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Inhibition of CNS Remyelination by the Presence of Semaphorin 3A

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Failure of oligodendrocyte precursor cell (OPC) differentiation has been recognized as the leading cause for the failure of myelin regeneration in diseases such as multiple sclerosis (MS). One explanation for the failure of OPC differentiation in MS is the presence of inhibitory molecules in demyelinated lesions. So far only a few inhibitory substrates have been identified in MS lesions. Semaphorin 3A (Sema3A), a secreted member of the semaphorin family, can act as repulsive guidance cue for neuronal and glial cells in the CNS. Recent studies suggest that Sema3A is also expressed in active MS lesions. However, the implication of Sema3A expression in MS lesions remains unclear as OPCs are commonly present in chronic demyelinated lesions. In the present study we identify Sema3A as a potent, selective, and reversible inhibitor of OPC differentiation *in vitro*. Furthermore, we show that administration of Sema3A into demyelinating lesions in the rat CNS results in a failure of remyelination. Our results imply an important role for Sema3A in the differentiation block occurring in MS lesions.

Introduction

Chronic demyelination in multiple sclerosis (MS) represents a significant challenge to clinical neurology. Chronic demyelination results from failed attempts of myelin regeneration, or "remyelination," and leaves axons chronically denuded. As demyelinated axons are prone to injury (Nguyen et al., 2009) and degeneration (Irvine and Blakemore, 2008), chronic demyelination is likely to significantly contribute to the clinical burden of the disease.

Although the mechanisms involved in CNS remyelination have been an intense focus of research, no treatments currently exist that are able to promote myelin regeneration in clinical disease. Remyelination in the CNS is mediated by a multipotent adult stem-precursor cell population commonly referred to as oligodendrocyte precursor cells (OPCs) (Dubois-Dalcq et al., 2008; Franklin and ffrench-Constant, 2008). Successful remyelination relies on the recruitment of OPCs into areas of demyelination, their subsequent engagement of denuded axons and their differentiation into mature oligodendrocytes.

Systematic assessment of MS lesions revealed that in most chronic demyelinated lesions OPCs are present (Kuhlmann et al., 2008). Therefore the processes involved in OPC differentiation seem to be more vulnerable than the processes involved in OPC recruitment. In contrast to the clinical situation, experimental studies have demonstrated that OPC differentiation occurs efficiently in animal models of CNS remyelination. To explain the discrepancies between the efficiency of OPC differentiation and remyelination in experimental models and human disease, the concept has emerged that MS lesions contain specific OPC differentiation-inhibiting factors. So far a number of OPC differentiation inhibitors have been indentified in MS lesions (Wang et al., 1998; Charles et al., 2002; John et al., 2002; Back et al., 2005; Fancy et al., 2009; Nakahara et al., 2009). Adding to the group of inhibitors, we were recently able to show that unknown proteins in myelin debris (myelin-associated inhibitors) that accumulate as a consequence of demyelination also inhibit remyelination by inhibiting OPC differentiation (Kotter et al., 2006; Syed et al., 2008; Baer et al., 2009).

Semaphorins comprise a large family of molecular guidance cues, defined by their semaphorin (Sema) domain and further distinguished by distinct protein domains including thrombospondin, Ig-like, and basic C-terminal domains. Little is known about the role of semaphorins in the context of oligodendrocyte biology. A previous study has associated Sema3A with negative guidance cues acting on OPC migration and diverting OPC processes (Spassky et al., 2002). Furthermore, a recent postmortem study investigated the expression of Sema3A in brains of individuals suffering from MS and controls (Williams et al., 2007). While control white matter and chronic MS lesions did not show any evidence of Sema3A expression, an upregulation of Sema transcripts was detected in active MS lesions. OPCs present in active MS lesions expressed the Sema receptor neuropilin-1 (NP-1) indicating that OPCs become reactive to Sema3A. How-

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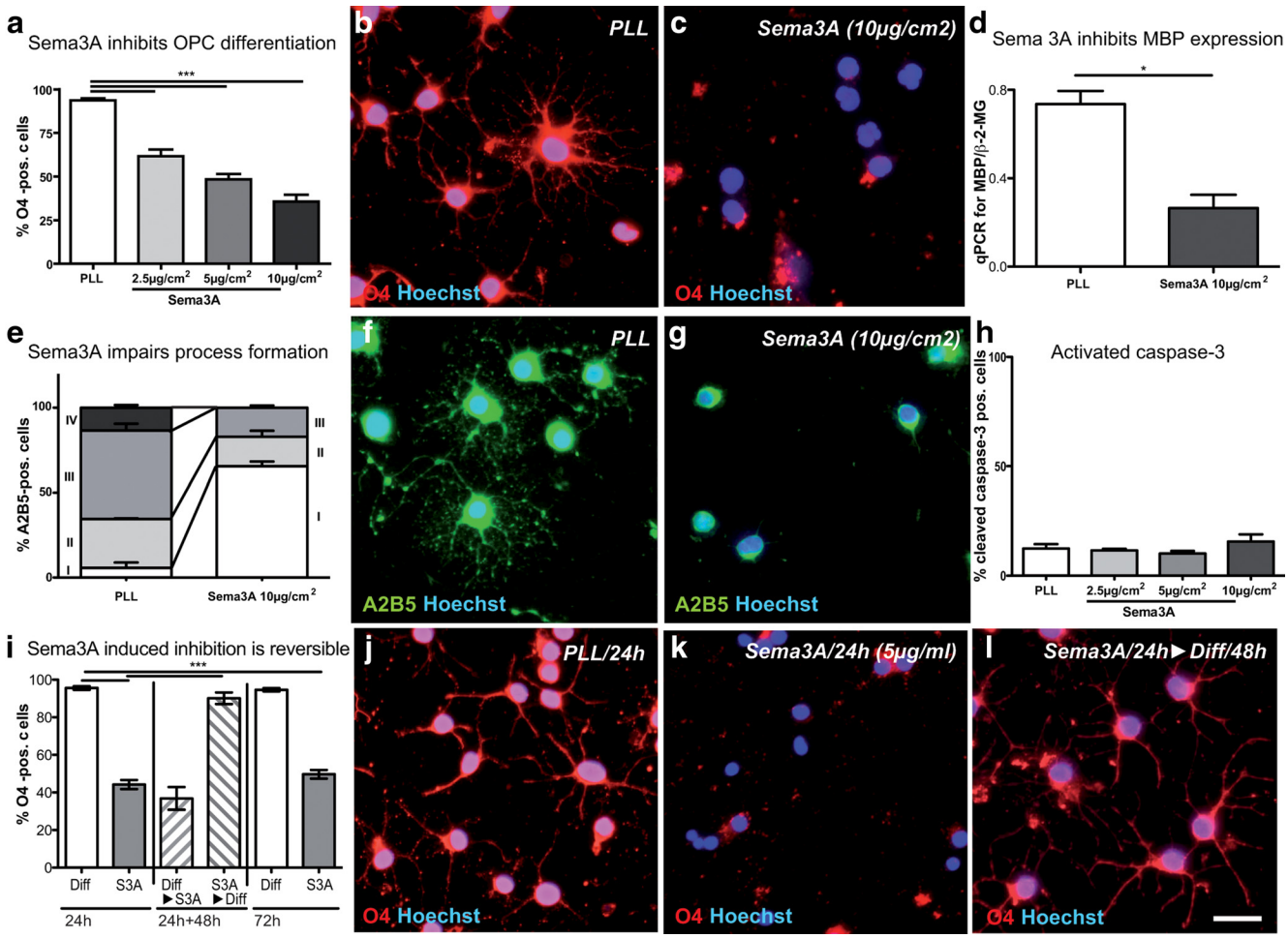

Figure 1. ***a–c***, OPCs plated on Sema3A substrates (protein/cm ^{2}) display a concentration-dependent downregulation of O4 expression after 2 d of culture in differentiation medium. ***d***, Furthermore, RT-qPCR demonstrates that Sema3A inhibits the expression of MBP mRNA. ***e–g***, The presence of Sema3A also induces a reduction of the complexity of OPC processes and an increase of earlier morphological stages compared with OPCs plated on control substrate (I, mono/bipolar; II, primary; III, secondary; IV, membranous branches). ***h***, Sema3A selectively affects OPC differentiation as no differences in cell death were detected by activated caspase-3 staining. ***i–l***, The inhibition of OPC differentiation by Sema3A is reversible. Whereas the majority of OPCs in differentiation medium are O4-positive after 24 h (Diff/24 h), addition of Sema3A (S3A/24 h) inhibits O4 expression. Cells remained inhibited as long as Sema3A was present in the medium (S3A/72 h). In contrast, 48 h following replacement with non-Sema3A-containing medium OPCs (Sema3A/24 h▶Diff/48 h "withdrawal") display O4 immunoreactivity corresponding to levels of control cells (Diff/24 h and Diff/72 h). Conversely, replacement of differentiation medium with Sema3A-containing medium after 24 h results in an inhibition of OPC differentiation (Diff/24▶Sema3A/48 h). Scale bar, 30 μm. In this and subsequent figures, error bars represent SEM.

ever, Sema3A expression in demyelinated lesions did not prevent the recruitment of OPCs.

The experiments summarized in the present article identify Sema3A as a regulator of OPCs *in vitro* whose presence induces a potent, selective, and reversible inhibition of OPC differentiation. Furthermore, we tested the hypothesis that Sema3A inhibits CNS remyelination by administering recombinant Sema3A into experimentally demyelinated lesions. Our data indicate that the presence of Sema3A is able to induce a failure of CNS remyelination without affecting the presence of OPCs.

Materials and Methods

Preparation of primary OPC cultures. Primary OPC cultures were isolated from neonatal Sprague Dawley (postnatal day 0–2) rat forebrains following a standard protocol (Baer et al., 2009). Differentiation was induced by Sato's medium supplemented with 0.5% FCS. Only cultures with >94% purity were used.

In preliminary experiments that have lead to the present study, OPCs were cultured in medium conditioned by Sema3A-AP (alkaline phosphatase)-expressing Hek 293 cells (kindly provided by Prof. James Fawcett, University of Cambridge, Cambridge, UK); the Sema3A-AP construct is described by Giger et al. (1998) and was a generous gift from Prof. Joost Verhaagen, Netherlands Institute for Neuroscience (Amsterdam, The Netherlands). OPCs react to Sema3A-AP at the concentrations commonly used (supplemental Fig. 1*a*, available at www.jneurosci.org as supplemental material) (Schlomann et al., 2009). OPCs react to commercially available recombinant Sema3A-Fc (R&D Systems) at corresponding concentrations (5 μg/ml⇔44.24 nM). For the purpose of limiting any confounding factors all subsequent experiments were performed using recombinant Sema3A-Fc from the commercial source. Thus, either Sema3A substrates were prepared by incubation of poly-L-lysine (PLL)-coated slides with recombinant Sema3A-Fc or, alternatively, recombinant Sema3A-Fc was added to Sato's differentiation medium. To assess whether the effects of Sema3A are reversible cells were first cultured in Sema3A containing differentiation medium. After 24 h the medium was replaced with Sema3A-free differentiation medium and the cells were cultured for further 48 h (Fig. 1*i*).

Immunocytochemical analysis. OPCs were seeded onto PLL- or Sema3A-coated 8-well chamber slides (2×10^4 cells/well). Following differentiation (48 h), cells were stained with O4 (1:100; Calbiochem;) or A2B5 antibodies (1:100; Millipore Corporation; Cy3-conjugated secondary antibody 1:100 dilution, Jackson ImmunoResearch) (Syed et al., 2008; Baer et al., 2009). To assess OPC differentiation the percentage of $O4^+$ cells to >100 4′,6-diamidino-2-phenylindole dihydrochloride-stained nuclei per experiment for each condition in randomly selected

eye fields was determined. To assess the morphological phenotype of OPCs, >100 A2B5 stained cells were categorized as follows: stage I: mono/bipolar; stage II: multipolar, primary branched; stage III: multipolar, secondary branched; stage IV: secondary branched cells with membranous processes. At least three independent experiments were conducted. Neuropilin-1 (NP-1) expression by cultured OPCs was assessed using NP-1 (1:200; Abcam; Alexa Fluor 488-conjugated secondary antibody 1:500 dilution) and A2B5 (1:100; Millipore Corporation; Alexa Fluor 548-conjugated secondary antibody 1:500). Cells were visualized on a LSM 5 Pascal confocal microscope (Zeiss).

Assessment of cell viability and apoptosis. Fragmented DNA was detected by terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick end labeling (TUNEL) assay (Promega) and the percentage of apoptotic nuclei was determined (Baer et al., 2009). To confirm that Sema3A does not affect cell survival in addition to TUNEL assays the percentage of active caspase-3-positive cells was determined (active caspase-3 antibody, Abcam, 1:300; Alexa Fluor 488- or 548-conjugated secondary antibody, Invitrogen, 1:300).

Quantitative reverse transcriptase PCR. Total RNA was extracted using RNeasy Mini Kit (Qiagen). Quantitative PCR (qPCR) was conducted as previously outlined (Baer et al., 2009) on an Applied Biosystems 7500HT Fast Real-time PCR system. Values are represented as myelin basic protein (MBP)/β2-microglobulin ratios. Triplicate measurements were made on three biological replicates.

Induction of focal demyelination. All experiments were conducted in accordance with animal welfare regulations of the German animal protection law, the state of Lower Saxony (Niedersächsisches Landesamt für Verbraucherschutz und Lebensmittelsicherheit (license: RKO_033/2008) and Max Planck Institute guidelines.

Female Sprague Dawley rats (180–200 g) were anesthetized with ketamine (75 mg/kg) and xylazine (10 mg/kg) and positioned in a stereotactic instrument (custom made). Demyelination was induced bilaterally by stereotactic injection of ethidium bromide (0.01%, 4 μl) into the caudal cerebellar peduncle (CCP) (10.4 mm caudal, ±2.6 mm lateral and 7.07 mm ventral to bregma) with slight modifications to the method detailed previously (Kotter et al., 2006) (supplemental Fig. 2*a*, available at www.jneurosci.org as supplemental material).

For continuous local delivery of proteins into demyelinated lesion, a 30 gauge modified mouse osmotic pump connector cannula with dummy wire (tubing length of 6.1 mm below the cannula pedestal; Plastics One Inc.) was placed at the same coordinates and attached to the skull (Loctite 454).

Infusion of Sema3A into demyelinated lesions. Ten days following lesion induction osmotic pumps (0.5 μl/h; 14 d, Alzet 2002, Durect Corporation) were filled with Sema3A (R&D Systems; 50 μg/ml in PBS) or PBS according to the manufacturers instructions. The surgical wound was reopened, the dummy wire removed and the osmotic pumps were connected and implanted subcutaneously. To verify efficient delivery of Sema3A into the target area, a dye experiment was performed (supplemental Fig. 2*b*, available at www.jneurosci.org as supplemental material). To test whether Sema3A alters the appearance of normal white matter, Sema3A was infused into naive CCPs. Sema3A neither affected the presence of myelin sheaths nor induced any other visible effects in normal white matter (supplemental Fig. 2*c*, available at www.jneurosci.org as supplemental material).

In situ *hybridization.* The expression of a number of marker mRNA species in demyelinated lesions was examined by *in situ* hybridization with digoxigenin-labeled cRNA probes. *In situ* hybridization probes for macrophage scavenger receptor type B (MSR-B), platelet-derived growth factor receptor-α (PDGFR-α), Nkx2.2, and proteolipid protein (PLP) were a gift from Prof. R. J. M. Franklin, University of Cambridge (Cambridge, UK). The *in situ* hybridization probe for Sema3A was kindly provided by Prof. J. Verhaagen, Netherlands Institute for Neuroscience.

Animals were perfused with 4% paraformaldehyde (PFA) via the left ventricle. The tissue was extracted, postfixed in 4%PFA, cryoprotected in 30% sucrose and snap frozen. *In situ* hybridizations were conducted on cryostat sections (15 μm) using established protocols (Kotter et al., 2006).

For two-color *in situ* hybridization (ISH), the sections were hybridized with two riboprobes, one labeled with digoxigenin (DIG) and the second with FITC. Transcripts of the DIG labeled probe were visualized with AP-conjugated anti-DIG antibody and nitroblue tetrazolium/BCIP (5-bromo-4-chloro-indoyl phosphate) (Roche), producing a dark blue deposit. Subsequently AP was inactivated by incubation in maleic acid buffer (65°C, 30 min) and the FITC-labeled probe visualized with AP-conjugated anti-FITC antibody and 2-(4-iodophenyl)-5-(4-nitrophenyl)-3-phenyltetrazolium chloride/BCIP (Sigma-Aldrich), resulting in magenta deposits.

Lesions were identified on digital images of solochrome cyanine-stained sections, and the lesion area was determined using ImageJ 1.43b (http://rsb.info.nih.gov/ij/). The same program was used to determine the number of stained cells within the lesions on digitized adjacent sections. To rule out effects attributable to the size of the lesion, the density of PLP-positive cells was assessed in the PBS group by linear regression (GraphPad, Prism). The inclusion of lesions $<0.4\ mm^2$, which were strongly populated with PLP-positive cells, resulted in a significant non-zero slope indicating an effect of the lesion size on the cell density. This may be explained by the fact that in small lesions recruitment of OPCs and the clearance of myelin debris is more efficient and thus remyelination becomes more efficient compared with larger lesions. No significant correlation between the size of the lesion and the density of PLP-positive cells was observed in lesions $>0.4\ mm^2$ area. Consequently, only lesions $>0.4\ mm^2$ were included in the analysis.

Immunohistochemistry. Tissue was processed as for ISH. Sections were incubated in sodium citrate (10 mM, microwave preheated), permeabilized, and blocked with PBS, 5% serum 0.3% Triton X-100 (1 h, room temperature), and incubated with primary antibody in PBS, 2% serum (overnight, 4°C). For neuropilin-1 immunohistochemistry (IHC) the permeabilization step was omitted. and the sections were blocked in PBS, 10% serum (1 h, room temperature) and incubated with primary antibody diluted in PBS, 2% serum (overnight, 4°C). Following a washing step (PBS), sections were incubated with appropriate Alexa 488- or 594-conjugated secondary antibodies (1:500, Invitrogen). Nuclei were visualized with Hoechst 33342 and the slides mounted with Fluor Save (Calbiochem). Images were acquired on a Confocal LSM 5 Pascal (Zeiss) microscope.

The following antibodies were used: polyclonal anti-Olig-2 (1:1000; Millipore), polyclonal anti-neuropilin-1 (NP1, 1:300; Abcam), polyclonal GFAP (1:500; Dako), monoclonal Nkx2.2 (1:300; Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA), monoclonal CC1 (1:300; Calbiochem). For combinations of ISH with IHC the ISH step was conducted first followed by IHC using the protocols provided (Arnett et al., 2004).

Histological analysis of remyelination. To assess the extent of remyelination tissue was fixed in 4% glutaraldehyde, osmicated and processed into resin (TAAB Laboratories) (Kotter et al., 2006). Sections (1 μm) were stained with methylene blue and Azur-II. Remyelination was assessed by rank analysis with researchers blind to experimental groups. Lesions with the greatest extent of remyelination were assigned the highest rank value.

Electron microscopy. Ultrathin sections (50 nm) containing the lesions were stained with aqueous 4% uranylacetate and lead citrate. The sections were visualized on an electron microscope (Leo EM912AB; Zeiss) and images were taken using an on-axis 2048 × 2048 charge-coupled device camera (Proscan, Schering).

Oil Red O staining. Oil Red O (Sigma) working solution was prepared by adding 20 ml of H_2O to 30 ml of 1% Oil Red O in isopropanol. Sections were stained for 10 min then washed for 4 min and counterstained in Carrazi's hematoxylin for 4 min. Following a 4 min wash in H_2O, the sections were differentiated in 0.5% aqueous hydrochloric acid for 7 s and again washed in water for 10 min. Finally, the slides were mounted using an aqueous mounting medium. Representative images of Oil Red O stained lesions were digitized and blindly ranked with the highest staining density receiving the highest rank value.

Data analysis. Data were analyzed using GraphPad software (Prism). Multiple group comparisons were conducted using one-way ANOVA followed by Dunnett's *post* test. To detect cellular differences between Sema3A-treated and PBS-infused animals and for analysis of qPCR data two-tailed Student's *t* tests were conducted. For rank analysis a two-tailed nonparametric Mann–Whitney test was used.

Table 1. List of proteins tested for their role in OPC differentiation

Protein	Biological function	Cellular location
Myelin-associated glycoprotein (MAG)	Member of the Ig superfamily binding sialic acid (Kelm et al., 1994; Crocker et al., 1998); one of several white matter inhibitors of neurite outgrowth *in vitro* and axonal regeneration *in vivo* (Filbin, 2003); inhibits microtubule assembly by a Rho-kinase-dependent mechanism (Mimura et al., 2006). Deletion of MAG induces a developmental delay in OPC differentiation and myelin formation (Pernet et al., 2008).	Membrane
Nogo-A	Myelin-associated neurite outgrowth inhibitor. Nogo-A plays a role both in the restriction of axonal regeneration after injury and in structural plasticity in the CNS of higher vertebrates (Prinjha et al., 2000; Fournier et al., 2001). Deletion of Nogo-A induces a developmental delay in OPC differentiation and myelin formation (Pernet et al., 2008).	Membrane
Oligodendrocyte myelin glycoprotein (OMgp)	A glycosyl phosphatidyl inositol-linked protein and minor component of both CNS and PNS myelin. OMgp expression is also found in cells extending processes to nodes of Ranvier that may prevent aberrant sprouting from the node (Huang et al., 2005). OMgp may also exert antiproliferative effects on OPCs and induce OPC differentiation (Vourc'h and Andres, 2004)	Membrane
Brevican	Highly abundant proteoglycan in normal brain (Yamaguchi, 2000).; contributes to the nonpermissive environment for axon regeneration (Quaglia et al., 2008).	Extracellular matrix Membrane Secreted
Netrin-1	Major neuronal guidance cue (Serafini et al., 1994); can provide both attractive and repulsive cues to neurons, depending on the receptors present and cellular context.	Extracellular matrix
	Early studies have indicated that netrin-1 acts as chemorepellent for optic nerve-derived OPCs (Sugimoto et al., 2001). Similarly, it has been shown that addition of netrin-1 to the medium is able to repel OPCs in transmigration assays as well as negatively regulate OPC process formation during a time interval ranging from 30 min to 16 h following exposure (Jarjour et al., 2003). However, the same group subsequently reported that netrin-1 does not affect the differentiation of immature or mature OPCs *in vitro* (observation period, 24 h). Moreover, data included in our paper demonstrate that netrin-1 is able to exert positive effects on process formation, branching, and myelin-like sheet formation (Rajasekharan et al., 2009). The temporal switch from netrin-1-mediated chemorepulsion and process inhibition to process elaboration occurring in OPCs was subsequently shown to be dependent on RhoA signaling (Rajasekharan et al., 2009). The present experiment assessed the OPC response following 48 h of exposure to netrin-1 substrates and mirrors the results reported following prolonged exposure to netrin-1 (≥24 h) (Rajasekharan et al. 2010; Rajasekharan et al., 2009).	Secreted
Myelin oligodendrocyte glycoprotein (MOG)	Target antigen for the experimental autoimmune encephalitis (Lebar et al., 1986); present on the outermost lamellae of mature CNS myelin. Its late appearance during myelinogenesis suggests that it contributes to myelin maturation or maintenance (Amor et al., 1994; Genain et al., 1999).	Membrane

Results

Sema3A induces a dose-dependent inhibition of OPC differentiation

We set out to assess the effects of a number of proteins on OPC differentiation that are known to act as lesion-associated inhibitors of axon regeneration. For this purpose we used a well established assay (Robinson and Miller, 1999; Baer et al., 2009) in which the expression of O4 was assessed on primary OPCs cultured on substrates prepared from various molecules as summarized in Table 1 and by Syed et al. (2008). Of the molecules tested only the presence of Sema3A resulted in a reduction of differentiating O4-positive OPCs (Fig. 1*a*; supplemental Fig. 1*a–c*, available at www.jneurosci.org as supplemental material). The inhibitory effect of Sema3A was confirmed in three independent experiments assessing O4 antigeneity ($n = 3$; one-way ANOVA: $p \leq 0.0001$; Dunnett's *post* test PLL (93.67 ± 1.202%) vs Sema3A (2.5 $\mu g/cm^2$, 61.67 ± 3.844%; 5 $\mu g/cm^2$, 48.33 ± 3.180%, 10 $\mu g/cm^2$, 35.70 ± 3.874%; $p < 0.05$) and on the basis of quantitative reverse transcriptase (RT)-PCR-based assessment of the expression of MBP mRNA relative to β2-microglobulin (Fig. 1*d*; $n = 3$; PBS, 0.73 ± 0.05967, Sema3A, 0.26 ± 0.05967; *t* test, $p = 0.0051$). Furthermore, the presence of Sema3A OPCs also induced a reduction in the complexity of OPC processes (Fig. 1*e–g*).

To rule out that the decrease of O4 expression mediated by Sema3A was a result of cell death or proliferation cleaved caspase-3 staining (Fig. 1*h*), TUNEL assays (supplemental Fig. 1*d*, available at www.jneurosci.org as supplemental material), and cell counts were conducted (data not shown). As neither active caspase-3 staining [Fig. 1*h*; $n = 3$, one-way ANOVA: $p = 0.3422$, Dunnett's *post* test, PLL (12.48 ± 1.982%) vs Sema3A (2.5 $\mu g/cm^2$, 11.65 ± 0.7112%; 5 $\mu g/cm^2$, 10.21 ± 1.164%; 10 $\mu g/cm^2$, 15.67 ± 3.277%: $p > 0.05$) nor TUNEL assay ($n = 3$, one-way ANOVA: $p = 0.124$, Dunnett's *post* test PLL (14 ± 1.155%) vs Sema3A (2.5 $\mu g/cm^2$, 14.57 ± 1.374%; 5 $\mu g/cm^2$, 23.00 ± 8.021%; 10 $\mu g/cm^2$, 27.00 ± 3.464%): $p > 0.05$] showed significant differences, we concluded that Sema3A selectively inhibits the differentiation of OPCs into oligodendrocytes.

The differentiation block induced by Sema3A is reversible

To determine whether the inhibitory effect induced by Sema3A is reversible, Sema3A was added to differentiation medium of purified OPC cultures. After 24 h one part of the cells were fixed and stored for further analysis. The medium of the remaining cells was replaced with either Sema3A-free or Sema3A-containing medium and the cells cultured for further 48 h. Assessment of O4 immunoreactivity confirmed that O4 expression was inhibited as long as Sema3A was present in the medium (Fig. 1*i–l*). However, following removal of Sema3A (Sema3A/24 h▶Diff/48 h) the inhibition was lost and the cells were able to differentiate ($n = 3$; one-way ANOVA: $p < 0.0001$, Dunnett's *post* test Sema3A/24 h (14 ± 1.155%) vs control differentiation medium (Diff/24 h) (95.59 ± 0.9515%), Sema3A/24 h▶Diff/48 h (withdrawal) (90.12 ± 3.084%), and Diff/72 h (94.67 ± 0.8819%); $p < 0.05$; Sema3A/24 h vs Diff/24▶Sema3A/48 h (36.83 ± 6.022%), and Sema3A/72 h (49.67 ± 2.281%); $p > 0.05$).

Assessment of the effects of Sema3A in demyelinating lesions

A recent postmortem study demonstrated that Sema3A is expressed in nonmyelinating MS lesions at a phase, which might be critical for the outcome of endogenous efforts of remyelination (Williams et al., 2007). We therefore hypothesized that Sema3A inhibits OPC differentiation *in vivo* and consequently impairs CNS remyelination. To test this we adopted a well established model of CNS remyelination based on stereotactic delivery of

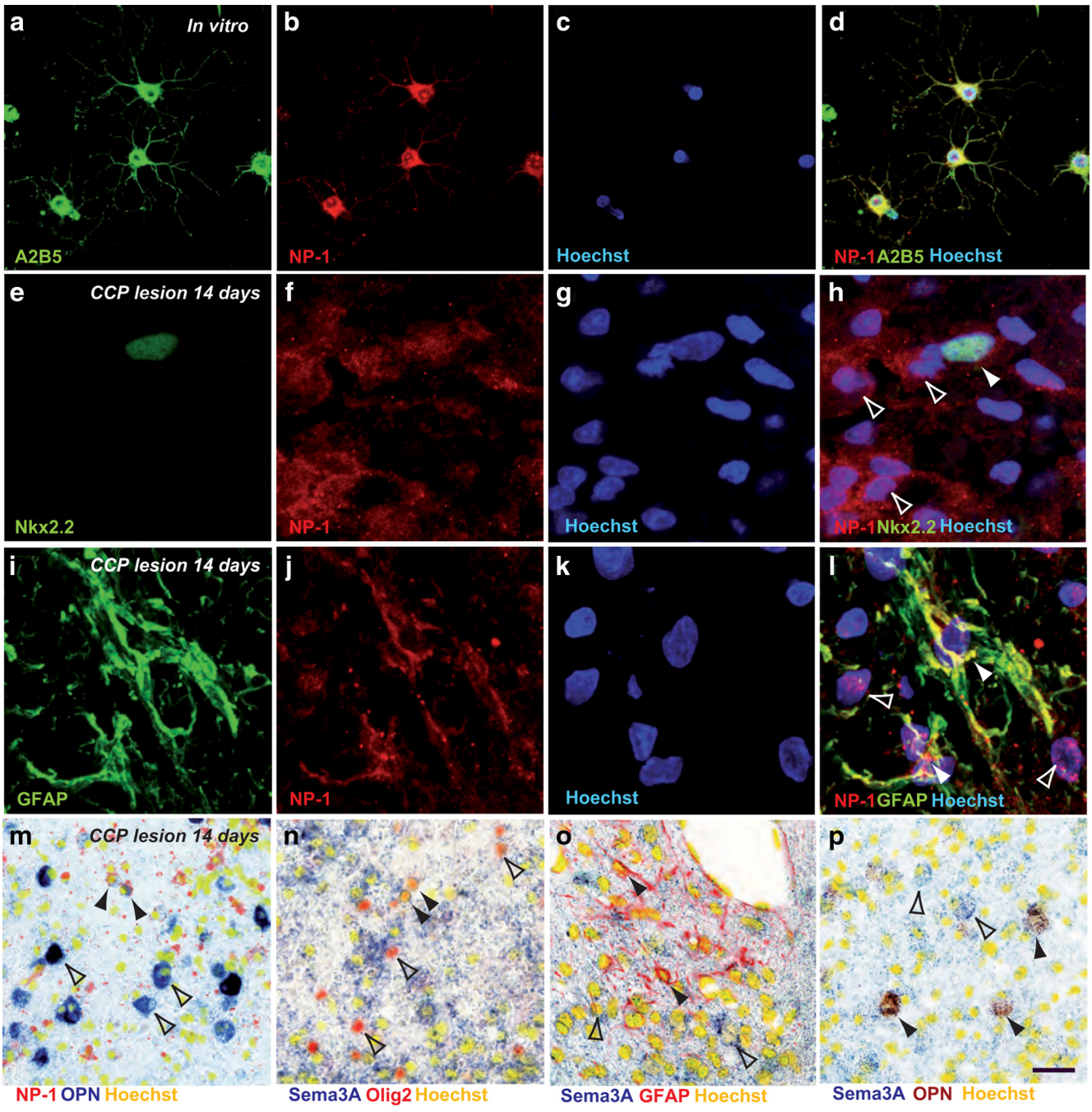

Figure 2. ***a–d***, Cultured OPCs express the Sema3A receptor NP-1. ***e–h***, Apart from other cell types (outline arrowheads) NP-1 antigeneity was also detected on Nkx2.2-positive OPCs in 14 d post-lesion induction (white arrowheads). ***i–m***, Further cell types expressing NP-1 include astrocytes (white arrowhead; outline-arrowheads: non-GFAP-positive NP-1-expressing cells) (***i–l***) and osteopontin-mRNA-expressing microglia/macrophages (***m***). However, NP-1 was only detected in microglia/macrophages expressing OPN at low levels (black arrowheads), whereas cells expressing OPN at high levels did not express NP-1 (outline-arrowheads). In addition, the endogenous expression of Sema3A was assessed 14 d post-lesion induction. ***n***, Whereas low-olig-2-expressing OLCs (black arrowheads) expressed Sema3A mRNA, Sema3A was not detected in high-olig-2-expressing OLCs (open arrowheads). ***o***, GFAP-immunopositive astrocytes showed only weak expression of Sema3A mRNA transcripts (black arrowheads). ***p***, In contrast, OPN-mRNA-positive microglia/macrophages seemed a prominent source of Sema3A (black arrowheads). (Outline arrowheads in ***p***, ***m***, Sema3A-expressing cells that did not coexpress GFAP or OPN, respectively.) Scale bar (in ***p***): ***a–d***, 20 μm; ***e–h***, 8 μm; ***i–l***, 8 μm; ***m–p***, 30 μm.

ethidium bromide into the CCP. In this model OPCs are recruited into demyelinated areas within 5 d (Woodruff and Franklin, 1999; Kotter et al., 2006). The first evidence of remyelination in this model can be observed 10 d following lesion induction at the lesion border. To assess the effects of Sema3A on remyelination Sema3A-Fc was stereotactically infused from day 10 post-lesion induction (supplemental Fig. 2*a*,*b*, available at www.jneurosci.org as supplemental material). At 28 d the lesion size was assessed. No significant changes in the lesion size were detected between the groups (supplemental Fig. 3, available at www.jneurosci.org as supplemental material).

Expression of NP-1 and Sema3A in CCP lesions

To confirm that OPCs are able to react to Sema3A the expression of the Sema3A receptor NP-1 was assessed in purified OPC cultures as well as in CCP lesions (Fig. 2). Colocalization of NP-1

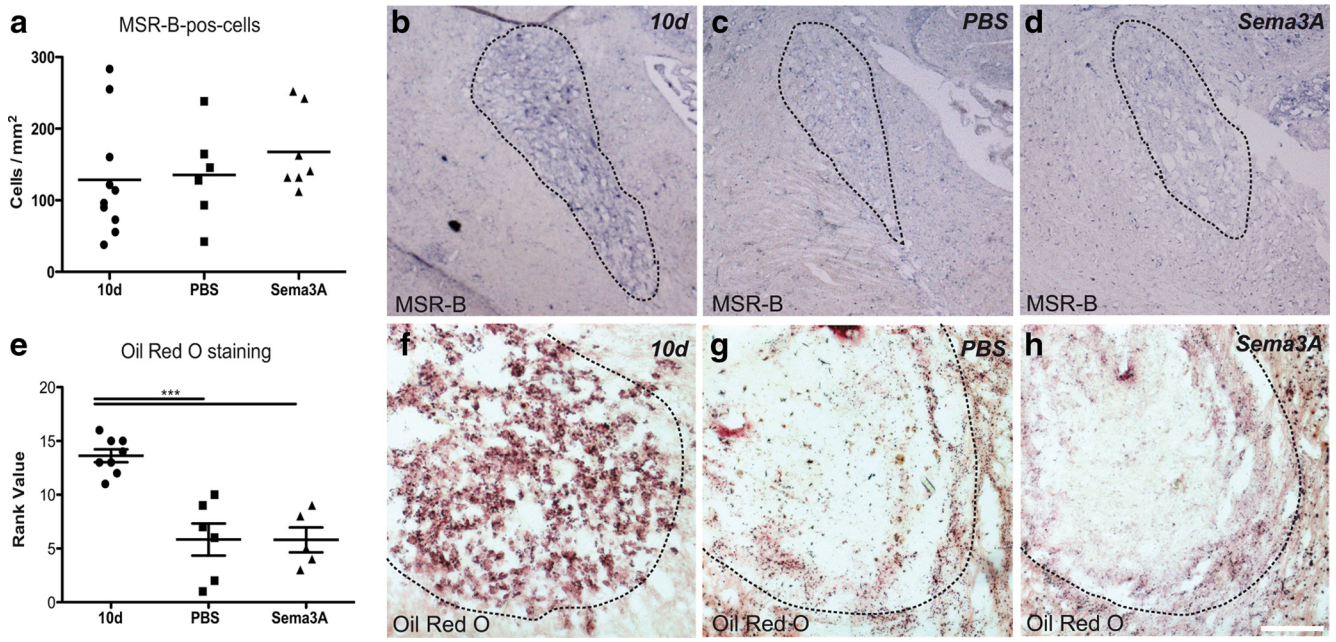

Figure 3. ***a–d***, Sema3A infusion did not alter the presence of MSR-B macrophages. ***e–h***, Furthermore, Oil Red O staining did not show any differences in the phagocytic activity between Sema3A-infused and control animals. Scale bar (in ***h***): ***b–d***, 50 μm; ***f–h***, 30 μm.

antigeneity and A2B5 in culture and the presence of NP-1$^+$/Nkx2.2$^+$ cells in control lesions at 14 d confirmed that OPCs express NP-1 both *in vitro* (Fig. 2*a–d*) and *in vivo* (Fig. 2*e–h*). [Nkx2.2 is a transcription factor associated with early stages of OPC differentiation and a marker of OPC activation (Fancy et al., 2004; Kotter et al., 2006).] Apart from OPCs, we also found evidence of NP-1 expression by GFAP-positive astrocytes (Fig. 2*l*) and by a subpopulation of osteopontin (OPN) mRNA-expressing macrophages/microglia (Fig. 2*m*). NP-1-expressing macrophages/microglia were smaller and expressed OPN at lower levels than NP-1-negative macrophages/microglia, and thus NP-1 expression potentially is able to differentiate between cells at different stages or from different origins.

To identify endogenous sources of Sema3A in native CCP lesions combinations of *in situ* hybridization for Sema3A and immunohistochemistry for Olig-2 (OPCs) and GFAP (astrocytes), and double-*in situ* hybridizations for Sema3A and MSR-B (macrophages/microglia) were conducted. While Sema3A transcripts often colocalized with oligodendrocyte lineage cells (OLCs) expressing Olig-2 transcripts at low levels we were unable to detect Sema3A expression in OLCs expressing Olig-2 at high levels with certainty. Furthermore, Sema3A expression by GFAP-positive astrocytes and OPN-positive macrophages was noted (Fig. 2*n–p*).

Sema3A infusion does not alter the macrophage response

Cells of the immune response and especially macrophages play an important role in the process of remyelination (Kotter et al., 2001). To assess whether Sema3A infusion alters the number of macrophages within the lesion *in situ* hybridization for MSR-B was conducted (Fig. 3*a–d*). Although it has been shown that Sema3A is able to induce apoptosis in monocyte-derived macrophages (Ji et al., 2009), in the present experiment Sema3A did not alter the presence of macrophages (10 d: $n = 10$, 128.7 ± 25.91 cells/mm^2; Sema3A: $n = 7$, 167.7 ± 21.33 cells/mm^2; PBS controls: $n = 6$, 135.3 ± 27.13 cells/mm^2).

In addition to the presence of macrophages we assessed the phagocytic activity in the lesions on the basis of Oil Red O stained neutral lipids (Fig. 3*e–h*) (Kotter et al., 2005). Whereas high levels of Oil Red O staining were detected at day 10 ($n = 8$, mean rank value 13.63 ± 0.5957) indicative of ongoing phagocytosis, no differences between Sema3A-treated ($n = 5$, mean rank value 5.83 ± 1.493) and control lesions ($n = 6$, mean rank value 5.8 ± 1.158) were found 28 d post-lesion induction.

Sema3A infusion does not alter the presence of OPCs in demyelinated lesions

OPCs in demyelinated lesions were visualized by *in situ* hybridization for PDGFR-α (Fig. 4*a–d*) (Kotter et al., 2006). No differences were found between Sema3A-infused lesions ($n = 7$; 155.8 ± 10.10 cells/mm^2), lesions before infusions at day 10 ($n = 11$; 143.1 ± 20.80 cells/mm^2) and PBS-treated controls ($n = 8$; 155.0 ± 19.09 cells/mm^2). Similarly, *in situ* hybridization for Nkx2.2, showed no differences between the treatment groups (Fig. 4*e–h*; PBS: $n = 5$, 159.1 ± 28.84 cells/mm^2; Sema3A: $n = 7$, 176.5 ± 19.44 cells/mm^2). Finally, the presence of apoptotic cells was assessed by immunohistochemistry for activated caspase-3 (aCas3) and Nkx2.2. No differences were detected between Sema3A- and PBS-infused animals (supplemental Fig. 4, available at www.jneurosci.org as supplemental material; total aCas cells: 10 d: $n = 3$; 55.50 ± 12.84 cells/mm^2; PBS: $n = 5$; 84.46 ± 11.45 cells/mm^2; Sema3A: $n = 5$; 82.68 ± 11.45 cells/mm^2; aCas3$^+$/Nkx2.2$^+$ cells:10 d: $n = 3$; 32.50 ± 5.08 cells/mm^2 PBS: $n = 5$; 27.09 ± 3.73 cells/mm^2; Sema3A: $n = 5$; 32.21.5 ± 3.0 cells/mm^2).

Sema3A inhibits the lineage progression of OPCs in demyelinated lesions

To assess OPC differentiation *in situ* hybridization for PLP was conducted (Fig. 4*i–l*). Sema3A infusion significantly decreased the density of PLP-positive cells in the lesions ($n = 7$, 497.3 ± 127.9 cells/mm^2) compared with PBS-infused controls ($n = 7$, 911.0 ± 112.3 cells/mm^2). Levels of PLP-positive OLCs following infusion of Sema3A infusion were comparable to those found in 10 d controls ($n = 9$, 506.4 ± 75.40 cells/mm^2), a time point at

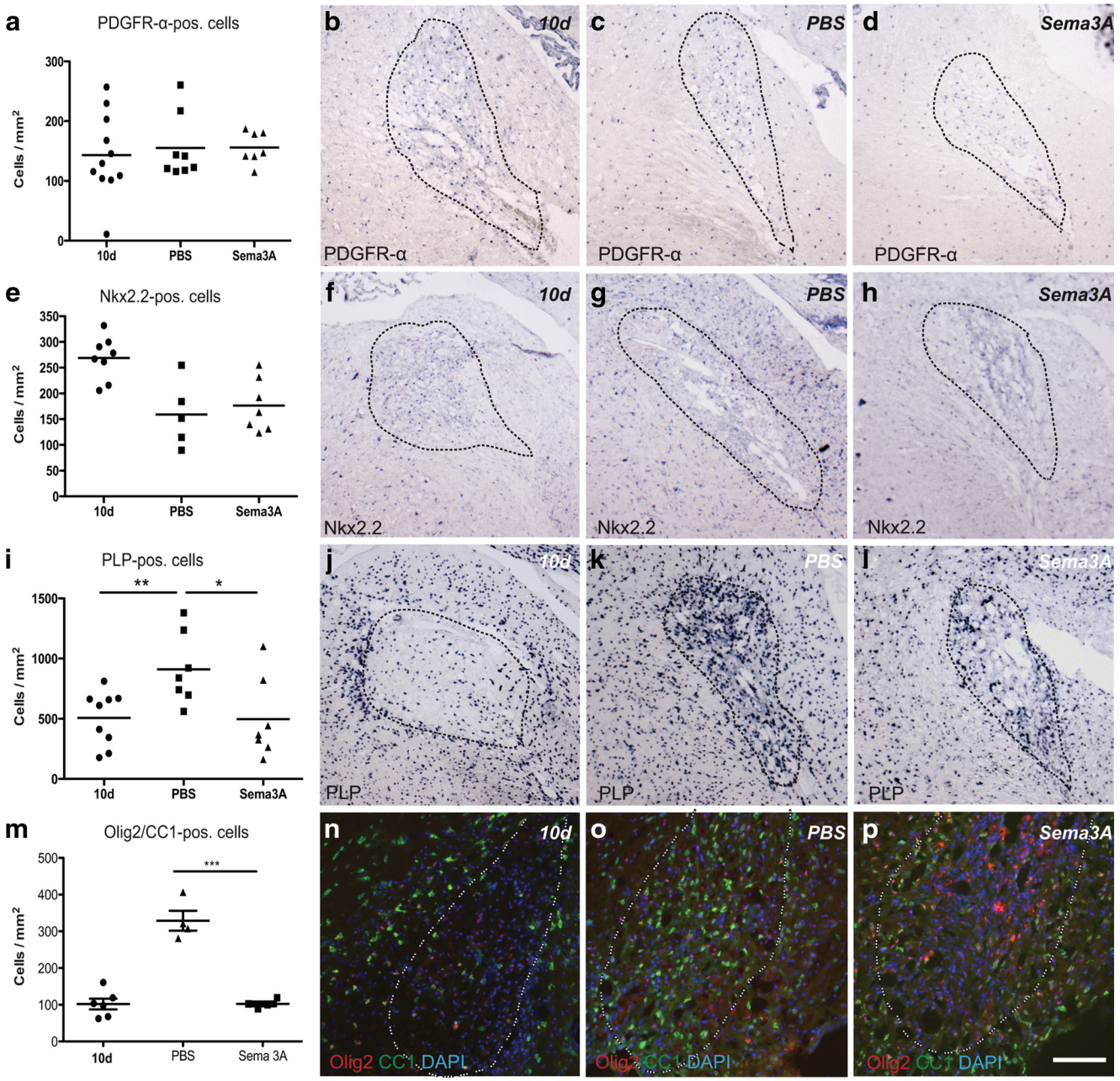

Figure 4. ***a–d***, *In situ* hybridization for PDGFR-α showed comparable OPC densities within lesions of Sema3A-treated animals and controls 28 d after lesion induction. ***e–h***, Similarly, levels of Nkx2.2-positive cells, a marker of OPC activation, were comparable in both experimental groups at 28 d. ***i–l***, Assessment of OPC differentiation on the basis of ISH for PLP demonstrated a significant reduction of OPC differentiation in Sema3A-infused lesions. ***m–p***, These findings were confirmed by quantification of CC1/Olig2-immunopositive mature oligodendrocytes in the lesion. Scale bar (in ***p***): ***b–l***, 50 μm; ***n–p***, 30 μm.

which lesions display minimal amounts of remyelination indicating that differentiation was inhibited. On the contrary, the number of PLP-positive OLCs was significantly increased in PBS-controls at 28 d (Fig. 4*i*; one-way ANOVA: $p = 0.0109$; Dunnett 10 d vs PBS and Sema3A: $p < 0.05$).

To confirm that Sema3A induces a differentiation block in OPCs immunolabeling for CC1, a marker of mature oligodendrocytes, was conducted (Fig. 4*m–p*; one-way ANOVA: $p < 0.0001$; Dunnett's *post* test 10 d vs PBS and Sema3A: $p < 0.05$). The number of $CC1^+/Olig2^+$ cells increased significantly in PBS-treated animals ($n = 4$, 328.9 ± 27.1 cells/mm^2) at day 28 compared with the 10 d group ($n = 6$, 101.8.9 ± 14.71 cells/mm^2). This increase was absent in Sema3A-treated animals where the levels of $CC1^+/Olig2^+$ cells remained comparable to the levels detected in the 10 d group ($n = 4$, 102.5 ± 6.245 cells/mm^2).

Sema3A inhibits CNS remyelination

Finally, the extent of remyelination was assessed 28 d post-lesion induction on semithin resin sections (Fig. 5*a–d*). While remyelination in control animals was almost complete with the majority of axons being surrounded by thin myelin sheaths (Blakemore, 1974), in Sema3A-infused lesions the large majority of axons remained demyelinated. Where remyelination occurred, it was confined to the border of the lesion. Remyelination was compared between the groups by investigator-blinded rank analysis (Kotter et al., 2006). The results confirm a strong impairment of remyelination (Mann–Whitney *U*: $p = 0.0005$)

in Sema3A-infused animals ($n = 8$; mean rank value 11 ± 3.207) compared with controls ($n = 10$; mean rank value 19.10 ± 3.665). It is important to note that Sema3A infusion does not affect the integrity of mature myelin sheaths as has been demonstrated by administration of Sema3A into naive CCPs (supplemental Fig. 2*c*, available at www.jneurosci.org as supplemental material).

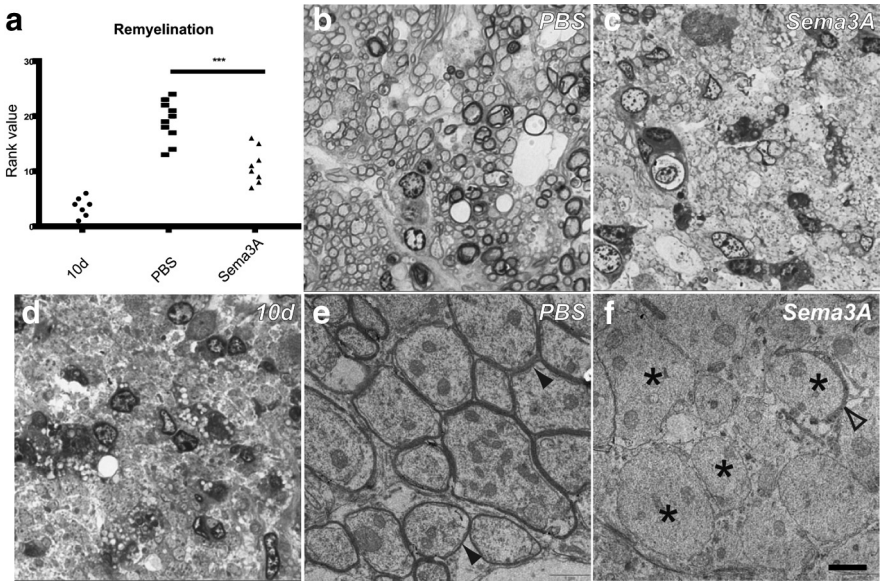

Figure 5. ***a***, The extent of remyelination was assessed by rank analysis of methylene blue and Azur-II-stained semithin-sections. ***b–d***, Sema3A infusion induced a highly significant reduction of remyelination compared with PBS controls. Whereas remyelination in controls was nearly complete 28 d post-lesion induction (***b***), the majority of axons in Sema3A-infused lesions remained demyelinated (***c***), resembling lesions at 10 d before Sema3A infusion (***d***). ***e***, EM analysis used to establish the stage at which remyelination was arrested confirmed that most axons in control lesions were remyelinated by oligodendrocytes (black arrowheads). ***f***, Although axons of Sema3A-treated animals were abundant and intact on electron micrographs they remained demyelinated (stars). Some axons were contacted by cellular processes that were arrested at a premyelinating state and failed to produce compacted myelin (outline arrowhead). Scale bar (in ***f***): ***b–d***, 30 μm; ***e***, ***f***, 1 μm.

Sema3A arrests OPCs at a premyelinating state

To confirm the light microscopic observations and for further descriptive studies the samples were assessed by electron microscopy (Fig. 5*e*,*f*). As expected most axons in PBS-infused lesions were surrounded by thin myelin sheaths, which are characteristic of remyelination mediated by oligodendrocytes. Schwann cell-mediated remyelination, which is characterized by thicker myelin sheaths that often are associated with a nucleus resulting in the appearance of a signet ring, was also occasionally observed. In contrast, in Sema3A-infused animals the majority of axons remained denuded. Although a proportion of axons were contacted by cellular processes resembling those of oligodendrocytes, these failed to form new compact myelin sheaths. The bulk of myelin debris in Sema3A-treated animals was cleared and only occasionally structures reminiscent of myelin debris were found.

Discussion

Numerous experiments have demonstrated that remyelination is a reliable regenerative response of the adult CNS and that significant experimental manipulations are required to induce even a transient impairment of myelin regeneration *in vivo*. In search for an explanation for the discrepancies between efficient remyelination in experimental models and inefficient remyelination in human disease, the concept has emerged that MS lesions contain specific OPC differentiation-inhibiting factors. Possible candidates include polysialylated neural cell adhesion molecule (PSA-NCAM) (Charles et al., 2000, 2002), hyaluronan (Back et al., 2005), Wnt (Fancy et al., 2009), and Notch-1 (Wang et al., 1998; John et al., 2002; Zhang et al., 2009) as well as unknown molecules in myelin debris (Kotter et al., 2006; Baer et al., 2009). To identify further OPC-differentiation inhibitors potentially relevant for the failure of remyelination in MS, we assessed the effects of a number of OLC-related proteins on differentiating OPCs. From this Sema3A emerged as a potential candidate. Although Sema3A is not considered a myelin protein per se it can be expressed in cells of the oligodendrocyte lineage (Williams et al., 2007).

Expression of Sema3A and NP1 in remyelinating lesions

To confirm endogenous Sema3A expression in the CCP model *in situ* hybridization was conducted. As previously (Williams et al., 2007), several sources of Sema3A were detected, including astrocytes, microglia/macrophages, and in a subpopulation of cells of the oligodendrocyte lineage. While Sema3A transcripts often co-localized with low-Olig-2-expressing cells Sema3A mRNA could not be detected in high-Olig-2-expressing cells. Differences in Olig-2 expression are thought to discriminate between activated and quiescent OPC population in remyelinating lesions (Fancy et al., 2004). Detection of Sema3A mRNA in low-Olig-2-expressing OPCs may therefore indicate that Sema3A expression is specific to earlier or more quiescent OPC stages where it could potentially function as a regulator of OPC density.

In addition expression of the semaphorin receptor NP-1 was assessed. As suggested by previous studies (Williams et al., 2007) NP-1 is expressed by OPCs in culture and following experimental demyelination, thus confirming the ability of OPCs to react to the presence of Sema3A. Furthermore, NP-1 expression was noted in astrocytes and cells of the macrophages/microglia lineage. NP-1-expressing macrophages/microglia appeared to be smaller and to express OPN at lower levels than NP-1-negative macrophages/microglia. These differences in the expression of NP-1 may again point to differences between different cellular states, e.g., phagocytotic macrophages versus earlier stages, or to cells from different origins.

Class 3 semaphorins as regulators of OPC migration

On the basis that Sema3A exerts repulsive effects on OPC migration it has been proposed that Sema3A prevents repopulation of demyelinated lesions with remyelination-competent cells (Spassky et al., 2002). However, OPCs are commonly present in MS lesions and thus expression of Sema3A does not generally prevent their recruitment into areas of demyelination (Wolswijk, 1998, 2000; Chang et al., 2000, 2002; Kuhlmann et al., 2008). A potential explanation for this discrepancy is provided by the fact that another member of class 3 semaphorins, namely semaphorin 3F (Sema3F), is expressed in demyelinated lesions (Williams et al., 2007). Sema3F has been recognized as a chemoattractant and mitogenic factor for OPCs (Spassky et al., 2002). Similar to Sema3A, Sema3F expression is restricted to active MS plaques and not found in chronic MS lesions or normal white matter.

However, important differences with respect to the expression of Sema3A and Sema3F have been identified as increased levels of Sema3F expression were found in highly inflammatory lesions, where CD68-positive cells represent >15% of the perilesional cells. In contrast, the highest levels of Sema3A expression were associated with lower levels of inflammation. These findings suggest that while attractive cues may dominate in early inflammatory lesions these give way to aversive cues during later stages. Conversely, if the recruitment or differentiation of OPCs at early stages is insufficient, a dysbalance favoring Sema3A expression may have the potential to interfere with both OPC recruitment and differentiation and thus contribute to remyelination failure in MS.

Sema3A as an inhibitor of OPC differentiation

Earlier studies suggest that Sema3A induces a reversible reduction of process complexity in adult OLCs (Ricard et al., 2001) and causes redirection of OLC growth cones away from Sema3A (Cohen et al., 2003). Our finding that the presence of Sema3A is able to inhibit the differentiation of OPCs *in vitro* prompted us to ask whether Sema3A is able to exert a similar effect in remyelinating lesions. Infusion of recombinant Sema3A 10 d after experimental lesioning induced a failure of CNS remyelination that was associated with an inhibition of OPC differentiation.

The levels of Sema3A present in demyelinating lesions are unknown and it is therefore not possible to assess how the amount of Sema3A infused compares with levels present in MS lesions. However, infusion of Sema3A in the present series of experiments did not reach toxic levels as first, Sema3A did not influence the lesion size, second no differences were detected in the numbers of macrophages, OPCs and the level of apoptotic cells in the lesions, and third, infusion of Sema3A into normal white matter at the same concentration did not induce any detectable abnormalities.

The results of our monoculture assay demonstrate a direct and specific effect of Sema3A on OPC differentiation. However, as the pool of mature oligodendrocytes increases strongly during remyelination and as this might require the recruitment of additional OPCs, Sema3A may potentially affect the recruitment of OPCs at late stages. Although our *in vitro* findings seem to translate directly into the situation *in vivo* it is possible that Sema3A exerts additional indirect effects on OPCs in remyelinating lesions. Sema3A is known to induce a variety of cellular responses including apoptosis, redirection of processes, and exerting negative guidance cues in diverse cell types such as astrocytes, macrophage lineage cells, and neurons (Pasterkamp et al., 1998; De Winter et al., 2002; Spassky et al., 2002; Zhou et al., 2008; Ji et al., 2009).

The physiologic function of Sema3A in OPCs remains speculative. Sema3A secreted by axons, macrophages or OPCs themselves may provide aversive guidance cues, which help to regulate the number of OPCs in a certain area. Furthermore, the inhibitory effects of Sema3A on OPC differentiation may be part of a regulatory mechanism signaling that an OPC has already engaged with a certain stretch of an axon and prevent further OPCs from attempting to form a myelin sheath at the same location.

Sema3A is expressed at critical stages of demyelination in disease

Whereas expression of the known OPC differentiation inhibitors PSA-NCAM (Charles et al., 2000, 2002) and hyaluronan (Back et al., 2005) in MS lesions is associated with chronic stages of demyelination, similar to Notch-1 (John et al., 2002), upregulation of Sema3A transcripts was detected in late active MS lesions (Williams et al., 2007). At this stage the presence of cells capable of reacting to Sema3A, as judged by their expression of the Sema3A receptors neuropilin-1 and 2, is restricted to the plaque and periplaque area. In contrast neuropilin-1/2-positive cells seem to be absent in normal appearing white matter. On the basis that remyelination in MS proceeds in a similar way as experimental remyelination, Sema3A seems to be expressed at a critical stage, at which OPCs recruited into demyelinated lesions may be prevented from differentiating into myelin forming oligodendrocytes.

Semaphorin signaling as a potential target for promoting CNS remyelination

The context in which Sema3A is expressed in MS together with the finding that Sema3A is able to inhibit CNS remyelination *in vivo*, implies that Sema3A signaling may be a relevant target for promoting remyelination in demyelinating disease. There are several levels at which this could be achieved. These include antibody-mediated neutralization of Sema3A epitopes or pharmacological modulation of down-stream signaling effectors. A number of drugs that are able to modulate signaling effectors of Sema3A including RhoA (Baer et al., 2009) are currently being used or clinically evaluated for the treatment of other diseases such as cancer. It will be important to establish their efficacy to promote remyelination in preclinical models of remyelination. Given the clinical availability of such drugs, a potential translation into clinical disease only seems a small step ahead.

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