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

Introduction

The adult pattern of neuronal 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 that 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 aminoterminal 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 that are expressed in the developing vertebrate nervous system in patterns that suggest they play complex roles in neural development (Medina et al., 2004; Oster et al., 2003; Jones et al., 2002; Adams et al., 1996; Skaliora et al., 1998). The class 5 semaphorin Sema5A can function as an inhibitory cue that collapses both cultured fibroblasts and retinal ganglion cell (RGC) growth cones and 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 that influence the response of growth cones to guidance cues (Condic et al., 1999; Diefenbach et al., 2000; Hopker et al., 1999;
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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 that 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 that bind to glycosaminoglycans and interact with receptors on the surface of neuronal growth cones (Anderson et al., 1998; Britts 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 that 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 that originate in the habenula nucleus (Hb) and project along the boundary between prosomere 1 and prosomere 2 (Figure 1A). The secreted repellent Sema3F is expressed in prosomere 1 (Funato et al., 2000; Sahay et al., 2003), and an unidentified membrane-anchored inhibitory activity is localized to prosomere 2 (Funato et al., 2000). The combination of 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 colocalizes with neuropilin-2 (Npn2), a Sema3F receptor known to be expressed in Hb neurons (Figure 1B and Supplemental Figure S2E at http://www.neuron.org/cgi/content/full/44/6/961/DC1/; Chen et al., 1998; Giger et al., 1998). Sema5A antisera (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 (Figures 1D and 1E). An alkaline phosphatase (AP)-tagged Sema5A ectodomain fusion protein (AP-5Aαα) 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 projec-
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Figure 1. Sema5A is Important for the Proper Development of the Fasciculus Retroflexus

(A) Schematic lateral view of the developing rat 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.

(D and E) 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-5Aecto) binds both to FR axons (open arrowheads) and to prosomere 2 (outlined area).

(G–I) E13.5 rat organotypic diencephalon explants stained with antibodies against TAG-1. Compared to explants treated with control IgG (G), explants treated with Sema5A function-blocking antibodies (H) show significantly more FR fibers (open arrowhead) crossing inappropriately into prosomere 2 (I). Results are expressed as the mean number of fibers per explant crossing into prosomere 2 ± SEM. The letters in the graph legends correspond to the letters in the panels. DIV, days in vitro. *p < 0.001, Student’s t test. All scale bars equal 100 μm.

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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; mean number FR fibers in prosomere 2 ± SEM; IgG, 4.79 ± 0.79; α5A, 10.6 ± 1.08; p < 0.001, Student’s t test). However, the organization and width of the MTT was not significantly disrupted in the presence of Sema5A antibodies compared to IgG controls (Figures 1G, 1H, and 1J; mean width of MTT ± SEM; α5A, 42 ± 2.9 μm; IgG, 35 ± 3.29 μm; p > 0.07, Student’s t test). These results show that Sema5A is critical for proper FR development in an in vitro model that 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,
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.

(F) Constructs used in this study. The purple circles represent alkaline phosphatase (AP).

(G) Quantification of data in (A)–(E). The scale bar equals 100 μm.
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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 (Figures 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 Figures S3F, S3G, and S3N). To determine if the Sema5A function-blocking antibodies act on Hb axons or on the membrane substrate, prosomere 2 membranes were preincubated with these antibodies prior to preparing the membrane stripes, washed extensively to remove unbound antibody, and 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 (Figures 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 pretreated with control IgG (Figures 3C and 3E; \( p < 0.001 \), Mann-Whitney test).

Taken together, these results demonstrate that Sema5A is an endogenous membrane-anchored guidance cue 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 required for the binding of AP-tagged Sema5A to FR axons, embryonic brain sections were treated with heparinase. We found that AP-5A ecto binding to the FR was substantially reduced by heparinase treatment (Figures 4C and 4D) but was not eliminated by proteinase K treatment (Supplemental Figures S1C and S1D). 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 membrane stripes and instead extended preferentially on the neutralized control stripes (Figures 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 Figures S3D, S3E, and S3M; 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.

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Taken together, these results demonstrate that Sema5A is an endogenous membrane-anchored guidance cue that is expressed in prosomere 2 and serves as a repellent for Hb axons.

**Figure 3. Sema5A Is an Endogenous Prosomere 2 Repellent for Hb Axons**

(A and 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 and 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 indicate treatments added to the culture medium.

(E) Quantification of the stripe assay data in (A)–(D). The scale bar equals 100 μm.
Figure 4. Heparan Sulfate Proteoglycans Mediate the Permissive Effects of Sema5A

(A and B) E15.5 sagittal rat 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 ecto 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 affect Sema5A function, while treatment with xyl-NM (K), which interferes with HS biosynthesis, abolishes the attractive effects of Sema5A.

(L, M, and O) Analysis of the FR phenotype in EXT1 mutant mice, which are defective in HS biosynthesis. AP-Sema3F binding to horizontal brain 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). Results are expressed as the mean number of discrete FR fascicles seen in the horizontal plane per hemisphere ± SEM. Mice from three separate litters were analyzed. *p < 0.001, Student’s t test. All scale bars equal 100 μm.

(N) Quantification of data in (G)–(K).
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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 E15 rat FR axons, as detected by the antibody Syn3C.

(B) Sema5A 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 (C), an identical amount of ethanol vehicle was added to cultures treated with 0 M xyl-NM as was added to cultures treated with 100 M xyl-NM. Results are expressed as the mean of normalized 3G10 pixel intensities (3G10 pixel intensity/Syn3C pixel intensity) for 20–25 axon segments for each concentration of xyl-NM ± SD. *p < 0.001, Student’s t test. All scale bars equal 100 μm.

(D.B.K., A.L.K., and R.J.G., data not shown). More specifically, we found that the binding pattern of a fusion protein consisting of AP linked to the first four TSRs of Sema5A (AP-TSR1-4) is identical to the binding pattern of AP-5Aecto (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 S1K). 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 (Figures 4D and 4F), nor did this treatment affect the binding of AP-Sema3F to the FR (Supplemental Figures S3G and S3H). 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 specifically 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 (Figures 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 Figures S3H, S3I, and S3N).

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 (Figures 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-xylolides are a class of compounds that 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 (Figures 4K and 4N; p < 0.001, Mann-Whitney test). 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 (Figures 4J and 4N). We also noted that in the presence of xyl-NM, FL-5A-containing stripes became modestly but significantly inhibitory for Hb axons (Figures 4K and 4N; p < 0.001, Mann-Whitney test; see Discussion). These results show that HSPGs are required cell autonomously 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 (Figures 5D and 5G) and in prosomere 2 membranes (Figure 5B). We determined that Sema5A and syndecan-3 physically associate endogenously, as Sema5A coimmunoprecipitates 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 (Figures 5C–5I; the mean of normalized 3G10 pixel intensities [3G10 pixel intensity/Syn3C pixel intensity] ± SD; 0 μM, 0.78 ± 0.10; 25 μM, 0.53 ± 0.13; 50 μM, 0.38 ± 0.10; 100 μM, 0.29 ± 0.15; p < 0.001, Student’s t test). Vehicle treatment alone did not affect 3G10 or Syn3C labeling. These results show that xyl-NM prevents the attachment of HS GAGs to syndecan-3, without affecting core protein expression levels.

Sema5A expressed on early FR axons might serve as an 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 sections were labeled with AP-Sema3F, the binding of which does not depend on intact HS (Supplemental Figures S3G and S3H). In mutant mice where aloxP-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 (Figures 4L, 4M, and 4O; mean number of fascicles per hemisphere ± SD, as seen in horizontal sections; EXT1+/−, 1.0 ± 0; EXT1−/−, 2.25 ± 0.36; p < 0.001, Student’s 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 Sem5A to function as an inhibitory cue? Given the specific binding of AP-5Aext to prosomere 2 in brain 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-5Aext to prosomere 2 is resistant to proteinase K treatment, raising the possibility that glycosaminoglycans account for the binding of Sema5A to prosomere 2 (Supplemental Figures S1C and S1D). Because the binding of AP-5Aext and AP-TR5–14 to prosomere 2 was also resistant to heparinase treatment, this suggested the involvement of a different glycosaminoglycan (Figures 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 colocalized 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,
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Figure 6. Sema5A Binds to Chondroitin Sulfate Glycosaminoglycans, and Chondroitin Sulfate Proteoglycans Promote the Fasciculation of FR Axons

(A and B) CSPGs are coexpressed 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). (A) and (B) are adjacent sections to the Sema5A in situ hybridizations in Figures 1D and 1E, respectively.

(C–F) Sema5A binds to CS GAGs in situ. AP-5Aecto 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. Uniform carpets of membranes derived from postnatal day 0 rat cortex were applied to polycarbonate filters precoated with BSA or CSPG mixture. On filters precoated with BSA (G), Hb axons were highly defasciculated. In contrast, axons growing on filters precoated with escalating concentrations of CSPGs (H–J) grew progressively more fasciculated. (K) Quantification of the data in (G)–(J). All scale bars equal 100 μm.

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-5Aecto and AP-TSR1-4 to CS-rich regions of the diencephalon without affecting binding to the FR (Figures 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 Figures S1I and S1J). 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. CSPGs expressed in prosomere 2 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 that had been precoated with a commercial preparation of embryonic chicken brain CSPGs (Ernst et al., 1995) or
BSA. On the BSA substrate, Hb axons were highly defasciculated, rarely growing as thick bundles (Figure 6G). However, as the concentration of CSPG mixture increased to 100 μg/ml, Hb axons became progressively and significantly more fasciculated than explants grown on filters precoated with BSA (Figures 6H−6K; p < 0.001, Mann-Whitney test). 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 that had only modest effects on Hb axon fasciculation would influence the function of 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 precoated with 50 μg/ml of the CSPG mixture. Remarkably, we observed that Sema5A was converted from an attractive to an inhibitory cue for Hb axons when presented together with substrate bound CSPGs (Figures 7B and 7I). In contrast, when polycarbonate filters were precoated with 50 μg/ml BSA, Sema5A remained an attractive cue for Hb axons (Figures 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 (Figures 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 precoated 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 colonized with CSPGs in prosomere 2. In contrast, when expressed on FR fibers that 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 (Figures 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 S3K), 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 S3L). Taken together, these results show that CSPGs are extrinsic cues that 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 (Figure 7H). However, explants treated with vehicle alone had significantly fewer axons projecting into prosomere 2 (Figures 7G, 7H, and 7I; mean number FR fibers in prosomere 2 ± SEM; vehicle, 3.6 ± 1.25; ch’ase, 7.9 ± 2.0; p < 0.007, Student’s t test). There was no significant difference in the development of the MTT between vehicle- and chondroitinase-treated explants (Figures 7G, 7H, and 7K; p > 0.7, Student’s 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 soma domain. While the oligomerized Sema5A soma 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. 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 well positioned in the diencephalon to influence critical axon guidance events underlying the formation of the FR (Bovolenta and Fernaudo-Espinoza, 2000; Skaliora et al., 1998; this study). However, Sema5A alone is not sufficient to explain the inhibitory properties of prosomere 2. Rather, a specific developmental environment that exists in prosomere 2 causes Sema5A to function as an inhibitory cue for FR axons. We demonstrate that CSPGs are a critical fea-
Proteoglycans Regulate Sema5A Function

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 precoated with BSA (A), assays prepared on polycarbonate filters precoated 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.

(G, H, J, and K) E13.5 rat 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 arrowhead) crossing inappropriately into prosomere 2 (J). Results are expressed as the mean number of fibers per explant crossing into prosomere 2 ± SEM. The width of the MTT (closed arrowhead) was not significantly different between vehicle and chondroitinase treatments (K). Results are expressed as the mean width of the MTT (in μm) ± SEM. *p < 0.001, Student’s t test. All scale bars equal 100 μm.

(I) Quantification of data in (A)–(F).

ature of this developmental environment that 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 has 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
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(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 neurally expressed HSPGs. Finally, netrin-1 expressed in the ventral diencephalon serves as an attractant that draws FR axons ventrally (Funato et al., 2000).

In the adult, the FR is composed of a heterogeneous population of axons that 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 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 is 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 respond differently to Sema5A, perhaps as a result of expressing a different complement 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; this study). 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.

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/faminin 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 can each 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 how CSPGs modulate the function of surrounding axon guidance 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 kinase C and the small GTPase Rho in neurons (Sandvig et al., 2004; Sivasankaran et al., 2004). A second possibility is that CSPGs promote an interaction between Sema5A and a repulsive receptor. It has been shown recently that Plexin-B3 is a high-affinity Sema5A receptor that mediates Sema5A repulsive and attractive responses in cultured cells (Artigiani et al., 2004). However, we and others do not find that CSPGs promote an interaction between Sema5A and a repulsive receptor. In addition, plexin B3 transcript is expressed only at low levels, if at all, in the Hb during the time of FR development and is not upregulated in Hb explants (R.J.G., D.B.K., A.L.K., unpublished observations). Finally, CSPGs may interfere with the interactions between Sema5A and cell surface receptor components, such as HSPGs, which mediate Sema5A attraction.

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 coreceptors that 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 HS PG 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., 1998). 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 that regulate the shedding of HSPGs such as syndecan-3 from the cell surface may affect the function of axon guidance cues.
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, 2004). 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.

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., 1996). 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 hr. For protease treatment, slides were immersed in proteinase K (10 μg/ml; Invitrogen) in PBS for 4 min 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%), heat-inactivated BSA (15%), glucose (40 mM), glutamine (2 mM), and gentamicin (12.5 μg/ml). Chondroitinase (200 × 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 3 hr 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 hr later. Control rabbit IgG was added to the cultures to a concentration of 100 μg/ml initially, then again at 40 μg/ml 36 hr later. After 72 hr 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 α5A-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 postnatal day 0 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 hr, then visualized with the vital dye calcein AM (5 μg/ml; Molecular Probes).

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 hr at 4°C; heparinase (0.1 U/ml) in buffer H 2 hr at 37°C; chondroitinase (0.1 U/ml) in buffer C 2 hr at 37°C; and proteinase K (10 μg/ml) in PBS 5 min 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 precoated, 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 hr 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 hr at 4°C, rinsed, then treated with chondroitinase (0.25 U/ml) in buffer C for 2 hr 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 S4. 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), and Syn3C (1:750; gift from M. Sheng, MIT). Primary antibodies were detected with the appropriate AlexaFluor secondary antibody (1:600; Molecular Probes).

Explants to be stained with the antibody 3G10 were fixed in 4% PFA for 6 hr, then rinsed extensively in PBS. These explants were incubated with heparanase (0.2 U/ml in buffer H for up to 8 hr at 37°C. The explants were then washed extensively in PBS, postfixed for 3 hr in 4% PFA, then immunostained as above. 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 five 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 min at RT. Sections to be stained with the 3G10 antibody were first incubated with heparinase for up to 8 hr at 37°C. Slides were washed in PBS, blocked for 1 hr in 5% PBS 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), and 3G10 (1:200).

Immunoprecipitation
Prosmere 2 membranes were solubilized in modified RIPA (mRIPA) buffer (50 mM HEPES [pH 7.0], 1 mM MgCl2, 1% NP-40, 0.25% N-deoxycholate plus protease inhibitors) and immunoprecipitated with the Syn3C antibody as described with some modifications (Hsueh and Sheng, 1999). Protein samples were immunoblotted using standard methods with the following antibodies: AP (1:5000) (American Research Products), Sema5A (1:1000), and Syn3Ec (1:3000; gift from A. Oohira, Aichi Human Service Center).

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References


