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Invariant Sema5A inhibition serves an ensheathing function during optic nerve development

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SUMMARY

Retinal axon pathfinding from the retina into the optic nerve involves the growth promoting axon guidance molecules L1, laminin and netrin 1, each of which governs axon behavior at specific regions along the retinal pathway. In identifying additional molecules regulating this process during embryonic mouse development, we found that transmembrane Semaphorin5A mRNA and protein was specifically expressed in neuroepithelial cells surrounding retinal axons at the optic disc and along the optic nerve. Given that growth cone responses to a specific guidance molecule can be altered by co-exposure to a second guidance cue, we examined whether retinal axon responses to Sema5A were modulated by other guidance signals axons encountered along the retinal pathway. In growth cone collapse, substratum choice and neurite outgrowth assays, Sema5A triggered an invariant inhibitory response in the context of L1, laminin, or netrin 1 signaling, suggesting that Sema5A inhibited retinal axons throughout their course at the optic disc and nerve. Antibody-perturbation studies in living embryo preparations showed that blocking of Sema5A function led to retinal axons straying out of the optic nerve bundle, indicating that Sema5A normally helped ensheath the retinal pathway. Thus, development of some CNS nerves requires inhibitory sheaths to maintain integrity. Furthermore, this function is accomplished using molecules such as Sema5A that exhibit conserved inhibitory responses in the presence of co-impinging signals from multiple families of guidance molecules.

Key words: Visual system, Retinal ganglion cell, Pathfinding, Semaphorin, Growth cone, Mouse

INTRODUCTION

The proper wiring of axons to correct targets is essential for development of the nervous system. This process is orchestrated by axon guidance molecules that act either as attractants and support growth, or as inhibitory signals that steer axons away from incorrect pathways and targets. The semaphorins are the largest family of inhibitory axon guidance molecules, consisting of seven distinct classes, and are defined by a characteristic 500 amino acid semaphorin (Sema) domain near the N terminus. Invertebrate semaphorins include classes I and II, while vertebrate semaphorins consist of classes III-VII and encompass over 15 members (Raper, 2000). Previous work in vertebrates has focused on Class III semaphorins. These secreted guidance molecules contribute to development of cranial nerves, cortical architecture, and dorsal root ganglia sensory axon pathways (Messersmith et al., 1995; Polleux et al., 1998; Taniguchi et al., 1997). However, the majority of vertebrate semaphorins, such as class IV, V and VI, are transmembrane molecules, and their function in vivo is not well understood.

While semaphorins are best known for their inhibitory activity during development, a number of reports have provided evidence for the opposite function. Studies in grasshopper have shown that transmembrane Sema-1a functions as an attractive guidance molecule in different regions of the limb bud (Wong et al., 1999). In the mammalian cortex, Sema3A has been reported to inhibit cortical axon growth but attract dendritic growth from the same cell population (Polleux et al., 2000). Similarly, growth cones can switch their response to a given guidance molecule from attraction to repulsion, and vice versa, depending on signaling from a second guidance molecule (Hopker et al., 1999; Song and Poo, 1999). In vertebrates, L1, a guidance molecule of the Ig superfamily, has been reported to switch growth cone response to secreted Sema3A from inhibition to attraction (Castellani et al., 2000). It is not clear whether a similar type of response switching occurs for transmembrane semaphorins. If so, axon responses to transmembrane semaphorins might continually change along a neural pathway as growth cones sequentially encounter signals from a variety of axon guidance molecules.

Amongst the vertebrate semaphorins, Sema5A and Sema5B have an unusual pairing of protein domains. The extracellular domain of these two semaphorins contains seven thrombospondin (TSP) type-1 repeats in addition to the sema domain (Adams et al., 1996). Given that thrombospondin type-1 repeats have been reported to promote neurite outgrowth and

neuronal adhesion (Adams and Tucker, 2000), the pairing of a typically inhibitory sema domain with TSP repeats poses an intriguing question as to how class V semaphorin functions during neuronal development.

In the visual system, the optic nerve consists of the axons of retinal ganglion cells (RGCs), and conveys retinal information to CNS targets. During embryonic development, the optic nerve is formed as RGC axons extend from their cell bodies centrally towards the optic disc, pass through the optic disc, and travel down the optic stalk towards the brain. Some of the axon guidance molecules contributing to this process have been identified. These include L1, which is expressed by retinal axons, and is involved in axon fasciculation in the retina (Brittis et al., 1995). L1 is also highly expressed by retinal axons traversing the optic nerve, chiasm and optic tract, suggesting that a large number of retinal axon pathfinding tasks operate in the context of L1 signaling. A second retinal axon guidance molecule is netrin 1, which is expressed by the group of neuroepithelial cells that form a collar surrounding the optic disc. Netrin 1 is essential for promoting retinal axon growth through the disc into the optic nerve (Deiner et al., 1997). In approximately the same region, retinal axons are also thought to interact with laminin 1, which co-operates with netrin 1 to help steer retinal axons into the optic nerve (Hopker et al., 1999). Given that L1, netrin 1 and laminin 1 are all involved in retinal axon exit at the optic disc leading to optic nerve formation, these three guidance molecules can potentially modulate how retinal growth cones respond to other guidance cues in the retinal pathway.

To identify additional guidance molecules mediating optic nerve development, we examined expression patterns of known guidance molecules including semaphorins. Of eight semaphorins studied, we found that Sema5A was specifically expressed at the optic disc, and along the optic nerve, in a pattern suggestive of a role in retinal axon pathfinding. Functional assays revealed that Sema5A consistently triggered an inhibitory response in embryonic retinal growth cones when tested in the presence of signaling from other relevant guidance molecules. Antibody mediated disruption of Sema5A function resulted in retinal axon defasciculation, and axons straying away from the optic nerve. Our results indicated that transmembrane Sema5A acts as a continually present inhibitory sheath, encasing the retinal pathway as RGC axons carry out multiple pathfinding tasks involving different sets of axon guidance molecules.

MATERIALS AND METHODS

Recombinant proteins and cell lines

Three recombinant mouse Sema5A-Fc fusion proteins were produced. (1) full-length Sema5A extracellular domain (ECD-Fc), (2) Sema5A semaphorin domain (Sema-Fc) and (3) the seven thrombospondin repeats of Sema5A (TSP-Fc). cDNAs encoding each region were cloned into mammalian expression vectors pEx.Fc (Exelixis) or pSectag (Invitrogen), in frame with the human IgG-Fcγ domain. HEK 293 cell lines secreting each recombinant protein were grown for 5 days in Optimem (Gibco), and the supernatants harvested. Fc-tagged proteins were isolated by protein-A chromatography (Amersham Pharmacia), and protein concentrations determined by Coomassie Blue staining and comparison with bovine serum albumin (BSA) standards. Protein purity was verified using silver staining. Fc control protein was prepared as described previously (Birgbauer et al., 2001). The cDNA encoding the extracellular domain of human L1 (aa 1-1120) was cloned into pEX.Fc, and recombinant L1-Fc protein was isolated and characterized as described for Sema5A proteins.

Full-length Sema5A cDNA was cloned into the pEX-myc vector, and a stably transfected HEK 293 cell line was isolated. Immunoblots using membrane preparations from transfected cells showed a single band of the expected size (140 kDa) by anti-myc staining, and by staining using an anti-Sema5A antibody (see below).

In situ hybridization

E12-E18 wild-type (C57/B6) mouse embryo brains were fixed in 4% paraformaldehyde for 60 minutes, and 10 μm cryostat sections cut for in situ hybridization as previously described (Birgbauer et al., 2000). Digoxigenin-labeled sense and antisense RNA probes for mouse Sema3A, 3C, 3E, Sema4A, 4C, Sema5A, 5B, and Sema6A between 250-400 bases in length were taken from the 3′ end of these genes. An additional Sema5A in situ probe (bp 201-2065) and Sema5B probe (bp 337-2497) were also used. These probes spanned the Sema-domains and TSP repeats of both molecules.

Embryonic tissue culture

Retinal tissue was obtained from E14 mouse embryos harvested from anesthetized timed pregnant C57/B6 mice (detection of vaginal plug was counted as day 0). Laminin (Gibco) was coated onto polylysine covered dishes at 5 μg/ml for collapse assays, and at 2.5 μg/ml for netrin 1-dependent neurite outgrowth assays (see below). To use L1 as a substratum, polylysine-covered dishes were coated with anti-human Fc antibody (5 μg/ml; Jackson Immunochemicals). Anti-human Fc-coated dishes were then blocked with 0.25% BSA, and L1-Fc (5 μg/ml) was applied. All coatings were performed at 37°C for 2 hours. L1-Fc coated dishes were washed with PBS three times and used for explant cultures. Explants were maintained at 37°C, 5% CO_2 in F12 medium and N2 supplement (F12/N2 medium) (Gibco).

Retina-optic nerve preparations

The retinal eye cup and a length of optic nerve were removed from E14 mouse embryos. Three quarters of the retina was cut away, leaving a retinal wedge and the attached optic nerve stump (see Fig. 4A). Retina-optic nerve preparations were cultured overnight on L1-coated dishes, and held in place by a flap of fine wire mesh (#203025-A, Small Parts Inc.) attached to the culture dish by Sylgard (Dow Chemical).

Growth cone collapse assays and substratum choice assays

Collapse assays were performed in 8-well coverglass chamberslides (Fisher), ECD-Fc, Sema-Fc, TSR-Fc or Fc were diluted in F12/N2 medium, and added to wells containing retinal explants at final concentrations of 2.5 nM-100 nM. Anti-human Fc antibody (Jackson Immunochemical) was added at 7.5 μg/ml to multimerize the Fc-tagged recombinant proteins. After exposure to reagents at 37°C in 5% CO_2 for 30 minutes, retinal explants were fixed with 4% paraformaldehyde. Growth cones were stained using Texas Red-Phalloidin (Molecular Probes), and scored for collapsed or expanded morphology. Substratum choice assays were performed as previously described (Birgbauer et al., 2001). The responses of growth cones extending on laminin (5 μg/ml) encountering a substratum region containing laminin (5 μg/ml) and 20 nM ECD-Fc were analyzed. Growth cone stops or turning away were considered as positive responses. A small amount of Cy3-labeled antibody was included along with ECD-Fc as a fluorescent marker. ECD-Fc was substituted with 20 nM heat-inactivated ECD-Fc (74°C for 20 minutes) in control experiments. E14 RGC axons show minimal response to Fc protein in substratum choice assays (Birgbauer et al., 2001).

Time-lapse microscopy

35 mm coverslip dishes (MakTek) containing retinal explants or retina-nerve preparations were overlayed with pre-warmed mineral oil

(Sigma) and maintained at 37°C on a microscope stage incubator with CO_2 influx. Time-lapse images of growth cones were captured at 1 minute intervals with a CCD camera (PXL2, Photometrics) using Hoffman optics and Deltavision image acquisition software (API). Baseline growth was recorded for 20-45 minutes before reagent application. Recombinant proteins and anti-Fc antibody were diluted to a volume of 100 µl, and then added by gel-loading pipette to cultures underneath the mineral oil to achieve a recombinant protein concentration of 12.5 nM. Following reagent application, growth cone behavior was recorded for an additional 45-80 minutes.

Neurite outgrowth assays

Supernatant from netrin 1 expressing HEK 293 cells was concentrated using centriprep-10 concentrator columns (Amicon). Netrin 1 concentration was determined by Coomassie Blue staining and comparison with BSA standards. In netrin 1-dependent outgrowth assays, laminin was used at 2.5 µg/ml (threshold for reliable outgrowth was 4 µg/ml) and netrin 1 (100 ng/ml) was added to the culture medium. Anti-DCC monoclonal antibody (Oncogene) was used at 1:150 dilution.

Co-culture assays of retinal explants with either Sema5A-myc 293 cells or parental 293 cells were performed in laminin-coated 35 mm coverslip dishes (MakTek). 293 cells were grown to either 20% or 40% confluence. Retinal explants obtained from GFP-transgenic E14 mouse embryos (Jackson Lab) were cultured overnight on top of the laminin and 293 cells in F12/N2 medium with netrin 1 supplementation. Total neurite outgrowth was determined after fixation in 4% paraformaldehyde and tracing the fluorescent neurites on a video monitor. After conversion to digital images, total neurite length was quantified using Adobe Photoshop.

Antibody production and characterization

A rabbit polyclonal antibody was raised against bacterially expressed Sema5A without the thrombospondin-type 1 repeats (aa 1-544 fused with 939-974). This coding sequence was inserted into pTrcHis 2B (Invitrogen), in frame with a C-terminal myc epitope and a poly-histidine (His_6) tag. Recombinant protein was produced in *E. coli* (strain BL21(DE3)pLysS) after IPTG induction for 3 hours at 30°C. Bacteria were then lysed, and recombinant protein affinity purified on a NiNTA agarose column (Pharmacia). Antigen was injected into rabbits by Covance Inc. (Berkeley). Anti-Sema5A rabbit immune and pre-immune serum were precipitated using ammonium sulfate, and dialyzed against F12 medium. Fab fragments were made using papain digestion, and separated from Fc fragments and uncut antibodies by protein A chromatography (Pierce).

Anti-Sema5A was used at 1:400 in western blots to demonstrate immunoreactivity against recombinant ECD-Fc. Live 293 cells expressing Sema5A-myc were stained for 1 hour at 37°C with anti-Sema5A (1:100) in culture medium. After 3 washes with pre-warmed culture medium, they were fixed in 4% paraformaldehyde, and visualized using a Cy3 donkey anti-rabbit antibody (Jackson Immunochemicals).

Retinal immunostaining

Live E14 retinas were isolated along with a short segment of optic nerve, and incubated with anti-Sema5A antibody (1:100) in F12/N2 medium for 2-16 hours at 37°C. After three washes with warm culture medium, retinas were incubated Cy2 donkey anti-rabbit antibody (1:500) (Jackson Immunochemicals) for another 2-16 hours. Retinas were then washed three times, fixed in 4% paraformaldehyde for 1 minute, and mounted whole. Control retinas were processed in the same manner using pre-immune serum. The pattern of immunoreactivity was analyzed using a confocal microscope (Pascal LSM, Zeiss).

Sema5A function blocking experiments

For optic nerve experiments, live tissue preparations containing the retinas, optic nerves, and the optic chiasm were dissected from E14 embryos (Sretavan and Reichardt, 1993). Preparations were cultured for 8-10 hours at 37°C in the presence of anti-Sema5A or pre-immune serum (1:100) in F12/N2 medium. Tissue preparations were then transferred into 2 ml of antbody-free F12/N2 medium, cultured overnight, and fixed with 4% paraformaldehyde for 24 hours. Retinas were removed, and a DiI crystal placed at the optic disc using a glass micropipette. After overnight incubation in 4% paraformaldehyde at 37°C, optic nerves were examined using rhodamine optics on a fluorescent microscope (Microphot-SA, Nikon) or a confocal microscope (Pascal LSM, Zeiss).

For intra-retinal function blocking experiments, gestational day 14 pregnant mice were anesthetized with xylazine/ketamine mixture, and a mid-abdominal incision made to expose the uterine sacs. A small incision was made through the uterine wall to reveal one eye of an embryo. Micropipettes loaded with Fab fragments (1 µg/µl in F12 medium) were used for intraocular injections, and an estimated 2-4 µg of antibody was delivered into each eye. Up to four embryos were injected in each animal. Uterine incisions were closed with 8-0 sutures, and the abdominal wall and overlying skin closed with 4-0 sutures. After a further 24 hours of in utero development, injected embryos were harvested and fixed in 4% paraformaldehyde for 24 hours. A DiI crystal was placed in the peripheral retina of treated eyes, and after 6-8 hours at 37°C, retinas were mounted whole, and visualized using standard fluorescence optics.

RESULTS

Sema5A mRNA expression in the developing retinal pathway

To better understand the molecular basis of retinal ganglion cell axon guidance and optic nerve development, we carried out in situ hybridization analysis to identify axon guidance molecules expressed in the developing retinal pathway. Included were probes corresponding to eight different members of the semaphorin gene family, covering both secreted and transmembrane semaphorins (Table 1).

A number of semaphorins were found in the developing mouse retina during the period of active RGC neurogenesis and axon outgrowth through the optic disc into the optic nerve (Table 1). The mRNAs corresponding to Sema3A, Sema3C, Sema3E and Sema6A were found in the retinal ganglion cell layer. However, only mRNA corresponding to Sema5A, a class V transmembrane semaphorin, was detected at the developing optic disc and optic nerve. Sema5A mRNA was expressed by the optic disc neuroepithelial cells, which as a group surrounded the RGC axons exiting from the retina (Fig. 1A,B). Previous work has shown that this population of neuroepithelial cells expresses netrin 1, which plays a major role in guiding axons into the optic nerve (Deiner et al., 1997). Sema5A mRNA was also found in neuroepithelial cells that line the embryonic optic stalk connecting the retina to the ventral diencephalon (Fig. 1A,E). Expression was however absent at the midline region including the optic chiasm, and absent within the diencephalon. Sema5A mRNA was present at the optic disc and optic stalk from E12-E16, during the period of active retinal axon growth through these areas, but was absent from these regions by E18 (data not shown). Sema5A mRNA expression was also observed in the lamina propria under the olfactory epithelium (Fig. 1G), and in the walls of the lateral ventricles and developing striatum (data not shown). It is of interest that mRNA for Sema5B, the other

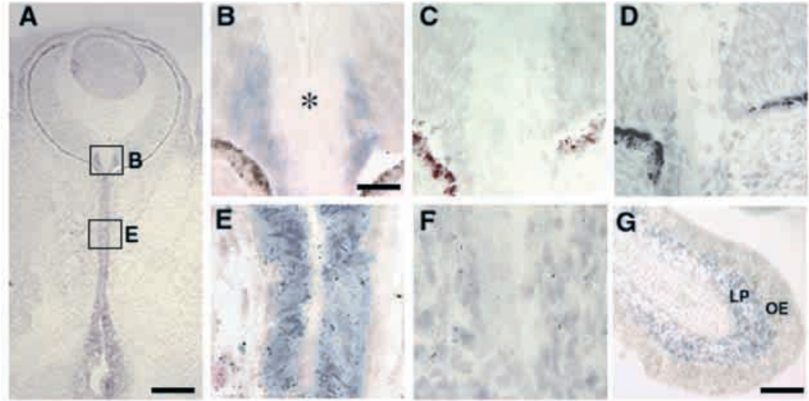

Fig. 1. Expression of Sema5A mRNA. (A) Sema5A in situ hybridization in the E14 mouse retinal pathway. Sema5A was expressed at the optic disc and along the developing optic nerve to the ventral diencephalon. The boxed areas are shown at higher magnification in B and E. (B) Sema5A mRNA expression at the optic disc. Hybridization signal using an antisense probe was present in neuroepithelial cells that flank the exiting retinal axons (*). As a group, these Sema5A-positive neuroepithelial cells formed a collar around the retinal axon bundle. (C) Hybridization at the optic disc using the Sema5A sense probe. (D) Hybridization at the optic disc using the Sema5B probe showed no signal. (E) Sema5A mRNA expression in the developing optic nerve. Note the presence of hybridization signal in neuroepithelial cells flanking the central core containing retinal axons. (F) Hybridization in the optic nerve using the Sema5A sense probe. (G) Sema5A mRNA expression in the lamina propria (LP) under the olfactory epithelium (OE). Scale bars: (A) 150 μm; (B-F) 25 μm; (G) 75 μm.

Table 1. Semaphorin mRNA expression in E13-E15 mouse retina and optic nerve

Semaphorin	RGCs	Optic disc/nerve
Secreted		
Sema3A	++	–
Sema3C	++	–
Sema3E	++	–
Transmembrane		
Sema4A	+	–
Sema4C	–	–
Sema5A	–	+++
Sema5B	+	–
Sema6A	+++	–

known vertebrate Class V semaphorin, was not detected in the developing retinal pathway (Fig. 1D). The pattern of Sema5A mRNA expression placed this molecule in regions traversed by embryonic retinal axons, and suggested a role in retinal axon guidance.

The presence of Sema5A mRNA in the optic nerve and at the optic disc, a major exit point for retinal axons, was at odds with the fact that vertebrate semaphorins are generally considered inhibitory axon guidance molecules. Given that growth cone responses have been reported to switch from inhibition to attraction in the context of signaling from a second guidance molecule (Castellani et al., 2000; Hopker et al., 1999), Sema5A function during retinal pathway development needed to be determined. Since axon pathfinding through the optic disc into the optic nerve is known to involve L1, netrin 1 and laminin 1, we tested retinal axon responsiveness to Sema5A in the context of signaling from each of these molecules.

Sema5A inhibits retinal growth cones on laminin

A secreted recombinant Sema5A protein was generated consisting of the extracellular portion of Sema5A, encompassing both its semaphorin and thrombospondin domains, fused to the human IgG Fc domain (ECD-Fc) (Fig. 2A,B). Addition of 12.5 nM ECD-Fc, oligomerized with 7.5 μg/ml anti-Fc antibody, resulted in the collapse of 68% of RGC growth cones cultured on laminin within 30 minutes (Fig. 2C). Increases in ECD-Fc concentration, or duration of exposure, did not further increase the response rate (Fig. 2C and data not shown). Oligomerization was required for maximal response, and was used in all further collapse assays. In control experiments, axons treated with oligomerized human Fc protein responded at a rate of 12% (n=89, P<0.001) (Fig. 2C).

Given that the extracellular domain of Sema5A collapsed retinal axons, we next tested whether this protein when used as a substratum could create a barrier to neurite growth. A substratum choice assay was used, in which growth cones extending on laminin (5 μg/ml) were confronted with a test substratum containing laminin (5 μg/ml) and either ECD-Fc (20 nM) or heat-inactivated ECD-Fc (20 nM). Growth cone responses upon reaching the test substratum included stopping at the border, turning to avoid the area, or growing freely across the border into the labeled region (Fig. 2D,E). We found that 58% of growth cones stopped at, or turned away from, ECD-Fc borders (n=105), while only 36% of growth cones (n=169) behaved similarly at heat-inactivated ECD-Fc borders (P<0.001) (Fig. 2F).

The ability of Sema5A to inhibit retinal axons when expressed as a transmembrane molecule on cell surfaces was also tested. To do so, we measured RGC axon outgrowth in the presence of stably transfected cells expressing full-length Sema5A. Retinal explants placed on top of untransfected 293 cells growing at 20% confluence on a laminin substratum resulted in a mean total neurite length of 5.3 mm per explant (n=25 explants). In the presence of Sema5A-myc-transfected cells at the same confluence, the mean total length was reduced by 30% to 3.7 mm per explant (n=25 explants, P<0.001) (Fig. 2G-I). An increase in the density of 293 cells seeded onto the laminin-coated dishes to 40% confluence resulted in a mean total neurite length of 2.1 mm per explant for untransfected cells (n=9 explants), while Sema5A-myc-transfected cells resulted in a mean total neurite length of only 1.3 mm per explant (n=9 explants, P=0.011) (Fig. 2I). These results indicated that retinal axons extending on laminin were inhibited by Sema5A presented either as a soluble protein fragment in collapse assays, as immobilized protein in substratum choice assays, or as a cell surface protein in neurite outgrowth studies.

Sema5A inhibition is maintained on a L1 substratum

The extracellular fragment of human L1 was used as a substratum for retinal axons in Sema5A growth cone collapse

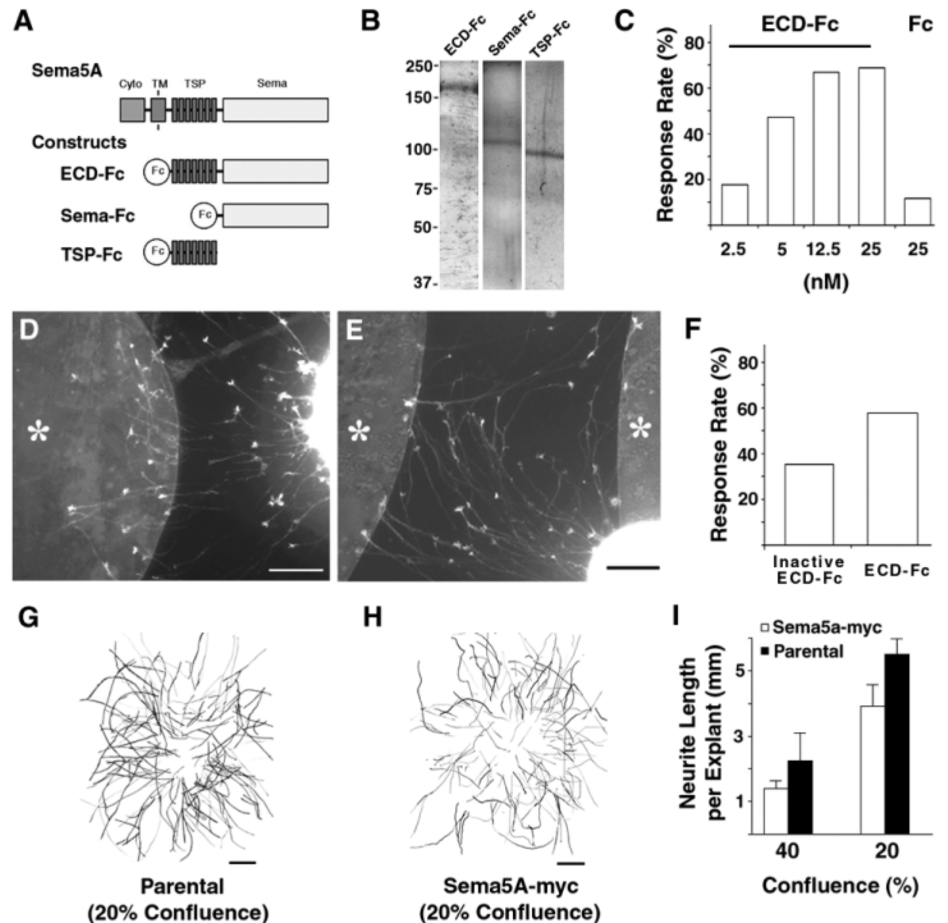

Fig. 2. Recombinant Sema5A proteins inhibit RGC axons on a laminin substratum. (A) Domain structure of Sema5A, and diagrams of three Sema5A extracellular domain constructs. (B) Silver staining of the three Sema5A recombinant proteins (ECD-Fc, sema-Fc, TSP-Fc). (C) The percentage of growth cones on a laminin substratum exhibiting collapse in response to increasing amounts of oligomerized ECD-Fc. (D,E) Examples of growth cone responses to a substratum choice assay. (D) Retinal axons grew freely into regions containing heat-inactivated ECD-Fc (*), but (E) avoided entering regions containing active ECD-Fc (*). (F) The response rate of growth cones encountering a border of either heat-inactivated ECD-Fc or ECD-Fc. (G,H) Composite images showing the pattern and density of axon outgrowth for 25 explants grown on a laminin substratum in the presence of parental 293 cells (G), or Sema5A-myc expressing 293 cells (H), at 20% confluence. (I) Mean total neurite outgrowth per explant on a laminin substratum in the presence of HEK 293 cells transfected with full-length Sema5A-myc or parental cells. At both 20% and 40% confluence, explants grown in the presence of cells transfected with Sema5A-myc exhibited less neurite outgrowth. Scale bars: (D,E) 100 μm; (G,H) 250 μm.

assays. After a 30 minute exposure to 12.5 nM ECD-Fc, 74% of growth cones extending on L1 collapsed (n=148). By comparison, Fc treatment alone resulted in the collapse of only 5% of retinal growth cones (n=178, P<0.001) (Fig. 3B). Time lapse microscopy confirmed that ECD-Fc-treated growth cones extending on L1 (n=30) underwent a loss of filopodia and lamellopdial structures characteristic of growth cone collapse. Typical growth cone collapse involves contraction from an open to a collapsed morphology, but interestingly, growth cones extending on L1 displayed an intermediate stage in which growth cones appeared to lose lamellopodia and consisted mainly of filopodial-like structures (Fig. 3A). These intermediate growth cones subsequently progressed to the fully collapsed, stick-like morphology.

Activities of the semaphorin and TSP domains

Class V semaphorins are unique in that they contain both a sema domain and seven thrombospondin type-1 repeats in their extracellular domain. To investigate the possible function of each sub-domain, recombinant proteins consisting of the semaphorin domain alone fused to an Fc tag (Sema-Fc), or the thrombospondin repeats fused to an Fc tag (TSP-Fc), were purified (Fig. 1A,B) and used in collapse assays. The ability of the sema domain alone to trigger growth cone collapse appeared to be reduced compared to the intact extracellular domain. 12.5 nM Sema-Fc led to only 13% growth cone collapse (n=93), compared to the 74% collapse response observed when 12.5 nM of the entire extracellular fragment was used (Fig. 3C). An increase of Sema-Fc to 100 nM resulted in a 62% collapse rate (n=103), suggesting that the sema domain alone was approximately 8-10 fold less potent as an inhibitor (Fig. 3C).

Application of TSP-Fc up to 100 nM resulted in a collapse rate of only 12% (n=146), which was not different to the 10% response rate seen after application of recombinant Fc control protein (n=52) (Fig. 3C). TSP-Fc was also tested as a growth substratum at concentrations up to 20 mg/ml, but failed to support axon outgrowth from embryonic retinal explants. This data indicated that the inhibitory activity of transmembrane Sema5A lay within its sema domain, as has been described for secreted Sema3A (Koppel et al., 1997). Additionally, the full inhibitory potential required activity supplied by other regions in the extracellular domain.

Sema5A function in the presence of netrin 1

The mRNA expression pattern for Sema5A was remarkably similar to that of netrin 1. To determine whether exposure to netrin 1 modified retinal growth cone responses to Sema5A, we carried out Sema5A collapse assays in the presence of soluble netrin 1, using retinal axons extending on either L1 or laminin.

Retinal explants were cultured overnight on an L1 substratum in medium containing 100 ng/ml netrin 1, a concentration previously shown to effectively promote RGC

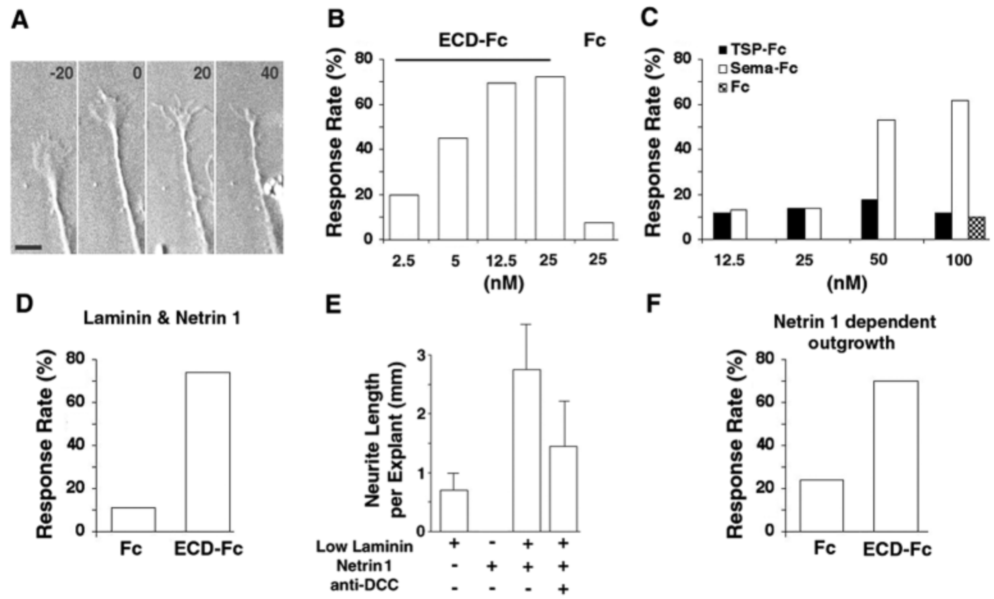

Fig. 3. Growth cone responses to Sema5A in the presence of L1 and netrin 1. (A) Timelapse sequence showing a retinal growth cone extending on an L1 substratum and exhibiting collapse in response to application of oligomerized ECD-Fc. (ECD-Fc was applied at t=0). Numbers at top left are minutes elapsed. During collapse, axons on L1 tended to show an intermediate stage characterized by the shrinkage of the growth cone into a branched structure (t=20). Growth cones eventually progressed to a fully collapsed morphology (t=40). (B) The percentage of growth cones on L1 substratum exhibiting collapse in response to increasing amounts of oligomerized ECD-Fc. (C) The percentage of growth cones on L1 substratum exhibiting collapse in response to increasing amounts of oligomerized sema-Fc or TSP-Fc. (D) The percentage of growth cones extending on laminin in the presence of netrin 1 responding to oligomerized ECD-Fc. The presence of netrin 1 did not alter the ability of ECD-Fc to mediate growth cone collapse. (E) Assay of netrin 1-dependent outgrowth. Sub-optimal levels of laminin resulted in little axon outgrowth (column 1). 100 ng/ml of netrin 1 by itself was not able to support outgrowth (column 2). The combination of sub-optimal laminin levels and 100 ng/ml netrin 1 resulted in robust outgrowth (column 3). This netrin 1-dependent outgrowth was reduced by the addition of an antibody against the netrin receptor DCC (column 4). (F) Axons dependent on netrin 1 for outgrowth remained responsive to ECD-Fc-mediated growth cone collapse. Scale bar: (A) 10 μm.

axon outgrowth in collagen gels (Deiner et al., 1997). ECD-Fc was then added to a final concentration of 12.5 nM for 30 minutes, and the resulting growth cone morphology was analyzed. Sema5A in the presence of netrin 1 resulted in collapse of 74% of RGC growth cones (n=252), same as the response rate of 74% following ECD-Fc treatment without netrin 1 in the culture medium (Fig. 3D). Addition of Fc alone in the presence of netrin 1 resulted in the collapse of 11% of growth cones (n=116, $P<0.001$) (Fig. 3D).

In a second set of experiments, we examined the effects of ECD-Fc on retinal neurites that were more directly dependent on netrin 1 for outgrowth. Retinal explants were cultured on a dish coated with 2.5 μg/ml laminin, a sub-optimal level for reliable axon outgrowth. However, the addition of 100 ng/ml netrin 1 to these cultures elicited reproducible retinal axon outgrowth. Netrin 1 dependence was demonstrated by the fact that addition of an antibody blocking the function of the Netrin receptor DCC attenuated this increased neurite outgrowth (Fig. 3E). In this netrin 1-dependent outgrowth assay, ECD-Fc collapsed 70% of RGC growth cones (n=135) compared to only 24% in Fc-treated controls (n=77, $P<0.001$) (Fig. 3F). Together, this data indicated that netrin 1 was not capable of modulating the inhibitory effects of Sema5A on RGC axons. Thus, Sema5A appeared to maintain its inhibitory function as RGC axons encountered the retinal pathfinding molecules L1, laminin or netrin 1.

Sema5A inhibition during navigation in the optic nerve

One feature of Sema5A mRNA expression was its presence along the entire course of the developing optic nerve. Given that exposure to guidance molecules at specific pathfinding regions can silence receptors and eliminate the ability of a growth cone to respond to a different guidance molecule (Stein and Tessier-Lavigne, 2001; Zou et al., 2000), we investigated whether RGC axons, after passing through the optic disc, were altered in their responsiveness to Sema5A.

Retina-optic nerve preparations consisting of a portion of retina connected to the optic nerve were cultured on L1 (Fig. 4A). Retinal axons that had grown through the optic disc region readily extended from the cut end of the optic nerve (Fig. 4B) and their responsiveness to Sema5A was recorded using time-lapse microscopy. Axons growing out of the optic nerve were sensitive to Sema5A, with 57% of growth cones collapsing in

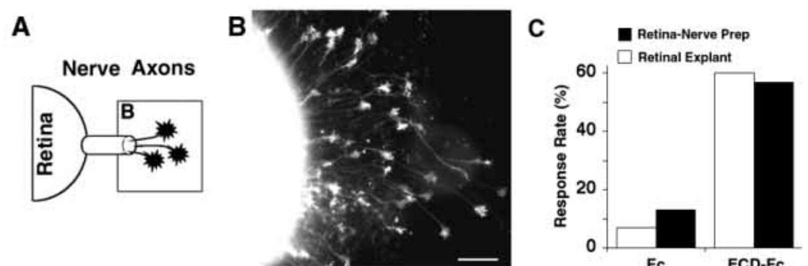

Fig. 4. Sema5A inhibition of post-optic disc retinal axons. (A) Diagram of the retina-optic nerve preparation. (B) Retinal axons and growth cones extending from the cut optic nerve of a retina-optic nerve preparation (boxed region in A), stained with TexasRed-Phalloidin. (C) The response rate of growth cones from retina-optic nerve preparations and standard retinal explants to oligomerized ECD-Fc. Growth cone responses were observed with time-lapse microscopy. Scale bar, (B) 50 μm.

response to ECD-Fc (n=30), compared to only 12% of Fc-treated growth cones (n=24, P=0.002) (Fig. 4C). Additionally, we used video microscopy to test the response rate of axons from retinal explants. In this video-assay, growth cones from retinal explants had a 60% response rate, and there was no significant difference in the response rates between ECD-Fc-treated axons growing from retinal explants or retina-optic nerve preparations (60% versus 57%) (Fig. 4C). Results from these assays suggested that after growth through the optic disc, retinal axons remained sensitive to Sema5A during their course along the optic nerve.

Localization of Sema5A protein in retinal pathway

The results thus far indicated that Sema5A inhibited RGC axons, and that this inhibition was maintained in the presence of co-impinging signals from several relevant guidance molecules. To understand how this inhibition contributed to retinal pathway development, we examined Sema5A protein localization using a polyclonal antibody. This antibody recognized recombinant ECD-Fc on western blots, and a single band of the appropriate size for Sema5A (135 kDa) from embryonic retinal membrane preparations (Fig. 5A). Furthermore, the antibody also resulted in membrane staining of living cells transfected with Sema5A-myc, but not of untransfected cells (Fig. 5B and data not shown).

Retinal immunostaining using this anti-Sema5A antibody required the use of unfixed tissues. In E14 mouse retinas, immunoreactivity was detected as a ring at the perimeter of the optic disc, encircling RGC axons passing through the optic disc (Fig. 5C,D). Immunostaining was absent from the central region of the optic disc that contained retinal axons and the neuroepithelial cell processes known to display netrin 1 protein on their surfaces (Deiner et al., 1997). This pattern of protein localization, together with the inhibitory activity of Sema5A on retinal axons, raised the possibility that Sema5A prevented RGC axons from straying away from the optic disc and the optic nerve.

Perturbation of Sema5A function results in axon guidance errors

To test the role of Sema5A in development of the retinal pathway, anti-Sema5A antibody, or Fab preparations, were applied intraocularly to embryos in utero and to living embryonic tissue preparations containing the optic nerve. In control experiments, anti-Sema5A antibody (1:100) was effective in blocking Sema5A-mediated growth cone collapse of retinal axons extending on L1 in vitro. In the presence of anti-Sema5A antibody, growth cone collapse rates after ECD-Fc exposure dropped from 74% (n=148) (Fig. 3A) to 32% (n=121, P<0.001) (Fig. 6A). Pre-immune serum did not curb Sema5A-mediated growth cone collapse, and resulted in a 70% response rate (n=95) (Fig. 6A).

Horizontal slice preparations of embryonic mouse brains, including the retinas and the optic nerves, were cultured in either anti-Sema5A antibody (1:100) or pre-immune serum (1:100) for 8-10 hours, and then grown overnight. In anti-Sema5A-treated optic nerves (n=26), 35% exhibited pathfinding errors compared with no occurrence of errors among optic nerves incubated in pre-immune serum (n=22, P=0.007) (Fig. 6B). The guidance errors ranged from mild cases in which a small number of axons were observed straying from the main optic nerve bundle (Fig. 6D) to more severe cases in which fascicles of axons were observed veering off from their normal path (Fig. 6E,F).

Anti-Sema5A Fab fragments injected into eyes of E14 mouse embryos in utero led to retinal axon guidance errors in 4 of 18 treated retinas. The phenotypes were mild, and consisted of stray axons leaving the optic disc region and projecting aberrantly for short distances within the retina itself (Fig. 6G,H). In sum, disruption of Sema5A function resulted in axons straying from the main retinal pathway, and this effect was more prominent in the optic nerve than at the optic disc.

DISCUSSION

In the present study, we found that while a number of semaphorins were expressed within the embryonic mouse retina, only Sema5A was specifically expressed at the optic disc and along the optic nerve during active retinal axon outgrowth. In growth cone collapse and neurite outgrowth assays, Sema5A triggered inhibitory responses in embryonic retinal axons. Furthermore, this inhibition was maintained when exposure to Sema5A was systematically paired with signaling mediated by L1, netrin 1, or laminin; three guidance molecules that govern axon pathfinding in the region of the optic disc and optic nerve. This suggested that retinal axons continually responded to Sema5A inhibition as they progressed through the developing retinal pathway. Immunostaining in live tissues using an anti-Sema5A antibody confirmed mRNA in situ hybridization results, and showed that Sema5A protein was present as a ring surrounding retinal axons in the visual pathway. Antibody perturbation studies resulted in retinal axons straying from the optic disc and optic nerve defasciculation, supporting the notion that Sema5A acts as an inhibitory sheath ensuring proper development of the optic nerve.

Sema 5A inhibition and L1, laminin, netrin 1 signaling

A major pathfinding task for retinal axons is exiting the retina by growing through the optic disc into the optic nerve. This guidance event is mediated by netrin 1 (Deiner et al., 1997), and occurs in the presence of L1 (Deiner et al., 1997) and laminin (Hopker et al., 1999). Given that Sema5A was also present at the optic disc and nerve, we examined how combinations of the axon guidance molecules L1, netrin 1 and laminin affected the ability of retinal axons to respond to Sema5A. The results showed that Sema5A clearly inhibited retinal growth cones in the presence of all guidance molecule combinations tested, including laminin or L1 alone, and pairings of netrin 1 with L1 or laminin. Thus, retinal growth cones reacted to Sema5A as an inhibitory molecule even while receiving growth supporting co-impinging signals from members of multiple families of attractive axon guidance molecules.

Neurite outgrowth in the presence of laminin and netrin 1

The assay testing retinal axon responses to Sema5A under the influence of both netrin 1 and laminin involved culturing retinal explants on a laminin at a level that alone was too low

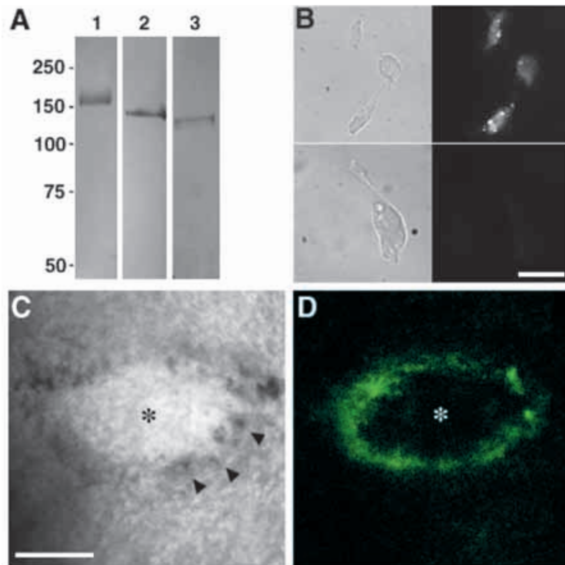

Fig. 5. Anti-Sema5A antibody characterization and Sema5A localization at the optic disc. (A) In immunoblots, the Sema5A antibody recognized ECD-Fc protein (lane 1), Sema5A-myc from transfected 293 cells (lane 2), and yielded a band of the expected size for Sema5A (135 kDa) from E14 retina (lane 3). (B) Anti-Sema5A staining of live Sema5A-myc-expressing 293 cells. Top row shows Sema5A-myc-expressing cells stained with Anti-Sema5A, and the bottom row shows Sema5A-myc cells similarly treated with pre-immune serum. (C) Bright-field image of the optic disc. Arrowheads point to retinal pigment epithelium cells (* marks the center of the optic disc). (D) Same optic disc as in C. Sema5A immunoreactivity was localized to the peripheral rim of the optic disc. Image compiled from optical sections through a depth of 25 μm at the optic disc. Scale bars: (B) 25 μm; (C-D) 50 μm.

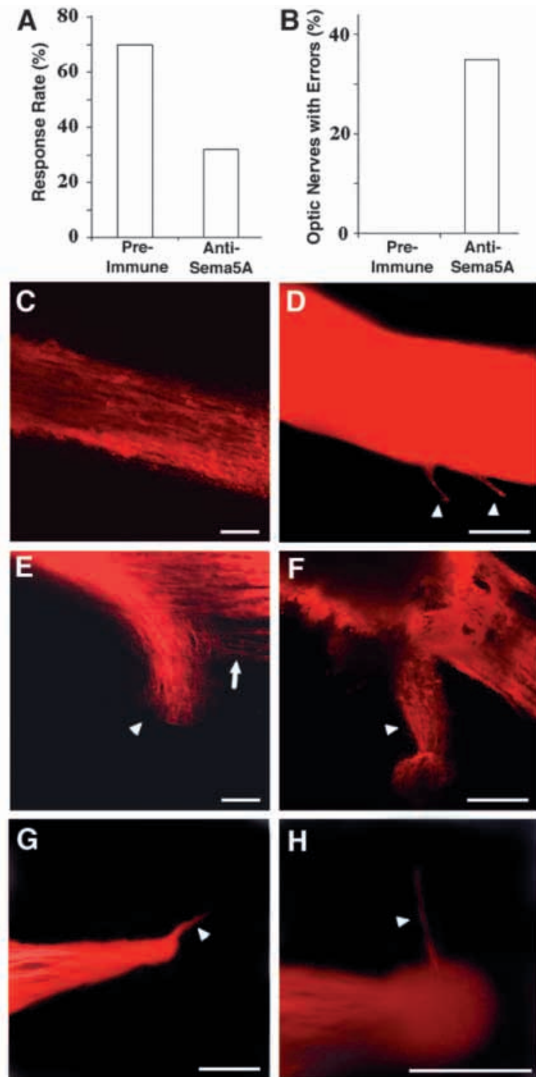

Fig. 6. Sema5A function blockade and RGC axon pathfinding errors. (A) ECD-Fc-mediated retinal growth cone collapse on an L1 substratum in the presence of anti-Sema5A antibody or pre-immune serum. (B) The incidence of axon guidance errors in optic nerves treated with anti-Sema5A antibody. (C) A normal, DiI-labeled E14 optic nerve showing retinal axons contained within the retinal pathway. The retina and optic disc are towards the left. (D-F) Examples of axon guidance errors in anti-Sema5A-treated optic nerves. The retina is towards the left. (D) A mild phenotype: a pair of short axons tipped with growth cones (arrowheads) are straying from the optic nerve bundle. (E) A more severe error showing a bundle of axons (arrowhead) veering away from the main portion of the optic nerve. Note that some aberrantly projecting axons are growing parallel to the optic nerve (arrow). (F) A large fascicle of axons near the optic disc (arrowhead) have split away from the main optic nerve bundle, and terminate in a knot-like structure. (G,H) Examples of stray RGC axons within the retina (arrowheads). Unlike normal axons, stray axons fail to grow completely through the optic disc, and extend inappropriately within the retina. Scale bars: (C-H) 50 μm.

to produce reliable axon outgrowth, but which resulted in consistent neurite extension after the addition of soluble netrin 1. The dependence of this assay on both laminin and netrin 1 was demonstrated by the fact that netrin 1 supplementation alone without the laminin substratum supported no retinal axon outgrowth, and the fact that the outgrowth induced by the laminin/netrin 1 combination was decreased by an antibody against the Netrin receptor DCC (Fig. 2E). The ability of this laminin/netrin 1 combination to augment neurite outgrowth was somewhat unexpected given a previous study reporting that in the presence of laminin 1, *Xenopus* retinal growth cone response to netrin 1 was switched from attraction to repulsion (Hopker et al., 1999). One explanation for this difference is the micropipette delivery of a netrin 1 point source to *Xenopus* axons, while in the present study netrin 1 was bath applied, and did not expose neurites to a netrin 1 gradient. A second possibility is a difference in laminin levels. The substratum laminin level in the present study by itself did not reliably support retinal axon growth in vitro. In contrast, *Xenopus* growth cones were first grown on effective concentrations of laminin 1, and then tested with netrin 1 gradients. If this second explanation is correct, then whether switching occurs in vivo may depend critically on the precise concentration of particular axon guidance molecules to specific growth cones.

Effect of L1 on responses to Sema5A

Several aspects of Sema5A action on retinal axons such as effective concentration, localization of inhibition to the sema domain, and increased effectiveness after oligomerization,

were consistent with previous studies of semaphorin function (Koppel et al., 1997; Koppel and Raper, 1998; Xu et al., 2000). One difference, however, was the regulation of responses to semaphorins by the Ig superfamily guidance molecule L1. Cortical axons, like retinal axons, express abundant L1 on their surfaces in vivo, and likely carry out numerous pathfinding tasks in the presence of L1 signaling. In a study in which cortical axons were exposed to Sema3A in the presence of L1-Fc, growth cones did not collapse, but instead maintained an open morphology (Castellani et al., 2000), suggesting that growth cones responding to L1 signaling were freed of Sema3A-mediated inhibition. This was not the case for Sema5A, as retinal growth cones responded to Sema5A inhibition in the presence of L1. Furthermore, concentrations of Sema5A required to trigger growth cone collapse were similar for retinal axons grown on laminin or L1. These results raise the possibility that the growth cone signaling cascades triggered by Sema3A and Sema5A are different, and cross-talk with L1 signaling exists in the case of Sema3A but not Sema5A. Interestingly, it appears that L1 influences Sema3A signaling by interacting directly with the Sema3A receptor neuropilin 1 (Castellani et al., 2000). Currently, known families of semaphorin receptors include the neuropilins and the plexins (Raper, 2000), however, specific receptors for transmembrane semaphorins have not yet been identified.

Function of thrombospondin type-1 repeats

A unique aspect of class V semaphorins is the seven thrombospondin type-1 repeats in their extracellular domains. While TSP repeats in other molecules have been shown to promote axon growth and to mediate neural attachment (Adams and Tucker, 2000), these results showed that TSP repeats combined with a semaphorin domain clearly resulted in a molecule with overall inhibitory activity for retinal neurite outgrowth. In addition, the TSP repeat domain by itself did not trigger growth cone collapse, nor did it support retinal axon outgrowth. Given that the intact Sema5A extracellular domain, which included the TSP region, was a more potent inhibitor of retinal axons than the sema domain alone, it is possible that TSP repeats contribute to ligand-receptor binding, a known function for the non-sema domains of other semaphorins (Feiner et al., 1997). However, given that TSP repeats have also been implicated in protein binding (Adams and Tucker, 2000), an additional possibility is that Sema5A associates with other proteins through the TSP repeats and these protein complexes represent an as yet unrecognized aspect of Sema5A function.

Localization of Sema5A

In situ hybridization and immunostaining showed that Sema5A was expressed by the group of optic disc neuroepithelial cells that surround the retinal axon bundle exiting the retina. These same cells are known to extend netrin 1-bearing processes radially towards the center of the optic disc (Deiner et al., 1997). Although expressed by the same cell population, Sema5A protein was localized mostly to the cell bodies of the neuroepithelial cells arrayed at the periphery of the optic disc, and Sema5A protein was not detected on the netrin 1-bearing processes. This suggested that Sema5A may be specifically excluded from neuroepithelial cell processes, possibly through anchoring of transmembrane Sema5A via its cytoplasmic domain.

An inhibitory sheath for optic nerve development

Blocking Sema5A function resulted in retinal axon defasciculation from the main axon bundle of the optic nerve. Although some cases showed sizable retinal axon bundles leaving the optic nerve, other preparations had only a few stray axons, and in many anti-Sema5A-treated nerves, no errors were noted. This variation in occurrence and severity of pathfinding errors could be due to the use of a function-blocking antibody over a relatively short time period; a necessary limitation to maintain the health of tissue preparations. A second possibility is the presence of other inhibitory molecules such as Slit proteins in the developing optic nerve (Niclou et al., 2000) serving a similar ensheathing function.

In principle, axon defasciculation from the optic nerve in the face of anti-Sema5A antibodies could be interpreted as a loss of growth promoting/fasciculation activity rather than loss of Sema5A-mediated inhibition. However, given the in vitro evidence that Sema5A inhibited retinal axon outgrowth and caused growth cone collapse, combined with the evidence that Sema5A mRNA and protein were found surrounding the optic nerve bundle, we favor the model that Sema5A served as an inhibitory sheath. Furthermore, since new retinal growth cones entering the optic nerve generally extend along the external surface of the axon bundle (Reese et al., 1991; Williams et al., 1991), a Sema5A sheath places this inhibitory molecule in a direct position to contain the growth of newly arriving axons.

Maintaining inhibition during pathfinding

Previous studies have shown that receptor signaling cascades activated by guidance molecules can interact and modulate growth cone responses. These interactions set up a logic system in which a growth cone's response to multiple cues presented simultaneously is not merely the sum of the individual guidance forces, and creates increased functionality given a limited repertoire of guidance molecules. During pathfinding however, there are instances, as axons grow over long distances, where it may be useful to maintain a constant responsiveness to a single guidance molecule while accomplishing sequential pathfinding tasks involving various others. In such cases, the nervous system could utilize molecules such as Sema5A that appear to trigger a conserved response in growth cones in the face of co-impinging signals from multiple families of guidance molecules.

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