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Notch Receptor Activation Inhibits Oligodendrocyte Differentiation

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Summary

In this study, we show that oligodendrocyte differentiation is powerfully inhibited by activation of the Notch pathway. Oligodendrocytes and their precursors in the developing rat optic nerve express Notch1 receptors and, at the same time, retinal ganglion cells express Jagged1, a ligand of the Notch1 receptor, along their axons. Jagged1 expression is developmentally regulated, decreasing with a time course that parallels myelination in the optic nerve. These results suggest that the timing of oligodendrocyte differentiation and myelination is controlled by the Notch pathway and raise the question of whether localization of myelination is controlled by this pathway.

Introduction

During mammalian development, oligodendrocyte precursor cells differentiate into oligodendrocytes, which subsequently myelinate axons on a predictable schedule. For example, in the developing rat cervical cord, the expression of myelin-specific glycolipids and myelin-basic protein starts in the ventral funiculus on postnatal day 1 (P1), followed by the fasciculus cuneatus and the ventrolateral funiculus on P2, but myelin proteins are not expressed in the corticospinal tract and the Lissauer tract until P11 (Schwab and Schnell, 1989). The timing of specific CNS pathways is so precise and characteristic that the age of human fetuses can be determined accurately simply by assessing which pathways have myelinated (Friede, 1973). These observations suggest that a highly localized signaling mechanism regulates the timing of oligodendrocyte differentiation and myelination.

We have focused on the rat optic nerve because it is a simple part of CNS white matter that contains primarily three types of cells—oligodendrocytes, oligodendrocyte precursor cells (OPCs, previously termed O2As), and astrocytes—in addition to the axons of retinal ganglion cells (RGCs) (Raff et al., 1983a, 1983b, 1983c; Miller et al., 1985; Richardson et al., 1990). OPCs migrate through the optic chiasm into the optic nerve beginning at about embryonic day 16 (E16) (Small et al., 1987). Despite this early appearance of OPCs in the nerve, the first oligodendrocyte is not generated until 1 week later at about P1 (Miller et al., 1985), and it is not until about P6 that rapid generation of oligodendrocytes begins (Barres et al., 1992; Barres and Raff, 1994), concurrent with the onset of myelination (Matheson, 1970a, 1970b; Skoff et al., 1976, 1980; Skoff, 1978). These observations suggest that the timing of oligodendrocyte differentiation may control the timing of myelination.

In this paper, we have asked whether the Notch pathway, an important signaling pathway that controls cell differentiation, could play a role in the timing of oligodendrocyte differentiation. The Lin12/Notch receptor family has been shown to regulate the differentiation of many cell types in *Drosophila*, including cells in their eyes, bristles, sensillae, muscles, and wings (Muskavitch and Hoffmann, 1990; Muskavitch, 1994; Artavanis et al., 1995). *Drosophila* Notch interacts with membrane-bound ligands, including Delta (Vassin et al., 1985) and Serrate (Fleming et al., 1990). Four vertebrate homologs of Notch genes have now been cloned from many species (reviewed by Fortini and Artavanis, 1993; Artavanis et al., 1995; Weinmaster, 1997), where their structure and function are largely conserved (reviewed by Weinmaster, 1997). The ligands that bind to and activate these different Notch receptors belong to the DSL family defined by the invertebrate ligands Delta, Serrate, and Lag2 (Weinmaster, 1997). Notch receptor activation by these ligands generally inhibits the differentiation of vertebrate cells (Lewis, 1996). For example, Jagged1, which encodes a Serrate-like membrane-spanning protein, has been shown to suppress muscle differentiation in vitro through activation of Notch1 (Lindsell et al., 1995) and to suppress differentiation of immune cells (Robey et al., 1996), and a Delta homolog inhibits neurogenesis in *Xenopus* (Chitnis et al., 1995). So far, however, there is no evidence that the Notch pathway regulates gliogenesis, and in the nervous system it is thought to specifically regulate neuronal development (Nye et al. 1994; Henrique et al., 1997; Kintner, 1997, Soc. Neurosci., abstract).

To determine whether Notch signaling plays a role in oligodendrocyte development in the CNS, we have first analyzed the expression of Notch1 and its ligands Jagged1 and Delta1 in the developing rat optic nerve. Here, we show that Notch1 is expressed by oligodendrocytes and their precursor cells in the developing rat optic nerve and that Jagged1 is expressed on RGC axons. Jagged1 is developmentally downregulated with a time course that parallels myelination. These expression patterns are consistent with the hypothesis that Jagged1 in retinal ganglion cells signals to Notch1 on surfaces of OPCs to inhibit their differentiation into oligodendrocytes. In support of this possibility, we demonstrate that Jagged1, or a soluble form of Delta1, strongly inhibits oligodendrocyte differentiation in culture.

Results

Notch Expression in the Developing Rat Optic Nerve

To determine whether glial cells in the developing rat optic nerve express Notch receptors, we used in situ hybridization to examine cryosections for the presence of

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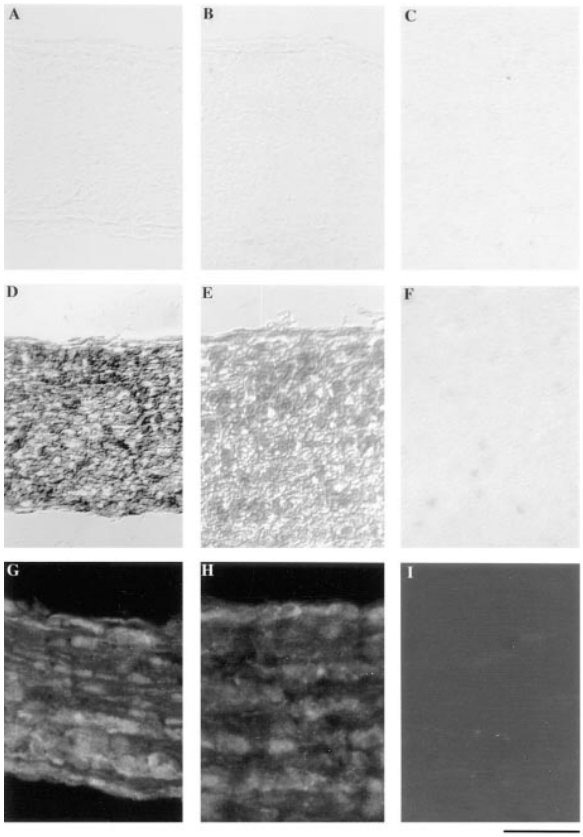

Figure 1. Expression of *Notch1* mRNA and Protein in the Rat Optic Nerve

Perfusion-fixed and frozen developing and adult optic nerves were sectioned longitudinally and labeled by in situ hybridization with a digoxygenin-conjugated *Notch1* antisense RNA probe (D–F) or with a sense probe (A–C), or by immunostaining with an affinity-purified anti-Notch1 rabbit antiserum (93-4), followed by an FITC-conjugated goat anti-rabbit IgG antibody (G–I). Background labeling with the sense probe was minimal in P1 (A), P8 (B), and P60 (C) optic nerve cryosections. A high level of *Notch1* mRNA and immunoreactivity were expressed by nearly all cells at P1 (D and G), a subset of cells at P8 (E and H), and only a few cells at P60 (F and I). Scale bar, 50 μm.

Notch1 mRNA using a digoxigenenin-conjugated Notch1 antisense oligonucleotide (see Experimental Procedures). *Notch1* mRNA was abundant in P1 optic nerve; at this age, glial cells along the edge of the nerve were strongly labeled, suggesting that at least some of the labeled cells were astrocytes (Figure 1D, see below). In addition, high levels of *Notch1* mRNA were expressed throughout the nerve. The number of cells expressing *Notch1* mRNA decreased in P8 nerves, and in the adult was limited to only a few cells (Figures 1E and 1F). To determine whether Notch1 protein was also present, we used a rabbit antiserum directed against the intracellular domain of Notch1 to stain longitudinal cryosections of rat optic nerve (see Experimental Procedures). Immunoreactivity showed a similar pattern to the mRNA signal; nearly all of the glial cells in the P1 optic nerve were immunoreactive, but this number began to decrease at P8, and little staining was present in adult tissue (Figures 1G–1I).

To determine the identity of the Notch1-expressing

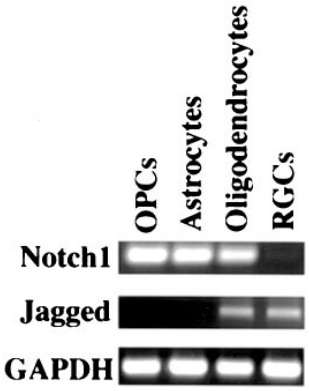

Figure 2. RT-PCR Analysis of *Notch1* and *Jagged1* mRNAs in Acutely Isolated, Purified Populations of Neurons and Glia

PCR analyses were performed on mRNA isolated from purified populations of retinal ganglion cells (RGCs), oligodendrocytes, oligodendrocyte precursor cells (OPCs), and astrocytes using specific oligonucleotide pairs corresponding to the sequences for a *GAPDH* control gene (20 cycles) and *Notch* pathway genes (30 cycles). The amplified DNA fragments were separated on 1.2% agarose gels and visualized by ethidium bromide staining.

cells, we purified each of the main glial cell types from P8 optic nerve cell suspensions and retinal ganglion cells from P8 retinal cell suspensions by immunopanning and examined their mRNA, which was extracted after isolation and prior to culture, by RT-PCR (see Experimental Procedures). Whereas the level of glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) mRNA was similar in all four cell types, a prominent 500 base pair band corresponding to rat *Notch1* mRNA was detected in OPCs and oligodendrocytes but was not detectable in the retinal ganglion cells (Figure 2). A relatively high signal was also detected from astrocyte mRNA (Figure 2).

To determine the identities of the cell expressing Notch1 proteins and to further characterize the specificity of the anti-Notch1 antiserum in our preparation, we performed Western blot studies on extracts of purified cultures of each cell type, using a polyclonal antiserum directed against the intracellular domain of Notch1. A 300 kDa band representing the full-length Notch1 protein was observed in the OPCs and in the oligodendrocytes, as well as a 110 kDa band corresponding to the transmembrane domain (Figure 3A; Blaumueller et al., 1997). A small amount of the 300 kDa and 110 kDa Notch1 proteins was also detected in the astrocyte extracts. Notch1 protein was not detected in the RGC extracts (Figure 3A).

To determine whether any of the Notch receptor protein expressed in the oligodendrocytes, OPCs, and astrocytes was on the cell surface where it could be activated by ligand binding, we stained unpermeabilized cultures with an anti-Notch1 rabbit antiserum that is directed against an extracellular domain. Immunoreactivity was observed on the surface of the oligodendrocytes and OPCs, a lower level of immunoreactivity was found on the surfaces of astrocytes, but no staining was detectable on the RGCs (Figures 4A and 4C). Interestingly, despite the low Notch signals present in adult optic nerve cryosections, Notch1 immunoreactivity was

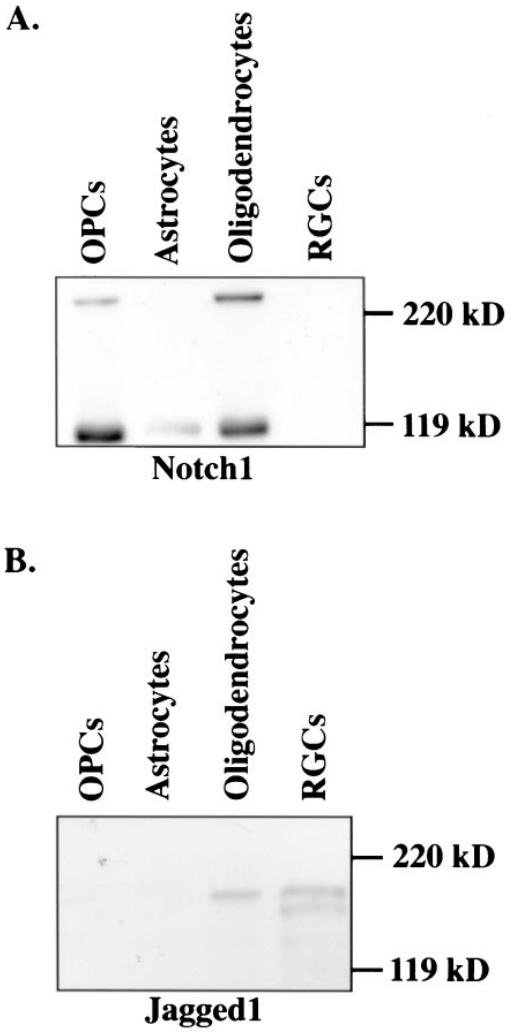

Figure 3. Western Blot Analysis of Notch1 and Jagged1 in Acutely Isolated, Purified Populations of Neurons and Glia

Western blots were prepared from extracts of purified RGCs, oligodendrocytes, OPCs, and astrocytes and stained with the anti-Notch1 antiserum (93-4), which binds to the intracellular domain (A), or with the anti-Jagged1 antibody (J59-1), which binds to the intracellular domain (B). Notch1 protein was detected in OPCs, oligodendrocytes, and astrocytes, whereas Jagged1 protein was detected in oligodendrocytes and RGCs.

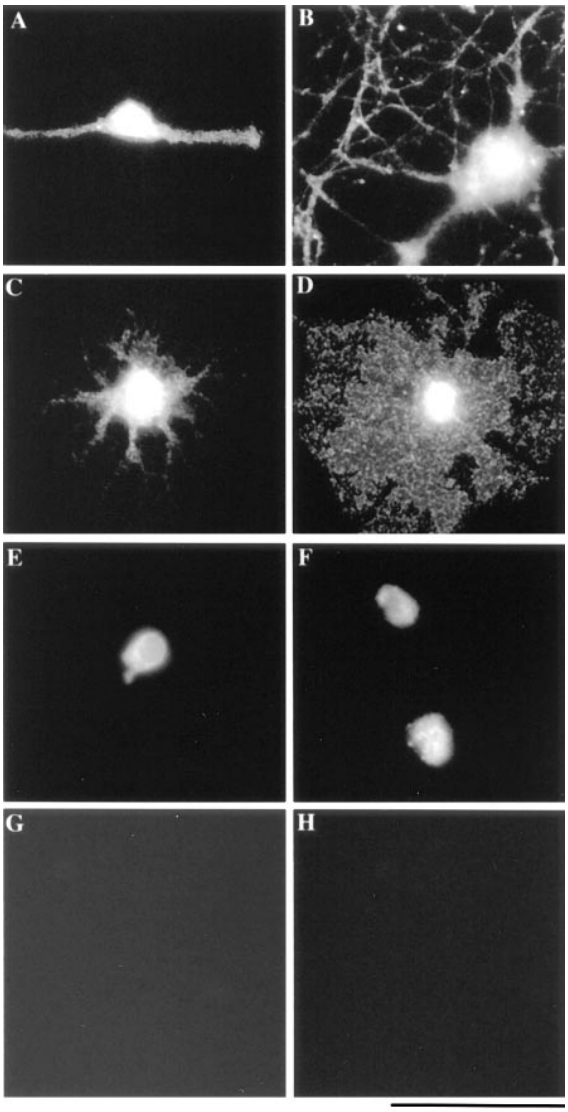

Figure 4. Notch1 and Jagged1 Immunoreactivity in Neuronal and Glial Cultures

Purified retinal ganglion cells, oligodendrocytes, and perinatal P8 OPCs were cultured in serum-free medium for 3 days, and acutely purified adult P60 OPCs were cultured for 1 hr. They were immunostained with an anti-Notch1 antiserum (5261) (A, C, and E) or with an anti-Jagged1 antiserum (J59-1) (B, D, and F), followed by an FITC-conjugated anti-rabbit IgG antibody. Preimmune sera of 5261 (G) and J59-1 (H) were used as negative controls. Notch1 immunoreactivity was detected on the P8 OPCs (A), oligodendrocytes (C), and P60 OPCs (E). Jagged1 immunoreactivity was detected on the RGCs (B) and oligodendrocytes (D) and on P60 adult OPCs (F). Scale bar, 50 μm.

also detected on surfaces of OPCs purified from adult optic nerve (Figure 4E). Together, these results show that *Notch1* mRNA and Notch1 protein are not expressed by RGCs but are expressed by developing optic nerve oligodendrocytes, OPCs, and astrocytes in vitro and in vivo.

Expression of Notch Ligands in the Developing Optic Nerve and Retina

We next addressed whether glial cells in the developing optic nerve or RGCs in the developing retina express the Notch1 ligands Jagged1 or Delta1. We examined cryosections by in situ hybridization using digoxigenin-conjugated antisense oligonucleotide probes as previously described (Lindsell et al., 1995, 1996; see Experimental Procedures). At P1, most cells in the RGC layer of the rat eye were labeled heavily, as were cells in the inner nuclear layer and photoreceptor layer, as previously reported (Bao and Cepko, 1997) (Figure 5D). The inner plexiform layer, which consists primarily of neuronal processes, including dendritic processes of RGCs, was not labeled. By P8, the number of RGCs expressing Jagged1 was markedly decreased, as was their level of expression (Figure 5E). In the adult (P60) retina, Jagged1 expression was nearly absent (Figure 5F). In the P8 optic nerve, a small number of Jagged1-expressing cells were

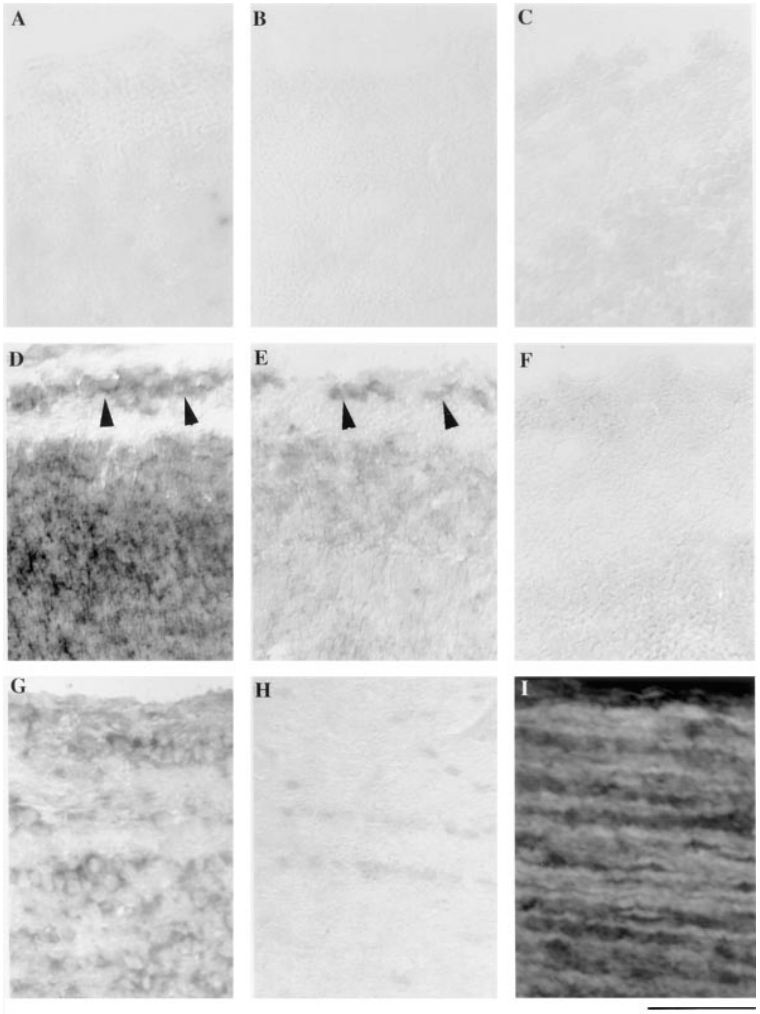

Figure 5. Expression of *Jagged1* mRNA and Jagged1 Protein in the Rat Eye and Optic Nerve

Perfusion-fixed and frozen developing and adult retinas and optic nerves were sectioned and labeled by in situ hybridization with a digoxygenin-conjugated *Jagged1* antisense RNA probe (D–H) or with a sense probe (A–C), or by immunostaining with an anti-Jagged1 rabbit antiserum, directed against an intracellular domain, followed by an FITC-conjugated goat anti-mouse IgG antibody (I). Background labeling with the sense probe was minimal in P1 (A), P8 (B), and P60 (C) eye cryosections. A high level of *Jagged1* mRNA was expressed by most retinal cells, including retinal ganglion cells (arrow) at P1 (D), but levels of mRNA were significantly decreased in retinal cells, including retinal ganglion cells (arrow) by P8 (E), and in only a few cells at P60 (F). In the optic nerve, some glial cells expressed *Jagged1* mRNA at P8 (G), and columns of cells expressing low levels of *Jagged1* mRNA were present at P60 (H). In the P8 optic nerve, Jagged1 immunoreactivity was detected primarily along axons (I). Scale bar, 50 μm.

observed (Figure 5G), and in the adult optic nerve, low levels of Jagged1 expression were still observed, particularly in longitudinal columns of glial cells suggestive of oligodendrocytes (Figure 5H).

To determine which cell types expressed *Jagged1* mRNA, we examined extracts from acutely isolated, purified populations of each P8 glial cell type and P8 RGCs by RT-PCR. A specific *Jagged1* signal was detected from mRNA isolated from RGCs and oligodendrocytes but was not detectable either in OPCs or in astrocytes (Figure 2). *Delta1* mRNA was not detected in any of the glial cell populations or RGCs (data not shown).

To determine whether the glia or RGCs made Jagged1 protein, we performed Western blots on extracts of cultures of purified cells using a rabbit antiserum directed against the intracellular domain of Jagged1. A 170 kDa band was detected in the RGC and oligodendrocyte extract but was not detectable in astrocytes or OPCs (Figure 3B). The Notch ligand Delta1 was not detectable in any of the glial cell types or the RGCs by Western blot analysis (data not shown). To determine the cellular localization of the Jagged1 protein, we immunostained cultures of oligodendrocytes and RGCs using the same antibody used for the Western blotting experiments. Jagged1 immunoreactivity was present on the oligodendrocytes and their processes but not on perinatal OPCs or astrocytes (Figure 4D). A low level of Jagged1 immunoreactivity was also detected on OPCs purified from adult optic nerve (Figure 4F). Jagged1 immunoreactivity was present along the axons of RGCs and extended all the way to, and included, the growth cones (Figure 4B). Jagged1 immunoreactivity was also present in a longitudinal pattern consistent with axonal staining in cryosections of P8 optic nerve examined by confocal microscopy (Figure 5I). Together, these results show that *Jagged1* mRNA and Jagged1 protein are not present in developing OPCs or astrocytes but are present in RGCs and oligodendrocytes and adult OPCs in vitro and in vivo.

Effects of Notch1 Activation on Oligodendrocyte Differentiation

When Notch1-expressing myoblasts are cultured together with a stably transfected Jagged1-expressing L cell line, their differentiation into myotubes is inhibited. Thus, to determine whether Notch1 receptor activation inhibits the differentiation of OPCs into oligodendrocytes, we adapted this coculture system. We cultured

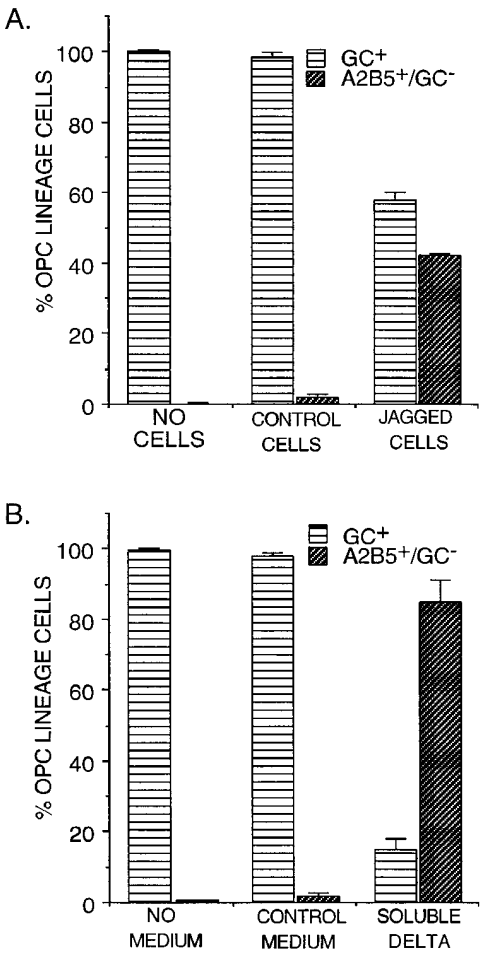

Figure 6. Effect of Notch Receptor Activation on Oligodendrocyte Differentiation

(A) Purified P8 OPCs were cultured on top of monolayers of Jagged1-expressing cells (L-SN3T-9) or cells of the parental strain (L-tk$^-$) for 3 days.

(B) Purified P8 OPCs were cultured for 3 days in the presence of conditioned medium with or without a soluble Delta. The percentage of oligodendrocytes and OPCs was determined by immunostaining; OPCs were A2B5$^+$/GC$^-$ and oligodendrocytes were GC$^+$.

purified P8 OPCs on top of a layer of L cells that expressed Jagged1, or a layer of the parental L cells that did not express Jagged1, and then measured the percentage of purified P8 OPCs that differentiated into oligodendrocytes over 3 days of culture. When the OPCs were cultured in serum-free medium in the absence of mitogens and L cells, they nearly all differentiated into highly process-bearing GC$^+$ oligodendrocytes within three days, as expected (Figure 6A). Similarly, when the OPCs were cocultured on top of a monolayer of parental L cells, which do not express Jagged1, they nearly all differentiated into oligodendrocytes. However, when OPCs were cocultured with the Jagged1-expressing L cells, over 40% of them failed to differentiate into oligodendrocytes and retained the bipolar morphology and A2B5$^+$/GC$^-$ antigenic phenotype of OPCs (Figure 6A). Similar results were obtained when a different oligodendrocyte-specific antibody, myelin basic protein (MBP), was used to assess differentiation. These results are consistent with the idea that Jagged1 expressed on the surface of the L cells is interacting with Notch1 receptors on the surface of the OPCs to inhibit their differentiation.

To confirm and extend these observations that Notch signaling inhibits oligodendrocyte differentiation, we next studied the effects of a soluble form of the Notch ligand Delta1. Soluble forms of Lin12/Notch ligands have previously been shown to be functional (Fitzgerald and Greenwald, 1995; Li et al., 1998). Based on these data, we designed and generated a soluble form of Delta1 (see Experimental Procedures) that binds specifically to the Notch1 receptor (C. H., D. N., and G. W., unpublished data), and made stably transfected cell lines that express and secrete this form. OPCs incubated with this soluble form of Delta1 showed upregulation of CBF-1 *trans*-activity (200%) and *HES5* mRNA (Figure 10; also, see Experimental Procedures), two downstream events in the Notch signaling pathway (Hsieh et al., 1996; Weinmaster, 1997). We cultured purified P8 OPCs in medium conditioned by either the parental control cells or the soluble Delta1-secreting cells and then measured the percentage of OPCs that differentiated into oligodendrocytes over 3 days of culture. As described above, when the OPCs were cultured in serum-free medium in the absence of mitogens and conditioned medium, they nearly all differentiated into highly process-bearing GC$^+$ oligodendrocytes within 3 days, as expected (Figures 6B, 7A, and 7B). Similarly, when the OPCs were cultured in conditioned medium from the parental cells, which do not secrete the soluble form of Delta1, they nearly all differentiated into oligodendrocytes (Figures 6B, 7C, and 7D). However, when the OPCs were cultured in the conditioned medium containing the soluble form of Delta1, almost 90% of them failed to differentiate into oligodendrocytes (Figure 6B) and retained the bipolar morphology and A2B5$^+$/GC$^-$ antigenic phenotype of OPCs (Figures 7E and 7F). These results provide strong evidence that activation of the Notch pathway powerfully inhibits oligodendrocyte differentiation.

To further confirm that activation of the Notch pathway inhibits oligodendrocyte differentiation, we transfected OPCs with a truncated constitutively active form of the Notch1 receptor (Shawber et al., 1996b; also, see Experimental Procedures). OPCs transfected with this truncated form of Notch1 showed upregulation of CBF-1 *trans*-activity of over 20-fold (S. W. et al., unpublished data). When the OPCs were cultured in serum-free medium in the absence of mitogens, they all differentiated into highly process-bearing MBP-positive oligodendrocytes within 3 days (data not shown). Similarly, the OPCs transfected either with the expression vector alone (data not shown) or with a β-galactosidase–encoding vector all differentiated into MBP-positive oligodendrocytes (Figures 8A, 8B, and 8C). However, nearly all of the OPCs transfected with the vector encoding constitutively active Notch1, as assessed by immunostaining, failed to differentiate into MBP-positive oligodendrocytes and retained the bipolar or multipolar morphology of OPCs (Figures 8D, 8E, and 8F). Taken together, these findings show that activation of the Notch pathway either by two different ligands, Jagged1 and a soluble form of Delta1, or by a constitutively active truncated form of Notch receptor, inhibits oligodendrocyte differentiation.

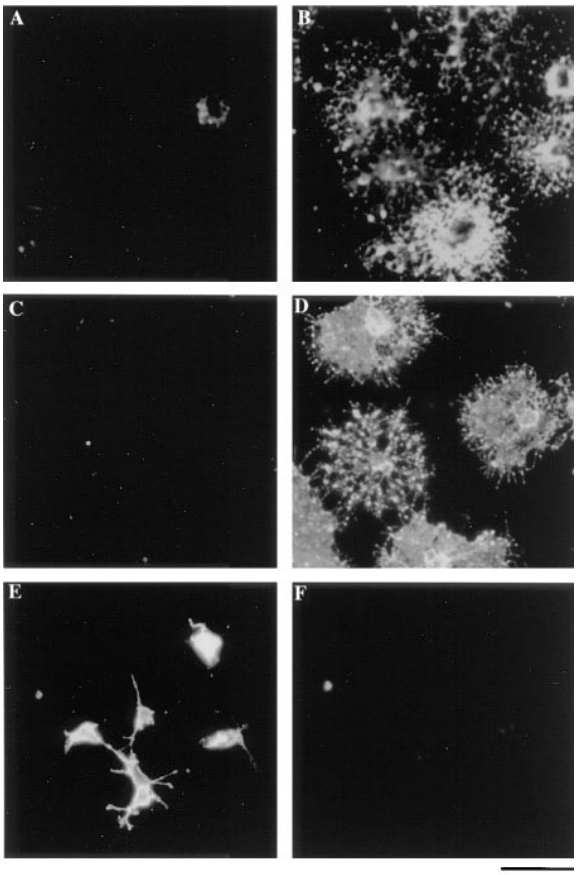

Figure 7. Effects of a Soluble Form of Delta1 on Oligodendrocyte Differentiation

Purified P8 OPCs were cultured either alone (A and B), with conditioned medium from control 293T cells (C and D), or with conditioned medium from a soluble Delta-Fc–producing cell line (E, F) in serum-free medium. After 3 days, the cultures were double-immunostained with both the A2B5 (A, C, and E) and GC (B, D, and F) monoclonal antibodies. In the absence of Delta1, A2B5$^+$ OPCs differentiated into GC$^+$ oligodendrocytes, whereas in the presence of Delta1, many OPCs did not differentiate. Scale bar, 50 μm.

Because there is an obligate relationship between proliferation and differentiation in the oligodendrocyte lineage, it was possible that Jagged1 blocked oligodendrocyte differentiation by stimulating OPC proliferation, as do mitogens such as platelet-derived growth factor (PDGF). To test this possibility, we determined whether Jagged1 stimulated bromodeoxyuridine (BrdU) incorporation into the OPCs. When BrdU (10 μM) was added to the OPC cultures in the presence of Jagged1, no BrdU-positive cells could be detected; in contrast, under the same conditions PDGF strongly stimulated BrdU incorporation into the cells (data not shown). Thus, Jagged1 does not stimulate DNA synthesis in the OPCs. OPCs stimulated by Jagged1, however, were still capable of taking up BrdU and proliferating. When PDGF was added to the culture medium and BrdU was added to the culture medium for 2 hr, ~20% of the OPCs incorporated BrdU in both the presence and absence of Jagged1. These results show that Jagged1 does not inhibit oligodendrocyte differentiation by stimulating proliferation of the OPCs, but instead acts by uncoupling proliferation from differentiation, arresting OPCs at an early stage of differentiation.

Effect of Notch1 Activation on Oligodendrocyte Maturation

The in situ hybridization studies showed that oligodendrocytes stop expressing Notch1 receptors as they mature. However, when OPCs differentiate into newly formed oligodendrocytes, they continue to express Notch1 receptors for at least several days, raising the question of whether further oligodendrocyte maturation could be prevented by activation of these Notch receptors. To test this possibility, we studied whether the Notch pathway activation by the soluble form of Delta1 affected the expression of the myelin-associated proteolipid protein (PLP). Newly formed oligodendrocytes, which express the glycolipid GC and MBP do not express PLP until 3 days after differentiation (reviewed by Skoff and Knapp, 1990). When purified OPC cultures were grown without mitogens for 2 days, nearly all of the cells differentiated into GC$^+$/PLP$^-$ newly formed oligodendrocytes (Figures 9A and 9B). After 4 more days in culture, nearly all became PLP-expressing mature oligodendrocytes. Similarly, when conditioned medium from control cells that did not secrete soluble Delta1 was added to the newly formed oligodendrocytes, they all became GC$^+$/PLP$^+$ after 4 days (Figures 9C and 9D). However, when conditioned medium containing soluble Delta1 was added, the majority of cells remained GC$^+$/PLP$^-$ over the 4 day culture period (Figures 9E and 9F). These results show that Notch signaling strongly blocks newly formed oligodendrocytes from differentiating into mature oligodendrocytes. Notch receptor activation, however, does not revert newly formed oligodendrocytes back into OPCs, even in the presence of mitogens (data not shown).

Discussion

Notch Receptor Activation Inhibits Oligodendrocyte Differentiation

Activation of the Notch pathway inhibits the differentiation of a wide variety of vertebrate cell types including muscle cells, lymphocytes, and neurons (Austin et al., 1995; Chitnis et al., 1995; Dorsky et al., 1995; Lindsell et al., 1995; Robey et al., 1996; Henrique et al., 1997). It has been reported that Notch signaling does not inhibit gliogenesis (Nye et al., 1994). These studies, however, did not utilize primary glial cells but rather focused on the effects of Notch activation on P19 embryonic carcinoma cells that can be induced to differentiate into neural cells by retinoic acid. In these P19 cultures, Notch activation inhibited neurogenesis but did not inhibit gliogenesis, as assessed by antibodies to the astrocyte-specific protein glial fibrillary acidic protein (GFAP). Our studies, however, demonstrate that activation of the Notch pathway powerfully inhibits oligodendrocyte differentiation and raise the question of whether it might also inhibit astrocyte development, as we found that astrocytes in the developing optic nerve express Notch1 receptors. Notch activation generally acts as a restrictive rather

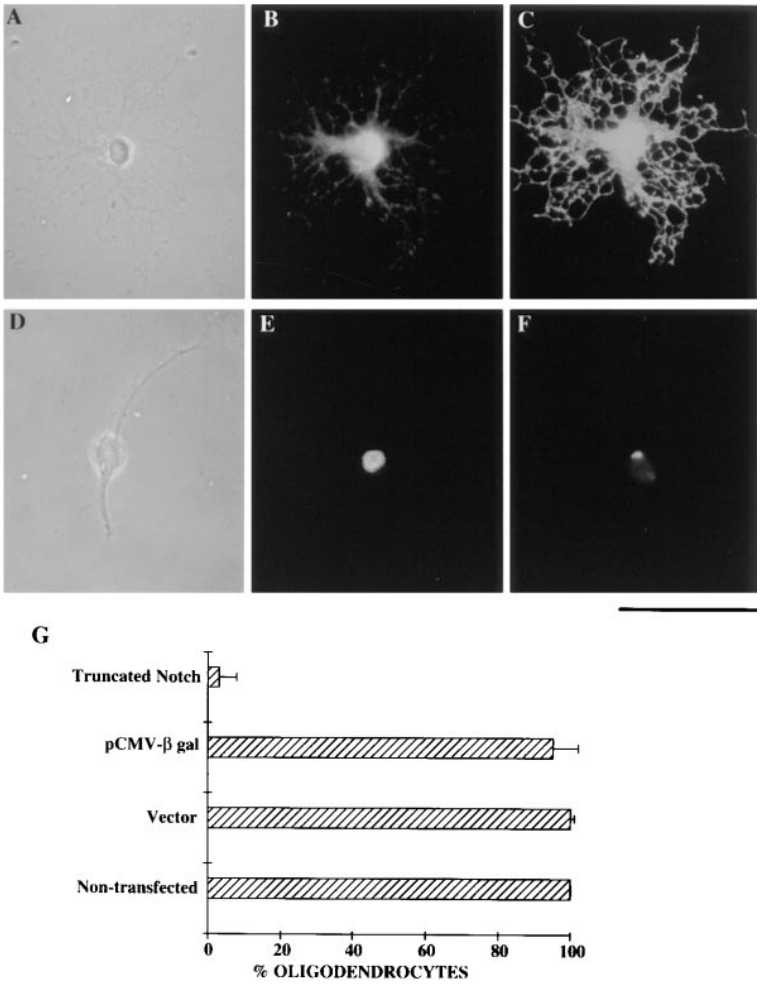

Figure 8. Effects of a Constitutively Active Form of Notch1 on Oligodendrocyte Differentiation

Purified P8 OPCs were transfected either with *pcDNA3-FCDN1* encoding a truncated constitutively active form of Notch1 (D, E, and F) or with a vector encoding β-galactosidase (A, B, and C) for 24 hr and cultured in the absence of mitogens for 3 days. The cultures were immunostained with an anti-MBP monoclonal antibody (C and F) and an anti-Notch1 cytoplasmic domain polyclonal antibody 93-4 (B). OPCs expressing a constitutively active form of Notch1 (E) failed to differentiate into MBP-positive oligodendrocytes (F) and retained bipolar OPC morphology (C). OPCs expressing β-galactosidase (B) all differentiated into highly process-bearing (A), MBP-positive (C) oligodendrocytes. The averages of transfected cells that differentiated into MBP^+ oligodendrocytes in each condition are shown as mean ± SEM (G) ($n = 3$). Scale bar, 50 μm.

than instructive signal to keep cells at the current developmental stage, thus preventing them from responding to other environmental cues (reviewed by Artavanis et al., 1995). Consistent with this function, our findings show that Notch receptor activation inhibits OPCs from differentiating into oligodendrocytes, although it does not affect the ability of the cells to survive and divide in response to stimulation by PDGF. Inhibition of differentiation by Notch activation is not due to any ability of the Notch pathway to influence a cell's ability to respond to instructive signals, as the differentiation of oligodendrocytes is constitutive. OPCs differentiate into oligodendrocytes by default in serum-free medium in the absence of specific inducing signals upon withdrawal from the cell cycle (Raff et al., 1983b; Barres et al., 1992). Notch pathway activation also inhibits the constitutive maturation of oligodendrocytes that have been newly generated from OPCs. Thus, although it is unclear how Notch activation blocks oligodendrocyte differentiation, it is clear that this inhibition is not simply the result of the inability of OPCs to respond to instructive environmental cues.

The differentiation of OPCs into oligodendrocytes can also be prevented by mitogen stimulation. There is an obligate relationship between proliferation and differentiation in the oligodendrocyte lineage (Temple and Raff, 1986). In response to mitogens, OPCs divide but do not differentiate; when mitogens are withdrawn, the OPCs withdraw from the cell cycle and differentiate into oligodendrocytes that can myelinate but not divide. Because of this relationship, it has not hitherto been possible to maintain OPCs in an undifferentiated state in culture in the absence of mitogens. Remarkably, however, Notch receptor activation is not mitogenic yet prevents OPCs from differentiating when mitogens are withdrawn. Thus, Notch pathway activation uncouples the relationship between OPC proliferation and differentiation. In the myoblast lineage, proliferation and differentiation are thought to be coupled by sequestration of helix-loop-helix (HLH) proteins, such as MyoD, by phosphorylated retinoblastoma protein (Gu et al., 1993). Thus, Notch activation might block OPC differentiation simply by sequestering constitutively expressed HLH proteins, as has previously been suggested for myoblasts (Kopan et al., 1994).

The Developmental Regulation and Localization of Notch1 and Jagged1 Suggest a Role in Timing of Oligodendrocyte Differentiation and Myelination

Much attention has been focused on a lateral inhibitory mechanism that allows a layer of initially equivalent

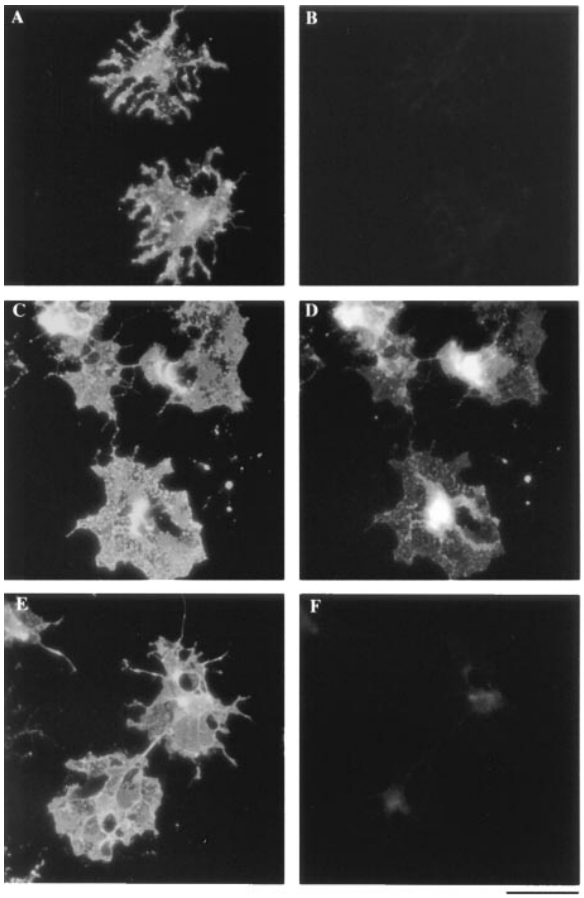

Figure 9. Effect of Notch Activation on Newly Formed Oligodendrocyte Differentiation

Purified P8 OPCs were first cultured in the absence of mitogens for 2 days (A and B) and then cultured either in conditioned medium from control 293T cells (C and D) or in conditioned medium from 293T cells that secreted a soluble Delta for an additional 4 days (E and F). The cultures were double-immunostained with both GC (A, C, and E) monoclonal and PLP (B, D, and F) polyclonal antibodies. In the absence of soluble Delta, most newly formed GC^+/PLP^- oligodendrocytes (A and B) differentiated into GC^+/PLP^+-mature oligodendrocytes (C and D), whereas in the presence of soluble Delta, the majority of them remained GC^+/PLP^- (E and F). Scale bar, 50 μm.

multipotential stem cells, each expressing Notch and Delta, to establish separate identities (reviewed by Chitnis, 1995; Henrique et al., 1997). In contrast, OPCs are not multipotential and are thought to exclusively give rise to oligodendrocytes in vivo. Although in cultures containing fetal calf serum, nearly all OPCs will differentiate into type-2 astrocytes (Raff et al., 1983b), recent evidence suggests that this occurs only rarely if at all in the optic nerve (Fulton et al., 1992). Thus, while Notch receptor activation can regulate fate selection in some cases, its potential role in controlling the oligodendrocyte lineage seems most likely to be restricted to regulating when and where oligodendrocytes are generated. Moreover, as opposed to a Notch–Delta signaling interaction occurring between neighboring undifferentiated cells, our findings suggest that a Notch ligand Jagged1 expressed on an already differentiated cell type, retinal ganglion cells, may interact with Notch1 receptors of undifferentiated oligodendrocyte lineage cells.

The nearby spatial localization of the Notch receptor and Jagged1 in the developing rat optic nerve is strongly suggestive of a potential functional interaction in vivo. Jagged1 protein is localized in axons, where it is in an ideal position to interact with Notch receptors on the surfaces of OPCs and therefore to inhibit their differentiation. Direct proof awaits the development of reagents that can be used to interrupt the function of the Notch receptor or its ligands in vivo. Because of the early lethality of transgenic mice lacking Notch or its ligands, it has not yet been possible, to date, to confirm the in vivo role of this pathway in any mammalian tissue studied.

What might be the point of inhibiting OPCs from differentiating into oligodendrocytes? OPCs need to migrate long distances from their germinal zones to enter white matter throughout the brain; as oligodendrocytes cannot migrate, preventing premature differentiation of OPCs might be crucial for ensuring that they successfully make it to their final destination. Recent studies have shown that OPCs migrate in the optic nerve along the axons of RGCs (Ono et al., 1997), where they are likely to be stimulated by Jagged1. The downregulation of Jagged1 expression by RGCs temporally correlates well with the onset of myelination in the optic nerve around P6, as well as with the maturation of immature astrocytes that begin to express a variety of new proteins after P6, such as ciliary neurotrophic factor (CNTF) and connexin 43 (Stockli et al., 1991; Konietzko and Muller, 1994). Although increasing levels of T3 postnatally enhance myelination (Barres et al., 1994; Ahlgren et al., 1997), a systemic hormonal change cannot by itself explain the different timing of myelination in different white matter pathways. Myelination of axons in a given pathway generally occurs a few days after they reach their target (Schwab and Schnell, 1989), raising the question of whether downregulation of Jagged1 in axons occurs in response to target innervation. Consistent with this possibility, Jagged1 expression in cultured RGCs is sustained for weeks in the absence of their target cells (S. W. and B. A. B., unpublished data).

Although we have focused on the role of Notch signaling in the CNS, several recent findings point to a similar role for this pathway in regulating myelination in the PNS. Both the *Notch1* and *Notch2* receptor genes were originally isolated from rat Schwann cells (Weinmaster, 1991; Weinmaster et al., 1992). Cell surface labeling experiments have shown that Notch1 is present on the surface of rat Schwann cells in culture (G. W., unpublished data). Moreover, embryonic dorsal root ganglion (DRG) neurons express both Jagged1 and Jagged2 (Shawber et al., 1996a). Myelination of the PNS occurs rapidly over the first postnatal week; thus, it will be of great interest to determine whether DRG neurons normally downregulate their expression of Notch ligands around the time of birth.

The Notch Pathway May Regulate Adult OPC Development

It has been suggested that the Notch pathway may control the rate at which stem cells progress into differentiation in order to avoid exhausting the stock of stem cells

(Henrique et al., 1997); such a role would make good sense in ensuring that oligodendrocyte generation from OPCs can be successfully sustained over the first 2 postnatal months in the developing rat brain. In addition, it has been hypothesized that even after development is completed, the Notch pathway may help maintain some mammalian stem cells and precursor cells in adult tissues by preventing them from differentiating, thus allowing for the possibility of new cell generation in renewing or damaged tissues (Artavanis et al., 1995). Our findings provide evidence that strongly supports this possibility. Small numbers of OPCs persist in the adult rat optic nerve, where, unlike their perinatal counterparts, they do not differentiate and only rarely divide (ffrench-Constant and Raff, 1986; Wolswijk and Noble, 1989; Shi et al., 1998). Because of the obligate relationship between proliferation and differentiation in the oligodendrocyte lineage, the persistence of OPCs that do not differentiate but also do not divide in the adult optic nerve has been puzzling. Our observations provide a way that this quiescent state can be achieved, as we have shown that OPCs stimulated by Jagged1 in the absence of mitogens do not divide or differentiate. Not only do OPCs express Notch1 receptors, but mature oligodendrocytes express Jagged1, suggesting that as sufficient numbers of oligodendrocytes are generated, they signal nearby OPCs to stop generating oligodendrocytes. This would ensure that all of the OPCs do not differentiate, preserving a reservoir of precursor cells in the adult optic nerve that might be able to generate new oligodendrocytes after injury or disease (renewal of oligodendrocytes in healthy adult tissues does not occur). An extrinsic signaling mechanism, however, is not sufficient by itself to account for the phenotype of adult OPCs. We have recently directly compared the properties of highly purified perinatal and adult OPCs and found that they differ intrinsically; adult OPCs divide and differentiate three times more slowly than do perinatal OPCs in the same culture medium (Shi et al., 1998), raising the question of whether the small amounts of Jagged1 expressed by the adult OPCs activate in *cis* their own Notch receptors.

Does the Notch Pathway Control the Localization of Myelination?

It is not obvious why some axons become myelinated and others do not. As myelination only enhances the conduction rate of axons greater than a diameter of $\sim$0.5 μm (Rushton, 1951), it has been proposed that the diameter of an axon governs whether or not myelination by oligodendrocytes occurs (Friede, 1973; Voyvodic, 1989). For instance, the axons of rat cerebellar granule neurons, which form the parallel fibers of the molecular layer, are $<$0.4 μm in diameter and are not myelinated, whereas nearly all rat RGC axons are 1 μm in diameter and are myelinated. Remarkably, however, OPCs but not oligodendrocytes are found in unmyelinated CNS pathways, such as in the molecular layer of the cerebellum (Levine, 1989; Peters et al., 1991). A simple explanation for these observations, consistent with our present results, is that persistent expression of Jagged1 or another Notch ligand on some adult axons prevents OPCs from differentiating into oligodendrocytes in these pathways. The small diameter of these axons would be a consequence of the loss of an oligodendrocyte signal that promotes radial growth of axons (Sanchez et al., 1996). Consistent with this possibility, OPCs are prevented from differentiating into oligodendrocytes in cultures of cerebellum, which consist primarily of cerebellar granule neurons, even in the presence of serum-free medium (Levine, 1989), suggesting that cerebellar granule neurons may express Jagged1 or a related ligand. Determining whether the expression pattern of Notch ligand expression in the adult brain correlates with myelination status will be an important next step. It will also be important to determine whether aberrant expression of Notch ligand limits the amount of remyelination that occurs after injury or demyelinating disease.

Experimental Procedures

Step-by-step protocols for all procedures are available upon request.

Reagents

Recombinant human basic fibroblast growth factor (bFGF), PDGF, and neurotrophin 3 (NT-3) were obtained from Peprotech (New Jersey). Insulin was obtained from Sigma. Recombinant trophic factors were generously provided by Regeneron (human BDNF, CNTF).

Purification of Retinal Ganglion Cells, Optic Nerve OPCs, and Type-1 Astrocytes by Sequential Immunopanning

Purified cell populations were obtained essentially as previously described (Barres et al., 1988, 1992, 1993, 1994; Meyer-Franke et al., 1995; Shi et al., 1998). Briefly, P8 retinas or neonatal optic nerves were obtained from Sprague-Dawley rats (Simonsen Labs, California) and were incubated at 37°C for 30 min in a papain solution (15 units/ml for retina or 30 units/ml for optic nerve; Worthington) in an Earle's Balanced Salt Solution (EBSS, Gibco) containing L-cysteine. For adult O2A purification, optic nerves were obtained from P70 Sprague-Dawley rats and incubated at 37°C for 60 min in a collagenase solution (333 units/ml, Sigma), followed by two 20 min incubations in trypsin (30,000 units/ml, Sigma) dissolved in Ca^{2+}/Mg^{2+}-free DPBS (Gibco). The enzymes were neutralized by the addition of fetal calf serum (FCS). The tissue was then triturated sequentially in a solution containing ovomucoid (2 mg/ml, Boehringer-Mannheim), DNase (0.004%, Sigma) and bovine serum albumin (BSA) (1 mg/ml, Sigma) to yield a suspension of single cells. After centrifugation at 800 $\times$ g, the cells were rewashed in a high ovomucoid/BSA solution (10 mg/ml each).

For RGC purification, the retinal suspension was incubated in anti-rat macrophage antiserum (Axell, 1:100) for 20 min, centrifuged, resuspended in PBS, and incubated on two 150 mm anti-rabbit IgG panning plates sequentially at room temperature for 45 min each. The nonadherent cells were removed with the suspension, filtered through 15 μm Nitex mesh (Tetko), and placed on the Thy1.1 (T11D7e2, American Type Culture Collection, TIB 103) panning plate. After 45 min, the plate was washed with PBS. Cells on the panning dish were incubated with 4 ml of a trypsin solution (0.125%, Sigma) for 10 min in a 10% CO_2 incubator at 37°C. The cells were dislodged by gently pipetting trypsin solution around the plate. A 30% FCS solution (10 ml) was added to inactivate the trypsin, and the cells were spun and collected.

For oligodendrocyte precursor purification, the optic nerve cell suspension was resuspended in 7 ml PBS containing insulin (5 μg/ml, Sigma) and filtered through Nitex mesh. The cell suspension was first placed on the anti-RAN-2 (IgG, Bartlett et al., 1981) plate for 30 min at room temperature, after which the nonadherent cells were transferred to the anti-GC (IgG, Ranscht et al., 1982) dish to deplete the oligodendrocytes. The nonadherent cells were then transferred to the A2B5 dish to collect the OPCs. After 45 min, this plate was washed eight times with 10 ml of PBS, and the OPCs

were removed from the plate by incubating with trypsin solution and collected as above.

The purification procedure for optic nerve type-1 astrocytes utilized a similar sequential immunopanning protocol. Optic nerve cell suspensions from P2 rats were panned on a dish coated with the MRC-OX7 Thy1.1 antibody to deplete the suspension of meningeal fibroblasts, a dish coated with A2B5 and GC antibodies to deplete the suspension of OPC lineage cells, and a final dish coated with the C5 monoclonal antibody (Miller et al., 1984) to select remaining neuroepidermal-derived cells.

Culture of Purified Retinal Ganglion Cells, OPCs, Oligodendrocytes and Type-1 Astrocytes

Approximately 10,000 purified retinal ganglion cells were cultured on glass coverslips in 24-well plates (Falcon) coated with poly-D-lysine (PDL) (70 kDa, 10 μg/ml; Sigma), followed by merosin (2 μg/ml, Telios/Gibco) in 100 μl of a serum-free medium containing Neurobasal (Brewer et al., 1993), bovine serum albumin (BSA), selenium, putrescine, thryoxine, tri-iodothyronine, transferrin, and progesterone (B-S medium) (modified from Bottenstein and Sato, 1979, as previously described in Lillien and Raff, 1990), pyruvate (1 mM), glutamine (1 mM), insulin (5 μg/ml), CNTF (1 ng/ml), BDNF (10 ng/ml), and forskolin (5 μM).

Approximately 5,000 purified OPCs were cultured on glass coverslips in 24-well plates (Falcon) that had been coated with PDL in a serum-free medium containing Dulbecco's modified Eagle medium (DMEM). The serum-free medium included BSA, selenium, putrescine, transferrin, progesterone, pyruvate (1 mM), glutamine (1 mM), insulin (5 μg/ml), CNTF (1 ng/ml), PDGF (10 ng/ml), and NT-3 (1 ng/ml). For in vitro differentiation of oligodendrocytes, PDGF and NT-3 were omitted from the culture medium. For type-1 astrocytes, PDGF, CNTF, and NT-3 were omitted and human bFGF (1 ng/ml) was added.

In Situ Hybridization

The Notch1 (SN6–7) and Jagged1 (SN3ED) plasmids were linearized and transcribed with either T3 or T7 RNA polymerase to obtain the antisense or sense probes. Probes were labeled nonradioactively by digoxigenin-labeled nucleotides according to the instructions of the manufacturer (Boehringer Mannheim Biochemicals).

In situ hybridization procedures were performed essentially as described by Schaeren-Wiemers and Gerfin-Moser (1993). Perinatal rats were decapitated and eyeballs and optic nerves quickly removed by dissection. The tissue was fixed in 4% paraformaldehyde for 2 hr and stored in 30% sucrose/PBS at 4°C overnight. Cryosections (7–15 μm) were prepared from Tissue-Tek embedding medium block and collected on Superfrost Plus slides (Fisher Scientific, Pittsburgh, PA). The tissue sections were baked at 56°C for 20 min, fixed in 4% paraformaldehyde at 4°C for 30 min, treated with 0.5 μg/ml proteinase K for 15 min, and acetylated for 10 min. Prehybridization was performed at 56°C for 2 hr with 75 μl hybridization buffer (50% formamide, 5× SSC, 1× Denhardts, 10% Dextran Sulfate, 100 ng/ml salmon sperm DNA, and 500 ng/ml bakers yeast tRNA) per section. The prehybridization buffer was aspirated and 75 μl of 1 μg/ml probe in hybridization buffer was added. Hybridization was performed overnight at 56°C in a 5× SSC–humidified chamber. Slides were washed in 5× SSC twice for 10 min each, followed by two stringent washes at 65°C in 0.2× SSC for 15 min each. The tissue sections were rinsed in buffer B1 (buffers B1–B5 from Boehringer Mannheim dig detection kit), blocked in buffer B2 for 2 hr, incubated for 3 hr at 37°C with anti-Dig antibody diluted 1:500 in buffer B2, rinsed twice with buffer B1 for 15 min, and equilibrated in buffer B3 for 5 min. The color development was performed at room temperature overnight in buffer B4 containing 1 mM levamisole (Sigma) and stopped by washing the slides with buffer B5. Coverslips were mounted on the slides using AQUA-MOUNT (Lerner Laboratories, Pittsburgh, PA).

RT-PCR Analysis

Poly(A)$^+$ RNA was purified from the last panning dish (A2B5 for OPCs, GC for oligodendrocytes, C5 for type-1 astrocytes, and T11D7 for RGCs) using Oligo-dT-cellulose chromatography (Qiagen). For *HES* RT-PCR, purified P8 OPCs were incubated with conditioned medium from either parental 293T cells, or cells secreting a soluble form of Notch ligand Delta-Fc, for 24 hr before mRNA isolation. mRNA was reverse transcribed using Superscript II (Gibco) with a random hexamer (10 μM) as a primer in a 35 μl reaction containing 1× Superscript II RT buffer, 10 μM each of dATP, dTTP, dCTP, and dGTP, and 20 U of RNasin (Gibco). After 2 hr at 42°C, the reaction was terminated by adding 365 μl of H_2O and boiling for 2 min. For PCR amplification, specific oligodendrocyte primer pairs (0.5 μM each) were incubated with 1 μl of cDNA and 1 U of Taq polymerase (Perkin Elmer) in a 20 μl reaction mixture that included 1× Taq buffer and 100 μM each dATP, dCTP, dGTP, and dTTP. Typical cycle parameters were 1 min at 94°C, 1 min at 59°C, and 3 min at 72°C for 20–30 cycles, followed by a cycle at 72°C for 10 min. The whole reaction was then fractionated on 1.2% regular agarose gel, and the PCR product was visualized by ethidium bromide staining.

The primers for RT-PCR analysis are: rat *Notch1*, 5′ primer, GCAG CCACAGAACTTACAAATCCAG, and 3′ primer, TAAATGCCTCTGG AATGTGGGTGAT; rat *Notch2*, 5′ primer, GCTGTCCTCTTCATGCT GCA, and 3′ primer, AGCAGAAGTCAAGACAGTC; rat *Jagged1*, 5′ primer, CCACAAAGGGCACGGCGAGTG, and 3′ primer, TGCATGG GGTTTTTGATTTGG; rat *Delta1*, 5′ primer, AAGAAGGGGATCCTGG GGAA, and 3′ primer, GCATGCTGATATCTGCTGACAGG; rat *HES1*, 5′ primer, AGCCAACTGAAAACACCTGATT, and 3′ primer, GGAGTT TATGATTAGCAGTGG; rat *HES5*, 5′ primer, GCCGCTGGAAGTGGT AAA, and 3′ primer, CGCATCAACAGCGCATT; and rat *GAPDH*, 5′ primer, ATTGTCAGCAATGCATCCTGCA, and 3′ primer, AGACAAC CTGGTCCTCAGTGTA.

Immunofluorescence Staining for Primary Cultures and Optic Nerve Sections

After fixation with 4% paraformaldehyde for 10 min at room temperature, cells were incubated for 30 min in a 50% goat serum solution containing 1% BSA and 100 mM L-lysine to block nonspecific binding and Triton 0.4% to permeabilize the membrane. To stain the surface antigens of O2A lineage cells, cells were incubated in monoclonal A2B5 antibody and/or in monoclonal anti-GC antibody, followed by fluorescein-coupled goat anti-mouse IgG (Jackson, 10 μg/ml). To stain the Notch pathway genes, cells were incubated with polyclonal anti-Notch, anti-Jagged1, and anti-Delta antibodies, followed by fluorescein-coupled goat anti-rabbit IgG (Jackson, 10 μg/ml). The coverslips or slides were mounted in Citifluor on glass slides, sealed with nail varnish, and examined in a Zeiss Axioskope fluorescence microscope. Specificity of the Notch1 antibodies (93-4 and 5261) was previously determined by Western blot analysis and immunohistochemistry on C2C12 cells (Shawber et al., 1996b). Specificity of anti-Jagged1 (J59-1) and anti-Delta1 (88-C-6) antibodies was determined by Western blot analysis and immunostaining on L cells expressing rat Jagged1 or Delta1 (G. W. et al., unpublished data).

After perfusion with 4% paraformaldehyde, optic nerves were transferred to 30% sucrose/PBS and frozen in O. C. T. compound (Tissue-Tek). Cryosections (7 μm) were collected on gelatin-coated (Sigma) slides. For immunostaining, slides were dried at 37°C for 30 min and incubated for 1 hr in a 50% goat serum solution containing 1% BSA and 100 mM L-lysine to block nonspecific binding and Triton 0.4% to permeabilize the membrane. Sections were incubated with polyclonal antibodies against different Notch pathway genes, respectively, followed by fluorescein-coupled goat anti-rabbit IgG (Jackson, 10 μg/ml). The slides were mounted in Citifluor, sealed with nail varnish, and examined in a Zeiss Axioskope fluorescence microscope.

Western Blot Analysis

Cultured OPCs, oligodendrocytes, type-1 astrocytes, and RGCs were harvested in enzyme-free sample buffer containing 65 mM Tris-HCl (pH 6.8), 0.75% SDS, 5% β-mercaptoethanol and 10% glycerol with 20 mM phenylmethylsufonyl fluoride (PMSF), soybean trypsin inhibitor, pepstatin, and aprotinin (10 mg/ml). Extracts of 100,000 cells (50,000 for Jagged1-expressing cell line) were electrophoresed through 8% SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membrane. Polyclonal antibodies against Notch pathway genes were incubated with the blots, and anti-rabbit peroxide (Boehringer Mannheim Biochemicals) was used to visualize the proteins.

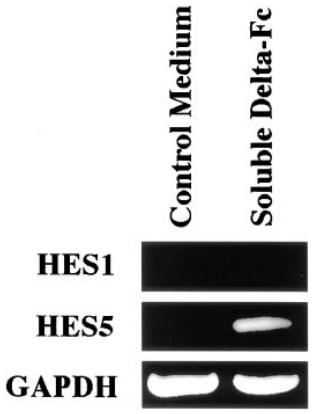

Figure 10. RT-PCR Analysis of *HES1* and *HES5* mRNAs in Soluble Delta-Fc–Treated Purified OPC Cultures

PCR analyses were performed on mRNA isolated from purified populations of OPCs incubated in conditioned medium from either 293T parental cells or cells secreting a soluble Notch ligand Delta-Fc, using specific oligonucleotide pairs corresponding to the sequences for a *GAPDH* control gene (20 cycles) and for *HES* genes (30 cycles). The amplified DNA fragments were separated on 1.2% agarose gels and visualized by ethidium bromide staining.

Preparation of Cocultures

To assess the effects of Notch signaling on OPC development, we plated ~250,000 Jagged1-expressing cells (L-SN3T-9) or nontransfected cells of the parental strain (L-tk$^-$) onto PDL-coated coverslips in a 24-well plate and allowed the cells to grow to confluence in DMEM/10% FCS without additional trophic factors. The coverslips were washed extensively in DPBS (Gibco) and incubated in Sato-medium with insulin (5 mg/ml) prior to coculturing. Fifty thousand purified OPCs were plated onto the monolayer of cells and grown in Sato-medium with insulin for 3 days.

Treatment with a Soluble Form of Delta1

A Delta1/Fc fusion construct was engineered to encode the extracellular domain of rat Delta1 (amino acids 1–487, GenBank accession number U7889), which was fused in-frame with human IgG-Fc lacking the hinge region (encodes 1–217 amino acids of the Fc domain) using a PCR-overlap strategy. The *Delta-Fc* cDNA sequences were subcloned into the mammalian expression vector *pcDNA3* (Invitrogen), and the correct coding sequences were confirmed by DNA sequencing. The details of this construction are available upon request. OPCs transfected with a luciferase reporter construct containing four CBF1 binding elements (Hsieh et al., 1996) showed increased luciferase activity upon treatment with this soluble form of Delta1, and RT-PCR analysis showed that OPCs treated with the same soluble ligand had increased expression of *HES5* mRNA (Figure 10).

Stable human 293T cell lines overexpressing Delta-Fc were generated using calcium phosphate cotransfection of *pcDNA3-D1Fc* and the hygromycin resistance gene, along with selection in 400 μg/ml hygromycin (Sigma). Positive Delta1-Fc–secreting cell lines were identified by Western blot analysis of whole-cell lysates and conditioned medium, as previously described (Shawber et al., 1996a). Delta1-Fc–conditioned media were harvested from Delta1-Fc–expressing 293T cells cultured in DMEM alone every 2 days. Conditioned media from parental 293T cells were prepared in the same manner and used as a negative control. The conditioned medium was concentrated 20-fold with a Centriplus-30 filter (Amicon, Beverly, MA) at 4°C, and diluted 1 to 10 in the serum-free culture medium. Five thousand OPCs were plated per well in a PDL-coated, 24-well tissue culture plate. Cells were fed with the same medium every 2 days for the entire culture period.

Transfection of OPCs with a Constitutively Active Form of Notch1

The cytoplasmic portion of the rat Notch1 receptor (amino acids 1747–2531) was inserted into a mammalian expression vector *pcDNA3* (Invitrogen) to create a constitutively active form of *Notch1*, *pcDNA3-FCDN1*. OPCs transfected with this form of Notch1 showed >20-fold upregulation of CBF-1 *trans*-activity (S. W. et al., unpublished data). Purified OPCs were grown in PDL-coated 60 mm culture dishes in the presence of mitogens until reaching 40% confluency. *pcDNA3-FCDN1* cDNA (4 μg) and lipofectamine (10 μl, 2 mg/ml stock, Gibco) were gently mixed and incubated at room temperature for 45 min. *pcDNA3* and a vector-encoding β-galactosidase were also incubated with lipofectamine as controls. Each DNA-lipofectamine mixture was diluted with 2 ml fresh OPC growth medium containing mitogens before adding to the cultures. Twenty-four hours after the start of transfection, the cells were subcultured into 24-well culture dishes in OPC growth medium, minus mitogens, and grown for an additional 3 days. The OPCs transfected with the constitutively active form of Notch1 were assessed by the characteristic nuclear staining with an anti-Notch1 polyclonal antibody (93-4). The differentiation of the oligodendrocytes was determined by immunostaining with an anti-MBP monoclonal antibody.

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