Notch1 control of oligodendrocyte differentiation in the spinal cord

Stéphane Genoud,1 Corinna Lappe-Siefke,2 Sandra Goebbels,2 Freddy Radtke,3,4 Michel Aguet,3 Steven S. Scherer,5 Ueli Suter,1 Klaus-Armin Nave,2 and Ned Mantei1

1Department of Biology, Institute of Cell Biology, Swiss Federal Institute of Technology, ETH-Hönggerberg, CH-8093 Zürich, Switzerland
2Department of Neurogenetics, Max-Planck-Institute of Experimental Medicine, D-37075 Göttingen, Germany
3Swiss Institute of Experimental Cancer Research, CH-1066 Epalinges, Switzerland
4Ludwig Institute for Cancer Research, Lausanne Branch, University of Lausanne, CH-1066 Epalinges, Switzerland
5Department of Neurology, University of Pennsylvania Medical Center, Philadelphia, PA 19104

We have selectively inhibited Notch1 signaling in oligodendrocyte precursors (OPCs) using the Cre/loxP system in transgenic mice to investigate the role of Notch1 in oligodendrocyte (OL) development and differentiation. Early development of OPCs appeared normal in the spinal cord. However, at embryonic day 17.5, premature OL differentiation was observed and ectopic immature OLs were present in the gray matter. At birth, OL apoptosis was strongly increased in Notch1 mutant animals. Premature OL differentiation was also observed in the cerebrum, indicating that Notch1 is required for the correct spatial and temporal regulation of OL differentiation in various regions of the central nervous system. These findings establish a widespread function of Notch1 in the late steps of mammalian OPC development in vivo.

Introduction

Signaling through the Notch pathway is involved in various cell fate decisions, in particular in maintenance of precursor cells in an undifferentiated state until they are competent to respond to inductive cues (for reviews see Artavanis-Tsakonas et al., 1995; Robey, 1997; Weinmaster, 1998). In this context, Notch signaling plays a key role in tissue-autonomous fate determination in central nervous system (CNS)* development, in a process referred to as a lateral inhibition (Artavanis-Tsakonas et al., 1999), and also regulates mammalian neurogenesis (Lütolf et al., 2002). A role of Notch signaling in the regulation of terminal neural differentiation also has been found in the development and remodeling of dendrites (Berezovska et al., 1999; Franklin et al., 1999; Sestan et al., 1999; Redmond et al., 2000).

*Abbreviations used in this paper: β-gal, β-galactosidase; CNP, 2’,3’-cyclic nucleotide 3’-phosphodiesterase; CNS, central nervous system; GFAP, glial fibrillary acidic protein; MAG, myelin-associated glycoprotein; MBP, myelin basic protein; NeuN, neuronal nuclear antigen; OPC, oligodendrocyte precursor cell; OL, oligodendrocyte; PDGFR-α, PDGF receptor α; Plp, proteolipid protein.

Key words: ectopic expression; brain; Cre-lox P; newborn; apoptosis

In vertebrate glial cell development, the Notch pathway has been implicated in a number of crucial events. Activated Notch promotes the formation of radial glia in the fetal forebrain (Gaiano et al., 2000; Chambers et al., 2001), Schwann cells in dorsal root ganglia (Wakamatsu et al., 2000), and Müller glia in the retina (Furukawa et al., 2000). In cell culture, even transient activation of Notch strongly promotes the differentiation of adult hippocampus-derived multipotent progenitors into astroglia (Taniyagi et al., 2001), and of neural crest stem cells into the Schwann cell lineage (Morrison et al., 2000).

However, the oligodendrocyte (OL) lineage appears to respond somewhat differently. Activation of Notch signaling suppresses rather than promotes the differentiation of OLs from multipotent progenitor cells (Gaiano et al., 2000; Taniyagi et al., 2001). Furthermore, a potential regulatory role of the Notch pathway in mammalian oligodendrocyte precursor cell (OPC) differentiation has been suggested by cell culture studies showing that OPCs derived from the postnatal rat optic nerve could be inhibited in their differentiation by incubation with Notch ligands (Wang et al., 1998).

How proliferation and differentiation are regulated in the OL lineage is only partially understood (Barres et al., 1992; Barres and Raff, 1994; Raff et al., 1998; Kondo and Raff, 2000a,b; Qi et al., 2001; Wang et al., 2001; Zhou et al.,...
The Journal of Cell Biology

The basis of morphology and molecular phenotype, cells of the OL lineage can be grouped into OPCs, immature OLs (premyelinating), and mature OLs (myelinating cells; Gard and Pfeiffer, 1990; Hardy and Reynolds, 1991; Armstrong et al., 1992; Miller, 1996). OPCs of the developing spinal cord are generated along the length of the neural tube from a narrow zone in the ventral region of the neuroepithelium (Noll and Miller, 1993; Pringle and Richardson, 1993; Yu et al., 1994). Determination of the OL lineage is promoted by the transcription factors Olig1, Olig2, and Nkx2.2 (Lu et al., 2001; Zhou et al., 2001; Fu et al., 2002), but the exact nature of the interplay among them is not clear. OPCs arising in the ventral spinal cord subsequently proliferate and disperse throughout the developing CNS (for reviews see Miller, 1996; Richardson et al., 1997; Spassky et al., 2000).

Postmitotic OLs appear first in the ventral funiculus and later in the dorsal and lateral funiculi, the large axonal bundles that become the white matter tracts of the spinal cord (Jordan et al., 1989; Yu et al., 1994). However, it remains an open question how OPCs are able to migrate through an already established neuronal system around, over, and along the axons they may ultimately myelinate, before differentiating at the appropriate time point (Blaschuk and ffrench-Constant, 1998). Progression past the OPC stage is strongly delayed and reduced in Nkx2.2−/− mice, suggesting a possible role for this transcription factor in later development of OLs (Qi et al., 2001). Ectopic OPCs arising on overexpression of Notch1 together with Olig2 in chick spinal cord do not mature, which is consistent with a negative role for Notch1 in late stages of differentiation (Zhou et al., 2001). In contrast, Givogri et al. (2002) have reported a transient increase of myelination in some parts of the CNS during the early postnatal weeks in heterozygous-null Notch1 mutants. In vitro studies and correlative evidence based on the regulation of Notch1 and its ligand Jagged1 in the developing optic nerve have also implicated the Notch pathway in the control of the timing of OPC differentiation (Wang et al., 1998). This is a tantalizing idea supporting a model in which loss of responsiveness to growth factors may permit OPC differentiation, but local cues (e.g., the Notch pathway) regulate the timing of final maturation in different myelinated tracts (Blaschuk and ffrench-Constant, 1998). Unfortunately, further support for this model has not been particularly forthcoming over the last years and several crucial questions remain open: is the observed function of Notch signaling in the control of rat OPC differentiation restricted to the optic nerve or is it a general phenomenon throughout the rodent CNS? Is the observed effect specifically Notch1 dependent or may other members of the Notch family be involved (Notch 2–4; Lindsell et al., 1996; Irvin et al., 2001)? Are the findings with isolated OPCs in vitro directly transferable to the in vivo situation, in particular given the recent reappraisal that OL development is under stringent axonal control and the proposed interactions of OPCs with mature OLs via the Notch pathway in tissue (Wang et al., 1998; Barres and Raff, 1999; Casaccia-Bonnefille, 2000)? The “acid test” to answer these questions requires an analysis in a physiologically accurate system in which specifically Notch1 has been reduced or eliminated. Transgenic mice provide such a proper setting. Notch1 knockout mice were not informative, however, due to early embryonic lethality (Swiatek et al., 1994; Conlon et al., 1995). Thus, in this study we have selectively inhibited Notch1 signaling using a conditional Notch1 knockout mouse strain (Radtke et al., 1999). Our data demonstrate a crucial function of Notch1 in late steps of OL differentiation in the spinal cord and suggest a similar function in the brain.

**Results**

We have used a “floxed” allele of Notch1, in which the exon encoding the signal sequence (Fig. 1 A) is flanked by loxP sites (Radtke et al., 1999) for conditional gene ablation. Recombination mediated by the Cre recombinase excises the region between the lox sites, thereby inactivating the gene. Cre was expressed under the control of regulatory elements known to be active in the OL lineage, either those of the Cnp gene (with the Cre coding region inserted into the endogenous gene; unpublished data) or of the proteolipid (Plp) gene (as a transgene driving Cre; unpublished data) (Fig. 1 A). Newborn mice homozygous for the floxed Notch1 allele and carrying either the Cnp-Cre (Cnp-Cre ΔΔ) or the Plp-Cre (Plp-Cre ΔΔ) allele were outwardly normal, nursed, moved, breathed, and responded to mechanical stimulation, but usually survived only a few hours after birth. Recombination outside the CNS was assessed in Cnp-Cre ΔΔ mice by reporter gene expression (for method, see next paragraph) and was observed in various organs whose development is known to be affected by Notch signaling, including kidney (McLaughlin et al., 2000), liver (Nijjar et al., 2001), lung (Ito et al., 2000), and pancreas (Apelqvist et al., 1999). These findings could explain the early death of the Cnp-Cre ΔΔ animals. A small number of Cnp-Cre ΔΔ individuals survived longer, exhibited only modest defects (smaller in size, poor sense of balance, and partially closed eyes), and could be kept until adulthood. In contrast, Plp-Cre ΔΔ mice that survived after birth had to be killed because of severe growth retardation and motor defects. Some Cnp-Cre ΔΔ and Plp-Cre ΔΔ mice had strikingly enlarged lateral ventricles at birth (unpublished data).

We focused our studies on the spinal cord because OL development has been extensively studied in this structure. To determine where and when Cre recombinase was produced in the spinal cord in our Cre-transgenic mice, ROSA26 reporter (R26R) mice (Soriano, 1999) were crossed with mice carrying Cnp-Cre (yielding Cnp-Cre R26R) or Plp-Cre (yielding Plp-Cre R26R). This approach allowed lineage tracing via detection of the LacZ product β-galactosidase (β-gal; Fig. 1 a). Sections were cut at the forelimb level of doubly transgenic mice at embryonic day 13 (E13), and β-gal was detected by histological X-gal staining. In Cnp-Cre R26R mice (Fig. 1 b, enlarged in d), X-gal–positive cells were observed within the spinal cord in the ventral ventricular zone, in a pattern consistent with recombination in OPCs as indicated by PDGF receptor-α (PDGFR-α) in situ hybridization (Fig. 1 e). This restricted expression in OPCs was expected based on the reported endogenous 2′,3′-cyclic nucleotide 3′-phosphodiesterase (CNP) expression in the rat (Yu et al., 1994) and the analysis of transgenic mice under the control of Cnp regulatory elements (Gravel et al., 1998;
Precocious appearance of OLs in the spinal cord at E17.5

Next, we examined spinal cords at E17.5, shortly before OLs start to accumulate in substantial numbers in the ventral fiber tracts of the mouse thoracic spinal cord. Initial X-gal staining in Cnp-Cre Δ/Δ R26R (Fig. 2 a) and Cnp-Cre Δ/wt R26R (Fig. 2 b) embryos showed no major differences in the pattern of recombined cells in the homozygous (Δ/Δ) compared with heterozygous (Δ/wt) mice. We examined the cellular composition of the neural tube in Cnp-Cre Δ/Δ versus control (lox/lox) mice using immunostaining and in situ hybridization for markers characteristic of neurons, astrocytes, OPCs, and immature OLs. We used antibodies against neuronal nuclear antigen (NeuN; Fig. 2, c and d), neurofilament (unpublished data), and β-tubulin III (unpublished data) for neurons, glial fibrillary acidic protein (GFAP) for astrocytes (Fig. 2, e and f), and detected PDGFR-α by in situ hybridization for OPCs (Fig. 2, i and j). None of these markers showed significant differences between mutant and control mice. The number of neurons and astrocytes was indirectly quantitated by determining the average overall intensity of NeuN and GFAP immunostaining of 10 representative sections from three embryos of each genotype. Staining and image processing were performed identically for sections of mutant and control animals. No significant differences were obtained, when intensity was calculated either per whole spinal cord cross section (Fig. 2, g and h) or per unit area (unpublished data). The size of the white matter area (i.e., that part of the spinal cord outside the area stained by NeuN) also did not differ significantly (38 ± 7.4 relative units for Cnp-Cre Δ/Δ vs. 42 ± 6.0 for control, P = 0.27, t test). However, for immature OLs, the situation was quite different. The number of cells expressing high levels of PLP/DM20 mRNA (Fig. 2, k and l) was strongly increased in the spinal cord of mutants compared with control animals, both in the gray and in the future white matter (PLP/DM-20 has been described to be expressed at earlier times in the ventral ventricular zone, but expression outside this region is characteristic of differentiated OLs; Richardson et al., 2000). An in-situ hybridization (e). (b) Arrowheads indicate the nerve roots and dorsal root ganglia that are part of the PNS. Expression in the Plp-Cre line used is much broader, affecting most cells of the spinal cord (c). V, ventricle. Bars: 10 μm.
creased number of differentiating OLs was also observed by immunostaining for myelin-associated glycoprotein (MAG; Fig. 2, m and n). We found 10-fold more perinuclear MAG-positive cells per section compared with controls (Fig. 2 p). Interestingly, this increased number of immature OLs was not the result of an increased number of OPCs (PDGFR-α mRNA–positive cells; Fig. 2 o). Furthermore, no significant increase in proliferating cells was observed using phosphorylated histone H3 as a marker for cells in the S phase of the cell cycle (11.1 ± 5.2 positive cells per section in mutant animals vs. 10.1 ± 3.9 in control animals).

**Ectopic appearance of immature OLs in the gray matter at birth**

Dramatic changes in the number and spatial distribution of OLs were also evident in spinal cords of newborn (P0) Cnp-Cre Δ/Δ compared with control (lox/lox) mice (Fig. 3, compare c and e with d and f). Many immature OLs were abnormally located in the gray matter. This altered distribution was seen along the entire length of the spinal cord in mutant mice (unpublished data). We quantified the MAG-positive cells showing perinuclear staining (mature OLs; Fig. 3 i), using their perinuclear staining to distinguish them from myelinating mature OLs (Trapp et al., 1997). After excluding the mature OLs at the ventral margin of both mutant and control spinal cords (Fig. 3, e and f; see also MBP staining for mature OLs in Fig. 3, g and h for comparison), there were approximately sixfold more immature OLs present in mutanats as compared with controls (Fig. 3 l). Similar observations were made in Plp-Cre Δ/Δ mice (53 ± 19 vs. 5 ± 4 in mutant and control, respectively). At E17.5, we found no difference between the numbers of cells positive for PDGFR-α mRNA in Cnp-Cre Δ/Δ and control mice (Fig. 3, a, b, and k).
Increased apoptosis of prematurely differentiated OLs
Although we observed ectopic perinuclear MAG-positive OLs in both thoracic (unpublished data) and cervical spinal cord (Fig. 3, g and h), even though the latter is at a more advanced stage of differentiation than the thoracic segment. However, some staining was observed in the future white matter of both mutant and control animals (Fig. 3, g and h). These observations raised the possibility that the ectopic immature OLs might be cleared by programmed cell death. To test this hypothesis, we double stained sections from Cnp-Cre mice at P0 for TUNEL (Gavrieli et al., 1992) and MAG (Fig. 3 j). Significantly more TUNEL-positive dying cells were found in mutant spinal cords: 14.9 ± 3.9 per section in mutants versus 7.8 ± 2.3 per section in controls (P < 0.001, n = 17, t test). Further, a higher percentage of MAG-positive cells were TUNEL-positive in mutant mice (Fig. 3 m). Note that most, but not all, of the dying MAG-positive immature OLs were localized in the gray matter. Together, these results suggest that the ectopic and precociously appearing immature OLs are eliminated before they fully differentiate into MBP-positive mature OLs.

Efficiency of Notch1 ablation
Because our multiple efforts to directly show elimination of the Notch1 protein failed due to a lack of reliable reagents, we demonstrated that Cre recombinase had been active in the differentiating OLs. We prepared short-term cultures of cells isolated from the spinal cord of single Cnp-Cre R26R mice homozygous for the floxed Notch1 allele (Δ/Δ). Heterozygous littermates (Δ/wt) were used as controls. The cells were first stained with X-gal (Fig. 4, a and b) and then for O4 (Fig. 4, c and d), a marker for both immature and mature OLs (Hardy and Reynolds, 1991). 89 out of 100 and 71 out of 100 O4-positive cells derived from homozygous and heterozygous animals, respectively, were also positive for lacZ-gal, demonstrating expression of Cre recombinase and efficient recombination of the R26R allele within the O4-positive cell population. To directly demonstrate Notch1 recombination, DNA was isolated from spinal cords of newborn mice and analyzed by Southern blotting (Fig. 4 e). Quantification by PhosphorImager analysis revealed ~20–25% recombination in Cnp-Cre whole spinal cord, after normalization to DNA isolated from control lox/0 mice.

Early stages of OPC development are normal in Notch1 mutant embryos
Because expression of Cre recombinase starts as early as E11.5 in Cnp-Cre R26R mice (unpublished data), we examined the early development of OPCs in Cnp-Cre Δ/Δ mice. PDGFR-α mRNA–positive OPCs were found at E12.5 in the neural tube in homozygous (Δ/Δ) as well as in heterozygous (Δ/wt) mice (unpublished data). At E14.5, the PDGFR-α mRNA-positive OPCs were dispersed throughout the cross section (Fig. 5, a and b, and their number was similar in mutants and controls (Fig. 5 g). MAG-positive cells were not detected at this time point (Fig. 5, a and b). Thus, early
development of the OL lineage appeared normal in Cnp-Cre Δ/Δ mice. The distribution of motoneurons showed no major alterations in Cnp-Cre Δ/Δ mice at E12.5 (unpublished data), E14.5 (Fig. 5, e and f, arrows), and E17.5 (Fig. 2, a and b, arrows), as judged by immunostaining for the markers Isl1 and Isl2 (Tsuchida et al., 1994; Pfaff et al., 1996) and X-gal staining in Cnp-Cre Δ/Δ R26R mice.

Precocious differentiation of OLs in the forebrain

We asked next whether precocious OL differentiation is confined exclusively to the spinal cord. Thus, we examined the forebrain, where OL differentiation starts slightly later compared with the spinal cord. As in the spinal cord, ablation of Notch1 in Cnp-Cre Δ/Δ mice led to a strong increase in the number of perinuclear MAG-positive immature OLs compared with controls, with a large fraction (≈50%) found in the gray matter (Fig. 6, a–c). However, we did not observe precocious differentiation using antibodies against MAG in the optic nerve of P0 mutant animals (unpublished data), likely because OL differentiation starts later in the optic nerve.

Discussion

In the present paper, we used defined conditional gene ablation to address the question of how the OL lineage is affected when Notch1 function is specifically eliminated in vivo. We provide direct evidence that Notch1 is critically important for correct temporal and spatial differentiation of OLs from the precursor to the immature stage in the mouse spinal cord by demonstrating that ablation of Notch1 in OPCs leads to ectopic production of prematurely differentiated immature OLs. Most of the prematurely differentiated OLs were found in the gray matter at P0 where immature OLs are usually very scarce in control animals. Apoptosis was strongly increased among the precociously differentiated cells. However, the number of OPCs was not altered at the ages examined, and early OL development appeared normal. Precocious differentiation was also observed in the anterior
forebrain, suggesting that Notch1 signaling may play an important role in OL development throughout the CNS.

An important issue is whether the observed effects of Notch1 deletion on OPC differentiation depend directly on deletion within the OPCs themselves or are an indirect consequence of deletion in some other cell type. We used Cre-mediated recombination driven by Cre recombinase inserted into the Cnp locus. In agreement with published reports on CNP regulation and according to our own data, in the developing spinal cord Cre recombinase was expressed exclusively by early oligodendrocyte progenitors and motoneurons. Given the pattern of X-gal and immunostaining at E17.5, Cre is likely not to be expressed in astrocytes (Fig. 2, compare b with f). Development of neurons appears normal in the spinal cords of mutant mice (Fig. 2, c, d, and g). However, we cannot exclude subtle effects such as altered trophic support or abnormal electrical signaling from the periphery. Electrical activity of neurons has been shown to influence proliferation of OPCs (Barres and Raff, 1993) and the process of myelination (Demerens et al., 1996), but we are not aware of evidence that such electrical activity influences differentiation of OPCs into immature OLs for review see Barres and Raff, 1999). OPCs develop normally in explant cultures from spinal cords of mutant mice (Fig. 2, c, d, and g). These results are quantitated in panel a, which shows the mean number of cells (±SD) with perinuclear MAG staining. Bar: 20 μm.

Almuth β-gal expression was seen as early as E11.5, the first immature OLs were not found until E17.5. Several possibilities may be envisaged to explain this delay. Persistence of Notch1 protein might render the cells effectively Notch1-negative (Rechsteiner and Rogers, 1996). Alternatively, ablation of Notch1 might have an immediate effect, but progress through the developmental program might require several days (Durand and Raff, 2000). This would be consistent with the clonal analyses of purified precursor cells isolated from P7–8 (Barres et al., 1994) and E18 rat optic nerve (Gao et al., 1998) suggesting that a cell intrinsic program plays an important role in determining when OPCs stop dividing and differentiate. Finally, Notch1 may inhibit the transition from OPCs to immature OLs only relatively late in development, shortly before E17.5 in the mouse spinal cord.

Why do many of the prematurely differentiated OLs in Notch1 mutants die? Immunostaining with late differentiation markers and assessment of apoptosis suggest that precociously differentiated OLs cannot terminally differentiate and are unable to survive in the long term if they arise at the wrong place and time, most likely due to missing appropriate survival cues. Similar conclusions were reached by Calver et al. (1998). Overexpression of PDGF led to the appearance of an excess of immature OLs at ectopic sites in the gray matter of transgenic spinal cords, due to an excess production of OPCs, but the OLs survived in large numbers only in white matter. In our study, the number of precursors was normal, and it was precocious and uncontrolled differentiation that led to a high number of immature OLs in the gray matter. However, in both cases, these cells were eliminated by apoptosis, and mature OLs accumulated only in the white matter. It has been suggested that OPCs differentiate preferentially in fiber tracts (Hardy and Friedrich, 1996). However, our results and those of Calver et al. (1998) indicate that progenitor cells can differentiate both in gray and white matter, but survive in large numbers only in white matter.

The increase in immature OLs was not accompanied by a decrease in the number of OPCs. It has been proposed that expansion of OPCs is regulated by a density-dependent mechanism (Zhang and Miller, 1996), which could have served to prevent depletion of the OPC pool in our experiments. We did not observe a significant increase in the number of proliferating cells. However, if the OLs live considerably longer than the cell cycle time of the OPCs which ranges from ~30 h at E13 to ~100 h at E17; van Heyningen et al., 2001), the expected increase in proliferation rate might have escaped detection. We also considered the possibility that Notch ablation might have produced MAG-positive OLs that were also still positive for early markers. We tested this by coimmunostaining for MAG and the early marker NG2, but found that the MAG-positive cells in the gray matter were NG2 negative (unpublished data).

Our analysis demonstrates that Notch1 is required for the regulation of the differentiation of OPCs to OLs, and thus contributes to the timing of oligodendrocyte differentiation in vivo. It has been suggested that this timing might be controlled by an intrinsic mechanism that allows a single OPC to undergo controlled cell cycle arrest and to differentiate in vitro without neurons (Temple and Raff, 1986). Our data and the observations by Wang et al. (1998) show that additional regulatory mechanisms that are mediated by direct cell–cell interactions via the Notch1 receptor and its ligands throughout the CNS are functional in this process.
most probably involves interactions between the axon and OPCs but there might also be a contribution by interactions between mature OLs and OPCs (Wang et al., 1998). The intracellular mechanisms that underlie precocious OL differentiation in the Notch conditional knockout animals remain unknown at this stage. The finding that maturation of OLs is strongly inhibited in the absence of the transcription factor Nkx2.2 (Qi et al., 2001) suggests the possibility that this transcription factor or one of its regulatory targets is antagonized by the Notch signaling pathway.

Very recently, examination of mutants heterozygous for a Notch1-null allele has provided evidence for an effect of Notch1 on myelination (Giovetti et al., 2002). Several changes in the pattern of myelination were observed, including premature myelination in the cortex, increased myelination of small caliber axons in the optic nerve, and ectopic myelination in the molecular layer of the cerebellum. Some of these changes may reflect premature differentiation of OPCs, such as we describe here but in the heterozygous-null setting, there also seem to be effects on survival of OLs and on the choice of axons to be myelinated.

Cell–cell interactions mediated by Notch signaling are likely to play a pivotal role in development as part of a local regulatory circuit that forms the basis for the asynchronous myelination of the CNS and its different fiber tracts. The final trigger for OL differentiation is likely to be a down-regulation of the axonal component of the Notch1–ligand interaction, possibly linked to electrical activity (Rogister et al., 1999). Likewise, OPCs in the adult nervous system might be held in the undifferentiated state via this regulatory system. If correct, inhibition of Notch1 might be an effective way of activating these quiescent cells for repair in demyelinating diseases like multiple sclerosis. This is particularly warranted by the fact that lesions in multiple sclerosis are not starved of OPCs as previously thought, but rather that other factors, possibly including activated Notch signaling, are responsible for the failure of remyelination (Solanky et al., 2001).

Materials and methods

Mice

Mice carrying LoxP-flanked Notch1 alleles have been described previously (Radke et al., 1999). Recombination between the LoxP sites reduces expression of Notch1 protein to undetectable levels, and no shorter fragments are seen (Wolfer et al., 2001). Mice carrying the Cnp-Cre allele have been described previously (line PLCD) and were provided by Dr. P. Soriano (Soriano, 1999). Embryos were generated for animal experiments were approved by the Veterinary Office of the Canton of Zurich (permit 113/99).

For most experiments, we compared Cnp-Cre ΔΔ or LoxP-Cre ΔΔ animals with loxlox littermates as a control. Findings were reproduced from at least three animals of each genotype and from at least two different litters. Spinal cord sections were cut at the forelimb level in embryos and at the thoracic level in newborns, unless otherwise indicated. The lateral ventricles were used as landmarks for the brain sections.

X-gal histochemistry, in situ hybridization, immunofluorescence, and TUNEL staining

Timed pregnant mothers were killed, and embryos were isolated, fixed for 1–2 h in 4% paraformaldehyde, incubated for a few hours to overnight in 30% sucrose, embedded in OCT (TissueTech), and immediately frozen on dry ice. Newborns were anesthetized by intraperitoneal injection of a pentobarbital solution, and then perfused with a 0.9% saline solution followed by 4% ice-cold paraformaldehyde. Brains and spinal cords were isolated, fixed for 24–72 h in the same fixative at 4°C, incubated in 30% sucrose overnight, and embedded in OCT. 20-μm (embryos) or 5-μm (newborn)–frozen sections were thaw-mounted onto Superfrost slides (Mettler) and air dried.

For the X-gal staining, the sections were incubated overnight in a PBS solution containing 5 mM potassium ferrocyanide, 5 mM potassium ferrocyanide, 2 mM magnesium chloride, and 2 mM X-gal (Calbiochem). In situ hybridizations were performed with digoxigenin-labeled RNA probes overnight at 72°C in buffer containing 50% formamide and detected using an anti–DIG-AP antibody according to the manufacturer’s instructions (Roche Diagnostics). The PDGFR-α plasmid was gifts from Dr. P. Soriano, T.M. Jessell, and B. Zalc, respectively. Immunohistochemistry was performed overnight at 4°C with antibodies against NeuN (1:100; Chemicon Inc.), GFAP (1:500; Accurate), β-tubulin III (1:300; Sigma-Aldrich), neurofilament 160 (1:20; Sigma-Aldrich) and phosphorylated histone H3 (1:100; Upstate Biotechnology). Antibodies against MBP (1:500), PLP/DM20 (1:500), Isl1/2 (1:500), and MAG (1:500) were provided by Drs. N. Baumann, I.R. Griffiths, T.M. Jessell, and J.L. Salzer, respectively. Secondary antibody incubations (1:300; Jackson ImmunoResearch) were performed for 1 h at room temperature.

Apoptotic cell death was analyzed by TUNEL staining using biotin-labeled UTP and an FITC-conjugated streptavidin complex according to the manufacturer’s instructions (Roche Diagnostics). Images were collected using an Axio microscope (Carl Zeiss Microimaging, Inc.) in conjunction with a ProgRes 3008 (Jenoptik) or Hamamatsu CCD camera. Image processing was performed with Adobe Photoshop 5.0 software. Staining intensity was measured with NIH Image 1.62 software. The number of cells expressing PDGFR-α mRNA or showing perinuclear MAG staining was determined by counting all cells in at least 10 sections.

Primary cell dissociation

Spinal cords were individually isolated from newborn animals by incubation for 20 min at 37°C in 200 μl DME medium (Life Technologies) containing 2 mg/ml collagenase type IV (Worthington Biochemical Corp.), 1.2 mg/ml hyaluronidase type IV-S (Sigma-Aldrich) and 0.3 mg/ml trypsin inhibitor (Sigma-Aldrich). After trituration, dissociated cells were plated onto poly-c-lysine (Sigma-Aldrich)–coated dishes (35 mm; Corning) in Eagle’s medium with 10% FCS (Sera-Tech) and maintained at 37°C in 5% CO2 overnight. These cells were used for X-gal and O4 staining (Fig. 4, a–d). The carcasses were genotyped by PCR.

Southern blot and quantification

Spinal cord was isolated from newborn animals and cells were mechanically dissociated as previously described (Milner and Frenich-Constant, 1994). Southern blotting (5 μg EcoRI-digested genomic DNA to a Hybridma-N+ membrane (Amersham Biosciences) was performed essentially as described by Radke et al. (1999), using as probe a 750-bp BamHI-EcoRI fragment derived from the 5' upstream region of the Notch1 locus. The probe was hybridized at 42°C, and the signals quantified using a PhosphorImager (Storm 820; Molecular Dynamics).

We thank Drs. L. Sommer and V. Taylor for many helpful discussions and valuable advice, as well as for critically reading the manuscript. We are grateful to Drs. N. Baumann, I.R. Griffiths, T.M. Jessell, W.D. Richardson, J.L. Salzer, and B. Zalc for supplying us with plasmids and antibodies. We thank Kathrin Mannigel and Roger Scab for animal care.

This work was supported by the Swiss National Science Foundation and the Fifth European Community Framework Programme (Control of specification and migration of oligodendrocytes).

References

Apelqvist, A., H. Li, L. Sommer, P. Beatus, D.J. Anderson, T. Honjo, M. Hrabe de Angelis, U. Lendahl, and H. Edlund. 1999. Notch signalling controls pan-creatic cell differentiation. Nature. 400:877–881.
Armstrong, R.C., H.H. Dorn, C.V. Kufta, E. Friedman, and M.E. Dubois-Dalcq. 1992. Pre-oligodendrocytes from adult human CNS. J. Neurosci. 12:1538–1547.
Notch signaling in oligodendrocyte precursors | Genoud et al. 717

Arataviv-Tsakonas, S., K. Matsuno, and M.E. Fortini. 1995. Notch signaling. Science. 268:225–232.
Arataviv-Tsakonas, S., M.D. Rand, and R.J. Lake. 1999. Notch signaling: cell fate control and signal integration in development. Science. 284:770–776.
Barres, B.A., and M.C. Raff. 1993. Proliferation of oligodendrocyte precursor cells depends on electrical activity in axons. Nature. 361:258–260.
Barres, B.A., and M.C. Raff. 1994. Control of oligodendrocyte number in the developing rat optic nerve. Neuron. 12:355–364.
Barres, B.A., M.C. Raff. 1999. Axonal control of oligodendrocyte development. J. Cell Biol. 147:1123–1128.
Barres, B.A., I.K. Hart, H.S. Coles, J.F. Burne, J.T. Voyerodice, W.D. Richardson, and M.C. Raff. 1992. Cell death in the oligodendroglial lineage. J. Neurobiol. 23:1221–1230.
Barres, B.A., M.A. Lazar, and M.C. Raff. 1994. A novel role for thyroid hormone, glucocorticoids and retinoic acid in timing oligodendroglial development. Development. 120:1097–1108.
Berezovska, O., P. McLean, R. Knowles, M. Frosh, S.E. Lux, and B.T. Hyman. 1999. Notch1 inhibits neurite outgrowth in postmitotic primary neurons. Neuroscience. 93:433–439.
Blaschuk, K.L., and C. ffrench-Constant. 1998. Developmental neurobiology: notch is tops in the developing brain. Curr. Biol. 8:R334–R337.
Calver, A.R., A.C. Hall, W.P. Yu, F.S. Walsh, J.K. Heath, C. Betsholtz, and W.D. Richardson. 1998. Oligodendroglial population dynamics and the role of PDGF in vivo. Neuron. 20:869–882.
Casacasa-Bonelli, P. 2000. Cell death in the oligodendroglial lineage: a molecular perspective of life/death decisions in development and disease. Glia. 29:124–135.
Chambers, C.B., Y. Peng, M. Nguyen, N. Gaiano, G. Fishell, and J.S. Nye. 2001. Spatiotemporal selectivity of response to Notch1 signals in mammalian forebrain precursors. Development. 128:689–702.
Chandross, K.J., R.I. Cohen, P. Paras, Jr., M. Gravel, P.E. Braun, and L.D. Hudson. 1999. Identification and characterization of early glial progenitors using a transgenic selection strategy. J. Neurosci. 19:759–774.
Conlon, R.A., A.G. Reaume, and J. Rossant. 1995. Notch1 is required for the coordinate segmentation of somites. Development. 121:1535–1545.
Demeter, C., B. Stankoff, M. Logak, P. Anglade, B. Allinquant, F. Couraud, B. Zalc, and C. Lubbertz. 1996. Induction of myelination in the central nervous system by electrical activity. Proc. Natl. Acad. Sci. USA. 93:9887–9892.
Durand, B., and M. Raff. 2000. A cell-intrinsic timer that operates during oligodendrocyte development. Bioessays. 22:64–71.
Franklin, J.L., B.E. Berechid, F.B. Cuttering, A. Presente, C.B. Chambers, D.R. Foltz, A. Ferreira, and J.S. Nye. 1999. Autonomous and non-autonomous regulation of mammalian neurite development by Notch1 and Delta1. Curr. Biol. 9:1448–1457.
Fu, H., Y. Qi, M. Tan, J. Cai, H. TakedaYoshi, M. Nakafuku, W.D. Richardson, and M. Qiu. 2002. Requirement for LIM homeobox gene Isl1 in motor neuron generation reveals an essential role for insulin-like growth factor-I receptor signalling in the developing spinal cord. Development. 129:681–693.
Furukawa, T., S. Mukherjee, Z.Z. Bao, E.M. Morrow, and C.L. Cepko. 2000. rax, a basic helix-loop-helix transcription factor, regulates the neuroendocrine differentiation of fetal mouse pulmonary epithelium. Development. 127:3913–3921.
Jordan, C., V. Friedrich, Jr., and M. Dubois-Dalot. 1989. In situ hybridization analysis of myelin gene transcripts in developing mouse spinal cord. J. Neurosci. 9:248–257.
Kondo, T., and M. Raff. 2000a. Basic helix-loop-helix proteins and the timing of oligodendrocyte differentiation. Development. 127:2989–2998.
Kondo, T., and M. Raff. 2000b. The Id4 HH1 protein and the timing of oligodendrocyte differentiation. EMBO J. 19:1998–2007.
Lindsell, C.E., J. Jouster, G. deShoos, A. Gonsal, and G. Weinmaster. 1996. Expression patterns of Jagged, Delta1, Notch1, Notch2, and Notch3 genes identify ligand-receptor pairs that may function in neural development. Mol. Cell. Neurosci. 8:14–27.
Lu, Q.R., L. Cai, D. Rowitch, C.L. Cepko, and C.D. Stiles. 2001. Ecotopic expression of Olig1 promotes oligodendrocyte formation and reduces neuronal survival in developing mouse cortex. Nat. Neurosci. 4:975–974.
Lu, Q.R., T. Sun, Z. Zhu, N. Ma, M. Garcia, C.D. Stiles, and D.H. Rowitch. 2002. Common developmental requirement for Olig function indicates a motor neuron/oligodendrocyte connection. Cell. 109:75–86.
Lütolf, S., F. Radtke, M. Aguet, U. Suter, and V. Taylor. 2002. Notch1 is required for neuronal and glial differentiation in the cerebellum. Development. 129:373–385.
McLaughlin, K.A., M.S. Rones, and M. Mercola. 2000. Notch regulates cell fate in the developing pronephros. Dev. Biol. 227:567–580.
Miller, R.H. 1996. Oligodendrocyte origins. Trends Neurosci. 19:92–96.
Miller, R.H., J. Payne, L. Milner, H. Zhang, and D.M. Orentas. 1997. Spinal cord oligodendrocytes develop from a limited number of migratory highly proliferative precursors. J. Neurosci. Res. 50:157–168.
Miller, R.H., and C. ffrench-Constant. 1994. A developmental analysis of oligodendroglial lineages in primary cells: changes in alpha v-associated beta subunits during differentiation. Development. 120:3497–3506.
Morrison, S.J., S.E. Perez, Z. Qiao, J.M. Verdi, C. Hicks, G. Weinmaster, and D.J. Anderson. 2000. Transient Notch activation initiates an irreversible switch from neurogenesis to gliogenesis by neural crest stem cells. Cell. 101:499–510.
Nijjar, S.S., H.A. Crosby, L. Wallace, S.G. Hubsher, and A.J. Strain. 2001. Notch receptor expression in adult human liver: a possible role in bile duct formation and hepatic neovascularization. Hepatology. 34:1184–1192.
Noll, E., and R.H. Miller. 1993. Oligodendrocyte precursors originate at the ventral ventricular zone dorsal to the ventral midline region in the embryonic rat spinal cord. Development. 118:563–573.
Pfaff, S.L., M. Mendelson, C.L. Stewart, T. Edlund, and T.M. Jessell. 1996. Requirement for LIM homeobox gene Id1 in motor neuron generation reveals a motor neuron-dependent step in interneuron differentiation. Cell. 84:309–320.
Pringle, N.P., and W.D. Richardson. 1994. A singularity of PDGF alpha-receptor expression in the dorsolateral axis of the neural tube may define the origin of the oligodendrocyte lineage. Development. 117:525–533.
Qi, Y., J. Cai, Y. Wu, R. Wu, J. Lee, H. Fu, M. Rao, L. Sussel, J. Rubenstein, and M. Qiu. 2001. Control of oligodendrocyte differentiation by the Nkx2.2 homeodomain transcription factor. Development. 128:2723–2733.
Radtke, F., A. Wilson, G. Stark, M. Bauer, J. van Meerwijk, H.R. MacDonald, and M. Aguet. 1999. Deficient T cell fate specification in mice with a reduced inactivation of Notch1. Immunity. 10:547–558.
Raff, M.C., B. Durand, and F.B. Gao. 1998. Cell number control and timing in animal development: the oligodendrocyte cell lineage. Int. J. Dev. Biol. 42:263–267.
Rechsteiner, M., and S.W. Rogers. 1996. PEST sequences and regulation by proteolysis. Trends Biochem. Sci. 21:267–271.
Redmond, L., S.R. Oh, C. Hicks, G. Weinmaster, and A. Ghosh. 2000. Nuclear Notch1 signaling and the regulation of dendritic development. Nat. Neurosci. 3:340–40.
Richardson, W.D., N.P. Pringle, W.P. Yu, and A.C. Hall. 1997. Origins of spinal cord oligodendrocytes: possible developmental and evolutionary relationships with motor neurons. Dev. Neurosci. 19:58–68.
Richardson, W.D., H.K. Smith, T. Sun, N.P. Pringle, A. Hall, and R. Woodruff. 2000. Oligodendrocyte lineage and the motor neuron connection. Glia. 29: 136–142.

Robey, E. 1997. Notch in vertebrates. Curr. Opin. Genet. Dev. 7:551–557.

Rogister, B., T. Ben-Hur, and M. Dubois-Dalcq. 1999. From neural stem cells to myelinating oligodendrocytes. Mol. Cell. Neurosci. 14:287–300.

Sestan, N., S. Artavanis-Tsakonas, and P. Rakic. 1999. Contact-dependent inhibition of cortical neurite growth mediated by notch signaling. Science. 286: 741–746.

Solanki, M., Y. Maeda, X. Ming, W. Husar, W. Li, S. Cook, and P. Dowling. 2001. Proliferating oligodendrocytes are present in both active and chronic inactive multiple sclerosis plaques. J. Neurosci. Res. 65:308–317.

Soriano, P. 1999. Generalized lacZ expression with the ROSA26 Cre reporter strain. Nat. Genet. 21:70–71.

Spassky, N., C. Goujet-Zalc, E. Parmantier, C. Olivier, S. Martinez, A. Ivanova, K. Ikenaka, W. Macklin, I. Cerruti, B. Zalc, and J.L. Thomas. 1998. Multiple restricted origin of oligodendrocytes. J. Neurosci. 18:8331–8343.

Sun, T., N.P. Pringle, A.P. Hardy, W.D. Richardson, and H.K. Smith. 1998. Pax6 influences the time and site of origin of glial precursors in the ventral neural tube. Mol. Cell. Neurosci. 12:228–239.

Temple, S., and M.C. Raff. 1986. Clonal analysis of oligodendrocyte development in culture: evidence for a developmental clock that counts cell divisions. Cell. 44:773–779.

Timsit, S., S. Martinez, B. Allinquant, F. Peyron, L. Puelles, and B. Zalc. 1995. Oligodendrocytes originate in a restricted zone of the embryonic ventral neural tube defined by DM-20 mRNA expression. J. Neurosci. 15:1012–1024.

Trapp, B.D., A. Nishiyama, D. Cheng, and W. Macklin. 1997. Differentiation and death of premyelinating oligodendrocytes in developing rodent brain. J. Cell Biol. 137:459–468.

Tsuchida, T., M. Ensini, S.B. Morton, M. Baldassare, T. Edlund, T.M. Jessell, and S.L. Pfaff. 1994. Topographic organization of embryonic motor neurons defined by expression of LIM homeobox genes. Cell. 79:957–970.

van Heyningen, P., A.R. Calver, and W.D. Richardson. 2001. Control of progenitor cell number by mitogen supply and demand. Curr. Biol. 11:232–241.

Wakamatsu, Y., T.M. Maynard, and J.A. Weston. 2000. Fate determination of neural crest cells by NOTCH-mediated lateral inhibition and asymmetrical cell division during gangliogenesis. Development. 127:2811–2821.

Wang, S., A.D. Sdrulla, G. diSibio, G. Bush, D. Noziger, C. Hicks, G. Weinmaster, and B.A. Barres. 1998. Notch receptor activation inhibits oligodendrocyte differentiation. Neuron. 21:63–75.

Wang, S., A. Sdrulla, J.E. Johnson, Y. Yokota, and B.A. Barres. 2001. A role for the helix-loop-helix protein Id2 in the control of oligodendrocyte development. Neuron. 29:603–614.

Weinmaster, G. 1998. Notch signaling: direct or what? Curr. Opin. Genet. Dev. 8:436–442.

Wolfer, A., T. Bakker, A. Wilson, M. Nicolas, V. Ioannidis, D.R. Littman, P.P. Lee, C.B. Wilson, W. Held, H.R. MacDonald, and F. Radtke. 2001. Inactivation of Notch 1 in immature thymocytes does not perturb CD4 or CD8T cell development. Nat. Immunol. 2:235–241.

Yu, W.P., E.J. Collarini, N.P. Pringle, and W.D. Richardson. 1994. Embryonic expression of myelin genes: evidence for a focal source of oligodendrocyte precursors in the ventricular zone of the neural tube. J. Neurosci. 14:353–362.

Zhang, H., and R.H. Miller. 1996. Density-dependent feedback inhibition of oligodendrocyte precursor expansion. J. Neurosci. 16:6886–6895.

Zhou, Q., G. Choi, and D.J. Anderson. 2001. The bHLH transcription factor Olig2 promotes oligodendrocyte differentiation in collaboration with Nkx2.2. Neuron. 31:791–807.