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Notch Signaling Promotes Astrogliogenesis Via Direct CSL-Mediated Glial Gene Activation

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In the developing central nervous system (CNS), Notch signaling preserves progenitor pools and inhibits neurogenesis and oligodendroglial differentiation. It has recently been postulated that Notch instructively drives astrocyte differentiation. Whether the role of Notch signaling in promoting astroglial differentiation is permissive or instructive has been debated. We report here that the astrogliogenic role of Notch is in part mediated by direct binding of the Notch intracellular domain to the CSL DNA binding protein, forming a transcriptional activation complex onto the astrocyte marker gene, glial fibrillary acidic protein (GFAP). In addition, we found that, in CSL–/– neural stem cell cultures, astrocyte differentiation was delayed but continued at a normal rate once initiated, suggesting that CSL is involved in regulating the onset of astrogliogenesis. Importantly, although the classical CSL-dependent Notch signaling pathway is intact and able to activate the Notch canonical target promoter during the neurogenic phase, it is unable to activate the GFAP promoter during neurogenesis. Therefore, the effect of Notch signaling on target genes is influenced by cellular context in regulation of neurogenesis and gliogenesis.

Key words: Notch; Delta; CSL proteins; neural stem cells; astrocytes

The mammalian cerebral cortex originates from a single layer of neuroepithelial cells (Bayer and Altman, 1991). These common progenitor cells (also referred to as *neural stem cells*; NSC) proliferate and sequentially produce the three major cell types of the brain: neurons, astrocytes. and oligodendrocytes (Qian et al., 2000). In rat and mouse cortices, neuro- and glio-genesis occur over a 2 week period perinatally, with birth marking the end point for neurogenesis and the beginning of glial differentiation (Qian et al., 2000). Throughout the developing nervous system, common progenitor cells exhibit this temporal fate switch in which they appear first to enter a neurogenic phase by turning on a gene expression program specific for neurogenesis, then later switch into a gliogenic phase by activating distinct patterns of gene expression specific for glial differentiation. Environmental cues as well as cell-intrinsic properties play active roles in this fate switch process (Sun et al., 2001; Takizawa et al., 2001).

The molecular mechanisms underlying cell fate specification in multipotent progenitor cells in the developing central nervous system (CNS) are beginning to be illuminated. Neurogenic basic helix-loop-helix (bHLH) transcription factors, including neurogenin1/2 and Mash1, not only promote neuronal cell fate specification but also inhibit precocious glial differentiation both in vitro and in vivo (Furukawa et al., 2000; Nieto et al., 2001; Novitch et al., 2001; Satow et al., 2001; Sun et al., 2001; Zhou et al., 2001). On the other hand, the cytokine leukemia inhibitory factor (LIF) promotes astroglial differentiation in vitro and in vivo via the receptor (LIFRβ and gp130 subunits)-activated JAK-STAT signaling pathway (Johe et al., 1996; Bonni et al., 1997). The role of LIF in astrocyte differentiation in vivo is substantiated by a number of knockout studies. In LIF–/–, LIFRβ–/–, gp130–/–, and STAT3–/– mice, astrocyte differentiation is impaired (Bugga et al., 1998; Koblar et al., 1998; Nakashima et al., 1999a; Fan, Sun and Levy, unpublished observations). These data strongly suggest that the JAK-STAT pathway is one of the major astrogliogenic pathways during CNS development. In addition to the JAK-STAT pathway, Notch signaling may also play a role in astrogliogenesis.

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Despite the inhibitory effects of Notch signaling on neuronal and oligodendroglial differentiation, this signaling pathway has recently been reported to instructively drive satellite glial cell differentiation in peripheral neural crest stem cells and to promote astrocyte differentiation in adult hippocampal NSCs (Morrison et al., 2000; Tanigaki et al., 2001).

Notch is an ancient protein used by organisms ranging from worms and flies to vertebrates in controlling multiple aspects of development (Ghysen et al., 1993; Artavanis-Tsakonas et al., 1995, 1999). During mammalian cerebral cortical development, Notch signaling is implicated early on in prevention of precocious neurogenesis/preservation of progenitor pools. At a slightly later time, it causes a transient cell cycle arrest. Further along in development, at perinatal stages, Notch signaling increases progenitor proliferation and astrocyte differentiation, and postnatally it promotes dendritic branching while inhibiting dendritic growth of neurons (Sestan et al., 1999; Gaiano et al., 2000; Redmond et al., 2000; Chambers et al., 2001; Noctor et al., 2001). These pleiotropic effects of Notch signaling are likely cell context dependent.

The Notch gene encodes a 300 kD, single transmembrane protein that is constitutively cleaved by a convertase of the furin family to generate a heterodimeric cell surface receptor (Blaumueller et al., 1997; Logeat et al., 1998; Rand et al., 2000). Upon binding to Notch ligands (Delta and Jagged), the heterodimeric Notch undergoes two sequential proteolytic cleavage events, first by tumor necrosis factor-α-converting enzyme (TACE) and then by γ-secretase, the enzymatic function of which requires proteins such as presenilin1/2 and nicastrin (De Stooper et al., 1999; Song et al., 1999; Struhl and Greenwald 1999; Ye et al., 1999; Brou et al., 2000; Yu et al., 2000). Cleavage by γ-secretase causes the Notch intracellular domain (NICD) to be released from the membrane, allowing its translocation to the nucleus (Artavanis-Tsakonas et al., 1995, 1999; Kopan and Turner, 1996; Weinmaster, 1997). Through its RAM domain and ankyrin repeats, NICD associates with the DNA binding protein CSL (for CBF-1, suppressor of hairless, Lag-1, or RBPj-κ) and the CSL partner SKIP, turning CSL-SKIP from a transcriptional repressor into an activator, stimulating the transcription of target genes, such as Hes1 (for hairy and enhancer of split) and Hes5, that function to inhibit neurogenesis (Brou et al., 1994; Kageyama and Nakanishi, 1997; Ohtsuka et al., 1999; Zhou et al., 2000; Beatus et al., 2001). In addition to this highly conserved, canonical CSL-dependent Notch signaling pathway, recent findings in *Drosophila* and mammalian systems suggest the existence of novel Notch signaling pathways that do not require CSL-family proteins (Shawber et al., 1996; Ligoxygakis et al., 1998; Ordentlich et al., 1998; Brennan et al., 1999; Nofziger et al., 1999; Zecchini et al., 1999; Ramain et al., 2001; Bush et al., 2001; Yamamoto et al., 2001).

Although Notch signaling clearly can enhance astrocyte differentiation, it is not clear whether its role is an instructive or permissive one. One possibility is that, by inhibiting neurogenesis, Notch signaling preserves the progenitor pool so that remaining cells can respond to other astrogliogenic cues and subsequently differentiate into astrocytes. Alternatively, Notch might instructively trigger an astrogliogenic pathway by directly activating the transcription of astroglia-specific genes. Through studying the molecular mechanisms by which Notch signaling activates glial gene expression, we have obtained evidence to support a role for the canonical CSL-dependent pathway in direct control of glial genes. Moreover, we found that this direct CSL-dependent pathway is nonfunctional on a glial-reporter gene during the neurogenic period, suggesting the existence of additional modulations of this Notch astrogliogenic signaling pathway that are likely cell context dependent.

MATERIALS AND METHODS

Rat and Mouse Cortical NSC Culture and the Differentiation Assay

Embryonic day 13 (E13) Long Evans rat or E11.5 CD1 mouse cortices were dissected out in Hank's balanced salt solution (HBSS; Gibco-Invitrogen, Grand Island, NY). Cortices were isolated and dissociated mechanically by trituration using a flame-polished pasture pipet. Dissociated cells were plated onto polyornithine (PO; 15 μg/ml in H_2O)- and fibronectin (FN; 2 μg/ml in phosphate-buffered saline; PBS)-coated 10 cm dishes at a density of 1 million cells/dish in serum-free medium [DMEM/F12 supplemented with B27 supplement, penn/strep (50 μg/ml and 50 U/ml, respectively), and basic fibroblast growth factor (bFGF; 10 ng/ml; Becton Dickinson, San Jose, CA)]. bFGF at a final concentration of 10 ng/ml was added to the culture every day to expand the progenitor cell population. After 5–7 days in culture, the cells were scraped off the plate and plated onto a new PO/FN-coated plate at a density of 1 million cells/dish and cultured in the same condition as the primary cells. After two passages (1–2 weeks), the cultures were relatively homogenous, composed predominantly of nestin-positive multipotent neural progenitor/stem cells (Sun et al., 2001). For immunocytochemistry analyses of differentiated NSCs, cells at various passages were plated on PO/FN-coated glass coverslips at a density of 25,000–50,000 cells per well in a Costar 24 well plate. bFGF was added once at the time of plating. Usually, 4 days after culturing, without further bFGF supplement, cells were fixed and subjected to immunostaining.

DNA Constructs and Reagents

Antibody-clustered Delta1, a Notch ligand, was obtained as follows: 293T-con-Fc and 293T-Delta1-Fc cell lines were cultured in 10 cm dishes with DMEM-high-glucose medium containing 10% fetal bovine serum, penn/strep, and glutamine. Hygromycin B (200 μg/ml) was used to select the Fc-expressing cells (Morrison et al., 2000). When cells became confluent, the culture medium was replaced by 5 ml DMEM-high-glucose medium without serum and conditioned for 5 days. The conditioned medium was concentrated in Amicon Centriplus Cen-

trifugal Filter Devices (Millipore, Bedford, MA) with a molecular weight cutoff of 10 kD. After one wash with 5 ml fresh DMEM/F12 medium, the conditioned medium (CM) was concentrated 50–100-fold. The concentrated CM was used at a 1:50 dilution. The goat anti-human Fc antibody (1.8 mg/ml; catalog No. 109-005-098) was used at 100× as described by Morrison et al. (2000).

The deletion series of GFAP-pGL2, GFAP-pGL3, and the dominant interfering form of STAT3 expressing constructs were previously described (Bonni et al., 1997; Sun et al., 2001). The CMV-pRL or TK-pRL *Renilla* luciferase constructs, used as transfection controls in the dual luciferase assays, were obtained from Promega (Madison, WI). The CSL-pGL3 construct, the series of NICD wild-type and mutant constructs, and the wild-type and the dominant interfering form of CSL expressing constructs have been previously described (Shawber et al., 1996; Bush et al., 2001). The γ-secretase inhibitor DAPT, obtained from Dr. Michael Wolfe, Harvard University, was used at a final concentration of 5 μM. The CSL binding site mutation of the GFAP-pGL3 construct was made through the Quick-Change kit (Stratagene, La Jolla, CA). A *Xenopus* CSL expression construct with T7 promoter at its 5′ region was obtained from the Weinmaster laboratory and was used for in vitro transcription and translation (TNT kit from Promega) to generate CSL proteins for electrophoretic mobility shift assay (EMSA). The EMSA was performed as described previously (Sun et al., 2001). Human recombinant LIF (used at 50 ng/ml) was purchased from R&D Systems (Minneapolis, MN).

Dual Luciferase Reporter Assay

To study the promoter activity, NSCs were plated onto PO/FN-coated 96 well plates at a density of 2–4 million cells/plate. Cells were cotransfected with promoters of interest driving the firefly luciferase (pGL2 or pGL3) and either a constitutively active TK promoter or a CMV promoter driving the *Renilla* luciferase (TK-pRL, CMV-pRL) as internal controls for transfection efficiency. The Fugen-6 transfection method (from Roche) was used. Usually, 24 hr after transfection, cells were lysed, and promoter activities were assayed using the Promega dual-luciferase assay kit.

Immunocytochemistry

Cells were routinely fixed with methanol/acetone (v/v 1:1) at room temperature for 2 min. After PBS washes, cells were permeablized in PBS with 0.4% Triton X-100 at room temperature for 30 min to 1 hr or at 4°C overnight. For staining, the fixed and permeablized cells were incubated in a blocking buffer, Tris-buffered saline (TBS) with 0.02% Tween-20 plus 10% milk and 1% normal goat serum, at room temperature for 1 hr. Primary antibodies were diluted with a dilution buffer [TBS with 0.02% Tween-20 plus 3% bovine serum albumin (BSA)] and added to the cells after incubation with the blocking buffer. Cells were usually incubated with primary antibodies at 4°C overnight and were then washed with PBS before incubation with flourescent dye-conjugated secondary antibodies. This incubation was performed at room temperature for 2 hr. After the final PBS wash, coverslips were mounted onto glass slides with a mounting solution containing an antiflourescent bleaching reagent, n-propyl-gallate (5% in PBS:glycerol, 1:1). A monoclonal mouse anti-GFAP antibody was used at 1:400 to stain astrocytes. A mouse monoclonal anti-S100β antibody (Sigma, St. Louis, MO; 1:400) was also used to label astrocytes. A polyclonal rabbit anti-nestin (a gift from Dr. Mckay at NINDS, used at 1:2,000) antibody was used to stain neuoepithelial precursor cells. DAPI staining was used to label nuclei. Bromodeoxyuridine (BrdU) labeling was used to monitor cell proliferation. Briefly, cultured cells were incubated with 10 μM BrdU overnight in culture before fixation. After fixation with 4% paraformaldehyde or methanol/acetone, cells were postfixed with 70% ethanol, permeablized with 0.4% Triton X-100, treated with 2 N HCl, then neutralized with $Na_2B_4O_7$. After PBS washes, the staining procedure continued as described above. The rat anti-BrdU antibody (Harlan Sera Labs, Indianapolis, IN; used at 1:500) was used to label BrdU. Cy3- or Alexa-conjugated goat anti-rabbit antibodies and Cy2- or Cy3-conjugated goat anti-mouse antibodies were all purchased from Jackson Immunoresearch (West Grove, PA). The biotinolated goat anti-rat antibody was used for BrdU staining. Cy3-conjugated streptavidin was used to visualized the biotinolated secondary antibody.

ES Cell-Derived NSC Cultures

Wild-type and RBP-J/CSL(–/–) ES cells, obtained from Dr. Honjo's laboratory (Japan), were cultured to generate neural stem cells (Oka et al., 1995). The procedure is as follows: ES cells were first cultured in DMEM/F12-B27 serum-free medium at a density of 40,000/ml in an uncoated tissue culture flask with 50 ng/ml LIF for 1 week. After 1 week in LIF, most cells form spheres, which contain many nestin-positive neural epithelial progenitor/stem cells (passage 0; P0). These spheres were then plated onto PO/FN-coated 10 cm plates and cultured in serum-free medium supplemented every day with 10 ng/ml bFGF for an additional 1 week. These cells were considered passage 1 (P1) cells. During this week, NSCs migrate out of the spheres and form a monolayer. From then on, the P1 cells were scraped off the plate, triturated, and plated onto new PO/FN-coated plates and cultured as described for cortical NSCs. During each passage, a cohort of cells was subjected to differentiation as described above for cortical NSCs. Another portion of the cells was cryopreserved. To compare the differentiation status of ES cell-derived NSCs (ES-NSCs) at different passages, cryopreserved ES-NSCs at different passages were thawed and differentiated at the same time.

RESULTS

Delta1-Fc Promotes Astrocyte Differentiation and Activates the GFAP Promoter

Delta1-Fc has been described previously and used to inhibit the differentiation of oligodendrocyte precursor cells (OPC) and to induce the differentiation of nonmyelinating Schwann cells through ligand-dependent activation of Notch signaling (Wang et al., 1998; Morrison et al., 2000). In 2–3 week bFGF-propagated E13 rat or E11.5 mouse cortical NSC cultures, treatment with antibody-clustered Delta-Fc led to enhanced astrocyte differentiation compared with cells treated with antibody-clustered control Fc (con-Fc; Fig. 1A–C). NSCs were

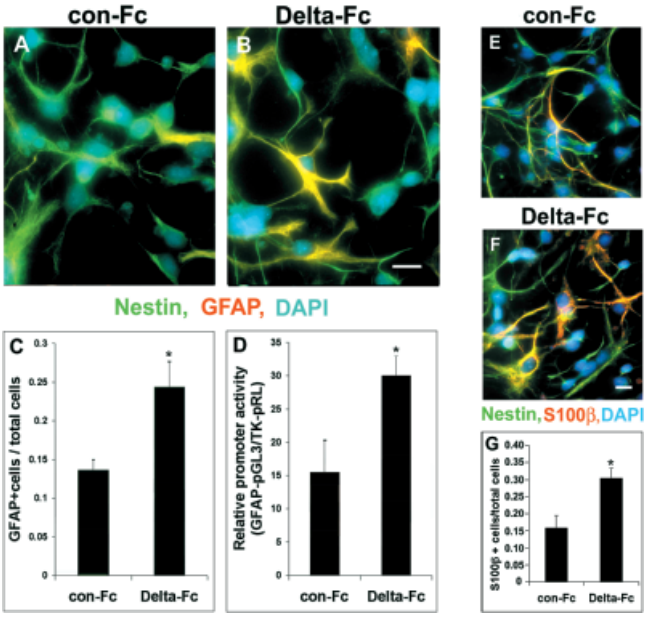

Fig. 1. Delta-Fc promotes astroglial differentiation and activates the GFAP promoter in E13 rat cortical NSCs; 2–3 week bFGF-propagated E13 rat cortical NSCs were cultured in medium supplemented with either Delta1-Fc (**B**) or control-Fc (**A**) for 4 days. Cells were then fixed and doubly labeled with an antibody (Ab) against a neuroepithelial progenitor marker, nestin (red), and an Ab against an astrocyte marker, GFAP (green). DAPI was used to stain the nuclei (blue). The quantification of the proportion of cells that differentiated into astrocytes is shown in **C**. In **D**, 2–3 week bFGF-propagated E13 rat cortical NSCs were transfected together with the GFAP promoter-reporter construct and the transfection control, the TK promoter-reporter construct. At the time of transfection, cells were treated with either con-Fc or Delta1-Fc. Twenty-four hours after transfection/treatment, promoter activities were measured and are shown in D. **E,F:** The same rat NSC cultures treated with con-Fc or Delta-Fc for 4 days were fixed and stained with an antibody against S100β (Sigma). The quantification of the data is presented in **G** ($\star P < 0.05$; n = 5 in C, n = 4 in D, n = 5 in G). Scale bars = 19 μm.

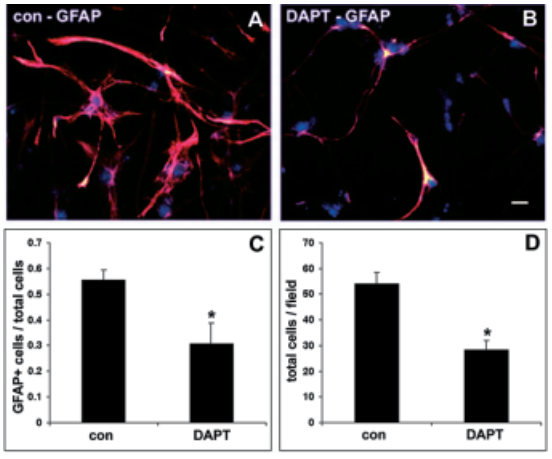

Fig. 2. A γ-secretase inhibitor, DAPT, inhibits endogenous astrocyte differentiation in E11.5 mouse cortical NSCs; 2–3 week bFGF-propagated E11.5 mouse cortical NSCs underwent differentiation upon withdrawal of bFGF in the presence and absence of DAPT (5 μM) for 4 days. Cells were fixed and stained with an antibody against GFAP (red in **A,B**). The proportion of cells that differentiated into astrocytes in the presence and absence of DAPT is shown in **C**. Total cell numbers per optic field are shown in **D** ($\star P < 0.05$; n = 5 in C,D). Scale bar = 19 μm.

routinely labeled with an antibody against a neuroepithelial precursor marker, the intermediate filament protein nestin (Fig. 1A,B). After Delta-Fc treatment for 4 days, the number of GFAP-positive, stellate-appearing astrocytes increased almost two-fold (Fig. 1B,C). In addition, we found that the number of cell positive for another astrocyte marker, S100β, also increased almost two-fold, indicating that activation of the Notch signaling pathway enhances astroglial differentiation in embryonic cortical NSCs.

Delta-Fc not only enhances astrocyte differentiation of cortical NSCs, it also activates transcription from the 1.9 kb rat GFAP promoter (Bonni et al., 1997). Two or three week bFGF-propagated rat cortical NSCs were transfected with an astrocyte-specific reporter that contains the 1.9 kb GFAP promoter sequence upstream of the fly luciferase cDNA. Immediately after transfection, cells were treated with Delta-Fc or con-Fc, and 24 hr later luciferase activity was measured as a readout for the GFAP promoter activity. As shown in Figure 1D, treatment of Delta-Fc led to a twofold induction of the GFAP reporter construct, suggesting that the 1.9 kb GFAP promoter sequence contains responsive elements for Notch signaling.

Previous reports on Notch signaling inducing astroglial or nonmyelinating Schwann cell differentiation were all based on gain-of-function studies. To address whether the endogenously activated Notch pathway is involved in astrocyte differentiation of NSCs, we treated 2–3 week bFGF-propagated cortical NSCs with a γ-secretase inhibitor, DAPT, at a concentration of 5 μM to prevent the γ-secretase cleavage of Notch and its resultant downstream signaling. Despite the fact that γ-secretase also cleaves amyloid precursor protein (APP), we assume that the effects of DAPT on astrocyte differentiation are through inhibition of Notch signaling, because APP has not been implicated in astrogliogenesis. Cortical NSCs were allowed to differentiate upon withdrawal of the mitogen bFGF. When cultures were treated with DAPT for 4 days, there was a decrease in the percentage of cells that differentiated into astrocytes (Fig. 2A,B). The extent of astrocyte differentiation was measured by the percentage of GFAP-positive cells over total cells (Fig. 2C). DAPT treatment led to a reduction of astrocyte differentiation even though this drug also caused a decrease in total cell numbers (Fig. 2D). Although the reduction in cell numbers might result from a nonspecific cytotoxicity of the drug, no obvious cytotoxicity in 24 hr DAPT-treated cultures was observed (see below). Therefore, it seems likely that DAPT inhibition of Notch signaling caused a

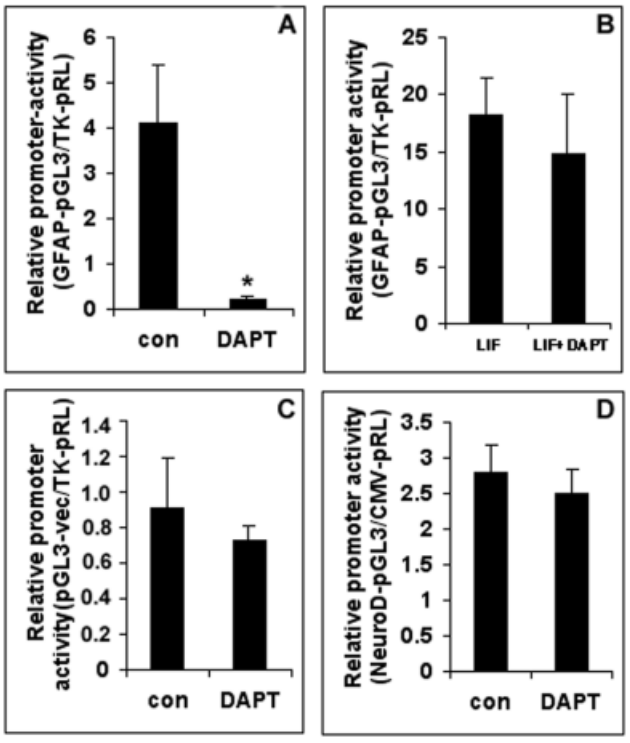

Fig. 3. DAPT inhibits the GFAP promoter activity; 2–3 week bFGF-propagated E11.5 mouse cortical NSC were transfected with the GFAP or neuroD promoter-reporter construct (**A,B,D**). The pGL3 luciferase empty vector and the neuroD promoter-reporter were used as controls to assess the toxicity of DAPT (**C**,D). Immediately after transfection, cells were treated with DAPT, LIF, or both. Twenty-four hours after transfection or drug treatment, cells were lysed and subjected to dual luciferase assays. Either the TK-pRL or the CMV-pRL was used as an internal control for transfection efficiency. Normalized promoter activities are shown ($\star P < 0.05$; n = 6 for all).

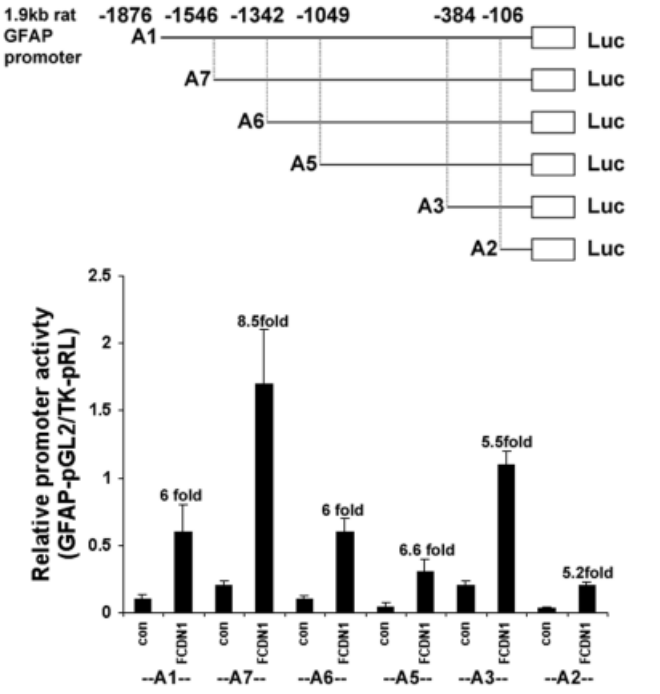

Fig. 4. Activated Notch (FCDN1) activates the entire deletion series of the GFAP promoter; 2–3 week bFGF-propagated E13 rat cortical NSCs were cotransfected with activated Notch (NICD/FCDN1) and a deletion series of the GFAP promoter-reporter construct. Twenty-four hours posttransfection, cells were lysed, and a dual luciferase assay was performed. The relative promoter activities of each promoter construct are shown, and the number-fold induction of the promoters by FCDN1 is indicated in the bar graph (all of the FCDN1-caused increases in promoter activities were statistically significant at $P < 0.05$).

decrease in cell proliferation and/or cell survival, resulting in reduced numbers of total cells.

Consistently with the inhibition of astrocyte differentiation, DAPT treatment also led to a significant reduction in transcriptional activation from the 1.9 kb GFAP promoter (Fig. 3A). Since DAPT treatment did not cause a reduction of luciferase activity when cells were transfected with the pGL3 vector construct or the neurogenic neuroD promoter-luciferase construct, the reduction of the promoter activity is unlikely due to the potential cytotoxicity of the drug (Fig. 3C,D). Interestingly, DAPT-induced inhibition of the GFAP promoter was not detected when cells were treated with the potent astrocyte-inducing factor LIF (Fig. 3B), suggesting that the LIF-induced JAK-STAT pathway can override the effect of DAPT on astroglial differentiation. Therefore, either the JAK-STAT pathway is downstream of the Notch pathway or it is a parallel, but more potent, astroglialgenic pathway. Taken together, our data suggest that the endogenous Notch pathway is involved in astrocyte differentiation of bFGF-propagated cortical NSCs in culture.

Notch Signaling Enhances the Activation of the GFAP Promoter in Part Via Direct CSL-Mediated Transcriptional Activation

To investigate the mechanism by which Notch signaling enhances GFAP promoter activity, we performed a promoter-deletion analysis to identify the DNA elements within the GFAP promoter that are responsive to Notch signaling. To obtain a more robust induction of the GFAP promoter-reporter gene for promoter-deletion analysis, a previously described ligand-independent, constitutively activated form of Notch (FCDN1), instead of the ligand Delta-Fc, was used. Overexpression of FCDN1 in adult hippocampal NSCs has been shown to instructively induce astroglial differentiation (Tanigaki et al., 2001). FCDN1 overexpression in our NSCs also appears to enhance endogenous GFAP expression (data not shown). When the FCDN1 expression construct and a deletion series of the GFAP promoter-luciferase reporter constructs were cotransfected into cortical NSCs, as shown in Figure 4, the entire deletion series was responsive to activated Notch (FCDN1). Strikingly, the A2 construct that contains only 106 bp of the promoter region immediately upstream of the transcription initiation site was activated

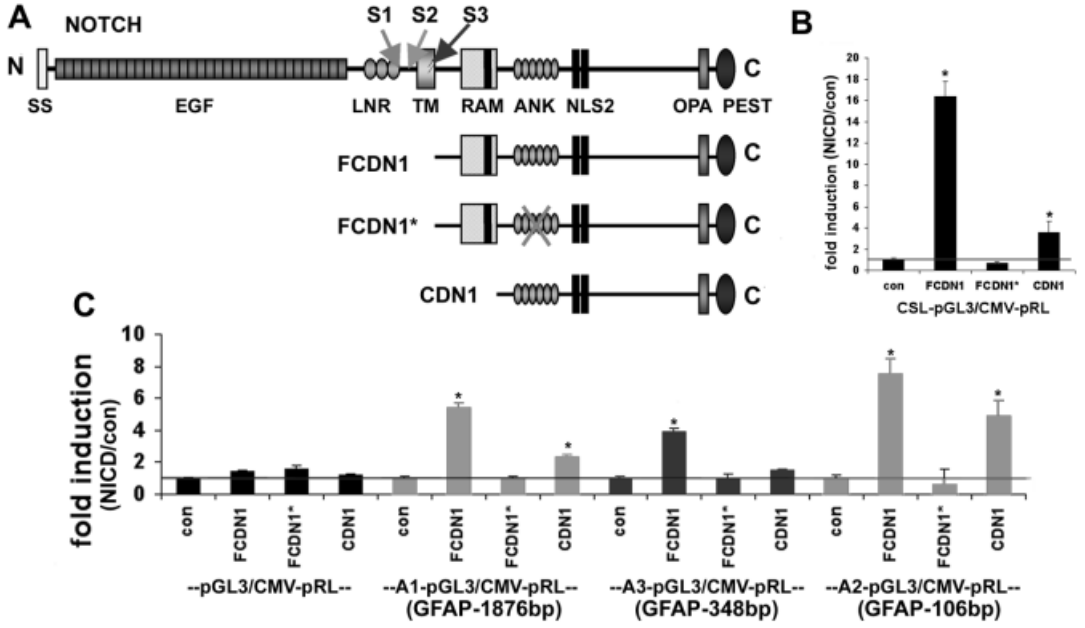

Fig. 5. Activated Notch (FCDN1) activates the GFAP promoter partly depending on CSL. **A:** Diagram of the structure of Notch with its intracellular domain (NICD/FCDN1) and two mutants of NICD that do not interact with CSL (the ankyrin repeat mutant FCDN1★ and the RAM domain deletion mutant CDN1). The functions of FCDN1, FCDN1★, and CDN1 on the CSL promoter in 2–3 week bFGF-propagated E11.5 mouse cortical NSC are shown in **B**. Their actions on the GFAP (full 1.9 kd promoter, A1), A3, and A2 (see Fig. 5) promoters are shown in **C**. The pGL3 luciferase reporter empty vector was used as a control (C; $\star P < 0.05$ vs. control in the same promoter set, n = 24).

by FCDN1. This 106 bp promoter segment does not contain any putative CSL binding sites, suggesting that activated Notch may act on this 106 bp promoter segment and activate gene transcription either directly through a CSL-independent mechanism or indirectly through a CSL-dependent mechanism. Moreover, additional Notch-responsive elements may also exist more 5′ within the 1.9 kb GFAP promoter.

To examine further whether Notch signaling activates the GFAP promoter through the canonical CSL-dependent pathway, we used two mutant forms of FCDN1, one with a mutation in the ankyrin repeats (FCDN1★) and the other missing most of the Ram domain (CDN1; Fig. 5A; Shawber et al., 1996). The ankyrin repeat mutant does not activate Notch-responsive genes because it fails to bind SKIP, a critical partner for CSL in mediation of transcription (Zhou et al., 2000). The Ram domain promotes a strong interaction between CSL and the Notch intracellular domain. Removal of the Ram domain sequence as occurs in the CDN1 construct results in weak activation of CSL (Shawber et al., 1996; Nofziger et al., 1999; Redmond et al., 2000). In mouse cortical NSCs, as expected, FCDN1★ failed to activate the Notch canonical target promoter, namely, the synthetic CSL promoter, and CDN1 only weakly activated the CSL promoter (Fig. 5B).

In a manner similar to that of the synthetic CSL promoter, the GFAP promoter was activated effectively by FCDN1, weakly by CDN1, but not by FCDN1★ (Fig. 5C). This induction in luciferase activity is promoter specific, because none of these Notch1 mutant constructs increased luciferase activity when coexpressed with the pGL3 luciferase reporter vector construct (Fig. 5C). This finding suggests that a CSL-dependent mechanism is involved in mediating the effect of Notch on the GFAP promoter, because FCDN1 is more effective than CDN1, and FCDN1★ is inactive. Interestingly, with the 106 bp A2 construct that does not contain any putative CSL binding sites, CDN1 appears to be relatively more active (Fig. 5C). This suggests that the mechanism by which Notch signaling activates the 106 bp promoter segment might differ from that involved in activation of the 1.9 kb GFAP promoter.

Sequence analysis of the 1.9 kb GFAP promoter identified a putative CSL binding site located at a −183 bp (5′-TTCCCAGG-3′) to −176 bp position (considering transcription initiation site at the +1 position. Substitution mutation of this putative CSL binding site (TT*CC*CAGG to TT*AA*CAGG) rendered FCDN1 less effective at activating the mutant promoter (Fig. 6A). Moreover, using EMSA, we demonstrated that an in vitro-transcribed and -translated *Xenopus* CSL protein indeed can bind to the putative CSL element within the GFAP promoter (TT*C-C*CAGG; Fig. 6B). When the element was mutated into TT*AA*CAGG, the *Xenopus* CSL failed to bind to the mutated DNA element (Fig. 6B). Taken together, these data suggest that FCDN1 transcriptionally activates GFAP through direct interactions with CSL and the CSL binding

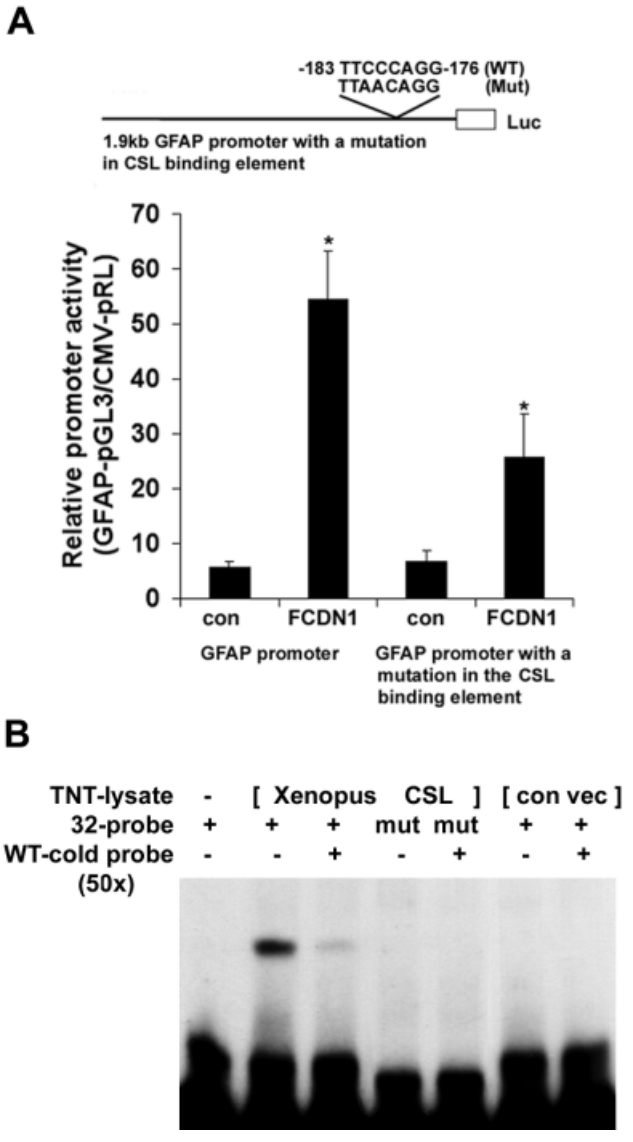

Fig. 6. Mutation of the –183 bp putative CSL binding site in the GFAP promoter renders the promoter less responsive to activated Notch (FCDN1). The putative CSL binding site in the GFAP promoter at the –183 bp position (5′-TT*C*CCAGG3-′) was mutated into (5′-TT*AA*CAGG3-′). **A:** In 2 week bFGF-propagated mouse cortical NSCs, the mutated GFAP promoter is less responsive to activated Notch (FCDN1; $^{\star}P < 0.05$ for the FCDN1 group comparison between wild-type promoter and mutant promoter; n = 6). **B:** EMSA showing binding of in vitro-translated and -transcribed *Xenopus* CSL (using the Promega TNT kit) to the putative CSL binding site within the GFAP promoter. Note that empty expression vector for the *Xenopus* CSL expression construct was also used in the TNT reaction and served as a control for in vitro-translated and -transcribed protein lysate in the EMSA. Both wild-type probe (5′CAGAGTCAAGGGTTT**C-CTGGGAA**CACCAGCCTGGCTTCAC3′) and mutant probe (5′CAGACTCAAGGGTTT**CCTG*TT*AA**CACCAGCCTGGCTT-CAC3′) were used in the EMSA.

element within the GFAP promoter. Moreover, additional mechanisms might function in Notch activation of the GFAP promoter, insofar as CSL binding sites were not identified in the 106 bp DNA fragment.

Astrocyte Differentiation Is Delayed in RBP-J Mutant (CSL–/–) NSCs

The identification of the CSL binding site in the GFAP promoter suggests that CSL is one of the major effectors for the astrogliogenic Notch pathway. To examine whether CSL is involved in astrocyte differentiation of NSC, we used RBP-J–/– (CSL–/–) ES cells to produce CSL mutant NSCs. The advantages of using ES cell-derived monolayer cultures of CSL–/–NSC is that it bypasses the early lethality of the RBP-J knockout animals, allowing us to study the influence of CSL deficiency on astrocyte differentiation. We established our own protocol of generating NSCs from ES cells, based on the methods described in two previous reports (Okabe et al., 1996; van der Kooy, 2001). Interestingly, ES cell-derived NSCs first go through a neurogenic period, later switching to an astrogliogenic period, indicating that the sequential onset of neuronal and glial differentiation is preserved in these cultures (Y.E.S.'s laboratory, unpublished results). RBP-J–/– (CSL–/–) ES cell-derived NSC were delayed for about 2 weeks in astrocyte differentiation (Fig. 7A,B). However, even though astrocyte differentiation in CSL–/– cells was delayed, once initiated, the rate and the extent of astroglial differentiation were not significantly lower than those of wild-type control cells. This finding suggests that, although CSL-mediated Notch signaling is likely involved in astrocyte differentiation, there does not appear to be an absolute requirement of CSL for astroglial differentiation of NSC. To confirm that the mutant cells were in fact RBPJ–/– (CSL–/–), we stained the cells for β-galactosidase, because the second CSL allele was disrupted through insertion of the β-Gal gene. The cells used in this study showed nuclear β-galactosidase immunoreactivity, confirming their CSL–/– identity (Fig. 7C). In addition to the delay in astrocytic differentiation, later passages of CSL–/– cells appeared to proliferate more slowly than their wild-type counterparts (Fig. 7D,E). This reduction in proliferation, however, cannot account for the delay in astrocyte differentiation, because this reduction occurred after the onset of astrogliogenesis in both wild-type and CSL–/– cells. Taken together, these findings suggest that CSL, likely by mediating Notch signaling, regulates NSC proliferation and promotes astrocyte differentiation.

Astrogliogenic Notch and JAK-STAT Pathways Are Inhibited in Early/Neurogenic Progenitor Cells

In the developing mammalian nervous system, neurogenesis precedes gliogenesis. Consistently with this sequence, the glial differentiation program appears to be actively suppressed during the neurogenic period (Y.E.S.'s laboratory, unpublished data). Notch is classically considered as an anti-differentiation factor involved in inhibiting

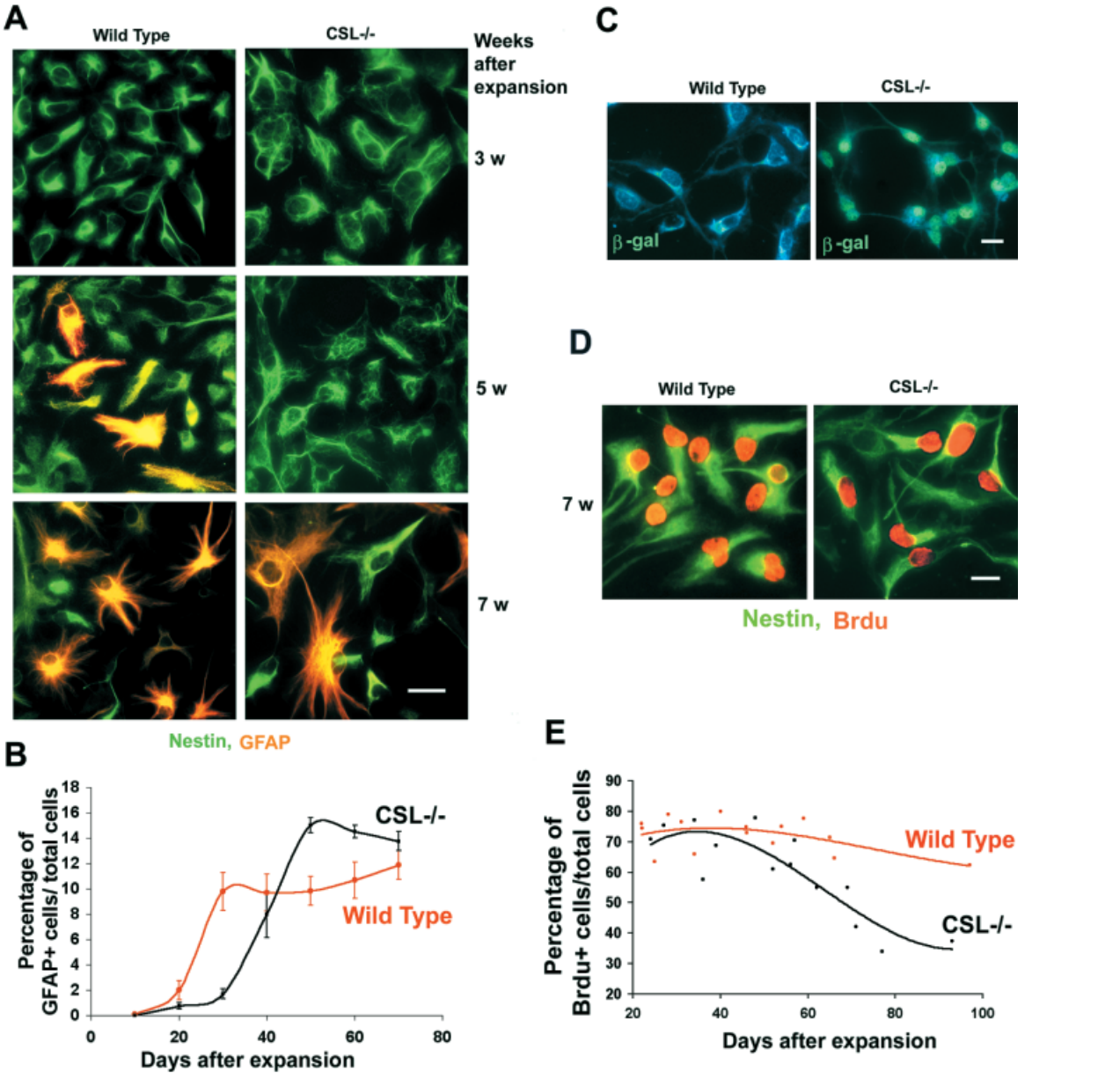

Fig. 7. In CSL−/− ES-NSCs, astroglial differentiation is delayed and cell proliferation is decreased in long-term-expanded cells. Both wild-type and RBP-J/CSL−/− ES cells were cultured in suspension for 1 week in the presence of LIF. At the end of LIF treatment, cells were considered as 0 days of expansion. LIF-treated cells were then moved onto PO/FN-coated dishes and cultured in FGF-containing serum-free medium designed to expand NSCs. When the cells became 80% confluent, they were passed onto several new dishes to allow continuous expansion. After several days of expansion, cells were allow to differentiate upon mitogen withdrawal. After 4 days of differentiation, cells were fixed with methanol/acetone and subjected to immunostaining with antibodies against nestin and GFAP (**A**). Astrocyte differentiation was measured by the percentage of cells becoming GFAP positive. **B** is a quantification graph of the astrocyte differentiation potential in wild-type and CSL−/− NSCs that have been expanded for different periods of time (for each time point, n = 4; SEs are also presented). In CSL−/− ES cell-derived NSCs, astrocyte differentiation is delayed for about 1–2 weeks. **C:** Wild-type and CSL−/− ES cell-derived NSCs were stained with an anti-β-Gal antibody. CSL−/− cells display nuclear β-Gal staining, confirming their CSL−/− identity. ES cell-derived NSCs were expanded for 7 weeks, then labeled with BrdU for 24 hr. The BrdU staining is shown in **D**. **E** is the quantification of 24 hr BrdU labeling of ES cell-derived NSCs that have been expanded for different periods of time (overlapping raw data are presented and a polynomial, order of 3, curve fit was used). A decrease in BrdU incorporation is obvious in CSL−/− cells (E). Scale bars = 19 μm.

neurogenesis and preserving progenitor pools. It is clear that Notch activation does not always lead to astrocyte differentiation, at least not during the neurogenic period. For example, in the early developing CNS, the Notch pathway is activated to suppress neuronal differentiation. This is illustrated in mutant mice deficient in Notch pathway genes (RBP-J–/– or Notch1–/–), which show increases in expression of the neurogenic bHLH genes, including neurogenin1 and -2 and Mash1 (de la Pompa et al., 1997; Ma et al., 1998). The increased expression of the neurogenic bHLH genes is indicative of precocious or enhanced neurogenesis. Since Notch signaling is activated during neurogenesis at a time when astrocyte differentiation is not occurring, it seems likely that the astrogliogenic function of Notch is suppressed during the neurogenic period or in early neurogenic progenitor cells.

Activation of the astrogliogenic JAK-STAT pathway and GFAP expression are repressed in early/neurogenic progenitor cells (<4 day cultured E11.5 mouse cortical cells). During the neurogenic period, progenitor cells in short-term (<4 days) mouse E11.5 cortical cultures produce only neurons even when treated with LIF. Moreover, the JAK-STAT pathway is poorly activated in <4 day cultured cortical progenitor cells isolated during the neurogenic period (mouse E11.5) compared with cells from >4–5 days E11.5 cortical cultures, when astrocyte differentiation can be first detected (He and Sun, unpublished observation). Importantly, LIF triggered activation of the Ras-MAP kinase pathway is similar in 1–2 day and in 4–5 day cultured E11.5 cortical cells, suggesting that the LIF receptor is expressed and functional in cells after 1–2 days in vitro (DIV; He and Sun, unpublished observation). Consistently with the idea that the JAK-STAT pathway regulates GFAP expression, we found that the GFAP promoter is inactive in 1–2 day cultured E11.5 mouse cortical progenitor cells when the JAK-STAT pathway can only be poorly activated but becomes active and inducible by LIF after 4–5 days of culturing (Fig. 8A). We previously reported that, in E17 rat primary cortical cultures, the STAT binding element, located 1,523 bp upstream of the transcription initiation site (–1,523 bp; 5′-*TTCC*GAGAA-3′ –1,515 bp) within the GFAP promoter, is critical for the JAK-STAT pathway to activate this promoter (Bonni et al., 1997). Mutation of this STAT binding site to *CCAA*GAGAA renders the promoter non-responsive to LIF stimulation in 4–5 day cultured moue E11.5 cortical cells (Fig. 8A). However, in long-term (>3 weeks) bFGF-expanded NSC cultures, mutation of the STAT binding site within the GFAP promoter allowed low levels of LIF activation of the mutant promoter. bFGF-elicited NSC expansion correlates with reduced neurogenic potential and increased astrogliogenic potential of NSCs, so this finding suggests, that in the more gliogenic/long-term-expanded NSCs additional mechanisms were in operation for LIF-mediated glial differentiation (Fig. 8B). Similarly to the effect of LIF, FCDN1 in short-term (1–2 day)-cultured/neurogenic progenitors led to activation of the Notch

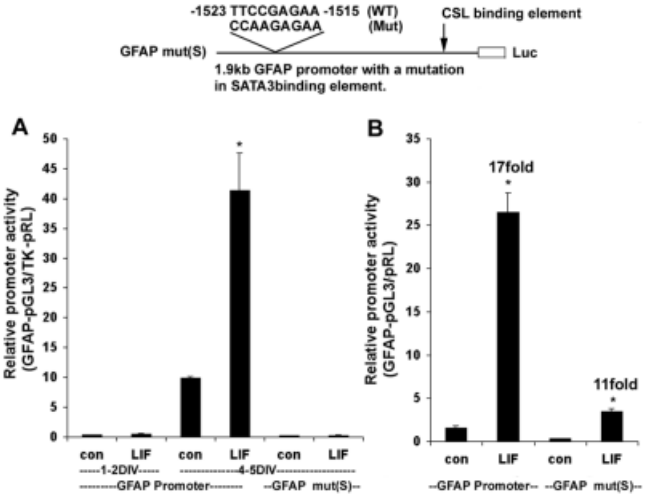

Fig. 8. The LIF-induced GFAP promoter activation is suppressed in early/neurogenic cortical neural progenitor cells. The GFAP promoter-reporter construct or the promoter with a mutation at the –1,523 bp STAT binding site was introduced into short-term/1 day cultured E11.5 mouse cortical primary cells, most of which were in the neurogenic state. Twenty-four hours after transfection and LIF stimulation, cells were lysed and subjected to the dual luciferase assay. In 1–2 day cultured cortical primary cells, LIF failed to induce the GFAP promoter; however, when the promoters were introduced into the same cells cultured for 4 days, LIF activated the wild-type promoter but not the STAT binding mutant promoter [GFAP mut(S); **A**]. In long-term (>3 weeks) bFGF-propagated E11.5 mouse cortical NSCs, LIF is capable of causing an induction of the GFAP mut(S) promoter (**B**), suggesting that additional sites on the GFAP promoter became LIF responsive in older cortical NSCs ($\star P < 0.05$ vs. controls; n = 12 for all).

canonical target, the synthetic CSL promoter, but not the GFAP promoter (Fig. 9A). However, long-term (about 2 weeks) culturing of NSC (old) to achieve a more gliogenic state allowed FCDN1 to activate both the CSL and the GFAP promoters (Fig. 9B).

Relationship Between the JAK-STAT and the Notch Signaling Pathway During Astrocyte Differentiation

To date, three major factors have been shown to promote astrocyte differentiation in NSCs. They are LIF, bone morphogenetic protein (BMP), and Notch signaling (Bonni et al., 1997; Nakashima et al., 1999b; Tanigaki et al., 2001). It is known that the LIF effect is mediated by the JAK-STAT pathway, and BMP works synergistically with LIF through the formation of a STAT-p300-Smad1 transcription coactivating complex on the STAT binding site (–1,523 bp) in the GFAP promoter (Nakashima et al., 1999b). The intricacies of the Notch astrogliogenic signaling pathway are unclear. Here we showed that Notch signaling activates glial gene expression in part via a direct CSL-mediated transcription activation, although additional mechanisms appear to be involved. To address the relationship between the astrogliogenic effects of the JAK-

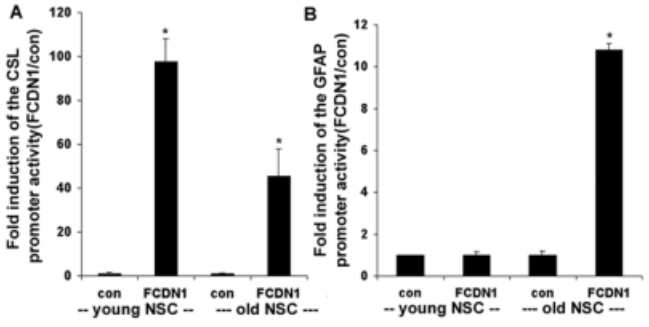

Fig. 9. Activated Notch induced-GFAP promoter activation is suppressed in early cortical neural progenitor cells. In short-term (1–2 day)-cultured neurogenic mouse E11.5 cortical cells (young NSC), activated Notch, via the canonical CSL-dependent Notch signaling pathway, turns on transcription at the Notch canonical target promoter CSL (**A**). In the same cells, however, Notch activation fails to turn on transcription of the astrocyte-specific promoter GFAP promoter, which also contains a CSL binding site (**B**). After long-term (about 2 weeks) expansion, the same NSCs (old NSC) are competent for Notch to activate the transfected GFAP promoter (B; $\star P < 0.05$ vs. controls; n = 12 for all).

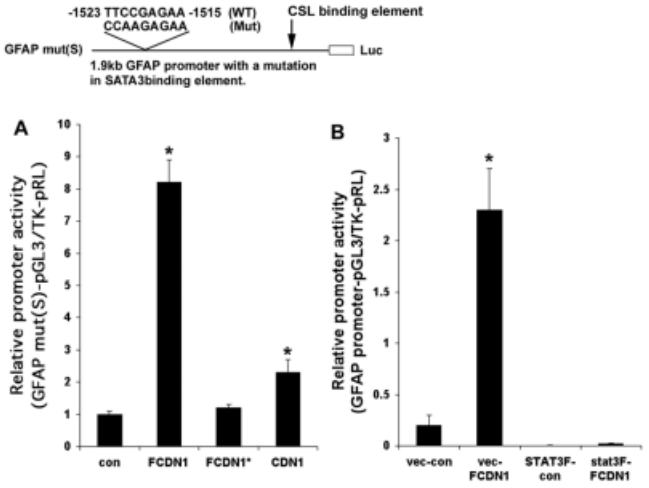

Fig. 10. Inactivation of the JAK-STAT pathway attenuates the astrogliogenic function of Notch, even though the –1,523 bp STAT binding site in the GFAP promoter does not seem to be required for Notch to activate the GFAP promoter. In 1–2 week bFGF-propagated NSCs, activated Notch (FCDN1) induced the GFAP promoter with the –1,523 bp STAT binding site mutated (**A**). However, when a dominant interfering form of STAT3, STAT3F, was expressed in the same cells, the effect of FCDN1 on the wild-type GFAP promoter was significantly suppressed (**B**; $\star P < 0.05$ vs. and the STAT3F groups; n = 12 for all).

STAT and the Notch pathways, we determined whether mutation of the CSL binding site within the GFAP promoter would still allow for activation by LIF and whether the GFAP promoter with a STAT binding mutation can be activated by FCDN1 in 1–2 week cultured E11.5 mouse cortical NSCs (Fig. 10A). Mutation of the CSL binding site within the GFAP promoter did not disrupt LIF activation of the promoter (data not shown), suggesting that the JAK-STAT pathway does not rely on the CSL-binding site to activate the GFAP promoter. In long-term (1–2 weeks) bFGF-propagated NSC cultures, FCDN1 also activates the STAT3 mutant promoter as effectively as, if not more effectively than, the wild-type promoter (Figs. 5C, 10A). This is consistent with previously postulated effects in adult hippocampal NSCs (Tanigaki et al., 2001). Mutation of the STAT binding site within the GFAP promoter, however, eradicates only direct STAT binding. STAT might still affect the transcriptional activation of GFAP through interaction with other transcription complexes, which could be independent of the STAT binding element within the promoter. To examine whether STAT proteins are involved in regulating the Notch astrogliogenic pathway, we introduced a dominant interfering form of STAT3, STAT3F, into 1–2 week bFGF-propagated NSCs. STAT3F functions as a dominant interfering form of STAT by permanently associating with LIF receptors and inhibiting endogenous STATs from being recruited to the receptors and activated through phosphorylation. In the presence of STAT3F, FCDN1 had a minimal effect on activation of the promoter (Fig. 10B), suggesting that activation of the JAK-STAT pathway might be required for Notch signaling to turn on the GFAP gene effectively.

DISCUSSION

Notch signaling is used by invertebrates and vertebrates to control multiple aspects of development (Artavanis-Tsakonas et al., 1995, 1999). Notch signaling functions in a wide range of cellular environments to elicit multiple developmental outcomes appropriate to the cellular context. Historically, Notch signaling has been postulated to function both in the preservation of progenitor pools and in the control of cell fate specification (de la Pompa et al., 1997; Wang et al., 1998). Recently, it was postulated that Notch signaling instructively promotes nonmyelinating Schwann cell and astrocyte differentiation (Morrison et al., 2000; Tanigaki et al., 2001). The mechanism by which Notch signaling activates astrocyte-specific gene expression is not known. It is debated whether Notch signaling might play a permissive role by inhibiting neuronal and oligodendroglial fates and thereby allowing progenitor cells to become astrocytes by default or whether Notch signaling may directly activate the astrocyte differentiation program. Our study on the mechanisms by which Notch activation regulates the astrocyte-specific GFAP gene expression suggests that Notch signaling directly induces astroglial differentiation through the downstream effector CSL (Brou et al., 1994; Christensen et al., 1996). Moreover, our data indicate a direct link between Notch activation at the cell surface and astroglial gene activation in the nucleus. In support of a role for CSL-dependent Notch signaling in regulating astrocyte differentiation, CSL mutant NSCs were delayed in astrogliogenesis. However, it is worth noting that CSL-

deficient cells still undergo astrocyte differentiation, indicating that CSL-mediated Notch signaling is not absolutely required for astrocyte differentiation.

Our data also indicate that Notch activation of the astrocyte-specific GFAP promoter is developmentally controlled. Although Notch signaling activates both the GFAP and the synthetic CSL promoter, only the latter is activated during neurogenesis. It is well known that neurogenesis precedes glial differentiation in the developing CNS (Qian et al., 2000). During the neurogenic period, the astrocyte differentiation program is actively suppressed. We found that neither LIF nor Notch signaling can trigger astrocyte differentiation during neurogenesis (Y.E.S.'s laboratory, unpublished observations). The mechanisms that prevent precocious gliogenesis are beginning to be elucidated. Thus far, two properties intrinsic to the neurogenic progenitor cells have been identified that appear to suppress precocious astroglial differentiation. One such factor is the expression of neurogenic bHLH factors such as neurogenin1/2 and Mash1 (Nieto et al., 2001; Sun et al., 2001). The other factor is DNA methylation of glia-specific genes (Takizawa et al., 2001; Fan and Sun, unpublished results). Both of these two factors suppress the activation and function of the astrogliogenic JAK-STAT pathway (Sun et al., 2001). Whether and how these two factors function to inhibit Notch-mediated astrogliogenesis have remained to be determined. Moreover, additional mechanisms involved in inhibiting the Notch astrogliogenic pathways during neurogenesis may exist. Many questions remain, such as why during the neurogenic period Notch activates the synthetic CSL promoter but not the GFAP promoter, even though both contain a CSL binding site. Perhaps CSL binds to the GFAP promoter during both neurogenesis and astrogliogenesis but becomes active only during the latter. It will be important to determine whether NICD and CSL form a complex on the GFAP promoter during the neurogenic period. In addition, it is known that, without Notch activation, CSL and its binding partner SKIP function to repress gene transcription by recruiting SMRT or NcoR and histone deacetylases (HDACs; Zhou et al., 2000). Upon Notch activation, CSL-SKIP in turn binds to NICD and recruits MAML1, the human homolog of Mastermind, or p300 to activate gene transcription (Wu et al., 2000; Oswald et al., 2001). It is possible that, during the neurogenic period, a strong DNA repression complex is formed at the CSL binding site on the GFAP promoter and that derepression cannot be achieved by Notch activation. During astroglial differentiation, the repression complex might be less stable and the expression of MAML1 might be increased, allowing Notch signaling to turn CSL into a transcriptional activator. Studying these questions will advance our understanding of regulation of the astrogliogenic Notch pathways.

Although the astrogliogenic function of Notch is inhibited during the neurogenic period, Notch signaling functions to suppress neurogenesis during this period. For example, Notch signaling activates the transcription of Hes1 and Hes5 genes to inhibit precocious neurogenesis (de la Pompa et al., 1997), slowing the depletion of the progenitor pool, resulting in multiwave neurogenesis (Ma et al., 1998). In addition to inducing astrocyte differentiation, in our ES-derived NSC culture system, CSL-mediated Notch signaling also appears to control the proliferation of long-term-expanded NSCs. In this regard, the specific cellular context is critical for Notch to confer specific biological functions.

Two lines of loss-of-function studies presented here using the γ-secretase inhibitor and the CSL−/− ES-NSCs indicated that the endogenous CSL-dependent Notch signaling pathway is involved in astrocyte differentiation of NSCs. However, it does not appear to be absolutely required for astrogliogenesis. The JAK-STAT pathway seems to be the major astrogliogenic pathway during CNS development, as indicated by the deficiency of astrocyte differentiation in knockout mice with various components of the LIF-triggered JAK-STAT pathway. This pathway appears to be able to override or compensate for the effects of Notch deficiency on astrocyte differentiation in culture. Whether the CSL-dependent Notch pathway is involved in in vivo astrocyte differentiation during development remains to be determined through generation and subsequent analysis of conditional knockouts of various components in the Notch signaling pathway.

It was previously proposed that the Notch astrogliogenic pathway is independent of the JAK-STAT pathway (Tanigaki et al., 2001). This notion, however, was based on the observation that expression of activated Notch does not lead to STAT phosphorylation/activation and that the mutation of the GFAP promoter within the STAT binding element does not perturb activation of the promoter by Notch signaling. In this study, we have shown that, when a dominant negative form of STAT3 was introduced into NSCs, Notch signaling failed to activate the GFAP promoter effectively. This suggests that, although the STAT binding element in the GFAP promoter is not involved in the mechanism by which NICD activates the GFAP promoter, the STAT proteins might still affect the Notch astrogliogenic pathway through protein–protein interactions. Consistently with this notion, Notch signaling usually induces astrocyte differentiation in progenitor cells that are more gliogenic, in which the JAK-STAT pathway is already elevated (He and Sun, unpublished observation).

Interestingly, the A2 promoter-reporter construct that contains only the 106 bp DNA fragment at the 3′ end of the GFAP promoter can also be activated by Notch signaling. Within this 106 bp segment, no putative CSL binding site was identified, so the mechanism by which Notch signaling activates this promoter segment is not obvious. Either a CSL-dependent pathway might function indirectly on this 106 bp DNA segment or an unknown CSL-independent mechanism might be involved.

In summary, this study identified a CSL binding site within the astrocyte-specific GFAP promoter that is important for Notch activation of this glial gene. Therefore,

the canonical CSL-dependent Notch pathway might directly function to activate astrocyte differentiation. Loss-of-function experiments in this study suggested that the endogenous CSL-mediated Notch pathway is involved in regulating astrocyte differentiation but does not appear to be an absolute requirement for astrogliogenesis. Importantly, our data showed that, although CSL was activated by Notch signaling during neurogenesis, Notch activation of the GFAP promoter did not occur at this time, suggesting that there is a suppression of Notch activation of CSL on the GFAP promoter during neurogenesis. Therefore, Notch does not unconditionally instruct astrocyte differentiation in the mammalian developing CNS.

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