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Function of Notch1 signaling in oligodendrocyte development and CNS remyelination

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TABLE OF CONTENTS

1 SUMMARY

All the neurons and glial cells of the central nervous system are generated from the epithelial cells (neuroepithelium) that line the walls of the neural tube. The production of these different cell types is highly organized in space and in time. Among the last cell type to be specified, the oligodendrocytes differentiate from migratory precursor cells that arise from specific zones of neuroepithelium. A fundamental challenge of developmental neurobiology is to understand the molecular mechanisms that give rise to the differentiation of distinct populations of cells. Results from knockout animals as well as in vitro studies have allowed identification of several key factors regulating the complex process of oligodendrocyte differentiation and maturation. However, the full characterization of the signals and how these mechanisms are orchestrated in vivo remain elusive.

The goal of this work was to assess the role of Notch1 signaling in the development and function of the oligodendrocyte lineage. I used a Cre/lox system for oligodendrocyte-specific deletion of the Notch1 receptor. Through use of conditional knockout mice I showed in the first part of my thesis that Notch1 is critically important for establishing the correct temporal and spatial pattern of differentiation of oligodendrocytes in the spinal cord. Despite the loss of Notch1 in oligodendrocyte precursor cells, early development of the oligodendrocyte lineage appeared normal: In both mutant and wild-tpye, oligodendrocyte precursors arising in the ventral neural tube subsequently proliferated and dispersed throughout the gray and white matter of the developing spinal cord. The number of oligodendrocyte precursor cells was not altered at the ages examined. However, at embryonic day 17.5 and at postnatal day 0, a loss of Notch1 led to the appearance of an excess of immature oligodendrocytes at ectopic sites in the gray matter of mutant spinal cords. Premature oligodendrocyte differentiation was observed both in the spinal cord and in the cerebrum, indicating that Notch1 is required for the correct timing of oligodendrocyte differentiation in various regions of the central nervous system. Precociously differentiated oligodendrocytes in the gray matter were eliminated by apoptosis, although Notch1$^{-/-}$ oligodendrocytes could differentiate and survive in vitro. This suggests that some extracellular factors, present only in the white matter, are

critical for oligodendrocyte survival in vivo. These results demonstrate conclusively a crucial function of specifically Notch1 for differentiation of oligodendrocyte precursors in the spinal cord and suggest a similar function in the brain (Genoud et al., J. Cell Biol., in press).

In the second part of my thesis, I showed that the lack of Notch1 expression in oligodendrocyte precursors or developing oligodendrocytes does not alter the localization of myelin within the first two weeks of spinal cord development and in adult animals. Furthermore, no myelinating oligodendrocytes were observed within the molecular layer of the cerebellum, a region which contains oligodendrocyte precursor cells but no myelinated axons. Precocious appearance of myelinating oligodendrocytes was also not detected. Finally, I addressed the functional importance of Notch1 in the process of remyelination in the central nervous system, and used a model in which continuous cuprizone (bis-cyclohexanone oxaldihydrazone) intoxication of adult mice leads to perturbation and death of mature oligodendrocytes. Although caution must be taken in generalizing these data because of the small number of mice analyzed, I failed to show a direct role for Notch1 in the regulation of central nervous system remyelination. Further studies are being carried out with the aim of better understanding the cellular changes occurring after Notch1 deletion in a lesion.

2 RÉSUMÉ

Toutes les cellules neuronales et gliales du système nerveux central sont générées à partir de cellules épithéliales (neuroépithélium) qui bordent les parois du tube neural. La production de ces différents types cellulaires est extrêmement bien structurée dans l'espace et dans le temps. Parmi le dernier type cellulaire à être spécifié, les oligodendrocytes se différentient à partir de cellules précurseurs migratrices qui ont pour origine des zones spécifiques du neuroépithélium. Un défi fondamental de la neurobiologie du développement consiste à comprendre les méchanismes moléculaires qui entraînent la différentiation de populations distinctes de cellules. Des résultats obtenus à partir d'animaux "knockout" ainsi que des études in vitro ont permis l'identification de plusieurs facteurs clés qui régulent le processus complexe de la différentiation et maturation des oligodendrocytes. Cependant, la caractérisation complète des signaux et la manière dont ces méchanismes sont orchestrés in vivo restent encore à découvrir.

Le but de cette étude consista à évaluer le rôle de la signalisation de Notch1 dans le développement et la fonction de la lignée oligodendrocytaire. J'ai utilisé un système Cre/lox pour la délétion spécifique du récepteur Notch1 dans les oligodendrocytes. En utilisant des souris "knockout" conditionnel, j'ai montré dans la première partie de ma thèse que Notch1 joue un rôle décisif dans le contrôle temporel et spatial de la différentiation des oligodendrocytes dans la moëlle épinière. Malgré l'absence de Notch1 dans les cellules oligodendrocytaires précurseurs, les premiers stades de développement de la lignée oligodendrocytaire se sont avérés normaux: aussi bien dans les souris mutantes que dans les souris sauvages, les cellules oligodendrocytaires précurseurs qui proviennent du tube neural ventral ont par la suite proliféré et se sont dispersés dans la matière grise et matière blanche de la moëlle épinière en développement. Le nombre de cellules oligodendrocytaires précurseurs n'a pas été modifié à tout âge examiné. Cependant, au 17.5ème jour embryonnaire ainsi qu'à la naissance, une absence de Notch1 a conduit à un excédent d'oligodendrocytes immatures de façon ectopique dans la matière grise des moëlles épinières mutantes. Une différentiation prématurée des oligodendrocytes a été observée dans la moëlle épinière et

dans le cerebrum, indiquant que Notch1 est requis pour le minutage précis de la différentiation des oligodendrocytes dans differentes régions du système nerveux central. Les oligodendrocytes prématurément différentiés dans la matière grise ont été éliminés par apoptose, bien que les oligodendrocytes n'exprimant pas Notch1 aient été capables de se différencier et de survivre in vitro. Cela suggère la présence critique de facteurs extracellulaires, et ce uniquement dans la matière blanche, afin d'assurer la survie des oligodendrocytes in vivo. Ces résultats démontrent de façon conclusive une fonction cruciale de spécifiquement Notch1 dans la différentiation des cellules oligodendrocytaires précurseurs de la moëlle épinière et suggèrent une fonction analogue de Notch1 dans le cerveau (Genoud et al., J. Cell Biol., sous presse).

Dans la seconde partie de ma thèse, j'ai montré que l'absence de Notch1 dans les cellules oligodendrocytaires précurseurs ou dans les oligodendrocytes en phase de développement n'affecte pas la localisation précise de la myélinisation durant les deux premières semaines de développement de la moëlle épinière et chez les animaux adultes. De plus, aucun oligodendrocyte en train de myéliniser n'a été détecté dans la couche moléculaire du cervelet, une région qui contient des cellules oligodendrocytaires précurseurs mais pas d'axones myélinisés. L'apparition précoce d'oligodendrocytes producteurs de myéline n'a également pas été observée dans plusieurs régions du système nerveux central. Finalement, j'ai examiné l'importance de la fonction de Notch1 dans le processus de remyélinisation du système nerveux central. Pour cela, j'ai utilisé un modèle, grâce auquel une intoxication continue de cuprizone (N,N-oxalylbis(cyclohexanone-hydrazone)) dans la souris adulte conduit à une perturbation et à la mort des oligodendrocytes mûrs producteurs de myéline. Bien que la prudence s'avère nécessaire à généraliser ces résultats dûs à un nombre restreint de souris analysées, je n'ai pu démontrer un rôle direct de Notch1 dans la régulation de la remyélinisation du système nerveux central. De plus amples études sont en train d'être poursuites afin de mieux comprendre les changements moléculaires se produisant à la suite d'une délétion de Notch1 dans une lésion.

2 INTRODUCTION

Part 1 of my Ph.D thesis is an expanded version of Genoud et al., J. Cell Biol., in press. Oligodendrocyte precursors originate from neuroepithelial cells of the ventricular zones, at early stages during embryonic life. They then proliferate and migrate long distances away from these zones and populate the developing central nervous system to form white matter throughout the brain, spinal cord and optic nerve. Final differentiation into myelin-forming oligodendrocytes does not occur in the mouse until several days after the appearance of the first precursors. Because mature oligodendrocytes cannot migrate, preventing premature differentiation of progenitors is therefore crucial for ensuring that they successfully make it to their final destination. How proliferation, migration and differentiation are regulated in the oligodendrocyte lineage is only partially understood (Barres et al., 1992; Barres and Raff, 1994; Kondo and Raff, 2000a; Kondo and Raff, 2000b; Qi et al., 2001; Raff et al., 1998; Wang et al., 2001; Wang et al., 1998; Zhou et al., 2001).

2.1 Origin and differentiation of the oligodendrocytes

2.1.1 The oligodendrocyte precursor cells

On the basis of morphology and molecular phenotype, cells of the oligodendrocyte (OL) lineage can be grouped into three main groups, i.e. oligodendrocyte precursor cells (OPCs), immature OLs (premyelinating), and mature OLs (myelinating cells) (Armstrong et al., 1992; Gard and Pfeiffer, 1990; Hardy and Reynolds, 1991; 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).

In the telencephalon, there are now several lines of evidence indicating that OLs originate also there from ventral subsets of neuroepithelial precursors (Richardson et al., 2000)and reference therein). These conclusions were first suggested by following the expression of specific markers of oligodendrocytes, some of which are transcripts of future protein components of myelin (2',3'-cyclic nucleotide 3'-phosphohydrolase (CNP), myelin basic protein (MBP), proteolipid protein (PLP)) (Ikenaka et al., 1992; Peyron et

al., 1997; Pringle and Richardson, 1993; Timsit et al., 1995; Timsit et al., 1992; Yu et al., 1994) and then by transplantation studies (Richardson et al., (2000) and reference therein).

Two major classes of transcription factors have emerged as determinants of neuron versus glial fate determination and of neuronal subtype specification: the basic-helix-loop-helix (bHLH) factors (Vetter and Brown, 2001) and homeodomain (HD) factors (Jessell, 2000), respectively. In the spinal cord, a combinatorial code of HD transcription factors specifies the regional identity of progenitor domains along the dorso-ventral axis (Briscoe et al., 2000; Jessell, 2000). Recently, a subclass of neural bHLH factors, called *Olig* genes (Lu et al., 2000; Takebayashi et al., 2000; Zhou et al., 2000), has been identified. In the mouse, there are two *Olig* genes, Olig1 and Olig2, that are specifically expressed in oligodendrocyte precursors (Lu et al., 2000; Zhou et al., 2000). In collaboration with the HD factor Nkx2.2, Olig1/2 promote specification of oligodendrocyte precursors (Qi et al., 2001; Zhou et al., 2000) and are thought to be downstream from Sonic Hedgehog signaling (Lu et al., 2000). In chick embryos, just before OPCs are produced from the motorneuron domain (MN), expression of Nkx2.2 is dorsally expanded into the MN domain (Figure 2.1A, B). In mouse embyos, Nkx2.2 expression is not dorsally expanded and the $Olig2^+$ OPCs acquire Nkx2.2 expression after migration (Fu et al., 2002). Although it is generally accepted that OPCs are derived from the ventral neuroepithelium, it is not yet clear whether OLs are derived from one or several different progenitors. Zhou et al., (2001) and Fu et al., (2002) have proposed that in the spinal cord there may be separate lineages of oligodendrocyte progenitors that are $Nkx2.2^+$, $Olig2^+$ and $Nkx2.2^+$, $Olig2^-$, respectively, with distinct origins in the chick and mouse ventricular zone. In the chicken spinal cord, one population emigrates from the zone of overlap between these two factors, while the other population emigrates more ventrally (Figure 2.1 B). In the mouse spinal cord, the $Nkx2.2^+$ cells that are generated from the Nkx2.2 region migrate however relatively slowly and remain in the ventral gray matter until E16.5 (Fu et al., 2002). Although previous studies have indicated that all $Nkx2.2^+$ cells are OPCs, but not astrocytes or neurons (Xu et al., 2000), this issue still remains unclear as there is no final proof that the $Nkx2.2^+$, $Olig2^-$ cells can give rise to differentiated OLs. Similarly, in the developing brain expression studies with different markers have revealed the presence of a discrete population of cells which express

mRNA encoding myelin proteolipid protein (PLP) and/or its alternatively-spliced isoform DM-20, but not PDGFR alpha, an early marker for OPCs (Spassky et al., 1998). OPCs arising in the ventral neural tube subsequently proliferate and disperse throughout the developing spinal cord (reviewed in ref. Miller, 1996; Richardson et al., 1997; Spassky et al., 2000) (Figure 2.1C). It remains an open question, however, 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 (Blaschuk and ffrench-Constant, 1998).

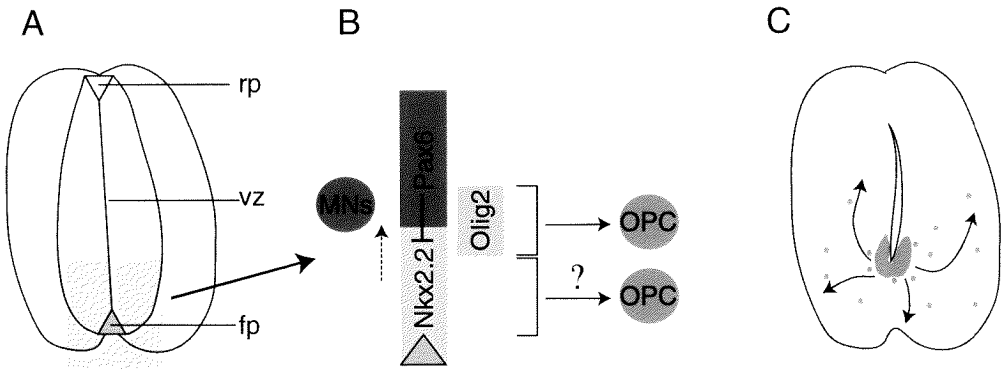


Figure 2-1: Origin, specification and migration of the oligodendrocyte precursor cells.

A) Ventral origin of the OPCs in the spinal cord. B) Summary of spatio-temporal changes in Olig2 and Nkx2.2 expression and their overlap leading to oligodendrocyte precursors production in chick embryos (the overlap is symbolized by an arrow). Note that in mouse embryos, the coexpression of Olig2 and Nkx2.2 will occur only after the migration of OPCs. C) Migration of OPCs throughout the spinal cord. rp, roof plate; vz, ventricular zone; fp, floor plate; MNs, somatic motorneurons. ? in B) suggests that some oligodendrocyte precursors (i.e. those that are Nkx2.2+ and Olig2-) can be specified in the absence of Olig2. Adapted from Lu et al., (2000) and Zhou et al., (2001)

2.1.2 The differentiation of oligodendrocytes

In the mouse, the earliest born oligodendrocytes appear at E14.5 along the midline of the medulla, the medullary raphe (Hardy and Friedrich, 1996). Oligodendrocytes appear subsequently along the spinal cord in a rostrocaudal wave of differentiation, 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). Postmitotic OLs appear in the forebrain only at birth.

Maturation of OLs proceeds through very distinct stages which can be identified by dramatic changes in cell morphology and by various markers, some of which are characteristic myelin components. Numerous stages have been reported (Figure 2.2), but they can be simplified and placed into two main groups: an immature stage (also called premyelinating stage), where OLs are postmitotic but do not myelinate yet, and a mature myelinating stage. Note that differentiation of mouse OLs is similar to that of the rat after the stage at which O4 is acquired, whereas mouse progenitors show greater variety at the level of progenitor and pre-oligodendrocyte stages, both in their morphology and in the expression of markers, such as A2B5 and GD3 (Fanarraga et al., 1995).

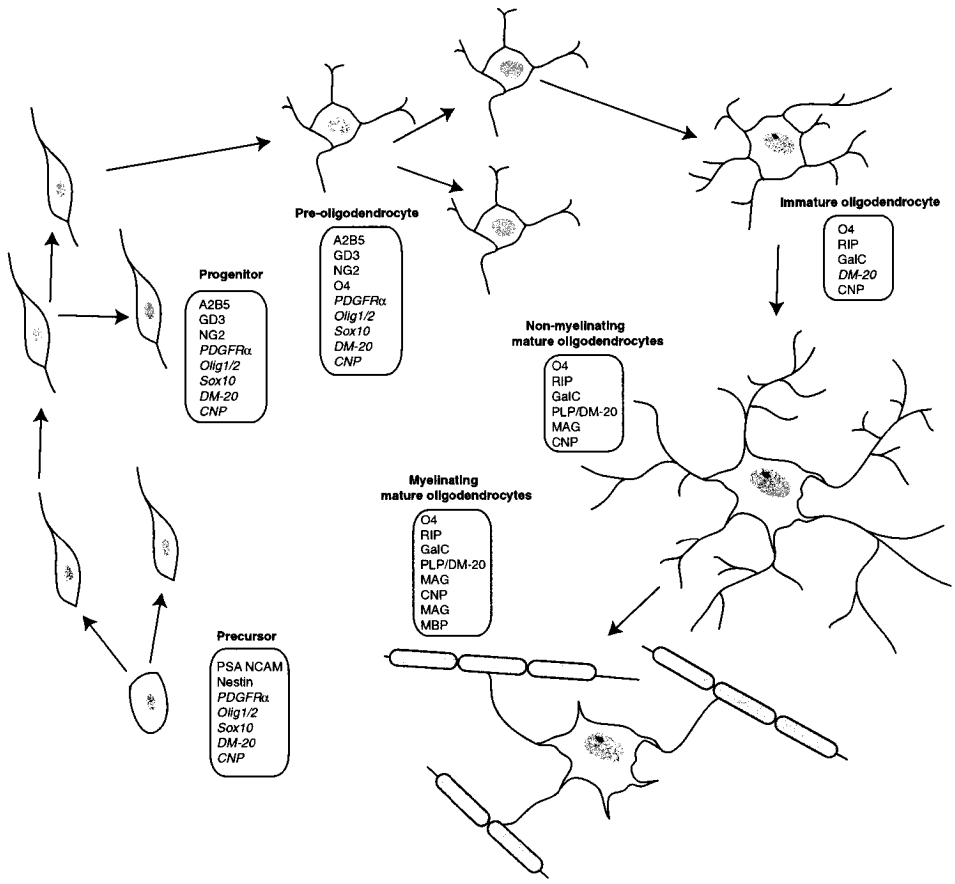


Figure 2-2: Schematic representation of the developmental stages of cells of the oligodendrocyte lineage.

Schematic drawing of the morphological and antigenic progression from precursor cells to myelinating mature oligodendrocytes, through progenitors, preoligodendrocytes, and immature nonmyelinating

oligodendrocytes in the rat. Stage-specific markers are boxed. RNAs are in italics. Adapted from Baumann et al., (2001) (Baumann and Pham-Dinh, 2001).

2.2 Mechanisms of oligodendrocyte differentiation

2.2.1 Cell-intrinsic and cell-extrinsic regulation

The timing of OL differentiation is thought to depend on both intracellular mechanisms and extracellular signals. Interestingly, oligodendrocyte precursor cells cultured with mitogenic factors such as platelet-derived growth factor (PDGF) and hydrophobic signals such as thyroid hormone (TH) can divide only a limited number of time before they stop proliferating and differentiate. Furthermore, OPCs cultured at 33°C rather than 37°C proliferate more slowly but differentiate sooner, after fewer cell divisions (reviewed by Raff et al., (1998)). Finally, differentiation into O4-positive pre-oligodendrocytes was postponed by 4-5 days in the telencephalon in comparison with the spinal cord. This delay between determination and differentiation appears to be intrinsic to telencephalic OLs, as it was not shortened by diffusible or cell-cell contact factors present in the spinal cord (Spassky et al., 2001). Together, these studies suggested that an intrinsic clock (also called timer) is operating to control OL differentiation, and it does so by counting time but not cell divisions (Gao et al., 1998; Temple and Raff, 1986). Although the timing mechanism is apparently built into each OPC, it depends on extracellular signals to operate normally: In their absence, OPCs seem to divide indefinitely (for at least 16 days) in response to mitogens (Barres et al., 1994; Tang et al., 2001).

Hydrophobic signals, such as thyroid hormone, glucocorticoids and retinoic acid, have been found to be involved in the differentiation of the oligodendrocyte lineage (Barres et al., 1994; Billon et al., 2001). Most of these studies have been performed in vitro. It is therefore extremely difficult to extrapolate to in vivo conditions, as multiple factors may act in concert to achieve the exquisitely fine regulation of the complex process of OL development. There is also evidence that the cyclin-dependent kinase (Cdk) inhibitor p27/Kip1 (p27) is apparently one element of the timer, as p27-deficient precursor cells undergo more divisions than normal in vitro (Durand et al., 1998) and OL differentiation is perturbed (Casaccia-Bonnefil et al., 1997). Finally, studies have shown that the Id2 and

Id4 helix-loop-helix (HLH) transcription factors (Kondo and Raff, 2000b; Wang et al., 2001), the homeodomain protein (HD) Nkx2.2 (Qi et al., 2001), and the basic helix-loop-helix (bHLH) factor Olig1 (Lu et al., 2001; Lu et al., 2002) play important roles in the control of OL differentiation. For example, 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 (Lu et al., 2001). However, it remains unclear how these different proteins and mechanisms are coupled.

2.2.2 The Notch signaling pathway

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 (reviewed in ref. Artavanis-Tsakonas et al., 1995; Robey, 1997; Weinmaster, 1998). In this context, Notch signaling plays a key role in tissue-autonomous fate determination in 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 the development and remodeling of dendrites (Berezovska et al., 1999; Franklin et al., 1999; Redmond et al., 2000; Sestan et al., 1999) has also been found.

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 (Chambers et al., 2001; Gaiano et al., 2000), 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 (Tanigaki et al., 2001), and of neural crest stem cells into the Schwann cell lineage (Morrison et al., 2000).

The OL lineage, however, 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; Tanigaki et al., 2001). Furthermore, a potential regulatory role of the Notch pathway in the regulation of mammalian OPC differentiation has been suggested by cell culture studies showing that OPCs derived from postnatal rat optic nerve could be inhibited in their differentiation by incubation with Notch ligands (Wang et al., 1998).

Ectopic OPCs arising upon overexpression of Notch1 together with Olig2 in chick spinal cord (Zhou et al., 2001) do not go on to mature, consistent with a negative role for Notch1 in late stages of differentiation (Zhou et al., 2001). 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 (Irvin et al., 2001; Lindsell et al., 1996))? 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 (Barres and Raff, 1999; Casaccia-Bonnefil, 2000; Wang et al., 1998)? 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 knock-out mice were not informative, however, due to early embryonic lethality (Conlon et al., 1995; Swiatek et al., 1994). Thus, in this study I have selectively inhibited Notch1 signaling using a conditional Notch1 knock-out mouse strain (Radtke et al., 1999). My data demonstrate conclusively,

for the first time, a crucial function of specifically Notch1 for differentiation of oligodendrocyte precursors in the spinal cord and suggest a similar function in the brain.

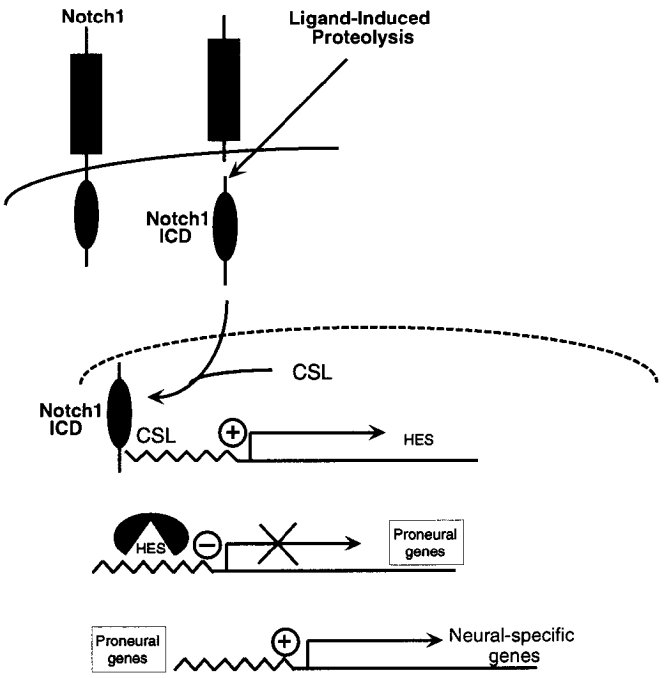


Figure 2-3: Basic scheme of Notch signaling in neurogenesis.

Upon ligand binding, the intracellular domain (ICD) of Notch is cleaved and translocates into the nucleus where it binds the transcription factor CSL (for mammalian CBF-1/ RBP-JK, *Drosophila* Su(H), and *C. elegans* Lag-1). The formation of this complex leads to the expression of the HES genes (the mammalian homologues of *Drosophila* hairy and Enhancer of Split proteins), whose expression inhibits the transcription of proneural genes.

3 RESULTS

3.1 Experimental strategy

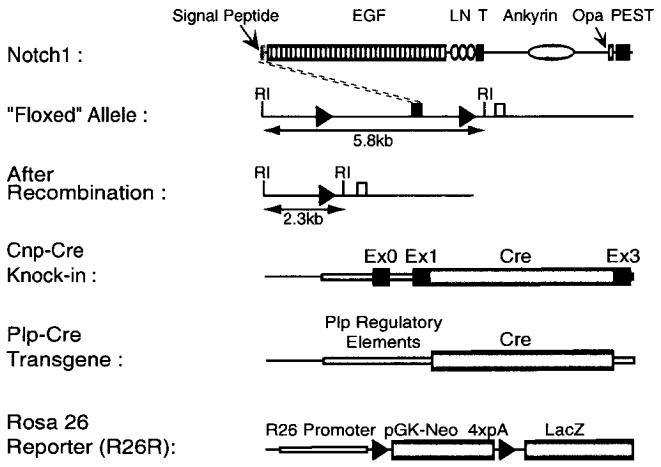
I have used a "floxed" allele of *Notch1*, in which the exon encoding the signal sequence (Figure 3.1) is flanked by *loxP* sites (Radtke et al., 1999) for conditional gene ablation. Recombination mediated by the Cre recombinase excises the region between the lox sites, thereby inactivating the gene. This strategy is very appealing, since it allows to study the function of a gene in a specific cell lineage. In the present study, *Notch1* was inactivated early in development of the oligodendrocyte lineage in the spinal cord. The consequences for the further development of the lineage were then studied in detail.

Cre was expressed under the control of regulatory elements known to be active in the OL lineage, either those of the 2',3'-cyclic nucleotide 3'-phosphodiesterase (*Cnp*) gene (with the Cre coding region inserted into the endogenous gene; Corinna Lappe-Siefke, Sandra Goebbels, Klaus-Armin Nave, Max-Planck-Institute, 37075 Göttingen, Germany, (unpublished), or of the proteolipid (*Plp*) gene (as a transgene driving Cre; S.G., C.L., K-A.N., unpublished) (Figure 3.1A). Newborn mice homozygous for the floxed *Notch1* allele and carrying either the *Cnp-Cre* (*Cnp-Cre Δ/Δ*) or the *Plp-Cre* (*Plp-Cre Δ/Δ*) allele were outwardly normal, nursed, moved, breathed, and responded to mechanical stimulation, but usually survived only a few hours after birth. A small number of *Cnp-Cre Δ/Δ* individuals survived longer, exhibited only modest defects (smaller in size, poor sense of balance, partially closed eyes), and could be kept until adulthood. In contrast, *Plp-Cre Δ/Δ* mice that survived after birth had to be sacrificed because of severe growth retardation and motor defects.

Figure 3-1: Schematic representation of the murine Notch1 protein and loxP/Cre-mediated deletion strategy (next page).

The Notch protein contains 2531 amino acid residues that encompass a signal peptide, 36 EGF repeats (EGF), three Lin/Notch domains (LN), a transmembrane domain (T), cytoplasmic ankyrin repeats, a polyglutamine stretch (Opa), and a PEST sequence. In the floxed *Notch1* allele (Radtke et al., 1999), the first coding exon is flanked by *loxP* sequences (gray triangles). After Cre-mediated recombination, a null allele is generated. Arrows indicate Eco RI fragments that differ in size between the floxed locus compared to the locus after deletion of the 3.5 kb segment flanked by *loxP* sites. The Cre recombinase gene was

inserted into the *Cnp* locus (*Cnp-Cre*) or is driven from *Plp/DM20* regulatory elements (Spassky et al., 1998) (*Plp-Cre*). The ROSA26 reporter mouse (Soriano, 1999) (*R26R*) was used to follow recombination events: Cre-mediated recombination deletes the neomycin phosphotransferase gene (PGK-neo) plus four polyadenylation sites (4xpA), activating the production of beta-gal encoded by LacZ. RI indicates Eco RI restriction sites.



3.2 Cre mediated recombination

3.2.1 *Cnp-Cre* mediated recombination

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*). This approach allowed lineage tracing via detection of the LacZ product beta-galactosidase (beta-gal; Figure 3.2A). Sections were cut at the forelimb level of doubly transgenic mice at embryonic day 13 (E13), and beta-gal detected by histological staining with the chromogenic substrate X-gal. In *Cnp-Cre R26R* mice (Figure 3.2A, enlarged in 2C), 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 platelet-derived growth factor receptor (PDGFR) alpha in situ hybridization (Figure 3.2D). This restricted expression in OPCs was expected based on the reported endogenous CNP expression in the rat (Yu et al., 1994) and the analysis of transgenic mice under the control of CNP-regulatory elements (Chandross et al., 1999;

Gravel et al., 1998). Additional recombination was observed in the ventral horns of the neural tube, presumably in developing motoneurons. Furthermore, X-gal positive cells were already found in the ventral ventricular zone as early as E11.5 (data not shown). By E17.5, the number of X-gal positive cells was increased substantially and the cells were dispersed throughout the spinal cord (Figure 3.4B), consistent with the expected proliferation and migration of OPCs (Miller et al., 1997).

In the forebrain, numerous X-gal positive cells were already detected at E11.5, but no recombined cells were detected within the subventricular zone (Figure 3.2B). Outside the CNS, recombination was observed at E17.5 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) (Figures 3.2E, 2F and not shown). These findings could explain the early death of the Cnp-Cre Δ/Δ animals.

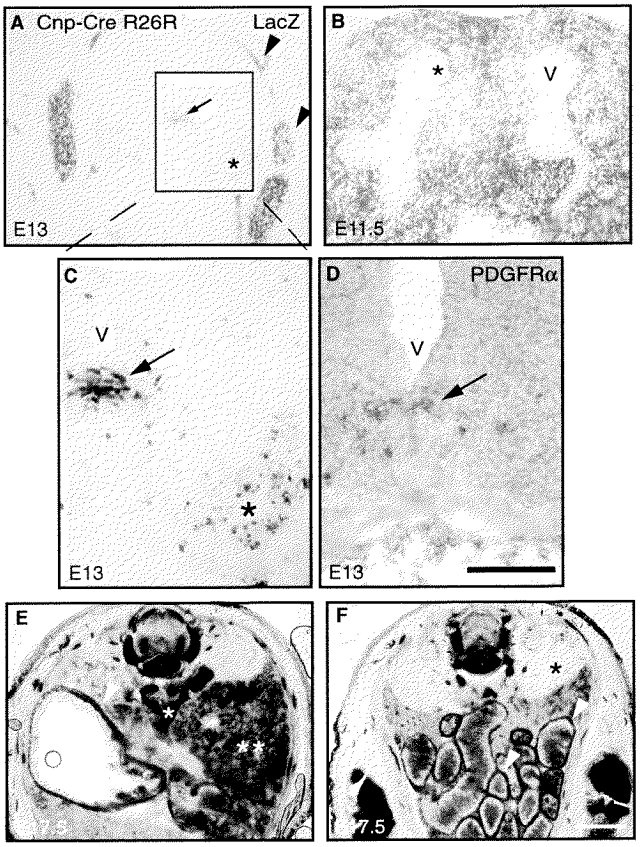


Figure 3-2: *Cnp-Cre*-induced beta-gal expression pattern (previous page).

Cnp-Cre mice were crossed with R26R reporter mice. Cells in which Cre had been expressed were detected by X-gal staining for beta-galactosidase activity in sections of E11.5, E13 and E17.5 embryos. At E11.5, extensive staining is detected in the forebrain, but not in the subventricular zone (B, asterisk). At E13 in the spinal cord, the Cnp-Cre line shows expression in the ventral ventricular zone (A, arrow) and motoneurons (C, asterisk); these regions are enlarged in panel (C). The area indicated by an arrow in A) and C) is the site of origin of the OPCs consistent with the expected endogenous CNPase expression pattern and confirmed by PDGFR alpha in situ hybridization (D). Arrowheads in A) indicate the nerve roots and dorsal root ganglia that are part of the PNS. Outside the CNS, some staining in the pancreas (asterisk), liver (double asterisk) and around the stomach (arrowhead) can be detected (E). Recombination also occurred in the intestines (F, arrowhead) and in the kidney (F, asterisk). Scale bar in D (for C, D) equals 10 μm. Scale bar in F equals 10μm (for A, B) and 40μm (for E, F). V, ventricle.

3.2.2 *Plp-Cre* mediated recombination

To determine the expression pattern of *Plp-Cre*, the same procedure was used as for *Cnp-Cre* (see above). *Plp-Cre* mice were crossed with mice carrying *R26R* (yielding *Plp-Cre R26R*) and recombination analyzed via detection of the LacZ product beta-galactosidase. At E9.5, X-gal positive cells start to appear in the neural tube of *Plp-Cre R26R* animals (Figure 3.3A, asterisk) and can be progressively seen along the whole length of the developing spinal cord (not shown). By E10.5, the number of X-gal positive cells was substantially increased in spinal cord sections cut at the forelimb level (Figure 3.3B and 3C). This is however most likely an artefact of the transgene insertion, since expression of the endogenous Plp gene is more restricted and starts only at E14.5 in the spinal cord (Timsit et al., 1995; Yu et al., 1994). Additional recombination was observed in the dorsal part of the spinal cord, presumably neural crest (Figure 3.3A, arrowheads), and in structures of the peripheral nervous system (Figures 3.3B, C, arrowheads). In order to better quantify the percentage of recombined cells within the spinal cord of *Plp-Cre R26R* animals, the following experiment was performed. The whole spinal cord of embryos isolated at E12.5 was carefully dissected and dorsal root ganglia cut off. The tissue was then dissociated, and the cells (resulted from single animals) plated and stained for X-gal (Figure 3.3D). 200 cells were counted and 87% of them were X-gal positive. Further analysis was therefore performed mainly with the *Cnp-Cre* mice, which show more restricted recombination in the oligodendrocyte lineage.

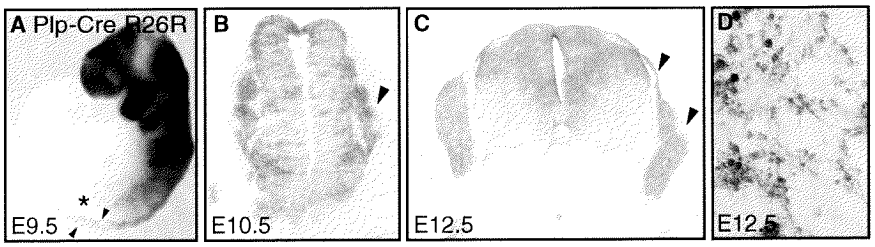


Figure 3-3: *Plp-Cre*-induced beta-gal expression patterns.

Plp-Cre mice were crossed with *R26R* reporter mice. Cells in which Cre had been expressed were detected by X-gal staining for beta-galactosidase activity in E9.5 whole-mount embryos (A) or sections of E10.5 (B) and E12.5 (C) embryos. The spinal cord of E12.5 embryos was dissected, and processed separately for each animal. The tissue was triturated to have a single cell suspension and the resulted cells were plated onto poly-D-lysine (PDL) coated dishes for overnight culture followed by X-gal staining (D). At E9.5, the *Plp-Cre* line shows expression in the dorsal part of the neural tube (A, arrowheads) but most of the spinal cord does not yet express the transgene (A, asterisk). Arrowheads in B) and C) indicate the nerve roots and dorsal root ganglia. Note the very high proportion of recombined cells within the spinal cord of *Plp-Cre R26R* animals (B,C,D).

3.3 Precocious appearance of OLs in the spinal cord at E17.5

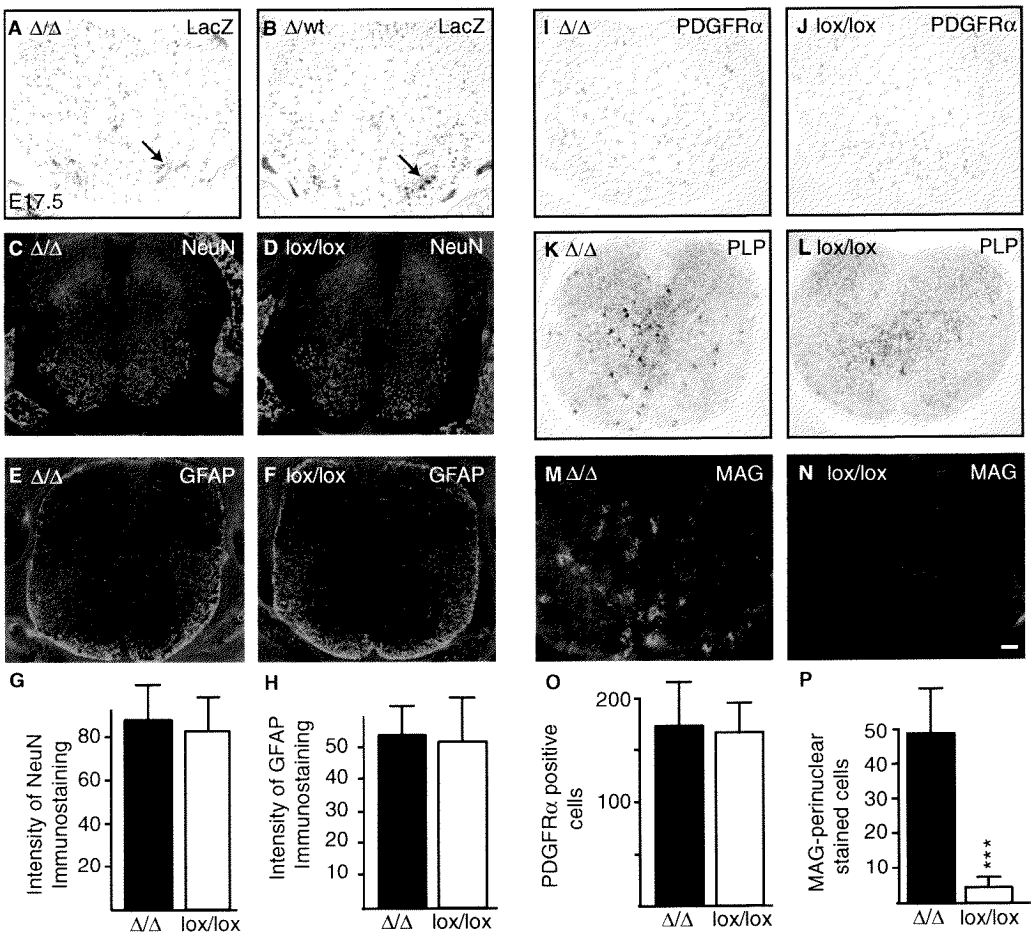
I focused my studies on the spinal cord because OL development has been extensively studied in this structure. I examined spinal cords at E17.5, shortly before OLs start to accumulate in substantial numbers in the ventral fiber tracts of the mouse thoracic spinal cord. Initial X-gal staining in *Cnp-Cre Δ/Δ R26R* (Figure 3.4A) and *Cnp-Cre Δ/wt R26R* (Figure 3.4B) embryos showed no major differences in the pattern of recombined cells in the homozygous (Δ/Δ) compared to heterozygous (Δ/wt) mice. I then 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. I used antibodies against neuronal nuclear antigen (NeuN; Figures 3.4C, D), neurofilament (data not shown) and beta-tubulin III (unpublished data) for neurons, glial fibrillary acidic protein (GFAP) for astrocytes (Figures 3.4E, F), and detected PDGFR-alpha by in situ hybridization for OPCs (Figures 3.4I, 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 3 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 (Figures 3.4G, H) or per unit area (data not shown). 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* versus 42±6.0 for control, p=0.27, Student's t-test). For immature OLs, however, the situation was quite different. The number of cells expressing high levels of PLP/DM20 mRNA (Figures 3.2k, l) was strongly increased in the spinal cord of mutants compared to control animals, both in the gray matter 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 (see discussion in Richardson (2000)). An increased number of differentiating OLs was also observed by immunostaining for myelin-associated glycoprotein (MAG) (Figures 3.4M, N). I found approximately ten-fold more perinuclear MAG-positive cells per section compared to controls (Figure 3.4P). Interestingly, this increased number of immature OLs was not the result of an increased number of OPCs (PDGFR-alpha mRNA-positive cells; Figure 3.4O). 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 versus 10.1 ± 3.9 in control animals).

Figure 3-4: Precocious differentiation of immature OLs in E17.5 spinal cord of Cnp-Cre Δ/Δ mice (next page).

A, B) Sections were cut from triple-transgenic animals carrying *Cnp-Cre*, *R26R*, and one (b) or two copies (a) of the floxed *Notch1* allele. X-gal stainings indicate recombination with a similar pattern in homozygous mutant (A, Δ/Δ) or heterozygous mutant (B, Δ/wt) spinal cords; motoneurons are indicated by arrows. Neurons, astrocytes, OPCs, and immature OLs were compared in *Cnp-Cre Δ/Δ* and control (*lox/lox*) spinal cords by immunohistochemistry (C–F, M, N) or in situ hybridization (I-L). Neurons, astrocytes and OPCs, assessed by the markers NeuN (C, D), GFAP (E, F) and PDGFR alpha (I, J), appear normal in conditional mutant animals (*Δ/Δ*) compared to control (*lox/lox*) littermates. Total image intensity was determined for ten sections of each genotype. For NeuN (G) we integrated only in the region of the gray matter and for GFAP (H) intensity was averaged over the whole spinal cord section. Intensities are in

arbitrary units. The number of cells positive for PDGFR alpha mRNA was similar in mutant and control animals (O). By contrast, the number of immature OLs, assessed by counting cells positive for perinuclear MAG immunoreactivity (M, N) is significantly increased in mutant compared to control spinal cord (panel P; mean +/- SD; ***, p<0.001 Student's t-test). The precocious differentiation was confirmed by in situ hybridization for Plp/DM20 (K, L). Scale bar in N equals 10 μm (for panels A-F, I-N).



3.4 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 to control (*lox/lox*) mice (Figures 3.5C and D, 5E 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 (data not shown). I quantified the MAG-positive cells showing perinuclear staining

(immature OLs; Figure 3.5H), 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 (Figures 3.5E, F; see also MBP staining for mature OLs in Figures 3.6A, B for comparison), there were approximately 6-fold more immature OLs present in mutants as compared to controls (Figure 3.5G). Similar observations were made in *Plp-Cre Δ/Δ* mice (53 ± 19 vs 5 ± 4 in mutant and control, respectively). As at E17.5, no difference was seen between the numbers of cells positive for PDGFR alpha mRNA in *Cnp-Cre Δ/Δ* and control mice (Figures 3.5A, B and I).

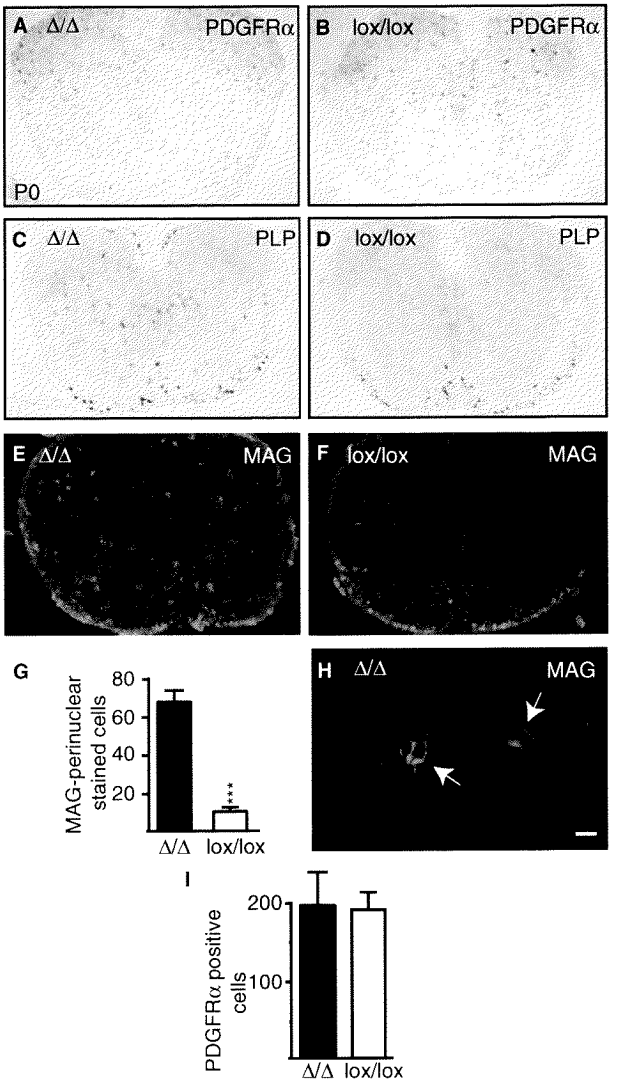


Figure 3-5: OPCs and immature OLs in P0 *Cnp-Cre Δ/Δ* spinal cord (previous page).

Sections of thoracic spinal cord of *Cnp-Cre Δ/Δ* or control (*lox/lox*) mice were labelled with PDGFR alpha cRNA or PLP cRNA (A-D), or with anti-MAG (E, F, H) antibodies. The number and location of PDGFR alpha-positive OPCs was similar in mutant and control animals (A, B, I). In contrast, in the gray matter of the spinal cord, there was a significant increase in ectopic immature OLs with extensive perinuclear MAG staining (H; quantified in G, mean +/- SD; ***, $p<0.001$ Student's t-test). Scale bar in H equals 100 μm for A-F, and 10 μm for H.

3.5 Increased apoptosis of prematurely differentiated OLs

Although I observed ectopic perinuclear MAG-positive OLs in *Cnp-Cre Δ/Δ* mice, staining for MBP (a late marker for myelinating OLs) was absent from the gray matter of both thoracic (not shown) and cervical spinal cord (Figures 3.6A, B), even though the latter is at a more advanced stage of differentiation than the thoracic segment. Some staining was, however, observed in the future white matter of both mutant and control animals (Figures 3.6A, B). These observations raised the possibility that the ectopic immature OLs might be cleared by programmed cell death. To test this hypothesis, I double-stained sections from *Cnp-Cre Δ/Δ* spinal cords at P0 for "TUNEL" (Gavrieli et al., 1992) and MAG (Figure 3.6C). 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; Student's t-test). Further, a higher percentage of MAG-positive cells were TUNEL-positive in mutant mice (Figure 3.6D). 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.

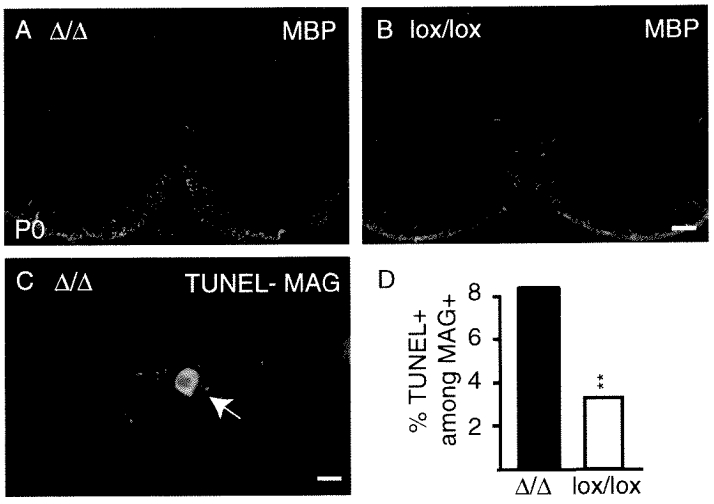


Figure 3-6: Mature OLs and apoptosis in P0 *Cnp-Cre Δ/Δ* spinal cord.

Sections of thoracic spinal cord of *Cnp-Cre Δ/Δ* or control (*lox/lox*) mice were labelled with anti-MBP (A, B) antibodies, or with MAG antibodies combined with TUNEL staining (C). The number and location of mature MBP-positive OLs in the developing white matter of the cervical spinal cord was similar (A, B). A subpopulation of the MAG-positive cells were TUNEL-positive (arrow in C). The percentage of apoptotic cells among perinuclear MAG-positive cells was significantly higher in mutant than in control spinal cord (D, 1077 cells counted in mutant mice and 303 cells in control mice; $\chi 2$ distribution; $\chi 2 = 86.29$; **, $p<0.005$). Scale bar in B equals 100 μm for A and B, and 10 μm for C.

3.6 Efficiency of Notch1 ablation

Since my multiple efforts to show elimination of the Notch1 protein directly failed due to a lack of reliable reagents, I demonstrated that Cre recombinase had been active in the differentiating OLs. I 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 (Figures 3.7A, B) and then for O4 (Figures 3.7C, D), a marker for both immature and mature OLs (Hardy and Reynolds, 1991). Eighty-nine of 100 and 71 of 100 O4-positive cells derived from homozygous and heterozygous animals, respectively, were also positive for beta-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 (Figure 3.7E). Quantification by PhosphorImager analysis revealed approximately 20 to 25% recombination in P0 *Cnp-Cre Δ/Δ* whole spinal cord, after normalization to DNA isolated from control *lox/0* mice. As a comparison, in E12.5 *Plp-Cre Δ/Δ* whole spinal cord, approximately 75 to 80% recombination was achieved (Figure 3.7F).

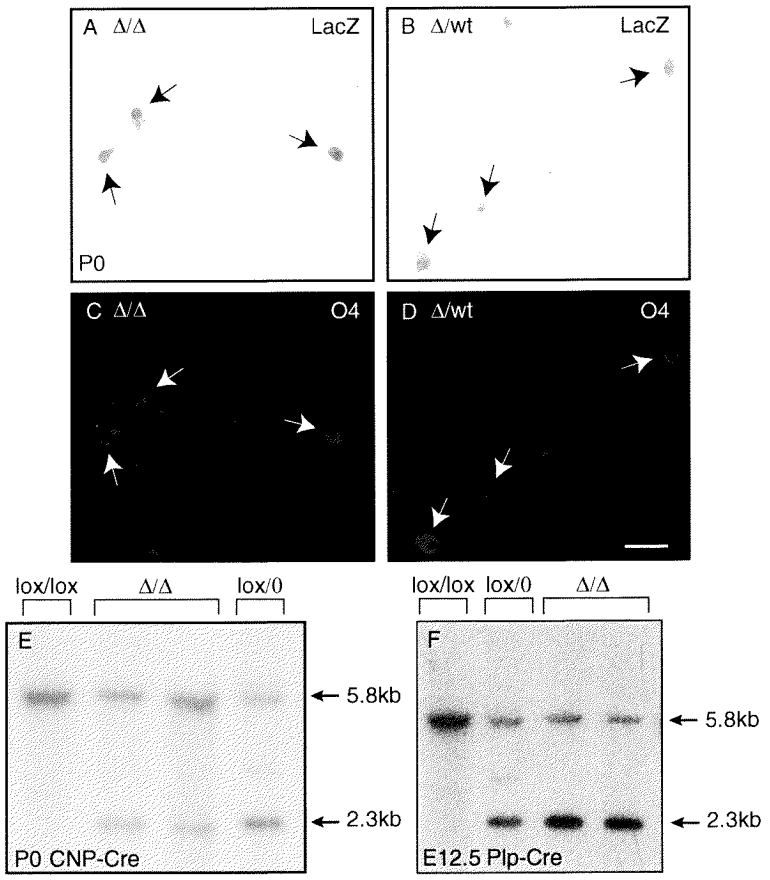


Figure 3-7: Recombination in cultured OL and efficiency of ablation of Notch1 in P0 *Cnp-Cre* versus E12.5 *Plp-Cre Δ/Δ* spinal cord.

Acutely dissociated cells isolated from individual P0 *Cnp-Cre R26R* mice homozygous for the floxed *Notch1* allele (A, C; Δ/Δ) or from *Cnp-Cre R26R* heterozygous littermates (B, D; Δ/wt) were stained for X-gal (A, B) and O4 (C, D). Arrows mark double-labeled cells. (E, F), Southern blot analysis using a probe derived from the 5' upstream region of the *Notch1* locus revealed a 5.8 kb fragment from the wild-type allele after digestion with Eco RI. After recombination a 2.3 kb fragment can be detected. DNA was isolated from the spinal cord of P0 animals carrying *Cnp-Cre* (E) or of E12.5 embryos carrying *Plp-Cre* (F). Genotypes of the animals used are indicated above the lanes.

3.7 Early stages of OPC development are normal in Notch1 mutant embryos

Since expression of Cre recombinase starts as early as E11.5 in *Cnp-Cre R26R* mice (Figure 3.2), I examined the early development of OPCs in *Cnp-Cre Δ/Δ* mice. PDGFR alpha mRNA-positive OPCs were found at E12.5 in the neural tube in homozygous (Δ/Δ) as well as in heterozygous (Δ/wt) mice (data not shown). At E14.5, the PDGFR alpha mRNA-positive OPCs were dispersed throughout the cross-section (Figures 3.8A, B), and their number was similar in mutants and controls (Figure 3.8G). MAG-positive cells were not detected at this time point (Figures 3.8C, D). 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 (data not shown), E14.5 (Figures 3.8E, F, arrows), and E17.5 (Figures 3.4A, B, arrows), as judged by immunostaining for the markers Isl1 and Isl2 (Pfaff et al., 1996; Tsuchida et al., 1994) and X-gal staining in *Cnp-Cre Δ/Δ R26R* mice.

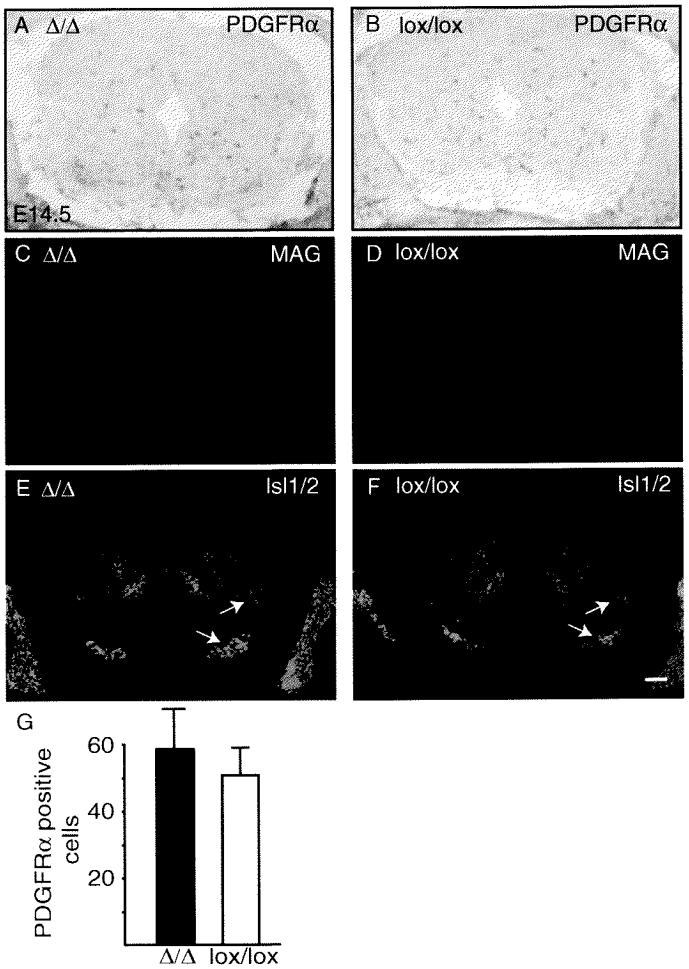


Figure 3-8: E14.5 spinal cord appears normal in *Cnp-Cre Δ/Δ* mice (previous page).

Transverse sections of E14.5 *Cnp-Cre Δ/Δ* and control (*lox/lox*) spinal cords following in situ hybridization (A, B) or immunohistochemistry (C-F). OPCs, OLs, and motoneurons were analyzed by the expression of PDGFR alpha mRNA (A, B), MAG (C, D), and Isl1/2, respectively. Comparable numbers of OPCs and no MAG-positive cells were observed. Motoneurons (arrows in E, F) appear normal; some interneurons and dorsal root ganglia are also marked by Isl1/2. Scale bar in F for A-F equals 10 μm.

3.8 Precocious differentiation of OLs in the forebrain

I asked next whether precocious OL differentiation is confined exclusively to the spinal cord. Thus, I examined the forebrain, where OL differentiation starts slightly later compared to 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 to controls, with a large fraction (approximately 50%) found in the gray matter (Figures 3.9A, B, C).). An increase in apoptotic cells, such as seen in the spinal cord, was also observed (237±42 per section in mutants versus 144±22 per section in controls). I did not observe, however, precocious differentiation using antibodies against MAG in the optic nerve of P0 mutant animals (data not shown), possibly because OL development is shifted to later times in the optic nerve.

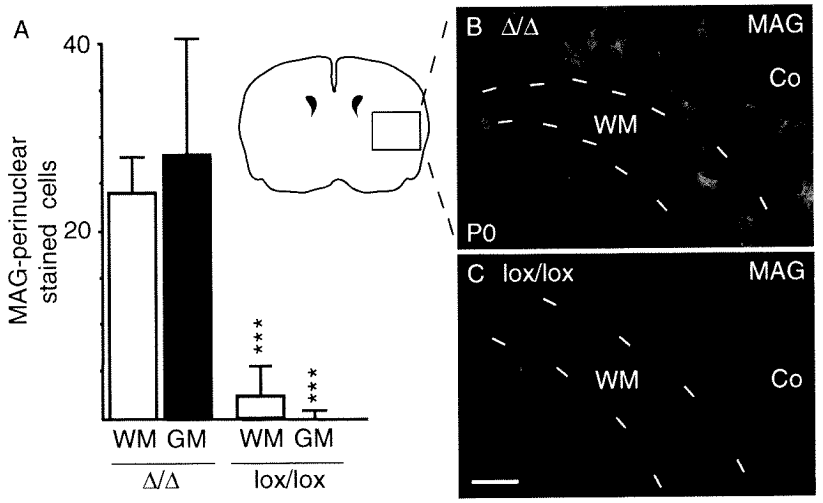


Figure 3-9: Precocious development of immature OLs in the cerebrum of P0 *Cnp-Cre Δ/Δ* mice (previous page).

Transverse sections of P0 cerebrum were immunostained for MAG. B) At P0, there were many immature (perinuclear MAG-positive) OLs present in the mutant brain (*Δ/Δ*), both in white matter (WM) and in gray matter (GM, here cortex, Co), whereas these were rare in the control (*lox/lox*) brain (C). These results are quantitated in panel (A), which shows the mean number of cells (+/- SD) with perinuclear MAG staining. Scale bar in B for B, C equals 20 μm.

3.9 Notch1$^{-/-}$ OPCs and OLs develop normally in vitro

The complexity of the in vivo environment might have masked an effect of Notch1 ablation in other aspects of OL development, such as proliferation of OPCs, apoptosis or OL maturation. To further investigate the requirement for Notch1 in the OL lineage, I therefore turned to an in vitro cell culture approach, in which OL development occurred in defined conditions.

3.9.1 Proliferation and apoptosis

Primary mixed brain cell cultures were prepared from the cerebral cortex of newborn *Cnp-CreΔ/Δ* animals. *Lox/lox* animals were used as control. OPCs were isolated by mechanical dissociation followed by a process of differential adhesion (Milner and Ffrench-Constant, 1994). Enriched OPCs were resuspended in DMEM supplemented with thyroid hormones (T3 and T4) to trigger differentiation of the OLs and cultured for three to five days (see Section 10 for details). To examine cell proliferation, bromodeoxyuridine (BrdU) was given at a final concentration of 10μM as single pulse. After 22 hours, the cells were fixed and double stained with antibodies against BrdU (to label cells that had been in S-phase) and NG2 (to label OPCs). Compared with controls, the fraction of proliferating oligodendrocyte precursors was similar in the *Cnp-CreΔ/Δ* cell culture (Table 3.10).

Next, I asked whether the increase in the level of apoptosis observed in vivo (Figure 3.6) is a cell intrinsic (and therefore associated directly with the loss of *Notch1*) or a cell extrinsic phenomenon. If the latter is true, it would imply that the presence or the lack of some factors (yet to be determined) is responsible for the death of the precociously differentiated OLs. Studies in the hematopoietic lineage showed that activated Notch signaling enhances cell survival in vitro (Jundt et al., 2002; Tan-Pertel et al., 2000), and

therefore suggest that the presence of Notch is necessary for survival in the hematopoietic cells. However, in my cell culture experiment I counted a small but similar number of apoptotic cells in the cell culture derived from *Cnp-CreΔ/Δ* animals as compared to *lox/lox* mice (Table 3.10). Since high insulin concentration can prevent OLs from dying, the final concentration was decreased ten fold (0.5μg/ml instead of 5μg/ml) in order to be close to physiological conditions. No differences were observed (data not shown), therefore suggesting that Notch1 *per se* is not required for oligodendrocyte survival in vitro. This also suggests that some external cues are required to prevent apoptosis of prematurely differentiated oligodendrocytes in vivo.

	CNP-Cre Δ/Δ	*Lox/lox*
(1)NG2+/BrdU+ cells	53%	65%
(2)TUNEL+ cells	1.5%	0.9%

(1) p= 0.5
(2) p= 0.5, Chi-squared test

Table 3-10: proliferation and apoptosis of OPCs in vitro.

OPCs were isolated and enriched from the cerebral cortex of *Cnp-Cre Δ/Δ* and *lox/lox* animals, as described in Material and Methods. Enriched OPCs were then plated on PDL-coated dishes and induced to differentiate by treatment for 3 days with thyroid hormones T3 and T4. 1) On the last day of hormone treatment, effect of *Notch1* ablation on OPC proliferation was determined by BrdU incorporation (22hr labeling) and NG2 immunostaining. Over 300 cells were counted. 2) Cells were stained for TUNEL activity to determine the level of apoptosis. 10 TUNEL-positive cells out of 683 were counted in mutant mice and 5 out of 564 cells in control mice ($\chi 2$ distribution; $\chi 2= 0.44$; $p=0.5$).

I showed that the loss of Notch1 leads neither to an abnormal rate of OPC proliferation nor to increased apoptosis in the oligodendrocyte cell culture. I haven't shown however that an activation of the Notch1 signaling pathway (and not only a loss of Notch1) would lead to similar conclusions. To address this point, I cultured OPCs derived from the cerebral cortex of newborn *Cnp-CreΔ/Δ* or *lox/lox* animals in the presence of a soluble

form of the Notch ligand Delta1 (Dll-1), that has been shown to be functional and able to specifically binds the Notch1 receptor (Wang et al., 1998/G Weinmaster, unpublished data). Although this preparation of Dll-1 can strongly promote Schwann cell differentiation from cultured rat neural crest cells (H-Y Lee, personnal communication), I was unable to inhibit OL differentiation under these same conditions, even though such an inhibition had been shown by Wang et al., 1998. Given the fact that the Dll-1 ligand was generated from a rat cDNA, the discrepancy between the results of Wang and mine might be a result of the different species used in these studies (rat *versus* mouse). This remains unknown and needs further investigations.

3.9.2 Maturation of OLs

A careful analysis of the ability of Notch1$^{-/-}$ OLs to differentiate up to a mature stage has been difficult using the *Cnp-Cre Δ/Δ* animals, because of their early lethality. To partially solve this problem, a cell culture system similar to that described in the previous paragraph was used. OPCs were isolated from the cerebral cortex of newborn *Cnp-CreΔ/Δ R26R* or *Cnp-CreΔ/wt R26R* animals. Cells were then cultured for seven days and allowed to differentiate in Sato medium (Milner and ffrench-Constant, 1994). X-gal staining was first performed to determine the number of recombined cells. In both mutant and heterozygous animals, I found a similar and relatively high percentage of recombined cells (around 76%). On sister plates, I performed immunohistochemistry using an antibody against the myelin-basic protein (MBP), a late marker of OLs. I also found a similar percentage of MBP-positive cells, i.e. 52% in mutant animals and 45% in heterozygous animals (Figure 3.11), therefore suggesting that Notch1 is not required for the maturation of oligodendrocytes in culture. We can't however exclude that Notch signaling has functions in the formation of a normal myelin sheath or in the correct pattern of myelin location in the adult central nervous system.

Therefore, to further determine whether Notch1 signaling could play a role in myelination, I conditionally ablated *Notch1* at other stages during OL development. These animals were fully viable, therefore making possible the examination of adult animals. These experiments are developed and discussed in the second part of my Ph.D thesis (see section 7).

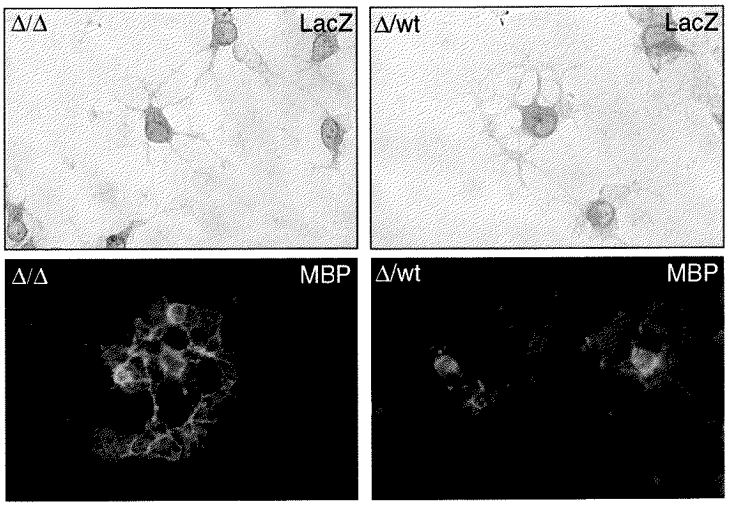


Figure 3-11: Maturation of OLs in vitro.

OPCs were isolated and enriched from the cerebral cortex of *Cnp-Cre Δ/Δ* and *Cnp-Cre Δ/wt* animals, as described in Material and Methods. Enriched OPCs were then plated on PDL-coated dishes and induced for differentiation for 7days in presence of the thyroid hormones T3 and T4. X-gal staining was performed to determine the percentage of recombined cells. The effect of *Notch1* ablation on the late stages of OL differentiation was determined by MBP immunostaining.

4 DISCUSSION

In the first part of my thesis, I have used defined conditional gene ablation to address the question of how the OL lineage is affected when Notch1 function is specifically eliminated in vivo. I 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. At P0, most of the prematurely differentiated OLs were found in the gray matter, where immature OLs are usually very scarce in control animals. Apoptosis was strongly increased among the precociously differentiated cells. The number of OPCs was, however, not altered at the ages examined, and early OL development appeared normal. Precocious differentiation was also observed in the forebrain, suggesting that Notch1 signaling plays an important role in OL development throughout the CNS.

4.1 Direct or indirect effects of Notch1 ablation

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. My study employed Cre-mediated recombination driven by Cre recombinase inserted into the *Cnp* locus. In agreement with published reports on CNP regulation and according to my 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 (compare Fig. 3.4B and 3.4F). Development of neurons appears normal in the spinal cords of mutant mice (Fig. 3.4C, D and G). However, I 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 I am not aware of evidence that such electrical activity influences differentiation of OPCs into immature OLs (for review see Barres and

Raff, (1999)). OLs develop normally in explant cultures from spinal cord of Isl1-/- mice (Sun et al., 1998), even though no motoneurons or V1 interneurons are produced (Pfaff et al., 1996). OLs also develop in hindbrain of Olig2-/- mutants (Lu et al., 2002), which lack somatic motoneurons. The most likely interpretation of my results is therefore that they reflect a direct effect of Notch1 deletion in OPCs.

4.2 Prematurely differentiated OLs in Notch1 mutants die before they mature

Why do many of the prematurely differentiated OLs in Notch1 mutants die? Immunostaining with late differentiation marker and assessment of apoptosis suggested that precociously differentiated OLs cannot terminally differentiate and are not able 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. (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 my 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 OPC differentiate preferentially in fiber tracts (Hardy and Friedrich, 1996). However, my results and those of Calver et al. (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.

Is it possible that the increased number of immature OLs in the mutant spinal cord reflects an enhanced cell survival rather than a precocious differentiation of oligodendrocytes? This is a plausible hypothesis (Notch signaling has been shown to promote cell survival in the hematopoietic lineage (Jundt et al., 2002; Tan-Pertel et al., 2000), but none of my experiments support this in the oligodendrocyte lineage. Several lines of evidence suggest that the signaling through Notch receptor mediates a differentiation rather than survival signal for emerging oligodendrocytes. For example, I

found an increased number of dying OLs in the spinal cord of mutant animals. Thus, if Notch signaling would promote survival of OLs, it would be paradoxical to detect an increased number of apoptotic cells. Furthermore, my results from the in vitro culture of Notch1 $^{-/-}$ OLs did not suggest a role of Notch1 in oligodendrocyte survival.

Some candidate genes have been suggested to play a role in terminal differentiation of OLs. Taking recent reports into account (Park et al., 2001; Qi et al., 2001; Stolt et al., 2002), the neuregulin receptor Erb2, the Sry-related high mobility group (HMG) box gene Sox10 and the homeodain transcription factor Nkx2.2 appear to be essential for terminal differentiation of oligodendrocytes. In the absence of Notch1, many oligodendrocytes died at an immature stage of differentiation. Could Notch1 be also required for the later stages of OL differentiation? This seems unlikely, since MBP, a marker for late differentiation, was present, albeit at a still low level, in the cervical spinal cord of both control and mutant newborn animals. In vitro studies demonstrated that Notch1 $^{-/-}$ OLs are capable of full differentiation, supporting the argument that disruption of Notch1 does not prevent OLs from terminal differentiation.

4.3 Additional regulatory mechanisms are likely to participate in the timing of OL differentiation

It should be noted that although beta-gal expression was seen as early as E11.5, I detected the first immature OLs only at E17.5. Several possibilities may be envisaged to explain this delay. Persistence of Notch1 protein might render the cells effectively Notch1-positive for some time after recombination has occurred. This appears unlikely since, although I was not able to directly assess the lifetime of the Notch1 protein, the presence of a PEST sequence near the C-terminus suggests that Notch1 turns over rapidly (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 rat optic nerve at 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. This mechanism allows a single OPC to undergo controlled cell cycle arrest and to differentiate in vitro without neurons (Temple and Raff, 1986). My 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. This 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). 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.

The intracellular mechanisms that underlies precocious OL differentiation in the Notch conditional knockout animals remain unknown at this stage. The finding that maturation of OLs in the spinal cord is strongly inhibited in the absence of the transcription factor Nkx2.2 (Qi et al., 2001) and Olig1 (Lu et al., 2002) suggests that these transcription factors or one of their regulatory targets are antagonized by the Notch signaling pathway. A direct effect on Nkx2.2 and Olig1 expression seems unlikely, since OPCs normally express Nkx2.2 and Olig1 throughout their lifetime (Lu et al., 2002; Qi et al., 2001; Soula et al., 2001; Xu et al., 2000; Zhou et al., 2001). However, an effect on the function of these transcription factors could be possible, since posttranslational mechanisms can regulate the timing of bHLH factor function, even for factors that are coexpressed (Moore et al., 2002). Whatever the mechanisms are, it is reasonable to postulate that oligodendrocyte differentiation would require the presence of multiple regulatory pathways, working independently but in concert to allow a fine control of the timing of OL differentiation.

4.4 OLs in the gray matter of the spinal cord: migration defect or in situ differentiation?

Would it be possible that the numerous OLs found within the gray matter had migrated out from the white matter after differentiation? Some experiments showed that migration and differentiation are separate events (Milner et al., 1996), in contrast to the situation with proliferation and differentiation that are tightly coupled. It is therefore possible that precociously differentiated oligodendrocytes still have the ability to migrate. Other studies have also shown that oligodendrocyte precursor cells are migratory cells, whereas differentiated OLs have lost their migratory potential during development (Milner et al., 1996; Noble et al., 1988; Small et al., 1987). Although difficult to prove one hypothesis

or the other, it seems more likely that the precociously differentiated OLs have developed directly within the gray matter of the spinal cord.

4.5 Regulation of the number of oligodendrocyte precursor cells

The increase in immature OLs was not accompanied by a detectable decrease in the number of OPCs. Zhou et al., (2001), as well as Fu et al., (2002), suggested the presence of multiple (at least 2) populations of OPCs in the spinal cord. If *CNP-Cre* would be active in only one of these suggested populations, the high number of the remaining oligodendrocyte precursor cells could therefore mask the absence of the Notch1$^{-/-}$ OPCs that have precociously differentiated. However, a similar pattern of expression of PDGFRα and βGal was observed in the embryonic spinal cord, and it has been demonstrated by antibody-mediated complement lysis that most or all OLs in the embryonic rat spinal cord are derived from PDGFRα- expressing progenitors (Hall et al., 1996). Although this does not exclude the possibility of multiple populations of PDGFR*a*-positive OPCs, it does not provide an explanation why the local density of OPCs remained constant.

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 my experiments. I 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 hr at E13 to ≈100 hr at E17; (van Heyningen et al., 2001), the expected increase in proliferation rate might have escaped detection. I also considered the possibility that Notch ablation might have produced MAG-positive OLs that were also still positive for early markers. I tested this by co-immunostaining for MAG and the early marker NG2, but found that the MAG-positive cells in the gray matter were NG2 negative (data not shown).

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 myelination is likely to be a down-regulation of the axonal component of the Notch1-

ligand interaction, probably 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 activated Notch signaling, are responsible for remyelination failure (Solanky et al., 2001).

5 OUTLOOK

5.1 The function of the Notch ligands in the differentiation of oligodendrocytes

The present study showed that the Notch1 receptor regulates the timing of OL differentiation in vivo. However, I have not provided any evidence of which signal could activate the Notch1 receptor, and therefore inhibit OL differentiation in the spinal cord and forebrain. The signal could be supplied by several Notch ligands: Delta1, Delta2, Jagged1 or Jagged2 are the prime candidates because of their CNS expression during embryogenesis (Beckers et al., 1999; Dunwoodie et al., 1997; Lindsell et al., 1996; Valsecchi et al., 1997). However, their detailed expression pattern remains unknown, most notably during late embryogenesis when OL differentiation begins. I therefore suggest to first carefully examine by in situ hybridization the temporal expression pattern of the Notch ligands. The ligand(s) playing a role in the regulation of OL differentiation is (are) expected to be highly expressed when OLs have not yet differentiated (e.g. between E12 and E19 in the mouse spinal cord) and to be downregulated when OL differentiation is starting (e.g around E19). In a next step, it is important to determine the cell types expressing these ligands. Studies from Wang et al., (1998) have shown that Jagged1 is localized on the retinal ganglion cells and suggested Notch1 to be the receptor. The nearby spatial localization of the Notch receptor and Jagged1 in the developing rat optic nerve is strongly suggestive of a potential functional interactions in vivo. However, because of the early lethality of transgenic mice lacking Notch ligands, it has not yet been possible, to determine which ligand interacts with Notch1 to keep the OLs in an undifferentiated state. The use of the Cre/lox system will allow this difficulty to be overcome. Note that the use of in vivo models to validate a model is particularly important, because the interaction of a receptor with its ligand can be modulated. For example, Lunatic fringe can either inhibit Notch signaling when activated by Jagged1, or potentiate Notch signaling when activated by another ligand, Delta1 (Hicks et al., 2000).

5.2 Identification of factors regulating OL survival in the white matter

One of the crucial questions that arise from my studies is: why can OLs that have precociously differentiated can not terminally differentiate, but rather die by programmed cell death at an early time during OL development? Barres et al., (1992) and Trapp et al., (1997) showed that about 50% of oligodendrocytes normally die in the developing rat optic nerve and about 20% in the developing cerebral cortex. This phenomenon was explained as a result of a competition for limiting amounts of survival signals, such as platelet-derived growth factor (PDGF), insulin-like growth factor (IGF), ciliary neurotrophic factor (CNTF) or neurotrophin-3 (NT-3). Although PDGF supports the survival of newly formed OLs, it can not support the survival of more mature OLs, which no longer express PDGF receptors (Hart et al., 1989; McKinnon et al., 1990). In 1993, Barres et al. (Barres et al., 1993) showed that CNTF promotes OL survival in vivo, and suggested that multiple signals may be required for the long-term survival of OLs. Although these data are reasonable for explaining the increased apoptosis observed in the spinal cord of *Cnp-Cre Δ/Δ* animals, the explanation is however not sufficient. Why do we not detect any mature MBP-positive OLs in the spinal cord gray matter where no competition is occurring? As an alternative or complementary explanation, it might be expected that the survival of OLs also depends on axons (Barres and Raff, 1994). If tetrodotoxin is injected into the eye to electrically silence the retinal ganglion cells and their axons, OLs do not die. Thus, the ability of axons to promote OL survival does not depend on electrical activity in the axons. On the other hand, purified neurons, but not neuron-conditioned culture medium, promote the survival of purified OLs in vitro, suggesting that mature OLs might have to contact axons to survive. The identity of this axon-dependent signal is, to date, unkown. It would be therefore very interesting to overexpress antiapoptotic factors such as Bcl2 in OLs of *Cnp-Cre Δ/Δ* individuals and examine whether OLs in the gray matter of the spinal cord can mature and myelinate. If the cells survive, what would be their further fate and developmental potential? Assuming there is a way to purify axons from the molecular layer of the cerebellum (no myelinated axons) and axons of the white matter tracts (myelinated axons) (Rozental et al., 1995), would it be then possible to determine their protein-expression profiling with the aim of identifying factors which are selectively expressed on myelinated fibers?

5.3 Notch signaling pathway in oligodendrogenesis

Notch signaling plays a key role in tissue-autonomous fate determination in 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). Upon binding to Notch ligands, the intracellular domain of Notch (ICN) translocates into the nucleus and forms a complex with the DNA-binding protein CBF-1 (also termed RBP-J and CSL). It then activates the expression of basic helix-loop-helix Hes genes (mammalian hairy and Enhancer-of-split homologues), which repress the expression of proneural genes, such as Mash-1 and Math-1. In the oligodendrocyte lineage, it is still unclear which genes exactly are involved downstream from this cascade. I therefore propose to use an in vitro culture of OPCs and by using biochemical studies, one might look for similarities between the Notch signaling pathway that regulates neurogenesis and that which regulates oligodendrogenesis.

5.4 Investigation of the Notch signaling pathway to regulate the maintenance of adult OPCs

In the present study, I provided 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 in newborn animals. But does Notch1 signaling play a similar role in the adult animals by maintaining and keeping OPCs in an undifferentiated stage?

I suggest first to culture adult OPCs in the presence or absence of a Notch ligand, such as a soluble form of Delta-1 (Dll-1), as described by Wang et al., (1998), and then to measure the percentage of differentiated OLs. If OL differentiation can be inhibited, I propose to use OPCs isolated from *Cnp-Cre Δ/Δ* animals (where specifically Notch1 is ablated) and to proceed similarly as above to prove the direct role of Notch1 signaling in this inhibition. I mentioned in the results that the soluble form of a rat Delta-1 was not able to inhibit the differentiation of OLs isolated from newborn mouse brain. One should however be able to overcome this problem by titrating the ligand concentration or by generating a mouse Dll-1 instead of a rat Dll-1. Indeed, a recent report indicated that the

multimeric state achieved through clustering Dll-1 ligands with αFc antibodies determines whether ligand binding to Notch results in activation or inhibition of downstream signaling (Hicks et al., 2002).

If positive the results can be extended by using defined conditional gene ablation to address the question of how the OL lineage is affected when Notch1 function is specifically eliminated in vivo in adult OPCs. To address this question we can generate a transgenic mouse which expresses a Tamoxifen-inducible Cre recombinase under the control of a specific OPC promoter like the one of the platelet-derived growth factor receptor alpha for example. Alternatively we can use the PlpCre ER mice which are described in the next section. There, I show that the Plp promoter is active in the NG2-positive OPCs (see next section Figure 2), making these mice a very valuable tool to elucidate the role of Notch signaling, or other proteins, in adult animals. A detailed expression study of Notch1-4 in the adult OL lineage might also turn out to be very useful, if functional compensation is provided by other Notch family members, which seems to be the case as seen in preliminary experiments using the Cnp-Cre mice (data not shown).

The results shown in the first part of my thesis described a role for Notch1 in the early steps of OL differentiation. But what about Notch1 function in myelination and most importantly in remyelination?

6 INTRODUCTION

In the central nervous system (CNS), mature oligodendrocytes produce myelin, which is wrapped around the axon of the neurons and is essential for rapid propagation of action potentials and for normal neurological function (Waxman, 1980). Myelin has a high lipid to protein ratio (75% lipid, 25% protein) and contains proteins such as proteolipid protein (PLP), myelin associated glycoprotein (MAG), myelin basic protein (MBP) and myelin oligodendrocyte glycoprotein (MOG). The main function of the lipid rich, multilayered myelin is to increase the conduction velocity of neuronal impulses from the neuronal cell body to the target cell. Myelination occurs during development caudorostrally in the brain and rostrocaudally in the spinal cord. The sequence of myelination is a stricly reproducible process for a given species. In the mouse, it starts at birth in the spinal cord. In brain, myelination is achieved in almost all regions around 45-60 days postnatally. The importance of myelin in human development is highlighted by its involvement in an array of different neurological diseases such as leukodystrophies and multiple sclerosis in the CNS and peripheral neuropathies in the peripheral nervous system (PNS).

6.1 Mechanisms regulating myelination in the CNS

Little is known about the mechanism of myelination or the signals that regulate this complex process. There are sequential steps involving the following: 1) recognition of the neurons that are to be myelinated, and the fact that axons and not dendrites are recognized 2) adhesion of the OL process to the axon 3) spiraling of the process around the axon. The timing of myelination is specific for a species and a region of the CNS, and concerns only certain nerve fibers and tracts (Schwab and Schnell, 1989). For example, long axonal projections that run along the length of the spinal cord, such as the corticospinal tract (CST) and cuneate and gracile fasciculi, develop more than one week apart (Schreyer and Jones, 1982). Furthermore, at a time when cuneate and gracile are composed predominantly of myelinated axons, the CST still contains predominantly elongating axons (Schwab and Schnell, 1989).

Outside of providing trophic factors to OLs, astrocytes may be important in relation to myelination. It as been shown that astrocytes induce OL processes to align with and adhere to axons, providing a novel role for astrocytes in controlling the onset of myelination (Meyer-Franke et al., 1999).

Although the mechanisms for triggering and directing myelinogenesis are not known, it is certain that neurons and their axons are required for the appropriate formation of the myelin sheath. Studies support the idea of a critical diameter for myelination, with a critical diameter of about 1μm determining whether axons in mouse sciatic nerve are myelinated (Friede and Samorajski, 1967). The situation might be slightly more complicated for OLs; these cells myelinate multiple axons, and individual OLs sometimes myelinate axons with different sheath thicknesses (Friedrich and Mugnaini, 1983; Waxman and Sims, 1984). Voyvodic et al. (1989) has demonstrated that sympathetic postganglionic axons, which are normally not myelinated, become myelinated when axon caliber is increased as a result of increasing the size of the peripheral target they innervate. But individual axons are not myelinated along their entire length simultaneously, indicating that additional factors or mechanisms are involved (Suzuki and Raisman, 1994).

Axonal electrical activity has also been shown to influence myelination by OLs (Demerens et al., 1996). If neuronal Na^+ channels are blocked by the selective Na+ channel blocker tetradotoxin, thereby inhibiting electrical activity, the number of myelinated fibers is decreased in vitro as well as in vivo in the optic nerve. In addition, by slowing the Na^+ channel inactivation with α-scorpion toxin, the duration and frequency of spontaneous action potentials is dramatically increased and myelination is enhanced.

Axonally derived signals appear to be required for several aspects for OL and myelin formation (for review see Barres and Raff, (1999)). One potential axonally derived signaling molecule critical for myelin formation is neuregulin (NRG)-1 (for reviews see Adlkofer and Lai, 2000; Carraway and Burden, 1995; Pinkas-Kramarski et al., 1998; Riese and Stern, 1998). The activity of NRG-1 are mediated through members of the erbB receptor tyrosine kinase family, which includes erbB2, erbB3, and erbB4, all three of which are expressed on oligodendrocytes (Park et al., 2001). In vitro studies demonstrate that axonally derived NRG in its membrane-associated form is capable of

activating erbB receptors (Vartanian et al., 1997), indicating that axonally associated NRG can mediate its effects as a consequence of direct axonal-glial interactions. Quite interestingly, erbB2 $^{-/-}$ oligodendrocytes can extend processes that contact neurites, but fail to form ensheathment tubes, suggesting a critical role for erbB2 signaling in axonal ensheathment and myelination.

A second potential axonally derived signaling molecule critical for myelin formation is Jagged-1 (Wang et al., 1998). Indirect evidence suggests that, in the optic nerve, the timing of OL differentiation and myelination is controlled by the Notch pathway. Retinal ganglion cells express Jagged1, a ligand of Notch1 receptor, along their axons. Jagged1 is developmentally downregulated in axons of retinal ganglion cells with a time course that parallels myelination, suggesting that Jagged1 signals to Notch1 on surfaces of OPCs to inhibit their differentiation into OLs. The downregulation of Jagged1 correlates well with the onset of myelination in the optic nerve. Consequently, as more and more of the optic nerve becomes myelinated, levels of both Jagged1 and Notch1 correspondingly decline. Myelination of axons in a given pathway generally occurs a few days after the axons reach their target (Schwab and Schnell, 1989), raising the question of whether downregulation of Jagged1 in axons occurs in response to target innervation, subsequently triggering the signal to begin myelination. In unmyelinated regions like the molecular layer (ML) of the cerebellum for example, Jagged or other Notch ligands may persist on the axons of the cerebellar granule neurons, which form the cerebellar ML, and continue to inhibit the generation of myelin-forming OLs. Indeed, Jagged1 expression on the granular neurons is maintained until adulthood (Givogri et al., 2002; Stump et al., 2002) and cerebellar OPCs express Notch1 (Givogri et al., 2002). Therefore, this would maintain these axons in an unmyelinated state and help maintain myelin patterns in the fully developed CNS. Consistent with this hypothesis, Givogri et al., (2002) observed in Notch1$^{+/-}$ animals some small-caliber axons located in the ML that were myelinated.

6.2 The neurotoxicant cuprizone as a model to study demyelination and remyelination in the central nervous system

Feeding of cuprizone (bis-cyclohexanone-oxaldihydrazone) to young adult mice induces massive demyelination in certain regions of brain, most prominently the cerebellar peduncles and corpus callosum (Blakemore, 1972; Blakemore, 1973; Hiremath et al., 1998; Ludwin, 1978; Morell et al., 1998). Other white matter tracts, such as those in the spinal cord are not affected by cuprizone (personal observations). Furthermore, removal of cuprizone from the diet of mice permits the study of remyelination. The readily apparent demyelination occurs without damage to cell types in the central nervous system other than oligodendrocytes (Blakemore, 1973; Cammer and Zhang, 1993; Fujita et al., 1990; Komoly et al., 1987; Suzuki and Kikkawa, 1969), but results in significant weight loss and can lead to the death or the sacrifice of the animal.

Cuprizone is a copper chelator and it is assumed although not proven that the binding of copper results in the observed pathology. It is thought that cuprizone-induced copper deficit might be detrimental to mitchondrial functions in brain (Venturini, 1973), leading to a disturbance of energy metabolism in oligodendroglia and cell function that causes demyelination. Why OLs should be more susceptible to copper deficit is not known, although an obvious hypothesis is that OLs have to maintain a vast expanse of myelin and this extraordinary metabolic demand can exhaust the cell if the demand cannot be met. Note however a challenge to this hypothesis since different white matter areas are differently affected by the treatment.

The basic model has been described in earlier literature, reviewed by Blackemore (1984) and Ludwin (1994). Feeding of cuprizone induces demyelination, in a time frame of weeks (between four and five for mice at 8-10 weeks of age); the exact time depends on dose of cuprizone and age of application. Some studies have been done with older mice, where demyelination was not observed until the eighth week (Blakemore, 1973; Cammer and Zhang, 1993; Ludwin, 1978). This is then closely followed by recruitment of microglia/macrophages and by phagocytosis of myelin. OPCs can still continue to proliferate and invade demyelinated areas. When the cuprizone challenge is terminated, an almost complete remyelination takes place in a matter of weeks (Figure 6.1). Any change in mouse strain requires recalibration of dose regimens, however, the model has

been successfully used only in mice; rats treated with 0.5-2% cuprizone did not develop demyelination (Love, 1988).

Other models, such as experimental allergic encephalomyelitis (EAE), or ethidium bromide or lysolecithin injection, have been extensively used for various studies. However, the lesions caused by the EAE models are sporadic, asynchronous and scattered. This lack of anatomical reproducibility of lesions between animals greatly hampers accurate and reliable assessments of changes in quantification of demyelination. Furthermore, results obtained with EAE models must be considered with respect to the immune system. For example, production of antibodies may prevent remyelination (Bornstein and Raine, 1970). On the other hand, these complexities of the EAE model mimic important human disorders and the situation may be closer to that which one has to face in such diseases. An advantage of the cuprizone model is that it allows to tease out the events most directly linked to demyelination and remyelination, bypassing some of the considerable complexities of the immune system. Another advantage provided by the cuprizone model is that it permits synchronous and consistent demyelination and remyelination. This reproducibility of the model with respect to time-course and pattern of demyelination greatly facilitates the testing of manipulations (e.g. transgenic/ pharmacological treatments) which may accelerate or retard the process of demyelination and/or remyelination. Ethidium bromide injection is not like cuprizone, which is selectively cytotoxic for mature oligodendrocytes, and so ethidium bromide treatment leads to several complex conditions (Schwann cell remyelination, remyelination possible only from external emigrated oligodendrocyte precursors). Finally, the lysolecithin model can produce localized lesions on favorable tracts with large axons, but requires considerable surgical skill and special apparatus.

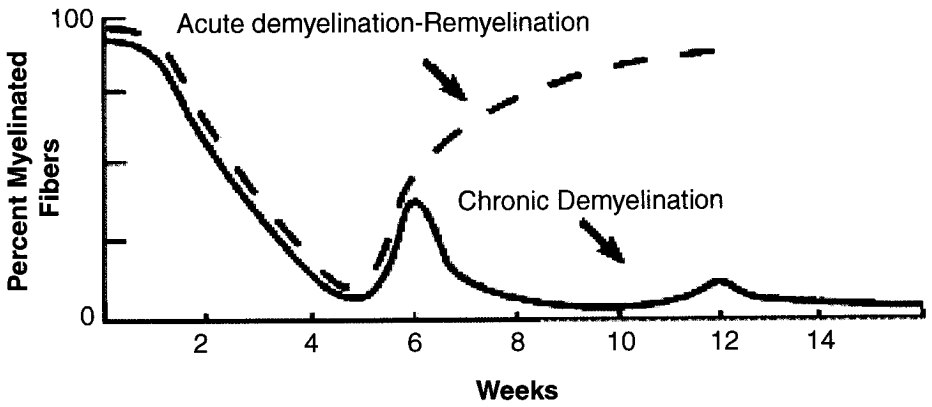


Figure 6-1: rate of demyelination and remyelination during acute and chronic exposure to cuprizone in the corpus callosum above the fornix (Matsushima et al., 2001).

6.3 Remyelination in the central nervous system

A number of genetic and/or inflammatory diseases may affect myelin formation (dysmyelinating diseases) or its maintenance (demyelinating diseases). Demyelinating diseases are more common and they constitute a significant fraction of neurological disorders with a variety of etiologies. In primary demyelination, the myelin itself and its supporting cells are damaged or degraded. In comparison, secondary demyelination results from axonal damage, thus interrupting the axon-glial interactions necessary for the maintenance of the myelin sheath. Quite interestingly, the adult nervous system retains the capacity to remyelinate without extrinsic measures (Gensert and Goldman, 1997; Ludwin, 1978; Yajima and Suzuki, 1979), but regenerated myelin never regains its normal thickness, and the normal linear relationship between axon and sheath thickness is also never regained (Ludwin, 1997). The capacity to remyelinate is not age-dependent, but studies in the rat spinal cord and cerebellar peduncle have demonstrated that remyelination in old adult rats proceeds over a more protracted time-course, taking up to forty days to completion, compared to twenty-eight days in the younger animals (Shields et al., 1999).

The mechanism underlying these processes is not well understood. First, it is not clear whether the mechanism of remyelination is identical to myelination. The origin and identity of endogenous cells that affect remyelination is still an unsolved issue. Whether

the remyelinating cells come from OLs or adult OPCs has been debated and appear to be resolving in favour of the latter (Gensert and Goldman, 1997). There are immature cycling cells endogenous to white matter, which respond to experimental demyelination by differentiating into myelinating OLs (Gensert and Goldman, 1997). Proliferation of mature OLs can also occur after a trauma of the nervous system (reviewed in Keirstead and Blakemore, 1999). However, no one has demonstrated that mature OLs become involved in remyelination.

The apparent ability of exogenous growth factors to promote remyelination in vivo (Cannella et al., 1998; McTigue et al., 1998; Woodruff and Franklin, 1997; Yao et al., 1995, McMorris et al., 1996) and the impairment of remyelination by growth factor antagonists (McKay et al., 1997) suggest that growth factors do play important roles in remyelination. In addition, factors involved in the inflammatory response can significantly help the remyelination process to proceed. The tumor necrosis factor alpha, a multipotent inflammatory cytokine that induces a wide variety of responses including apoptosis in some cells and proliferation in others, promotes the proliferation of oligodendrocyte precursor cells, which then develop into myelinating oligodendrocytes required for remyelination (Arnett et al., 2001). The receptor of another cytokine, the neurotrophic cytokine leukemia inhibitory factor, also improves remyelination, by preventing apoptosis of oligodendrocytes (Butzkueven et al., 2002). Finally, some other factors which are expressed in the oligodendrocyte lineage could play a key role in a demyelinated lesion: the integrins for example, which are also expressed in keratinocytes and are important for epidermal wound healing.

In this context, studies concerning the roles played by Notch1 in OL differentiation (Wang et al., (1998) and Genoud et al., (JCB, in press)) and the observation of specifically high Notch1 expression in ethidium bromide-induced demyelinating rat lesions (unpublished, M Stidworthy) may be particularly relevant. The signals which activate repair might thus concomitantly inactivate the Notch pathway. If so, the efficiency and effectiveness of repair might be dependent on inactivation of the Notch pathway. The demonstration of non-dividing OPCs in human MS lesions, in which chronic demyelination is present, suggests that inhibitory signals present in such lesions may prevent OPC differentiation into new myelin-forming OLs (Wolswijk, 1998). In MS, remyelination also occurs, but it is incomplete and poorly sustained (Prineas et al.,

1993; Raine and Wu, 1993). In addition, studies of transcription factor expression suggest that there is a delay in differentiation of OPCs in old rats compared to younger animals (Sim et al., 2002). Inhibitors of Notch activation may therefore offer novel therapies for demyelinating diseases.

In the second part of my thesis, my particular field of interest consisted of determining the possible role of Notch1 signaling in the maturation of OLs and in the process of remyelination in the central nervous system. A long term goal would be to define the similarities between the mechanisms regulating myelination and remyelination as well as the critical steps involved in the remyelination process, which might be promoted by drug delivery or by in vivo manipulations. It appears that the expression of the principle myelin proteins follows a similar sequence and pattern in both myelination and remyelination (Figure 6.2), and it would also seem likely that these processes involve regulation by the same transcription factors (Hudson et al., 1997). Therefore, to gain further understanding of the mechanisms involved in myelination, I have inhibited selectively Notch1 signaling in developing OPCs and OLs using a conditional Notch1 knock-out mouse strain (Radtke et al., 1999). It is shown that loss of Notch1 throughout the oligodendrocyte lineage during development and adulthood does not alter the pattern of myelin in various regions within the CNS and does not promote precocious myelin production in the cerebral cortex. The role of Notch1 in the mechanism of remyelination is however still unclear. We showed so far that abrogation of Notch1 function in the NG2-positive cells does not have a striking influence on the final outcome of remyelination. As discussed in section 8 and 9 further experiments with different schedule of tamoxifen administration are now in progress. The latter project was

peformed in callaboration with Dr. RF Franklin and M Stidworthy, Department of Clinical Veterinary Medicine, University of Cambridge, Cambridge, UK.

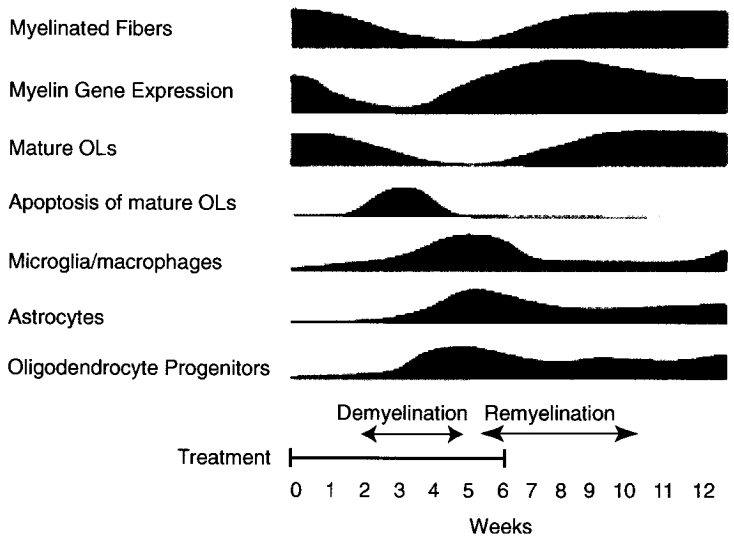


Figure 6-2: Summary of morphologic, cellular, and biochemical trends during cuprizone-induced demyelination followed by recovery.

The diagram was adapted from Mason et al., (2000).

7 RESULTS

In the following section, I used a strategy similar to that described previously, in which a floxed allele of Notch1 is flanked by *loxP* sites (Radtke et al., 1999) for conditional gene ablation (see Part 1, Figure 7.1). Recombination was mediated by three different Cre expressing mouse lines whose expression was driven by promoters known to be active at various steps during OL differentiation. Cre expression under the control of the *Cnp* promoter starts in the OPCs and has been described in Part 1. In the two other mouse lines Cre expression was under the control of either the *Plp* or *MBP* promoter, both known to be active in developing OLs in newborn mice. By excising Notch1 before OL differentiation or during OL differentiation, the aim of this study was to determine Notch1 function in OL maturation and myelin localization.

7.1 Cre-mediated recombination

7.1.1 *Plp-CreERT2* mediated recombination in neonatal animals

Cre was expressed under the control of regulatory elements of the *Plp* gene, known to be active in the OL lineage (Figure 7.1A). In this particular transgenic mouse, Cre is expressed as a fusion protein between Cre and a mutant form of the ligand-binding domain of the estrogen receptor (ERT2) (Feil et al., 1997). This mutation prevents binding of its natural ligand (17β-estradiol) at normal physiological concentrations, but renders the ERT2 domain responsive to 4-hydroxy (OH)-tamoxifen (TM) (D Leone et al., manuscript in preparation).

To determine where Cre recombinase was produced in our Cre-transgenic mice, *ROSA26* reporter (*R26R*) mice (Soriano, 1999) were crossed with mice carrying *Plp-CreERT2* (yielding *Plp-CreERT2 R26R*). Upon administration of tamoxifen, CreERT2 becomes functionally active and can therefore induce recombination. This approach allowed lineage tracing via detection of the LacZ product beta-galactosidase (beta-gal).

To test the potential use of the PlpCreERT2 line in the CNS of neonatal animals, the recombination specificity was examined following intraperitonal injection of tamoxifen into a breast feeding mother. Tamoxifen was administered at 1 mg per day (diluted in

90% sunflower oil/10% ethanol) for five consecutive days (from P0 to P5). We sacrified the mice two weeks or eight weeks after the last tamoxifen injection and beta-galactosidase activity was detected by histological X-gal staining (Figures 7.1B, C). A similar pattern was detected in *Plp-CreERT2 R26R* mice isolated two or eight weeks after TM injection. X-gal positive cells were observed within the white matter tracts of cerebellum (Figure 7.1C) and spinal cord (Figure 7.1D, dorsal view; asterisk, dorsal column; arrowhead, dorsal root ganglia from the PNS), but were absent from the forebrain (Figure 7.1C). This X-gal pattern closely parallels the appearance of mature OLs in the developing CNS (Ikenaka et al., 1992), therefore suggesting that in newborn animals, recombination is occurring during the late stage of OL differentiation.

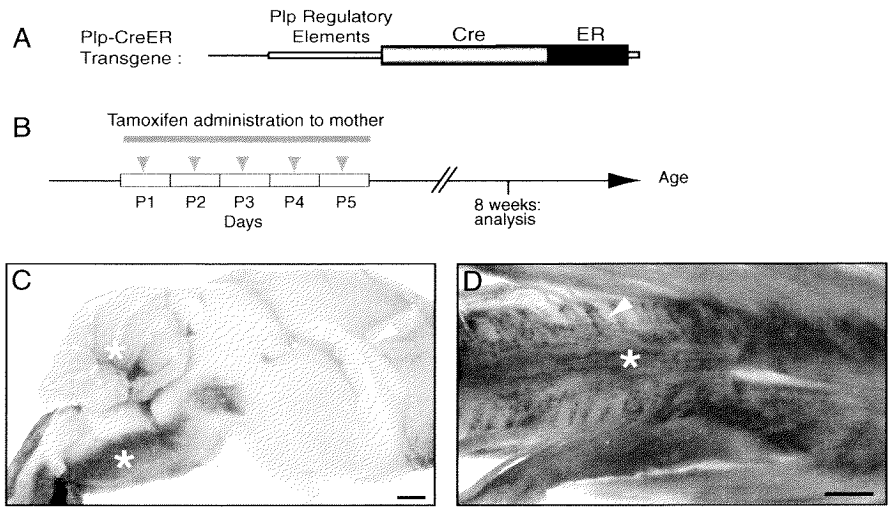


Figure 7-1: *Plp-CreERT2* mediated recombination after TM administration in neonatal animals.

The Cre recombinase gene is driven by the *Plp/DM20* regulatory elements (Spassky et al., 1998) (*Plp-CreERT2*), where Cre is a tamoxifen-inducible CreERT2 variant (A). *Plp-CreERT2* mice were crossed with *R26R* reporter mice. Tamoxifen was administered once a day from postnatal day one to day five in newborn animals through the mother (B). Cells in which Cre had been expressed were detected by X-gal staining for beta-galactosidase activity in eight weeks old animals. The *Plp-CreERT2* line shows expression in the cerebellum and brain stem (C, asterisk). Note the absence of recombination in the corpus callosum (arrowheads). In the spinal cord (D), the asterisk indicates the dorsal column and the arrowhead the dorsal root ganglia. Scale bars in C and D equal 1mm. (From D Leone et al., manuscript in preparation)

7.1.2 *MPB-Cre* mediated recombination

Cre was expressed under the control of regulatory elements of the myelin basic protein (*MBP*) gene (as a transgene driving Cre; M Miura, Department of Neuroanatomy, Osaka University, Japan) (Figure 7.2A). To determine when and where Cre recombinase was produced in our Cre-transgenic mice, *ROSA26* reporter (*R26R*) mice (Soriano, 1999) were crossed with mice carrying *MBP-Cre* (yielding *MBP-Cre R26R*). Sections were cut through the brain and spinal cord of doubly transgenic mice at postnatal day 4 (P4), P7 and P18, and beta-gal detected by histological X-gal staining. In *MBP-Cre R26R* mice, X-gal positive cells were observed at P18 within the white matter tracts of the cerebellum (Figure 7.2B) and of the spinal cord (Figure 7.2C), but very few in the forebrain (data not shown). Outside the area of the white matter tracts, X-gal positive cells were however absent, suggesting that MBP-Cre is selectively expressed in mature OLs. No staining was detected at P4 in the spinal cord and cerebellum of *MBP-Cre R26R* mice (data not shown). As comparison, note that activation of PlpCreERT2 between P0 and P5 led to widespread recombination in the white matter of the spinal cord and cerebellum (Figure 7.1C and D).

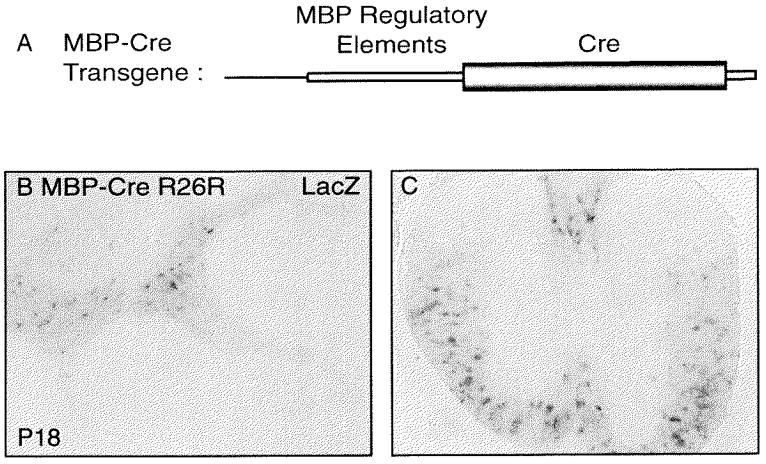


Figure 7-2: *MBP-Cre*-induced beta-gal expression patterns.

The Cre recombinase gene is driven from the *MBP* regulatory elements (A). *MBP-Cre* mice were crossed with *R26R* reporter mice. Cells in which Cre had been expressed were detected by X-gal staining for beta-galactosidase activity in sections of P18 mice. The *MBP-Cre* line shows specific expression in the white

matter tracts of the cerebellum (B, sagital sections) and spinal cord (C). Spinal cord sections were cut at the thoracic level.

7.2 Description and histological analysis of postnatal mutant animals

Homozygous deletion of *Notch1* caused perinatal lethality in mice carrying the *Plp-Cre* or the *Cnp-Cre* allele. I have however observed a variability in the penetrance of lethality, since a small percentage of these knockout animals live longer, ranging from a few more days to adulthood. *Cnp-Cre Δ/Δ* individuals survived longer and exhibited only modest defects (smaller in size, poor sense of balance, partially closed eyes, loss of hair). Histological analysis by hematoxylin/eosin staining of the brain and spinal cord of the *Cnp-Cre Δ/Δ* animals did not reveal any obvious abnormality (data not shown). In contrast, *Plp-Cre Δ/Δ* mice that survived after birth had to be sacrificed because of severe growth retardation and motor defects. The mice also displayed severe brain abnormalities: The lateral ventricles, as well as the 3rd and 4th ventricles, were greatly enlarged, there was extensive cell loss in the subcortical region, especially around the subventricular zone, and part of the hippocampus appeared to be missing (Figure 7.3 and not shown). *Plp-CreERT2 Δ/Δ* individuals that received TM between postnatal day 0 to P5 looked however fully normal (normal growth rate and behaviour). *MBP-Cre Δ/Δ* animals were also viable, but showed retarded growth and poor sense of balance with variability in the penetrance of the phenotypes. Interestingly, the observed phenotype was very similar to that of *Cnp-Cre Δ/Δ* adult animals. Histological analysis revealed that the brain and spinal cord of the adult *MBP-Cre Δ/Δ* were structurally normal (not shown).

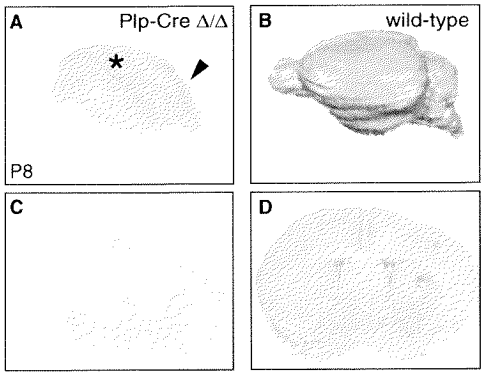


Figure 7-3: Severe defects in the brain of postnatal *Plp-Cre Δ/Δ* mice (previous page).

Following perfusion with 4% PFA, the brain of *Plp-Cre Δ/Δ* animals was carefully dissected, photographed and embeded for further analysis. In A, the arrowhead shows a reduction in the size of the cerebellum, and the asterisk a collapse of the hemispheres. On sections, hematoxylin/eosin staining shows extensive damage in the brain and strong hydrocephalus (C,D).

7.3 OL maturation and myelination

7.3.1 Notch1 ablation in oligodendrocyte precursor cells does not hinder OL maturation and myelin localization

A role of Notch signaling in the regulation of terminal OL differentiation and myelination has been found by the analysis of Notch1 receptor heterozygous null mutant animals (Notch1$^{+/-}$ (Givogri et al., 2002). To further address this interesting question, I specifically induced recombination in the OL lineage by using Cre-expressing mice, where Cre is produced either in the OPCs (*Cnp-Cre*) or in premyelinating/myelinating OLs (*PlpCreERT2* and *MBPCre*). In each case I analyzed the cerebral cortex, the cerebellum and the spinal cord, and performed X-gal staining to determine the level of recombination which occurred in the regions of interest.

I first examined *Cnp-Cre Δ/Δ* animals at P14, while OL maturation is still in process. Initial X-gal staining in *Cnp-Cre Δ/Δ R26R* (Figures 7.4A, C, E, G) and *Cnp-Cre Δ/wt R26R* (Figures 7.4B, D, F, H) mice showed no major differences in the number and pattern of recombined cells in the homozygous (*Δ/Δ*) as compared to heterozygous (*Δ/wt*) mice. I then performed immunohistochemistry with antibodies against MBP (not shown) and PLP/DM20 and examined the localization and extent of myelination in the cerebral cortex (Figure 7.4I), the cerebellum (Figure 7.4K) and the spinal cord (Figure 7.4M) of *Cnp-Cre Δ/Δ* versus *Cnp-Cre Δ/wt* animals (Figures 7.4J, L, N). The spatial and temporal pattern of myelination was similar between mutant and control mice. Similar experiments were repeated with older *Cnp-Cre Δ/Δ* animals (3–15 months old), and no abnormality in the localization of myelination was detected (Figures 7.4O, P). Of special interest, note the complete absence of PLP immunostaining in the molecular layer of the cerebellum of mutant and control animals at both P14 (Figures 7.4K, L, asterisk), and P427 (Figures 7.4O, P, asterisk), despite the presence of numerous X-gal positive cells in

this region in the adult (asterisk in Figures 7.4G, H). Few X-Gal positive cells were however observed in the molecular layer of P14 cerebellum (asterisk in Figures 7.4C, D).

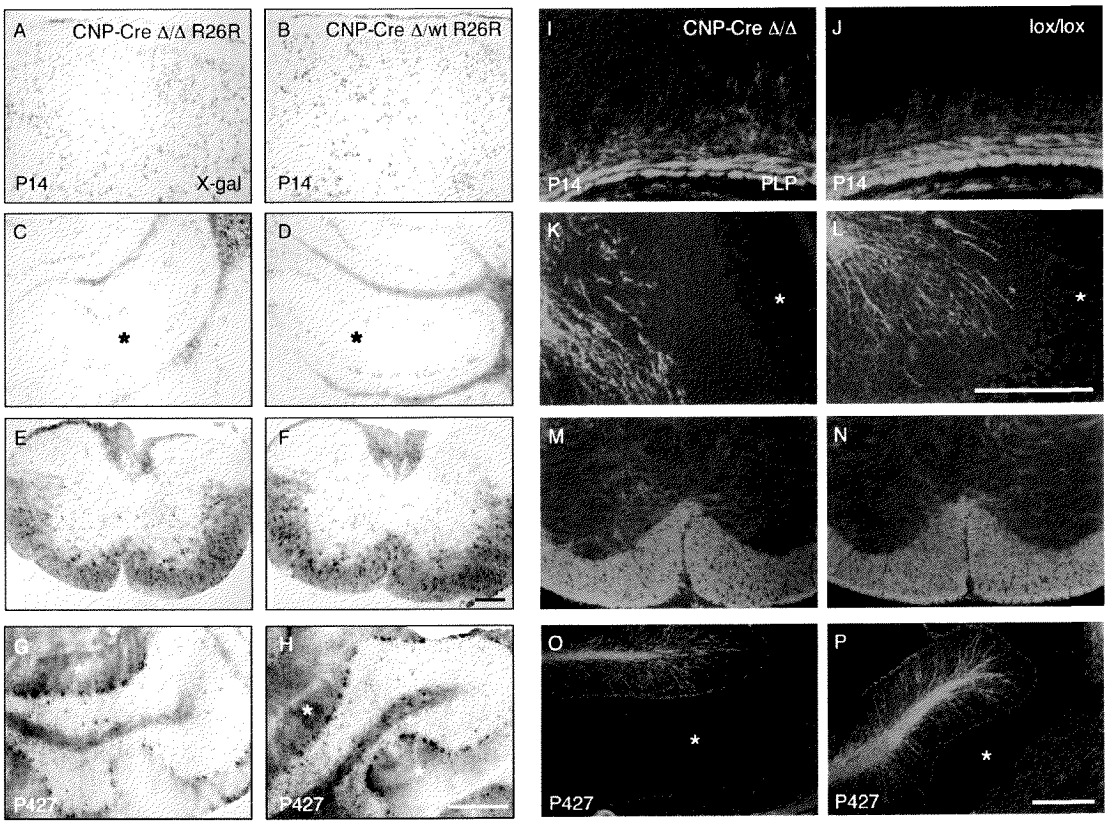


Figure 7-4: OL maturation and myelination in P14 and adult Cnp-Cre Δ/Δ mice.

A-H) Sections were cut from triple-transgenic animals carrying *Cnp-Cre*, *R26R*, and one (B,D,F,H) or two copies (A,C,E,G) of the floxed *Notch1* allele. X-gal stainings at P14 indicate recombination patterns in homozygous mutant (Δ/Δ) cerebral cortex (A), cerebellum (C), spinal cord (E, thoracic level) and in the corresponding heterozygous tissues (B, D, F, Δ/wt). The pattern of recombination was also analyzed in adult animals, in the cerebellum of homozygous mutant (G) and heterozygous (H) mice. The extent of OL maturation and pattern of myelination were then compared in *Cnp-Cre Δ/Δ* with control (*lox/lox*) sections by immunohistochemistry with an antibody against PLP/DM20. The asterisk shows the molecular layer of the cerebellum. In K and L, tissues were stained with DAPI to mark the cell nuclei. Scale bars equal 40 μm.

7.3.2 Notch1 ablation in premyelinating/myelinating oligodendrocytes does not hinder OL maturation and myelin localization

Next, I examined *Plp-CreERT2 Δ/Δ* and *MBP-Cre Δ/Δ* animals. In contrast to *Cnp-Cre* which is expressed in the OPC stage (Figure 3.2), Cre activity starts later in developing oligodendrocytes in the *Plp-CreERT2* mice (when tamoxifen is administered from P0 to P5) and in the *MBP-Cre* mice (Figures 7.1 and 2). Newborn *Plp-CreERT2 Δ/Δ* and control individuals received tamoxifen through the mother for a period of 14 days (one injection per day), and were sacrified at P15. *MBP-Cre* individuals were sacrified at P18. Initial X-gal staining in *MBP-Cre Δ/Δ R26R* (Figures 7.5A, C) and *MBP-Cre Δ/wt R26R* (Figures 7.5B, D) mice showed no major differences in the pattern of recombined cells in the cerebellum and spinal cord of homozygous (*Δ/Δ*) compared to heterozygous (*Δ/wt*) mice. I then performed immunohistochemistry with antibodies against MBP (not shown) and PLP/DM20 and examined the localization and extent of myelination in the cerebellum (Figures 7.5E, F) and spinal cord (Figures 7.5G-J). No obvious abnormality was detected. Similar results were observed with *Plp-CreERT2 Δ/Δ R26R* and *Plp-CreERT2 Δ/wt R26R* animals (data not shown).

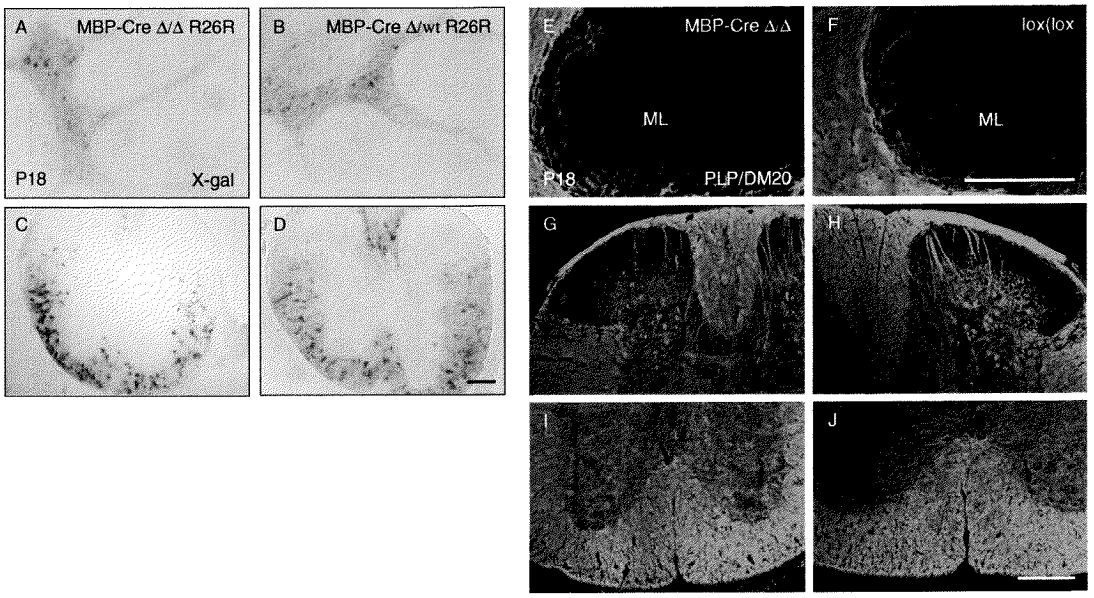


Figure 7-5: OL maturation and myelination in P18 MBP-Cre Δ/Δ mice (previous page).

A-D) Sections were cut from triple-transgenic animals carrying *MBP-Cre*, *R26R*, and one (B,D) or two copies (A,C) of the floxed *Notch1* allele. X-gal staining at P18 indicate recombination patterns in homozygous mutant (Δ/Δ) cerebellum (A) and spinal cord (C, thoracic level), and in the heterozygous corresponding tissues (Δ/wt, B, D). The extent of OL maturation and the pattern of myelination were then compared in *MBP-Cre Δ/Δ* with control (*lox/lox*) sections by immunohistochemistry with an antibody against PLP/DM20. G, H, dorsal horns of the spinal cord; I, J, ventral horns of the spinal cord. In E and F, tissues were stained with DAPI to mark the cell nuclei. ML, molecular layer. Scale bars equal 40 μm.

7.4 Investigation of the Notch1 signaling pathway during CNS remyelination using a cuprizone model

7.4.1 *Plp-CreERT2* mediated recombination in adult animals

To test the potential use of the PlpCreERT2 line in adult CNS, the recombination specificity was examined following intraperitonal injection of TM into eight to 15 week-old animals for five consecutive days (Figure 7.6A). Injections were performed twice a day with 1 mg TM. The mice were sacrified 10 to 20 days later and their forebrain dissected. Acutely dissociated cells were then plated and imunostaining was performed using antibodies against beta-galactosidase and NG2 to label recombined cells, and oligodendrocyte precursor cells (OPCs) respectively (Figure 7.6B). Around 75% of the NG2-positive cells, as measured and obtained from the brain of three different animals, were also positive for beta-gal, demonstrating expression of Cre recombinase and recombination of the LacZ reporter allele within the NG2-positive oligodendrocyte precursor population (Figure 7.6C). Cells with the typical bipolar morphology of OPCs were observed (Figure 7.6B), as well as cells with a more complex morphology, as

observed in more differentiated cells (data not shown). Recombination was also observed in mature OLs (D Leone et al., manuscript in preparation).

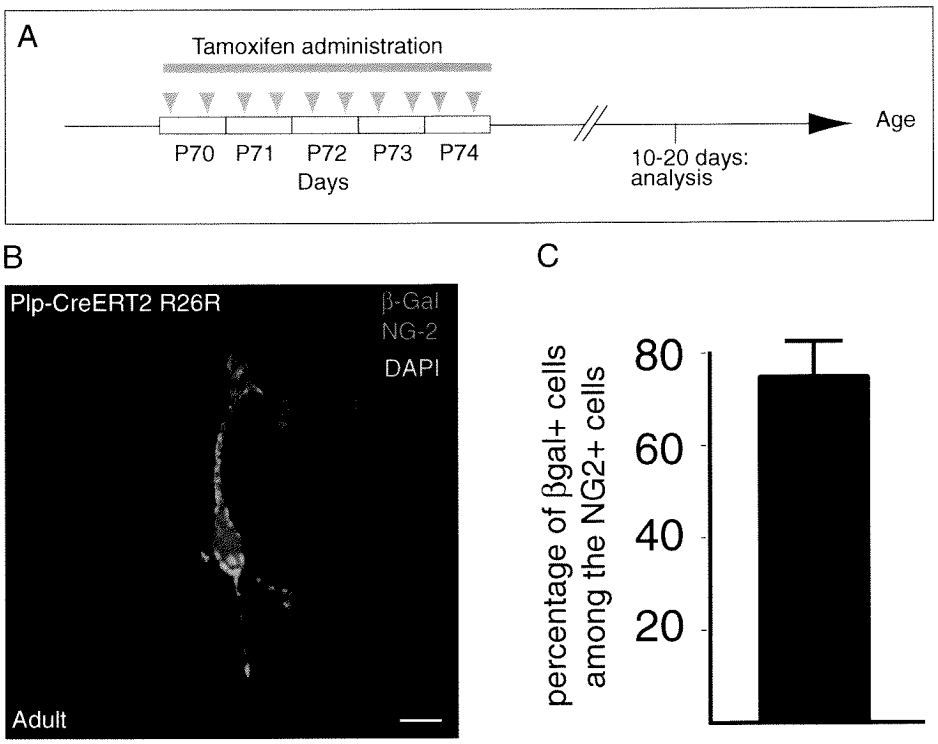


Figure 7-6: *Plp-CreERT2* mediated recombination after TM administration in adult animals.

Plp-CreERT2 mice were crossed with *R26R* reporter mice. Cells in which Cre had been expressed were detected by X-gal staining for beta-galactosidase activity in 10-15 weeks old animals. Tamoxifen was administered intraperitanally twice a day for five consecutive days and the mice sacrificed 10 to 20 days later (A). Acutely dissociated cells were isolated from the forebrain of *Plp-CreERT2 R26R* mice and stained for beta-galactosidase and NG2 (B). DAPI marks cell nuclei. The *Plp-CreERT2* line shows expression in around 75% of the NG2-positive cells (C). Scale bar equals 100μm.

7.4.2 Experimental strategy

Demyelination occurs after three weeks of cuprizone administration, and progresses to completion by five weeks (Hiremath et al., 1998). Removal of cuprizone at five weeks leads to partial remyelination one week later and to almost complete remyelination by week 7 (Mason et al., 2000; Morell et al., 1998). The peak accumulation of NG2-positive

precursor cells in the corpus callosum occurs during the last weeks of cuprizone treatment and drops to a lower and constant level when cuprizone is removed (Mason et al., 2000). To address the function of Notch1 in the process of remyelination in the CNS, we therefore started the tamoxifen administration at the beginning of week 4 and continue through week 6, in order to induce recombination in as many progenitors as possible (Figure 7.7). Animals were sampled at the end of the cuprizone treatment (i.e. at week 5) to determine the efficiency of demyelination. Animals were also sacrified one or two weeks later (i.e. at week 6 or week7), to assess for remyelination.

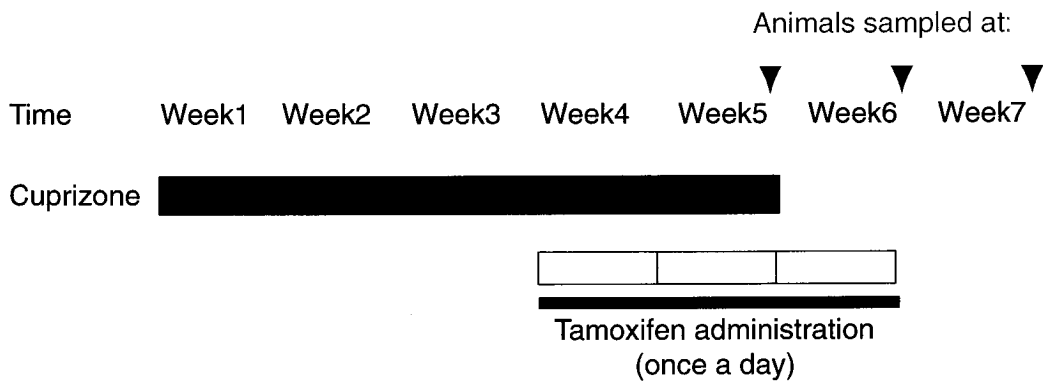


Figure 7-7: Summary of the experimental strategy.

Demyelination was achieved by feeding adult mice with cuprizone for five weeks, and the extent of remyelination was assessed one or two weeks later. Some animals were sacrified at the end of the cuprizone treatment to evaluate the level of demyelination. To induce recombination, tamoxifen was administered for three consecutive weeks, with a single tamoxifen dose each day.

7.4.3 Cuprizone induces constant and severe demyelination in the body and splenium of the corpus callosum

The corpus callosum is a heterogeneous white-matter tract that connects the cerebral hemispheres. It can be subdivided into three regions along the rostrocaudal axis, namely the genu, body and splenium. At week 5 (e.g. at the end of the cuprizone treatment), histological analysis of thin resin sections with a Toluine Blue stain revealed variability in the severity of demyelination in different anatomical regions of the corpus callosum (Figure 7.8). The ventral part of the splenium of the corpus callosum, at the transition to the body, was found to be most consistently and severely affected with fewest residual

myelin sheaths and the greatest cellularity. Although both the genu and part of the body demonstrated areas of partial and complete demyelination, these areas varied in size and severity between animals, making the selection of appropriate areas for detailed examination more difficult.

Electron microscopy (EM) examination of both the ventral splenium and the caudal part of the body of the corpus callosum confirmed that the splenium showed the most marked changes in comparison with normal mice (see Figure 7.10). In these regions, myelinated axons were virtually absent in the mice sacrified at the end of the cuprizone treatment. Further analysis was therefore performed in the area between the body and the splenium, close to the midline, because of its high density of myelinated fibers and because of its consistent and severe demyelination capacity upon cuprizone administration.

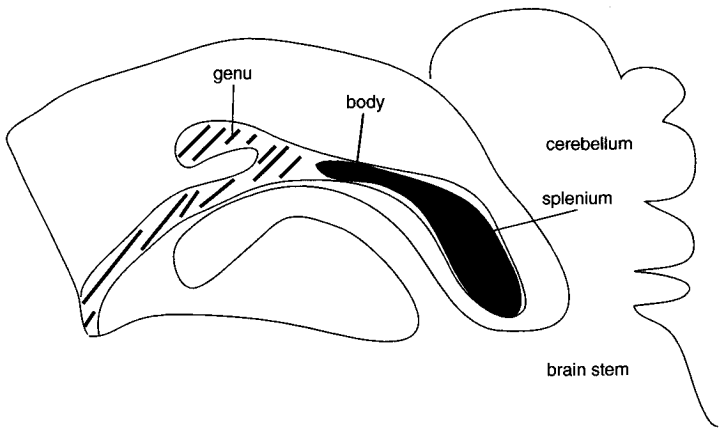


Figure 7-8: map of lesions in the corpus callosum following cuprizone treatment.

Sagittal representation of a midline brain section. Variety in the consistence and severity of the lesions was observed in the corpus callosum. Hatched lines represent regions where demyelination was not complete or not constant between different animals. Filled volume indicates severe and constant demyelination. From M Stidworthy.

7.4.4 Normal remyelination following cuprizone and tamoxifen treatment

Although only ultrastructure is definitive in the morphological assay, much information can be gained from resin/thin section histology with a Toluidine Blue stain. This method allows for semi-quantitative estimates of myelination status by subjective (but observer

blinded) "staging" (depending on the number of animals analysed). Mutant *PLP-CreER Δ/Δ* and control *lox/lox* animals were sacrified one or two weeks after the end of the cuprizone administration (e.g. week 5 or 6), perfused with glutaraldehyde, and tissue embedded in resin for thin sectioning and Toluidine Blue staining (Figure 7.9). Based on myelination rank of the body and splenium of the corpus callosum, no significant differences were observed when males were pooled together with females at either week 6 or week 7 ($p>0.1$, Mann-Whitney test). However, a nearly significant difference ($p=0.1$) was detected at week 7 between mutant *PLP-CreER Δ/Δ* and control *lox/lox* males, where the level of remyelination was lower in the mutant as compared to the control mice. Such a difference was not observed with females in this sample. This study is now being repeated with a larger number of males and using a different protocol (see Figure 9.1).

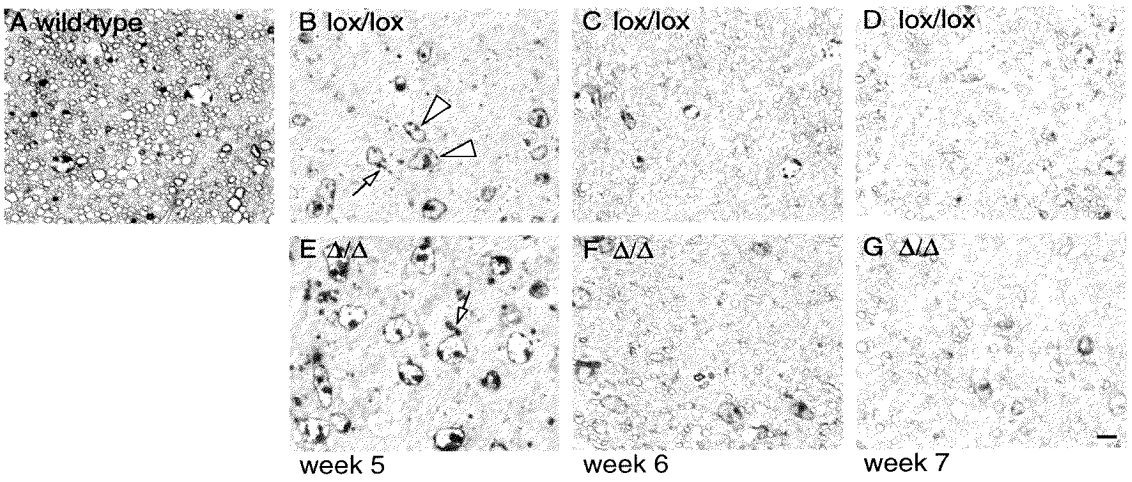


Figure 7-9: Toluidine Blue staining of the corpus callosum during remyelination.

Staining was performed on thin sagittal sections of the splenium of the corpus callosum. (A) shows typical myelination in the central nervous system. The time course of remyelination in control mice (B-D) and mutant mice (E-G) is similar. After five weeks of treatment with cuprizone (B, E), both exhibit almost complete demyelination, increased cellularity and the presence of macrophages (arrowheads in B), with myelin debris in their cytoplasm (arrows in B and E). By week 6 (one week without cuprizone) and week 7 (two weeks without cuprizone), both animals show significant remyelination (C, F). Scale bar in G equals 10μm.

Since the level of remyelination appeared to be similar between *PLP-CreER Δ/Δ* and *lox/lox* animals, we considered two possibilities: Complete demyelination may have failed in the splenium of the corpus callosum of some animals, therefore masking the level of remyelination, or the rate of remyelination may have been identical between *PLP-CreER Δ/Δ* and *lox/lox* animals. To distinguish these two possibilities, very tightly defined small brain regions were determined and analysed by EM. In a defined location it is possible to quantitatively determine various parameters related to presence of myelin. These include the number of axons per unit area and the distribution of their sizes, how many are myelinated, the thickness of the myelin sheath, and the relationship of thickness in relation to size of the axon (so called g-ratio). Since these values differ between an environment where remyelination is occurring or has occured and a normal, fully myelinated situation, we have been able to demonstrate that the rate of remyelination, when males and females are pooled together, does not differ between *PLP-CreER Δ/Δ* and *lox/lox* animals (Figure 7.10 and personal communications from M Stidworthy).

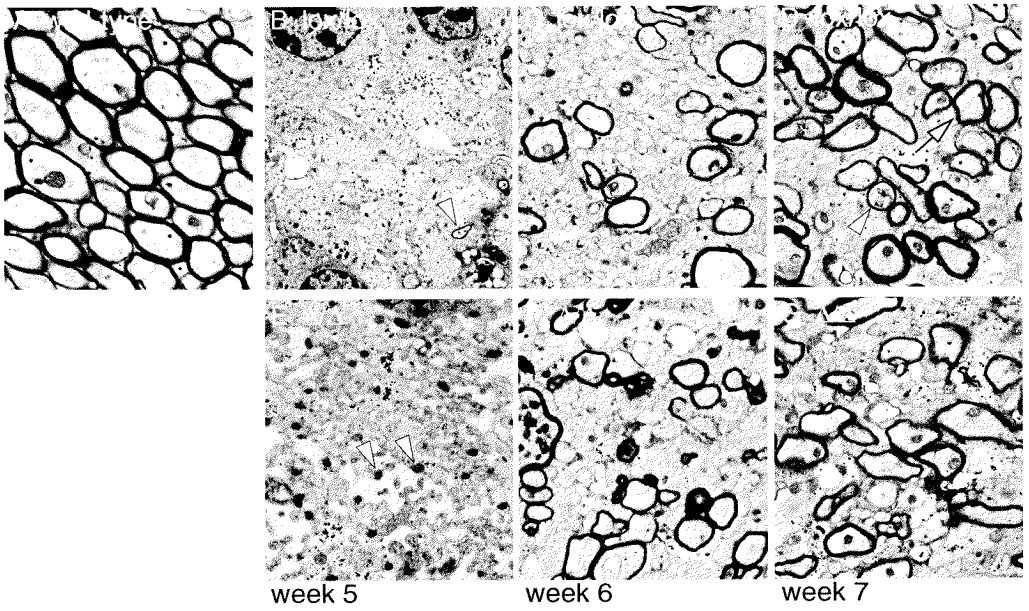


Figure 7-10: EM analysis of the corpus callosum during remyelination.

Electron microscopy was performed on sagital sections of the splenium of the corpus callosum. (A) shows typical myelination in the central nervous system. The time course of remyelination in control mice (B-D) and mutant mice (E-G) is similar. After five weeks of treatment with cuprizone (B, E), both exhibit almost

complete demyelination, with very few axons still myelinated (arrowhead in B). After demyelination, axons in control and mutant animals can shrink; the arrowheads in panel E most likely represent mitochondria within shrunken axons. By week 6 (one week without the toxin), both animals show significant remyelination (C, F). By week 7 (D, G), one can already observe thick myelin sheaths (arrow in D) as well as axons starting to be myelinated (arrowhead in D). From M Stidworthy.

7.4.5 Possible impairment of the early steps of OL development in a lesion

I then examined the cellular composition and recombination efficiency of the corpus callosum in *PLP-CreER Δ/Δ* versus control (*lox/lox*) mice sacrified one week after the end of the cuprizone treatment. To assess the efficiency of recombination, some white matter tracts known to be unaffected by cuprizone were chosen for comparison. Initial X-gal staining in these unaffected regions of *PLP-CreER Δ/Δ R26R* (Figure 7.11A) and *PLP-CreER 0/wt R26R* (Figure 7.11B) mice showed no major differences in the number of recombined cells in the homozygous (Δ/Δ) compared to heterozygous (0/wt) mice. However, when the corpus callosum was analyzed, a much reduced number of recombined cells was observed in homozygous mutant animals (Figure 7.11C) compared to heterozygous animals (Figure 7.11D). TUNEL staining suggested that these cells might have died by apoptosis, since a higher level of dying cells were observed in the corpus callosum of *PLP-CreER Δ/Δ* animals (data not shown). I then examined the cellular composition of the corpus callosum using immunostaining and in situ hybridization for markers characteristic of OPCs and proliferating cells. I used antibodies against the chondroitin sulfate proteoglycan NG2 (a marker for OPCs and reactive macrophages; Figures 7.11E, F) and histone H3 (for proliferating cells; Figures 7.11I, J), and detected platelet-derived growth factor alpha (PDGFRα) by in situ hybridization for OPCs (Figures 7.11G, H). During the inflammation that accompanies cuprizone-induced demyelination, NG2-positive OPCs rapidly accumulate, and upon removal of cuprizone, presumably differentiate into mature OLs. In null heterozygous mice, a high level of NG2-positive cells was maintained throughout the length of the corpus callosum (Figure 7.11F). However, the number of NG2 progenitors was strongly reduced in the corpus callosum of mice lacking Notch1, but not in other regions of the brain. This weak NG2 immunoreactivity correlates well with the reduced number of Xgal-positive cells and quite interestingly, PlpCreER was shown to be active in the NG2-

positive cells (see Figure 7.7). I then used PDGFR-alpha, another marker to label OPCs, and quite surprisingly noticed a strong upregulation of this marker in the corpus callosum of *PLP-CreER Δ/Δ* animals (Figures 7.11G vs H). This observation is in agreement with proliferative studies, where numerous mitotic cells were detected in mutant animals (Figures 7.11I vs J). Given that these data come from a single mutant and a single control mouse, these observations need careful consideration and are further discussed in the outlook.

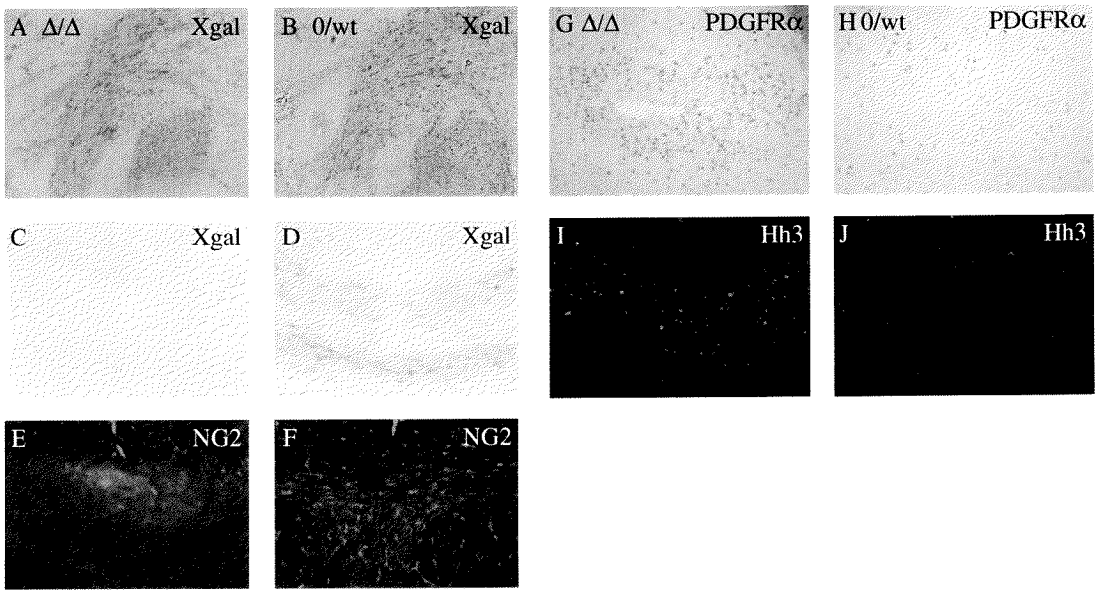


Figure 7-11: recombination efficiency and cellular events in the corpus callosum after the first week of remyelination.

Coronal sections of the corpus callosum of mutant animals (*PLP-CreER Δ/Δ R26R*) were compared with sections of heterozygous animals (*PLP-CreER 0/wt R26R).* To induce recombination tamoxifen was injected for three weeks (see Figure 7.7). Xgal stainings in A) and B) indicate a similar recombination efficiency in control white matter tracts (not affected by cuprizone) of *PLP-CreER Δ/Δ R26R* and *PLP-CreER 0/wt R26R* animals. The number of recombined cells within an affected region of the corpus callosum was, in contrast, much different (C and D). The number of OPCs was compared by immunohistochemistry (E, F) and in situ hybridization (G, H). NG2 immunostaining revealed a reduced number of NG2-positive cells in the corpus callosum of *PLP-CreER Δ/Δ R26R* (E) animals as compared to *PLP-CreER 0/wt* mice *R26R* (F).By contrast, PDGFR-alpha showed an opposite effect (G, H). Proliferation was also enhanced in mutant animals (I) compared to control animals (J).

8 DISCUSSION

A great deal is known about the structural organization of the myelin membrane and the role of myelin in the physiology of nerve fiber conduction (Chiu and Ritchie, 1984). However, despite considerable attention in the past 30 years, less is known about the mechanism of myelination and remyelination or the signals that regulate these complex processes. In the course of my Ph.D thesis, I have provided direct evidence that Notch1 is critically important for correct temporal and spatial differentiation of OLs from the precursor to the immature stage (Genoud et al., JCB, in press) (Figure 4, 5 and 9, Part 1). In the current work, I have investigated the role of Notch1 signaling later in development of OLs and in the localization of myelin, as well as in the process of remyelination after a brain lesion. Despite the loss of Notch1 in different stages along the OL lineage, the pattern of myelination was not disturbed in various regions of the CNS during the first two postnatal weeks and in the adult. Furthermore, although further investigations are still in progress, we so far failed to show a direct role of Notch1 in the remyelination process in the mouse brain.

8.1 Loss of Notch1 in the oligodendrocyte lineage does not hinder faithful localization of myelin

Oligodendrocytes stop expressing Notch1 receptors as they mature (Givogri et al., 2002; Wang et al., 1998). However, when OPCs differentiate into newly formed OLs, they continue to express Notch1 receptors. Since Notch pathway activation strongly blocks newly formed OLs from differentiating into mature OLs (Wang et al., 1998), this raised the question of whether further OL maturation and maybe myelination could be promoted by a decrease in Notch signaling.

Givogri et al., (2002) provided evidence that this could be the case in vivo, by observing a small number of myelinated fibers in the molecular layer of Notch1$^{+/-}$ P35 cerebella., but failed to detect abnormal myelination in adult animals. The fact that Notch1 was however ablated in every cell does not exclude the possibility that the observations reported there are indirect consequences of Notch deletion. I therefore used defined

conditional gene ablation to address the question of how myelination in the central nervous system is affected when Notch1 function is specifically eliminated in vivo.

Given my own interest in development, I have focused for the most part on two week-old mice, an age when myelination is already ongoing in multiple CNS regions, but not yet completely achieved. Notch1 was deleted in either the oligodendrocyte precursor cells (by using CNPCre), the premyelinating oligodendrocytes (by using PlpCreERT2) or in the mature oligodendrocytes (by using MBPCre). Given that MBP-positive cells have been shown to be co-labeled with markers for proliferation (Mathis et al., 2000), this suggests that deletion of Notch1 occurs during the early stage of maturation of oligodendrocytes, at a time when Notch1 receptors are still expressed in OLs from the optic nerve (Wang et al., 1998). In contrast to Givogri et al., (2002), I observed neither the presence of myelinated fibers in the ML of the cerebella in any of the three types of mice analyzed, nor an increased immunoreactivity of myelin proteins in the cerebral cortex. Together, my data indicate that establishment of the pattern of myelin is not altered in the absence of Notch1 in the OPCs and mature OLs. The observation of Givogri et al., (2002) most likely reflects a defect of Notch1 in neurons or astrocytes, both essential for myelination.

In *PDGF-A*$^{-/-}$ mice there is a substantial amount of OL death and hypomyelination. Conversely, in transgenic mice overexpressing PDGF-A in neurons, OPCs divide more than normally. This results in a large increase in OPCs, which eventually populate not only the white matter but also the grey matter of the spinal cord (Calver et al., 1998). These supernumerary OLs are later eliminated by programmed cell death, so that the number of OLs becomes normal in adult transgenic mice. These studies indicate that axonal signals adjust OL numbers according to neuron/axon numbers to obtain a full set of myelin internodes. I propose therefore that Notch signaling, together with other mechanisms (see discussion Part 1), controls the timing of OL differentiation, irrespective of whether the neurons of a particular region will be myelinated or not. Then, axonal contact might be critical for further stages of OL maturation and myelination, but independent of Notch1 signaling. Indeed, OLs are able to myelinate axons of different calibers when the proper conditions are offered, which suggests that myelination of a particular axon depends more on environmental or axonal regulatory signals than on an inherited OL cellular program (Fanarraga et al., 1998). ErbB2

signaling has been shown to play a critical role in axonal ensheathment and myelination in vitro (Park et al., 2001). Due to the early lethality of the erbB2$^{-/-}$ mice, it has until now not been possible to determine how myelination is affected in vivo in the absence of erbB2.

My data do not however exclude the possibility that other Notch receptors are important for controlling myelination. Jagged1 expression has been shown to correlate well with myelination (Wang et al., 1998), and Notch2 and Notch3 are also highly expressed in the adult CNS (Irvin et al., 2001). Jagged-1 could therefore interact with another Notch receptor to control the localization of myelin in the maturating CNS.

8.2 A reliable model to study remyelination using inducible conditional knockout mice

With regard to potential therapies for demyelinating disorders, it would be useful to develop a system to assay conditions and factors that promote remyelination in vivo. I propose for this purpose the model of demyelinated brain resulting from exposure of mice to cuprizone. With respect to the choice of model system, protocols needed to establish the temporal and spatial reproducibility of the demyelination induced by exposure to cuprizone have been refined by several groups (Blakemore, 1984; Ludwin, 1994; Morell et al., 1998). The present data have added an important finding regarding this model system; the results of my study demonstrate the constant and severe demyelination of the caudal part of the corpus callosum (splenium and body), suggesting that it is a reliable animal model with which to study demyelination and remyelination. The more rostral parts of the corpus callosum (e.g. the genu) are however not reliable in terms of constancy and severity of the demyelination. Note that the results likely depend on the dose of cuprizone, since we did not observed any demyelination in the superior cerebellar peduncle, as others have reported using a much higher dose (Blakemore, 1973; Ludwin, 1978). Note also that the lesion does not destroy axons, which allow us to restrict the study of the remyelination process to glial cells.

Having a precise temporal and cellular control of the recombination event will circumvent the problem of genes that may have several activities in different cell lineages and at different stages of development. Furthermore many genes are embryonic lethal. Here we describe a tool with which we can study the function of a gene in the

mature CNS during the process of remyelination, although this gene leads to embryonic lethality when deleted during embryogenesis. By using the Cre/lox system and an inducible version of the cre recombinase, we successfully mediated the recombination of beta–galactosidase during the two last weeks of the demyelination process and during the first week of remyelination. Tamoxifen administration can therefore be used to ablate activity of a gene at a certain stage of OL development and at a certain age. How rapidly is recombination detected in adult tissues? Time is unfortunately a limitation in the method, since β-gal activity assayed in a variety of tissues 24h after a single intraperitoneal injection of 9mg of tamoxifen injection into an adult was not sufficient to lead to a widespread distribution (Hayashi and McMahon, 2002). Two injections per day with 1 mg tamoxifen for 48h led also to very few Xgal-positive cells in the brain (data not shown). After 5 days and multiple injections, widespread recombination was observed (Hayashi and McMahon, (2002); Leone et al., manuscript in preparation).

8.3 The rate of remyelination is normal despite the lack of Notch1 expression in developing OPCs

Several studies have identified differentiating oligodendrocyte progenitors as the cells responsible for remyelinating lesions within the adult CNS (Gensert and Goldman, 1997; Mason et al., 2000). The factors responsible for the recruitment and differentiation of these progenitors in vivo have not been fully delineated. Repair of insult to the CNS has been increasingly attributed to indirect effects of the immune response, such as those elicited by TNFα (Arnett et al., 2001) and IL-1β (Mason et al., 2001). One other possible factor is the Notch1 receptor, which has been shown to regulate OPC differentiation in the developing CNS (Wang et al., (1998) and Part 1). This protein, in turn, is highly expressed in ethidium bromide-induced brain lesions, whereas other family members such as Notch2 and Notch3 are expressed only at a low level (M Stidworthy, personal communications). To address the functional importance of Notch1 in demyelination-remyelination, we used a model in which continuous cuprizone (bis-cyclohexanone oxaldihydrazone) intoxication of adult conditional knockout mice leads to death of mature oligodendrocytes.

The importance of OPC differentiation in remyelination has been highlighted in a study in which the age-related difference in remyelination has been attributed to an impairment

of both OPC differentiation and their recruitment to the site of the lesion (Sim et al., 2002). Differentiation-inducing signals may therefore be absent, or on the other hand, a Notch ligand such as Jagged may inhibit OPC differentiation via activation of Notch receptors. We first showed by Xgal staining that recombination occurred in the NG2 progenitor cells. We then evaluated a number of events - including demyelination, remyelination and the presence of OPCs - occurring after cuprizone exposure and found that the rate of remyelination (number of myelinated axons and myelin thickess in relation to the axon caliber (so called g-ratio)) was similar in animals with or without Notch1 expression.

8.4 Notch1 deletion leads to a depletion of NG2-positive cells in the corpus callosum

In many instances the NG2-positive cells are the first cells to react to damage, but these reactive changes are spatially restricted to the immediate area of lesion (Di Bello et al., 1999; Levine, 1994). The functional significance of this rapid response to brain injury is not well understood. It is possible that high levels of chondroitin sulphate proteoglycans, including NG2, may have a neuroprotective effect. However, the growth-inhibiting properties of NG2 (Dou and Levine, 1994) may also contribute to the creation of an environment that is no longer supportive of nerve regeneration and repair. Although caution must be taken in generalizing these data because of the small number of mice analyzed, NG2-positive cells were barely detected after the first week of remyelination within the area of demyelination. NG2 cells were detected at the border of these lesions and in adjacent normal-appearing white matter, so we consider it unlikely that they were overlooked for technical reasons. NG2-positive cells remain abundant throughout the adult rodent CNS (Levine et al., 1993; Nishiyama et al., 1996; Reynolds and Hardy, 1997) and have the potential to generate oligodendrocytes in vitro (Ffrench-Constant and Raff, 1986; Wolswijk and Noble, 1989). The use of this marker to provide unequivocal identification is however still controversial: are they part of the OL lineage? Can they give rise to new OLs following demyelination? Direct evidence that the OLs involved in remyelination differentiate from dividing NG2-positive progenitors is still missing (reviewed in Dawson et al., (2000)), but there is compelling evidence that NG2-positive cells are OPCs and are capable of differentiation into mature OLs in vivo. Furthermore,

the detection of NG2-positive cells in MS lesions has raised the possibility that they give rise to remyelinating oligodendrocytes in MS lesions (Chang et al., 2000). Quite interestingly, we observed a very low number of NG2 progenitor cells in the corpus callosum of the *Plp-CreERT2 Δ/Δ* brains, despite a normal level of remyelination in the group of mice used for morphological analysis. The absence of Il-1β leads to an accumulation of NG2-positive cells, but low remyelination capacity because OLs are not able to mature in the absence of IL-1β (Mason et al., 2001). The absence of TNFα however, leads to a reduction of NG2-positive cells, fewer OLs and as a consequence also to a low remyelination capacity (Arnett et al., 2001). It shows that one can not rely on the number of OPCs to determine the capacity for remyelination. Rather, the level of remyelination depends on the number of mature OLs. In our case, this would suggest that Notch1 keeps the NG2-positive OPCs in a resting state in the adult lesioned brain, similar to what happens during development (Part1). By removing Notch1 all NG2-positive cells would be free to differentiate into remyelinating OLs. It remains however unclear why this led to a depletion of the NG2-positive cells within the area of demyelination, whereas this is not observed during normal development in newborn animals (data not shown).

Very few Xgal-positive cells were detected in the corpus callosum of *Plp-CreERT2 Δ/Δ* individual. Since we do observe an increase of apoptosis in the area of this mouse, the differentiated oligodendrocytes might have been cleared by programmed cell death. If oligodendrocytes are killed, how is it possible to have a normal capacity for remyelination? A possible explanation for the depletion of NG2- and Xgal-positive cells in the corpus callosum of *Plp-CreERT2 Δ/Δ* individuals might be the following: since tamoxifen was injected while the mice were still being treated with cuprizone, it is quite possible that all NG2-positive cells had differentiated (because of the loss of Notch1) and then were killed by cuprizone. If so, OPCs that had not recombined during this time might have proliferated and compensated for the OPCs lost to differentiation. When cuprizone was removed, OPCs still expressing Notch1 could have normally differentiated and produced a similar level of remyelination as in control animals. Although tamoxifen was still administered during the first week of remyelination, a single injection per day for five days is generally not enough to induce widespread

recombination (D Leone, personal communications). Further experiments with different schedule of substance administration are now in progress (see section 9).

8.5 Absence of colocalization of NG2 and PDGFR-alpha

The first postnatal week marks the peak in the number of NG2 and PDGF alpha-receptor immunoreactive cells, as well as the peak in the level of expression and the extent of co-localization of the two molecules. In the mature brain, although NG2 and PDGF alpha-receptor are not as well co-localized at the subcellular level as they are in the younger brain, the majority of immunoreactive cells expressed both NG2 and the established oligodendrocyte lineage marker PDGFR-alpha throughout all stages of development (Nishiyama et al., 1996). In the corpus callosum of *Plp-CreERT2 Δ/Δ* individual, we quite interestingly detect an increase in the PDGFR-alpha-positive cells at the site of the lesion, but without a corresponding accumulation of NG2-positive cells. Cells that are NG2-negative and PDGFR-alpha positive have been suggested to be the earliest form of precursors committed to the oligodendrocyte lineage (Nishiyama et al., 1996). Given that the Plp promoter driving production of cre recombinase is activated in the NG2-positive stage, the depletion of the NG2-positive cells could be due to the loss of Notch1. This could lead to a higher rate of proliferation of the earliest oligodendrocyte precursors expressing PDGFR-alpha, explaining the increase in such cells. Consistent with this hypothesis, we also observe an increase in the number of proliferating cells in the corpus callosum of the *Plp-CreERT2 Δ/Δ* brain.

Note also that it has been reported that NG2 staining intensity can vary in different brains and in different areas from the same brain. NG2 expression may be affected by local and generalized CNS environments that vary according to immune system activation, blood-brain barrier changes, or local cytokine production (Chang et al., 2000). The reduction of NG2 immunoreactivity might therefore not reflect the exact number of OPCs , but rather some local differences due to cuprizone.

9 OUTLOOK

9.1 Investigation of other Notch family members

During myelination of the cerebellum, Jagged-1 expression is detected on the granule cells, which are located in the internal granular layer and which project throughout the molecular layer, an area where myelination does not occur. However, axons in the cerebellar white matter do not express Jagged-1 in young animals (Givogri et al., 2002; Stump et al., 2002). In the study of Bongarzone et al., (2002) Notch1 was shown to be expressed by cerebellar OPCs, therefore suggesting that Jagged/Notch signaling was preventing OPCs from differentiating in the molecular layer. I provided some evidence in the second part of my thesis that the loss of Notch1 does not hinder faithfull localization of myelin in various parts of the CNS. To test whether some compensation mechanisms (via Notch2 or Notch3) occur, I propose first to carefully determine the expression pattern of all Notch family members, at the temporal, spatial and cellular level. Then, we can use a novel technology, which provides a relatively simple and rapid method to study gene function. Although quite new, the morpholino antisense oligos have now been used with success in a variety of species, including mice (for review, see (Heasman, 2002)). This would allow to block messenger RNA translation of a single Notch member or even possibly all three Notch proteins in one single cell. Wild-type OPCs can therefore be cultured in vitro and after gene function blockage transplanted into the cerebellum of adult mice. The use of a specific marker (such as GFP or βGal) is critical to follow the cells and determine whether they are able to differentiate and myelinate some normally unmyelinated regions.

9.2 Modifications and repetition of the cuprizone experiment

As mentioned in the discussion, the timing of cuprizone and tamoxifen administration may not have been optimal in the initial remyelination experiment. We are now performing a second set of experiments, in which tamoxifen is administered only close to the end of the cuprizone treatment (Figure 1), with a protocol consisting of injecting tamoxifen twice a day. This protocol leads to a high frequency of recombination (D

Leone, personal communications). By repeating the experiment, we also want to confirm the data obtained in Figure 10 (immunohistochemistry, in situ hybridization and X-gal) and repeat the histological analysis using males only, because an almost significant difference was detected in the level of remyelination in male brains two weeks after the end of cuprizone (data not shown).

At that time of the first experiment we were not aware that the severity of demyelination could vary between defined areas of the corpus callosum of different animals. Therefore, by repeating the experiment and by cutting sagital brain sections (instead of coronal sections as we did for the first experiment), we will also have a better overview of the remyelination process at the cellular level and can achieve a better comparison with the EM data.

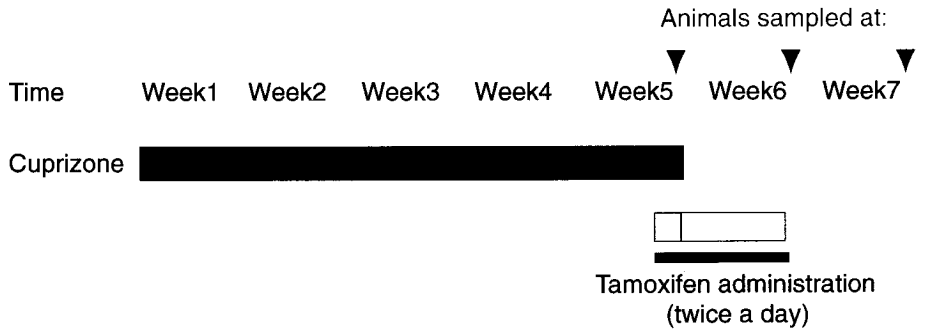


Figure 9-1: second protocol used to assess for a possible role of Notch1 in remyelination.

Complete demyelination is achieved by feeding mice with 0.2% cuprizone for five weeks. To induce recombination, tamoxifen is injected starting two days before the end of the cuprizone treatment and is continued during the first week of remyelination. Tamoxifen is administered twice daily.

9.3 Transplantation of Notch1$^{-/-}$ OPCs in a lesioned brain

As an alternative to study the functional role of Notch1 in the process of remyelination, Notch1$^{-/-}$ OPCs can be cultivated in vitro before being injected in the spinal cord white matter of adult rats. Lesions can be induced by injecting a small volume of ethidium bromide into the dorsal funiculus of the spinal cord. Intrinsic remyelination is prevented by exposure of the spinal cord to X-irradiation (detailed protocol in (Lobsiger et al., 2001)). OPCs are either derived from *CNP-Cre Δ/Δ* newborn brains or lox/lox animals,

where recombination is achieved in vitro after infection with an adenovirus encoding Cre. By doing so, we do not need to induce in vivo recombination with several tamoxifen injections. Therefore, OPCs have ablated Notch1 from the very beginning of the remyelination process, and most importantly, in all cells at the same time. However, this model does not provides the opportunity to modify Notch expression in a demyelinating lesion environment, because X-irradiation has prevented any possibility of endogeneous remyelination capacity. Furthermore, this does not provides the theoretical possibility of modifying Notch signaling at different time-points during lesion development.

10 MATERIAL AND METHODS

10.1 Mice

Mice carrying *LoxP*-flanked *Notch1* alleles have been described previously (Radtke et al., 1999). Recombination between the *LoxP* sites reduces expression of Notch1 protein to undectable levels (Wolfer et al., 2001). Mice carrying the Cre recombinase under the transcriptional control of the *Plp* (line PLCD) and *Cnp* regulatory elements will be described elsewhere. The *ROSA26* mouse reporter line was kindly provided by Dr. P. Soriano (Soriano, 1999). Embryos were generated by timed mating, counting the morning after pairing as E0.5. Procedures for animal experiments were approved by the Veterinary Office of the Canton of Zurich (permit 113/99).

For most experiments, I compared *Cnp-Cre* Δ/Δ or *Plp-Cre* Δ/Δ animals with *lox/lox* littermates as 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.

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

Timed pregnant mothers were euthanized, and embryos were isolated, fixed for one to two hours 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 (50mg/ml, 100µl per animal), then perfused with a 0.9% saline solution followed by 4% ice-cold paraformaldehyde. Brains and spinal cords were isolated, fixed for 24 to 72 hours in the same fixative at 4°C, incubated in 30% sucrose overnight, and embedded in OCT. Twenty or 5 µm frozen sections for embryos or newborn mice, respectively, were thaw-mounted onto Superfrost slides (Mettler) and air-dried.

For the Xgal staining, the sections were incubated overnight in a phosphate-buffered saline solution containing 5mM potassium ferrocyanid, 5mM potassium ferricyanid, 2mM magnesium chloride and 2mM X-gal (Calbiochem).

In situ hybridizations were performed with digoxigenin-labelled 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). Overnight hybridizations were performed with slides in a horizontal position, as described in the protocol from Dr. Verdon Taylor. The PDGFR alpha and PLP/DM20 plasmids were kind gifts from Drs. W. D. Richardson and B. Zalc, respectively (for the preparation of the probes, see table 10.1). Immunohistochemistry was performed overnight at 4°C with antibodies against NeuN (1:100; Chemicon), GFAP (1:500; Accurate), beta-tubulinIII (1:300; Sigma), neurofilament 160 (1:20; Sigma) 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 kindly provided by Drs. N. Baumann, I. R. Griffiths and K.A. Nave, T. M. Jessell, and J. L. Salzer, respectively. Secondary antibody incubations (1:300; Jackson Immunochemicals) were for one hour at RT (room temperature).

Immunohistochemistry in cell culture with antibodies against beta-galactosidase and NG2 was performed slightly differently. Fixation was performed in ice-cold 2% formaldehyde for 10 minutes, and the plates were then blocked in 10% NGS (normal goat serum) /PBS (phosphate buffer saline) for at least 10 minutes at RT. Anti-NG2 antibody (polyclonal, Chemicon) was used at a dilution of 1:300 and incubation was processed at 4°C ON. Following NG2 immunostaining and secondary antibody incubation (1hr at RT with an anti rabbit antibody coupled to cy3), cells were fixed in 3.7% formaldehyde/PBS (Fluka) for 10 minutes at RT and blocked in 10%NGS/0.3% triton. Staining for beta-galactosidase (1:200; polyclonal, Kappel) was then performed at 4°C ON and detected via an anti rabbit antibody coupled to FITC.

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 Axiophot microscope (Zeiss) in conjunction with a ProgRes 3008 (Jenoptik) or Hamamatsu (Hamamatsu Photonics) 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-alpha mRNA or showing perinuclear MAG staining was determined by counting all cells in at least ten sections.

	Sense/Anti sense	Restriction Enzyme	RNA poylmerase	Probe length (bp)
PDGFRα	Sense	BamHI	T3	1637
	Antisense	HindIII	T7	1637
PLP	Sense	XhoI	T7	around 2300
	Antisense	XbaI	T3	around 2300

Table 10-1: Preparation of the RNA probes used for in situ hybridizations.

10.3 Primary cell dissociation

Spinal cords were individually isolated from newborn animals by incubation for 20 min at 37°C in 200 ml DMEM medium (Life Technologies) containing 2 mg/ml collagenase type IV (Worthington Biochemical Corp.), 1.2 mg/ml hyaluronidase type IV-S (Sigma) and 0.3 mg/ml trypsin inhibitor (Sigma). After trituration, dissociated cells were plated onto PDL (poly-D-lysine, Sigma) coated dishes (35 mm; Corning) in Eagle's medium with 10% fetal calf serum (Sera-Tech) and maintained at 37°C in 5% CO2 overnight. These cells were then used for X-gal and O4 staining (Figures 3.6A-D). The carcasses were genotyped by PCR.

Adult brains were quickly removed to DMEM-Hepes (Sigma), forebrain was isolated and major blood vessels carefully peeled from the surface of each cerebral hemisphere. The tissue was transferred to a 60mm petri dish containing 1ml 0.25% trypsin in PBS and was thoroughly chopped with a razor blade. A further 2ml trypsin solution was added and the suspension transferred to a 75cm^2 tissue culture flask. A final 2ml trypsin solution was used to rinse the petri dish and DNase was added to give a final concentration of 50μg/ml. The flask was capped and incubated on a plate shaker at 37°C for fourty-five minutes. Following incubation, the contents of the flask were rinsed into a

15ml tube with DMEM+10% horse serum (HS) to make a final volume of 10ml. After centrifugation, the pellet was resuspended in 2-3ml DMEM+10% HS and the mixture split between two 15ml tubes for thorough trituration through a glass Pasteur pipette. Each tube was then filled with DMEM+10%HS, then allowed to stand for 5 minutes for clumps to settle. Next, the top 13-14ml of each tube was poured through a 40μm filter into a 50ml tube. The filtrate was then centrifuged at 1000 rpm for 5 minutes, the supernatant decanted and the pellet resuspended in 3 ml DMEM+10%HS supplemented with 10ng/ml PDGF-AA. A tenth of these cells were plated onto 35mm poly-D-lysine coated petri dishes (Corning) and maintained at 37°C in 6.5% CO2 for one to three days. These cells were used for immunostaining against NG2 and beta-galactosidase (Figure 7.2).

E12.5 embryos were dissected and a single cell suspension achieved by using the protocol for the isolation of oligodendrocyte precursors as described below. I then plated the cells ON at 37°C onto PDL-coated dishes in DMEM medium containing 10% FCS. These cells were used for XGal staining (Figure 3.3D).

10.4 Oligodendrocyte cell culture

Purified oligodendrocyte precursors were obtained by the method of Milner and ffrench-Constant (Milner and ffrench-Constant, 1994) with minor modifications. During the purification procedure, note the use of DMEM 10% horse serum for mouse cells instead of DMEM 10% fetal calf serum (FCS) used for rat cells. Note also that OPCs were cultured for 2 hours at 37°C in DMEM 10% FCS and then switched to Sato media for 5 or 7 days, without changing medium. 8000-10000 cells were plated per well (Chamber-slide Lab-Tek, life technologies). This method has been reported to yield greater than 95% oligodendroglial cells (oligodendrocytes and oligodendrocyte precursors)(Milner and ffrench-Constant, 1994).

10.5 Southern blot and quantification

Spinal cord was isolated from newborn animals or E12.5 embryos and cells were mechanically dissociated as previously described (Milner and ffrench-Constant, 1994). Southern blotting (5 μg of Eco RI-digested genomic DNA) to a Hybond-N+membrane

(Amersham) was performed essentially as described by Radtke et al. (Radtke et al., 1999), using as probe a 750 bp Bam HI–Eco RI 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).

10.6 Histological analysis

Animals were killed by CO2, then perfused with a 0.9% saline solution followed by 4% ice-cold paraformaldehyde. Brains were isolated, fixed for 12 to 72 hours in the same fixative at 4°C, incubated in 30% sucrose overnight, and embedded in OCT. Twenty µm frozen sections were thaw-mounted onto Superfrost slides (Mettler) and air-dried.
For hematoxylin staining, sections were first incubated for 45 seconds in a hematoxylin solution (Sigma), washed briefly 3 times in bidistilled water, and 30 seconds under tap water. This was followed by a brief wash in 70% ethanol/water and further incubation for 10 seconds in 1% eosin/90% ethanol solution. Finally, the sections were washed twice each in 80%, 95% ethanol/water and 100% ethanol. After incubation in xylol for twice ten minutes, the sections were directly embeded in Eukitt (Electron Microscopy Sciences, USA) and air-dried for 2 hours at RT.

10.7 Induction of demyelination and remyelination

To induce demyelination, we fed 12-13 weeks old mice with a diet of milled mouse chow containing 0.2% cuprizone (Sigma) for up to five weeks. Ten to 15% weight loss was observed, but no mice had to be sacrified. Hydrocephalopathy was observed in two mutant animals, which scould not be used for the experiment due to severe brain damage. During the recovery period, animals regained some weight and appeared to be at a normal activity level.

10.8 Collection of tissues for the analysis of remyelination

The brains of animals used for immunohistochemistry, in situ hybridizations and X-Gal staining were isolated and directly embedded in OCT. Twenty µm frozen sections were thaw-mounted onto Superfrost slides (Mettler) and air-dried. For immunohistochemistry and in situ hybridization experiments the sections were then fixed in 4%

paraformaldehyde/PBS (10mn, RT), and for the XGal staining in 0.3% glutaraldehyde/ PBS (Merck) (10mn, 4°C). The sections were then analyzed as described in section 10.2. Animals used for morphological analysis were perfused with 4% glutaraldehyde/0.008% calcium chloride/75mM sodium hydroxide/0.1M sodium dihydrogenorthophosphate buffer. The brains were then dissected and postfixed in the same fixative for several days. Further processing of the tissue has been carried out by our collaborators in Cambridge, UK (Dr. R. Franklin and M. Stidworthy).

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