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Notch1 and Jagged1 are expressed after CNS demyelination, but are not a major rate-determining factor during remyelination

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Summary

The reasons for the eventual failure of repair mechanisms in multiple sclerosis are unknown. The presence of precursor and immature oligodendrocytes in some non-repairing lesions suggests a mechanism in which these cells either receive insufficient differentiation signals or are exposed to differentiation inhibitors. Jagged signalling via Notch receptors on oligodendrocyte precursor cells (OPCs) inhibits their differentiation during development and the finding that both notch and jagged are expressed in multiple sclerosis lesions has fostered the view that this signalling pathway may explain remyelination failure. In this study, we show that Notch1 is expressed on adult OPCs and that there are multiple cellular sources of its ligand Jagged1 in a rodent model of remyelination. However, despite their expression, the lesions undergo complete remyelination. To establish whether Notch-jagged signalling regulates the rate of remyelination we compared their expression profiles in young animals with those in older animals, where remyelination occurs more slowly, but could find no correlation between expression and remyelination rate. Finally we found that OPC-targeted Notch1 ablation in cuprizone-treated *Plp-creER* Notch1$^{lox/lox}$ transgenic mice yielded no significant differences in remyelination parameters between knock-out and control mice. Thus, in contrast to developmental myelination, adult expression of Notch1 and Jagged1 neither prevents nor plays a major rate-determining role in remyelination. More generally, the re-expression of developmentally expressed genes following injury in the adult does not *per se* imply similar function.

Keywords: remyelination; notch; jagged; multiple sclerosis; oligodendrocyte

Abbreviations: EB = ethidium bromide; ER = estrogen receptor; GFAP = glial fibrillary acidic protein; Hh3 = histone h3; OPC = oligodendrocyte progenitor cell; Plp = proteolipid protein; PDGFαR = platelet-derived growth factor α receptor; RT–PCR = reverse transcription–polymerase chain reaction

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Introduction

Remyelination in the adult CNS involves a complex interplay of multiple signalling events, which, if inappropriately coordinated, lead to impairment in demyelinating disease such as multiple sclerosis (Franklin, 2002). Some lesions in multiple sclerosis patients contain oligodendrocyte progenitor cells (OPCs) (Scolding *et al*., 1998; Wolswijk, 1998; Chang *et al*., 2000; Maeda *et al*., 2001) or premyelinating oligodendrocytes (Chang *et al*., 2002), but remain chronically demyelinated without these cells differentiating into stable myelinating oligodendrocytes. Identifying factors that regulate differentiation is crucial to developing strategies for enhancing remyelination in these lesions. Notch signalling has emerged as an inhibitor of OPC differentiation that may contribute to remyelination failure (Wang *et al*., 1998; Genoud *et al*., 2002; Givogri *et al*., 2002; John *et al*., 2002).

The Notch family comprises conserved transmembrane receptors interacting with membrane-bound ligands in the Delta/Serrate/Jagged families (Lardelli *et al*., 1995; Weinmaster, 1997; Lewis, 1998; Artavanis-Tsakonas *et al*., 1999). Signalling is involved in developmental (de la Pompa *et al*., 1997; Vargesson *et al*., 1998; Favier *et al*., 2000; Singh *et al*., 2000; Zine *et al*., 2000; Gridley, 2001; Pear and Radtke, 2003), and

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adult regenerative processes (Lindner *et al*., 2001; Conboy *et al*., 2003; Mitsiadis *et al*., 2003). During CNS myelination, axonally expressed Jagged1 inhibits differentiation of OPCs via Notch1 receptors and its downstream effector Hes5 (Wang *et al*., 1998; Kondo and Raff, 2000), and plays a role in controlling the timing of OPC differentiation and myelination (Genoud *et al*., 2002; Givogri *et al*., 2002). It is proposed that this mechanism accounts for the failure of differentiation of OPCs in chronic multiple sclerosis lesions (John *et al*., 2002).

To understand the functional role of the Notch1 pathway in remyelination we have taken advantage of the contribution that delayed OPC differentiation makes to age-associated delay in remyelination (Sim *et al*., 2000). We hypothesized that Jagged1 expression by demyelinated axons may be prolonged in older animals, delaying differentiation of Notch1-expressing OPCs. To test this, we investigated expression patterns of Notch and its ligands during remyelination in young and old adult rats, exploiting the trigeminal system, where changes in neuronal gene expression in the trigeminal ganglion can be correlated with focal demyelination in the trigeminal tract (Fig. 1).

In further experiments, we used *Plp-creER* Notch1$^{lox/lox}$ transgenic mice to investigate remyelination following cuprizone intoxication. This overcame the phenotypic lethality of null homozygotes (Swiatek *et al*., 1994; Huppert *et al*., 2000) enabling the specific targeting of Notch ablation to OPC lineage cells during adult remyelination.

Materials and methods

Creation of demyelinating lesions

Procedures were undertaken in accordance with legislation on animal use in host institutions. Stereotaxic ethidium bromide (EB) injection was used to create focal areas of primary demyelination in the trigeminal tract of adult female Sprague–Dawley rats (Fig. 1A and B) (Shields *et al*., 1999; Woodruff and Franklin, 1999). Young (aged 8–10 weeks) and old (~9-month ex-breeder) rats were used to generate rapidly or slowly remyelinating lesions, respectively (Shields *et al*., 1999; Sim *et al*., 2002*b*). Young adult rats injected with saline served as controls.

Tissue processing

Animals were killed at 10 ($n = 5$ young, 4 old), 21 ($n = 7$ young, 5 old), 24 ($n = 5$ old animals only), 28 ($n = 5$ young, 4 old) and 40 ($n = 2$ young, 4 old) days after lesion induction, together with saline controls ($n = 2$ or 3 in each group). Animals were killed by pentobarbitone injection. Brains were removed, the separated hindbrain snap-frozen by immersion in isopentane at −30°C, and stored at −70°C. Transverse sections (12 μm) were cut by cryostat and thaw-mounted onto poly-L-lysine-coated slides. Air-dried sections were fixed for 5 min in cold buffered 4% paraformaldehyde, rinsed twice in PBS for 1 min, dehydrated in 70% ethanol for 5 min, then stored at 4°C in 96% ethanol (*in situ* hybridization), or at −70°C in airtight boxes with desiccant (immunolabelling).

In situ *hybridization*

Digoxigenin (DIG)-labelled riboprobes were made from linearized plasmid cDNA templates (gifts from Professor G. Weinmaster) for Notch1 (SN6-7; Weinmaster *et al*., 1991), Notch2 (H10-6; Weinmaster *et al*., 1992), Notch3 (5′PCR3′; Lindsell *et al*., 1995) and Jagged1 (SN3ED; Lindsell *et al*., 1995). *In situ* hybridization was performed as described (Fruttiger *et al*., 1999). RNA hybrids were visualized immunohistochemically with alkaline phosphatase-conjugated anti-DIG antibody (Roche Diagnostics). 5% v/v polyvinyl alcohol was added to the final colour reaction to increase sensitivity. Anti-sense and sense probes were run under standard conditions on both experimental and control tissues.

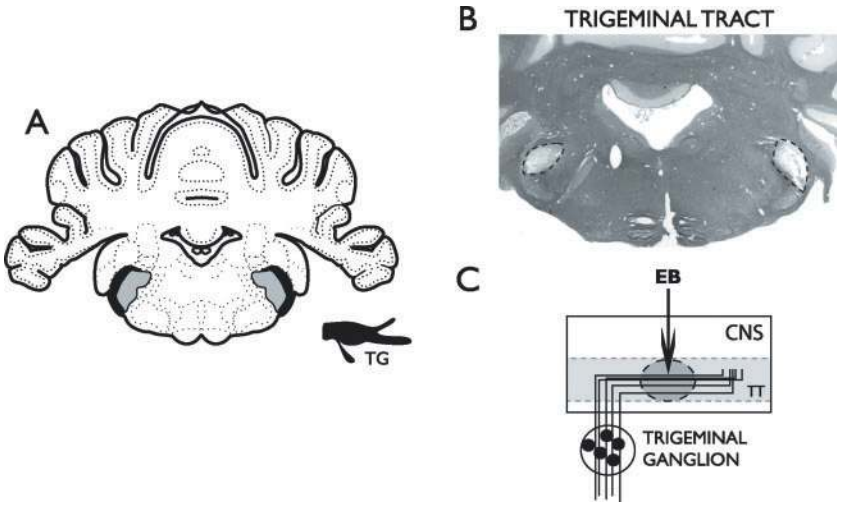

Fig. 1 (**A**) The trigeminal tract lesion model allows histological assessment of remyelinating lesions in the trigeminal tract (black area lateral to grey area representing the trigeminal nucleus), and changes in gene expression in the cell bodies of the demyelinated axons in the trigeminal ganglion (TG). (**B**) Bilateral demyelinating lesions were generated by injection of EB into the trigeminal tract (outlined by interrupted lines). (**C**) Axons in the trigeminal tract (TT) have their neuronal cell bodies exclusively in the trigeminal ganglion enabling clear localization and sampling.

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Quantification

Notch1-positive cells were counted by an observer, blinded to the animals' identities, using a Nikon E600 microscope at × 200 magnification. Four to ten transverse sections from the centre of each trigeminal tract lesion were sampled. Mean lesion areas were measured from three adjacent solochrome cyanin-stained serial sections using MCID 4.0 image analysis software. MCID 4.0 was also used to count cell nuclei within the demyelinated area. A mean density (cells/mm^2) was calculated for each animal based on measurement of both lesions (where bilateral), and then individual animal means were used to calculate group means ± SEM.

Immunolabelling

For Notch1 immunolabelling, polyclonal rabbit anti-rat antiserum against a 120 kDa band corresponding to the extracellular and transmembrane domains of Notch1 (a gift from Dr V. Taylor) was used. Detection utilized tyramide signal amplification (TSA Biotin System, NEN Life Science Products). Following quenching and blocking, sections were incubated with Notch1 antibody at 4°C overnight [1 : 3000 in Tris/sodium chloride/blocking agent (TNB) buffer]. Primary antibody was detected with HRP (horseradish peroxidase)-conjugated goat anti-rabbit antibody (DAKO; 1 : 100 in TNB), and signal amplified using biotinyl tyramide reagent (1 : 50 in amplification diluent) for 10 min at room temperature. Biotinylated tyramide was detected using streptavidin–FITC (fluorescein-isothiocyanate) 1 : 100 in TNB (1 h at room temperature). Notch1 labelling using this antibody and Notch1 mRNA detection by *in situ* hybridization using riboprobe SN6-7 localized similarly in serial sections of foetal rat brain (Fig. 2A and E).

For Jagged1 immunolabelling, cryostat sections were pre-treated with sodium dodecyl sulphate (SDS) for antigen retrieval (Brown *et al*., 1996; Robinson and Vandre, 2001). Sections were rehydrated, permeabilized and treated with 0.5% SDS for 5 min, before blocking and incubation with anti-Jagged1 antibody (4°C overnight, Santa Cruz, sc-6011; 1 : 100). Bound antibody was detected using donkey-anti-goat IgG-FITC antibody (Jackson Immunochemicals; 1 : 100). Specificity was confirmed by pre-absorption with Jagged-specific peptides (Fig. 5A–C), which abolished labelling of SDS-treated foetal tissues and adult cerebellum. Jagged1 antibody labelling and mRNA detection by *in situ* hybridization using riboprobe SN3ED localized similarly in ectodermal placodes in serial sections of foetal rat tissue (Fig. 5D and E) (Shawber *et al*., 1996).

For dual labelling experiments, Jagged1 or Notch1 labelling was followed by a range of primary antibodies: NG-2 (AB5320) 1 : 200 Chemicon; glial fibrillary acidic protein (GFAP) (Z0334) 1 : 200 DAKO; neurofilament (SMI31) 1 : 3000 Sternberger Monoclonals Inc.; P0 1 : 100 (a gift from Dr J. Archelos); ED-1 (MAB1435) 1 : 400, Chemicon; CD11b (Ox42, MCA275G) 1 : 100, Serotec. TRITC (tetrarhodaminine isothiocyanate)-conjugated donkey anti-rabbit or goat anti-mouse antibodies (Jackson; 1 : 100) were used. Hoechst 33342 (Molecular Probes) stained nuclei. Sections were imaged conventionally (Nikon E600 microscope, Coolpix 9000 digital camera) and by confocal microscopy (Leica confocal with TCS NT software), and images processed using Adobe Photoshop 6.0.

RNA extraction

Ganglia were removed, snap-frozen immediately in isopentane and stored at −70°C. Total RNA was extracted with TRIzol Reagent (Gibco BRL). Ganglia were homogenized with a glass homogenizer (Jencon Uniform) or minipestle (Kontes Pellet Pestle Motor). Extractions were stored at −70°C.

Reverse transcription–polymerase chain reaction (RT–PCR)

RNA was pretreated with amplification grade DNase1 (Gibco BRL), and reverse transcribed using the Superscript First-Strand Synthesis System (Gibco BRL). Thirty-five cycles of PCR were used to detect Notch ligand mRNA using Platinum Taq DNA Polymerase (Gibco BRL) and previously published or novel primers for rat Jagged1 (Wang *et al*., 1998), Jagged2, Delta1 and Delta3. Cyclophilin (Semple-Rowland *et al*., 1995) was to control for RNA integrity using 25 cycles of PCR. Products were extracted (Concert Gel Extraction System, Gibco BRL) and sequenced to confirm identity with rat Genbank sequences (Jagged1 NM_019147, Jagged2 U70050, Delta1 NM_032063, Delta3 AF084576).

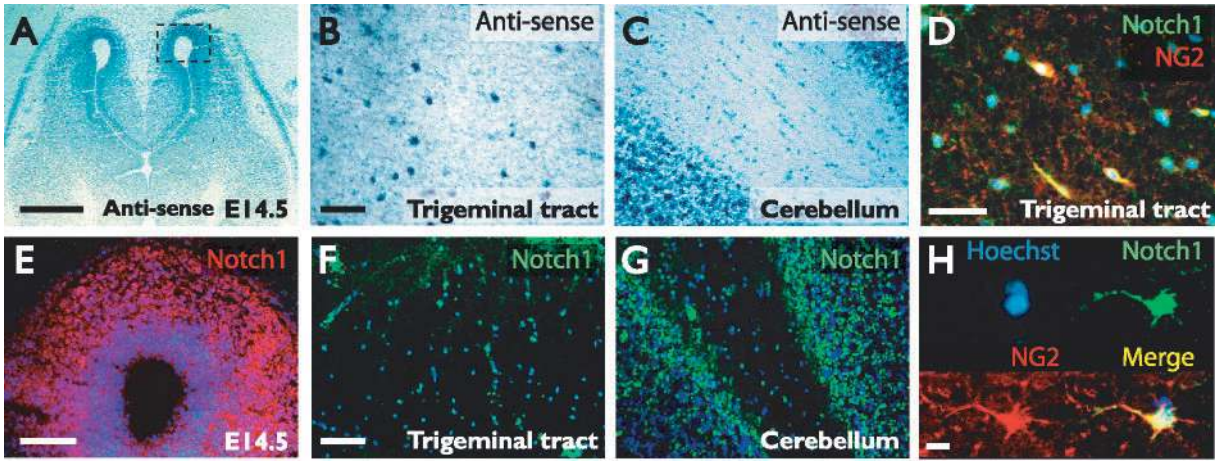

Fig. 2 Notch1 expression in the normal foetal and adult CNS. (**A** and **E**) Foetal brain (box in **A** denotes region depicted in **E**). (**B**, **D**, **F** and **H**) Normal trigeminal tract. (**C** and **G**) Granule layer of cerebellum and cerebellar white matter. Similar patterns of expression were observed using either anti-Notch1 polyclonal antibody (**E**–**G**) or anti-sense riboprobe for Notch1 (**A**–**C**). Dual labelling with anti-NG-2 antibody (**D** and **H**) identified Notch1-positive/NG-2-positive OPC in the normal adult trigeminal tract. Images are shown with Hoechst 33342 nuclear label. Scale bars: **A** = 1 mm; **B** (same as for **C**) and **F** (same as for **G**) = 100 μm; **D** = 50 μm; **E** = 200 μm; **H** = 8 μm.

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Transgenic mice

Plp-creER mice (Leone *et al*., 2003) were used to generate *Plp-creER* Notch1$^{lox/lox}$ mice (Genoud *et al*., 2002). PLP-expressing oligodendrocyte lineage cells in these mice excise floxed Notch alleles in the presence of tamoxifen. 'Knock-out' mice were compared with 'control' mice containing floxed Notch but lacking the *Plp-creER* construct. Additional 'knock-out' and 'heterozygote' (Notch$^{lox/wt}$) R26R reporter mice (Soriano, 1999) enabled the mapping of *Plp* expression in the presence or absence of a normal Notch1 allele.

Cuprizone administration and recombination

Two independent experiments were performed. Mice were fed 0.2% cuprizone in-feed for 5 weeks, and groups sacrificed at the end of week 5, then 7 and 14 days later, as described (Stidworthy *et al*., 2003). Two equivalent tamoxifen protocols were used (Leone *et al*., 2003). In the first experiment, 1 mg of tamoxifen was injected on 15 consecutive days, from the end of the third week of cuprizone (Stidworthy *et al*., 2003). In the second, 1 mg of tamoxifen was injected twice daily for 5 days from the day after cuprizone withdrawal.

Analysis of remyelination

Following perfusion with 4% glutaraldehyde, analysis of corpus callosum remyelination used an identical protocol to that described previously (Stidworthy *et al*., 2003).

Histological analysis of mouse tissues

X-gal histochemistry, PDGFαR (platelet-derived growth factor α receptor) *in situ* hybridization, immunofluorescence and TUNEL staining used published protocols (Genoud *et al.*, 2002). Antibodies against NG-2 and phosphorylated histone H3 (Upstate Biotechnology; 1 : 100) were used to label OPC and proliferating cells. Apoptosis was analysed by TUNEL staining using biotin-labelled UTP and FITC-conjugated streptavidin (Roche Diagnostics). NG-2 labelling intensity was measured from images of the body and splenium of the corpus callosum using NIH Image 1.62 software (Genoud et al., 2002). TUNEL-positive, Hh3-positive and PDGFαR-positive cells were counted within the sagittally sectioned midline corpus callosum.

Results

Notch1 mRNA and protein are expressed in normal adult rat cerebellum and hindbrain

Having first confirmed specific binding of both Notch1 riboprobe and antibody using embryonic rat brain (Fig. 2A and E) (Lindsell *et al*., 1996; Shawber *et al*., 1996) we examined Notch1 expression in normal adult CNS. Notch1 mRNA and protein were detected in small round glial cells throughout axon tracts of the white matter, both singly and in chains, suggestive of interfascicular oligodendrocytes (Fig. 2B, C, F and G). Some cells were confirmed as NG-2+ OPCs in the trigeminal tract (Fig. 2D and H). Purkinje cells, cells in the granular layer of the cerebellum, and the majority of neurons in brainstem and cerebellar nuclei also expressed Notch1 mRNA and protein. Signal was detected in the choroid plexus, *pia mater* of the meninges and a minor population of cells surrounding small blood vessels. The sense probe gave only scant deposits in cells in the granular layer of the cerebellum.

Notch1 mRNA and protein are expressed in remyelinating lesions of young and old rats

The reduced rate of remyelination in older animals is due in part to a delay in differentiation of recruited OPCs (Sim *et al*., 2002*b*). We hypothesized that this may reflect differences in the expression of Notch1, addressing this by comparing Notch1 expression patterns following EB-induced demyelination in the trigeminal tract of young adult and old adult female rats.

Compared with normal white matter, increases in numbers of Notch1 mRNA+ cells were observed at all time-points from 10 to 40 days after lesion induction, in both young and old animals (Fig. 3A and E–L). At day 10, positive cells were located predominantly around the lesion margins (Fig. 3E and I), but from 20 days were distributed throughout the remyelinating area. *In situ* hybridization with Notch2 and Notch3 probes labelled only a few scattered cells in the lesions (Fig. 3C and D). There was no labelling with sense riboprobes (Fig. 3B).

Protein distribution corresponded closely with that of mRNA expression (Fig. 3M–P). Cells expressing Notch1 protein accumulated around the lesion periphery at 10 days but was subsequently found towards the lesion centre. By 40 days the predominant Notch1+ component comprised a dense cluster of cells in the central portion of the lesion.

Notch1 is expressed by adult oligodendrocyte progenitors, subpopulations of oligodendrocytes, Schwann cells, macrophages and astrocytes within remyelinating lesions

Dual labelling studies using antibodies directed against NG-2 (an OPC marker), GFAP (an astrocyte marker), ED-1 (a microglia/macrophage marker), RIP (an oligodendrocyte marker) and P0 (a myelinating Schwann cell marker) were performed to identify Notch1+ cells. Despite the hypercellularity of the lesions, making quantification of individually labelled cells difficult, this revealed that the majority of cells in the lesions were Notch1+ /NG-2+ (Fig. 4A). Very few NG-2 cells did not also express Notch1.

A population of Notch1+ /RIP+ cells was found, initially located around the lesion periphery but more centrally from day 20 (Fig. 4D). At later survival times most RIP+ cells at the periphery of the lesion did not express Notch1. We have shown previously that, in the EB model, oligodendrocyte remyelination begins at the edge of the lesion and progresses towards the centre (Woodruff and Franklin, 1999; Sim *et al*., 2000). The relationship we found between Notch1 and RIP immunoreactivity suggested that Notch1 was expressed by RIP+ oligodendrocytes newly generated by OPCs, but subsequently lost from mature myelinating cells.

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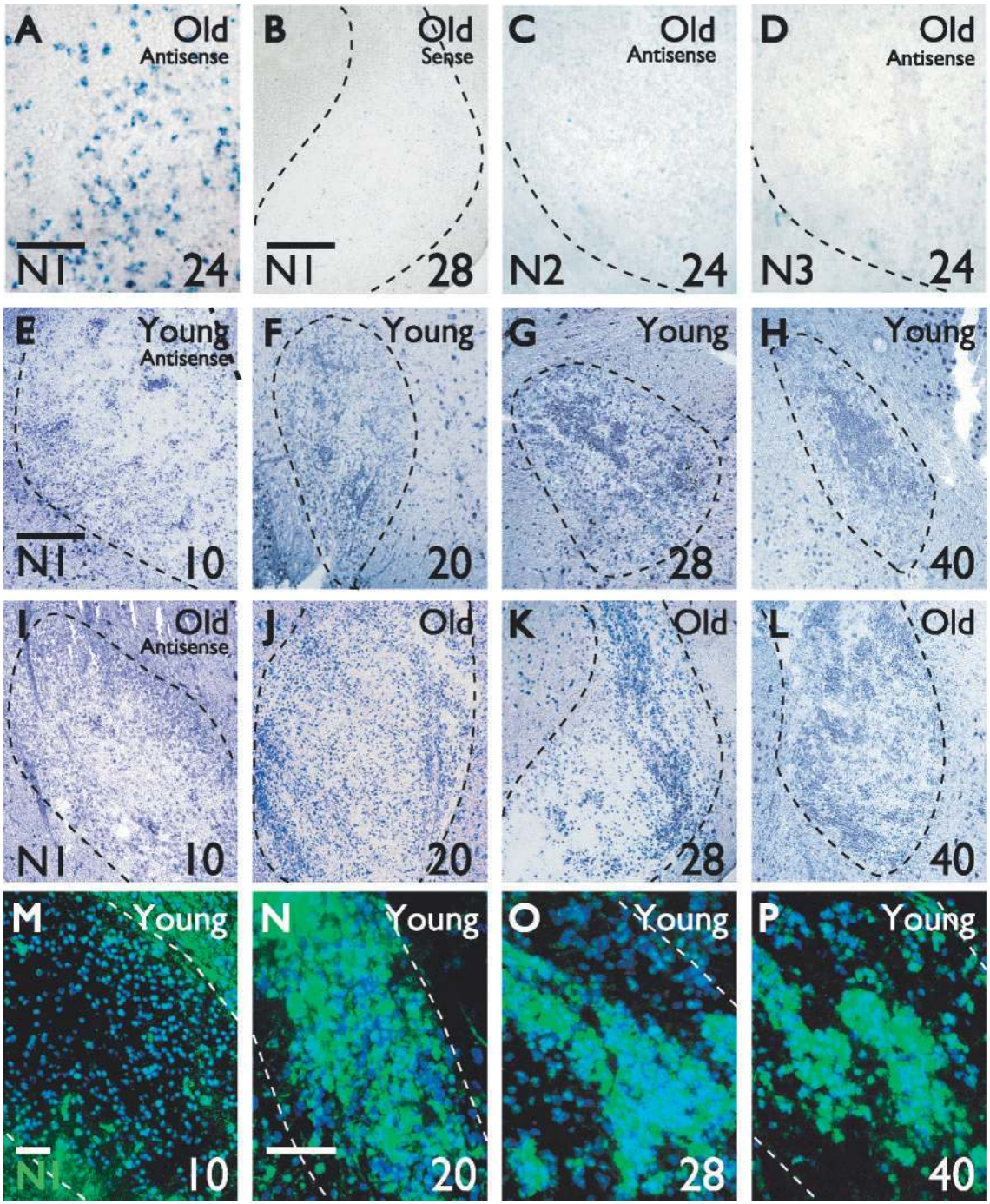

Fig. 3 Notch1 mRNA and protein expression in remyelinating white matter lesions of young and old adult rats. (**A**, **E–L**) *In situ* hybridization using anti-sense riboprobe for Notch1 mRNA on coronal cryostat sections of lesions from an old rat 24 days after lesioning (**A**), and from young (**E–H**) and old (**I–L**) adult rats at 10, 20, 28 and 40 days after lesioning. (**C** and **D**) *In situ* hybridization for Notch2 (N2) and Notch3 (N3) on coronal cryostat sections of lesions from old adult rats 24 days after lesioning. (**B**) *In situ* hybridization using sense riboprobe for Notch1 of lesion from old adult rat at 28 days. (**M–P**) Immunolabelling using anti-Notch1 polyclonal antibody with Hoechst 33342 nuclear label on coronal cryostat sections of remyelinating lesions from young adult rats at 10, 20, 28 and 40 days after lesioning. Scale bars: **A** = 50 μm; **B** and **E–L** = 200 μm; **C** and **D** and **M–P** = 100 μm. Interrupted lines indicate lesion margins.

At day 20, Notch1 mRNA+ cells were found as clusters in the centre of the lesion in both young and old animals. At later time-points these clusters were larger in size, eventually forming centrally located areas of intense signal (Fig. 3G–H and K–L). This pattern of expression closely resembled the behaviour of myelinating Schwann cells previously described in this model (Woodruff and Franklin, 1999; Sim *et al*., 2002*a*). Immunolabelling revealed these areas to be both Notch1+

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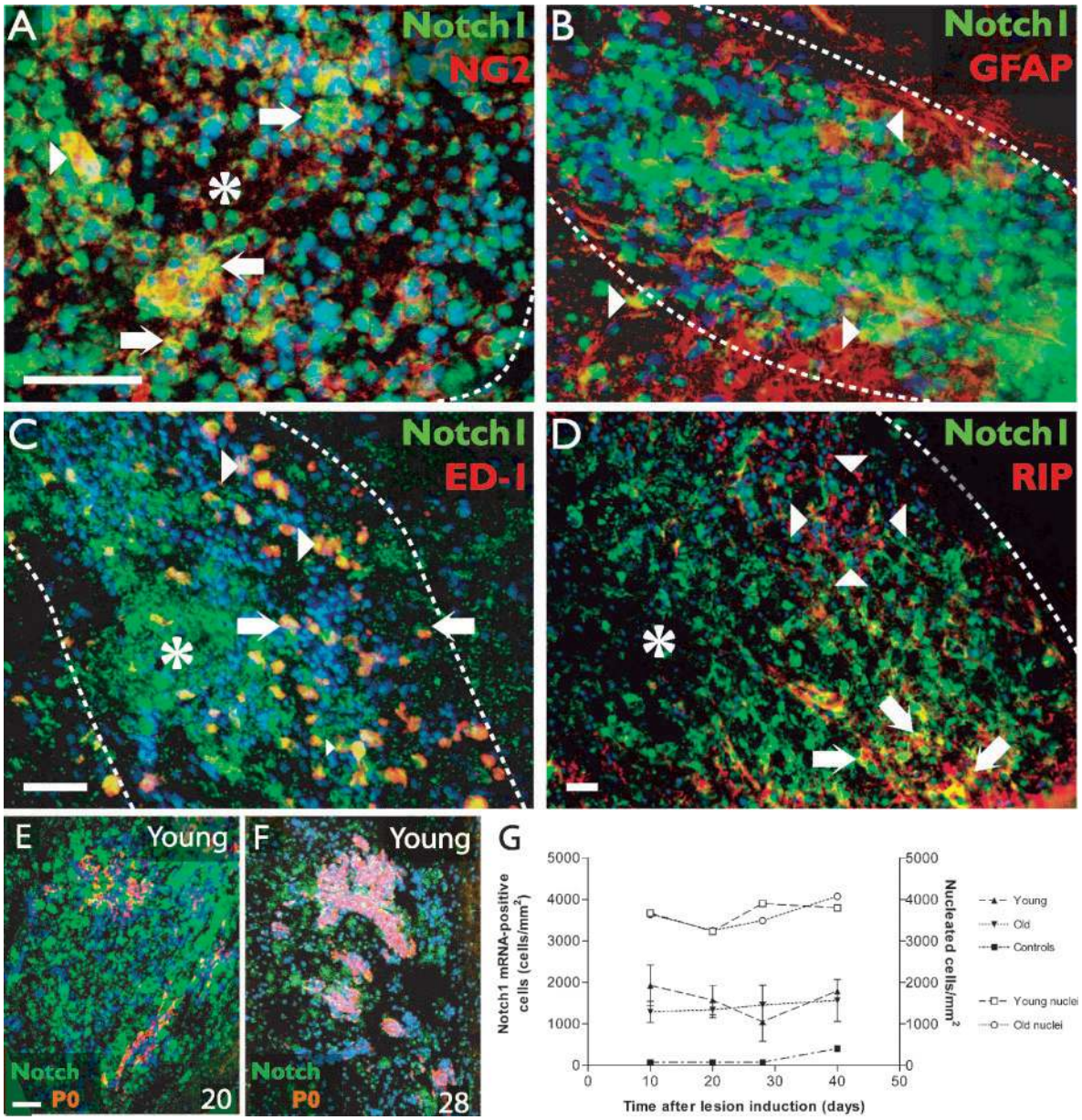

Fig. 4 (**A**–**F**) Notch1 protein labelling using anti-Notch1 polyclonal antibody (FITC secondary) on coronal cryostat sections of remyelinating lesions (interrupted lines indicate the lesion margins, asterisks denote the later remyelinating core) with: (**A**) anti-NG-2, arrows indicate clumps of NG-2+/Notch1+ progenitors, arrowhead indicates capillary endothelial labelling; (**B**) anti-GFAP, arrowheads indicate GFAP+/Notch1+ astrocytes at lesion margins and around blood vessels; (**C**) anti-ED-1, arrows delineate the remyelinating corona containing ED-1+/Notch1+ macrophages indicated by arrowheads; (**D**) anti-RIP, arrows and arrowheads delineate areas of RIP+/Notch1+ or RIP-/Notch1+ oligodendrocyte lineage cells, respectively; (**E** and **F**) anti-P0: Notch1+/P0- cells predominate across the lesion at 20 days (**E**), whilst Notch1+/P0+ Schwann cells clump in the lesion centres by 28 days (**F**). Merged images are shown with Hoechst 33342 nuclear label. Lesions illustrated are 20 (**B, D** and **E**), 28 (**A** and **E**) or 40 (**C**) days after induction in young (**B**–**F**) or old (**A**) adult rats. (**E**) and (**F**) are serial sections corresponding to Fig. 3F and G, respectively. Scale bars: **A**–**F** = 100 μm. (**G**) Quantification of Notch1 mRNA expression during remyelination in young and old rats. Control values were derived from saline-injected young animals. Values are expressed as group means ±SEM at each time-point in each age group. Nuclear counts indicate the total density of nucleated cells within the lesion area.

and P0+ and were presumably myelinating Schwann cells (Fig. 4E and F).

After the Notch1+/NG-2+ and Notch1+/P0+ cells, the next most numerous dual-labelled cells were those expressing Notch1 and ED-1 (Fig. 4C). These cells were distributed across the lesions at earlier time-points, and later found mainly around the edges of lesions. A small number of Notch1+/GFAP+ astrocyte processes were observed at lesion margins and surrounding blood vessels (Fig. 4B).

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The lesional density of Notch1-expressing cells is not significantly different between young and old animals

We next assessed whether there were quantitative differences in the patterns of Notch1 expression between young and old animals. Lesion area and total nucleated cell number were measured from solochrome cyanin-stained adjacent serial sections using an automated image analysis system. Notch1 mRNA+ cells were numerous at all time-points examined between 10 and 40 days after lesion generation (Fig. 4G). Although the identity of the cells expressing Notch1 mRNA was not established, the densities of Notch1 expressing cells were not significantly different between the two groups at any time-point despite differences in the rate of remyelination (Sim *et al*., 2002*b*).

mRNAs for the Notch ligands Jagged1 and Jagged2, but not Delta1 or Delta3, are expressed by trigeminal ganglia with normal, demyelinated and remyelinating trigeminal tracts

The axons in the trigeminal tract have their neuronal cell bodies in an accessible peripheral trigeminal ganglion, and a single demyelinating lesion will affect axons located only within that ganglion (Fig. 1C). Consequently, by generating demyelinating lesions within the tract and collecting the ipsilateral trigeminal ganglion, we could be certain to sample the relevant neuronal population. RT–PCR analysis of homogenates of trigeminal ganglia from histologically confirmed demyelinated tracts, as well as saline-injected and sham-operated controls, and normal young adult rats revealed constitutive expression of Jagged1 and Jagged2 mRNA within neurons extending axons into the trigeminal tract (Fig. 5, table). No Delta1 or Delta3 mRNA was detected at any time. No significant differences were apparent between young and old animals, or between different survival times. Only half of the ganglia were positive 10 days after lesion induction, but this was not a statistically significant difference by Fisher's exact test when compared with controls.

Patterns of Jagged1 mRNA and protein expressed in normal adult cerebellum and hindbrain are complementary to those of Notch1

To establish whether Notch ligands were expressed in adult brain regions in which we had examined Notch1 expression we undertook *in situ* hybridization using a Jagged1-specific antisense riboprobe. First, we verified the specificity of the probe in embryonic rat brain against previously published data (Fig. 5E) (Lindsell *et al*., 1995, 1996; Shawber *et al*., 1996). In the adult, Jagged1 mRNA was detected in cells in the molecular and granular layers of the cerebellum, in some Purkinje cells and neurons in the brainstem nuclei (not shown) and interspersed through white matter tracts (Fig. 5G, I and K).

Using Jagged1-specific antibodies, verified using blocking peptides (Fig. 5A–C), Jagged1+ cells were observed throughout myelinated axon tracts, both as small NG-2+ cells (Fig. 5 J and M) and in rows of cells likely to be interfascicular oligodendrocytes (Fig. 5F). In non-myelinated areas, such as the molecular layer of the cerebellum (Fig. 5A and H) and major nuclei of the brainstem and cerebellum (not shown), Jagged1 expression was widespread. In myelinated tracts, Jagged1 expression consistent with its expression by myelinated axons was also present, but the intensity was considerably less than that in non-myelinated areas (Fig. 5A, J and L). Intense Jagged1 labelling was also observed in a subset of cells in the cerebellar granule layer (Fig. 5B and F) consistent with published RT–PCR data (Solecki *et al*., 2001), as well as in Purkinje cells, the choroid plexus, the endothelia of many blood vessels, and the *pia mater*.

Jagged1 mRNA and protein are expressed predominantly by cells recruited to remyelinating lesions rather than by demyelinated axons

Within areas of demyelination, *in situ* hybridization revealed a Jagged1 mRNA labelling pattern similar to that observed for Notch1 (Fig. 6A–G). At 10 days, expression was predominantly in cells around the periphery of remyelinating lesions. At later time-points, Jagged1 expressing cells were distributed across the lesion, becoming concentrated centrally at 40 days. There were no clear differences between young and old animals. Sense probes did not give a signal (Fig. 6H).

Immunohistochemistry revealed widespread, albeit low level, expression of Jagged1 protein within remyelinating lesions at all time-points in both young and old rats (Fig. 6I–P). The intensity of labelling was initially diminished when compared with the unlesioned tract, particularly at the earliest survival (day 10) and in the centre of the lesions. At days 10 and 20 there was an increase in Jagged1+ cells at the edge of the lesion and, at later survival times, with greater abundance towards the lesion centre. Dual labelling studies with antibodies against NG-2, GFAP, ED-1, Ox42 (CD11b, a microglia/macrophage marker), P0 and phosphorylated neurofilament (present within axons) indicated that many Jagged1+ cells were also NG-2+ (Fig. 6K and N). Jagged1+/GFAP+ hypertrophic astrocyte processes were observed surrounding blood vessels and towards the lesion edge (Fig. 6L and O). Small numbers of cells expressed both ED-1 and Jagged1 (Fig. 6P). This may have resulted from the ingestion of Jagged1-labelled debris by macrophages. To resolve this, we also labelled cells with the membrane-associated macrophage marker Ox42, and were able to demonstrate cellular colocalization of Ox42 and Jagged1 by confocal microscopy (Fig. 6M). A further subpopulation of cells labelled with both Jagged1 and P0, indicating that myelinating Schwann cells also express Jagged1 (Fig. 6I). The intensity of Jagged1 labelling of axon profiles was reduced in lesions compared with the normal tract (Fig. 6J). This reduction in

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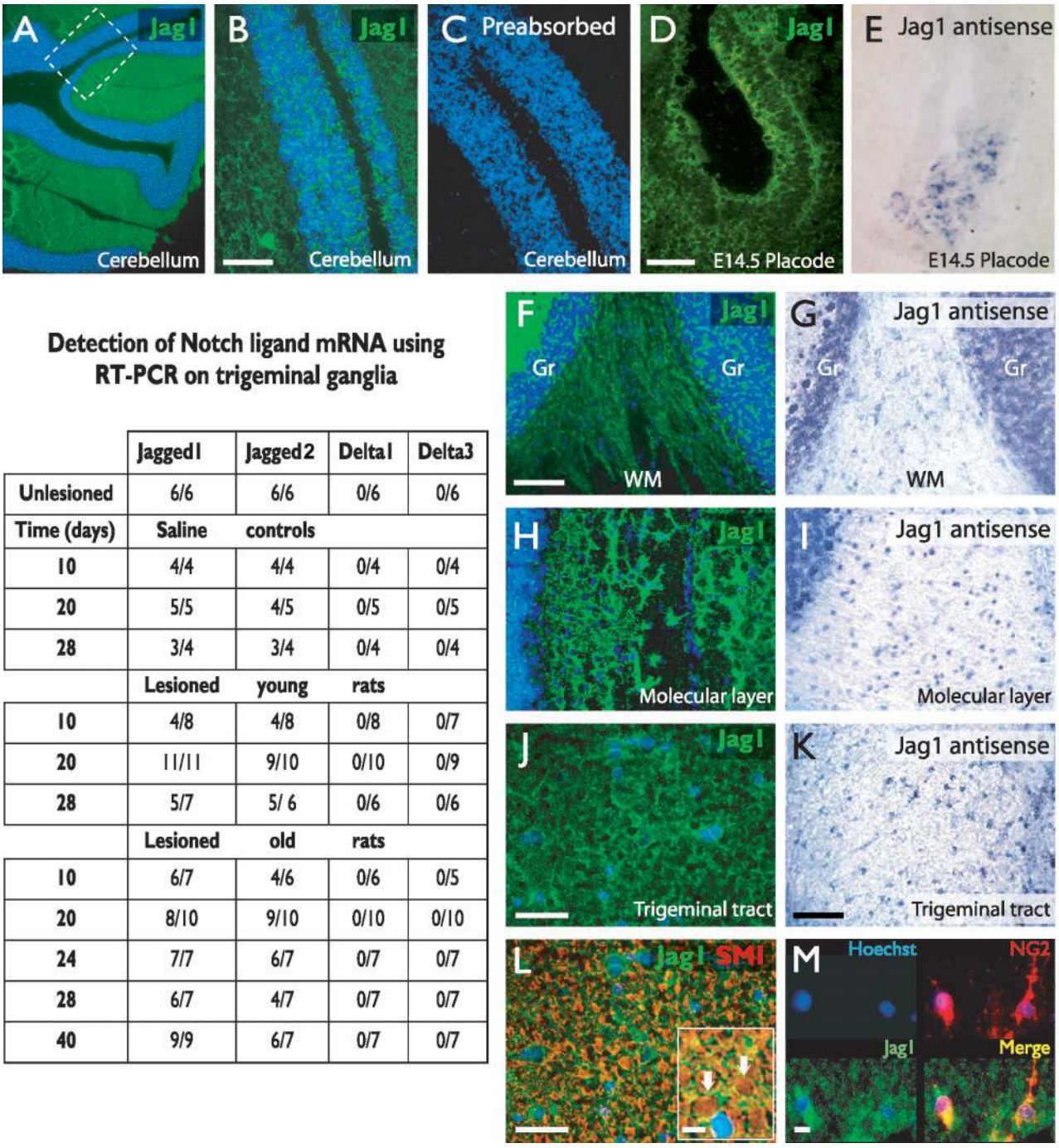

Detection of Notch ligand mRNA using RT-PCR on trigeminal ganglia

	Jagged1	Jagged2	Delta1	Delta3
Unlesioned	6/6	6/6	0/6	0/6
Time (days)	Saline	controls		
10	4/4	4/4	0/4	0/4
20	5/5	4/5	0/5	0/5
28	3/4	3/4	0/4	0/4
	Lesioned	young	rats	
10	4/8	4/8	0/8	0/7
20	11/11	9/10	0/10	0/9
28	5/7	5/ 6	0/6	0/6
	Lesioned	old	rats	
10	6/7	4/6	0/6	0/5
20	8/10	9/10	0/10	0/10
24	7/7	6/7	0/7	0/7
28	6/7	4/7	0/7	0/7
40	9/9	6/7	0/7	0/7

Fig. 5 Jagged1 and Jagged2, but not Delta1 and Delta3, mRNA are expressed in trigeminal ganglion neurons constitutively and during remyelination. **Table**: RT-PCR for Notch ligand mRNA on trigeminal ganglia derived from histologically confirmed demyelinated trigeminal tracts. Each value represents a single ganglion (derived from 3 normal or ⩾4 lesioned animals). The reductions in Jagged1 and 2 seen at 10 days in both groups were not statistically significant when compared with controls by Fisher's exact test. (**A**–**M**) Identical patterns of expression were observed using anti-Jagged1 polyclonal antibody (**A**–**D**, **F**, **H** and **J**) and anti-sense riboprobe for Jagged1 (**E**, **G**, **I** and **K**) on coronal cryostat sections of foetal or adult CNS. (**A**–**C**) Expression detected in the adult cerebellum (**A**) and inset (**B**) was abolished by preabsorption of Jagged1 antibody with a 5-fold excess of specific peptide (**C**). (**D** and **E**) Patterns of Jagged1 protein (**D**) and mRNA expression (**E**) corresponded in serial sections of foetal otic placode tissue. (**F**–**K**) Corresponding patterns of protein and mRNA expression were also found in cerebellar white matter and granular layers (**F** and **G**), cerebellar molecular layer (**H** and **I**), and normal trigeminal tract (**J** and **K**). (**L**) Large diameter myelinated fibres in the trigeminal tract, labelled with anti-phosphorylated neurofilament antibodies expressed Jagged1 on the axoplasmic surface (arrows). (**M**) Jagged1 antibody labelled NG-2+ OPCs. Images are shown with Hoechst 33342 nuclear label. Scale bars: **B** and **C** = 100 μm; **D** and **E** = 25 μm; **F**–**I** and **K** = 100 μm; **J** and **L** = 50 μm (**L** inset = 5 μm); **M** = 8 μm.

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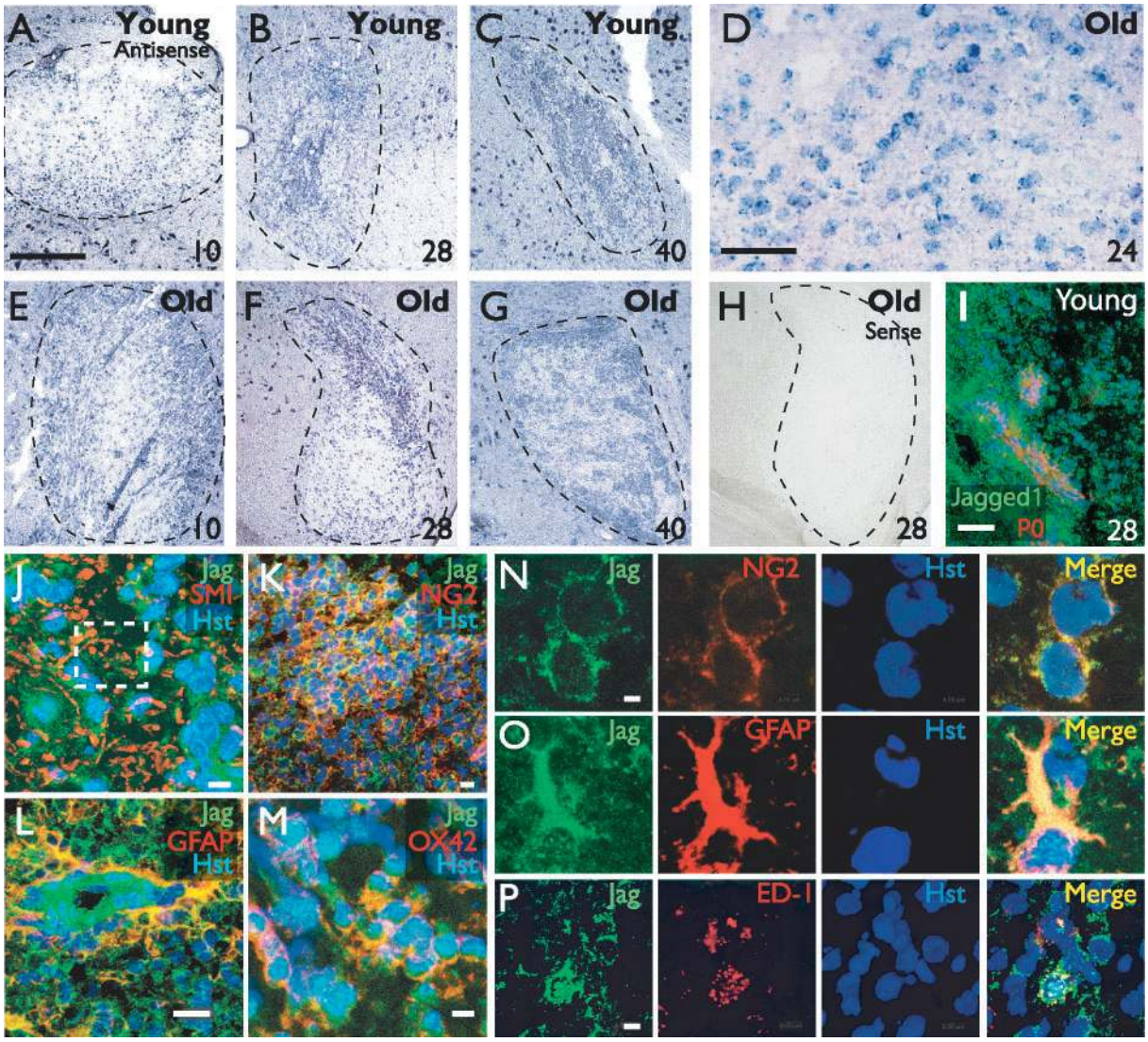

Fig. 6 Jagged1 mRNA and protein expression in remyelinating lesions. (**A**–**G**) *In situ* hybridization using anti-sense riboprobe for Jagged1 mRNA of remyelinating lesions from young and old adult rats at 10, 28 and 40 days (**A**–**C** and **E**–**G**), and old rat at 24 days (**D**). (**H**) *In situ* hybridization using sense riboprobe for Jagged1 mRNA. (**I**–**Q**) Jagged1 protein labelling using anti-Jagged1 polyclonal antibody (FITC secondary) on coronal cryostat sections of remyelinating lesions with: (**I**) anti-P0; (**J**) anti-phosphorylated neurofilament (SMI), box region of probable axonal Jagged1 colocalization; (**K** and **N**) anti-NG-2; (**L** and **O**) anti-GFAP; (**M**) anti-Ox42; (**P**) anti-ED-1. (**J** and **L**–**P**) Stacked confocal images. (**I** and **K**) Epifluorescent images. (**J**–**P**) Lesions depicted are 10 day in young (**L** and **O**), 20 day in young (**J**, **K** and **N**) and 20 day in old (**M** and **P**) rats. Merged images are shown with Hoechst 33342 nuclear label. (**I**) Serial section to **B**. Scale bars: **A**–**C** and **E**–**H** = 200 μm; **D** = 50 μm; **I** = 100 μm; **J** and **M** = 10 μm; **K** and **L** = 20 μm; **N** and **O** = 4 μm; **P** = 8 μm.

intensity, together with changes in axon morphology and neurofilament phosphorylation, made it difficult to unequivocally colocalize Jagged1 and neurofilament even with high-resolution confocal microscopy.

Histological and ultrastructural measures of remyelination show no significant differences between control and Notch1 conditional knock-out mice

Since there was no qualitative or quantitative difference in patterns of expression of Notch1 and Jagged1 between young and old animals, we inferred that Notch1 signalling was not a major determinant of the delayed OPC differentiation during slow remyelination in old rats (Sim *et al*., 2002*b*). To address further the role of Notch signalling during remyelination, we used adult Notch1 conditional knock-out mice in which demyelination was induced using cuprizone (Stidworthy *et al*., 2003). *Plp-creER* Notch1$^{lox/lox}$ ('knock-out') mice were compared with controls lacking the *Plp-creER* allele and thus expressing normal levels of Notch1 (Genoud *et al*., 2002; Leone *et al*., 2003). Remyelination during the 2 weeks after cuprizone withdrawal was assessed by the ranking of sections of the corpus callosum (Fig. 7A and B). In this method, the highest rank (1st) was given to

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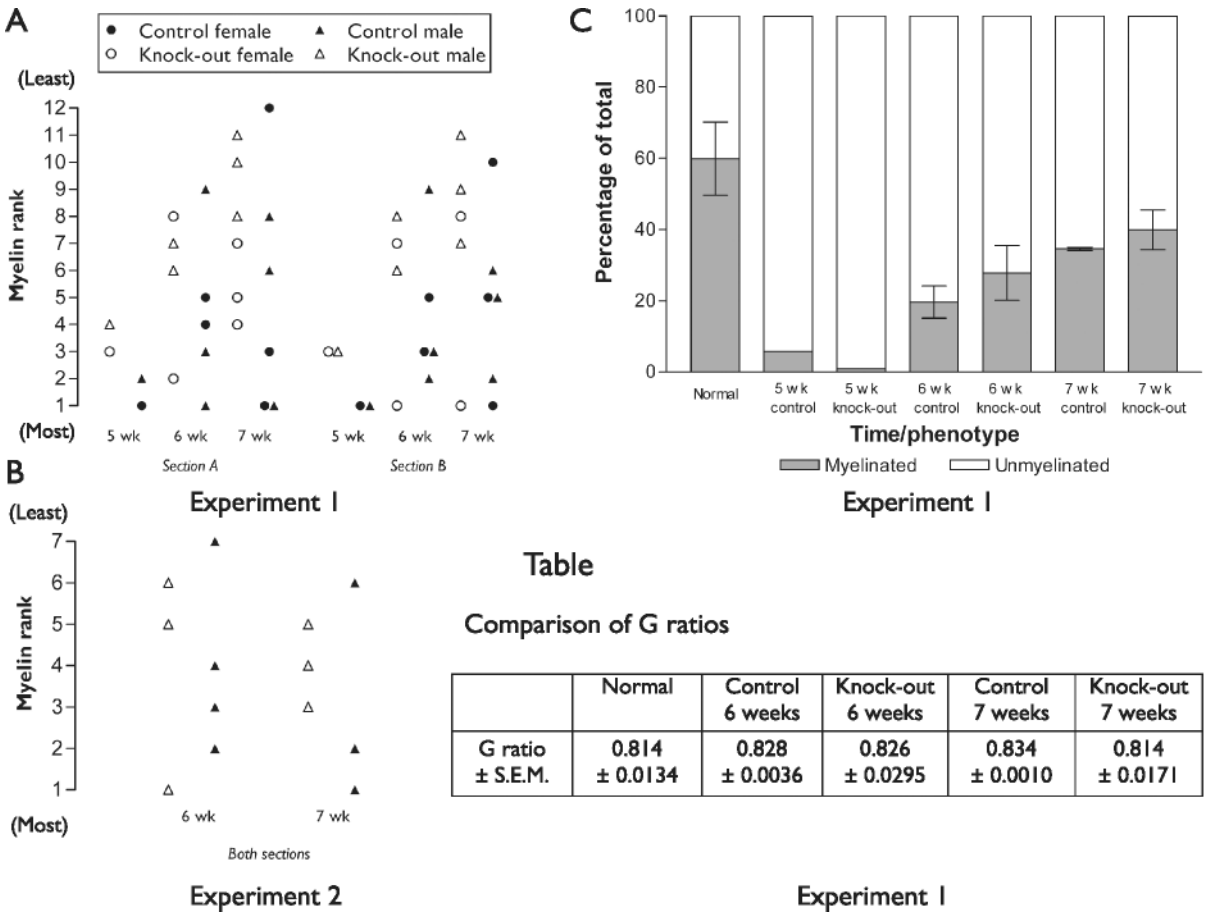

Table

Comparison of G ratios

	Normal	Control 6 weeks	Knock-out 6 weeks	Control 7 weeks	Knock-out 7 weeks
G ratio ± S.E.M.	0.814 ± 0.0134	0.828 ± 0.0036	0.826 ± 0.0295	0.834 ± 0.0010	0.814 ± 0.0171

Fig. 7 Histological and ultrastructural measures of early remyelination are not significantly different between Notch knock-out and control mice. (**A** and **B**) Myelination rank data for experiment 1 (**A**) using age-matched mice of mixed gender, and for experiment 2 (**B**) using male mice only. Comparison of the rank sums in each experiment using the Mann–Whitney test yielded no significant differences between groups. (**C**) Proportions of myelinated and unmyelinated axons show expected trends as remyelination proceeds, but there is no difference between knock-out and control animals. **Table**: Mean *G* ratio measurements for normal mice ($n = 4$), and for knock-out and control mice at 6 and 7 weeks in experiment 1. There is no significant difference between any of the groups.

the animal exhibiting the highest proportion of remyelination. If it was not possible to differentiate between two animals using this method then they were given the same rank. Two independent experiments were performed; the first using age-matched mice of mixed gender, the second using male mice only. Time-points were chosen to coincide with maximal OPC recruitment to areas of demyelination (Matsushima and Morell, 2001). In the first experiment (Fig. 7A) a single daily injection of tamoxifen was given from the beginning of week 4 of cuprizone treatment, continuing until the animals were sacrificed. Following ranking, groups were compared using the Mann–Whitney test. No significant differences were observed between groups at either time-point or location, between males or females, or within female-only groups. Comparison of males at 14 days identified more myelin in control mice, with a P value of 0.1, the minimum permissible P value with the available group sizes. A second experiment thus used males only. Induction of recombination was by twice-daily tamoxifen injection from the day after cuprizone ceased, a protocol previously demonstrated to have equivalent or superior recombination efficiency (Leone *et al*., 2003). This timing removed the possibility that OPC recruited during the demyelinating phase were eliminated by differentiation in the presence of cuprizone, obscuring a true difference in the rate of remyelination. Rankings were performed and revealed no differences between the control and knock-out groups (Fig. 7B).

To exclude the possibility that ranking was confounded by either small axon size or the degree of inflammation, proportions of myelinated and unmyelinated fibres in the body of the corpus callosum and the dorsal hippocampal region of the splenium were assessed ultrastructurally as previously described (Stidworthy *et al*., 2003). Proportions of myelinated and unmyelinated fibres followed predicted trends as remyelination proceeded, but there was no significant difference between the proportions in control or knock-out groups at any time (Fig. 7C). Such analysis would not identify differences in the remyelination rate manifest in the thickness of partially formed sheaths. Therefore, ultrastructural

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measurements of *G* ratio (the ratio of axon diameter to myelinated fibre diameter) were made at two locations in the corpus callosum (Stidworthy *et al*., 2003). Individual means were calculated from at least 100 axons from each animal, and used to generate group means for knock-out, control and untreated animals (Fig. 7, table). Means were compared by ANOVA. This revealed no significant differences in *G* ratio between the groups at any time-point.

Expression patterns of a LacZ reporter gene, NG-2, and markers of mitosis and apoptosis are similar between Notch1 knock-out mice and Notch1 heterozygote mice

To confirm that numbers and distributions of recombined Plp-positive cells were similar between mice in which Notch1 was ablated, and those in which a normal Notch1 allele remained, the distribution of *LacZ* reporter-expressing cells was compared between cuprizone-treated, age-matched female Notch1 knock-out animals and heterozygous animals expressing *Plp-creER* in combination with one normal Notch1 allele and the R26R allele (Leone *et al*., 2003). Distribution of *LacZ*-positive cells in both groups was similar to that described previously within myelinated and grey matter areas (Leone *et al*., 2003). In the demyelinated corpus callosum, patchy labelling was observed which paralleled the distribution of myelination and demyelination as previously characterized (Stidworthy *et al*., 2003) (Fig. 8A–D). No qualitative or quantitative differences were apparent between the two groups in the distribution or number of *LacZ*-positive cells, indicating that tamoxifen treatment and Cre-mediated recombination *per se* do not result in differences in Plp-positive OPC recruitment. Furthermore, *LacZ* expression is likely to denote recombination at all lox sites within a particular cell, and hence ablation of both Notch1 alleles in *Plp-creER* Notch1$^{lox/lox}$ mice. This conclusion was further supported by immunolabelling of serial sections from both groups for NG-2. Both were qualitatively similar, and yielded identical labelling intensities in the splenium and

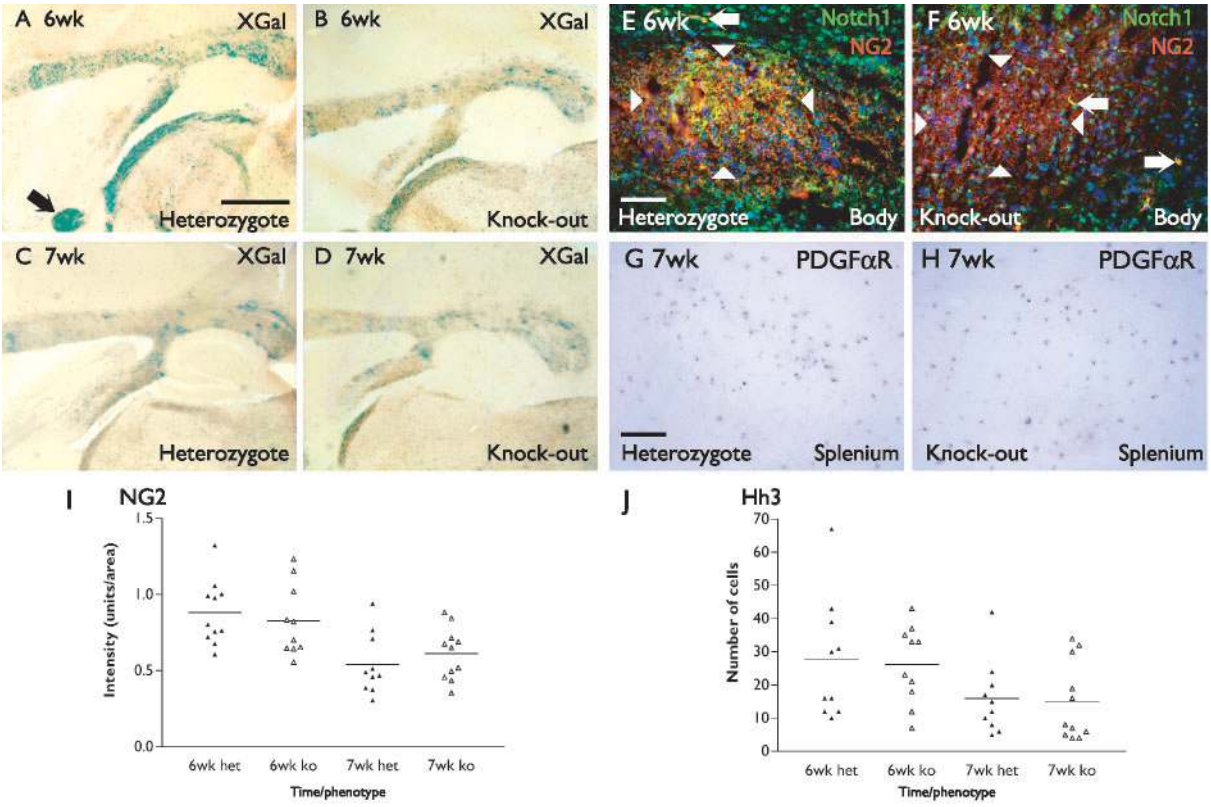

Fig. 8 Patterns of OPC recruitment and proliferation are similar in the corpus callosum of Notch1 knock-out and Notch1$^{lox/wt}$ heterozygote mice, despite a reduction in Notch1 expression. (**A**–**D**) Recruitment of recombined Plp-expressing cells, labelled with the marker gene *LacZ*, is similar into areas of demyelination in both heterozygote (**A** and **C**) and knock-out (**B** and **D**) mice. Arrow indicates myelinated rostral commissure labelled with X-gal. (**E** and **F**) Dual labelling of the remyelinating corpus callosum with antibodies against NG-2 and Notch1. NG-2 and Notch1 colocalize in areas of remyelination in heterozygote animals (**E**), whereas there are areas of NG-2+ cell recruitment without Notch1 labelling in knock-out mice (**F**). (**G**–**I**) Similar numbers of PDGFαR+ cells are recruited into the corpus callosum of heterozygote (**G**) and knock-out (**H**) mice, and there is no difference in the intensity of NG-2 labelling intensity between the two groups (**I**). (**J**) The number of proliferating cells in the remyelinating corpus callosum (identified by phosphorylated histone h3 expression) is not significantly different between heterozygote and knock-out mice. Scale bars: **A**–**D** = 1 mm; **E** and **F** = 100 μm; **G** and **H** = 50 μm.

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body of the corpus callosum when quantified by a method similar to that described previously (Genoud *et al*., 2002) (Fig. 8I). Comparison of OPC labelling by *in situ* hybridization for PDGFαR was similar (Fig. 8G and H). To identify differences in proliferation or cell death as a consequence of Notch1 ablation, serial sections of corpus callosum were labelled using an antibody against phosphorylated histone h3 (Hh3), a proliferation marker, and by the TUNEL method, a marker of apoptotic cell death. No significant differences in counts of Hh3-positive cells within the corpus callosum were detected between the groups at either time-point (Fig. 8J), implying that cellular proliferation is independent of genotype. Very few TUNEL-positive cells were seen and there was no difference between the groups (data not shown). Detailed histological, ultrastructural and immunohistochemical examination thus indicated no differences in the extent of remyelination, the proportion of remyelinated axons, the rate at which axons were remyelinated, or in OPC recruitment, proliferation and cell death within the corpus callosum of Notch1 knock-out mice and mice expressing Notch1.

Notch1 is expressed in remyelinating lesions of the mouse corpus callosum, and dual NG-2/ Notch1 labelling segregates differently between heterozygote and knock-out animals

Finally, sections of corpus callosum from each of the groups were labelled simultaneously with antibodies against Notch1 and NG-2. In control animals, Notch1 positivity was found both in the presence and absence of colocalized NG-2 positivity (Fig. 8E). In knock-out animals, however, the bulk of the Notch1 positivity did not overlap with areas of NG-2 labelling (Fig. 8F), further indicating the efficiency of recombination in the *Plp-creER* model.

Discussion

To study the role of the Notch–Jagged pathway in remyelination we have examined the expression of Notch and Jagged in an experimental model of demyelination that undergoes complete remyelination (Woodruff and Franklin, 1999; Penderis *et al*., 2003). First, we compared expression in young animals with that in old animals, where the slower rate of remyelination is largely due to a delay in OPC differentiation (Shields *et al*., 1999; Sim *et al*., 2000, 2002*b*). If this delay were attributable to Notch–Jagged signalling we would predict divergence in expression profiles between the two age groups, particularly at the later time-points, when remyelination in young animals is complete, but continuing in older animals (Shields *et al*., 1999; Sim *et al*., 2000, 2002*b*). Secondly, we used an inducible knock-out mouse, in which Notch1 ablation was confined to Plp-expressing cells in the presence of tamoxifen, to compare remyelination in the absence of Notch1.

Our findings of Notch1 expression in adult oligodendrocyte lineage cells correspond to those described in the adult optic nerve (Wang *et al*., 1998) and the adult forebrain (Irvin *et al*., 2001). We found that resting adult astrocytes do not express Notch1, suggesting Notch1 function in astrocytes is predominantly confined to lineage commitment in development (Morrison *et al*., 2000; Ge *et al*., 2002). However, following demyelination, expression was observed in small numbers of astrocytes at the lesion margins. Reports on neuronal expression are conflicting. Some demonstrate Notch1 in neurons of the adult cerebellum and neocortex (Higuchi *et al*., 1995; Berezovska *et al*., 1998; Mikami *et al*., 2001; Stump *et al*., 2002), and enteric nervous system (Sander *et al*., 2003). Others have failed to demonstrate significant expression in neurons in the adult striatum or in neuron-enriched embryonic cultures (Irvin *et al*., 2001). This may reflect anatomical variability, or differences in the sensitivities of methods employed. In our study there was good correlation in neuronal expression of both mRNA and protein by independent methods.

Using RT–PCR we demonstrated that two Notch ligands, Jagged1 and Jagged2, are expressed constitutively in the trigeminal ganglion, subsequently confirming expression of Jagged1 on myelinated axons using immunolabelling. The constitutive expression of Jagged1 on myelinated fibres contrasts with the developing optic nerve, in which axonal Jagged1 expression fell below the limits of detection as myelination proceeded (Wang *et al*., 1998). We did, however, confirm Jagged1 expression in NG-2-positive OPCs, and in oligodendrocytes, consistent with previous findings (Wang *et al*., 1998). The presence of Notch1 in post-mitotic neurons and oligodendrocytes, together with the ligand Jagged1 in axons and OPCs, suggests an ongoing role for Notch–Jagged signalling in the adult.

Hypotheses based on developmental recapitulation in adult regenerative processes often prove helpful. If regulation of remyelination in the adult trigeminal tract were to recapitulate the developmental pattern in the optic nerve, one would expect OPCs to express Notch1 and levels of Jagged1 expression to increase on demyelinated axons, subsequently declining as axons remyelinate. We confirmed that Notch1 is expressed by adult NG-2-positive OPCs recruited into remyelinating lesions. Although we did not unequivocally demonstrate functional activation of Notch1 in this study we think this likely to be the case since Notch1 expression is associated with increased expression of the Notch-associated transcription factor Hes5 (Kondo and Raff, 2000) in mouse models of toxin-induced demyelination (unpublished data). These cells populated the lesion from the periphery, a pattern previously described for PDGFαR-positive OPCs (Sim *et al*., 2002*b*). Throughout remyelination, RT–PCR confirmed Jagged1 and Jagged2 expression in trigeminal ganglia. However, the intensity of Jagged1 protein expression by demyelinated axons was low. Thus, in contrast to development, demyelinated axons are not a major source of Jagged1 in our experimental model or in multiple sclerosis tissue (John *et al*., 2002), and other cell types such as macrophages and reactive astrocytes provide a more

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abundant source. In addition, we noted Jagged1 expression by both NG-2-positive adult OPCs (suggesting that adjacent OPCs may exert mutually inhibitory effects on one another) and adult oligodendrocytes, as well as by myelinating Schwann cells. Jagged1 was originally cloned from a neonatal rat Schwann cell cDNA library (Lindsell *et al*., 1995), but expression during remyelination has not been reported. The alternative sources of ligand appear likely to overwhelm the axonal Jagged1 component, by virtue not only of their quantitative predominance, but also of their close temporal and spatial association with the Notch1-expressing population. The macrophage and reactive astrocyte presence is especially prominent during the early phases of remyelination, gradually subsiding as remyelination progresses. This suggests a mechanism in which Jagged1 expression by inflammatory cells and reactive astrocytes provides signals that by preventing premature differentiation of OPCs maintains them in a mitogen responsive state during the initial recruitment phase of remyelination. Consistent with this concept is the protracted macrophage presence in toxin lesions in old animals, which may contribute to the delayed OPC differentiation (Hinks and Franklin, 2000).

Using Notch1 knock-out mice, we were unable to identify differences during remyelination after cuprizone intoxication. The time-points were chosen to coincide with maximal OPC recruitment and differentiation within demyelinated areas of the corpus callosum (Matsushima and Morell, 2001). We also used protocols previously shown to induce high levels of recombination of floxed loci in *Plp-creER* mice (Leone *et al*., 2003). Using a range of histological measures of remyelination (Stidworthy *et al*., 2003), as well as immunolabelling techniques to identify OPC, cellular proliferation and apoptosis, we were unable to identify differences in the extent of remyelination, the proportion of remyelinated axons, the rate at which axons were remyelinated, or in OPC recruitment, proliferation and cell death within the corpus callosum. These findings support the conclusion that Notch signalling, either via Jagged or other ligands such as F3/Contactin (Hu *et al*., 2003) is not rate-determining during remyelination of toxin-induced demyelination. It would also seem that Notch signalling does not play a major role in determining OPC recruitment, proliferation or cell death, during the early stages of remyelination.

This study has allowed us to draw several conclusions regarding the role of Notch–Jagged signalling in CNS remyelination. First, since remyelination can proceed to completion despite widespread Notch–Jagged expression, our findings indicate that Notch–Jagged signalling in adult OPCs does not imply that remyelination will fail. Secondly, although similar signalling pathways may be used during remyelination the principal cellular protagonists may differ compared with developmental myelination. Lastly, since there were no quantitative differences in Notch1 expressing cells in slow and rapidly remyelinating lesions, or in the remyelination that occurred in mice in which Notch1 was excised in OPCs and in control mice, we conclude that Notch–Jagged signalling is not a rate-limiting determinant of remyelination in rodent models of demyelination.

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