Semaphorin 5A is a Bifunctional Axon Guidance Cue Regulated by Heparan and Chondroitin Sulfate Proteoglycans

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ABSTRACT

The response of neuronal growth cones to axon guidance cues depends on the developmental context in which these cues are encountered. We show here that the transmembrane protein semaphorin 5A (Sema5A) is a bifunctional guidance cue that exerts both attractive and inhibitory effects on developing axons of the fasciculus retroflexus, a fiber tract in the diencephalon associated with limbic function. The thrombospondin repeats of Sema5A physically interact with the glycosaminoglycan portion of both chondroitin sulfate proteoglycans (CSPGs) and heparan sulfate proteoglycans (HSPGs). CSPGs function as precisely localized extrinsic cues which convert Sema5A from an attractive to an inhibitory guidance cue. Therefore, glycosaminoglycan-bound guidance cues provide a molecular mechanism by which CSPGs inhibit axonal extension. Further, axonal HSPGs are required to mediate the permissive effects of Sema5A, suggesting that HSPGs are a component of a functional Sema5A receptor. Thus, neuronal responses to Sema5A are proteoglycan-dependent and interpreted according the biological context in which this membrane bound guidance cue is presented.
INTRODUCTION

The adult pattern of neural connectivity is established during development when axonal growth cones, follow stereotypical routes to their synaptic targets. The responsiveness of developing axons to guidance cues is subject to extensive modulation such that growth cones may react to the same guidance cue differently depending on the developmental context in which the cue is encountered (Yu and Bargmann, 2001). It is striking that growth cones tend to alter their responsiveness to surrounding guidance cues at precisely defined locations within the nervous system. This can be explained in part by intrinsic, age-related changes in developing neurons which regulate responsiveness to axon guidance molecules (Cai et al., 2002; Shewan et al., 2002). Alternatively, extracellular cues positioned at precise locations in the nervous system can also modulate growth cone behavior (Dickson, 2002; Hopker et al., 1999). Therefore, guidance cues do not act in isolation to define the routes taken by developing axons. Rather, the combination of guidance cues together with intrinsic and extrinsic factors that regulate the responsiveness of growth cones contributes to the proper development of neuronal circuits (Dickson, 2002; Song and Poo, 1999).

The semaphorins, a family of secreted and membrane-anchored proteins characterized by an amino-terminal semaphorin (sema) domain, play functionally conserved roles in axon guidance. The factors capable of modulating semaphorin function are only now being uncovered (Dickson, 2002; Yu and Bargmann, 2001). Class 5 semaphorins are integral membrane proteins which are expressed in the developing vertebrate nervous system in patterns that suggest they play complex roles in neural development (Medina, 2004 #1023; Oster, 2003 #911; Jones, 2002 #920; Adams, 1996 #1996).
The class 5 semaphorin, Sema5A, can function as an inhibitory cue which collapses both cultured fibroblasts and retinal ganglion cell growth cones, and which guides retinal projections to their proper targets in the brain (Artigiani et al., 2004; Goldberg et al., 2004; Oster et al., 2003). In addition, Sema5A has permissive effects on both cultured epithelial and endothelial cells leading to enhanced cell migration (Artigiani et al., 2004). Together, these findings suggest that Sema5A may also exert both inhibitory and permissive effects on developing axons, and raise questions regarding the mechanisms underlying these different functions.

Class 5 semaphorins are distinguished from other semaphorins by their domain organization, which consists of two clusters of type-1 thrombospondin repeats (TSRs) positioned C-terminal to the sema domain (Adams et al., 1996). While the role of TSRs in Sema5A-mediated axon guidance is poorly understood, TSR domains occurring in other proteins are known to mediate functional interactions with extracellular matrix (ECM) components (Adams and Tucker, 2000). The ECM is an important source of extrinsic cues which influence the response of growth cones to guidance cues (Condic et al., 1999; Diefenbach et al., 2000; Hopker et al., 1999; Nguyen-Ba-Charvet et al., 2001), raising the possibility that the Sema5A TSR domains mediate critical interactions with ECM components capable of modulating Sema5A function.

An intriguing feature of TSR domains is their ability to bind proteoglycans, a group of extracellular and cell surface proteins to which one or more highly charged sulfated glycosaminoglycan (GAG) side chains are covalently bound (Adams and Tucker, 2000). In the developing nervous system, proteoglycans predominately carry...
either chondroitin sulfate (CS) or heparan sulfate (HS) GAGs (Bovolenta and Fernaud-Espinosa, 2000).

Heparan sulfate proteoglycans (HSPGs) are a group of extracellular and cell surface proteins essential for proper axonal pathfinding during nervous system development (Bulow and Hobert, 2004; Walz et al., 1997; Wang and Denburg, 1992), and it is increasingly evident that the major mechanism by which HSPGs influence axon pathfinding is by regulating the function of axon guidance cues. While HSPGs affect several axon guidance cues including FGF, HB-GAM, Slits, and Anosmin/KAL-1 (Bulow and Hobert, 2004; Hu, 2001; Inatani et al., 2003; Irie et al., 2002; Johnson et al., 2004; Kinnunen et al., 1998; Steigemann et al., 2004; Walz et al., 1997), a role for these molecules in semaphorin mediated axon guidance has not been described.

Chondroitin sulfate proteoglycans (CSPGs) are a heterogeneous group of extracellular matrix molecules which influence the behavior of neuronal growth cones during development and, importantly, following CNS injury (Bovolenta and Fernaud-Espinosa, 2000; Morgenstern et al., 2002). However, the molecular mechanisms by which CSPGs affect neuronal growth cones are poorly understood. CSPGs are known to modulate the response of growth cones to other matrix components such as laminin (Condic et al., 1999). This raises the possibility that CSPGs are components of the developmental environment capable of regulating how growth cones respond to surrounding guidance cues. The biological activity of CSPGs may also be determined by distinct proteins which bind to glycosaminoglycans and which interact with receptors on the surface of neuronal growth cones (Anderson et al., 1998; Brittis and Silver, 1994; Emerling and Lander, 1996; Golding et al., 1999). Although CSPGs are known to
interact with growth factors, adhesion molecules, and other matrix components, the specific binding proteins capable of mediating the effects of CSPGs on neuronal growth cones remain to be identified (Bovolenta and Fernaud-Espinosa, 2000; Morgenstern et al., 2002).

We examine here the role of Sema5A in the development of the fasciculus retroflexus (FR), a diencephalic fiber tract, and find that Sema5A is a bifunctional guidance cue that exerts both attractive and inhibitory effects on these developing axons. We show that the TSR domains mediate critical regulatory interactions with sulfated proteoglycans that determine how Sema5A affects neuronal growth cones. HSPGs expressed on the surface of extending FR axons are required cell autonomously to mediate the permissive effects of Sema5A. In contrast, CSPGs serve as precisely localized extrinsic cues in the embryonic diencephalon which convert Sema5A from an attractive to an inhibitory cue for extending axons. These results define a molecular mechanism by which the guidance events underlying the development of the FR are controlled by interactions between Sema5A and both HSPGs and CSPGs.

RESULTS

Sema5A is required for the proper development of the fasciculus retroflexus

During development, the diencephalon becomes subdivided along the rostral-caudal axis into a series of morphological segments called prosomeres. Certain dorsal-ventral axon tracts form at the borders between prosomeres, suggesting that early pioneer axons utilize guidance cues expressed within these segments to establish a simple axon scaffold upon
which subsequent generations of axons selectively fasciculate and extend (Figdor and Stern, 1993). Two distinct types of guidance cues are thought to explain why these tracts form between prosomeres: repulsive cues expressed within the prosomeres that prevent axons from crossing into these areas, and attractive cues expressed on initial pioneer axons that promote the fasciculation of subsequent follower axons (Figdor and Stern, 1993).

The FR, which connects the limbic forebrain and the midbrain, is involved in controlling a variety of behaviors (Sutherland, 1982). The FR consists of axons which originate in the habenula nucleus (Hb) and project along the boundary between prosomere 1 and prosomere 2 (Figure 1A). The secreted repellant Sema3F is expressed in prosomere 1 (Funato et al., 2000; Sahay et al., 2003), and an un-identified membrane-anchored inhibitory activity is localized to prosomere 2 (Funato et al., 2000). The combination Sema3F and the unknown prosomere 2 repellent presumably restricts FR axons to the border between prosomere 1 and prosomere 2. The permissive cues expressed on early FR axons that promote fasciculation have not yet been identified.

Axons from the Hb nucleus pioneer the FR beginning around embryonic day 13 (E13) in rat (Funato et al., 2000), and Sema5A is expressed in two distinct locations in the diencephalon during this developmental period where it may be involved in discrete axon guidance events. As early as E13.5, Sema5A transcript is expressed in the Hb nucleus itself (D.B.K. and A.L.K., data not shown), and at E15.5 Sema5A message co-localizes with neuropilin-2 (Npn2), a Sema3F receptor known to be expressed in Hb neurons (Figure 1B, and Supplemental Figure 2E) (Chen et al., 1998; Giger et al., 1998).
Sema5A antiserum (Oster et al., 2003) also labels the axons of Hb explants grown in culture (Figure 1C).

In addition to its expression in Hb neurons, Sema5A is also expressed in prosomere 2 at E15.5 and tightly surrounds the FR as it projects ventrally (Figure 1D and 1E). An alkaline phosphatase (AP)-tagged Sema5A ectodomain fusion protein (AP-5A\textsuperscript{ecto}) labels both FR axons and prosomere 2, suggesting that Sema5A interacts with endogenous binding partners in these locations (Figure 1F; AP alone does not bind to e15.5 brain sections, data not shown).

To determine whether Sema5A is required for FR development, we examined organotypic diencephalon explants. Using TAG-1 as a marker for diencephalic fiber tracts, we found that the development of two projections, the FR and the mamillothalamic tract (MTT), closely resemble their development in vivo (Figure 1G). However, when diencephalic explants were cultured in the presence of Sema5A function-blocking antibodies directed against the sema domain (Oster et al., 2003), we found that FR axons were no longer restricted to the boundary between prosomere 1 and prosomere 2, and often did not reach their targets in the ventral midbrain (Figure 1H). Significantly more FR fibers crossed inappropriately into prosomere 2 in the presence of Sema5A antibodies compared with control IgG (Figure 1I; p < 0.001, student t-test). However, the organization and width of the MTT was not significantly disrupted in the presence of Sema5A antibodies compared to IgG controls (Figure 1G, 1H, and 1J; p > 0.07, student t-test). These results show that Sema5A is critical for proper FR development in an in vitro model which closely resembles in vivo diencephalon development.
Sema5A expressed in 293 cells functions as an attractant

To investigate how Sema5A specifically influences developing FR axons, we employed an in vitro membrane stripe assay in which Hb neurons were grown on a substrate consisting of alternating stripes of experimental and control membranes (Tuttle et al., 1995; Walter et al., 1987). Axons extending from Hb explants used in the stripe assays recapitulate the expression of proteins expressed on FR axons in vivo, including TAG-1, DCC, and Npn2 (Supplemental Figure 2A-2D). Stripe assays were prepared in which the experimental stripe contained cell membranes from HEK 293 cells transfected with a full-length Sema5A (FL-5A) construct, while the control stripe contained membranes from cells that had been transfected with GFP. In these and subsequent assays the control and experimental stripes also contained perinatal neocortical membranes, a highly permissive substrate for the outgrowth of Hb axons. We found that Hb axons strongly preferred to extend on stripes containing FL-5A compared to stripes containing GFP (Figure 2A, 2B and 2G; \( p < 0.001 \), Mann Whitney test), demonstrating that Sema5A can function as an attractive axon guidance cue.

We next investigated the structural determinants underlying Sema5A function by generating transmembrane Sema5A constructs lacking either the sema or TSR domains (Figure 2F). In stripe assays in which Sema5A TSRs linked to the transmembrane domain (TSR-TM) was in the experimental membranes, Hb axons showed a significant preference for TSR-TM stripes, compared to experiments in which GFP was in the experimental stripe (Figure 2A, 2C, and 2G; \( p < 0.001 \) Mann Whitney test). We next prepared stripe assays in which the experimental membranes contained the Sema5A sema domain linked to the transmembrane domain (sema-TM) and found that Hb axons grew
uniformly over both experimental and control stripes (Figure 2D and 2G). Since the inhibitory activity of Sema5A and other semaphorins is enhanced by multimerization (Oster et al., 2003; Yu and Bargmann, 2001), we asked whether clustering the Sema5A sema domain would reveal an inhibitory effect on Hb axons. We fused the C-terminus of Sema-TM to the assembly subunit of cartilage oligomeric matrix protein (COMP), a domain capable of forming pentameric \( \alpha \) helical coiled coils (Tomschy et al., 1996). We found that indeed Hb axons avoid stripes containing the pentamerized sema-COMP, as compared to experiments in which GFP was in the experimental stripe (Figure 2A, 2E and 2G; \( p < 0.001 \) Mann Whitney test). These results, together with the known inhibitory effects of Sema5A on retinal axons (Goldberg et al., 2004; Oster et al., 2003), demonstrates that Sema5A is a bi-functional guidance cue.

**Sema5A is an endogenous prosomere 2 repellent**

If Sema5A functions strictly as an attractive cue it should promote the outgrowth of FR fibers into prosomere 2, where Sema5A transcript is strongly expressed. Because FR fibers instead strictly avoid prosomere 2, we wondered whether Sema5A might function differently in distinct locations. Therefore, we prepared stripe assays in which the experimental stripes contained intact prosomere 2 membranes while the control stripes contained prosomere 2 membranes that had been briefly treated with proteinase K, heat, or trypsin to neutralize axon guidance proteins. In the presence of control rabbit IgG, Hb axons avoided the intact prosomere 2 membrane stripes and instead extended preferentially on the neutralized control stripes (Figure 3A and 3E), suggesting that an endogenous repellent resides in the prosomere 2 membranes. It is unlikely that the
prosomere 2 membranes were contaminated with Sema3F (the only other known repellent of Hb axons) since Hb explants taken from Npn2 null mice also avoided intact prosomere 2 membrane stripes (Supplemental Figure 3D, 3E, and 3M) (Funato et al., 2000; Sahay et al., 2003). Western blots of prosomere 2 membranes using the Sema5A antibody revealed a prominent 130 kDa band, the predicted molecular weight of Sema5A (Figure 5B). These data confirm the presence of an endogenous membrane bound FR repellent in prosomere 2 (Funato et al., 2000) and suggest that Sema5A present in prosomere 2 membranes may account for this activity.

To address this possibility, Sema5A function-blocking antibodies were added to stripe assays in which intact prosomere 2 membranes were in the experimental stripe and proteinase K treated prosomere 2 membranes were in the control stripes. The Sema5A antibodies substantially reduced the inhibitory activity of prosomere 2 membranes compared to cultures treated with control IgG (Figure 3A, 3B, and 3E; p < 0.001, Mann Whitney test). Importantly, the Sema5A antibodies showed some specificity for interfering with Sema5A function since they did not significantly affect Sema3F-mediated repulsion of Hb axons (Supplemental Figure 3F, 3G, and 3N). To determine if the Sema5A function-blocking antibodies act on Hb axons or on the membrane substrate, prosomere 2 membranes were pre-incubated with these antibodies prior to preparing the membrane stripes, washed extensively to remove unbound antibody, then used to prepare the experimental stripe. The control stripe contained untreated prosomere 2 membranes. Axons grew preferentially on the membrane that had first been neutralized with Sema5A antibodies (Figure 3D and 3E), suggesting that Sema5A located in the prosomere 2 membranes inhibits Hb axon extension. By comparison, Hb axons showed no preference
for prosomere 2 membranes pre-treated with control IgG (Figure 3C and 3E; p < 0.001, Mann Whitney test). Taken together, these results demonstrate that Sema5A is an endogenous membrane-anchored guidance cue which is expressed in prosomere 2 and which serves as repellent for Hb axons.

**HSPGs mediate Sema5A attraction**

Since TSR domains can functionally interact with glycosaminoglycans, we explored the possibility that the axon guidance functions of Sema5A depend on interactions with sulfated proteoglycans. We first asked if HSPGs are found on FR axons during development. We found that at E15.5 the FR, and other diencephalic fiber tracts, are strongly labeled by the monoclonal antibody 3G10; this antibody is directed against an HS neo-epitope revealed by treatment with heparinase III, a bacterial enzyme which digests specific types of HS GAGs (Figure 4A and 4B) (David et al., 1992). Further, axons extending from Hb explants are also strongly positive for HS (Figure 5E). To test whether intact HSPGs are required for the binding of AP-tagged Sema5A to FR axons, embryonic brain sections were treated with heparinase. We found that AP-5A\textsuperscript{ecto} binding to the FR was substantially reduced by heparinase treatment (Figure 4C and 4D) but was not eliminated by proteinase K treatment (Supplemental Figure 1C and 1D). By generating AP-tagged Sema5A deletion constructs (Figure 2F), we found that the TSR domains account for the majority of binding both to FR and to prosomere 2 (D.B.K., A.L.K., and R.J.G., data not shown). More specifically, we found that a fusion protein consisting of AP linked to the first four TSRs of Sema5A (AP-TSR1-4) recapitulated the binding pattern of AP-5A\textsuperscript{ecto} (Figure 4E), while a construct consisting of AP linked to the
last three TSRs of Sema5A (AP-TSR5-7) does not label brain sections (Supplemental Figure 1K). The binding of AP-TSR1-4 to the FR is similarly heparinase-sensitive (Figure 4F). Heparinase treatment did not, however, substantially affect the binding of AP-5Aecto or AP-TSR1-4 to prosomere 2 (Figure 4D and 4F), nor did this treatment affect the binding of AP-Sema3F to the FR (Supplemental Figure 3G and 3H). These results show that the TSR domains of Sema5A mediate binding to HS GAGs located specifically on FR axons, and they raise the possibility that Sema5A and HSPGs functionally interact.

To address whether HSPGs are functionally involved in Sema5A-mediated axon guidance, stripe assays were prepared in which the experimental membrane stripes contained FL-5A and the control stripes contained GFP, and heparinase was added to the culture medium to disrupt HS GAGs. We found that Hb axons no longer displayed a preference for the Sema5A containing stripes, in contrast to vehicle treatment which did not diminish the attractive effects of Sema5A (Figure 4G, 4H, and 4N; p < 0.001 Mann Whitney test). Heparinase treatment shows some specificity for Sema5A-mediated guidance since it does not significantly reduce Sema3F-mediated repulsion of Hb axons (Supplemental Figure 3H, 3I, and 3N).

We next asked whether HSPGs are required cell autonomously to mediate the attractive effects of Sema5A. First, membranes in the full-length Sema5A stripe assay were treated with heparinase and then washed extensively before the stripe assay was prepared. In these experiments, the Hb axons still preferred to grow on the Sema5A containing stripes (Figure 4I and 4N), suggesting that HS on Hb axons, not HS in the membrane substrate, mediates Sema5A function. Next, we employed a pharmacological approach to interfere with HSPG biosynthesis. β-D-xylosides are a class of compounds
which derail proteoglycan biosynthesis by serving as alternate substrates for GAG assembly; GAGs are attached to the exogenous xyloside instead of the proteoglycan core protein (Fritz et al., 1994). When used in the FL-5A stripe assay, napthalenemethanol-β-D-xyloside (xyl-NM), which serves as an alternate substrate for both CS and HS GAGs, significantly reduced the preference of Hb axons for the Sema5A containing stripes. In contrast, cis/trans-decahydro-2-naphthol-β-D-xyloside (xyl-decalin), which serves as an alternate substrate only for CS GAGs, had no significant effect on the preference of Hb axons for Sema5A containing stripes (Figure 4J, 4K, and 4N; p < 0.001 Mann-Whitney test). We also noted that in presence of xyl-NM, FL-5A containing stripes became modestly inhibitory for Hb axons (see Discussion). These results show that HSPGs are required cell autonomously on the surface of Hb axons to mediate the permissive effects of Sema5A, supporting a role for HSPGs as a component of the Sema5A receptor.

To confirm the effect of xyl-NM on HS biosynthesis in Hb axons, we examined how this drug affects the glycosylation of a specific HSPG. Using a candidate approach, we identified the transmembrane HSPG syndecan-3 as a specific Sema5A binding partner. Syn3C antibodies, which recognize the intracellular domain of syndecan-3, (Hsueh and Sheng, 1999) strongly labeled FR fibers during critical periods of axon pathfinding (Figure 5A). Syndecan-3 is also strongly expressed on Hb axons in vitro (Figure 5D and 5G) and in prosomere 2 membranes (Figure 5B). We determined that Sema5A and syndecan-3 physically associate endogenously, as Sema5A co-immunoprecipitates with the syn-3C antibodies, but not with control IgG (Figure 5B). These results allowed us to further examine the effects of xylosides on HS biosynthesis specifically in Hb axons. Hb explants grown in culture were treated with xyl-NM and
then double-labeled with two antibodies: Syn3C, which recognizes only the syndecan-3 core protein, and 3G10, which recognizes only the HS component of HSPGs. The ratio of 3G10 to Syn3C staining on Hb axons was used as a measure of the degree to which xyloside treatment interferes with the attachment of HS GAGs to the syndecan-3 core protein. In a dose-dependent fashion, xyl-NM selectively diminished 3G10 staining without affecting Syn3C staining (Figure 5C-5I). Vehicle treatment alone did not affect 3G10 or Syn3C labeling. These results show that xyl-NM effectively prevents the attachment of HS GAGs to syndecan-3, without affecting core protein expression levels.

Sema5A expressed on early FR axons might serve as attractive substrate upon which subsequent follower axons selectively fasciculate, and therefore intact HSPGs expressed on these later FR axons would be essential for mediating the permissive effects of Sema5A. To begin to test this hypothesis, we examined mice in which HS biosynthesis was genetically disrupted within the central nervous system. EXT1 is an enzyme required for the polymerization of HS, and mice lacking this enzyme express HSPG core proteins, including syndecan-3, devoid of HS side chains (Inatani et al., 2003). In order to visualize FR axons in E17 - E18 EXT1 mutant mice, brain section were labeled with AP-Sema3F, the binding of which does not to depend on intact HS (Supplemental Figure 3G and 3H). In mutant mice where a loxP-modified EXT1 allele was selectively removed from the nervous system with nestin-Cre (Inatani et al., 2003), the FR was significantly more defasciculated in homozygous null embryos than in heterozygous siblings (Figure 4L, 4M, and 4O; p < 0.001 student t-test). This phenotype was 100% penetrant, occurring in both hemispheres of every EXT1-/- embryo examined. Taken together with our observations that Sema5A binding to the FR is dependent on
intact HS, and that Sema5A also plays a critical role in the proper development of the FR, these data suggest that an impairment in Sema5A signaling causes the FR defasciculation observed in *EXT1* null embryos.

**CSPGs convert Sema5A from an attractive to an inhibitory guidance cue**

While HSPGs expressed on the surface of FR axons mediate the permissive effects of Sema5A, what are the factors in prosomere 2 that cause Sema5A to function as an inhibitory cue? Given the specific binding of AP-5A<sup>ecto</sup> to prosomere 2 in brains sections, we considered the possibility that this endogenous binding partner might represent a precisely localized extrinsic cue capable of modulating Sema5A function (Figure 1F). Importantly, the binding of AP-5A<sup>ecto</sup> to prosomere 2 is resistant to proteinase K treatment, raising the possibility that glycosaminoglycans account for the binding of Sema5A to prosomere 2 (Supplemental Figure 1C and 1D). Because the binding of AP-5A<sup>ecto</sup> and AP-TSR1-4 to prosomere 2 was also resistant to heparinase treatment, this suggested the involvement of a different glycosaminoglycan (Figure 4C - 4F). We asked whether chondroitin sulfates accounted for the binding of Sema5A to prosomere 2 by first labeling brain sections with the monoclonal antibody CS-56, which detects specific types of CS GAGs (Rhodes and Fawcett, 2004). We found that CS and *Sema5A* are strikingly co-localized in prosomere 2 (compare Figure 6A to Figure 1D, and Figure 6B to Figure 1E; these are adjacent sets of sections). In the sagittal view, CS-56 immunoreactivity and *Sema5A* are seen to form a molecular boundary in prosomere 2 which FR axons do not violate (Figure 6A). In the horizontal view, CS-56
immunoreactivity and *Sema5A* message are seen to surround the FR (Figure 6B). Importantly, CS-56 staining is not present on FR axons themselves.

To test whether intact CSPGs are required for the binding of AP-tagged Sema5A to prosomere 2, brain sections were treated with chondroitinase ABC, a bacterial enzyme that destroys CS polymers (Rhodes and Fawcett, 2004). This treatment reduced the binding of both AP-5A<sup>ecto</sup> and AP-TSR1-4 to CS rich regions of the diencephalon without affecting binding to the FR (Figure 6C - 6F). Further, AP-TSR1-4 binds directly to chondroitin sulfate-A and chondroitin sulfate-C purified from cartilage and immobilized on nylon membranes (D.B.K. and A.L.K., data not shown). Chondroitinase digestion did not, however, affect the binding of AP-Sema3F to cryosections (Supplemental Figure 1I and 1J). These results demonstrate a direct interaction between the TSRs of Sema5A and the CS component of CSPGs. Thus, Sema5A binds to two separate classes of GAGs expressed in two separate locations: CS expressed in prosomere 2, and HS expressed on FR axons. Further, CSPGs are expressed in prosomere 2, a location where these ECM molecules are in a position to influence Sema5A function.

We next investigated how CSPGs influence the outgrowth of Hb axons. Uniform carpets of neonatal cortical membranes were applied to polycarbonate filters which had been pre-coated with a commercial preparation of embryonic chicken brain CSPGs (Ernst et al., 1995). At low concentrations of CSPG mixture (10 µg/mL), Hb axons were highly defasciculated, rarely growing as thick bundles (Figure 6H). However, as the concentration of CSPG mixture increased to 100 µg/ml, Hb axons became progressively more fasciculated, eventually growing almost exclusively as thick bundles (Figure 6I - 6K). Thus, Hb axons are able to extend over substrates containing a relatively high
concentration of CSPGs. Rather than strictly inhibiting the outgrowth of Hb axons, CSPGs instead appear to drive the fasciculation of these fibers.

We next asked how a concentration of CSPG mixture which had only modest effects on Hb axon fasciculation would influence the function of heterologously expressed Sema5A. Stripe assays in which the experimental stripe contained full length Sema5A membranes and the control stripe contained GFP membranes were applied to polycarbonate filters that had been pre-coated with 50 µg/ml of the CSPG mixture. Remarkably, we observed that heterologously expressed Sema5A was converted from an attractive to an inhibitory cue for Hb axons when presented together with substrate bound CSPGs (Figure 7B, and 7I). In contrast, when polycarbonate filters were pre-coated with 50 µg/mL BSA, Sema5A remained an attractive cue for Hb axons (Figure 7A and 7I; p < 0.001, Mann-Whitney test). To confirm that the CS component of this CSPG mixture was critical for regulating the function of Sema5A, polycarbonate filters were first coated with the CSPG mixture and then treated with chondroitinase ABC to specifically destroy CS GAGs. Stripe assays were then prepared on these filters in which the experimental stripe contained full-length Sema5A membranes and the control stripe contained GFP membranes. Under these conditions, Sema5A remained an attractive substrate (Figure 7C and 7I). Importantly, since Hb axons avoid stripes containing both Sema5A and CSPGs and instead extend on stripes containing CSPGs alone, Sema5A also plays a critical modulatory role by dramatically increasing the intrinsic inhibitory activity of CSPGs.

To test the specificity of CSPG modulation, stripe assays in which the experimental stripe contained TSR-TM and the control stripes contained GFP were
prepared on polycarbonate filters that had been pre-coated with CSPG mixture. CSPGs did not convert TSR-TM to an inhibitory substrate, showing that CSPGs specifically regulate the function of full length Sema5A (Figure 7D). Taken together, these data suggest that Sema5A functions as a repellent cue for FR axons when co-localized with CSPGs in prosomere 2. In contrast, when expressed on FR fibers which are devoid of CSPGs, Sema5A likely functions as an attractive cue.

These data predict that Sema5A in prosomere 2 membranes should function as an attractive substrate if CS GAGs are eliminated. To test this prediction, stripe assays were prepared in which the experimental lane contained prosomere 2 membranes that had been treated with chondroitinase, while the control lanes contained prosomere 2 membranes treated with vehicle. We observed that Hb axons indeed grew preferentially on the chondroitinase treated prosomere 2 membranes (Figure 7E, 7F, and 7I). As a control, stripe assays were prepared in which the experimental stripe contained HEK 293 cell membranes treated with chondroitinase, and the control stripe contained vehicle treated HEK 293 membranes. Axons from Hb explants displayed no preference for either stripe (Supplemental Figure 3K), showing that chondroitinase treatment specifically affects prosomere 2 membranes. We confirmed that CS is present in the prosomere 2 membrane stripes, as detected by the CS-56 antibody, and also that chondroitinase treatment effectively abolishes CS-56 staining (Supplemental Figure 3L). Taken together, these results show that CSPGs are extrinsic cues which modulate Sema5A function, converting this guidance cue from an attractive cue to an inhibitory cue.

To directly assess the role of intact CSPGs in the development of the FR, organotypic diencephalon explants were cultured in the presence of chondroitinase. We
observed that the FR was disrupted in these experiments, with axons inappropriately projecting into prosomere 2. However, explants treated with vehicle alone had significantly fewer axons projecting into prosomere 2 (Figure 7G, 7H, and 7J; p < 0.007, student t-test). There was no significant difference in the development of the MTT between vehicle and chondroitinase treated explants (Figure 7G, 7H and 7K; p > 0.7, student t-test). These results demonstrate that both CSPGs and Sema5A play a crucial role in FR development.

DISCUSSION

Modulation of guidance cue function is essential in order for a limited repertoire of cues to orchestrate the development of multiple complex circuits. We show here that Sema5A is a bifunctional guidance cue regulated by sulfated proteoglycans, and that Sema5A is likely to play an essential role in the proper formation of the FR. Sema5A TSR domains play important roles in both the attractive and inhibitory functions of Sema5A by mediating interactions with different types of glycosaminoglycans. TSRs are necessary and sufficient for the permissive effects of Sema5A and they function independently of the sema domain. While the oligomerized Sema5A sema domain is necessary and sufficient for the inhibitory effects of Sema5A, the TSRs also play a critical role in inhibition since these domains mediate binding to CSPGs, an interaction that converts Sema5A from an attractive to an inhibitory cue for Hb axons. Together these results demonstrate that the permissive effects of Sema5A on Hb axons result from interactions with axonally expressed HSPGs, while the inhibitory effects of Sema5A depend on interactions with CSPGs. Therefore the nature of a growth cone's response to Sema5A
depends on the types of sulfated proteoglycans present in the developmental environment.

**Guidance events underlying FR development**

Both Sema5A and CSPGs are positioned in the diencephalon to influence critical axon guidance events underlying the formation of the FR (Bovolenta and Fernaud-Espinosa, 2000; Skaliora et al., 1998). However, Sema5A alone is not sufficient to explain the inhibitory properties of prosomere 2. Rather, a specific developmental environment which exists in prosomere 2 causes Sema5A to function as an inhibitory cue for FR axons. We demonstrate that CSPGs are a critical feature of this developmental environment which regulates Sema5A function.

In addition to serving as an inhibitory cue, our results suggest that Sema5A also plays an attractive role in maintaining the FR as a tightly fasciculated bundle. Since FR fibers are devoid of CS, Sema5A on these axons likely functions as a permissive substrate. Thus, FR pioneer axons expressing Sema5A may establish a permissive tract upon which later follower axons fasciculate in order to reach their targets. The establishment of fiber tracts by pioneer axons is known to be a critical feature of nervous system development in both invertebrates and vertebrates (Reichert and Boyan, 1997). An array of guidance events have now been described that explain the location, morphology, and targeting of the FR. Sema3F expressed in prosomere 1 together with CSPGs and Sema5A expressed in prosomere 2 likely serve to channel the FR between these two prosomeres (Funato et al., 2000; Sahay et al., 2003). Sema5A expressed on FR axons is likely to promote the highly fasciculated morphology of the FR through
interactions with neuronally expressed HSPGs. Finally, netrin-1 expressed in the ventral diencephalon serves an attractant the draws FR axons ventrally (Funato et al., 2000).

In the adult, the FR is composed of a heterogeneous population of axons which project to spatially distinct locations in the ventral diencephalon. For example, fibers from the medial Hb leave the FR in the ventral diencephalon to innervate the interpeduncular nucleus, while the fibers from the lateral Hb proceed either caudally to the paramedian midbrain, laterally to the substantia nigra, or rostrally to the median forebrain bundle (Sutherland, 1982). While the mechanisms that cause specific axons to leave the FR and innervate their targets are unknown, one possibility is that the conversion of Sema5A from a permissive to an inhibitory cue at precise locations along FR axons contributes to the adult innervation patterns of this fiber tract.

The attractive effects of Sema5A on Hb axons contrasts with the visual system, where Sema5A is strictly inhibitory for RGC axons (Goldberg et al., 2004; Oster et al., 2003). It is possible that Hb and RGC axons simply respond differently to Sema5A, perhaps as a result of expressing a different compliment of receptors. Consistent with this idea, syndecan-3 is expressed only at low levels in the retina during early development but is strongly expressed on FR axons during this period (Inatani et al., 2002). Interestingly, CS are distributed in the retina and along the optic tract in a pattern very similar to Sema5A (Brittis and Silver, 1994; Oster et al., 2003) (D.B.K. and A.L.K. unpublished observations), raising the possibility that modulatory interactions between Sema5A and CSPGs are important for inhibitory guidance events underlying proper targeting of retinal projections.
HSPGs are required for the permissive effects of Sema5A

We show here that HSPGs are directly involved in regulating semaphorin-mediated axon guidance. Our observation that HSPGs are required cell-autonomously for the attractive effects of Sema5A on Hb axons suggests that an HSPG, possibly syndecan-3, is one component of a functional Sema5A receptor on the surface of FR growth cones. HSPGs are known to function as obligate co-receptors which catalyze interactions between soluble ligands and their signaling receptors (Bernfield et al., 1999). HSPGs can also bind insoluble ligands such as cell surface proteins and ECM components, and this may more closely resemble the interaction between a cell surface HSPG and the transmembrane protein Sema5A (Bernfield et al., 1999). Importantly, binding of insoluble ligands by syndecan family members is known to promote interactions between the cytoplasmic domains of the syndecan and the actin cytoskeleton (Bernfield et al., 1999; Kinnunen et al., 1998).

Xylosides serve as alternate substrates for HS biosynthesis in place of the intended core proteins, and are secreted into the extracellular environment instead of being anchored to the cell surface (Miao et al., 1995). As such, in the presence of xylosides, HS are essentially translocated from the cell surface to the pericellular environment, where they exhibit functional activity (Miao et al., 1995). Interestingly, xyl-NM had the subtle effect of converting Sema5A from an attractive substrate to a slightly inhibitory substrate. This suggests that the translocation of HS polymers from the cell surface to the ECM may also regulate Sema5A function. Syndecans are known to be shed from the cell surface into the pericellular environment through a highly regulated, protease-dependent process (Bernfield et al., 1999). Therefore, endogenous
mechanisms which regulate the shedding of HSPGs such as syndecan-3 from the cell surface may affect the function of axon guidance cues.

**CSPGs regulate Sema5A function**

Although CSPGs are well known inhibitors of neurite outgrowth, the expression of these molecules in regions of the brain through which axons project during development demonstrates that growth cones exhibit variable responses to CSPGs (Bovolenta and Fernaud-Espinosa, 2000). We observe that CSPGs are not strictly inhibitory for the outgrowth of Hb neurons but instead promote the fasciculation of these fibers. The outgrowth of sensory axons on mixed CSPG/laminin substrates is also characterized by fasciculation (Snow et al., 2003). What are the factors that affect how growth cones respond to a complex environment rich in CSPGs? Experimental studies have shown that the species of CSPG, the relative balance of permissive and inhibitory substrates, the particular type of neuron, and the stimulus history of an axon each can contribute to how a growth cone responds after encountering CSPGs (Bovolenta and Fernaud-Espinosa, 2000; Condic et al., 1999; Snow and Letourneau, 1992; Snow et al., 2003). Our results suggest the novel possibility that the response of growth cones also depends on the complement of guidance cues in the surrounding environment, and how CSPGs modulate the function of those cues.

What are the molecular mechanisms by which CSPGs modulate Sema5A function? One possibility is that CSPGs affect the levels of intracellular second messengers known to be important for regulating growth cone behavior. For example, CSPGs elevate intracellular Ca$^{2+}$ levels in axonal growth cones, and also activate protein
CSPGs utilize bound guidance cues to affect axons

CSPGs belong to a group of myelin and glial scar associated inhibitory molecules, including Nogo, myelin associated glycoprotein (MAG), oligodendrocyte myelin glycoprotein (OMgp), and semaphorins all of which may be involved in preventing axon regeneration following CNS injury (Sandvig et al., 2004). While considerable progress has been made in identifying the receptor components of Nogo, MAG, OMgp, and the semaphorins, the mechanisms by which CSPGs initiate signaling events on the growth cone surface are poorly understood. One important insight stems from the observation that the biological activity of CSPGs, both during development and following CNS injury, resides in part with the glycosaminoglycan side chains (Rhodes and Fawcett,
Further, in vitro studies raise the possibility that the effects of CSPGs on growth cones are not attributable to the CSPGs per se, but rather to distinct CS-binding proteins (Emerling and Lander, 1996). Our results show that CS-bound Sema5A enhances the intrinsic inhibitory activity of CSPGs, providing one molecular explanation for how CSPGs inhibit the extension of Hb axons. This suggests that the effects of CSPGs on neurons is determined by the repertoire of CS-bound proteins. Enzymatic destruction of CS-GAGs by chondroitinase is among the few acute molecular manipulations known to promote partial functional recovery following experimental spinal cord lesions (Morgenstern et al., 2002). Since Sema5A may be one factor that limits regeneration of RGC axons (Goldberg et al., 2004), our results suggest that interactions between CSPGs and axon guidance molecules, including Sema5A, interfere with axonal extension following CNS injury.
Experimental Procedures

Animals. Pregnant Sprague Dawley rats were from Charles River, and the vaginal plug date was designated embryonic day zero (E0).

In situ hybridization. In situ hybridizations were performed on fresh frozen embryos as described (Giger et al., 1996). The Sema5A TSR domain probe was against bp 1680 - 3200.

Section binding. Binding of AP-tagged ligands to fresh frozen sections was performed as described (Giger et al., 1998). For enzymatic treatments, heparinase III (0.1 U/ml; Sigma; all enzyme units given in Sigma units) in buffer H (50 mM HEPES, pH 7.6, 0.1% BSA plus mammalian protease inhibitor cocktail from Sigma) or chondroitinase ABC (0.1 U/ml; Sigma) in buffer C (50 mM Tris, pH 8.0, 40 mM sodium acetate, 0.1% BSA plus protease inhibitors) were applied to the slides and incubated at 37°C for 2 hours. For proteinase treatment, slides were immersed in proteinase K (10 µg/ml; Invitrogen) in PBS for 4 minutes at room temperature (RT).

Diencephalon explants. The diencephalon and midbrain from E13.5 rat pups were dissected in L-15 medium into left and right hemispheres, and placed on Biocoat collagen I inserts (Becton Dickinson) ventricle side down. The explants were cultured in Optimem I (60% ; Invitrogen), Ham's F-12 (25%), heat inactivated FBS (15%), glucose (40 mM), glutamine (2 mM), gentamicin (12.5 µg/ml). Chondroitinase (200X stock in 50
mM Tris, pH 8.0, 40 mM sodium acetate, 50% glycerol) was added to the culture medium to a concentration of 0.25 U/ml initially, then again 36 hours later. Control cultures were treated with vehicle alone. IgG-purified whole Sema5A antibody (Oster et al., 2003) was added at a 100 fold dilution to the cultures initially, then again at a 250 fold dilution 36 hours later. Control rabbit IgG was added to the cultures to a concentration of 100 µg/ml initially, then again at 40 µg/ml 36 hours later. After 72 hours in culture, explants were fixed overnight in 4% PFA and labeled with TAG-1 antibodies (Developmental Hybridoma Bank) as described (Shirasaki et al., 1996). Fiber tracts were called agenic if they could not be clearly identified after TAG-1 staining. The FR and MTT were agenic in 30% and 38% of αSema5A treated explants, respectively, versus 20% and 30% of IgG treated explants, respectively. The FR and MTT were agenic in 32% and 55% of chondroitinase treated explants, respectively, versus 22% and 39% of vehicle treated explants, respectively.

**Membrane stripe assay.** Membrane stripe carpets were prepared as described (Walter et al., 1987). Membranes were isolated by sucrose gradient centrifugation as described (Tuttle et al., 1995). Each stripe contained, in addition to the experimental or control membranes, a 50% contribution of P0 cortex membranes. The explants were cultured in Neurobasal (Invitrogen) supplemented with B-27 (Invitrogen), glutamine (2 mM), and gentamicin (12.5 µg/ml) for 72 hours, then visualized with the vital dye calcein AM (5 µg/ml; Molecular Probes).

Heparinase (200X stock in 50 mM HEPES, pH 7.6, 50% glycerol) was added to the cultures to a final concentration of 0.2 U/ml initially, then again at 36 hours and 48
29 hours later. Sema5A antibodies were added to the culture medium at a 100 fold dilution 12-16 hours after plating, and control explants were treated with 100 µg/ml IgG purified pre-immune rabbit serum. Xyl-NM and xyl-decalin (200X stocks in ethanol; xylosides were gifts from J.D. Esko, UC San Diego) were added to the culture medium to a concentration of 50 µM initially, and again 24 hours later to a concentration of 25 µM. Control explants for each condition were treated with the appropriate vehicle.

For membrane treatments prior to the preparation of stripe assays, the following reagents were added to membranes for the indicated times. Sema5A antibodies (1:100 dilution) or control IgG (100 µg/ml) for 3 hours at 4°C. Heparinase (0.1 U/ml) in buffer H for 2 hours at 37°C. Chondroitinase (0.1 U/ml) in buffer C for 2 hours at 37°C. Proteinase K (10 µg/ml) in PBS for 5 minutes at RT. After each treatment, the membranes were washed in cold PBS plus protease inhibitors (PBS+), and the concentration of the membranes was determined.

For experiments in which polycarbonate filters were pre-coated, embryonic chicken CSPG mixture (Chemicon) or BSA solution were diluted in PBS+ to the indicated concentrations, spotted onto the center of a dry polycarbonate filter, and allowed to bind for 3 hours at 4°C. In some experiments, following absorption of the CSPG or BSA mixtures, the polycarbonate filters were blocked in 5% FBS in PBS+ for 1 hour at 4°C, rinsed, then treated with chondroitinase (0.25 U/ml) in buffer C for 2 hours at 37°C. Following each of these treatments the filter was rinsed in PBS+, and the membrane stripes were applied as described above.
**Scoring.** Scoring of the stripe assays was performed blind by an observer who was unaware of the experimental conditions, and who assigned scores based on the scoring template in Supplemental Figure 4. Scores were assigned by comparing the outgrowth of axons on the experimental stripe, usually marked with fluorescent beads, to the control stripes. Stripe assays were assigned to one of seven categories on a scale from -3 to +3. Diencephalon explants were scored by drawing a line between the apex of the mesencephalic flexure ventrally and the apex of the epithalamus dorsally. This line passes through the central region of prosomere 2. Individual axons and fascicles that crossed this line were counted. To measure the MTT, a line was drawn perpendicular from the midpoint of the line connecting the mesencephalic flexure to the epithalamus. The width of the MTT was determined at the point where this line crossed the MTT.

**Collagen explants.** Hb explants from E15.5 rat pups were embedded in a matrix of rat tail collagen mixed with matrigel (BD Biosciences) and cultured for 3 days in stripe assay growth medium. Collagen cultures were fixed and stained with antibodies as previously described (Giger et al., 1998). The following primary antibodies were used: DCC (1:750; gift from L. Richards, University of Maryland, and H. Cooper, Royal Melbourne Hospital), TAG-1 (1:35), 2H3 (1:75; Developmental Hybridoma Bank), 3G10 (1:200; Seikagaku), Syn3C (1:750; gift from M. Sheng, MIT). Primary antibodies were detected with the appropriate AlexaFluor secondary antibody (1:600; Molecular Probes).

Explants or fresh frozen brain section to be stained with the antibody 3G10 were fixed in 4% PFA for 6 hours or 10 minutes, respectively, then rinsed extensively in PBS. These explants were incubated with heparinase (0.2 U/ml) in buffer H for up to 8 hours at
37°C. The explants were then washed extensively in PBS, post-fixed for 3 hours in 4% PFA, then immunostained as below. Living Hb explants were labeled with Sema5A antibodies as described with some modifications (Oster et al., 2003).

For imaging of double labeled explants, image acquisition settings were adjusted to avoid maximal pixel intensity then maintained between images from the same experiment. Four or five axon segments from at least 5 explants per condition were outlined using Image J software, and the average pixel intensity in the outlined area was measured in the green channel (3G10) and red channel (Syn3C).

**Immunocytochemistry.** Cryosections from fresh frozen embryos were fixed with 4% PFA in PBS for 10 minutes at RT. Slides were washed in PBS, blocked for 1 hour in 5% FBS plus 0.3% Triton X-100 in PBS. Slides were incubated overnight at 4°C with the following antibodies: DCC (1:750), Syn3C (1:750), CS-56 (1:350), 3G10 (1:500).

**Immunoprecipitation.** Prosomere 2 membranes were solubilized in modified RIPA (mRIPA) buffer (50 mM HEPES, pH 7.0, 1mM MgCl₂, 1% NP-40, 0.25% Na deoxycholate plus protease inhibitors) and immunoprecipitated with the Syn3C antibody as described with some modifications (Hsueh and Sheng, 1999). Protein samples were western blotted using standard methods with the following antibodies: AP (1:5000) (American Research Products), Sema5A (1:1000), Syn3ec (1:3000; gift from A. Oohira, Aichi Human Service Center).
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Figure Legends

Figure 1. **Sema5A is important for the proper development of the fasciculus retroflexus (FR).** (A) Schematic lateral view of the developing diencephalon showing that the fasciculus retroflexus (FR) originates in the habenula (Hb) nucleus and extends between prosomere 1 (pros1) and prosomere 2 (pros2). Prosomere 1 expresses the repellent Sema3F, while prosomere 2 expresses an unknown membrane attached repellent activity. (B) Horizontal brain section showing that Sema5A transcript is found in the Hb nucleus (open arrowheads). (C) Axons extending from E15.5 Hb explants are strongly labeled by Sema5A antibodies. Sagittal (D) and horizontal (E) brain sections show that Sema5A transcript is expressed in prosomere 2 (filled arrowheads) adjacent to the FR (open arrowheads). (F) The alkaline phosphatase tagged ecto-domain of Sema5A (AP-5A\textsubscript{ecto}) binds both to FR axons (open arrowheads) and to prosomere 2 (outlined area). (G-H) E13.5 organotypic diencephalon explants stained with antibodies against TAG-1. Compared to explants treated with control IgG (G), explants treated with Sema5A function blocking antibodies (α5A) (H) show significantly more FR fibers (open arrowhead) crossing inappropriately into prosomere 2 (I). The width of the MTT (closed arrowhead) was not significantly different between αSema5A and IgG treatments (L). The letters in the graph legends correspond to the letters in the panels. DIV, days in vitro. All scale bars equal 100 μm.

Figure 2. **Sema5A expressed in HEK 293 cells is a permissive substrate for Hb axons.** (A-E) Structure/function analysis of Sema5A. Compared to membranes collected
from HEK 293 cells transfected with GFP (A), membranes from full length Sema5A (FL-5A) transfected cells (B) are a permissive substrate for Hb axons. (C) The permissive effects of Sema5A localize to the thrombospondin repeats. While the sema domain alone (D) has no effect on Hb axons, when oligomerized with the cartilage oligomeric matrix protein (COMP) assembly domain (E), the sema domain becomes an inhibitory cue for Hb axons. For all membrane stripe assays in this study, the horizontal boxes at the top of each assay indicate the composition of experimental and control stripes, with the experimental stripe designated by the left-most box. Panel (F) shows the constructs used in this study. The purple circles represent alkaline phosphatase (AP). Panel (G) is the quantification of data in panel A-E. The scale bar equals 100 µm.

**Figure 3. Sema5A is an endogenous prosomere 2 repellent for Hb axons.** (A-B) Stripe assays in which the experimental stripe contains intact prosomere 2 (pros2) membranes, and the control stripe contains prosomere 2 membranes that have been inactivated with brief proteinase K (protK) treatment. In experiments to which control IgG was added to the culture medium (A), Hb axons avoid the intact prosomere 2 stripes, while experiments treated with Sema5A function blocking antibodies (B) show reduced inhibition by intact prosomere 2 membranes. (C-D) Inactivation of prosomere 2 membranes with antibodies. Prosomere 2 membranes treated with control IgG (C) show no reduction in inhibitory activity, while prosomere 2 membranes treated with Sema5A antibodies (D) show significantly reduced inhibition, as Hb axons readily extend on antibody neutralized membranes. Horizontal boxes with triangles indicate a treatment to the membranes performed before stripe assay was assembled. Vertical boxes on the left
Figure 4. Heparan sulfate proteoglycans (HSPGs) mediate the permissive effects of Sema5A. (A-B) E15.5 sagittal brain sections stained with antibody 3G10, which recognizes a heparan sulfate (HS) neo-epitope revealed by heparinase (hep'ase) treatment. Sections treated with vehicle (A) show low levels of staining, but following heparinase treatment (B), FR fibers (open arrowheads) and blood vessels are strongly labeled. (C-F) Sema5A interacts with HS in situ. AP-5A<sub>ecto</sub> binding to the FR (C; open arrowheads) is reduced in an adjacent section treated with heparinase (D). AP-TSR1-4 binding to FR (E) is also eliminated in an adjacent section treated with heparinase (F). Note that the binding to prosomere 2 (outlined area) persists after heparinase treatment in (D) and (F). (G-K) Intact HSPGs are required cell autonomously for the attractive effects of Sema5A. Compared to control cultures treated with vehicle (G), cultures to which heparinase is added to the culture medium (H) show diminished Sema5A-mediated attractive effects. Treatment of the membranes alone with heparinase (I) has no effect on Sema5A function, suggesting that HSPGs on Hb axons are critical. Treatment of explants with the control xyloside xyl-decalin (J) does not effect Sema5A function, while treatment with xyl-NM (K), which interferes with HS biosynthesis, abolishes the attractive effects of Sema5A. Panel (N) shows the quantification of data in panels (G-K). (L and M) Analysis of the FR phenotype in EXT1 mutant mice, which are defective in HS biosynthesis. AP-Sema3F binding to horizontal brains sections from E17.5 mice heterozygous (L) or null (M) for a loxP modified EXT1 allele shows that the FR is significantly more
defasciculated in *EXT1* null animals (O). Mice from three separate litters were analyzed. All scale bars equal 100 µm.

**Figure 5. Xylosides render cell surface proteoglycan core proteins devoid of HS side chains on Hb axons.** (A) The transmembrane HSPG syndecan-3 is expressed on FR axons, as detected by the antibody Syn3C. (B) Sema 5A physically associates with syndecan-3. Both Sema5A and syndecan 3 are present in prosomere membranes, and the Syn3C antibody immunoprecipitates Sema5A from these membranes. A control IgG does not immunoprecipitate Sema5A. Syn3ec is a distinct antibody against the syndecan 3 ecto-domain. (C-I) The effect of xyl-NM treatment on HS biosynthesis in Hb axons. Hb explants were cultured in the presence of vehicle or xyl-NM then double labeled with two antibodies: Syn3C (red) which labels only the syndecan-3 core protein, and 3G10 (green) which labels only the HS component of HSPGs. (C) Quantification of the effects of xyl-NM. Xyl-NM reduced the normalized amount of axonal 3G10 labeling in a dose dependent manner. In panel C, an identical amount of ethanol vehicle was added to cultures treated with 0 µM xyl-NM as was added to cultures treated with 100 µM xyl-NM. All scale bars equal 100 µm.

**Figure 6. Sema5A binds to chondroitin sulfate (CS) glycosaminoglycans (GAGs), and CS proteoglycans (CSPGs) promote the fasciculation of FR axons.** (A-B) CSPGs are co-expressed with *Sema5A* and surround the developing FR. CS GAGs are detected with the antibody CS-56 (red), and the FR is labeled with antibodies against the receptor DCC (green). Panel A and B are adjacent sections to the *Sema5A in situ*
hybridizations in Figure 1D and 1E, respectively. (C-F) Sema5A binds to CS GAGs in situ. AP-5A<sup>ecto</sup> binds to regions of prosomere 2 (outlined area) that are rich in CS (C), and this binding is reduced in adjacent sections treated with chondroitinase (ch'ase) (D). AP-TSR1-4 binding to prosomere 2 (E) is also reduced by chondroitinase treatment in an adjacent section (F). Note that binding to the FR (open arrowheads) is spared in (D) and (F). (G-K) CSPGs have dose-dependent effects on the fasciculation of Hb axons. On polycarbonate filters pre-coated with BSA (G), Hb axons were highly defasciculated. In contrast, axons growing on filters pre-coated with escalating concentrations of CSPGs (H-J), grew progressively more fasciculated. (K) Quantification of the data in panels (G-J). All scale bars equal 100 µm.

**Figure 7. CSPGs switch Sema5A from an attractive to an inhibitory cue and are necessary for proper FR development.** (A-F) CSPGs functionally regulate Sema5A. Compared to membrane stripe assays prepared on polycarbonate filters pre-coated with BSA (A), assays prepared on polycarbonate filters pre-coated with embryonic brain CSPGs show that Sema5A switches from an attractive to an inhibitory substrate (B). Enzymatic destruction of CS with chondroitinase abrogates the ability of CSPGs to switch Sema5A function (C). The presence of the sema domain is critical for the repulsive effects of Sema5A, as CSPGs do not convert TSR-TM from an attractive to a repulsive substrate (D). Treatment of prosomere 2 membranes with chondroitinase causes these membranes to switch from being inhibitory (E) to being permissive (F). Horizontal boxes below the stripe assays indicate the treatments to the polycarbonate filter upon which the stripe assays were prepared. Panel (I) is the quantification of data
in panels (A-F). (G-H) E13.5 organotypic diencephalon explants showing that intact CSPGs are necessary for the proper development of the FR. Compared to explants treated with vehicle (G), explants treated with chondroitinase (H) show significantly more FR fibers (open arrowheads) crossing inappropriately into prosomere 2 (J). The width of the MTT (closed arrowhead) was not significantly different between vehicle and chondroitinase treatments (K). All scale bars equal 100 µm.
Supplemental Figure Legends

Supplemental Figure 1. In Situ Hybridization and Section Binding Control

Experiments.  (A) An ISH probe against the sema region of Sema5A reveals a pattern of transcript expression identical to a probe against the TSR region (compare to Figure 1D). (B) The TSR domain sense probe does not specifically label brain sections. (C-J) The effect of proteinase K, heparinase III, or chondroitinase ABC treatment on section binding. Heparinase III destroys specific types of HS GAGs, and chondroitinase ABC destroys specific types of CS GAGs (Ernst et al., 1995). Binding of AP-5A<sup>ecto</sup> to brain sections (C) is not eliminated in adjacent sections treated with proteinase K (D). However, binding of AP-Sema3F to brain sections (E), is abolished in adjacent sections treated with proteinase K (F). Binding of AP-Sema3F to brain sections (G) is not affected in adjacent sections treated with heparinase (H). Binding of AP-Sema3F to brain sections (I) is not affected in adjacent sections treated with chondroitinase (J). A fusion protein consisting of AP linked to the last three thrombospondin repeats of Sema5A (AP-TSR5-7) does not bind to brain sections (K). (L) Rat, human, and Drosophila alignments showing conservation of basic residues in the third anti-parallel β-strand in the fourth TSR of Sema5A (<i>rattus</i> and <i>homo</i>) and Sema5c (<i>Drosophila</i>). This region likely corresponds to the binding face of the fourth TSR of class 5 semaphorins. TSRs with clusters of positively charged amino acids on their binding face are best able to interact with the negatively charged carboxylate and sulfate groups of glycosaminoglycans (Tan et al., 2002; Tzarfaty-Majar et al., 2001). In rat Sema5A, the fourth TSR and to a lesser
extent the second TSR have a number of positively charged residues in positions predicted to be on the binding face of the domain. The scale bar equals 100 microns.

**Supplemental Figure 2. Characterization of Hb Explants.** Axons extending from Hb explants in vitro recapitulate the normal expression of TAG-1 (A), DCC (B), and neuropilin-2 (C) as revealed by binding of AP-Sema3F. Importantly, FR axons in vivo do not express neurofilament (as detected by the monoclonal antibody 2H3; D.B.K. and A.L.K., data not shown), and Hb explants in vitro do not aberrantly express this protein (D). *Neuropilin-2* ISH (E) marks the location of Hb neurons. This section is adjacent to the section shown in Figure 1B. Scale bars equal 100 microns.

**Supplemental Figure 3. Control Membrane Stripe Assays.** Heat inactivation (A), trypsinization (B), or proteinase K treatment (Figure 3A) all similarly inactivate the endogenous repellant activity of prosomere 2 membranes. As these three treatments all have the same effect, this suggests that the pattern of axon outgrowth observed in these assays is not the result of a permissive epitope unmasked in the treated membranes. Rather, it is more likely that an endogenous repellent is inactivated by these treatments. (C) Hb axons showed no preference for the experimental or control stripes in assays in which the experimental membranes were proteinase K treated prosomere 2 and the control membranes came from GFP transfected HEK 293 cells. (D - I) Sema3F control experiments. Sema3F does not account for the inhibitory activity of prosomere 2 membranes as Hb axons from *Npn2* +/− mice (D) and *Npn2* −/− mice (E) similarly avoided prosomere 2 membranes. Mice from three separate litters were analyzed. Neither control
IgG (F) nor the Sema5A function blocking antibody (G) interferes with the inhibition of Hb axons by membranes derived from HEK 293 cells transfected with Sema3F. Neither vehicle (H) nor heparinase treatment (I) significantly interferes with Sema3F mediated inhibition of Hb axons. (J) The Sema5A function blocking antibody is not serving as a permissive substrate for Hb axons, as addition of this antibody to membranes from GFP transfected HEK 293 cells has no effect on Hb axons. (K) The effect of chondroitinase treatment on prosomere 2 membranes shows some specificity as treatment of GFP transfected HEK 293 cell membranes with chondroitinase has no effect on outgrowth of Hb axons. (L) Chondroitin sulfates, as detected by the antibody CS-56, are present on prosomere 2 membranes, and treatment with chondroitinase eliminates CS-56 labeling. Panel M shows the quantification of the data in panels (A-E). Panel N shows the quantification of the data in panels (F-K). Scale bar equals 100 microns.

**Supplemental Figure 4. Scoring Template for Stripe Assays and Fasciculation Assays.** (A) Scoring template for stripe assays. A score of -3 was assigned to stripe assays in which axons avoided the experimental stripe almost exclusively, and a score of +3 was assigned to assays where axons grew on the experimental stripes almost exclusively. A score of zero indicates the axons grew equally on control and experimental stripes. Statistical significance was determined by the Mann Whitney rank sum test. (B) Scoring template for the fasciculation assays. The effect of CSPGs on Hb axons was scored by an observer who was given this scoring template and asked to rate the degree of fasciculation by comparing each explant to the template. All scoring was
performed blind by an independent observer who was unaware of the experimental conditions.
Supplemental Material and Methods

cDNAs and constructs. Rat Sema5A was cloned from an oligo-dT primed e15 Sprague Dawley rat spinal cord/DRG cDNA library, and the double strand sequence was determined. The TSR domain ISH probe construct consists of bp 1680 - 3200 in pBluescript, and for the preparation of anti-sense probe, this construct was linearized with XbaI and transcribed with T3 RNA polymerase. For sense probe, this construct was linearized with ClaI and transcribed with T7 RNA polymerase. The sema domain ISH probe construct consists of bp 1 - 1679 in pBluescript, and for the preparation of anti-sense probe, this construct was linearized with ClaI and transcribed with T7 RNA polymerase. The FLSema5A construct consists of the codons for aa 22-1074 subcloned by PCR into pSecTag B (Invitrogen). The TSR-TM construct consists of the codons for aa 533 - 1074 subcloned by PCR into pcDNA1.1 (Invitrogen). The Sema-TM construct consists of the codons for aa 22 - 532 (the sema domain) fused by PCR to the codons for aa 1021-1074 (the last 13 aa of TSR 7 plus the transmembrane and cytoplasmic domains) into pSecTag B. The Sema-COMP construct consists of the codons for aa 27-83 of rat COMP fused by PCR to the C-terminus of the Sema-TM construct. All of the AP tagged constructs were creating by fusing the following portions of Sema5A by PCR to an N-terminal human placental alkaline phosphatase in pcDNA1.1: AP-5A<sup>ecto</sup>, the codons for aa 22 - 970; AP-TSR1-4, the codons for aa 533-783; AP-TSR5-7, the codons for aa 784-940. The AP tagged human Sema3F construct has been described previously (Sahay et al., 2003).
**In Situ Hybridization.** Embryos younger than e15 were fixed for 5 hours at 4°C in freshly prepared 4% PFA, then cryoprotected in PBS plus 10% sucrose before freezing. Sections from these embryos were treated with 10 µg/ml proteinase K prior to hybridization. Probes for *in situ* hybridization were synthesized according to (Giger et al., 1996). Following *in vitro* transcription, probes were purified using Chromaspin 100 columns (Clontech) then hydrolyzed by alkaline hydrolysis to an average length of 400 bp. The hybridization buffer was from Dako

**Membrane Preparation and Stripe Assays.** Prosomeres from E15.5 rat pups were dissected under incident light using the location of the FR and the MTT as landmarks. P0 cortex membranes were derived from approximately the caudal third of the cortical wall, with the hippocampus removed. Tissues were dissected in L-15 medium then transferred to approximately 5 volumes of cold tissue homogenization buffer (10 mM Tris, pH 7.4, 1.5 mM CaCl$_2$ plus protease inhibitors), broken up by passing repeatedly through a blue tip, and homogenized by passing twice through a 27 gauge needle. HEK 293 EBNA cells were scraped into cold PBS, pelleted, and homogenized in approximately 5 volumes of cold cell homogenization buffer (15 mM Tris, pH 7.4, 1.5 mM CaCl$_2$ plus protease inhibitors) by passing twice though a 27 gauge syringe. The membrane fraction was separated by centrifugation at 50K x g on a sucrose step gradient (5% and 50% sucrose in tissue homogenization buffer with protease inhibitors added to the 5% sucrose). Membranes at the interface were collected, washed once in cold PBS+, and resuspended in PBS+. The concentration of each membrane solution was determined follows:
membranes were diluted 600 fold in 2% SDS, the absorbance was determined at 220 nm, and an OD reading of 0.2 was defined as 80X concentration.

HEK 293 cell membranes were prepared fresh for each experiment. Membranes from brain tissues were diluted 2 fold in glycerol and stored at -80°C until use. When needed, frozen membranes were thawed on ice, washed 2 times in cold PBS+, then resuspended in PBS+. For trypsinization experiments, membranes were resuspended in 0.25% Trypsin-EDTA (Invitrogen) and incubated for 5 min at RT. For heat treatment experiments, membranes were resuspended in HBS (50 mM HEPES, pH 7.0, 120 mM NaCl) and incubated at 65°C for 10 minutes.

Hb explants were dissected from E15.5 embryos, and held in place on the membrane carpets with strips of nitrocellulose. Images were acquired by confocal microscopy.

**HEK 293 EBNA cell transfections and ligand production.** HEK 293 EBNA cells plated on poly-lysine coated plastic were maintained in DMEM/10% FBS supplemented with penicillin/streptomycin. Cells were transfected using Lipofectamine 2000 (Invitrogen) overnight in DMEM/1% FBS, then returned to normal maintenance medium. Cells were allowed to express transfected cDNA for 48 hours before membranes were collected. DNA and Lipofectamine concentrations were according to manufacturers recommendations. For ligand production, 15 cm plates were transfected overnight in Optimem I supplemented with 1% FBS and 25 mM glucose. The following day the medium was changed to Optimem I supplemented with 3% FBS and 25 mM glucose. Cells were maintained for up to four days, by exchanging 5 mls of old medium for 5 mls
of fresh medium every 24 hours. Medium was collected and concentrated up to 10 fold with Amicon YM-30 concentrators. The concentration of AP ligand was quantified by comparing the activity against a calibrated Kit-AP standard (Genhunter). AP ligand was frozen at -80°C.

**Section Binding.** 18 to 25 µm cryosections from fresh frozen embryos were mounted on glass slides and maintained at 4°C to prevent desiccation while sectioning the embryo. Slides were frozen at -20°C for 30 minutes, then fixed for 10 minutes by immersion in methanol cooled to -20°C. The slides were rinsed 3 times in PBS, then blocked for 1 hour in HBH (1X Hank's balanced salt solution with calcium and magnesium (Invitrogen), 0.5% BSA, 50 mM HEPES, pH 7.0) plus 10% FBS. Subsequently, the slides were incubated with 1 nM AP tagged ligand for 1.5 hours at room temp in a humidified chamber, washed 5 times in HBH, fixed in 65% acetone/4% formalin/50 mM HEPES, pH 7.0 for 2 minutes at RT, then rinsed 3 times in HBS. Endogenous alkaline phosphatase was inactivated at 65°C for 1.5 hours then the slides were incubated with BCIP/NBT color substrate. Before enzymatic treatments, cryosections were first fixed in methanol at -20°C for 10 minutes.

**Immunostaining of Diencephalon and Hb Explants.** Explants were blocked in PBS plus 1% Triton X-100 (PBST) and 10% FBS (blocking buffer) for 2 hrs at RT, then incubated with TAG-1 diluted 35 fold in blocking buffer overnight at 4°C. The explants were washed for up to 6 hours in PBST plus 1% FBS with multiple changes of buffer. Explants were then incubated in an anti-IgM secondary antibody (1:600; Molecular
Probes) in blocking buffer for 3 hours at RT. Explants were washed for the next 24 hours with several changes of PBST. Explants were cleared in 60% glycerol and imaged by confocal microscopy.

For Hb explants stained with the Sema5A antibody, living explants were incubated with antibody (1:100 dilution) in culture medium for 4 hours, washed with several changes of growth medium over 2 hours, then incubated with secondary antibody diluted in growth medium for 4 hours. The cultures were washed with several changes of growth medium over 2 hours, then fixed in 4% PFA. For cultures treated with xyl-NM, this compound was added once at the time of plating at the indicated concentration.

For cultures stained with AP-Sema3F, living explants were incubated with 1 nM ligand for 2 hours, washed with several changes of growth medium over 6 hours, then fixed for 5 minutes in 4% PFA, 65% acetone, 20 mM HEPES, pH 7.0. Endogenous AP was inactivated at 65°C for 90 minutes.

**Immunoprecipitation.** Prosomere membranes were solubilized in mRIPA buffer for 30 minutes at 4°C, and the insoluble material was pelleted by centrifugation for 10 minutes at 14K x g. Solubilized extract (1 mg total) was pre-cleared with 40 µl of a 50% slurry of protein G agarose (Amersham Biosciences) for 30 minutes at 4°C. Twenty microliters of pre-cleared solubilized extract was removed and used as the input lane for Western blotting, while the remainder of the extract was incubated with Syn3C antibody (3 µg) or control rat IgG (3 µg) overnight at 4°C with rotation. Following this, 60 µl of the 50% protein G agarose slurry was added to the mixture and incubated for 2 hours at 4°C. The
precipitate was washed 3 times in mRIPA and bound proteins were released from the beads by boiling in SDS sample buffer.
Supplemental References


No response to the reviewers was required.
Figure 2, Kantor et al.
Figure 3

A. Control IgG
B. ProtK
C. IgG
D. α5A
E. Bar graph showing percent of total score. 
- A, n=17
- B, n=15
- C, n=19
- D, n=15

SCORE

(more inhibitory)

(more permissive)
Figure 4, Kantor et al.
Figure 5, Kantor et al.
Figure 6, Kantor et al.
Figure 7

Figure 7, Kantor et al.
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**Supplemental Data Fig 1**

Class 5 sema 4th TSR
third antiparallel β-strand

rat CKARLPDPNLLEVGRQRIEMRYC
hom CKARLADPNLLEVGRQRIEMRYC
dro CRATSPDSSVRIGLPKEESRNC
Supplemental Figure 3, Kantor et al.
Supplemental Figure 4, Kantor et al.

A

B

Supplemental Figure 4, Kantor et al.