Neuron, Vol. 14, 949–959, May, 1995, Copyright © 1995 by Cell Press

Semaphorin III Can Function as a Selective Chemorepellent to Pattern Sensory Projections in the Spinal Cord

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Summary

Distinct classes of primary sensory neurons in dorsal root ganglia subserve different sensory modalities, terminate in different dorsoventral locations in the spinal cord, and display different neurotrophin response profiles. Large diameter muscle afferents that terminate in the ventral spinal cord are NT-3 responsive, whereas small diameter afferents subserving pain and temperature are NGF responsive and terminate in the dorsal spinal cord. Previous in vitro studies showed that the developing ventral spinal cord secretes a diffusible factor that inhibits the growth of sensory axons. Here we show that this factor repels NGF-responsive axons but has little effect on NT-3-responsive axons. We also provide evidence implicating semaphorin III/collapsin, a diffusible guidance molecule expressed by ventral spinal cord cells, in mediating this effect. These results suggest that semaphorin III functions to pattern sensory projections by selectively repelling axons that normally terminate dorsally.

Introduction

Mammalian dorsal root ganglia (DRG) comprise several functionally distinct classes of primary sensory neurons mediating different types of somatosensory information (Brown, 1981; Willis and Coggeshall, 1991). Different classes have characteristic physiological properties and axonal diameters, and project axons and axon collaterals to second order neurons in the spinal cord and medulla. The pattern of projections within the spinal cord is highly stereotyped, with axons entering the dorsal horn from the dorsal funiculus and extending ventrally to laminar termination sites that are characteristic for each class. For example, small diameter afferents involved in thermoreception and nociception terminate in the dorsal-most laminae (I and II), larger diameter afferents involved in detection of nonnoxious stimuli terminate in deeper laminae (III and IV) in the dorsal horn, and the large group Ia muscle spindle afferents, which mediate the monosynaptic stretch reflex, terminate on motoneurons in the ventral spinal cord (Rexed, 1952; Eccles et al., 1957; Burke and Eklund, 1977; Christensen and Perl, 1970; Light and Perl, 1979). During development, these sensory axons reach the dorsal funiculus several days before sending collaterals into the spinal cord. In the rat, the larger diameter afferents then project to intermediate and ventral termination sites starting at embryonic days 14–15 (E14–E15), and small diameter afferents project to laminae I and II starting at E16 (Windle and Baxter, 1936; Smith, 1983; Altman and Bayer, 1984; Snider et al., 1992; Zhang et al., 1994; I. Silos-Santiago and W. Snider, personal communication).

The mechanisms that direct the projections of axons from neurons in DRG to different dorsoventral termination sites in the spinal cord are largely unknown. Permissive and attractive guidance cues are known to direct axonal projections in a variety of systems (reviewed by Goodman and Shatz, 1993; Tessier-Lavigne, 1994), and it is possible that different laminae are sources of attractive factors for distinct classes of axons. For example, neurotrophin 3 (NT-3) is expressed by motoneurons and has been proposed as a candidate for attracting Ia afferents to the ventral horn (discussed by Zhang et al., 1994).

In addition, axons can be guided by both short- and long-range inhibitory or repulsive cues (e.g., Kapfhammer and Raper, 1987; Walter et al., 1987; Bandtlow et al., 1990; Davies et al., 1990; Raper and Grunewald, 1990; Moorman and Hume, 1990; Pini, 1993; Nose et al., 1994; Matthes et al., 1995; Colamarino and Tessier-Lavigne, 1995). Previous studies have implicated inhibitory mechanisms in shaping axonal projections into the spinal cord. Peterson and Crain (1982) showed that axons from cultured DRG selectively failed to invade explants of ventral spinal cord in long-term culture. More recently, Fitzgerald et al. (1993) showed directly that the ventral spinal cord from E14–E15 rat embryos secretes a diffusible factor that has an inhibitory effect on the growth of axons from DRG at a distance in vitro. In both sets of experiments, the inhibitory effect of the ventral spinal cord was demonstrated on axons that grew out of DRG in response to nerve growth factor (NGF). These NGF-responsive axons correspond to the small diameter axons that terminate in superficial laminae (I and II) of the dorsal horn (Ruit et al., 1992; Mu et al., 1993; see below). The effect of the ventral spinal cord factor on other classes of axons that are not NGF responsive and that terminate more ventrally was not determined. It therefore remained unclear from this study whether the likely role of the factor was to delay ingrowth of all afferents into the spinal cord until the appropriate developmental stage or, alternatively, to prevent selectively the small diameter axons from ever approaching the

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ventral spinal cord. In addition, the molecular identity of the factor was not determined.

One family of molecules that comprises candidates for inhibitory guidance cues is the semaphorins (Kolodkin et al., 1992, 1993; Luo et al., 1993). The first member of the family, semaphorin I (Sema I, formerly fasciclin IV), a transmembrane semaphorin in insects, has been implicated in guiding pioneer axons in the grasshopper limb bud (Kolodkin et al., 1992). Other members of the semaphorin family were subsequently identified by sequence similarity in Drosophila melanogaster (D-Sema I and D-Sema II) and in human (H-Sema III). Independently, a member of this family, collapsin, was identified in chicken on the basis of its ability to cause collapse of growth cones from neurons in cultured DRG (Luo et al., 1993). Although there appear to be many distinct members of the semaphorin family in vertebrates (see Results), collapsin and H-Sema III appear to be species homologs. Unlike Sema I, Sema II and Sema III/collapsin lack transmembrane domains and are likely to be secreted and to act as diffusible factors. Recent evidence has shown that Sema II can function in vivo as a chemorepellent for motor axons in Drosophila (Matthes et al., 1995). The fact that Sema III/collapsin causes sensory growth cone collapse in vitro suggests that it too may function as a chemorepellent, in this case for vertebrate sensory axons.

To determine the role of Sema III/collapsin in directing the central projections of axons from neurons in DRG, we have focused on the development of these projections in rodents. We isolated a murine homolog of this molecule (hereafter referred to as Sema III), and examined its expression pattern in relation to primary sensory afferent projections in mouse and rat. High levels of *semaIII* mRNA expression were detected in the ventral spinal cord at appropriate developmental stages, and COS cells secreting Sema III were found to mimic the inhibitory effect of the ventral spinal cord on axons extending in response to NGF. These results suggest that Sema III contributes to mediating the long-range inhibitory effect described by Fitzgerald et al. (1993).

In addition, we have further extended these observations by examining whether Sema III has effects on other classes of neurons in DRG. For this, we took advantage of the fact that NT-3 but not NGF evokes outgrowth in vitro of muscle afferents that terminate in the ventral spinal cord (Hory-Lee et al., 1993; see also Davies, 1986; Hohn et al., 1990; and Discussion). In contrast to their effects on NGF-responsive axons, we have found that cells secreting Sema III do not affect the growth of axons from explants grown in NT-3. Together, our results suggest that Sema III secreted by ventral spinal cord cells contributes to patterning sensory projections in the spinal cord by creating an exclusion zone for axons that terminate dorsally, without preventing the ingrowth of axons that terminate ventrally.

Results

The Inhibitory Effect of Ventral Spinal Cord Explants Is Selective for NGF-Responsive Axons

The inhibitory effect of ventral spinal cord cells was previously demonstrated by culturing E14 rat lumbar DRG at a distance from explants of E14 rat ventral spinal cord in three-dimensional collagen gels in medium containing NGF. Outgrowth from the DRG was strongly inhibited on the side of the explants, but was unaffected by explants of dorsal spinal cord (Fitzgerald et al., 1993). NGF is expected to evoke axon outgrowth only from thermoreceptive and nociceptive neurons, which project to laminae I and II in the dorsal spinal cord (Ruit et al., 1992; Zhang et al., 1994; Snider, 1994). To determine whether the ventral spinal cord can inhibit outgrowth of sensory axons that project to other laminae, we focused on axons that are responsive to the neurotrophin NT-3. These include Ia muscle spindle afferents that project to the ventral spinal cord, and perhaps also some axons projecting to other laminae (see Discussion), but not laminae I and II.

We first sought to replicate the observations of Fitzgerald et al. (1993) by coculturing E14 rat DRG at distances of 150–950 μm from target explants of E14 rat dorsal or ventral spinal cord in collagen gels, in the presence of NGF. In agreement with these authors, we found that the pattern of outgrowth from DRG cultured alone or with dorsal spinal cord was radially symmetric (data not shown), whereas outgrowth from DRG cultured with ventral spinal cord explants was reduced on the side of the explant and directed away from it (Figure 1A). Quantification of the effect by comparing the extent of neurite outgrowth toward the ventral spinal cord explant (Figure 1C, proximal quadrant) to that away from the explant (Figure 1C, distal quadrant) showed that the inhibition was highly significant ($p < .0001$; Table 1).

Although in some cases the axons from DRG in the proximal quadrant appeared to contact some axons from the ventral spinal cord explant (e.g., Figure 1A), in most cases the inhibition was observed in the absence of such contact (data not shown), indicating, as previously described by Fitzgerald et al. (1993), that the effect is mediated by a diffusible factor secreted by ventral spinal cord cells. This is further supported by the observation that the inhibitory effect decreased roughly linearly with increasing distance between the DRG and the ventral spinal cord explants (Figure 2). This observation suggests that the ventral spinal cord secretes limiting amounts of the factor and that its concentration within the collagen matrix decreases with distance. A clear inhibitory effect was detected at considerable distances, as the extent of neurite outgrowth from the proximal side of each of the DRG was shorter than that from its distal side for all cultures in which the explants were separated by less than 800 μm (Figure 2; and data not shown). The in vivo distance at E14 between the ventral spinal cord and the dorsal funiculus (where the NGF-responsive axons are located) is about 200 μm.

We next examined the effects of ventral spinal cord explants on neurites extending from DRG cultured with NT-3. In all experiments, DRG were taken exclusively from brachial (forelimb) regions, which contain large numbers of NT-3-responsive neurons (DRG at nonlimb levels have a much smaller cohort of such neurons; Hory-Lee et al., 1993). In contrast to their effect on axons growing out of DRG in the presence of NGF, ventral spinal cord explants

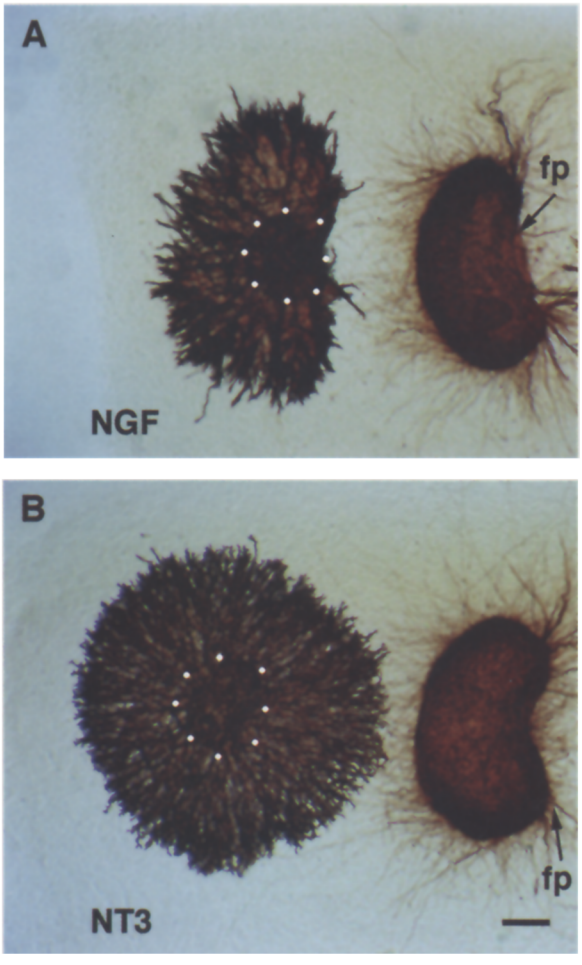

Figure 1. The Embryonic Ventral Spinal Cord Has a Long-Range Inhibitory Effect on NGF-Responsive but Not NT-3-Responsive Sensory Axons

(A and B) E14 rat DRG from brachial (forelimb) levels (outlined with dots) were cultured for 40 hr at a distance from ventral spinal cord explants (on right-hand side of figure) in collagen gels in medium containing 50 ng/ml NGF (A) or 50 ng/ml NT-3 (B). Ventral spinal cord explants were positioned with floor plate (fp) to the right. Cultures were stained with the anti-neurofilament antibody NF-M. Neurites growing from DRG cultured with NGF but not NT-3 were redirected away from the ventral spinal cord explant. Bar, 200 μm.

(C) Schematic representation of the method used to quantitate neurite outgrowth. The field surrounding each of the DRG was divided into four quadrants, and the distance from the explant to the neurite front was measured in the proximal and distal quadrants, as described in Experimental Procedures.

had no apparent effect on the radial profile of axons growing out of DRG in the presence of NT-3 (see Figure 1B; Table 1). Dorsal spinal cord explants also did not affect axons growing out in the presence of NT-3 (data not shown). Although no difference was observed in the lengths of neurites extending toward or away from the ventral spinal cord explants, in a few cocultures the density of neurites extending toward the explant appeared less than the density of those extending away (data not shown), raising the possibility that the NT-3-responsive population is heterogeneous and that the ventral spinal cord affects a subpopulation of these axons. In most cocultures no such difference was apparent. Thus, most if not all NT-3-responsive axons that grow out of DRG in these cultures appear to be unaffected by the inhibitory factor secreted by ventral spinal cord explants.

Sema III Is Expressed in the Ventral Spinal Cord during the Period of Sensory Axon Ingrowth

Because Sema III/collapsin can cause collapse of growth cones from neurons in DRG in vitro (Luo et al., 1993), we next examined whether it was expressed in the developing spinal cord in a manner consistent with a role in influencing sensory axon projections. To determine the expression of Sema III in the spinal cord of rodents, we isolated a cDNA comprising the murine Sema III coding sequence (except the first 318 bp; see Experimental Procedures). The predicted product is 95% and 90% identical to H-Sema III and chick collapsin sequences, respectively (see Experimental Procedures). Figure 3 shows the alignment of portions of the ~500 amino acid semaphorin domains (Kolodkin et al., 1993) of mouse and human Sema III. In addition, a search of the dbEST database (see Experimental Procedures) identified 11 human expressed sequence tags that appear to code for at least four (including H-Sema III) and as many as nine human proteins with semaphorin domains. The relation of these sequences to Sema III and previously identified insect semaphorins is also shown in Figure 3.

In an initial set of experiments, we used a coding region probe to examine the expression of *semaIII* mRNA by in situ hybridization in transverse sections of spinal cord from mouse embryos. Expression was detected very transiently in the roof plate region of the neural tube at early stages of neural tube formation (E9.5 in the mouse), but was then not detectable in the spinal cord until E11.5. From that time on, transcripts were detected in the ventral spinal cord until E18, the latest time examined (data not shown). The level of expression was high between E12.5 and E14.5 and decreased steadily thereafter (data not shown).

Because the timing of sensory afferent ingrowth has been studied in most detail in the rat, we also examined the expression of *semaIII* mRNA in rat embryos. Similar expression patterns were observed in rat and mouse embryos of comparable developmental stages. At E14 in the rat (which corresponds to E12.5 in the mouse), a high level of expression was detected in the ventral spinal cord,

Table 1. Neurite Outgrowth from DRG Cocultured with E14 Ventral Spinal Cord Explants or with COS Cell Aggregates

Culture Condition	Mean Outgrowth ± SD (μm)		Number of Explants per Experimental Condition	Two Sample t Test Assuming Unequal Variance (p value)
	Proximal Quadrant	Distal Quadrant		
With ventral spinal cord				
NGF 50 ng/ml	179 ± 125	360 ± 75	47	<.0001
NT-3 50 ng/ml	375 ± 112	411 ± 117	50	NS
With control COS cells				
NGF 50 ng/ml	324 ± 70	317 ± 63	21	NS
NT-3 50 ng/ml	442 ± 81	450 ± 86	22	NS
With COS cells secreting Sema III				
NGF 50 ng/ml	26 ± 36	303 ± 102	24	<.0001
NT-3 50 ng/ml	413 ± 81	457 ± 91	24	NS

NS, not significant ($p > .05$).

excluding the floor plate region (Figures 4A and 4B). The expression was not uniform, being highest in the ventricular zone and next highest in the developing motor columns; expression also appeared to extend into the dorsal horn for a short distance (Figure 4A). A similar pattern was observed at all axial levels of the spinal cord examined at this stage (data not shown). The level of expression subsequently decreased without change in the pattern of expression; significant though lower expression was still detected at E18 (Figures 4C and 4D), when muscle afferents have reached the ventral horn and after NGF-responsive axons have started to invade laminae I and II (see Discussion).

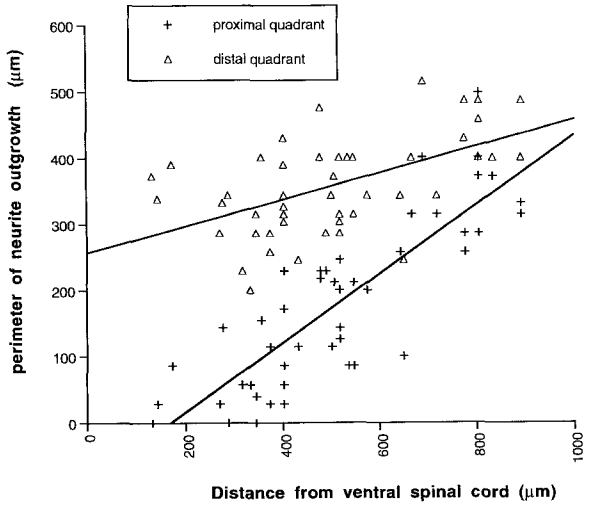

Figure 2. The Degree of Inhibition of NGF-Responsive Neurites by Ventral Spinal Cord Explants Decreases with Distance

DRG were cultured with ventral spinal cord explants in medium containing 50 ng/ml NGF as shown in Figure 1A. Length of outgrowth in the proximal quadrant (crosses) and the distal quadrant (triangles) (quantified as shown in Figure 1C) was plotted as a function of the distance between the proximal edges of the ventral spinal cord explant and the DRG. A strong correlation was observed between the distance separating the explants and the length of the outgrowth from the proximal quadrant (line fitted by linear regression; $r = 0.84$). Only a very modest correlation was found for the distance separating the explants and the length of the outgrowth in the distal (control) quadrant ($r = 0.54$).

Interestingly, Fitzgerald et al. (1993) reported that the ventral spinal cord inhibitory activity was still present but at lower levels in E18 embryos. Thus, the pattern and level of *semaIII* mRNA expression are consistent with Sema III mediating the inhibitory activity.

COS Cells Secreting Sema III Selectively Repel NGF-Responsive Axons

Previous studies have established that collapsin/Sema III causes collapse of axons from DRG when applied acutely in culture (Luo et al., 1993), but they have not determined its effects on axons from DRG in chronic cocultures like those with ventral spinal cord (see Figure 1A), nor have they determined which classes of neurons in DRG are affected. To test directly whether Sema III can contribute to the inhibitory effects of the ventral spinal cord, we cultured DRG together with aggregates of COS cells that had been transfected with a H-Sema III expression construct, and which were shown by immunoblotting to secrete Sema III protein (see Experimental Procedures).

We first examined the effect of cells secreting Sema III on NGF-responsive axons. When DRG were cultured in collagen gels in medium containing NGF, COS cells secreting Sema III dramatically reduced the amount of outgrowth on the proximal side of the DRG (i.e., in the proximal quadrant) without effect on the axons in the distal quadrant (Figure 5B; Table 1). Control COS cells had no apparent effect on the axons (Figure 5A; Table 1). Contrary to what was observed with ventral spinal cord, the degree of inhibition by COS cells secreting Sema III was similar regardless of the distance separating the cells from the explants (in the range 200–950 μm; data not shown), suggesting that the amount of Sema III that reaches the DRG in these experiments is far above threshold for the inhibitory effect.

These results suggest that Sema III contributes to the inhibitory effect of ventral spinal cord. To attempt to correlate further the sites of expression of Sema III with the ventral spinal cord inhibitory activity, we took advantage of the observation that *semaIII* transcripts were not detected in floor plate cells (see Figure 4). Microdissected floor plate from E14 embryos had no effect on NGF-responsive axons (data not shown; similar results have

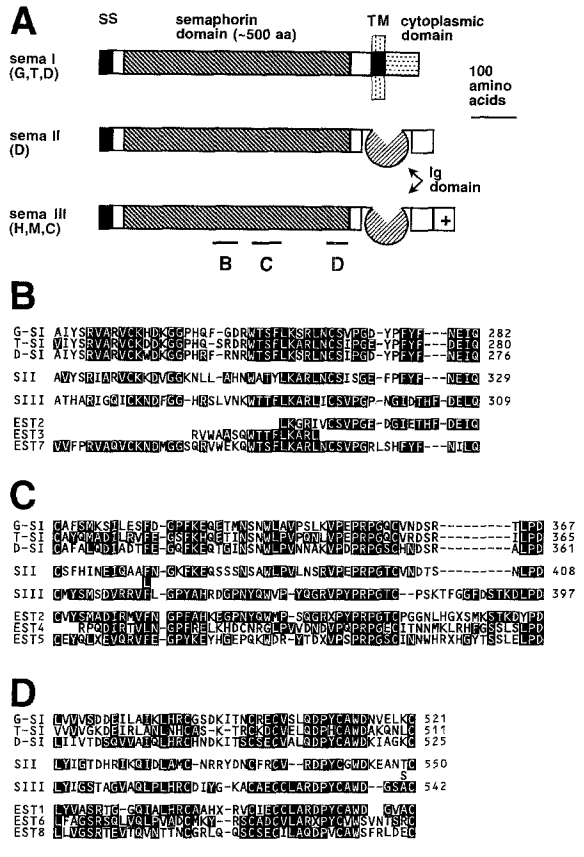

Figure 3. Comparison of the Murine Sema III Sequence with the Sequences of Other Insect and Vertebrate Semaphorins

(A) Diagram of structures of Sema I (a transmembrane semaphorin identified in three insect species: grasshopper [G], Tribolium [T], and Drosophila [D]), Sema II (a secreted semaphorin identified in Drosophila), and Sema III (a secreted semaphorin previously identified in human and chick, and reported here in mouse). All proteins have a signal sequence (SS; closed areas) and share an ~500 amino acid (aa) conserved semaphorin domain (hatched areas). The secreted proteins have a single immunoglobulin domain (Ig). In addition, Sema III has a basic domain at its extreme carboxyl terminus (+). The regions of the semaphorin domain whose sequences are compared in (B), (C), and (D) are labeled.

(B–D) Comparison of the amino acid sequences of the three insect Sema I proteins (G-SI, T-SI, and D-SI), the Drosophila Sema II (SII) protein, the human Sema III protein (SIII), and sequences encoded in 8 human expressed sequence tags (EST1–EST8) from the dbEST database (see Experimental Procedures). Where the murine Sema III sequence diverges from the human, the residue in the murine sequence is shown immediately above the corresponding residue in the human sequence. Some of the EST sequences contain probable frame shifts and were adjusted accordingly. The EST sequences appear to code for at least three and as many as eight distinct human members of the semaphorin family, in addition to Sema III. Boxed letters are all residues found in the ESTs that are identical to the corresponding residue in at least one of the previously known semaphorin proteins. Note that the EST sequences appear to fall into different subclasses. EST7 is most closely related to the three insect Sema I sequences. EST1–EST5 are most closely related to Sema III. EST6 and EST8 are most closely related to each other and show hallmarks of both Sema I and Sema III. The sequence shown for each EST is only a portion of the sequence available in the database. In addition to the eight ESTs shown here, dbEST also contains a portion of the Sema III sequence. Accession numbers: EST1, T48905 and T49107; EST2, Z21993; EST3, Z28925; EST4, Z45329; EST5, Z25335; EST6, T08621; EST7, T64205; EST8, T09073.

been obtained by G. Kwiat and M. Fitzgerald [1993, Soc. Neurosci., abstract]), indicating that the inhibitory factor is found only in the portion of the ventral spinal cord that expresses *semaIII*.

In contrast to their effect on NGF-responsive axons, COS cells secreting Sema III had no apparent effect on the outgrowth of NT-3-responsive axons from DRG (Figure 5D; Table 1). The density of axons projecting toward the COS cells also appeared in all cases to be unaffected (data not shown). The absence of an inhibitory effect of the COS cells on the NT-3-responsive axons was further suggested by the observation that axons that reached the COS cells could actually invade the aggregates (e.g., see Figure 5D). Control COS cells, like *semaIII*–expressing cells, had no effect on NT-3-responsive axons in this assay (Figure 5C; Table 1). Thus, Sema III functions as a selective diffusible repellent of NGF-responsive axons without apparent effect on NT-3-responsive axons.

Discussion

When the axons of different classes of primary sensory neurons enter the spinal cord during development, they project ventrally from the dorsal funiculus to different laminar termination sites along the dorsoventral axis that are appropriate for their specific modalities. Such a pattern of specific projections might in principle be controlled by differential attractive signals, repulsive signals, or a balance of both. Little is known, however, about the identity and function of the guidance molecules that direct this patterning. Here we provide evidence that one of these molecules is Sema III/collapsin. We show that Sema III is a selective chemorepellent of a specific set of sensory axons that terminate dorsally and suggest that it functions in vivo to help prevent those axons from projecting ventrally (Figure 6).

Sema III Is a Ventral Spinal Cord-Derived Repellent for NGF-Responsive Sensory Axons

Fitzgerald et al. (1993) showed that ventral spinal cord explants from E14 rats secrete a diffusible factor that has an inhibitory action on sensory axons that grow out in response to NGF. Our results implicate Sema III in mediating this effect. We found that *semaIII* transcripts are present in the ventral spinal cord at E14 in rats and that COS cells secreting Sema III can mimic the inhibitory effect of ventral spinal cord explants. The detailed spatial and temporal pattern of *semaIII* mRNA expression is consistent with Sema III being the inhibitor. Within the ventral spinal cord, neither the inhibitory activity nor *semaIII* transcripts are expressed by floor plate cells. Moreover, the level of *semaIII* transcripts expressed by ventral spinal cord cells decreases by E18, consistent with the decrease in inhibitory activity at that age reported by Fitzgerald et al. (1993). Thus, Sema III is likely to mediate in whole or in part the activity in ventral spinal cord explants. In our assay, COS cells secreting Sema III were more effective than ventral spinal cord explants; we presume that this simply reflects a higher level of secretion of Sema III by the COS cells.

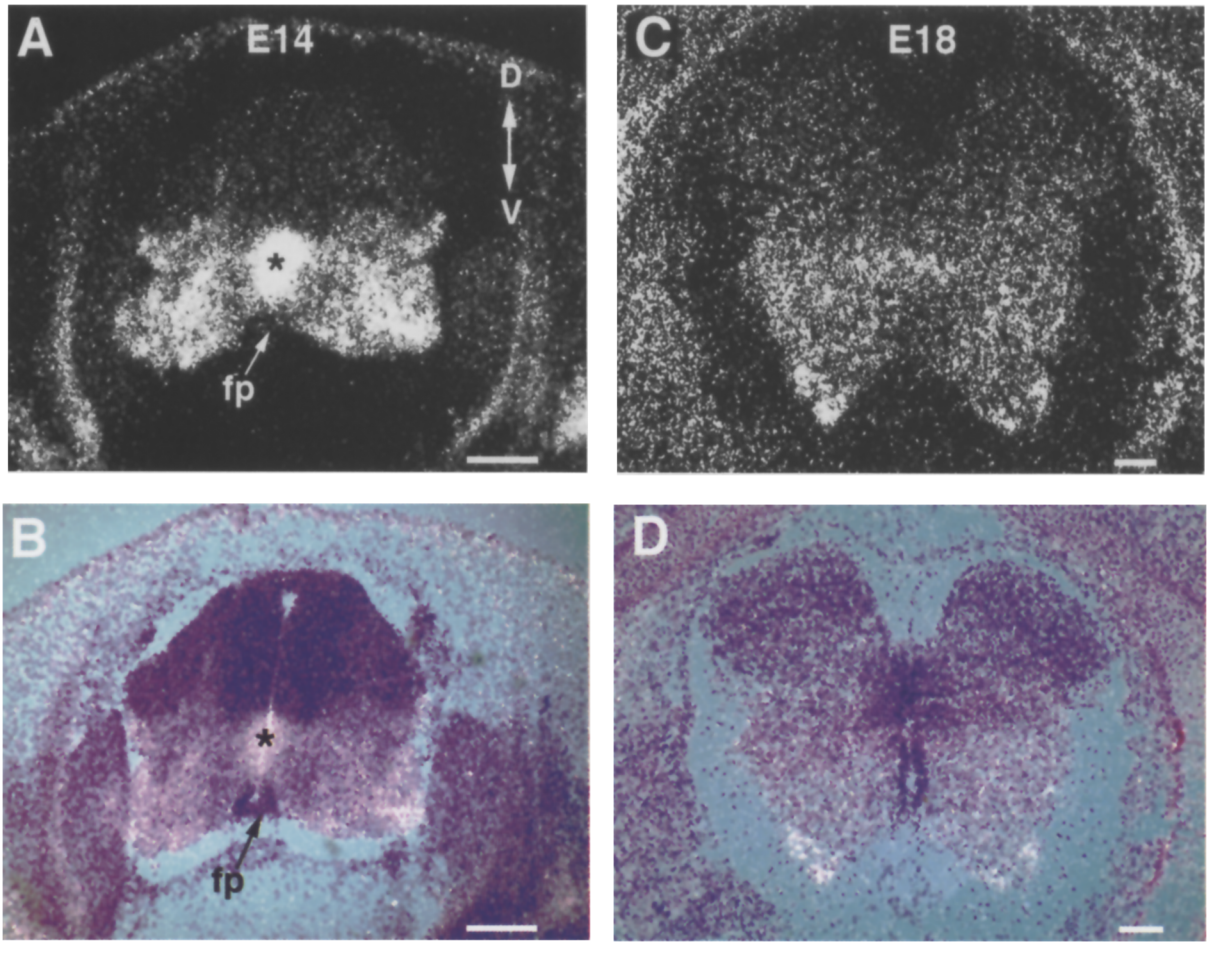

Figure 4. Expression of *semaIII* mRNA in the Developing Rat Spinal Cord

(A and B) Dark-field (A) and bright-field (B) views of a transverse section through the spinal cord of an E14 rat embryo hybridized with an antisense *semaIII* riboprobe, showing high levels of *semaIII* transcripts in the ventral half of the spinal cord. Asterisks indicate intense hybridization in the ventricular zone. No expression was detected in the floor plate (fp). Hybridization was also observed in a stripe of cells just lateral to the DRG. Hybridization was not observed with the corresponding sense probe, except in the surface ectoderm; counts of silver grains overlying the surface ectoderm at E18 failed to reveal any difference in the intensity of labeling between sense and antisense, suggesting that the apparent ectodermal hybridization is artifactual (data not shown). Double-headed arrow indicates section orientation (D, dorsal; V, ventral).

(C and D) Dark-field (C) and bright-field (D) views of a transverse section through the spinal cord of an E18 rat embryo processed in the same way as the section shown in (A). The pattern of expression of *semaIII* transcripts is similar to that observed at E14, but the level of expression is lower.

Bars, 100 μm.

The factor secreted by ventral spinal cord explants was previously described as inhibitory, but our results appear more consistent with this factor (and Sema III) being a chemorepellent rather than an outright blocker of outgrowth of NGF-responsive axons. Extensive outgrowth was observed on the side of the DRG facing ventral spinal cord explants or COS cells secreting Sema III, but these axons extended parallel to the target, appearing to have turned away from it (see Figure 1A and Figure 5B). This effect was particularly pronounced with COS cells secreting Sema III, which could prevent axons from projecting toward them at a distance of over 900 μm, but which did not appear to have affected the length of axons that were growing parallel to the target even when as close as 200–400 μm (see Figure 5B; and data not shown). Thus, a source of Sema III does not block the growth of axons in its vicinity, provided the axons are growing away from the source.

The cellular events involved in repulsion of these axons by Sema III have not been determined. Collapsin (the Sema III homolog in chick) causes rapid collapse of growth cones of axons from chick DRG when added acutely to these axons (Luo et al., 1993); the recombinant H-Sema III used in our studies has similar effects (unpublished data). However, when growth cones are exposed to a localized but nondiffusible source of collapsing signal (in the form of chick brain extract enriched in collapsin that is immobilized on beads), these growth cones often turn away from the source without collapsing following filopodial contact with the source (Fan and Raper, 1995). It is

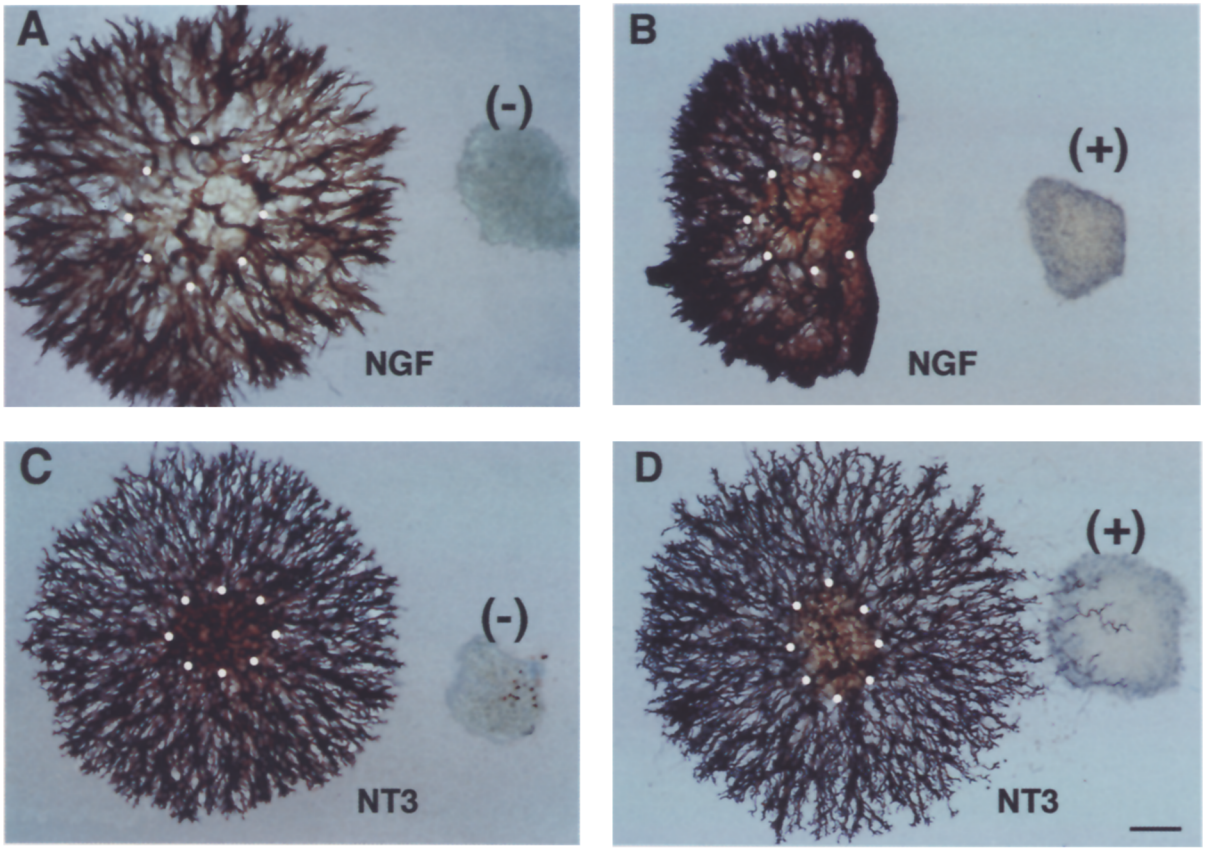

Figure 5. COS Cells Secreting Sema III Inhibit NGF-Responsive but Not NT-3-Responsive Sensory Axons

E14 rat DRG from brachial (forelimb) levels (outlined with dots) were cultured for 40 hr at a distance from aggregates of control COS cells (−) (A and C) or COS cells secreting recombinant H-Sema III (+) (B and D) in medium containing 50 ng/ml NGF (A and B) or 50 ng/ml NT-3 (C and D). Cultures were stained with the anti-neurofilament antibody NF-M. Outgrowth from DRG cultured with NGF but not NT-3 is repelled by COS cells secreting Sema III. Bar, 200 μm.

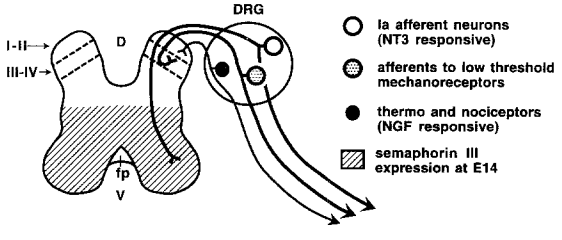

Figure 6. Trajectory of NGF-Responsive Axons and NT-3-Responsive Ia Afferents in Relation to *semaIII* Expression in the Embryonic Rat Spinal Cord

Group Ia afferents (NT-3-responsive; open circle) enter the spinal cord from the medial aspect of the dorsal funiculus starting around E14. NT-3-responsive axons also include large diameter afferents that relay information to the cerebellum via Clarke's column (which is located at intermediate levels along the dorsoventral axis in the thoracic and high lumbar spinal cord) or the lateral cuneate nucleus in the medulla (not shown; see text). Axons from NGF-responsive neurons (closed circle) project into the spinal cord starting around E16 and target the dorsal-most laminae I and II. *semaIII* transcripts are detected in the ventral spinal cord during this period. It is proposed that Sema III protein emanating from the ventral spinal cord contributes to targeting the NGF-responsive axons to laminae I and II. An additional class of afferents is those from low threshold mechanoreceptors (stippled circle), which enter the spinal cord medially, overshoot their targets, and then turn upward to terminate in laminae III and IV. The neurotrophin dependence of these cells is uncertain; our experiments do not address the role, if any, of Sema III in directing their projections (diagram adapted from Snider, 1994).

possible that growth cone collapse occurs only when many filopodia are exposed simultaneously and rapidly to a step change in concentration of the factor, such as is achieved in the collapse assay. Whether collapse occurred in our experiments where axons were chronically exposed to a presumed gradient of the factor and, indeed, whether collapse in response to Sema III occurs in vivo remain to be determined.

A Role for Sema III in Patterning Sensory Axon Projections in the Spinal Cord

Distinct classes of sensory axons project to different termination sites in the spinal cord, and our results indicate that Sema III affects only a subset of these axons. In the rat, the first axons project from DRG to the developing dorsal funiculus 2 days before entering the gray matter at E14–E15 (Windle and Baxter, 1936; Altman and Bayer, 1984). A similar waiting period is observed in other species (e.g., Knyihar et al., 1978; Lee et al., 1988; Mendelson et

al., 1992). The axons that enter into the spinal cord at this time include the Ia afferents that project to the ventral horn (Kudo and Yamada, 1987; Ziskind-Conhaim, 1990). This early projecting population also likely includes the afferents from the low threshold mechanoreceptors that terminate in intermediate regions of the dorsal horn in laminae III and IV (Kudo and Yamada, 1987; Snider et al., 1992; Zhang et al., 1994). Small diameter afferents involved in pain and temperature perception, which terminate in the dorsal-most laminae (I and II), only enter the gray matter later, starting at E16 (Snider et al., 1992; Zhang et al., 1994; I. Silos-Santiago, personal communication). These small diameter neurons have later birthdates than the larger ones (Altman and Bayer, 1984), which may in part explain their delayed entry into the spinal cord.

We have found that Sema III repels all NGF-responsive axons in our assay without apparent effect on NT-3-responsive axons. The NGF-responsive axons studied here are almost certainly the small diameter afferents that terminate in laminae I and II (Ruit et al., 1992; Zhang et al., 1994). The NT-3-responsive axons are likely to include the Ia afferents that project to the ventral spinal cord (Hory-Lee et al., 1993). These axons also likely include other large diameter afferents that relay information to the cerebellum (reviewed in Snider, 1994). At lumbar and thoracic regions, these afferents send collaterals to a specialized nucleus in the thoracic spinal cord called Clarke's column, positioned roughly midway along the dorsoventral axis. At the brachial levels used in our studies, these afferents project to a nucleus in the brainstem (the lateral cuneate nucleus), not the spinal cord. Interestingly, although Sema III never appeared to affect the NT-3-responsive axons, ventral spinal cord explants occasionally caused an apparent reduction in the density of this axonal population on the side of the explants, without any effect on the length of those present (see Results). One possibility is that an inhibitory factor distinct from Sema III is produced by the ventral spinal cord and affects a subpopulation of NT-3-responsive axons, perhaps those normally destined for Clarke's column or the lateral cuneate nucleus. Finally, our studies have not addressed whether Sema III plays a role in directing the projections of afferents from the low threshold mechanoreceptors, which terminate in laminae III and IV. Culture conditions that can selectively elicit the outgrowth of these axons have not been determined, and molecular genetic studies suggest that they may even be neurotrophin independent at this stage of development (reviewed in Snider 1994; see also Zhang et al., 1994). It is therefore unclear from our experiments whether Sema III affects this class of axons.

Our results suggest that Sema III secreted by ventral spinal cord cells patterns sensory projections by restricting the ventral growth of NGF-responsive axons that terminate in laminae I and II, without effect on axons that terminate in the ventral spinal cord. However, at least three different models for the role of Sema III are compatible with our data (see also Fitzgerald et al., 1993). First, if Sema III diffuses throughout the dorsal spinal cord, it may function as a waiting cue, preventing the ingrowth of NGF-responsive axons into laminae I and II until E16–E17. The down-regulation in expression of *semaIII* mRNA (and presumably also Sema III protein) would therefore be key in allowing these axons to enter. Second, Sema III protein may not normally reach the NGF-responsive axons in laminae I and II and may only be present more ventrally, to help redirect any of these axons that have mistakenly wandered beyond those laminae. Third, Sema III may be present in a gradient throughout the dorsal spinal cord that does not actually block ingrowth into the gray matter but does inhibit the progression of the NGF-responsive axons further ventral than laminae I and II. In this third model, Sema III is responsible for making the axons stop in laminae I and II, whereas in the first two, a separate cue is required for that purpose. Zhang et al. (1994) recently showed that injections of NGF in utero around E14–E16 cause the axons that normally terminate in laminae I and II to extend further into the dorsal horn. The axons could reach and even cross the midline, but they never projected into the ventral spinal cord. These results appear to favor the first two models over the third. Similar conclusions were recently drawn by Sharma et al. (1994) from studies of sensory projections into spinal cord explants in vitro.

Whatever the precise role of Sema III, several features of the projections of these axons indicate that other cues must also function with Sema III to guide them. The axons that project to the ventral spinal cord or laminae III and IV enter the spinal cord medially, while axons that project to laminae I and II enter laterally; the afferents from the low threshold mechanoreceptors initially overshoot laminae III and IV, then turn upward in a characteristic "flame-shaped appearance" (Ramón y Cajal, 1909; Scheibel and Scheibel, 1968). Specific cues must exist that constrain axons to these trajectories. It is also possible that the ventral spinal cord is the source of chemoattractant for the Ia afferents (discussed in Zhang et al., 1994).

Semaphorins: A Growing Family of Axon Guidance Molecules

Sema III/collapsin (Luo et al., 1993; Kolodkin et al., 1993) is a member of the semaphorin family, which comprises cell surface and secreted growth cone guidance molecules that are conserved from insects to humans (Kolodkin et al., 1993). Semaphorins are ~750 amino acids in length (including signal sequence) and are defined by a conserved ~500 amino acid extracellular semaphorin domain containing 14–16 cysteines, many blocks of conserved residues, and no obvious repeats (Kolodkin et al., 1993). The transmembrane semaphorins have an additional ~80 amino acid stretch, a transmembrane domain, and an 80–110 amino acid cytoplasmic domain. The secreted semaphorins have an additional ~20 amino acid stretch, a single immunoglobulin domain, and a 70–120 amino acid carboxy-terminal region. A search of the dbEST database has revealed the existence of a large family of semaphorins in humans (see Figure 3). In addition, two semaphorins are encoded in viral genomes (Kolodkin et al., 1993).

All available evidence thus far suggests that different members of the semaphorin family function as either cell surface or secreted chemorepellents or inhibitors of axon pathfinding, branching, or targeting. Although there are

similarities in how they function, there are also some interesting differences, and these differences may reflect either real differences in function or differences in the assays used (Kolodkin et al., 1992; Luo et al, 1993; Fan and Raper, 1995; Matthes et al., 1995; and results presented here).

The first identified member of the family was Sema I (formerly fasciclin IV; Kolodkin et al., 1992), a transmembrane Semaphorin in insects. Antibody-blocking experiments in the grasshopper limb bud have shown that expression of Sema I by a stripe of epithelial cells prevents Ti1 axons that encounter it from defasciculating and branching. However, Sema I is not an absolute inhibitor of growth, since the Ti1 growth cones grow on the Sema I–expressing cells (see Discussion in Kolodkin et al., 1993).

Sema II, a secreted semaphorin in Drosophila (Kolodkin et al., 1993), is transiently expressed in the embryo by a subset of neurons in the central nervous system and by a single large muscle during motoneuron outgrowth and synapse formation. To test the in vivo function of Sema II, transgenic Drosophila were created that generate ectopic Sema II expression by muscles that normally do not express it. The results show that in some cases Sema II can function to repel axons. In the case of a single identified motoneuron (RP3), however, Sema II inhibits the formation of its synaptic terminal arborizations without actually repelling it (Matthes et al., 1995).

Thus, in the developing organism, semaphorins appear capable of inhibiting branching (Sema I in grasshopper), influencing steering decisions (Sema I in grasshopper and Sema III in mammals), preventing axons from entering certain target regions (Sema II in Drosophila and perhaps Sema III in mammals), and inhibiting the formation of synaptic terminal arborizations (Sema II in Drosophila). The full range of effects of different or even individual semaphorin family members on developing axons remains to be determined. In particular, given the evidence that some guidance cues can have both repulsive and attractive effects (discussed in Goodman, 1994; Colamarino and Tessier-Lavigne, 1995), it will be important to determine whether any semaphorins function as attractants. Nothing is yet known about the identity of the receptors for semaphorin family members.

Emerging Diversity of Repulsive Guidance Molecules

It is not yet known how many semaphorins are encoded in the genome of any one species: there are at least two in Drosophila (Kolodkin et al., 1993) and four in human (Kolodkin et al., 1993; also see expressed sequence tag [EST] analysis in Figure 3), but this is probably an incomplete representation of the total number in either genome. Moreover, the semaphorins represent just one family of repulsive or inhibitory guidance molecules. A number of other molecules have been identified that can function in a repulsive or inhibitory fashion, including the cell surface proteins connectin (Nose et al., 1994) and myelin-associated glycoprotein (MAG; McKerracher et al., 1994; Mukhopadhyay et al., 1994), the diffusible protein netrin-1 (Colamarino and Tessier-Lavigne, 1995), the related extracellular matrix proteins tenascin and janusin/restrictin/J1-160/180 (Faissner and Kruse, 1990; Pesheva et al., 1993), and proteoglycans (see Snow et al., 1990). What is emerging is a picture of a great diversity of molecules that can repel or inhibit various aspects of axon growth, pathfinding, and targeting. The literature contains numerous cases of repulsion (e.g., Kapfhammer and Raper, 1987; Walter et al., 1987; Bandtlow et al., 1990; Davies et al., 1990; Raper and Grunewald, 1990; Moorman and Hume, 1990; Pini, 1993). It remains to be determined which of these events are mediated by members of known families of repellents or inhibitors, and whether other as yet unknown families also contribute to inhibitory and repellent interactions in the developing nervous system.

Experimental Procedures

Isolation and Sequence Analysis of Mouse *semaIII*

DNA cloning techniques were performed as described by Sambrook et al. (1989) unless otherwise stated. Mouse *semaIII* sequences were isolated by PCR, using as a template cDNA made to poly(A)$^+$ RNA isolated from whole mouse E14 brain. First, oligonucleotides (LSTH5 and LSTH3; see below) derived from the human *semaIII* (*H-semaIII*) sequence (Kolodkin et al., 1993) were used to amplify a 1300 bp fragment. This fragment was cloned into the SmaI site of pBluescript (Stratagene) generating pMSemaIIIA (which was used for RNA in situ analysis). An oligonucleotide (M3A) derived from the sequence of this 1300 bp fragment was used in conjunction with an oligonucleotide (M4B) derived from a more 3′ portion of the *H-semaIII* coding sequence (see below). This reaction generated a 970 bp fragment, which was directly cloned into pCR II (Invitrogen). Both cloned PCR fragments were sequenced on both strands using an ALF DNA Sequencer and Autoread reagents (Pharmacia). The composite *semaIII* sequence derived from these two PCR fragments encodes a protein sequence that is 666 amino acids in length and 95% identical at the amino acid level to H-Sema III, starting from amino acid 106 of H-Sema III to the end of the coding sequence. The *semaIII* sequence is incomplete at the 5′ end, missing ~59 amino acids at the start of the semaphorin domain and ~47 amino acids at the N-terminus of the protein. The high degree of similarity between available *semaIII* and *H-semaIII* coding sequences identifies the encoded protein Sema III as a homolog of H-Sema III and chick collapsin (Luo et al., 1993). The sequence of *semaIII* can be obtained via GenBank accession #L40484. Sequences described in Figure 3 were obtained by searching the dbEST database (Boguski et al., 1993; Adams et al., 1991) with previously identified semaphorin sequences. The PCR cycling conditions were: 35 cycles of 96°C, 30 s; 55°C, 1 min; 72°C, 3 min. Oligonucleotide sequences were: LSTH5-GGAAAAGACATCCCTGAAAGAA (corresponding to the *H-semaIII* DNA sequence encoding amino acids 107–113); LSTH3-CTGGGAATGACACGAACCCTA (encoding H-Sema III amino acids 532–538); M3A-ATGTTCATCGGAACAGAT (encoding Sema III amino acids MFIGTDV); and M4B-GACACTCCTGGGTGCCCTCTCAAA (encoding H-Sema III amino acids 764–771).

Production of Recombinant H-Sema III

A COS cell expression vector pCOS(LB)-BMN-myc was generated by modifying pMLP (which contains an SV40 ori, an E1A enhancer/MLP/tripartite leader, polylinker sequences, and DHFR and SV40 poly(A)$^+$ sequences; a gift from Lisa Brunet), through the addition in the polylinker of a sequence encoding a peptide from c-myc (EEQKLISEEDLLRKRREQLKHKLE) recognized by monoclonal antibody 9E10 (Evan et al., 1985). Three unique restriction sites were also inserted upstream of the c-myc epitope sequence to allow open reading frames to be cloned in-frame 5′ to the c-myc epitope. Primers derived from the *H-semaIII* sequence were used to amplify the *H-semaIII* open reading frame by PCR using Vent Polymerase (New England Biolabs), and the resulting amplification product was cloned into pCOS(LB)-BMN-myc to yield pCOS(*H-semaIII-myc*). COS cells were transfected with pCOS (*H-semaIII-myc*) using LipofectAMINE (GIBCO BRL). COS cell aggregates were prepared by the hanging drop method as previously described (Kennedy et al., 1994). Western analysis using 9E10 demon-

strated the presence of secreted myc-tagged *H-semaIII* in the medium conditioned by transfected COS cells.

RNA In Situ Hybridization

Rat embryos (E14) were obtained from timed pregnant rats (vaginal plug equivalent to E0; Simonsen Laboratories). Embryos were removed into L15 medium, embedded in OCT compound (Tissue Tek), flash-frozen in liquid nitrogen, and stored at −80°C. Cryostat sections (25 μm) were collected on Superfrost Plus slides (Fisher). Sense and antisense ^{35}S-labeled riboprobes corresponding to the 1300 bp coding region sequence of *semaIII* were generated with T3 and T7 RNA polymerase using pMSemaIIIA. Probes were base-hydrolyzed to an average size of 200 bp. For prehybridization, slides were brought to room temperature and fixed in 4% paraformaldehyde in 0.1 M PBS for 20 min, rinsed four times (5 min each) in PBS, and rinsed once in diethyl pyrocarbonate (DEPC)–treated water prior to dehydration through a graded ethanol series (50%, 80%, and 95%). Sections were digested with 1 mg/ml proteinase K for 20 min, refixed in 4% paraformaldehyde/0.1 M PBS, washed in PBS, washed in DEPC-treated water, dehydrated in ethanol, treated with 0.1 M tetraethylammonium and acetic anhydride, washed in DEPC-treated water, and dehydrated with ethanol. Sections were incubated with hybridization buffer containing 1 × 10^6 cpm/section of either sense or antisense riboprobe, incubated overnight at 55°C in a humidified chamber, washed twice (30 min each) in 2× SSC with 10 mM β-mercaptoethanol at room temperature, treated with 50 μg/ml RNase A in 2× SSC, 1 mM EDTA for 40 min, washed twice with 50% formamide in 2× SSC, 1 mM EDTA, 10 mM β-mercaptoethanol at 55°C (1 hr each), and washed in 0.2× SSC at 55°C and then with 0.1× SSC for 30 min at 55°C. Slides were dehydrated through an ethanol series, exposed to X-ray film, and processed for autoradiography as described (Luskin and Shatz, 1985).

Explant Cocultures

Spinal cord tissue and DRG from the brachial region were dissected from rat embryos (E14) obtained from timed pregnant rats and were embedded in collagen gels as described (Tessier-Lavigne et al., 1988). Cocultures consisted of DRG with either spinal cord (dorsal or ventral), floor plate, or COS cells (mock-transfected or transfected with pCOS(*H-semaIII-myc*)). Explants of the ventral spinal cord containing the floor plate were oriented with the floor plate side pointing away from the DRG, thus exposing the dorsal edge of the explants to the DRG. Explants were cultured for 36 hr in F12/N3 medium as described (Tessier-Lavigne et al., 1988), except that the medium contained only 0.5% heat-inactivated horse serum and was supplemented with either 50 ng/ml 2.5S NGF (Bioproducts for Science Inc.) or 50 ng/ml NT-3 (a generous gift from David Shelton, Genentech, Inc.).

Immunohistochemistry

After culture, explants were fixed with 4% paraformaldehyde in PBS for ~2 hr at room temperature. Immunostaining with a neurofilament-specific antibody (NF-M; 1:1000; Lee et al., 1987) and a horseradish peroxidase–conjugated secondary antibody (Boehringer-Mannheim; 1:400) was performed essentially as described (Kennedy et al., 1994), except that antibody incubations and washes were in PBS, 1% heat-inactivated goat serum, 0.1% Triton X-100. Explants were mounted in Aquamount (Lerner Laboratories) for analysis of neurite outgrowth.

Quantification of Neurite Outgrowth

DRG cultured in the presence of NGF or NT-3 displayed a radial pattern of neurite outgrowth. For quantitation, the region of neurite growth was divided into four quadrants, as diagrammed in Figure 1C. Neurite outgrowth into the collagen gel was measured from the outer border of each of the DRG to the outer perimeter of the bulk of neurofilament-stained neurites (thus measuring the perimeter at its region of highest density of staining). In instances where the perimeter was not entirely symmetric within a quadrant, the perimeter border was defined as the distance between the minimal and maximal radii. Statistical analysis was performed using Microsoft Excel 4.0.

Acknowledgments

Correspondence should be addressed to M. T.-L. We thank Sophia Colamarino for critical comments on the manuscript, Virginia Lee for the generous gift of NF-M, David Shelton for the generous gift of NT-3, and Jay Rehm, Krystyna Kilomanski, Caroline Esmurdoc, and Larry Nelson for technical assistance. We thank William Snider, Doug Wright, and their colleagues for communication of results prior to publication. E. D. L. was supported by NIH grant GM07618 through the UCSF MSTP program. E. K. M. was supported by the March of Dimes Birth Defects Foundation (to C. J. S.) and by Fight for Sight National Society to Prevent Blindness Postdoctoral Fellowship (PD93007). M. T.-L. was supported by the Lucille P. Markey Charitable Trust. A. L. K. was supported by NIH grant HD21294 (to C. S. G.). C. J. S. and C. S. G. are Investigators and M. T.-L. is an Assistant Investigator with the Howard Hughes Medical Institute.

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Received March 17, 1995; revised March 24, 1995.

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GenBank Accession Number

The accession number for the mouse Sema III reported in this paper is L40484.