expression of GFAP. In E14 cortical cultures treated for four days with CNTF, neurogenin expression reduced the number of GFAP-expressing astrocytes by 80% (Figures 1C and 1D). In addition, Ngn1-expressing cells display a neuronal-like morphology (small round cell bodies with one or two simple processes) that is quite distinct from the stellate GFAP-positive astrocytes. This implies that Ngn1 is not only regulating GFAP expression but may also affect other aspects of astrocyte differentiation.

The change in morphology is also seen when Ngn1 is expressed in neural stem cells in the presence of CNTF, which would normally cause the differentiation of virtually all of the stem cells into astrocytes (Figure



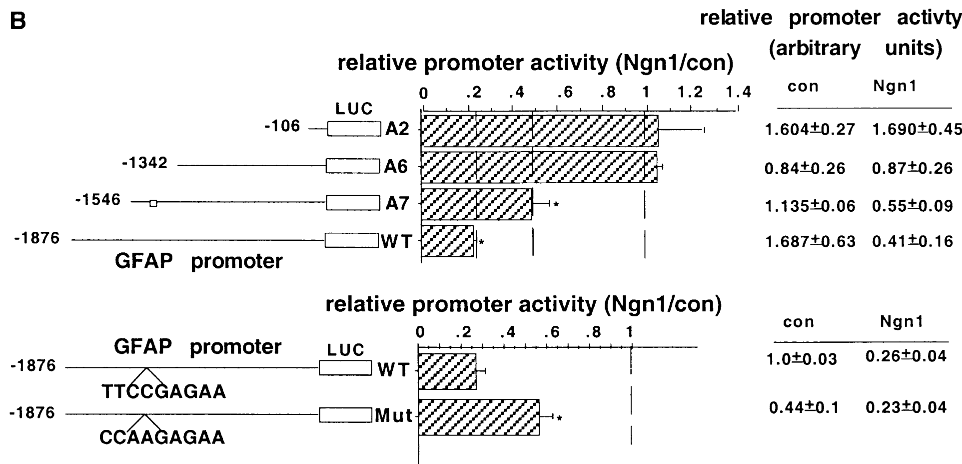


Figure 3. Two or More Non-E box Elements in the Promoter of a Glial-specific Gene Are Involved in the Transcriptional Repression of This Gene by Ngn1

The 1.9 kb rat GFAP promoter-luciferase-reporter construct was transfected, together with *Ngn1* or *Ngn1(nm)*/control cDNA, into 1-week-old E18 cortical astrocyte-enriched cultures. Ngn1 effectively inhibits the GFAP promoter even when the cells were treated with CNTF or BMP (A). Deletion and mutation analysis of the GFAP promoter indicates that two DNA fragments are required for Ngn1 to inhibit the promoter (B). Both fragments were found to lack E box or E box-like sequences. One of the fragments, however, contains a STAT binding site. Mutation of this site renders the GFAP promoter more resistant to the suppression by Ngn1 (B) (*; $p < 0.05$).

2A) (Johe et al., 1996). Some of the Ngn1-expressing cells express the neuronal markers TuJ1 (Figure 2A) and MAP2 (data not shown). The remaining Ngn1-expressing cells are TuJ1 negative, but nonetheless fail to display astrocytic morphology or GFAP expression. These findings suggest that the suppression of astrocyte differentiation in neural stem cells takes place before the induction of neuronal differentiation.

The neural stem cell cultures, because they are homogenous and display high (>90%) infection efficiency to adenoviruses, are amenable to studies of gene expression using Western (Figure 2B) and Northern (Figure 2C) blot analyses. We generated adenoviruses expressing either the Myc-tagged wild-type neurogenin (Myc-Ngn1) or the Myc-Ngn1(nm)/control, which expresses six repeats of the Myc tag but no Ngn1 protein. Western analysis indicates that Ngn1 inhibits the expression of a glial marker, GFAP, and induces the expression of neuronal-specific β-tubulin (TuJ1 antigen) (Figure 2B). By Northern analysis, we found that in CNS stem cells, Ngn1 inhibits the CNTF/LIF-induced upregulation of GFAP and another astrocyte marker s100β (Figure 2C), suggesting that Ngn1 inhibits various aspects of the glial differentiation program.

To investigate whether neurogenin can affect astrocyte-specific gene expression after astrocytes have already differentiated, we introduced Ngn1 into astrocyte-enriched cultures. Ngn1 overexpression clearly downregulates GFAP protein levels (Figure 2D). In the case of mature astrocyte cultures, neurogenin expression also caused a striking change in cell morphology and cell adhesion (Figure 2E). Though Ngn1 expression interferes with the astrocytic phenotype of mature astrocyte cultures, it is not capable of inducing neuronal-specific gene expression in this context. Therefore, inhibition of astrocyte differentiation by *Ngn1* is not the passive result of enhanced neurogenesis. Ngn1 appears to have two distinct roles: promoting neurogenesis and inhibiting glial differentiation.

Transcriptional Repression by Ngn1 Is Dependent on Non-E Box Promoter Elements

To understand the mechanism by which Ngn1 inhibits glial differentiation, we first examined whether Ngn1 expression leads to the downregulation of glial-specific

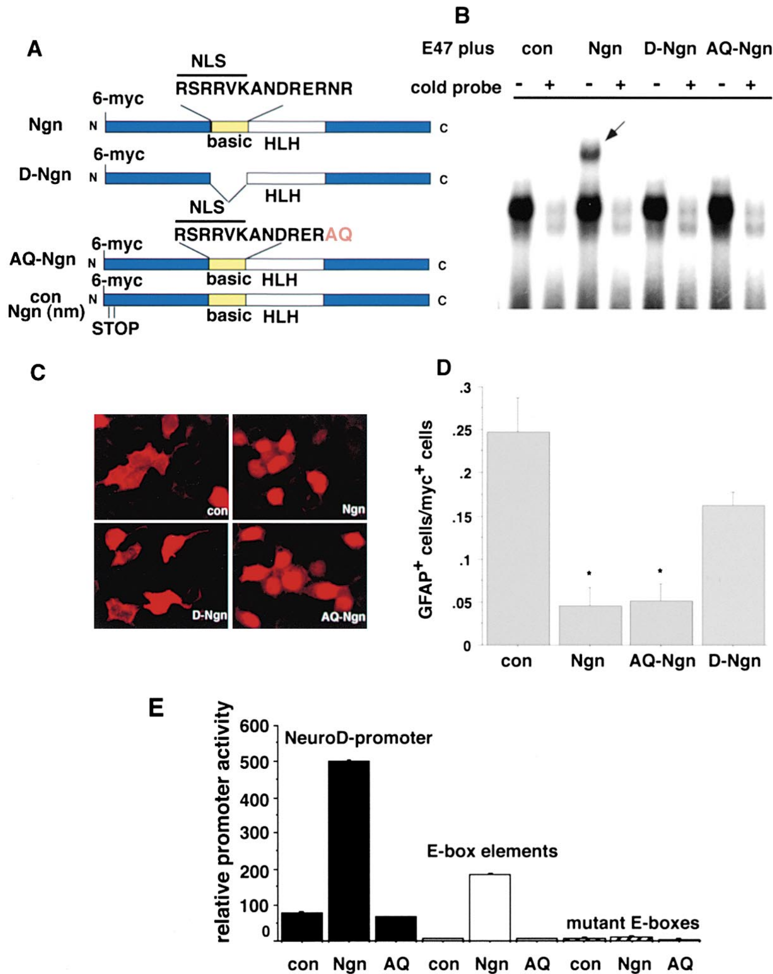


Figure 4. Ngn1 Inhibits Astrocyte Differentiation Independent of Its DNA Binding Domain

To examine whether Ngn1 can inhibit astrocyte differentiation without directly binding to DNA, we made two DNA binding mutants of Ngn1 (D-Ngn1 and AQ-Ngn1) (A). Both mutants failed to bind to the E box element (see arrow for shift) (B). While the D-mutation altered the subcellular localization of Ngn1, the AQ-mutation was expressed predominantly in the nucleus, as seen with wild-type Ngn1 (C). When these mutants were transiently transfected into CNS stem cells, AQ-Ngn1 inhibited astrocyte differentiation (D). Ngn1, but not AQ-Ngn1, activates both the full NeuroD promoter and the three E box elements of the NeuroD promoter, but not a promoter construct with all three E boxes mutated (E) (*; $p < 0.05$).

genes at the transcriptional level. Coexpression of Ngn1 significantly suppressed GFAP promoter activity, even when the cells were stimulated with glial-inducing factors such as CNTF/LIF or BMP (Figure 3A). Ngn1 similarly suppressed the GFAP promoter-luciferase reporter activity in neural stem cell cultures (data not shown). These data suggest that Ngn1, either directly or indirectly, represses the transcription of glial-specific genes.

Neurogenin might inhibit astrocyte gene expression in one of three ways. There might be E box sequences in astrocyte-specific promoters, and neurogenin might act as or recruit a transcriptional repressor to these sites. Alternatively, Ngn1 might function as a transcriptional activator (as it has classically been described), upregulating a glial repressor. Lastly, neurogenin might inhibit astrocyte differentiation independent of its ability to bind to DNA, for example, via a protein–protein interaction that sequesters an activator(s) of glial-specific gene expression. The results described below led us to favor the third of these possibilities.

We performed a detailed deletion and substitution mutation analysis of the GFAP promoter to identify DNA *cis*-acting elements that participate in the transcriptional repression by Ngn1. This analysis indicated that two regions of the GFAP promoter, −1876 bp to −1546 bp and −1546 bp to −1342 bp, are involved in the repression of the GFAP promoter by Ngn1 (Figure 3B). Neither of these two regions contains E box-like sequences, so we searched for other known DNA binding elements. Using Transcription Element Search Software, we identified a putative c-Ets1 site at −1685 bp, an AP1 site at −1525 bp, a STAT binding site at −1435 bp, as well as cryptic myogenin binding sites. A more detailed mutational analysis, involving deletions and substitutions of single and multiple sites, was undertaken. The results obtained suggest that, although the myogenin sites are not involved, a number of the other identified sites as well as several novel sites, are important for the repression of the GFAP promoter by Ngn1. Of these sites, mutation of the STAT binding element, previously known to be important for LIF/CNTF- and BMP-induced expression of this gene (Bonni et al., 1997; Nakashima et al., 1999), was most effective in reversing Ngn1 repression of the GFAP promoter activity (Figure 3B).

Though it appears that the STAT binding site is required for neurogenin to exert its full repressive effect on the GFAP promoter, we found that Ngn1 does not bind directly to the STAT binding element (data not shown). Furthermore, the fact that multiple DNA regulatory elements contribute to Ngn1 repression of the GFAP promoter suggests that Ngn1 might inhibit glial gene activation by interfering with a transcription cofactor that mediates the effects of multiple promoter bound transcription factors.

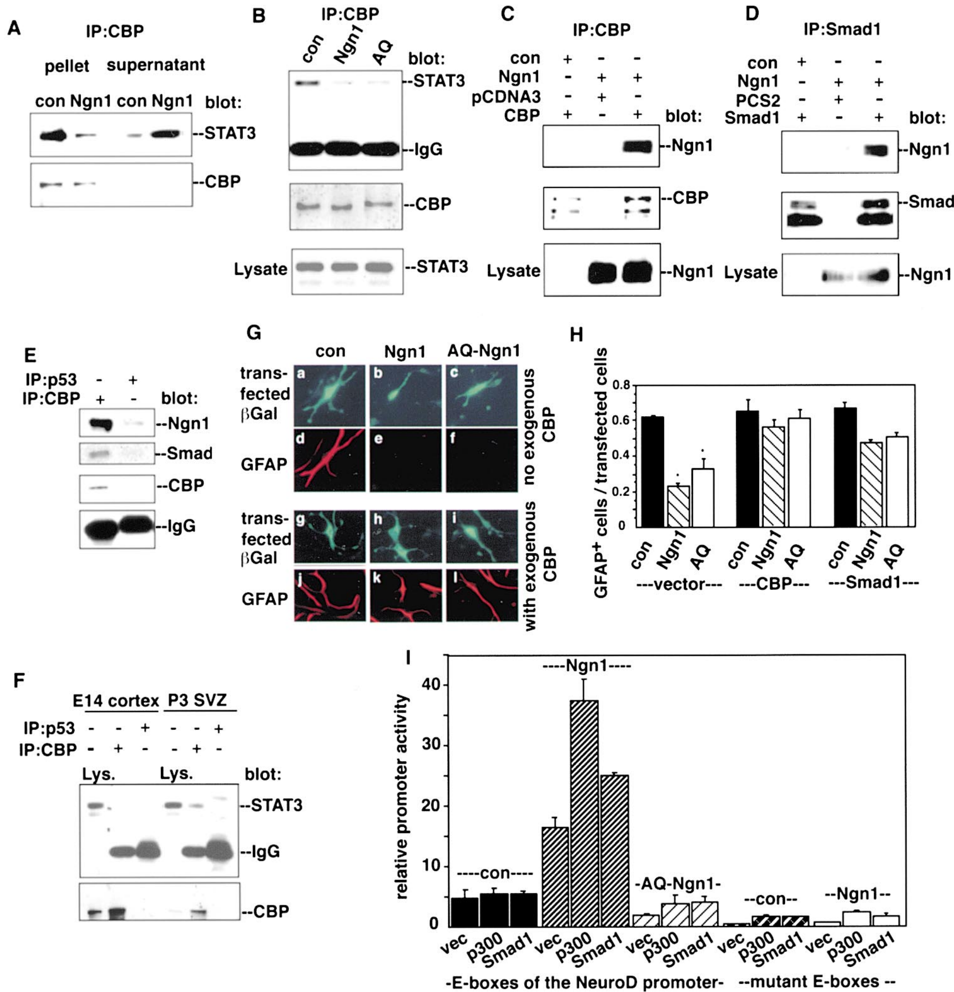


Figure 5. Ngn1 Sequesters the Transcriptional Coactivating Complex Composed of CBP/p300 and Smad1 from STAT Glial Differentiation Factors

In CNS stem cells, coimmunoprecipitation experiments show that STAT3 associates with CBP (A). Ngn1 expression, however, causes the disruption of the STAT3-CBP complex (A). When overexpressed in 293T cells, Ngn1 as well as AQ-Ngn1 inhibits the association between endogenous CBP and exogenous STAT3 (B), and Ngn1 itself associates with both exogenous CBP and exogenous Smad1 (C and D). Most importantly, in E14 rat cortex, endogenous CBP associates with endogenous Ngn1 and Smad1 (E), but not with STAT3, although STAT3 is expressed in E14 cortex (F). In P3 SVZ, where Ngn1 is not expressed and gliogenesis is ongoing, CBP co-IPs with STAT3 (F). An antibody against p53 was used as a negative control for the immunoprecipitation procedure (E and F). We examined whether the CBP/p300-Smad1 complex is limiting for cell differentiation, and found that overexpression of CBP and Smad1 rescues the Ngn1-mediated reduction in the number of astrocytes (G and H). Overexpression of p300 or Smad1 also enhances the transcriptional activation by Ngn1 on the E box elements of the NeuroD promoter (I) (*; $p < 0.05$).

A DNA Binding Mutant of Ngn1 that Is Incapable of Inducing Neuronal Differentiation Still Inhibits Astrocyte Differentiation

The finding that Ngn1 acts in part through the STAT binding element, yet does not bind this sequence, raised the possibility that the DNA binding function of neurogenin might not be required for inhibition of gliogenesis. The basic regions of Ngn1, Ngn2, and NeuroD, which are critical for DNA binding, are very similar (Sommer et al., 1996), and we found that Ngn1, in the presence of E47, can bind to the insulin E box in an electrophoretic mobility shift assay (EMSA), as can NeuroD (Peyton et al., 1994) and Ngn2 (Gradwohl et al., 1996).

To examine whether E box binding is required for Ngn1 to inhibit glial differentiation, we generated two DNA binding mutants of Ngn1 and examined their function in neuronal induction and glial inhibition. The first mutant is a deletion of the whole basic region of Ngn1 (D-Ngn1), and the second mutant contains a two amino acid substitution mutation in the C terminus of the basic region of Ngn1 (AQ-Ngn1). The AQ substitution has previously been shown to abolish the DNA binding activity of a related bHLH protein, NeuroD2 (Farah et al., 2000). Using the EMSA assay, we found that both D-Ngn1 and AQ-Ngn1 failed to bind to the E box element (Figures 4A and 4B).

When adenoviral vectors carrying either of these two Ngn1 mutants were introduced into neural stem cells, the Ngn1 mutants failed to induce neurogenesis (data not shown). However, given that there is a putative nu-

clear localization sequence (NLS) contained within neurogenin's basic region, it remained a possibility that these mutants were transcriptionally inactive because they could not enter the nucleus, not because the mutants were unable to bind to the E box. We examined the subcellular localization of the exogenous Myc-tagged wild-type and various mutant neurogenins in mammalian 293T cells, embryonic neural stem cells, and cortical progenitor cells. We found that wild-type Ngn1 predominantly localized to the nucleus. The subcellular localization of AQ-Ngn1 was indistinguishable from that of the wild-type Ngn1, whereas D-Ngn1 was distributed equally throughout the cell, as was also seen with the Ngn1(nm) mutant control (Figure 4C).

Both of the basic domain mutants were tested for their ability to inhibit astrocyte differentiation. We found that AQ-Ngn1, though unable to bind to the E box or induce neurogenesis, still effectively suppresses astrocyte differentiation (Figure 4D). As expected, AQ-Ngn1, unlike its wild-type counterpart, failed to activate the promoter of the Ngn1 target gene, NeuroD (Figure 4E) (Huang et al., 2000). This suggests that neurogenin can inhibit astrocyte differentiation independent of its DNA binding function, a function that is required for neuronal induction. In addition, the fact that D-Ngn1, which is not well localized to the nucleus, is not as effective as AQ-Ngn1 in suppressing glial differentiation suggests that the glial inhibition event occurs in the nucleus.

Ngn1 Sequesters a Transcriptional Coactivator Complex Composed of CBP/p300 and Smad1 and Blocks Its Interaction with STAT Glial Differentiation Factors

The finding that Ngn1 does not require a functional E box binding domain to suppress glial gene transcription suggests that Ngn1 may suppress glial differentiation without inducing a downstream glial repressor. In addition, the finding that multiple sites in the GFAP promoter together mediate the transcriptional repression by Ngn1 suggests that Ngn1 might interfere with a common transcriptional coactivator. Since the mutation of the STAT binding site renders the GFAP promoter partially resistant to suppression by Ngn1 (Figure 3B), we examined whether Ngn1 might block the transcriptional coactivators that are known to act at the STAT binding site.

It has previously been reported that CBP/p300 brings Smad1 to the GFAP promoter, and binds to STATs at the STAT binding element, to induce gene transcription (Nakashima et al., 1999). STATs and Smads do not appear to interact directly, but do so via CBP/p300. We hypothesized that Ngn1 might interfere with the recruitment of CBP/p300 to the promoter of astrocyte-specific genes by interfering with its ability to bind STAT proteins. In progenitor or stem cell cultures that are actively giving rise to astrocytes, endogenous CBP was found to interact with endogenous STAT3 (Figure 5A). However, this interaction is inhibited by ectopic expression of Ngn1 in these cells (Figure 5A). The ability of Ngn1 to inhibit the association between STATs and CBP is independent of its ability to bind DNA (Figure 5B), and might result from the fact that Ngn1 itself is capable of binding to both CBP and Smad1 (Figures 5C and 5D).

To exclude the possibility that the association between CBP-Smad1 and Ngn1 is a consequence of overexpression, we asked whether this association occurs in the developing cortex. We hypothesized that early in the development of the cortex, when neurogenin expression is at its peak, the neurogenins bind CBP and Smad1, thus inhibiting the ability of CBP-Smad1 to interact with STATs and to activate glial differentiation during the time of neurogenesis. To test this idea, we examined the association of CBP-Smad1, Ngn1, and STATs in extracts from rat E14 cerebral cortex. In these extracts, CBP was found to associate with both Ngn1 and Smad1 (Figure 5E), but not with STAT3 even though STAT3 is expressed at high levels in these extracts (Figure 5F). In contrast, we found that STAT3 did associate with CBP in protein extracts of postnatal day 3 (P3) cortical subventricular zone (SVZ) where the glial-producing precursor cells reside and at a time when neurogenin is no longer expressed (Figure 5F).

Neurogenin might not only sequester CBP-Smad1 away from glial promoters, but might use these coactivators to promote neuronal differentiation. We considered the possibility that the CBP and Smad1 transcriptional coactivators are limiting, such that the amount of coactivators present is only sufficient to promote differentiation of precursors into either neurons or astrocytes, but not both. To test this idea, we overexpressed CBP or Smad1 in cortical precursors in an attempt to reverse the suppressive effect of Ngn1 on glial differentiation. Overexpression of either CBP or, to a lesser extent Smad1, resulted in a significant rescue of glial differentiation from Ngn1 suppression (Figures 5G and 5H). Furthermore, we found that both CBP/p300 and Smad1 potentiate the transcription of a Ngn1-responsive promoter, suggesting that these two coactivators might be recruited to the Ngn1-E box transcription complex (Figure 5I). Taken together, these experiments suggest that Ngn1 sequestration of the CBP-Smad1 complex may account in part for Ngn1's ability to inhibit astrocyte differentiation.

Ngn1 Inhibits Glial Differentiation Partly by Inhibiting the STAT Pathway

In addition to its ability to sequester the CBP-Smad1 complex away from STAT3, we also found that Ngn1 inhibits the activation of the STAT signaling pathway. We have previously shown that in older cortical progenitors (E17/18) upon LIF/CNTF stimulation, the JAK-STAT signaling pathway is activated and the LIF/CNTF-treated cells rapidly give rise to astrocytes. This is in contrast to what is seen with younger cortical progenitors (E13/14), where LIF/CNTF stimulation leads to LIF receptor activation but nonetheless leads to poor STAT activation and no direct GFAP induction (M. N. V. and M. E. G., unpublished observations). Given that neurogenins are expressed at low levels in E17/18 cortical progenitors and at high levels in E13/14 progenitors, we considered the possibility that Ngn1 expressed in E13/14 progenitors might be inhibiting LIF-induced STAT phosphorylation in these cultures.

We measured the level of STAT activation using phospho-specific antibodies. In neural stem cells, LIF/CNTF stimulation triggers the phosphorylation of STAT1 and STAT3 at tyrosines 701 and 705, respectively. These

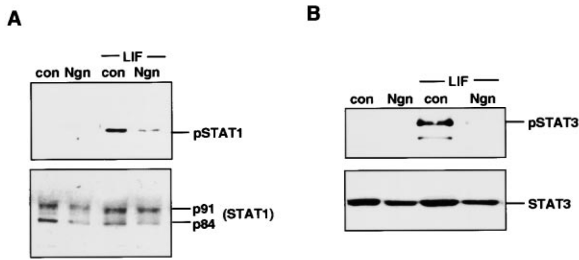


Figure 6. Ngn1 Inhibits Astrocyte Differentiation by Inhibiting the STAT Pathway

Both *Ngn1* and *Ngn1(nm)*/control adenoviral infected CNS stem cells were treated with LIF for 15 min. The activation of STAT1 and STAT3 was measured by Western blot analysis using phospho-specific antibodies pSTAT1. Wild-type Ngn1 significantly inhibited LIF-induced activation of both STAT1 and STAT3.

phosphorylation events are inhibited by the expression of exogenous Ngn1 (Figures 6A and 6B). This suggests that Ngn1, in addition to sequestering coactivators, directly blocks STAT signaling.

Ngn1 Can Convert BMP from a Glial-Inducing Cue to a Neuronal-inducing Factor

The observation that endogenous Ngn1 expression changes dramatically during early cortical development may explain not only the differing responses to LIF/CNTF at different stages of development, but also the differential responses to BMPs. In cortical progenitor cells, BMP has been reported to promote both neuronal and glial differentiation depending on the type of culture used. In E13/E14 cultures, BMP stimulation results in neuronal differentiation (Li et al., 1998; Mabie et al., 1999), whereas in E17/E18 cultures and neural stem cell cultures, BMPs induce astrocyte differentiation (Gross et al., 1996). Under culture conditions where BMPs induce astrocyte differentiation, neurogenin expression is low (Y. S. and M. E. G., unpublished observations).

In coimmunoprecipitation experiments, we found that activated Smad1 is bound to CBP regardless of whether CBP is associated with Ngn1 or with STAT1/STAT3 (Figure 5; Nakashima et al., 1999). We hypothesized that when neurogenin levels are high, BMP stimulation promotes the association of Smad1 and CBP (either independently or in combination) with neurogenin to induce neurogenesis. However, when endogenous neurogenin levels are low, as occurs in neural stem cells, exposure to BMPs induces the association of the Smad1-CBP complex with STAT proteins leading to astrocyte differentiation. To test this hypothesis, we assessed the effects of Ngn1 expression on the cells' response to BMP treatment. Neural stem cells were infected with a *Ngn1* or *Ngn1(nm)*/control adenovirus, and the differentiation of these cells was examined after BMP treatment. In Ngn1-expressing cells, BMP significantly increased neuronal differentiation, whereas in control cells, BMP induced astrocyte differentiation (Figures 7A–7D). These findings suggest that the level of neurogenin expression may determine whether BMP induces neuronal or glial differentiation.

Discussion

We have shown that neurogenin1, a bHLH transcription factor, can actively inhibit gliogenesis in a manner that is independent of its ability to induce neuronal differentiation. Ngn1 inhibits various aspects of astrocyte differentiation, such as the acquisition of an astrocytic morphology and the expression of cell type–specific markers such as GFAP and s100β, and it can do so without inducing neurogenesis. We have uncovered a novel mechanism of neurogenin action that does not rely on neurogenin's ability to bind to DNA via its basic domain. Instead, neurogenin appears to inhibit gliogenesis in at least two complementary ways: first, by binding and sequestering the transcriptional cofactors CBP and Smad1 and second, by blocking the activation of STAT1 and STAT3, signaling molecules that are critical inducers of astrocyte differentiation. Neurogenin's ability to both promote neurogenesis and inhibit gliogenesis may allow neurogenin to function as a potent neuronal commitment factor in a complex environment where extracellular stimuli may send conflicting cell fate–inducing signals.

Several findings suggest that the ability of ectopically expressed neurogenin to inhibit gliogenesis by sequestering the CBP-Smad complex reflects a function of endogenous neurogenin. First, endogenous neurogenin sequesters the CBP-Smad complex away from STATs in extracts from embryonic day 14 cortex, conditions where STATs and neurogenins are expressed at physiological levels. Second, during the development of the cortex, gliogenesis does not begin until neurogenin levels fall and STATs and CBP become capable of interacting with one another. Third, the absence of neurogenin and related proteins in knockout mice results in an inhibition of neurogenesis and increased glial differentiation consistent with the idea that a normal function of neurogenins is to inhibit glial differentiation (Nieto et al., 2001 [February issue of *Neuron*]) (Tomita et al., 2000).

Neurogenin Promotes Neuronal Differentiation and Inhibits Gliogenesis via Distinct Molecular Mechanisms

Cell fate specification involves the reciprocal activation of genes related to a particular cell fate and the suppression of genes of alternative fates. Many cell fate–specifying transcription factors can both positively regulate one fate and negatively regulate alternative fates (Anderson, 1993; Lehming et al., 1994; Tajbakhsh et al., 1996). However, the mechanisms by which these factors inhibit alternative fates are not well understood. A recent study demonstrates that the transcription factor Pit1 can recruit positive or negative transcription complexes to different cell type–specific promoters, depending on the spacing between the two Pit1 DNA binding elements (Scully et al., 2000). Our results suggest that the dual functions of Ngn1 in activating neuronal differentiation genes and suppressing glial-specific genes may reflect two independent processes that are mediated by distinct domains within the neurogenin protein. In particular, Ngn's suppression of gene expression appears not to require binding of Ngn1 to E box elements that are thought to be critical for Ngn1-dependent transcription.

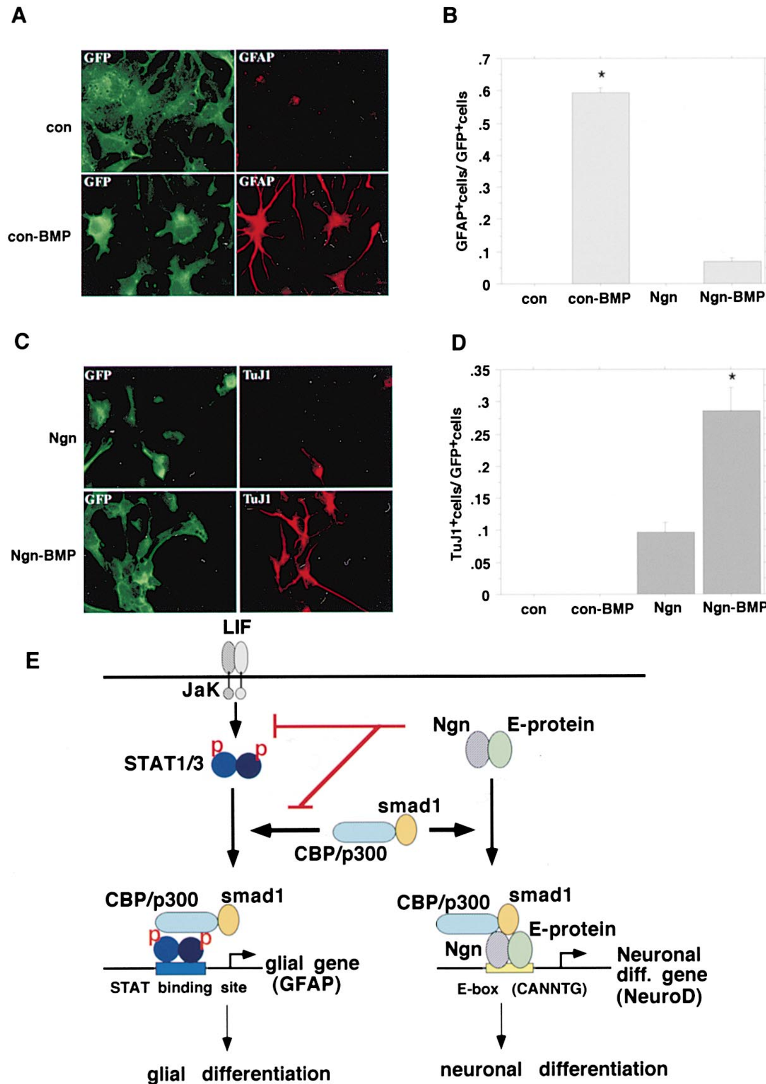


Figure 7. Ngn1 Converts BMP from a Glial-Inducing Cue to a Neuronal-Inducing Factor

Both *Ngn1* and *Ngn1(nm)*/control (con) adenoviral infected CNS stem cells were treated with BMP for two days. In Ngn1(nm)/control cells, BMP induced astrocyte differentiation (A and B). In Ngn1-expressing cells, BMP enhanced neuronal differentiation (C and D).

(E) Ngn1 inhibits glial differentiation by sequestering CBP-Smad1 away from glial-specific genes and by inhibiting the STAT1/STAT3 activation. CBP/p300 and Smad1, separately or together, associate with Neurogenin at neural-specific promoters (such as NeuroD), or are recruited to glial-specific genes (such as GFAP) by activated STAT1/STAT3, in the absence of neurogenins. Thus, neurogenin not only directly activates neuronal differentiation genes, it also inhibits glial gene expression (*; $p < 0.01$).

Ngn1 Inhibits Glial Differentiation by Sequestering the Transcriptional Cofactor CBP/p300

Many transcription factors require CBP/p300 in order to activate transcription, and there is evidence that the levels of CBP/p300 are limiting, i.e., that there is competition among the various families of transcription factors for CBP/p300 binding (Goodman and Smolik, 2000). For example, nuclear steroid receptors indirectly inhibit AP-1-dependent transcription by sequestering CBP/p300 away from AP-1 and onto sites where the nuclear receptors are bound (Kamei et al., 1996). Similarly, the anti-adenoviral actions of interferon are attributed to interferon's ability to activate STATs, which then sequester CBP/p300 away from the adenoviral transcription factor E1A (Horvai et al., 1997).

Our findings suggest that during early cortical development, endogenous Ngn1 associates with both CBP and Smad1, and that the presence of neurogenin blocks STAT binding to CBP. During the course of our study, it was reported that *Xenopus* neurogenin recruits CBP/p300 to the NeuroD promoter to activate transcription and induce neurogenesis. The characterization of the domains of CBP that interact with neurogenin revealed that both an N- and a C-terminal domain are involved (Koyano-Nakagawa et al., 1999). Interestingly, the neurogenin binding domains of CBP overlap with the STAT binding sites on CBP (but not with the Smad binding sites). This is consistent with our finding that neurogenin competes with STAT proteins for binding to CBP.

By sequestering CBP, neurogenin may not only inhibit STAT-mediated transcription, but may also inhibit the function of other CBP-dependent transcription factors. We have found that Ngn1 also inhibits AP-1-dependent transcription (Supplemental Data is shown on the *Cell* website at http://www.cell.com/cgi/content/full/104/3/365/DC1). This may be relevant to Ngn's ability to inhibit astrocyte differentiation since our analysis of the GFAP promoter identified multiple sites, including an AP-1 site, that contribute to neurogenin's inhibition of the GFAP promoter. Taken together, our findings and those of others suggest that CBP/p300 may orchestrate broad programs of gene expression that are relevant to cell fate determination. The effect of CBP/p300 on cell fate may then be determined by the relative binding affinity

and abundance of different transcription factors that either compete or cooperate with one another for binding to CBP/p300.

Neurogenin Inhibits STAT Phosphorylation

In addition to sequestering the CBP-Smad1 complex, neurogenin also inhibits the activation of astrocyte-specific genes by blocking STAT activation. The mechanism by which Ngn1 reduces the level of phospho-STAT1 and -STAT3 is unknown. We have preliminary data suggesting that the AQ-Ngn1 can also inhibit STAT phosphorylation, though not to the extent seen with wild-type Ngn1. This suggests that Ngn1 inhibits STAT phosphorylation only in part by a mechanism that is independent of Ngn1 binding to DNA. A more detailed characterization of the various mechanisms by which Ngn1 inhibits STAT phosphorylation is currently underway.

Neurogenin Expression May Determine the Timing of Neuronal and Glial Generation

The dual functions of neurogenins may help to explain why neurogenesis precedes and is dominant over glial differentiation during cortical development, even when gliogenic factors such as LIF or BMP are present (Shah and Anderson, 1997; Park et al., 1999). The completion of neurogenesis is marked by neurogenin downregulation, allowing progenitor cells to now respond to glial-inducing factors (LIF, BMPs) to produce astrocytes. In this way, the temporal control of neurogenin expression may orchestrate the sequential onset of cortical neuronal and glial differentiation. One would predict that in the absence of neurogenin, cortical gliogenesis might commence at an earlier time during development. One way of inhibiting neurogenic bHLH factors is by activating the Notch signaling pathway (Ma et al., 1996, 1997; Kageyama et al., 1997). When constitutively activated Notch is introduced into the developing cortex, this results in premature gliogenesis (Gaiano et al., 2000; Morrison et al., 2000).

Another way of inhibiting neurogenin function is to generate neurogenin null mutant mice. Unfortunately, the Ngn1/Ngn2 double knockout shows a dramatic upregulation of the related bHLH protein, Mash1, in cortical progenitor cells (Fode et al., 2000). Guillemot's group has recently analyzed the cortex of the Ngn2/Mash1 double knockout and found that the cortex of the double mutants has increased numbers of glial progenitors, and that astrocytes are generated earlier during development (Nieto et al., 2001 [February issue of *Neuron*]). In addition, in Math3/Mash1 double knockouts, precocious glial differentiation was observed (Tomita et al., 2000). These findings suggest that endogenous neurogenins and related bHLH proteins regulate the timing of glial production in the in vivo cortex.

Implications of This Work for Neural Stem Cell Studies

Multipotent neural stem cells have been isolated from many regions of the adult brain (Gage et al., 1995). Recent evidence suggests that the neural stem cells arise from ependymal and/or GFAP-positive cells (Doetsch et al., 1999; Johansson et al., 1999). It is possible that neurogenic bHLH factors are involved in suppressing the astrocytic features of the GFAP-positive cells so that these cells can give rise to neurons. However, in the adult brain, outside of the hippocampus and the subventricular zone (which gives rise to the olfactory rostral migratory stream), neural stem cells rarely give rise to neurons, possibly because these cells express very low levels of neurogenic bHLH factors. We propose that the manipulation of neurogenic bHLH factors within neural stem cells may provide a means of enhancing the ability of stem cells to generate large numbers of neurons that might be useful for treating neurodegenerative disorders or repairing the injured nervous system.

Experimental Procedures

Cell Culture

Timed pregnant Long-Evans rats (from Charles River) were used to prepare E13/14 or E17/18 cortical cultures as described in Bonni et al. (1997). Cortical stem cells were derived from E13/14 cortices, as described in Johe et al. (1996), and cells from a second or third passage were used for differentiation studies in the absence of bFGF. LIF/CNTF (50 ng/ml, Regeneron, and R&D Systems) or BMP7 (100 ng/ml) (Curis) were used for astrocyte differentiation. Mature astrocyte cultures were generated from passaged E17/18 cortical cells.

Retro- and Adenoviral Constructs and Infection Conditions

Ngn1 cDNA (obtained from Dr. Quifu Ma and Dr. David Anderson, Cal. Tech.) was cloned into a retroviral vector containing IRES-GFP and packaged accordingly. The Ngn1-expressing adenoviral constructs (the wild-type and mutants), all containing six myc tags at the N terminus, were cloned into an adenoviral shuttle vector containing a GFP expression cassette, pMZL6 (M. L. and M. E. G., unpublished data). Recombinant adenoviruses were made by co-transfection of the shuttle plasmids with the plasmid pBHG10 into HEK293 cells.

Immunocytochemistry and Antibodies Used

The immunostaining procedures are as described in Bonni et al. (1997). In addition, the following antibodies were used: a rabbit anti-GFAP antibody (Accurate), a rabbit anti-GFP antibody (Clontech), and a monoclonal anti-myc tag antibody (Santa Cruz, 9E10).

Transient Transfections and Luciferase Reporter Assays

Ngn1 and the various *Ngn1* mutants that we generated were all cloned into pcDNA3 (Invitrogen). The C-terminal flag-tagged CBP expression construct was provided by Dr. M. Rosenfeld (UCSD); the p300 expression construct by Dr. Xi He (Children's Hospital, Boston, MA); the Smad1 expression constructs by Dr. M. Whitman's lab (Harvard Medical School); and the NeuroD promoter series by Dr. M. J. Tsai (Baylor). For transient transfections, we used Fugen-6 (Boehringer Mannheim) for neural stem cells and calcium phosphate for primary cortical cultures and 293T cells. The dual luciferase assay (Promega) was done according to the company's recommendations.

Immunoprecipitation and Western Blot Analysis

For the coimmunoprecipitation assays, the cells were lysed in 0.7% NP40 lysis buffer and standard Western blot analysis was performed. The antibodies used for co-IP and Westerns were as follows: AC238, a mouse anti-CBP (gift from Drs. R Eckner & D. Livingston, Harvard Medical School); a rabbit anti-Smad1 (UBI); a mouse anti-p53 (Ab-6 from Oncogene); a mouse anti-STAT1 and mouse anti-STAT3 (Signal Transduction); and a mouse anti-GFAP (Sigma). The rabbit anti-Ngn1 antibody used to detect endogenous Ngn1 and the rabbit anti-phospho STAT1 and anti-phospho STAT3 were all generated in the Greenberg Lab.

Northern Analysis
Northern analysis was performed as described previously (Bonni et al., 1997). The s100β probe was generated using the s100β cDNA sequence in the GenBank.

In Vitro Transcription, Translation, and EMSA
The TNT-T7 kit (Promega) was used to in vitro transcribe and translate the various *Ngn1* cDNAs together with E47 cDNA (gift of Dr. A. Lassar, Harvard Medical School). The electric gel mobility shift assay (EMSA) was performed as described in (Peyton et al., 1994) using the RIPE3 probe.

Acknowledgments

We thank Qiufu Ma for providing us with the neurogenin1 construct, and for his many insightful discussions. Many thanks to Zuohua Zhang and Linda Hu for their support and technical advice with the neurogenin antibody. We thank Anne West, Janine Zieg, Qiufu Ma, David Anderson, and Gord Fishell for their critical reading of the manuscript and Adam Shaywitz for his helpful advice regarding p300/CBP. We also acknowledge Drs. D. Anderson, M. Rosenfeld, X. He, M. J. Tsai, I. Rozovsky, M. Whitman, J. Massagué, A. Lassar, R. Eckner, D. Livingston, F. Mariani, and R. Harland for providing critical reagents for our studies. Y. S. has been supported by both the Medical Foundation/Charles H. Hood Foundation and an NIH N.R.S.A. grant during the course of the study; M. N. V. is a Quan Fellow. This research has been supported by an NIH RO1 grant (CA43855) to M. E. G. and MRRC grant (NIHP30-HD 18655).

Received August 29, 2000; revised January 8, 2001.

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