Development/Plasticity/Repair

An Oligodendrocyte Lineage-Specific Semaphorin, Sema5A, Inhibits Axon Growth by Retinal Ganglion Cells

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In the mammalian CNS, glial cells repel axons during development and inhibit axon regeneration after injury. It is unknown whether the same repulsive axon guidance molecules expressed by glia and their precursors during development also play a role in inhibiting regeneration in the injured CNS. Here we investigate whether optic nerve glial cells express semaphorin family members and, if so, whether these semaphorins inhibit axon growth by retinal ganglion cells (RGCs). We show that each optic nerve glial cell type, astrocytes, oligodendrocytes, and their precursor cells, expressed a distinct complement of semaphorins. One of these, sema5A, was expressed only by purified oligodendrocytes and their precursors, but not by astrocytes, and was present in both normal and axotomized optic nerve but not in peripheral nerves. Sema5A induced collapse of RGC growth cones and inhibited RGC axon growth when presented as a substrate *in vitro*. To determine whether sema5A might contribute to inhibition of axon growth after injury, we studied the ability of RGCs to extend axons when cultured on postnatal day (P) 4, P8, and adult optic nerve explants and found that axon growth was strongly inhibited. Blocking sema5A using a neutralizing antibody significantly increased RGC axon growth on these optic nerve explants. These data support the hypothesis that sema5A expression by oligodendrocyte lineage cells contributes to the glial cues that inhibit CNS regeneration.

Key words: **regeneration; semaphorin; axon guidance; glia; axon growth; optic nerve**

Introduction

What is the relationship between axon repulsion in the developing nervous system and axon growth inhibition in the adult? When axons are severed in the postnatal or adult CNS, they sprout new growth cones but fail to elongate past the lesion site (Ramon y Cajal, 1928). This inhibition is caused, at least in part, by myelin-associated molecules such as Nogo, myelin-associated glycoprotein (MAG), and oligodendrocyte–myelin glycoprotein (OMgp) released at CNS injury sites. Blocking of these myelin-associated molecules, their receptors, or key downstream effectors allows a small percentage of axons to regenerate (Fournier et al., 2002; Schwab, 2002; Woolf, 2003; Sivasankaran et al., 2004).

During development, axons are guided by neuronal and glial molecules that attract and repel axonal growth cones (Goodman, 1996). For example, embryonic retinal ganglion cell (RGC) axons are repelled by Muller glia away from deeper retinal layers into the nerve fiber layer (Stier and Schlosshauer, 1995; Bauch et al., 1998); RGC axons are directed centrally in the retina away from chondroitin sulfate proteoglycans (CSPGs) (Brittis and Silver, 1995) and funneled into the optic nerve by a semaphorin sema5A (Oster et al., 2003); ipsilateral axons are repelled at the optic chiasm by slit1 and slit2 (Plump et al., 2002), ephrin-B (Nakagawa et al., 2000; Williams et al., 2003), and CSPGs (Chung et al., 2000); and temporal retinal axons are restricted to the anterior superior colliculus by ephrin-A2- and ephrin-A5-mediated repulsion in the posterior superior colliculus (Feldheim et al., 2000).

Do molecules that guide axons during development also contribute to axon inhibition in the postnatal or adult CNS? Such shared functions are plausible because both myelin-associated axon growth inhibitors in the adult CNS and developmentally expressed axon guidance molecules including semaphorins, ephrins, and slits share similar abilities to repel axons, collapse neuronal growth cones, and inhibit axon elongation *in vitro*. Furthermore, CSPGs may themselves serve both functions, because they repel axons during development, but they are also upregulated in the glial scar after injury (Pasterkamp et al., 1999; De Winter et al., 2002; Properzi et al., 2003), and their enzymatic removal leads to improved regeneration and functional recovery after injury in the adult (Moon et al., 2001).

In this study, we investigate whether semaphorins, which collapse growth cones and repel axons during development in the CNS (Puschel et al., 1995; Kobayashi et al., 1997; Bagnard et al.,

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1998; Polleux et al., 1998) and PNS (Luo et al., 1993), contribute to glial inhibition of regenerating axons in the CNS. Here we report that each glial cell type in the developing optic nerve expresses a distinct complement of semaphorins. One semaphorin, sema5A, is expressed by oligodendrocytes and their precursor cells in the developing optic nerve and inhibits axon growth by embryonic and postnatal RGCs *in vitro*. RGC axon growth on living postnatal and adult optic nerve explants was strongly inhibited, and blocking of sema5A-mediated inhibition using a neutralizing antibody (Ab) allowed a consistent increase in RGC axon regeneration on these explants. Thus CNS glial cells express a wide variety of semaphorin family members postnatally, and these studies provide evidence that sema5A contributes to the inhibition of CNS axon growth by oligodendrocyte lineage cells after injury.

Materials and Methods

Purification of primary neurons and glia. Step-by-step protocols for all procedures are available on request from barres@stanford.edu. RGCs from embryonic day 20 (E20) or postnatal day 7 (P7) Sprague Dawley (S/D) rats (Simonson Labs, Gilroy CA) were purified using sequential immunopanning for thy-1 to >99.5% purity as described previously (Barres et al., 1988). Similar immunopanning techniques were also used to purify astrocytes, oligodendrocytes, and oligodendrocyte precursor cells (OPCs) from optic nerves, excluding the optic chiasm (Barres et al., 1992, 1994; Barres and Raff, 1993).

RGCs were cultured in defined, serum-free growth media modified from Bottenstein (Bottenstein and Sato, 1979) designed to maximally support their survival and axon outgrowth (Meyer-Franke et al., 1995; Goldberg et al., 2002b), consisting of Neurobasal (Invitrogen, Gaithersburg, MD), bovine serum albumin, selenium, putrescine, triiodothyronine, transferrin, progesterone, pyruvate, glutamine, forskolin (5 μM), insulin (5 μg/ml; all from Sigma, St. Louis, MO), ciliary neurotrophic factor (10 ng/ml), and brain-derived neurotrophic factor (BDNF; 50 ng/ml) (both from Regeneron Pharmaceuticals). Cultures were maintained at 37°C in a humidified environment of 10% CO_2/90% O_2.

Analysis of semaphorin expression by RT-PCR. For analysis of cell type-specific expression, purified optic nerve glial cells were lysed directly from the final immunopanning dish into buffer RLT as per the RNeasy total RNA purification protocol (Qiagen, Hilden, Germany). For analysis of whole optic nerve expression after injury, intraorbital optic nerve crush axotomies were performed under anesthesia as described (Shen et al., 1999). Ten crushed optic nerves and 10 control nerves were collected 1 d later and homogenized using successively smaller gauge needles in buffer RLT, and total RNA was purified as above. Total RNA was reverse transcribed to cDNA using polyA primers and Superscript II (Invitrogen). Qualitative comparisons of expression level were based on PCR reactions stopped after 25, 30, and 35 cycles compared on 1.5% agarose Tris-acetate-EDTA gels. Expression levels were normalized on the basis of an assumption of equivalent actin mRNA expression. PCR primer sequences (generous gift of Exelixis Pharmaceuticals, South San Francisco, CA) were designed to 300–500 bp stretches from the 3′-untranslated regions of semaphorin genes, were blasted against the National Center for Biotechnology Information database to confirm unique specificity, and are available on request.

Generation of semaphorin proteins. Expression constructs containing either full-length secreted semaphorins or semaphorin extracellular domains fused with either a myc tag or the Fc domain of human Ig at the C terminus (Xu et al., 1998) were transfected using standard Lipofectamine 2000 protocols (Invitrogen) into human embryonic kidney (HEK)-293T cells (American Type Culture Collection). Similar semaphorin fusion proteins have been shown to be fully functional (He and Tessier-Lavigne, 1997; Kobayashi et al., 1997; Koppel et al., 1997; Shepherd et al., 1997). Cells transfected with an Fc construct alone were used as controls. After transfected cells recovered for 12 hr in DMEM containing 10% fetal calf serum, transfected HEK-293T cells were switched into serum-free RGC culture medium for 2 d. For some experiments, stable cell lines secreting semaphorin–Fc fusion proteins were derived by G418 selection. Conditioned semaphorin supernatants were collected and used immediately. Relative concentrations of the various semaphorin supernatants were estimated on Western blots using typical protocols and anti-Fc or anti-myc antibodies.

Collapse and substrate assays. To analyze the ability of semaphorins to collapse RGC growth cones, RGCs were purified and plated at a density of 100/mm^2 on glass coverslips (Carolina Glass) coated with poly-D-lysine (PDL) and mouse laminin (Sigma). After 24–48 hr in growth media at 37°C, semaphorin supernatants were added to the cultures for 30 min at 37°C, after which the coverslips were carefully perfused with warmed 4% paraformaldehyde for 20 min. Cultures were then transferred to staining boxes, fixed an additional 10 min, and permeabilized in 0.1% Triton, 1% BSA, 100 mM lysine for 5 min. Filamentous actin was labeled with fluorophore-bound phalloidin (Alexa-594 phalloidin, Molecular Probes, Eugene, OR) for 20 min, rinsed, counterstained with 4′,6′-diamidino-2-phenylindole (DAPI) to display nuclear morphology, mounted, and viewed under fluorescence (Nikon Diaphot). Growth cones with fewer than two filopodia and no lamellipodia were scored as collapsed. Each experiment tested each condition on triplicate coverslips. Collapse assays were performed four times, in two of which the observer was blinded to condition. A representative, blinded experiment is shown.

For substrate experiments, 35 mm Petri dishes were incubated overnight at 4°C with 4.8 μg goat anti-human Fc γ-chain specific (Jackson ImmunoResearch, West Grove, PA) in 2 ml of 50 mM Tris buffer, pH 9.5, rinsed three times with Dubelco's PBS, and incubated overnight at 37°C in semaphorin-Fc-conditioned media prepared as above. Dishes were subsequently coated with PDL and laminin as above. P8 RGCs were cultured on semaphorin-enriched substrates for 24 hr, after which calcein-AM (Molecular Probes), a vital dye, was added to visualize living neurons and their neurites. In each experiment, >50 RGC axons were imaged, and the longest axon per RGC was measured.

For myelin substrate experiments, rat CNS myelin was prepared as described previously (Norton and Poduslo, 1973; McKerracher et al., 1994). Briefly, adult rat brains were homogenized in 0.32 M sucrose in PBS and centrifuged through a 0.85 M sucrose cushion for 30 min at 75,000 × *g* at 4°C. The interface containing the myelin fraction was collected and washed three times with ddH_2O and further purified by centrifugation at 34,000 × *g* for 10 min at 4°C. Purified myelin was resuspended in ddH_2O and stored at −80°C. To prepare myelin substrates, glass coverslips (Carolina Glass) were coated with PDL, laminin, and purified myelin (2.2 μg per coverslip).

Immunofluorescence and Western blotting. RGCs were purified and cultured on glass coverslips for immunofluorescence and ligand binding studies. After fixation with 4% paraformaldehyde for 10 min in a 10% donkey serum solution containing 1% BSA and 100 mM L-lysine to block nonspecific binding and 0.4% Triton X-100 to permeabilize the membrane, fixed cells were incubated overnight at 4°C in goat polyclonal anti-neuropilin-1 (N-18), anti-plexin-B1 (N-18), or anti-plexin-C1 (N-17) (all 0.7 μg/ml; Santa Cruz Biotechnology, Santa Cruz, CA), followed by fluorescein-conjugated donkey anti-goat IgG (20 μg/ml; Jackson ImmunoResearch, West Grove, PA) for 1 hr at room temperature. The coverslips were mounted in Vectashield with DAPI, sealed with nail polish, and examined in a Nikon Diaphot fluorescence microscope.

For surface reactivity of neuropilin-1, the goat polyclonal anti-neuropilin-1 (N-18) used above was applied to fixed but nonpermeabilized RGCs for 30 min at room temperature, followed by secondary Ab incubation, mounting, and viewing as above. For ligand binding experiments, sema3A supernatant from HEK-293T cells was applied to fixed RGCs overnight at 4°C, followed by incubation with biotin-conjugated goat anti-human Fc (20 μg/ml; Jackson ImmunoResearch) and then with Cy5-conjugated streptavidin, each for 1 hr at room temperature, before mounting and viewing as above.

For immunostaining of optic nerves, optic nerves were dissected from freshly killed E20 and P8 S/D rats and P0 and P10 S100β–green fluorescent protein (GFP) transgenic mice (Lubischer et al., 2000; J. L. Lubischer, P. A. Krieg, W. J. Thompson, unpublished observations) and immediately fixed in 4% paraformaldehyde for 2 hr. The tissues were

cryoprotected in 30% sucrose, and 8–12 μm sections were cut and mounted on aminoalkylsilane-coated slides (Sigma). Sections were post-fixed in 4% paraformaldehyde for 10 min and blocked and permeabilized with 50% goat serum and 0.4% Triton X-100 for 40 min. Primary and secondary Ab incubations, mounting, and visualization were performed as above, using rabbit anti-sema5A (1:60–1:100 dilution) (Oster et al., 2003) and monoclonal mouse anti-CC1 (1:100 dilution; Oncogene Research Products) to detect oligodendrocytes.

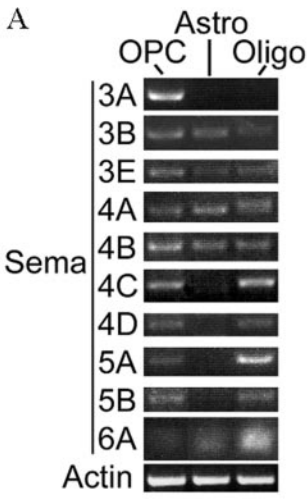
Western blotting was performed using typical protocols. Briefly, 25 μg of cell or tissue lysate collected in radioimmunoprecipitation assay buffer or of purified myelin or liver membranes was transferred to 0.45 μm nitrocellulose (Bio-Rad, Hercules, CA) and incubated for 2 hr with anti-sema5A antiserum (1:500 dilution) (Oster et al., 2003) or anti-Nogo-A antiserum (1:100; Transduction Labs, Lexington, KY) and 1 hr with horseradish peroxidase-conjugated secondary Ab (1:1000; Chemicon, Temecula, CA), both in 5% milk. Detection was performed using ECL (Amersham Biosciences, Arlington Heights, IL).

Living optic nerve axon growth assays. Optic and sciatic nerves from P4, P8, and adult (>4 months old) rats of various ages were explanted from acutely killed rats and transferred to nitrocellulose paper, which adhered strongly to the pial surface of the nerve. Using a microscalpel, the nerves were carefully sliced and opened longitudinally such that the pial surface wicked onto the nitrocellulose paper. At this point the paperbound nerve was placed into RGC culture medium with the now-exposed interior facing up as a culture substrate. Purified RGCs were labeled with the lipophilic fluorescent membrane tracer CM-DiI (Molecular Probes) for 15 min at 37°C plus 15 min at 4°C, rinsed three times in Dubelco's PBS, and plated onto these living optic nerves at a density of 100/mm^2 in 500 μl of growth media. In some experiments, polyclonal antibodies raised against sema5A (Oster et al., 2003) or against an irrelevant epitope (galactosidase, 5′-3′) were added at 1:50-:100 final dilutions. Axons were visualized and measured under fluorescence using a Nikon Diaphot microscope; survival of the RGCs and the optic nerve glial cells was confirmed by visualizing nuclear morphology with DAPI at the time of axon measurement. A minimum of 50 axons were counted per experiment, and experiments were repeated a minimum of three times.

Results

Expression of semaphorins by optic nerve glia

To find out whether glial cells express semaphorin family members, we purified astrocytes, oligodendrocyte precursor cells (OPCs), and oligodendrocytes by immunopanning to >99.9% purity from postnatal rat optic nerves (see Materials and Methods). We isolated the mRNA from acutely purified glial populations to minimize gene expression changes that may result from culture. Using primer pairs directed to 3′ untranslated portions of various semaphorin mRNAs, we performed a semiquantitative RT-PCR by stopping the PCR after 25, 30, and 35 cycles and comparing the intensity of the product bands separated by agarose gel electrophoresis. We specifically examined these cells for the expression of sema3A, 3B, 3E, 4A, 4B, 4C, 4D, 5A, 5B, and 6A and qualitatively compared expression levels in each cell population normalized to actin mRNA expression (Fig. 1*A*) (and data not shown). We found that each glial cell class expressed multiple semaphorin family members. Astrocytes expressed sema3B, 3E, 4A, 4B, and 6A but not 3A, 4C, 4D, 5A, or 5B. Oligodendrocytes also expressed these same semaphorins but in addition expressed sema4C, 4D, 5A, and 5B. OPCs expressed all of the semaphorin mRNAs that we looked for, including sema3A, which we did not see in oligodendrocytes or astrocytes. Sema4C, 4D, 5A, and 5B were restricted to oligodendrocyte lineage cells, being expressed by both OPCs and oligodendrocytes but not astrocytes (Fig. 1*B*). These results show that optic nerve glia express a variety of different semaphorin family members and that each optic nerve glial cell type expresses its own distinct complement of semaphorin family members.



Sema	OPC	Oligo	Astro
3A	XXX	-	-
3B	XX	X	XX
3E	XX	XX	X
4A	X	XX	XX
4B	XX	XX	XX
4C	XX	XXX	-
4D	XX	XX	-
5A	XX	XXX	-
5B	XX	XX	-
6A	X	XXX	XX
Actin	XXXX	XXXX	XXXX

Figure 1. Expression of semaphorins by purified optic nerve glia. Oligodendrocyte precursor cells (OPC), oligodendrocytes (oligo), and astrocytes (astro) were purified from optic nerves, recovered briefly in culture, and lysed for RNA recovery. Reverse transcription to cDNA was followed by PCR for specific semaphorins as marked. PCR cycle number was varied from 25 to 35 cycles to semiquantitatively estimate level of expression, which was normalized to actin mRNA detection (data not shown). *A*, RT-PCR products derived after 30 cycles of PCR separated by agarose gel electrophoresis. *B*, Summary of expression data: — represents undetectable and X-XXXX represents increasing expression.

Effect of semaphorins on RGC growth cones

We next investigated whether some of the semaphorins normally expressed by optic nerve glia are able to inhibit RGC axon growth. First, we investigated whether the various semaphorin proteins were able to acutely collapse RGC growth cones. HEK-293T cells were transiently transfected with expression vectors coding for soluble semaphorin–myc or –Fc fusion constructs and allowed to condition RGC growth medium for 2 d. To confirm that the semaphorin proteins were successfully expressed and secreted, these supernatants were examined by Western blot with an Ab against the myc or Fc domains and found to contain roughly comparable levels of each semaphorin (Fig. 2*A*). Supernatants with less soluble semaphorin by Western blot were concentrated by centrifugation through 30 kDa filters to ensure similar test conditions, and control supernatants were tested after concentration as well.

To determine whether these various semaphorins induced growth cone collapse, we next added the semaphorin-containing and control supernatants to cultures of purified RGCs for 20–30 min at 37°C, after which the RGCs were fixed and permeabilized. Staining with fluorophore-conjugated phalloidin identified filamentous actin and emphasized growth cone morphology, allowing us to score each growth cone as collapsed or not collapsed (open). In contrast to open growth cones (Fig. 2*B*), collapsed growth cones were clearly identifiable by their lack of filopodia or lamellipodia (Fig. 2*C*). When we counted the percentage of growth cones that collapsed in response to various semaphorins, we found that semaphorins 3A, 3B, 3C, 4A, 4B, 4D, and 6A did not significantly induce collapse compared with control conditions (Fig. 2*D*). Only sema5A was able to strongly and significantly induce collapse of RGC growth cones (Fig. 2*D*). The amount of collapse induced by sema5A, typically ~75%, was similar to that induced in postnatal RGCs by ephrins, as described previously (Shamah et al., 2001). No difference was observed between Fc- and myc-fusion constructs (data not shown). Taken together these results show that only the oligodendrocyte

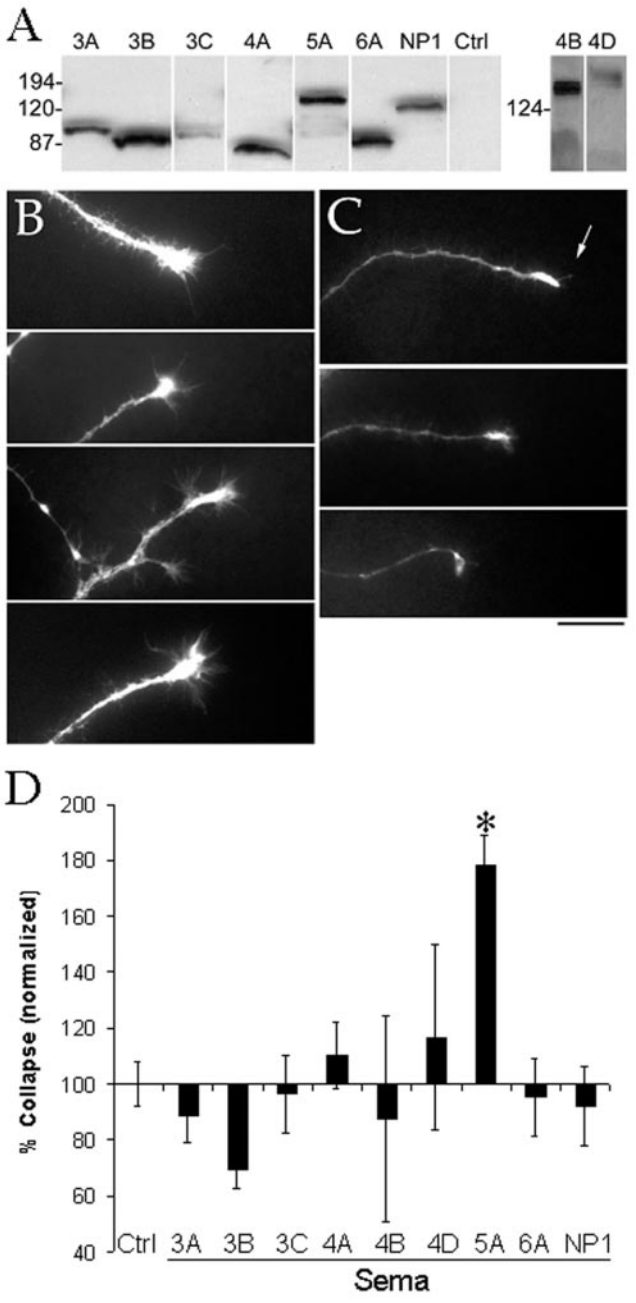


Figure 2. Effect of semaphorins on RGC growth cones. RGCs were purified and cultured overnight in growth media, exposed to various soluble semaphorins for 30 min at 37°C, and then fixed and stained with Alexa594-phalloidin to visualize growth cone morphology. Growth cones were scored as collapsed if they exhibited fewer than two filopodia and no lamellipodia. *A*, Semaphorin–Fc fusion constructs expressed in HEK-293T cell supernatants collected 2 d after transfection with various semaphorin–Fc fusion constructs were tested for the presence of secreted semaphorins by Western blot. Shown is an example Western blot using an Ab directed against the Fc domain. *B*, Examples of P8 RGC growth cones from control coverslips scored as open. *C*, Examples of P8 RGC growth cones from sema5A coverslips scored as collapsed. Note the single filopodium on one growth cone (arrow). *D*, P8 RGC growth cone collapse in response to various semaphorin, control, or neuropilin-1 (NP1) supernatants. Means ± SEMs are shown; $*p < 0.05$. Scale bar, 5 μm.

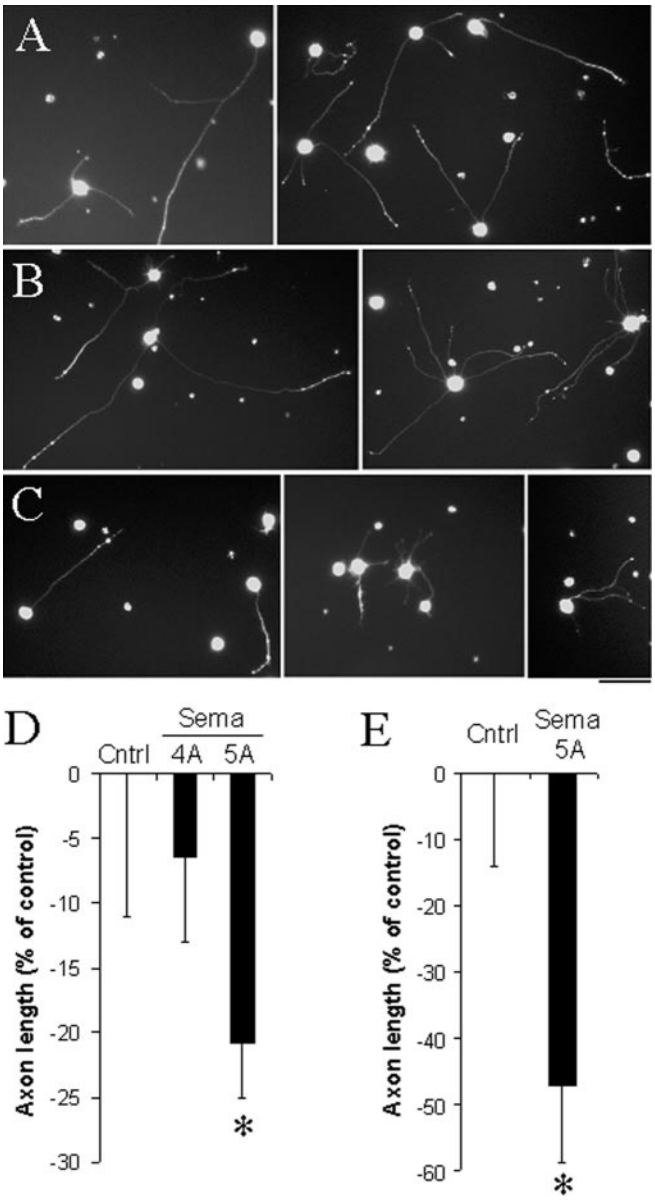


Figure 3. Effect of semaphorins on RGC axon elongation. RGCs were cultured on PDL–laminin substrates alone (*A*) or in the presence of sema3A (*B*) or sema5A (*C*). After 1 d, P8 (*D*) or E20 (*E*) RGC axon lengths were measured in the presence of semaphorin substrates and normalized to control cultures. For each semaphorin tested, 50–200 axons were measured per experiment. Means ± SEMs are shown; $*p < 0.05$. Scale bar, 50 μm.

lineage-specific semaphorin, sema5A, strongly induced growth cone collapse.

Effect of semaphorins on axon growth *in vitro*

The above results show that an acute presentation of sema5A strongly collapses RGC growth cones, raising the question of whether sema5A might also inhibit the rate of axon growth. To find out, we cultured purified RGCs on substrates of sema5A plus laminin, sema4A plus laminin, sema3A plus laminin, or laminin alone (Fig. 3*A–E*). Laminin was included to ensure adherence of RGCs to the coverslips and for promotion of their survival (Meyer-Franke et al., 1995). Because developing and regenerating RGCs must extend single axons over long distances through the optic nerve and tract, we measured the length of the longest axon on each neuron after 24 hr. We found that RGCs cultured on sema5A but not on sema3A or sema4A extended axons that were 20% shorter on average than controls (Fig. 3*A–D*) (and data not shown). When we examined the time course from 14 to 48 hr along which RGCs are inhibited on sema5A substrates, we found that inhibition was maximal at 24 hr (data not shown). Remarkably, even stronger inhibition was observed for E20 RGCs that

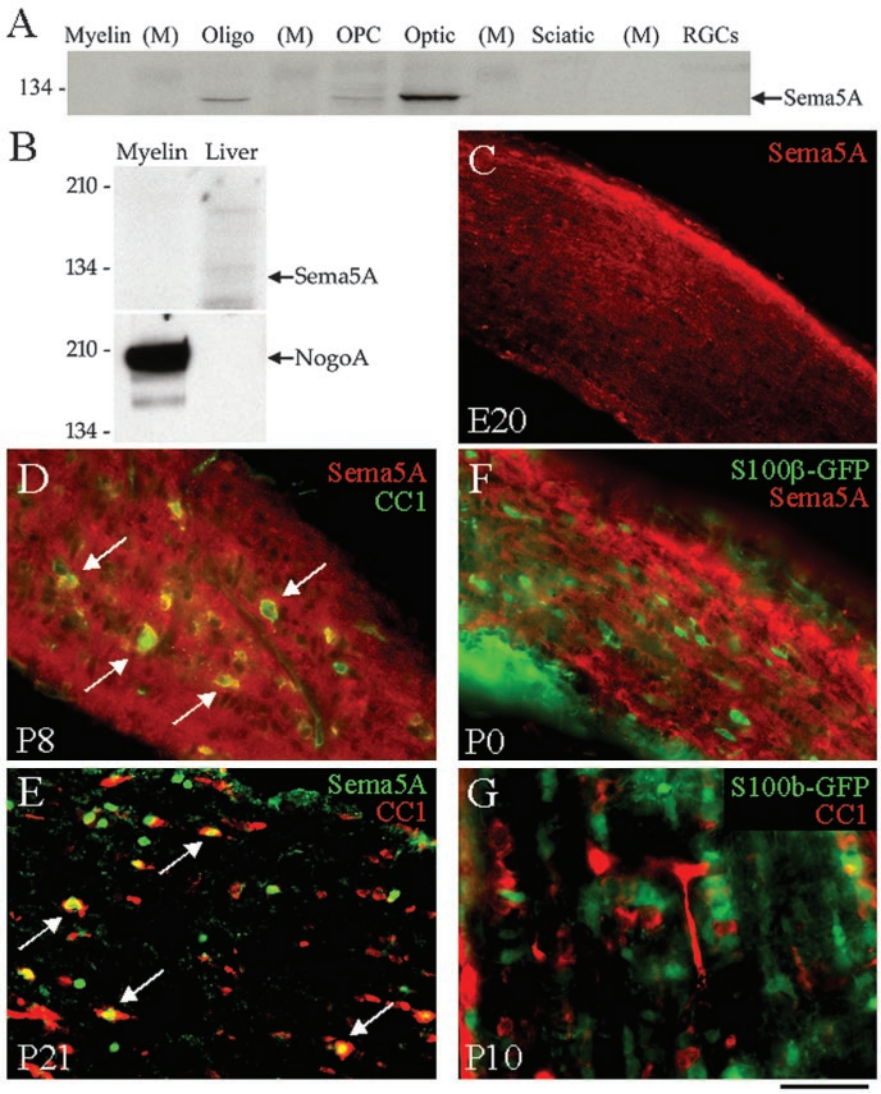


Figure 4. Expression of sema5A protein in the optic nerve. *A*, Western blot analysis of sema5A expression using myelin, oligodendrocyte, OPC, RGC, and optic and sciatic nerve extracts (25 μg protein per lane) separated by molecular mass markers (M). Sema5A bands were observed at the expected molecular weight (~130 kDa) using sema5A antisera (Oster et al., 2003). *B*, Western blot analysis of sema5A and NogoA expression in myelin and liver membranes. Using sema5A or NogoA antisera, NogoA staining was observed in myelin but not liver membranes, but reprobing did not reveal any Sema5A staining. *C–G*, Immunostaining of optic nerves from rat (*C–E*) or transgenic s100β-promoted GFP mouse (*F*, *G*) using antibodies to sema5A or CC1 (oligodendrocytes), or visualizing endogenous GFP fluorescence (astrocytes). Optic nerve ages are noted in the bottom left, and Ab colors are in the top right of each frame. Sema5A and CC1 were colocalized in yellow (*D*, *E*, arrows). Scale bars: *C*, *E*, *F*, 100 μm; *D*, 80 μm; *G*, 50 μm.

extended their axons nearly 50% slower in response to sema5A compared with control substrates (Fig. 3*E*). In preliminary experiments, a modest reduction in adult P28 RGC axon growth was also observed on sema5A substrates (data not shown). Cyclic nucleotide levels have been reported to regulate responsiveness to some semaphorin family members (Song et al., 1998); however, we did not detect any consistent effects of manipulating cAMP or cGMP levels on the ability of sema5A to inhibit axon growth (data not shown).

Expression of sema5A in the optic nerve

We next examined more closely the expression of sema5A protein. A band of the expected molecular weight corresponding to the sema5A protein (Oster et al., 2003) was observed by Western blot at high levels in optic nerve but not sciatic nerve (Fig. 4*A*). Furthermore, sema5A expression was found in both oligodendrocytes and oligodendrocyte precursor cells, whereas purified RGCs did not express sema5A at detectable levels (Fig. 4*A*). Because all lanes were loaded with an equal amount of protein, we attributed the lower sema5A signal in the purified glial cells compared with the optic nerve to the purification procedure, which uses papain and likely cleaves off surface proteins. When we looked for expression in CNS myelin, sema5A was not detectable at all, whereas NogoA was expressed at high levels in optic nerve, as well as in oligodendrocytes and OPCs (Fig. 4*A*,*B*) (and data not shown).

We also examined the expression pattern of sema5A *in vivo* by immunostaining optic nerve sections. The embryonic optic nerve demonstrated strong sema5A immunostaining along the pial cells lining the edge of the optic nerve (Fig. 4*C*), consistent with a previous study reporting embryonic RGCs directed by sema5A to elongate axons within the nerve (Oster et al., 2003). In P8 and P21 rat optic nerve, sema5A was colocalized with oligodendrocytes as identified by CC1 antisera and appeared to be throughout the cell bodies rather than specifically in myelin sheaths (Fig. 4*D*,*E*). In contrast, in P0 and P10 mouse optic nerve from transgenic mice in which astrocytes express GFP under the control of the s100β promoter (Lubischer et al., 2000), sema5A appeared to be specifically excluded from most of the GFP-positive astrocytes (Fig. 4*F*) (and data not shown). S100β-promoted GFP expression did not overlap with CC1 immunostaining of oligodendrocytes (Fig. 4*G*). Interestingly, although we did not observe sema5A protein in purified P8 RGCs by Western blot, E20 RGC axons did label strongly with sema5A antisera (data not shown). Thus sema5A protein is expressed in optic but not sciatic nerve and specifically in OPCs and oligodendrocytes by Western blot and immunostaining.

Expression of semaphorin receptors by RGCs

The specific sema5A receptor is not yet known, but we investigated whether several known semaphorin receptors are expressed by purified RGCs in culture. We used both Ab and ligand binding experiments (see Materials and Methods). Nearly all RGCs in culture were brightly neuropilin-1 immunoreactive (Fig. 5*A–C*). Because the antiserum was directed against the N-terminal domain of neuropilin-1, we also stained nonpermeabilized RGCs in culture. Bright punctate immunoreactivity was present along the surfaces of the RGCs (Fig. 5*C*). In permeabilized cultures, no RGCs stained with the plexin-B1-specific antiserum (Fig. 5*D*), consistent with their lack of response to sema4 family members (Figs. 2, 3) (also see Discussion). Most RGCs exhibited strong

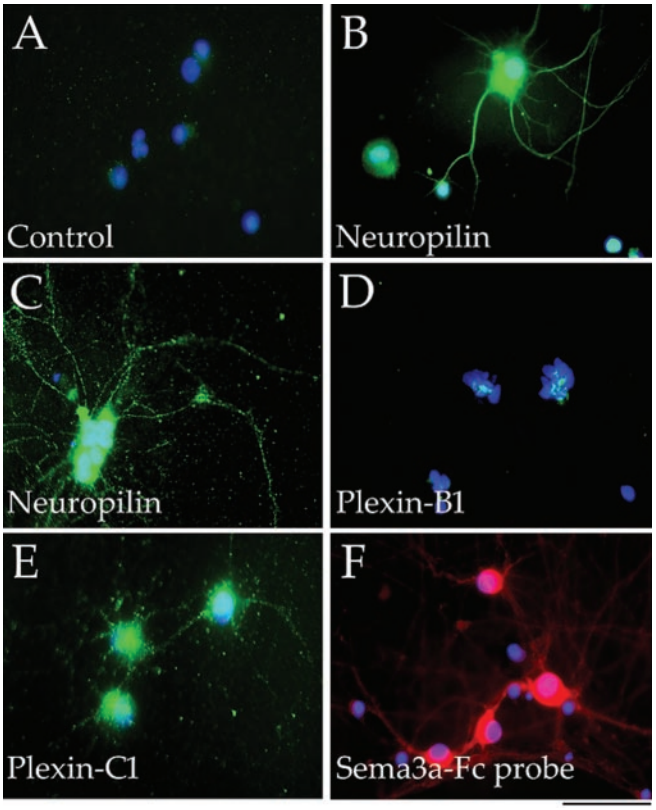


Figure 5. Expression of semaphorin receptors by purified RGCs. After 1 d in culture, RGCs were immunostained for various semaphorin receptors using no primary Ab (*A*, control) or antibodies against neuropilin-1 (*B*, *C*), plexin-B1 (*D*), or plexin-C1 (*E*). Surface immunostaining for neuropilin-1 (*C*) was performed on nonpermeabilized RGCs. *F*, A subset of purified RGCs bind sema3A on their surfaces, detected by secondary staining with biotin-conjugated goat anti-human Fc Ab and tertiary staining with Cy5-conjugated streptavidin. Scale bar, 50 μm.

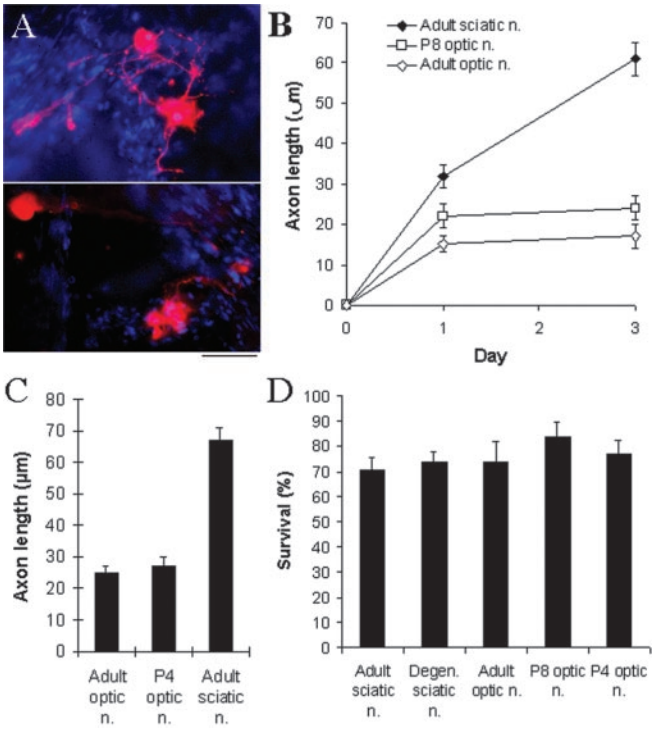


Figure 6. Postnatal retinal ganglion cell axon growth on living nerve explants. P8 RGCs were cultured on optic or sciatic nerves explanted and opened longitudinally as a culture substrate, in the presence of growth media containing trophic factors. *A*, RGC axons were visualized by DiI staining (red); glial cell nuclei in nerve explants (optic nerve shown here) were visualized by DAPI (blue). *B*, Longest axon per RGC was measured after culture on postnatal or adult optic or sciatic nerve after 1 or 3 d, as marked. *C*, RGC axon growth measured after 3 d on P4 optic and adult optic and sciatic nerves. *D*, Survival of RGCs on nerve explants as determined by cellular (DiI) and nuclear (DAPI) morphology. Means ± SEMs are shown. Scale bar, 50 μm.

plexin-C1 immunoreactivity (Fig. 5*E*). In addition, we analyzed RGC plexin expression by microarray analysis of gene expression using rat genechips (Affymetrix) (our unpublished data), which contain probes for neuropilin-1 and -2 as well as for plexin-B1 and -B3 (the latter having two separate probes currently annotated as "weak homology"). These data demonstrated that RGCs both embryonically and postnatally express neuropilin-1 but not -2, and neither plexin-B1 nor -B3 at the mRNA level, consistent with the immunostaining above. Thus by immunostaining and by mRNA analysis by microarray, RGCs express neuropilin-1 and plexin-C1 but not neuropilin-2, plexin-B1, or plexin-B3.

We also tested Fc forms of the various sema proteins for their ability to bind to RGCs. Consistent with the expression of neuropilin-1, a sema3A–Fc protein bound to the surface of nearly 100% of RGCs in culture (Fig. 5*F*). Sema4A, 5A, and 6A proteins did not show significant binding to RGCs using this immunofluorescence technique, either because their receptors are at low levels or absent or because their affinity is too low to detect using this approach (data not shown). Taken together, these results provide evidence that RGCs in purified cultures express at least two different semaphorin receptors: neuropilin-1 and plexin-C1 (see Discussion).

Ability of RGC axons to grow on optic nerve explants

We next investigated whether sema5A within the optic nerve might be inhibitory to regenerating RGCs. It is difficult to address this question *in vivo* because RGCs rapidly die after axotomy and it is difficult to deliver semaphorin-neutralizing antibodies into the CNS. We therefore took advantage of our ability to purify RGCs and to culture them in defined serum-free conditions that strongly promote their survival and axon growth (Meyer-Franke et al., 1995; Goldberg et al., 2002b). We adapted a novel model system in which we explanted optic and sciatic nerves from postnatal and adult rats and carefully opened them longitudinally, presenting the interior of the living nerve as a growth substrate for purified RGCs (see Materials and Methods). We cultured purified, DiI-labeled RGCs on living optic nerves explanted from P4 rats, an age at which oligodendrocytes have not yet begun to differentiate but many OPCs are present, from P8 rats after oligodendrocyte differentiation has just begun, and from fully myelinated adult rats (Skoff, 1981). RGC axons were found to extend axons along the nerve explants both longitudinally as well as in meandering, transverse directions (Fig. 6*A*). We compared the length of the longest axon from each RGC grown on these various nerves and found that axon growth was strongly inhibited on optic nerves of all ages (Fig. 6*B*). Sciatic nerve explants did not inhibit RGC growth as strongly as expected (Bray et al., 1991). Surprisingly, despite the absence of oligodendrocytes, the amount of inhibition on P4 optic nerves was not significantly different from the inhibition observed on P8 or adult optic nerves (Fig. 6*C*). In the absence of inhibition, P8 RGCs are normally able to extend their axons by, on average, 150 μm over 3 d (Goldberg et al., 2002a), yet they extended only ~25 μm when cultured on P4 optic explants, representing an inhibition of ~85% of RGC axon growth ability. This inhibition could not be attributed to

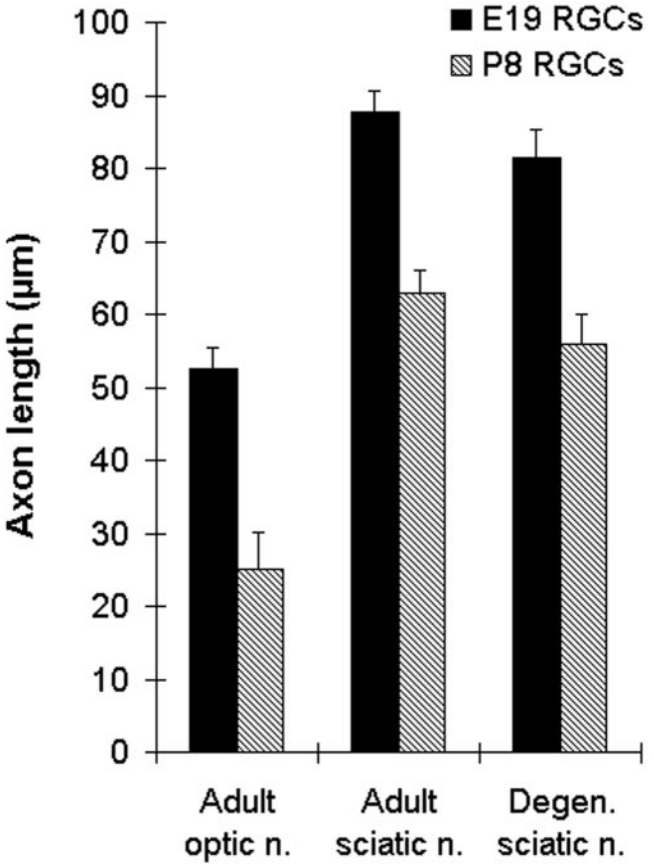


Figure 7. Embryonic RGC axon growth on optic nerve explants. E20 and P8 RGCs were cultured on optic and sciatic nerve explants as in Figure 6. Degenerated sciatic nerves were prepared by proximal axotomy of the adult sciatic nerve followed 1 week later by explanting and opening the distal segment of axotomized sciatic nerve as a culture substrate. After 3 d in culture, the longest axon per RGC was measured. Means ± SEMs are shown; $*p < 0.05$.

poor survival on explants, because the majority of cells survived when cultured on the explants (Fig. 6*D*), as they do when cultured directly on a laminin substrate (Meyer-Franke et al., 1995). We were unable to study the ability of RGCs to extend on embryonic optic nerve explants because the small size of embryonic nerves made this experiment technically impossible; however, the glial composition in E19 and P4 optic nerves is similar, consisting primarily of astrocytes and OPCs. Taken together, these results show that the growth of P8 RGCs is strongly inhibited when cultured on P4, P8, and adult optic nerve explants and provide evidence that optic nerve cues other than myelin-associated inhibitors are also inhibitory.

Embryonic neurons are reported to be generally much less sensitive to the effects of some growth inhibitors, such as Nogo (Fournier et al., 2002), than are postnatal and adult neurons. Are embryonic RGCs similarly sensitive to optic nerve inhibition? We cultured purified E20 RGCs on adult optic and sciatic nerves and found that they were also strongly inhibited (Fig. 7). Although the E20 RGCs were able to extend axons roughly twice as long as the P8 RGCs over a similar culture period, E20 RGCs are normally able to extend their axons 5- to 10-fold more rapidly than can P8 RGCs in purified cultures in the absence of glia (Goldberg et al., 2002a). Thus axon growth by both embryonic and postnatal RGCs is strongly inhibited when they are cultured on optic nerve explants.

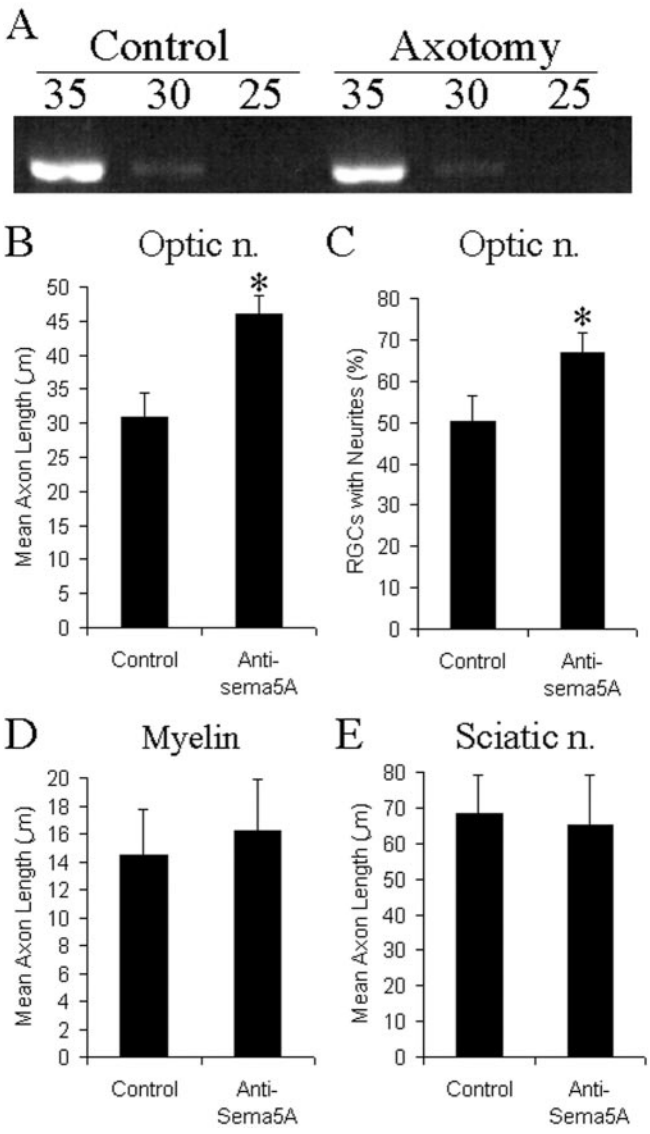


Figure 8. Effect of sema5A-mediated inhibition in optic nerve explants. *A*, Optic nerves axons were severed *in vivo* by optic nerve crush of P8 rats. After 18 hr, total RNA was collected from crushed and control optic nerves that were removed from killed animals. Reverse transcription to cDNA was followed by PCR for sema5A, varying the cycle number from 25 to 35 cycles to semiquantitatively estimate level of expression. No difference in expression of sema5A was found in crushed nerve samples. *B–E*, P8 RGCs were cultured on optic or sciatic nerve explants as in Figure 6, or on myelin substrates as labeled (see Materials and Methods), in the presence of anti-sema5A or control antisera. After 3 d the longest axon per RGC was measured. Means ± SEMs are shown; $*p < 0.05$.

Effect of sema5A-mediated inhibition in optic nerve explants

Finally, we used this explant culture system to investigate whether sema5A contributes to inhibition of RGC axon regeneration. We first investigated whether sema5A is upregulated after optic nerve injury. We severed RGC axons by optic nerve axotomy in P8 rats (see Materials and Methods). After 1 d, we isolated mRNA from the transected optic nerve. By RT-PCR, sema5A was still expressed at comparable levels before and after optic nerve axotomy (Fig. 8*A*). Thus sema5A is constitutively expressed regardless of the presence of axons or injury (Fig. 8*A*).

We next examined the role of sema5A in the optic nerve explant-mediated inhibition of RGC axon growth using a polyclonal antiserum directed against the extracellular domain of sema5A that has been shown previously to inhibit sema5A signal-

ing (Oster et al., 2003). We found that RGCs extended axons that were 50% longer on P8 optic nerve explants in the presence of the sema5A-blocking antiserum than in the presence of a control, irrelevant antiserum (Fig. 8*B*). This effect was a consistent and statistically significant improvement. Furthermore, a greater percentage of RGCs extended neurites in the presence of anti-sema5A antibodies (Fig. 8*C*). When we cultured RGCs on myelin substrates or on sciatic nerve, however, addition of the sema5A antiserum did not allow any greater axon outgrowth (Fig. 8*D*,*E*), consistent with the previous data demonstrating the absence of sema5A from myelin and peripheral nerve (Fig. 4). These data provide evidence that sema5A contributes to the inhibition of RGC axon growth on optic nerve explants.

Discussion

Sema5A collapses RGC growth cones and inhibits the rate of their elongation

These data support the hypothesis that sema5A, in addition to guiding the growth of developing RGC axons, contributes to the ability of glial cells to inhibit axon regeneration. Sema5A helps to guide embryonic RGCs, preventing them from growing out of the optic nerve sheath during development in explant cultures (Oster et al., 2003). In those experiments, sema5A was found to collapse growth cones extending from embryonic retinal explants and was localized to neuroepithelial cells surrounding retinal axons at the E14 optic disc and nerve. Our findings confirm the ability of sema5A to collapse embryonic RGC growth cones and inhibit their growth, and they extend these results by showing that sema5A is also inhibitory to postnatal RGCs, is produced by oligodendrocytes and their precursors, and contributes to the inhibitory environment of the injured postnatal optic nerve. RGCs demonstrated longer axon regeneration on optic nerve explants in the presence of sema5A-blocking antisera. We suggest that sema5A may similarly contribute to the failure of RGC regeneration after injury *in vivo*.

Although ours is the first evidence to suggest a functional role for semaphorins as axon growth inhibitors in the injured optic nerve, many previous studies have provided evidence that semaphorins other than sema5A (Oster et al., 2003) guide developing RGC axons. Sema4F is highly expressed by the embryonic optic nerve and can collapse E6 chicken retinal growth cones *in vitro* (Encinas et al., 1999), whereas in this study we show that the other class 4 semaphorins, 4A, 4B, and 4D, do not inhibit rat RGC axons. Similarly, sema3A can collapse *Xenopus* RGC growth cones (Campbell et al., 2001), and sema3E can collapse chick RGC growth cones (Steinbach et al., 2002), although secreted class 3 semaphorins do not inhibit rodent RGC axons (Luo et al., 1993; Giger et al., 1998a,b). Interestingly, we found that sema3A could bind to the surface of RGCs but was unable to collapse their growth cones or inhibit axon elongation, raising the question of why rodent RGCs express sema3A receptors (see below). Recent experiments suggest that anti-sema3A antibodies rescue adult RGCs from apoptosis after optic nerve axotomy (Shirvan et al., 2002). We have not observed any sema3A effect on RGC survival *in vitro* or in the presence of trophic factors (our unpublished observations). Therefore, sema5A is so far the first semaphorin demonstrated to inhibit developing rodent RGC axons.

Elsewhere in the CNS other semaphorins may play a similar role. For example, sema4D inhibits axons of sensory and cerebellar granule neuron from the postnatal brain, although it is not clear whether sema4D plays any similar role during development in these pathways (Moreau-Fauvarque et al., 2003). Similarly, sema3A is expressed in the spinal cord and inhibits ingrowing dorsal root ganglion (DRG) axons from overshooting their targets (Shepherd et al., 1997). Sema3A is induced after injury in the spinal cord, and is associated with the glial scar (Pasterkamp et al., 1999). Regenerating DRG neurons fail to cross these sema3A-expressing regions, even after a conditioning lesion that allows them to penetrate areas of other inhibitory molecules such as CSPGs (Pasterkamp et al., 2001). It is not yet known whether blocking sema3A will allow increased regeneration in the spinal cord *in vivo* or in postnatal explant cultures, but these data indicate that semaphorins specific to each region of the CNS may contribute to local failure of regeneration.

Optic nerve glia express distinct complements of semaphorins

A remarkable finding in our study was the breadth and specificity of semaphorins expressed by distinct classes of glial cells purified from rat optic nerve. Until recently, most studies of semaphorins concentrated on their roles in developing embryos, before the time that glial cells are generated. In embryos, neurons and neuroepithelial cells produce semaphorins (Huber et al., 2003). Surprisingly, most glial semaphorins did not inhibit RGC axons, suggesting that they have other, perhaps diverse functions in glial–neuronal and glial–glial signaling. For example, they may limit axon branching within the optic nerve. The oligodendrocyte lineage semaphorins including sema5A are of particular interest in this regard, because mutant mice lacking oligodendrocytes have an increase in axon collateralization (Colello and Schwab, 1994). Additionally, oligodendrocytes express semaphorin receptors, and sema3A has been reported to guide the migration of OPCs out of the optic chiasm into the optic nerve (Spassky et al., 2002; Cohen et al., 2003). High expression of semaphorins and their receptors by oligodendrocytes raises the possibility that these semaphorins might also help to coordinate glial spacing within the nerve, perhaps by inhibiting the motility of OPCs (Jefferson et al., 1997). Thus, our findings add to the growing evidence that semaphorins are likely to regulate glial development in addition to their well accepted roles in neuronal development.

Although oligodendrocyte-associated inhibitors described thus far have been demonstrated to be enriched in myelin, including Nogo, MAG, OMgp, and most recently sema4D (Moreau-Fauvarque et al., 2003), we did not find sema5A in myelin. First, sema5A was not detected by Western blot in purified myelin preparations. Second, immunostaining the postnatal optic nerve for sema5A and CC1 (an oligodendrocyte marker) was not suggestive of myelin sheath localization, but rather pointed toward cell body localization. Finally, RGC axons were greatly inhibited on purified myelin substrates, but this inhibition was not relieved with sema5A Ab. Thus, although we would not conclude that sema5A is specifically excluded from myelin, it does not appear to be particularly enriched there, and myelin preparations appear not to contain any immunologically or functionally detectable sema5A. Similarly, sema5A was not detected in peripheral sciatic nerve either immunologically by Western blot or functionally in sciatic nerve explant experiments in the presence of sema5A-blocking Ab. Thus, our data support the hypothesis that sema5A is a nonmyelin associated inhibitor that contributes to the failure of CNS regeneration, at least in the optic nerve.

What is the RGC receptor for sema5A?

The dual roles for sema5A during development and after injury in the CNS amplify the importance of identifying and characterizing its receptor or receptor complex. The discovery of sema-

phorin family members has far outstripped the identification of their receptors and downstream signaling pathways (Fiore and Puschel, 2003; Huber et al., 2003). For example, secreted, sema3 family members appear to bind either neuropilin-1 or -2 and transduce their signal in concert with a plexin family member (Takahashi et al., 1998, 1999; Winberg et al., 1998; Tamagnone et al., 1999), and class 4 transmembrane semaphorins thus far have three unrelated receptors, including plexin-B1, the nonplexin receptor CD72, and Tim-2 (Tamagnone et al., 1999; Kumanogoh et al., 2002; Swiercz et al., 2002). Sema3A and sema7A also signal via integrins (Pasterkamp et al., 2003; Serini et al., 2003), which greatly broadens the list of potential candidates for a sema5A receptor. RGCs exhibit bright neuropilin-1 and plexin-C1 immunoreactivity and express plexin-A1 and -A3 embryonically and plexin-A1, -A2, and -A3 postnatally *in vitro* and *in vivo*, but do not express plexin-B1 (Murakami et al., 2001; this paper). Thus the receptor for sema5A is currently unknown.

Can trophic stimuli overcome growth inhibitors?

An unanswered question is how axon growth stimulators interact with growth inhibitors. Neurotrophins such as NGF and BDNF decrease growth cone collapse of DRG neurons in response to sema3A (Dontchev and Letourneau, 2002), and by elevating cAMP levels neurotrophins can decrease myelin-associated inhibition of axon growth (Song et al., 1998; Cai et al., 1999). Conversely, sema3F collapses sensory neuron growth cones in part by antagonizing the activation of MAP kinase kinase and phosphatidyl inositol 3-kinase signaling pathways by NGF (Atwal et al., 2003). These findings support a model in which neither stimulators nor inhibitors are dominant, however. For example, neither sema5A (this paper) nor myelin (our unpublished observations) is sufficient to entirely overcome neurotrophic factor-stimulated growth of RGC axons, and exogenous addition of high levels of trophic factors has not been sufficient to reverse CNS inhibition of regeneration *in vivo* (Goldberg and Barres, 2000). In addition, we found that although embryonic RGCs were significantly inhibited by sema5A substrates, they maintained a significant growth advantage over P8 RGCs on optic nerve explants, suggesting that intrinsic axon growth ability may be critical to overcoming axon growth inhibitors (Goldberg et al., 2002a).

Sema5A may contribute to the ability of oligodendrocyte lineage cells to inhibit axon regeneration in the adult CNS

An appreciation for the specific glial subtypes expressing these inhibitory molecules is relatively new. We found that sema5A was expressed by oligodendrocytes as well as by OPCs, consistent with our data demonstrating that the explanted optic nerve inhibits axon regeneration at an age before oligodendrocytes have differentiated (P4). On adult optic nerve explants, myelin-associated inhibitors such as Nogo, MAG, and others could account for the significant inhibition that remains after blocking anti-sema5A. Sema5A was not expressed in myelin, however, and RGC axon growth inhibition by myelin was not reduced by sema5A-blocking antibodies. Our findings therefore add to the growing data that OPCs, as well as oligodendrocytes, may contribute to axon growth inhibition after injury, and that oligodendrocytes themselves are strongly inhibitory even in the absence of myelin. OPCs also express other inhibitory molecules such as CSPGs during development as well as after injury at the glial scar (Chen et al., 2002), and a number of other axon guidance molecules and receptors persist in the adult CNS, such as sema4F (Encinas et al., 1999), EphB3 (Miranda et al., 1999), and slit and robo (Marillat et al., 2002). It is interesting to speculate that all of these molecules may serve other important functions in the normal adult CNS but also may impair axon regeneration after injury. Thus semaphorins, particularly sema5A, may be among the oligodendroglial lineage-expressed molecules that block axon regeneration *in vivo*.

In conclusion, our data support the hypothesis that constitutive expression of sema5A in oligodendrocytes and their precursors contributes both to guiding RGC axons during normal development and to inhibiting RGC regeneration in the adult optic nerve. Because sema5A was not found in peripheral nerve, it may help to explain the difference between the ability of the mammalian CNS and PNS to regenerate after injury. Further identification and characterization of CNS molecules inhibitory to axon regeneration will be critical to understanding and overcoming the failure of CNS axon regeneration, and our data suggest that it will be important to continue to study other axon guidance molecules for roles in regenerative failure.

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