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Class 3 semaphorins influence oligodendrocyte precursor recruitment and remyelination in adult central nervous system

Gabrièle Piaton,[1] Marie-Stéphane Aigrot,[1] Anna Williams,[2] Sarah Moyon,[1] Vanja Tepavcevic,[1] Imane Moutkine,[1] Julien Gras,[1] Katherine S. Matho,[1] Alain Schmitt,[3] Heidi Soellner,[4] Andrea B. Huber,[4] Philippe Ravassard[1] and Catherine Lubetzki[1]

1 Université Pierre et Marie Curie, Centre de Recherche de l'Institut du Cerveau et de la Moelle épinière (CRICM), UMRS 975; Inserm U 975; CNRS, UMR 7225; Paris 75013, France
2 Multiple Sclerosis Centre, Centre for Inflammation Research, QMRI, Edinburgh, UK
3 Cochin Imaging Facility, Cochin Institute, Inserm U 1016; CNRS, UMR 8104; University Paris Descartes UMR-S 1016; Paris 75014, France
4 Institute of Developmental Genetics, Helmholtz Zentrum München-German Research Centre for Environmental Health, Munich/Neuherberg, Germany

Correspondence to: Catherine Lubetzki,
Université Pierre et Marie Curie,
Centre de Recherche de l'Institut du Cerveau et de la Moelle épinière (CRICM), UMRS 975;
Inserm U 975; CNRS, UMR 7225;
Paris 75013, France
E-mail: catherine.lubetzki@upmc.fr

Oligodendrocyte precursor cells, which persist in the adult central nervous system, are the main source of central nervous system remyelinating cells. In multiple sclerosis, some demyelinated plaques exhibit an oligodendroglial depopulation, raising the hypothesis of impaired oligodendrocyte precursor cell recruitment. Developmental studies identified semaphorins 3A and 3F as repulsive and attractive guidance cues for oligodendrocyte precursor cells, respectively. We previously reported their increased expression in experimental demyelination and in multiple sclerosis. Here, we show that adult oligodendrocyte precursor cells, like their embryonic counterparts, express class 3 semaphorin receptors, neuropilins and plexins and that neuropilin expression increases after demyelination. Using gain and loss of function experiments in an adult murine demyelination model, we demonstrate that semaphorin 3A impairs oligodendrocyte precursor cell recruitment to the demyelinated area. In contrast, semaphorin 3F overexpression accelerates not only oligodendrocyte precursor cell recruitment, but also remyelination rate. These data open new avenues to understand remyelination failure and promote repair in multiple sclerosis.

Keywords: semaphorin; oligodendrocyte precursor cell; recruitment; remyelination; gain and loss of function

Abbreviations: FACS = fluorescence-activated cell sorting; GFP = green fluorescent protein; PDGFR = platelet-derived growth factor receptor; NRP = neuropilin; Sema = semaphorin

Introduction

Multiple sclerosis is a chronic inflammatory demyelinating disease of the CNS and the first cause of acquired non-traumatic disability in young adults. Most treatments to date have been focused on the inflammatory component of the disease, with partial effect on the relapse rate, but only weak impact on the progressive phase, which mostly relates to inflammation-independent chronic demyelination and axonal damage (Compston and Coles, 2008). Spontaneous remyelination occurs in multiple sclerosis (Perier and Gregoire, 1965; Prineas and Connell, 1979; Raine and Wu, 1993). The newly formed myelin, characterized by a thin myelin sheath with short internodes, not only restores the rapid conduction of the nerve influx allowing functional recovery (Smith et al., 1981), but also prevents axonal damage and loss (Irvine and Blakemore, 2008). Oligodendrocyte precursor cells, which persist in the adult CNS, are the major remyelinating players. Remyelination can be extensive (Patrikios et al., 2006), but it is most often insufficient and limited to the periphery of the demyelinated lesions. Although astrogliosis, axonal damage or persisting inflammatory infiltrates may influence the outcome of myelin repair, a defect of oligodendrocyte precursor cells is likely to be a key issue. Multiple sclerosis demyelinated plaques vary in their oligodendroglial content (Lucchinetti et al., 1999). Some are characterized by the presence of immature oligodendroglial cells (Wolswijk, 1998; Maeda et al., 2001; Chang et al., 2002), suggesting local inhibitors of oligodendrocyte maturation or remyelination (Miller and Mi, 2007), whereas other demyelinated plaques lack oligodendrocyte precursor cells (Lucchinetti et al., 1999). This oligodendroglial depopulation, contrasting with the detection of oligodendrocyte precursor cells in the normal appearing white matter, may be the consequence of an impaired recruitment of oligodendrocyte precursor cells towards demyelinated areas (Piaton et al., 2009). This raises the question of the mechanisms involved in oligodendrocyte precursor cell recruitment in the adult CNS.

Oligodendrocyte precursor cells are migratory cells during development. Few guidance cues have been identified (de Castro and Bribian, 2005), either membrane bound or secreted. Two members of the secreted class 3 semaphorin (Sema) family, Sema3A and Sema3F, have an opposite effect on oligodendrocyte precursor cell migration during development: repulsive and attractive, respectively (Sugimoto et al., 2001; Spassky et al., 2002). These guidance cues bind to distinct obligate co-receptors neuropilin (NRP1 for Sema3A, NRP2 for Sema3F), which use Plexin-A (PlexinA) for signal transduction (Pasterkamp and Verhaagen, 2006). The potential influence of Sema3A and Sema3F in CNS demyelinating diseases was suggested by our previous study showing a strong upregulation of *Sema3A* and *Sema3F* messenger RNAs in multiple sclerosis post-mortem tissue, as well as in an experimental model of demyelination (Williams et al., 2007). Two important questions emerged from our initial observation that cues involved in developmental oligodendrocyte precursor cell migration are dysregulated after CNS demyelination. First, could the relative level of their expression influence oligodendrocyte precursor cell recruitment towards demyelinated areas, and second, would an increase in oligodendrocyte precursor cell recruitment result in a better myelin repair? Here we report that adult oligodendrocyte precursor cell migration is influenced by Sema3 signalling, and that after demyelination, oligodendrocyte precursor cell recruitment is increased by Sema3F overexpression or Sema3A downregulation. Finally, we provide evidence that this increased recruitment is associated with increased remyelination.

Materials and methods

DNA constructs and recombinant lentiviral production

Lentiviral vectors were first tested by *in vivo* injections in the mouse dorsal columns of the spinal cord, to select the vector infecting mostly astrocytic cells in this model. Cytomegalovirus and phosphoglycerate kinase promoters, in combination with either vesicular stomatitis virus or mokola virus glycoprotein envelope protein, were tested. The best astrocytic infection was obtained with cytomegalovirus and vesicular stomatitis virus. Green fluorescent protein (GFP)-tagged rat Sema3A (De Wit et al., 2005) and human Sema3F (Kusy et al., 2003) sequences were amplified by polymerase chain reaction with Pfx50^{TM} DNA polymerase (Invitrogen) using the following primers: GFP-SEMA3A sense: 5′ CACCATGGGCTGGTTCAC 3′; GFP-SEMA3A antisense: 5′ TCAGACACTCCTGGGTGCCCT 3′; GFP-SEMA3F sense: 5′ CACCATGCTTGTCGCCGGTCTT 3′; GFP-SEMA3F antisense: 5′ CCGCTTTACTTGTACAGCTC 3′. The resulting polymerase chain reaction products were cloned into pENTR^{TM}/D-TOPO® vector (Invitrogen) to generate GFP-SEMA3A and GFP-SEMA3F entry clones. Both inserts were completely sequenced to rule out polymerase chain reaction-induced mutations. Lentiviral vectors were prepared through LR Clonase® II Gateway recombination (Invitrogen) using each entry clone and the destination lentiviral vector pTRip cytomegalovirus-RFA (Gateway) ΔU3 (Russ et al., 2008) to generate pTRip cytomegalovirus-GFP-SEMA3A ΔU3 and pTRip cytomegalovirus-GFP-SEMA3F ΔU3. Lentiviral vector stocks were produced by transient transfection of human embryonic kidney (HEK) 293T cells with the p8.91 encapsidation plasmid (Zufferey et al., 1997), the vesicular stomatitis virus glycoprotein-G-encoding pHCMV-G plasmid (Yee et al., 1994) and the lentiviral recombinant vector as previously described (Zennou et al., 2000). Supernatants were treated with DNase I (Roche Diagnostic) prior to ultracentrifugation and the resulting pellet was resuspended in phosphate buffered saline, separated into aliquots and frozen at −80°C until use. The transduction efficiency of each vector stock was determined by fluorescence-activated cell sorting (FACS) analysis as previously described (Castaing et al., 2005). Expression and secretion of the guidance cues after gene transfer was checked in HEK 293T cells. The furin-dependent proteolytic processing (Adams et al., 1997) was notably confirmed by the decrease of cleaved form when a furin inhibitor was added in the culture medium.

Human embryonic kidney cell culture and transduction

HEK 293T cells were grown in Dulbecco's modified Eagle medium-Glutamax^{TM} (Gibco) supplemented with 10% foetal calf serum at 37°C in a 5% CO_2 environment. Eighty per cent confluent cells were transduced with *Sema3A-GFP*, *Sema3F-GFP* or *GFP*

lentiviral vector at a Multiplicity of Infection of 10. GFP-positive cells were FACS sorted (FACS Aria, Becton Dickinson) and used for all experiments using conditioned medium.

Fluorescence-activated cell sorting purification of GFP-positive adult oligodendrocyte precursor cells

Adult oligodendrocyte precursor cells were isolated from the brain and/or the spinal cord of 2-month old *PDGFRα::GFP* hemizygous mice (Klinghoffer *et al.*, 2002) in a two-step protocol. First, brain and spinal cord were dissected in Hanks buffer (Hanks 10×, Gibco), [0.01 M HEPES buffer, 0.75% sodium bicarbonate (Gibco), 1% penicillin/streptomycin]. After an enzymatic dissociation using papain (30 µg/ml in Dulbecco's modified Eagle medium-Glutamax™, with 0.24 µg/ml L-cystein and 40 µg/ml DNase I), oligodendroglial cells were isolated using a Percoll density gradient as previously described (Lubetzki *et al.*, 1991), except that the Percoll gradient was pre-formed by 30 min of centrifugation (20 000g) and the cells centrifuged for 15 min on the gradient. In a second step, GFP-positive cells were sorted by FACS (FACS Aria, Becton Dickinson) and collected in pure foetal calf serum, then resuspended in modified Bottenstein Sato medium (Dulbecco's modified Eagle medium containing 0.5% foetal calf serum, 2 mM L-glutamine, 10 µM insulin, 5 ng/ml sodium selenite, 100 µg/ml transferrin, 0.28 µg/ml albumin, 60 ng/ml progesterone, 16 µg/ml putrescine, 40 ng/ml triiodothyronine and 30 ng/ml L-thyroxine), before plating on poly-L-lysine (40 µg/ml, Sigma) coated glass coverslips (for immunostaining) or transwell culture inserts (for migration assay). On average, starting with four animals, ∼500 000 cells and 100 000 cells were obtained from brains and spinal cords, respectively.

Characterization of GFP-positive cells was performed by flow cytometry analysis. Cells were fixed with 4% paraformaldehyde and labelled, after the first step of the isolation procedure, with O4 or A2B5 antibody (refer to 'Primary antibodies' section) or control isotype (mouse IgM isotype, 0.5 mg/ml, BD Pharmingen), revealed by secondary antibody (phycoerythrin anti mouse IgM, 0.2 mg/ml, BD Pharmingen), each 30 min at 4°C in phosphate buffered saline, 1% bovine serum albumin and 2% goat serum. They were then analysed by flow cytometry using a LSR Fortessa flow cytometer (Becton Dickinson) and the Diva software. This analysis (results are the mean of two independent experiments) showed that GFP-positive cells express A2B5 (83.7 and 91.5% cells from brain and spinal cord, respectively) and O4 (81.3 and 89.3% cells from brain and spinal cord, respectively) (Supplementary Fig. 1).

Cell migration assay

GFP-positive FACS sorted cells were resuspended in Bottenstein Sato medium at a cell density of 10^6 cells/ml. Migration of GFP-positive cells was assayed using a Boyden transwell system (8 µm pore size, Corning Costar Co., USA), as previously described (Jarjour *et al.*, 2003). Before seeding, both sides of the transwell were coated overnight with poly-L-lysine (40 µg/ml, Sigma). Twenty microlitres of Bottenstein Sato medium containing 12 500 GFP-positive FACS sorted cells were added to the upper insert of a chamber for 30 min. Then 500 µl of either Bottenstein Sato medium containing Sema3A-Fc or Sema3F-Fc or human Fc [500 ng/ml, R&D, (Chen *et al.*, 2008)], or of 4-day conditioned medium from HEK 293T cells transduced with *Sema3A-GFP* or *Sema3F-GFP* or *GFP* lentiviral vector, was added to

the bottom chamber. Cells were fixed with 4% paraformaldehyde, 24 h after seeding and cells attached to the upper side of the membranes were thoroughly scraped off. Cells attached to the bottom side of the membranes were immunostained with anti-GFP antibody (refer to 'Immunostaining' section) and counted directly under the microscope (Zeiss Imager.Z1).

Animals and spinal cord injections

Animal care and experiments were carried out according to the European Community regulations and ethical policies. Animals were anaesthetized with an intraperitoneal injection of 20 mg/kg xylazine (Rompun, Bayer) and 80 mg/kg ketamine (Imalgene 500, Merieux).

Spinal cord injections were carried out at L1 level in 2-month old male OF1 mice (Charles Rivers). The vertebral column was fixed between metal bars on stereotaxic apparatus. The corresponding vertebral arch was removed, the meninges pierced and a pulled glass needle (10 µm diameter) was used to inject 1 µl of *Sema3A-GFP*, *Sema3F-GFP* or *GFP* lentiviral vector (P24 50–100 ng/µl) into the spinal cord, at a depth of 800 µm, just lateral to the central blood vessel, at a rate of 0.25 µl/min using a peristaltic pump. The needle was not disturbed for another 4 min to allow diffusion of the solution and then slowly withdrawn. The injection site was marked with carbon before replacing the vertebral arch and suturing. Lysophosphatidylcholine injections were performed 3 days after lentiviral injection: the suture and the vertebral arch were removed and 1 µl of lysophosphatidylcholine (Sigma, 1% in 0.9% NaCl) was injected at the marked site, with the same method of injection.

Immunostaining

For immunohistochemistry, animals were perfused with 4% paraformaldehyde in phosphate buffered saline. The spinal cords were dissected, cryo-protected in phosphate buffered saline/20% sucrose at 4°C overnight, frozen, embedded in optimum cutting temperature medium and 16 µm serial cryostat sections were performed. The slides were treated for 10 min with 100% ethanol at −20°C and after saturation in phosphate buffered saline 0.3% Triton X-100, 10% horse serum, 1 h at room temperature, the primary antibodies were incubated overnight at 4°C in phosphate buffered saline 0.3% Triton X-100, 5% horse serum. After washing, Alexa or DyLight-conjugated secondary antibodies were incubated for 90 min at room temperature. Secondary antibodies obtained from Molecular Probes (Invitrogen, Cergy-Pontoise, France) were used at a 1:1000 dilution, antibodies obtained from Jackson ImmunoResearch (Suffolk, UK) at a 1:400 dilution. Nuclei were stained with Hoechst solution (1 µg/ml), and sections mounted in Fluoromount-G (Clinisciences, France).

For myelin basic protein/adenomatous polyposis coli (CC1)/GFP triple detection, a five step protocol was used: (i) anti-GFP and anti-myelin basic protein primary antibodies overnight at 4°C; (ii) corresponding secondary antibodies 90 min at room temperature; (iii) 15 min fixation in 4% paraformaldehyde at room temperature; (iv) CC1 primary antibody incubation overnight at 4°C; and (v) anti-mouse IgG2b Alexa 594 90 min at room temperature. For Ki67 staining, saturation was performed at 37°C.

For immunocytochemistry, purified oligodendrocyte precursor cells from adult CNS cells were fixed with 4% paraformaldehyde for 15 min at room temperature, then incubated successively for 90 min in the primary and secondary antibodies. In all cases, controls were performed by omitting the primary antibody. Stainings were observed

using a fluorescence microscope Zeiss Imager.Z1. Pictures were acquired with AxioCam camera coupled to ApoTome module and analysed using ImageJ software. For confocal microscopy (Fig. 2A), z-stacks were acquired with a Leica inverted SP2 acousto-optical beam splitter, acousto-optical tunable filters confocal microscope and analysed using Fiji ImageJ software.

Primary antibodies

For immunostainings, primary antibodies were used at the following dilutions: anti-O4 (mouse monoclonal IgM, 1:5, hybridoma from I. Sommer), A2B5 antibody (mouse monoclonal IgM, 1:5, ATCC), anti-myelin basic protein (MBP, chicken IgY, 1:40, Millipore), anti-PDGFRα, (rat IgG2a, 1:800, BD Pharmingen), anti-GFP (rabbit polyclonal, 1:500, Invitrogen), anti-adenomatus Polyposis Coli (clone CC1; mouse IgG2b, 1:300, Calbiochem), anti-NRP1 (goat IgG, 1:50, R&D), anti-NRP2 (rabbit polyclonal, 1:50, Santa Cruz Biotechnology), anti-PlexinA1 (rabbit polyclonal, 1:20, Santa Cruz Biotechnology), anti-PlexinA2, A3 or A4 (rabbit polyclonal, 1:100, Abcam), anti-Sema3A (rabbit polyclonal, 1:50, Abcam), anti-Sema3F (rabbit polyclonal, 1:50, Millipore), anti-cleaved caspase 3 (rabbit polyclonal, 1:100, Cell Signalling Technologies), anti-Ki-67 (rabbit IgG, 1:500, Thermo Scientific) and anti-CD68 (rat IgG, 1:50, AbD Serotec).

Transmission electron microscopy

Animals were perfused 10 days after lysophosphatidylcholine injection with an intracardiac injection of 4% paraformaldehyde and 0.5% glutaraldehyde in phosphate buffer (pH 7.4). The spinal cords were post-fixed in the same fixative overnight at 4°C. Vibratome sections 100 μm thick of the injected site were performed and post-fixed in 0.025% glutaraldehyde overnight at 4°C. After rinsing in phosphate buffer, pH 7.4, they were incubated overnight at 4°C with anti-GFP antibody in phosphate buffer, pH 7.4, containing 0.01% Triton X-100 and 5% horse serum, preceded by 1 h saturation at room temperature with phosphate buffer, pH 7.4, containing 0.01% Triton X-100 and 10% horse serum. Horseradish-peroxidase coupled donkey anti-rabbit antibody (GE Healthcare, 1:300) was then incubated for 90 min at room temperature. After application of 3,3′-diaminobenzidine (Dako), only the sections with 3,3′-diaminobenzidine-positive cells were selected using light microscopy. These were then post-fixed in 3% glutaraldehyde in phosphate buffer, pH 7.4, for 24 h, washed, post-fixed with 1% osmium tetroxide in 0.1 M phosphate buffer and then gradually dehydrated in 70, 90 and 100% ethanol. After 10 min in a 1:2 mixture of epoxy propane and epoxy resin, the sections were embedded in gelatin capsules with freshly prepared epoxy resin and polymerized at 60°C for 24 h. Ultrathin sections of 90 nm were cut with an ultramicrotome (Reichert ultracut S), stained with uranyl acetate and Reynold's lead citrate (except for 3,3′-diaminobenzidine semi-quantification) and observed with a transmission electron microscope (JEOL 1011). Acquisition was performed with a Gatan ES1000W CCD camera.

Quantification of oligodendrocyte precursor cell recruitment and remyelination

Oligodendrocyte precursor cell recruitment was determined by quantifying the density of PDGFRα-expressing cells within the demyelinated lesion. In the gain of function experiments, given the variability of transduction efficacy (reflected by the number of GFP-positive cells and the real-time quantitative polymerase chain reaction data), we determined, in preliminary experiments, a threshold of transduction, with a cut-off corresponding to 400 transduced cells/mm^2, below which no effect of either *Sema3A-GFP* or *Sema3F-GFP* on oligodendrocyte precursor cell recruitment was detected 7 days after lysophosphatidylcholine injection. This criterion was then used for sample selection.

For ultrastructural quantification of remyelination, transduction efficacy was evaluated by 3,3′-diaminobenzidine positivity on ultrathin sections: grid squares (100 000 μm^2) within demyelinated lesions were selected after the evaluation of infectivity at ×100 000. Morphometric analysis was performed only on grid squares with high infectivity (>10 3,3′-diaminobenzidine-positive cells in a grid square), on a minimum of 20 fields of view at ×10 000, and consisted of quantifying normally myelinated axons (thick myelin sheath), axons remyelinated by oligodendrocytes (thin myelin sheath), axons remyelinated by Schwann cells (thicker myelin sheath with basal lamina) and naked axons (Fig. 6B).

Statistical analyses

All quantifications were performed blindly. Statistical analysis was performed using XLSTAT software. If data were normally distributed, parametric *t*-tests were performed to compare each group versus control, if not, non-parametric Mann–Whitney U-tests were performed.

Results

Adult oligodendrocyte precursor cells purified from brain and spinal cord express semaphorin 3 receptors

Analysis of class 3 semaphorin receptors was performed on FACS purified GFP-positive cells (Fig. 1A and B), corresponding to PDGFRα-positive adult oligodendrocyte precursor cells. As shown in the 'Materials and methods' section and in agreement with previous reports (Wolswijk *et al.*, 1990), these adult oligodendrocyte precursor cells, contrary to neonatal oligodendrocyte precursor cells, express the O4 marker (Supplementary Fig. 1). They were immunostained at 1 day *in vitro* with antibodies directed against Sema3 receptors: NRP1 and 2, and PlexinA1, A2, A3 (Fig. 1C), but not A4. Receptor expression was confirmed by real-time quantitative polymerase chain reaction using *NRP* and *PlexinA* specific sets of primers (data not shown). Therefore, like their embryonic counterparts, adult oligodendrocyte precursor cells express Sema3A and Sema3F receptors. However, in contrast to embryonic and neonate oligodendrocyte precursor cells (Okada *et al.*, 2007), adult oligodendrocyte precursor cells do not express PlexinA4.

Adult oligodendrocyte precursor cells are attracted by semaphorin 3F *in vitro*

Purified adult oligodendrocyte precursor cells were plated for 24 h onto the porous membrane of Boyden chambers, the bottom
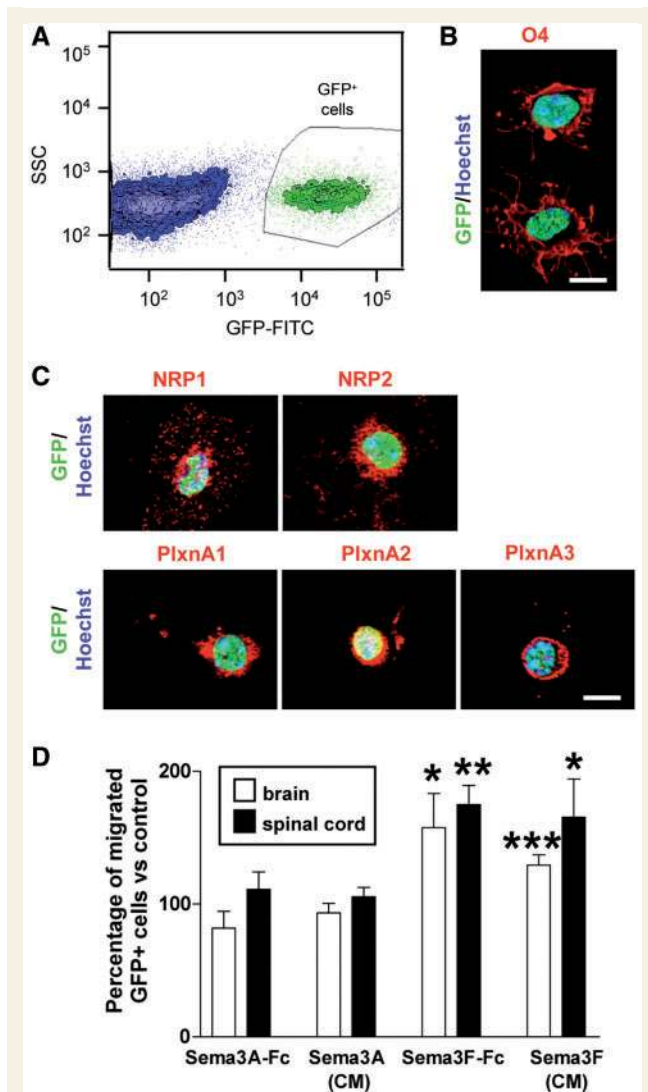
Figure 1 Adult oligodendrocyte precursor cells isolated from *PDGFRα::GFP* CNS express Sema3 receptors and are attracted by Sema3F. (**A**) FACS-sorting of GFP-positive cells from 2 month-old brain of *PDGFRα::GFP* hemizygous mice as a representative example. (**B**) Brain FACS isolated cells stained with anti-GFP and anti-O4 antibodies 1 day after isolation. GFP is nuclear due to the transgenic construction (Klinghoffer *et al.*, 2002) and cells display short processes. Scale bar = 5 μm. (**C**) Brain FACS isolated cells stained with anti-GFP combined with either anti-NRP1, anti-NRP2, anti-PlexinA1, anti-PlexinA2 or anti-PlexinA3 antibodies 1 day after isolation. Similar results were obtained with spinal cord FACS isolated cells. Scale bar = 5 μm. (**D**) GFP-positive FACS isolated cells from *PDGFRα::GFP* hemizygous mouse brain and spinal cord were plated onto the upper membrane of a Boyden transwell chamber in the presence, in the lower chamber, of either soluble Sema3 versus control human Fc (500 ng/ml), or conditioned medium (CM) from HEK 293T cells transduced with *Sema3-GFP* versus *GFP* lentiviral vector. Histogram represents the percentage (mean ± SEM) of GFP-positive cells having crossed the membrane after 24 h, normalized to the value of the parallel control. $n = 4$ per condition and experiment. *$P < 0.05$, ***$P < 0.001$ versus control (*t*-test).

compartment containing either Sema3A-Fc or Sema3F-Fc or control human Fc (Jarjour *et al.*, 2003). Both brain and spinal cord oligodendrocyte precursor cell migration were significantly 1.5-fold increased towards Sema3F-Fc (brain: $P = 0.006$, spinal cord: $P = 0.005$). Similar results were obtained with conditioned medium from HEK cells transduced with *Sema3A-GFP*, *Sema3F-GFP* or *GFP* lentiviral vector (brain: $P = 0.002$, $P = 0.023$) (Fig. 1D). Increased migration was not due to proliferation, as similar proportions of cells expressed the proliferation marker Ki67 after 24 h of treatment with Sema3F-Fc ($30.4 \pm 7.4\%$) or human Fc ($26.4 \pm 11.8\%$). In contrast, Sema3A added in the bottom compartment had no effect. These results show that Sema3F has a chemo-attractive effect on adult oligodendrocyte precursor cells, as for embryonic oligodendrocyte precursor cells (Spassky *et al.*, 2002).

Expression of semaphorin 3 and their receptors is increased after CNS demyelination

Expression of Sema3, NRP and PlexinA was analysed in the dorsal columns of the mouse spinal cord, both in control and in lysophosphatidylcholine-induced demyelinated conditions. Consistent with our previous data (Williams *et al.*, 2007), Sema3F and Sema3A proteins were upregulated after demyelination: Sema3F was increased early (2 days post-lesion), peaked at 7 days post-lesion and then returned to normal levels (14 days post-lesion), whereas Sema3A increase was delayed, with a peak 14 days post-lesion (Fig. 2B). After demyelination, recruitment of PDGFRα-positive oligodendrocyte precursor cells peaked 7 days after demyelination (with a 1000-fold increase in oligodendrocyte precursor cell density), corresponding to the peak of expression of Sema3F, and then remained stable (Fig. 2B). Oligodendrocyte precursor cells detected within the demyelinated lesion were mostly NRP2-positive at this recruitment peak ($83.8 \pm 8.5\%$). Neuropilin1-positive oligodendrocyte precursor cells were mainly detected later, $55.3 \pm 1.7\%$ of oligodendrocyte precursor cells expressing NRP1 14 days after demyelination (Fig. 2A and C). Among PlexinA receptors, $33.4 \pm 12.4\%$ of oligodendrocyte precursor cells expressed PlexinA1, $84.9 \pm 0.4\%$ expressed PlexinA2 and $50.3 \pm 8.8\%$ expressed PlexinA3 in uninjured dorsal columns, whereas, in accordance with *in vitro* data, PlexinA4 expression was not detected. PlexinA expression was unchanged 7 days after demyelination, $43.3 \pm 3.9\%$ expressing PlexinA1, $84.5 \pm 3.7\%$ expressing PlexinA2 and $65.8 \pm 5\%$ expressing PlexinA3. In summary, our results show that after demyelination, coincident with Sema3A and Sema3F increase, adult oligodendrocyte precursor cells upregulate corresponding receptors NRP1 and NRP2. As only a minority were found to express both receptors ($6.1 \pm 1.1\%$ in uninjured conditions, $25.1 \pm 4.9\%$ 7 days after demyelination), NRP1- and NRP2-expressing oligodendrocyte precursor cells may represent different subpopulations.
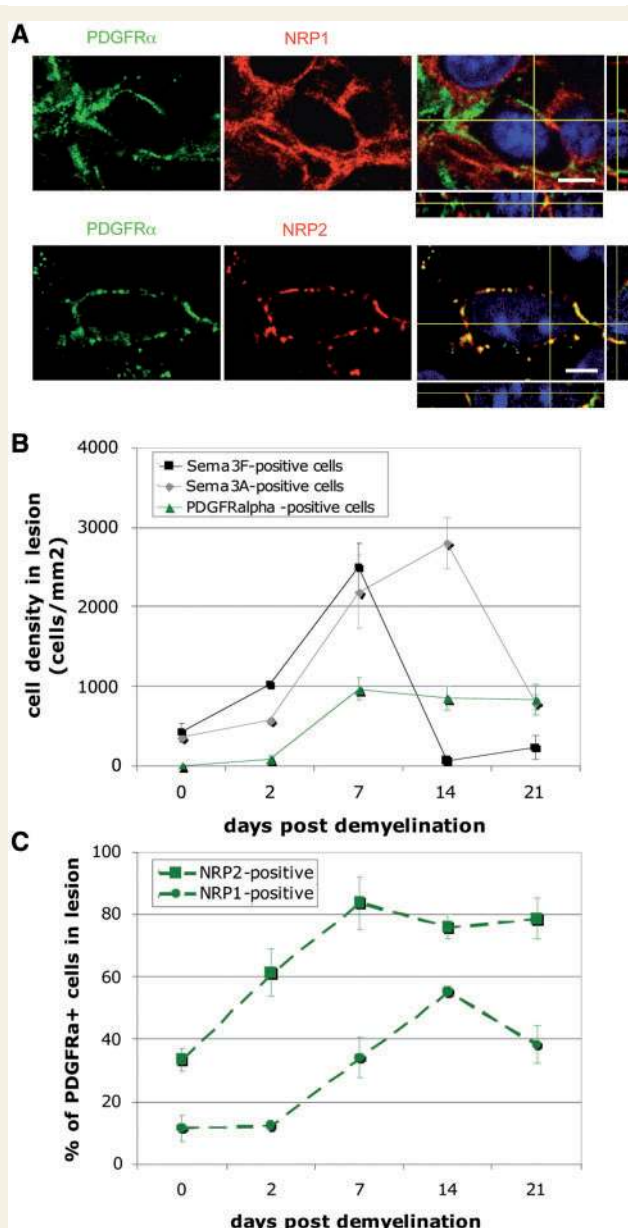
Figure 2 Class 3 semaphorin and neuropilin expression on adult oligodendrocyte precursor cells, are increased after demyelination. (**A**) Confocal images and z-stacks projections of transverse sections of demyelinated spinal cord dorsal columns, immunostained with anti-NRP1 or anti-NRP2 and anti-PDGFRα antibodies, 7 days after lysophosphatidylcholine injection. The two markers are colocalized on the same membrane. Scale bar: 5 μm. (**B**) Density (mean ± SEM, $n = 3$ per time point) of Sema3A-positive, Sema3F-positive and PDGFRα-positive cells in uninjured dorsal columns and within the lesion 2, 7, 14 and 21 days after lysophosphatidylcholine injection. (**C**) Density (mean ± SEM, $n = 3$ per time point) of NRP/PDGFRα double-positive cells in uninjured dorsal columns and within the demyelinated lesion 2, 7, 14 and 21 days after lysophosphatidylcholine injection.

Increased *in vivo* expression of semaphorin 3A or 3F influences adult oligodendrocyte precursor cell recruitment towards a demyelinated lesion

Gain of function experiments were designed through lentiviral mediated gene transfer, leading to overexpression of either Sema3A or Sema3F within and around a spinal cord demyelinated lesion. The GFP-tagged semaphorin constructs used for lentiviral vector production had been previously shown to be functional (Kusy *et al.*, 2003; De Wit *et al.*, 2005). The lentiviral vector was designed to efficiently transduce astrocytes, in order to get a local perilesional source of Sema3. Three days after transduction, GFP-positive cells were mainly GFAP-positive astrocytes, the remaining transduced cells being PDGFRα-positive (Fig. 3A–D). S*ema3* overexpression was confirmed by real-time quantitative polymerase chain reaction, corroborating the immunohistochemical detection of GFP-positive cells, and therefore transduction efficacy. Given its variability (Fig. 3E), quantification of oligodendrocyte precursor cell recruitment was only performed on samples selected for high transduction (refer to 'Materials and methods' section).

In non-demyelinated dorsal columns, oligodendrocyte precursor cell density was not affected by lentiviral vector transduction (*Sema3A-GFP*: 28.8 ± 6.3 PDGFRα-positive cells/section, $n = 3$, *Sema3F-GFP*: 34.8 ± 7.3 PDGFRα-positive cells/section, $n = 3$, and *GFP*: 38.3 ± 10.3 PDGFRα-positive cells/section, $n = 2$). After demyelination, oligodendrocyte precursor cell density was significantly increased in *Sema3F-GFP*-transduced lesions compared to *GFP*-transduced lesions (Fig. 4A and B). Two days after lysophosphatidylcholine injection, a 3-fold increase was detected ($P = 0.004$), and persisted for 7 days post-lysophosphatidylcholine injection ($P = 0.03$). In contrast, at both time points, significantly less PDGFRα-positive cells were quantified in *Sema3A-GFP*-transduced lesions (2 days: $P = 0.009$, 7 days: $P = 0.001$). The influence of both Sema3A and Sema3F overexpression was transient, with no difference in oligodendrocyte precursor cell density 14 days post-lesion (data not shown).

Interestingly, the amplitude of the migratory effect was infectivity-dependant, as illustrated using a subset of experiments in Fig. 4F. Sema3F-induced increase in oligodendrocyte precursor cell density was not related to increased proliferation as, 2 days post-lysophosphatidylcholine injection, no difference was detected in the percentage of PDGFRα-positive cells expressing Ki67 (Fig. 4C), consistent with the previous *in vitro* analysis. Sema3F effect was neither due to increased survival, as the same proportion of PDGFRα-positive cells expressed cleaved caspase 3 (Fig. 4D); similarly, no difference in survival was evidenced when FACS sorted adult oligodendrocyte precursor cells were treated *in vitro* for 24 h with Sema3F-Fc (1.6 ± 0.9%) or human Fc (1.8 ± 0.7%). Finally, Sema3A-induced decrease in oligodendrocyte precursor cell density was not related to increased apoptosis as, 7 days post-lysophosphatidylcholine injection, no difference was detected in the percentage of PDGFRα-positive cells
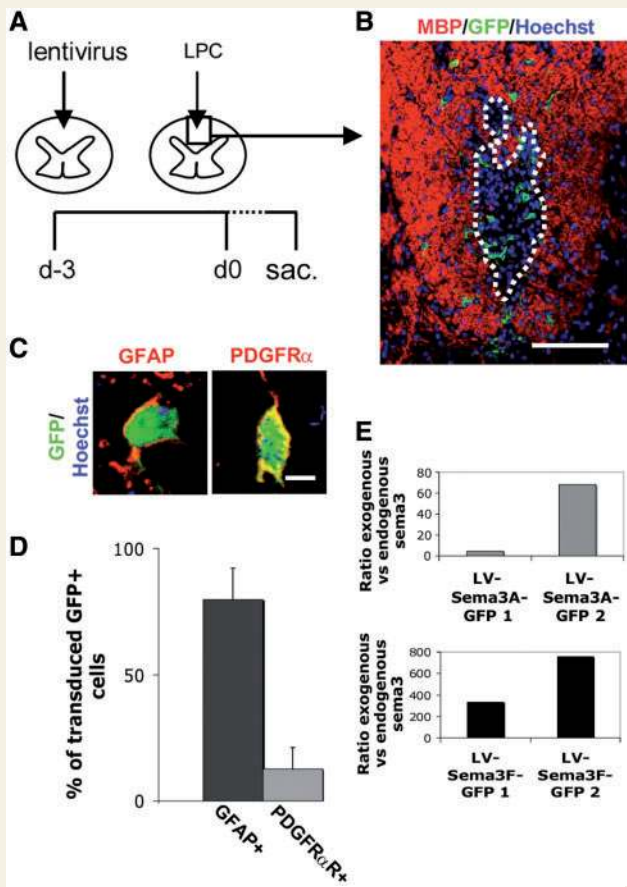
Figure 3 Lentiviral vector mediated gene transfer into a demyelinated lesion. (**A**) Schematic representation of the protocol: *Sema3A-GFP, Sema3F-GFP* or *GFP*-encoding lentiviral vector is stereotaxically injected into mouse dorsal columns of the spinal cord. Lysophosphatidylcholine (LPC) is injected at the same site 3 days later, and the mice sacrificed (sac) at different time points after lysophosphatidylcholine injection. (**B**) Transverse section of spinal cord dorsal column 7 days after lysophosphatidylcholine injection, stained with anti-myelin basic protein and anti-GFP antibodies: the demyelinated lesion, in the centre of the dorsal columns (delineated by a dotted line), contains green GFP-positive transduced cells. Scale bar = 100 µm. (**C**) Double stainings with anti-GFP and anti-GFAP or anti-PDGFRα antibodies on transduced spinal cord dorsal columns 3 days after lentiviral vector injection show that transduced cells are astrocytes and oligodendrocyte precursor cells. Scale bar: 5 µm. (**D**) Histogram presenting the percentage (mean ± SEM) of GFP-positive cells expressing GFAP or PDGFRα: transduced cells are mainly astrocytes, and to a lesser extent oligodendrocyte precursor cells. (**E**) Real-time quantitative polymerase chain reaction was performed on spinal cord blocks 3 days after transduction with either *Sema3A-GFP* or *Sema3F-GFP*, using primers specific for endogenous mouse *Sema3* and primers specific for *GFP* (allowing the detection of exogenous lentiviral *Sema3*). Histograms illustrate examples of ratios of exogenous versus endogenous levels of expression for *Sema3A-GFP*-transduced and *Sema3F-GFP*-transduced spinal cord dorsal columns (*n* = 2). Given the variability of the ligand level, only samples with high infectivity were used for quantification, as described in the 'Materials and methods' section.

expressing cleaved caspase 3 (Fig. 4E); similar results were obtained when FACS sorted adult oligodendrocyte precursor cells were treated *in vitro* for 24 h with Sema3A-Fc (1.6 ± 1.5%) or human Fc (1.8 ± 0.7%).

Loss of semaphorin 3A signalling favours oligodendrocyte precursor cell recruitment

In the *NRP1Sema-* transgenic mouse line, a mutation introduced in *NRP1* prevents Sema3A binding to NRP1, but still allows vascular endothelial growth factor binding (the other ligand of NRP1), avoiding angiogenic and vascular defects (Gu *et al.*, 2003) (Fig. 5A). An increased PDGFRα-positive cell density was found 7 days after lysophosphatidylcholine injection in homozygous *NRP1Sema-* compared with wild-type mice (*P* = 0.001), similar to the level previously achieved by Sema3F overexpression, suggesting that oligodendrocyte precursor cells are responding unhindered to the endogenous Sema3F signal (Fig. 5B). These loss of function experiments further support the chemo-repulsive role of Sema3A, influencing negatively adult oligodendrocyte precursor cell recruitment towards a demyelinated lesion.

Taken together, these data show that manipulation of class 3 semaphorin levels in a demyelinated acute lesion is sufficient to modify the time-course of oligodendrocyte precursor cell recruitment towards the lesion; Sema3F accelerates adult oligodendrocyte precursor cell recruitment through a chemo-attractive effect, whereas overexpression of Sema3A delays it through a chemo-repulsive effect.

Impact of oligodendrocyte precursor cell recruitment on remyelination

Having shown that the level of Sema3A or Sema3F influences oligodendrocyte precursor cell recruitment after demyelination transiently, we questioned the consequence of early remyelination. We first showed that, 14 days post-demyelination, Sema3F overexpression was associated with a 77% increase in the density of mature oligodendrocytes (expressing the adenomatus Polyposis Coli CC1 marker) within the demyelinated area (*P* = 0.0001) (Fig. 6A). This suggested that oligodendrocyte precursor cells attracted to the lesion by overexpressed Sema3F had matured locally. We next analysed remyelination at the ultrastructural level 10 days after lysophosphatidylcholine injection (Fig. 6B–D). Whereas there was no difference between animals receiving *Sema3A-GFP* lentiviral vector and controls, the density of axons remyelinated by oligodendrocytes was significantly higher in *Sema3F-GFP*-transduced lesions compared to *GFP*-transduced lesions (*P* = 0.036). Similarly, the density of demyelinated axons was lower in *Sema3F-GFP*-transduced lesions (*P* = 0.078) (Fig. 6D). In all groups, a few axons remyelinated by Schwann cells were detected, with no significant difference (not shown). To control for a possible additive pro-maturation action of Sema3F, we analysed the effect of Sema3F-Fc on spinal cord FACS sorted adult oligodendrocyte precursor cells. No difference in the proportion of
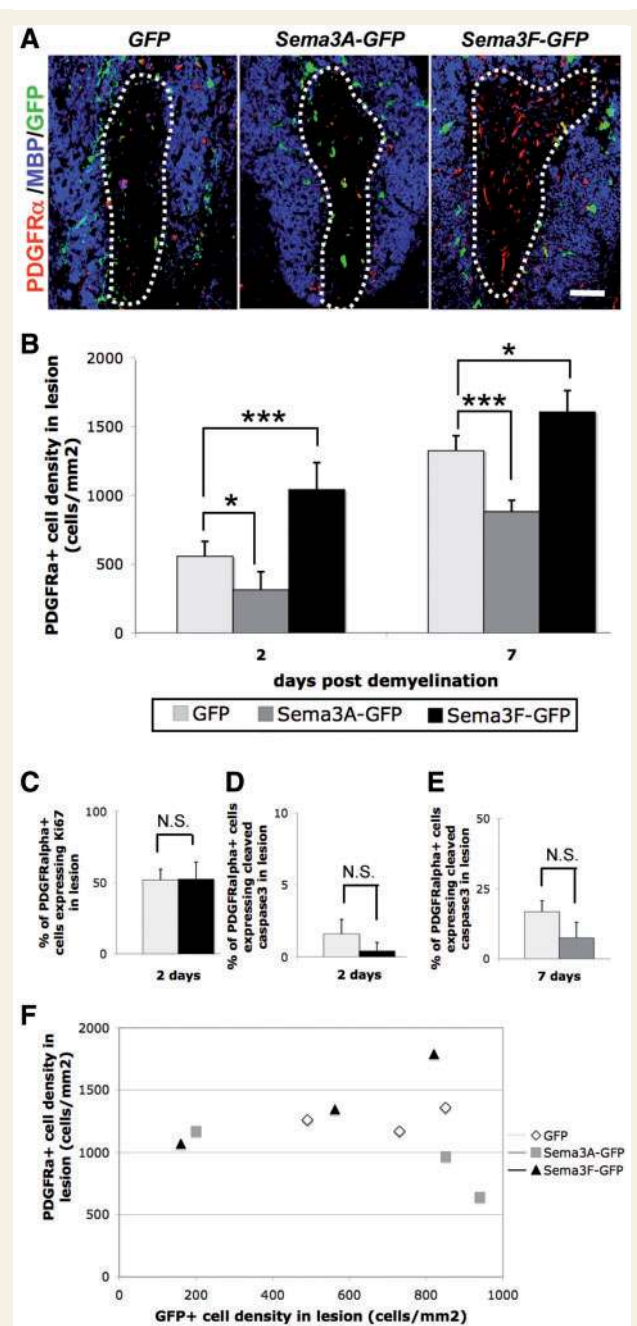
Figure 4 Sema3A and Sema3F overexpression influence oligo-dendrocyte precursor cell recruitment to a demyelinated lesion. (**A**) Triple-stainings with anti-GFP, anti-PDGFRα and anti-myelin basic protein antibodies on transverse sections of spinal cord dorsal columns transduced with *GFP*, *Sema3A-GFP* or *Sema3F-GFP* lentiviral vector, 2 days after lysophosphatidyl-choline injection. Dotted lines mark the boundaries of demyeli-nated lesions in the dorsal columns. Scale bar = 50 μm. (**B**) Density of PDGFRα-positive oligodendrocyte precursor cells within a demyelinated lesion transduced with *GFP*, *Sema3A-GFP* or *Sema3F-GFP* lentiviral vector, 2 and 7 days after lyspho-sphatidylcholine injection. At both time points, oligodendrocyte precursor cell density is significantly increased in lesions trans-duced with *Sema3F-GFP*, and significantly decreased in lesions transduced with *Sema3A-GFP*, compared to lesions transduced with *GFP*. Data are mean ± SEM. *n* = 5 (minimum) per

condition. *$P < 0.05$, ***$P < 0.001$, *t*-test versus control. (**C**) Percentage (mean ± SEM) of PDGFRα-positive cells expressing Ki67 in lesions transduced with *Sema3F-GFP* or *GFP* lentiviral vectors, 2 days post-demyelination. (**D**) Percentage (mean ± SEM) of PDGFRα-positive cells expressing cleaved caspase 3 in lesions transduced with *Sema3F-GFP* or *GFP* lenti-viral vectors, 2 days post-demyelination. (**E**) Percentage (mean ± SEM) of PDGFRα-positive cells expressing cleaved caspase 3 in lesions transduced with *Sema3A-GFP* or *GFP* len-tiviral vector, 7 days post-demyelination. (**F**) Graph presenting the infectivity-dependence of Sema3 effect in demyelinated le-sions, as a subset of experiments: density of PDGFRα-positive cells in lesion is presented as a function of the density of GFP-positive cells in the same lesion transduced with *GFP*, *Sema3A-GFP* or *Sema3F-GFP* lentiviral vector. Dots correspond to mean density for one animal. N.S = not significant.
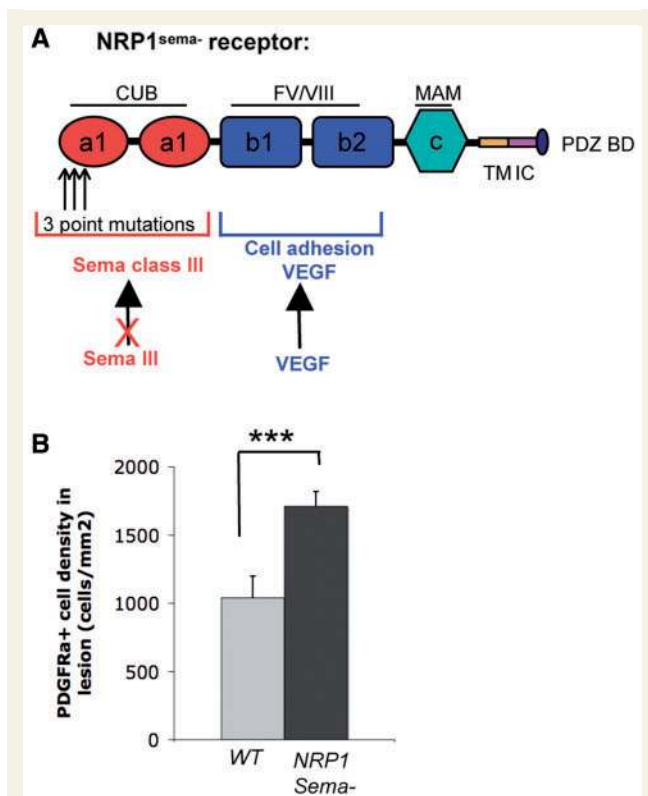
GFP-positive cells expressing myelin basic protein after 5 days *in vitro* (Sema3F-Fc: 92.3 ± 7.6%, human Fc: 97.3 ± 4.5%) or myelin oligodendrocyte glycoprotein after 10 days *in vitro* (Sema3F-Fc: 93.4 ± 2.8%, human Fc: 96.6 ± 1.2%) was de-tected. In summary, these results show that Sema3F overexpres-sion favours early remyelination and that the acceleration in myelin repair is largely due to an increase in the recruitment of myelin forming cells.

Discussion

In both multiple sclerosis and experimental models, remyelination efficiently protects axons from degeneration (Kornek *et al*., 2000; Irvine and Blakemore, 2008; Bruce *et al*., 2010). Hence, decipher-ing the molecular and cellular mechanisms involved in remyelina-tion is critical for building strategies aimed at enhancing repair. In this context, we had previously shown that Sema3A and Sema3F, known as guidance cues for embryonic oligodendrocyte precursor cells, were upregulated in multiple sclerosis and experimental de-myelination (Williams *et al*., 2007). Here, we addressed the ques-tion of the functional role that class 3 semaphorins play in oligodendrocyte precursor cell recruitment and remyelination. This was analysed in a focal, toxin-induced model of demyelin-ation. Unlike in multiple sclerosis, in this experimental model in-flammation and axonal loss are limited. However, such a simplified model is necessary to make scientific points that cannot be ad-dressed in an inflammatory and more complex model. On one hand, because semaphorins regulate T cell function (Takamatsu *et al*., 2010), experimental autoimmune encephalomyelitis models would not allow any clear conclusions to be drawn about their role in myelin regeneration. On the other hand, a model in which there is extensive axons loss would not be useful for studying remyelination. In addition, to our knowledge the mechanisms of regeneration are independent of the mechan-isms of tissue injury. What is discovered about remyelination biol-ogy from simple toxin models is therefore likely to be relevant to the entire range of models including immune-mediated ones.

We show that these guidance molecules act on adult oligo-dendrocyte precursor cell recruitment after demyelination, with

Figure 5 Sema3A loss of function results in increased oligo-dendrocyte precursor cell recruitment to a demyelinated lesion. (**A**) Schematic representation of mutated NRP1 in *NRP1Sema-* animals: *NRP1* gene carries a mutation in the binding site of semaphorin, preventing Sema3A binding. *Vascular endothelial growth factor* (VEGF) binding site is unaffected, avoiding angiogenic defects (Gu *et al.*, 2003). (**B**) Density of PDGFRα-positive cells within the lesion of wild-type (WT) mice (*n* = 4) and *NRP1Sema-* mice (*n* = 3) 7 days after lysophospha-tidylcholine injection. Results are mean ± SEM. ***P < 0.001 (Mann–Whitney U-test).

receptor CXCR2 was not found on oligodendrocyte precursor cells (Omari *et al.*, 2005) and the effect of CXCL1/CXCR2 signal-ling inhibition could be indirectly due to immunomodulation and/ or neuroprotection (Omari *et al.*, 2009). Epidermal growth factor and CXCL12 have been shown to promote migration of subven-tricular zone-derived cells and grafted neural stem cells respect-ively (Aguirre *et al.*, 2007; Carbajal *et al.*, 2010; Patel *et al.*, 2010). However their effect on parenchymal oligodendrocyte pre-cursor cells remains to be elucidated. Here, we provide the first evidence that Sema3A and Sema3F act on adult parenchymal oligodendrocyte precursor cell migration. While in control CNS, only a small proportion of adult oligodendrocyte precursor cells express neuropilin receptors, this percentage increases after de-myelination, with a strong and early upregulation of NRP2 and a lower and delayed expression of NRP1. These results allow us to hypothesize that, following acute demyelination, the delayed ex-pression of Sema3A may serve as a stop signal, after early increase of Sema3F has attracted a sufficient pool of future remyelinating cells. NRP1-positive cells detected within the lesion might be driven by other, non-Sema3, cues. The relative expression of Sema3A and Sema3F might therefore be one of the key factors controlling oligodendrocyte precursor cell recruitment after de-myelination and their unbalanced expression in multiple sclerosis lesions is likely to result in defective oligodendrocyte precursor cell recruitment (Franklin, 2002; Williams *et al.*, 2007).

Repair failure in multiple sclerosis has often been attributed to defective oligodendrocyte precursor cell differentiation into myeli-nating oligodendrocytes, due to local inhibitors present in demye-linated lesions, including astrocytic hyaluronan (Back *et al.*, 2005), activation of the oligodendroglial Notch/jagged pathway (John *et al.*, 2002; Zhang *et al.*, 2009), axonal or oligodendroglial LINGO-1 (Nogo-receptor interacting protein) (Mi *et al.*, 2009). Alternatively, axonal inhibitory factors are likely to act on axonal permissiveness to repair, such as adhesion molecule PSA-NCAM (polysialic acid-neural cell adhesion molecule) re-expressed on demyelinated axons in multiple sclerosis plaques (Charles *et al.*, 2002). Remyelination capacity was shown to be maintained after several experimental toxin-induced demyelinating insults, suggest-ing that successive episodes had not exhausted the oligodendro-cyte precursor cell pool (Penderis *et al.*, 2003). Increased oligodendrocyte precursor cell density within an lysophosphatidylcholine-induced demyelinated lesion, through transgenic overexpression of PDGF-A, the major oligodendrocyte precursor cell mitogenic and survival factor, in astrocytes, did not result in increased remyelination in *GFAP-PDGF-A*$^{+/-}$ mice (Woodruff *et al.*, 2004). These results had led to the concept that block of differentiation, rather than a defect in oligodendro-cyte precursor cell recruitment, was limiting remyelination cap-acity. Our results, in contrast, demonstrate for the first time that increased oligodendrocyte precursor cell recruitment can increase remyelination. This discrepancy between our results and the pre-vious study might be due to the potent mitogenic effect of sus-tained release of PDGF-A in *GFAP-PDGF-A*$^{+/-}$ mice, which might prevent oligodendroglial maturation, as suggested by the observa-tions of lesions full of closely packed cells resembling oligodendro-cyte precursor cells (Woodruff *et al.*, 2004). In contrast, in our experimental paradigm, recruited oligodendrocyte precursor cells
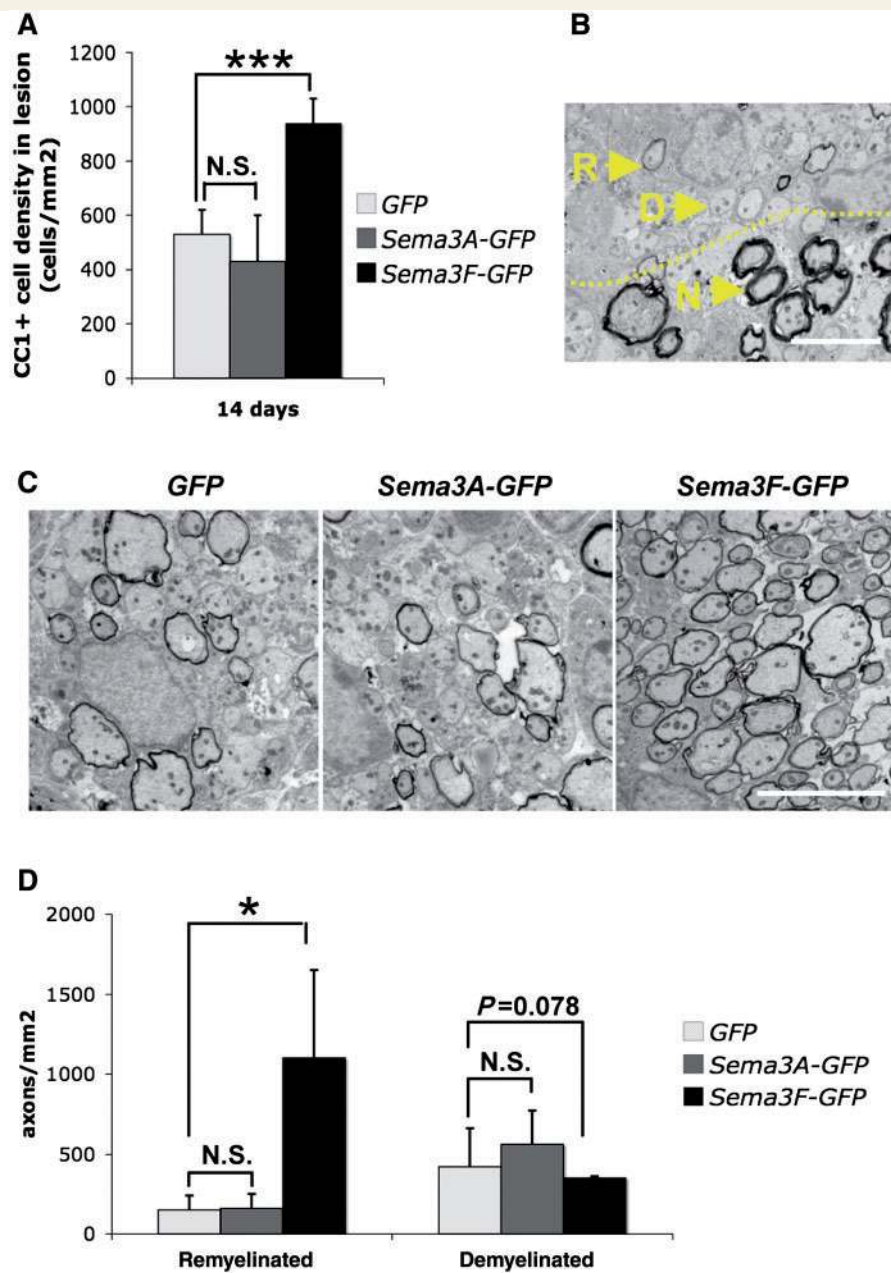
an attractive effect for Sema3F (both *in vitro* and *in vivo*) and a repulsive effect for Sema3A *in vivo*. The lack of *in vitro* effect of Sema3A may be due to the restricted percentage of adult cells expressing NRP1 in control conditions, compared to increased ex-pression after demyelination. Importantly, Sema3F overexpression also accelerates myelin repair, supporting the concept that facil-itating oligodendrocyte precursor cell access to lesions and/or increasing the pool of oligodendrocyte precursor cells can have functional consequences on the remyelination rate. Several oligo-dendrocyte precursor cell recruitment signals have been identified during development and include both secreted cues, such as semaphorins, netrin-1 (Jarjour *et al.*, 2003), chemokines such as CXCL1 (Tsai *et al.*, 2002) and CXCL12 (Dziembowska *et al.*, 2005), growth factors (PDGF, fibroblast growth factor) (Bribian *et al.*, 2006) and contact-mediated mechanisms such as extracel-lular matrix proteins, integrins, N-cadherins and ephrins (Prestoz *et al.*, 2004; Piaton *et al.*, 2009). In multiple sclerosis, CXCL1 is upregulated at the periphery of plaques, coincident with the ac-cumulation of oligodendrocyte precursor cells. However, its

Figure 6 Sema3F overexpression increases oligodendrocyte maturation and remyelination. (**A**) Density of CC1-positive mature oligodendrocytes within lesions transduced with *GFP* (*n* = 5), *Sema3A-GFP* (*n* = 6) and *Sema3F-GFP* (*n* = 6) lentiviral vectors, 14 days after lysophosphatidylcholine injection. *Sema3F-GFP*-transduced lesions contain more CC1-positive oligodendrocytes. Results are mean ± SEM. ***$P < 0.001$ (*t*-test versus control). (**B**) Electron micrograph of a lesion border, showing representative demyelinated (D), remyelinated by oligodendrocyte (R) and normally myelinated (N) axons in lesion, 10 days post-demyelination. Dotted line marks the boundary of the demyelinated lesion. Scale bar = 1 μm. (**C**) Electron microscopy of spinal cord lesions transduced with either *GFP* or *Sema3A-GFP* or *Sema3F-GFP* lentiviral vectors, 10 days post-lysophosphatidylcholine injection. Scale bar = 1 μm. (**D**) Density of remyelinated and demyelinated axons, within lesions transduced with *GFP* (*n* = 5), *Sema3A-GFP* (*n* = 4) and *Sema3F-GFP* (*n* = 3) lentiviral vectors, 10 days post-lysophosphatidylcholine injection. *Sema3F-GFP*-transduced lesions contain more remyelinated axons. Results are mean ± SEM. *$P < 0.05$ versus control (Mann–Whitney U-test). N.S = not significant.

differentiate into mature CC1-positive cells and become remyelinating oligodendrocytes. In addition, our results, showing increased remyelination in Sema3F-transduced spinal cords at 10 days post-lesion, are consistent with an acceleration of the repair

process due to early oligodendrocyte precursor cell recruitment. This acceleration of repair might have been missed in the other experiments where remyelination was quantified at later time points. Moreover, in a different model of cuprizone-induced

chronic demyelination, PDGF-A-induced increased density of oligodendrocyte precursor cells also correlated with improved remyelination through a pro-survival effect (Vana *et al.*, 2007), strengthening the concept that increasing the oligodendrocyte precursor cell population within a demyelinated area can promote repair. Taken together, these data suggest that not only a block of differentiation, but also a defect in oligodendrocyte precursor cell recruitment might participate in myelin repair failure. Finally, these different and not exclusive mechanisms impairing repair have to be replaced in the general context of age-related decline of remyelination efficacy and in particular age-related cell intrinsic changes (Rist and Franklin, 2008).

In acute models of demyelination, robust remyelination occurs, thus accelerating remyelination does not impact the final remyelination capacity. It might, however, be crucial in multiple sclerosis, where remyelination is in most cases insufficient. The importance of axonal damage in multiple sclerosis has been highlighted by several recent studies. Whereas the mechanisms might be many and not exclusive, demyelination is probably the major cause of axonal damage leading to loss of trophic support, mitochondrial dysfunction, excitotoxic mechanisms (Nave and Trapp, 2008) and re-expression of axonal surface inhibitory adhesion molecules (Charles *et al.*, 2002). The chronology of axonal damage after demyelination is unknown, but there appears to be a window of time during which axons are either undamaged or reversibly damaged (Crawford *et al.*, 2009). When this window is exceeded, irreversible axonal pathology occurs, leading to axonal loss and disability progression.

Our data, showing that manipulation of guidance cues influences oligodendrocyte precursor cell recruitment and remyelination leads to a proof of concept, namely that strategies to inhibit Sema3A and/or enhance Sema3F signalling in oligodendrocyte precursor cells in demyelinated lesions are likely to speed up remyelination and protect axons. In the future, these strategies will need, for translational development, to be evaluated in animal models more closely mimicking the clinical disease.

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Supplementary material

Supplementary material is available at *Brain* online.

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