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

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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 oblige 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 Ph20TM DNA polymerase (Invitrogen) using the following primers: GFP-Sema3A sense: 5′ CACCATGGCGCTTTAC 3′; GFP-Sema3A antisense: 5′ TCACGACCTCTGGTGCCTTCCC 3′; GFP-Sema3F sense: 5′ CACCATGCTTGTCGCCGGTCTT 3′; GFP-Sema3F antisense: 5′ CCGCTTTCACTTGACGCTTC 3′. The resulting polymerase chain reaction products were cloned into pEENTR/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., 1996) and the lentiviral recombinant cytomegalovirus vector pTRip U3 and pTRip U3 to select the vector infecting mostly astrocytic cells in this model. Human embryonic kidney cell culture and transduction

HEK 293T cells were grown in Dulbecco’s modified Eagle medium-GlutamaxTM (Gibco) supplemented with 10% foetal calf serum at 37°C in a 5% CO₂ 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 FACSorted (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-cysteine 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 0.9% foetal calf serum, 2 mM l-glutamine, 10 μM insulin, 5 mg/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 FACSorted cells were resuspended in Bottenstein Sato medium at a cell density of 10⁶ 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 transfused 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. Lyso- sphatidylcholine injections were performed 3 days after lentiviral injection: the suture and the vertebral arch were removed and 1 μl of lyosphatidylcholine (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 at 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 (Clinsciences, 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, 1:50, AbD Serotec).

Transmission electron microscopy

Animals were perfused 10 days after lyso phosphatidylcholine injection with a 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 15% 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 positivity on ultrathin sections: grid squares (100 000 µm²) 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 (thinner 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
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 ± 7.4%) or human Fc (26.4 ± 11.8%). In contrast, Sema3A added in the bottom compartment had no effect. These results show that Sema3F has a chemotactic 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 PDGFR\(^+\) 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 ± 8.5%). Neuropilin1-positive oligodendrocyte precursor cells were mainly detected later, 55.3 ± 1.7% of oligodendrocyte precursor cells expressing NRP1 14 days after demyelination (Fig. 2A and C). Among PlexinA receptors, 33.4 ± 12.4% of oligodendrocyte precursor cells expressed PlexinA1, 84.9 ± 0.4% expressed PlexinA2 and 50.3 ± 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 ± 3.9% expressing PlexinA1, 84.5 ± 3.7% expressing PlexinA2 and 65.8 ± 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 ± 1.1% in uninjured conditions, 25.1 ± 4.9% 7 days after demyelination), NRP1- and NRP2-expressing oligodendrocyte precursor cells may represent different subpopulations.

Figure 1 Adult oligodendrocyte precursor cells isolated from PDGFR\(^+\)-GFP CNS express Sema3 receptors and are attracted by Sema3F. (A) FACsorting of GFP-positive cells from 2 month-old brain of PDGFR\(^+\)-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 \( \mu \)m. (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 \( \mu \)m. (D) GFP-positive FACS isolated cells from PDGFR\(^+\)-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).
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).

Sema3 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.
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 lysophosphatidylycholine 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 lysophosphatidylycholine 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
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 detected. In summary, these results show that Sema3F overexpression 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, deciphering the molecular and cellular mechanisms involved in remyelination 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 demyelination (Williams et al., 2007). Here, we addressed the question 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 demyelination. Unlike in multiple sclerosis, in this experimental model inflammation and axonal loss are limited. However, such a simplified model is necessary to make scientific points that cannot be addressed 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 mechanisms of tissue injury. What is discovered about remyelination biology 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 oligodendrocyte precursor cell recruitment after demyelination, with

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 detected. In summary, these results show that Sema3F overexpression 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, deciphering the molecular and cellular mechanisms involved in remyelination 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 demyelination (Williams et al., 2007). Here, we addressed the question 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 demyelination. Unlike in multiple sclerosis, in this experimental model inflammation and axonal loss are limited. However, such a simplified model is necessary to make scientific points that cannot be addressed 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 mechanisms of tissue injury. What is discovered about remyelination biology 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 oligodendrocyte precursor cell recruitment after demyelination, with...
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 expression after demyelination. Importantly, Sema3F overexpression also accelerates myelin repair, supporting the concept that facilitating oligodendrocyte precursor cell access to lesions and/or increasing the pool of oligodendrocyte precursor cells can have functional consequences on the remyelination rate. Several oligodendrocyte precursor cell recruitment signals have been identified during development and include both secreted cues, such as semaphorins, netrin-1 (Jarjour et al., 2002), 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 extracellular 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 accumulation of oligodendrocyte precursor cells. However, its receptor CXCR2 was not found on oligodendrocyte precursor cells (Omari et al., 2005) and the effect of CXCL1/CXCR2 signaling 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 subventricular zone-derived cells and grafted neural stem cells respectively (Aguirre et al., 2007; Carbajal et al., 2010; Patel et al., 2010). However their effect on parenchymal oligodendrocyte precursor 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 demyelination, 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 expression 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 demyelination 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 myelinating oligodendrocytes, due to local inhibitors present in demyelinated 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, suggesting that successive episodes had not exhausted the oligodendrocyte precursor cell pool (Penderis et al., 2003). Increased oligodendrocyte precursor cell density within an lyso phosphatidylcholine-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-PDGFA-A/-/- mice (Woodruff et al., 2004). These results had led to the concept that block of differentiation, rather than a defect in oligodendrocyte precursor cell recruitment, was limiting remyelination capacity. 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 previous study might be due to the potent mitogenic effect of sustained release of PDGF-A in GFAP-PDGF-A-A/- mice, which might prevent oligodendroglial maturation, as suggested by the observations of lesions full of closely packed cells resembling oligodendrocyte precursor cells (Woodruff et al., 2004). In contrast, in our experimental paradigm, recruited oligodendrocyte precursor cells...
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., 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.

References


