

Neuropilin-2 Is a Receptor for Semaphorin IV: Insight into the Structural Basis of Receptor Function and Specificity

Roman J. Giger,[†] Erica Rowe Urquhart,[†]
Susan K. H. Gillespie, Dorothy V. Levengood,
David D. Ginty,^{*} and Alex L. Kolodkin^{*}
Department of Neuroscience
The Johns Hopkins University
School of Medicine
Baltimore, Maryland 21205-2185

Summary

Neuropilins bind secreted members of the semaphorin family of proteins. Neuropilin-1 is a receptor for Sema III. Here, we show that neuropilin-2 is a receptor for the secreted semaphorin Sema IV and acts selectively to mediate repulsive guidance events in discrete populations of neurons. *neuropilin-2* and *semaIV* are expressed in strikingly complementary patterns during neurodevelopment. The extracellular complement-binding (CUB) and coagulation factor domains of neuropilin-2 confer specificity to the Sema IV repulsive response, and these domains of neuropilin-1 are necessary and sufficient for binding of the Sema III semaphorin (sema) domain. The coagulation factor domains alone are necessary and sufficient for binding of the Sema III immunoglobulin- (Ig-) basic domain and the unrelated ligand, vascular endothelial growth factor (VEGF). Lastly, neuropilin-1 can homomultimerize and form heteromultimers with neuropilin-2. These results provide insight into how interactions between neuropilins and secreted semaphorins function to coordinate repulsive axon guidance during neurodevelopment.

Introduction

A central issue in developmental neurobiology concerns how growing axons of developing neurons are guided toward their appropriate target tissues. Recent work has identified several families of repellent and attractant cues that influence pathfinding decisions of neuronal growth cones (Tessier-Lavigne and Goodman, 1996). One large family of repulsive growth cone guidance cues is the semaphorins (Mark et al., 1997). Semaphorins include both transmembrane and secreted proteins, and all semaphorins possess a characteristic semaphorin (sema) domain of ~500 amino acids. In addition, all secreted semaphorins contain an immunoglobulin- (Ig-) like domain C-terminal to the sema domain. Secreted and transmembrane semaphorins are widely expressed in neuronal and nonneuronal tissues throughout development and into adulthood.

Recently, the axonal glycoprotein neuropilin-1 has been shown to be a cell surface receptor for the secreted semaphorin Sema III (He and Tessier-Lavigne, 1997; Kolodkin et al., 1997). Neuropilin-1 binds Sema III with high

affinity, it is expressed in all known Sema III-responsive neurons, it is present on the growth cones of Sema III-responsive neurons, and antibodies directed against its extracellular domain block the chemorepulsive and growth cone-collapsing activities of Sema III (reviewed by Kolodkin and Ginty, 1997). Further, growth cones of dorsal root ganglia (DRG) neurons isolated from mice with a targeted deletion of the *neuropilin-1* gene do not respond to Sema III (Kitsukawa et al., 1997). While these studies establish that neuropilin-1 is a Sema III receptor or the ligand-binding component of a Sema III receptor complex, it remains unclear how neuropilin-1 contributes to the propagation of the Sema III signal to the actin-based cytoskeleton within the growth cones of extending axons. Some clues to the answer of this question will come from detailed structure-function analysis of neuropilin-1. Neuropilin-1 has a large extracellular domain, a single transmembrane domain, and a very short cytoplasmic domain (reviewed by Fujisawa et al., 1997). While the neuropilin-1 cytoplasmic domain does not exhibit similarity to other known proteins, the neuropilin-1 extracellular domain is composed of two N-terminal domains similar to complement-binding domains (CUB domains, also called a1 and a2), two coagulation factor (V/VIII) domains (also called b1 and b2), and one C-terminal MAM (meprin, A5, μ) domain (also called c). The structural features of neuropilin-1 that contribute to ligand binding, multimerization, and signal propagation remain unknown.

During a search for additional semaphorin receptors, a neuropilin-1-related molecule, neuropilin-2, was identified, which is overall 44% identical to neuropilin-1 (Chen et al., 1997; Kolodkin et al., 1997). Neuropilin-2 has a domain structure that is strikingly similar to that of neuropilin-1. Moreover, multiple splice variants of neuropilin-2 exist that result in proteins containing distinct juxtamembrane regions, transmembrane domains, and cytoplasmic domains (Chen et al., 1997). The function of neuropilin-2 and its splice variants during nervous system development is unknown. *neuropilin-1* and *neuropilin-2* have distinct expression patterns in the developing nervous system (Kawakami et al., 1995; Chen et al., 1997; Kolodkin et al., 1997). For example, *neuropilin-1*, but not *neuropilin-2*, is highly expressed in neurons whose cell bodies are located in the DRG of embryonic day 14.5 (E14.5) rat embryos. Moreover, neuropilin-1 and neuropilin-2 bind to a distinct but overlapping subset of secreted semaphorins (Chen et al., 1997; Feiner et al., 1997). For example, the secreted semaphorin Sema IV/Sema 3F (here referred to as Sema IV) (Roche et al., 1996; Sekido et al., 1996; Xiang et al., 1996) binds with high affinity to both neuropilin-1 and neuropilin-2 (K_d s of ~1 nM and 0.1 nM, respectively). In contrast, Sema III binds to neuropilin-1 with high affinity (K_d of ~1 nM), but it binds poorly, if at all, to neuropilin-2. Taken together, it is likely that neuropilin-2, like neuropilin-1, is a receptor for a subset of secreted semaphorins.

In the present study, we show that neuropilin-2 is a functional receptor for Sema IV. Moreover, Sema IV and neuropilin-2 display strikingly complementary patterns

^{*}To whom correspondence should be addressed (e-mail: david.ginty@qmail.bs.jhu.edu [D. G.], alex.kolodkin@qmail.bs.jhu.edu [A. K.]).

[†]These authors contributed equally to this work.

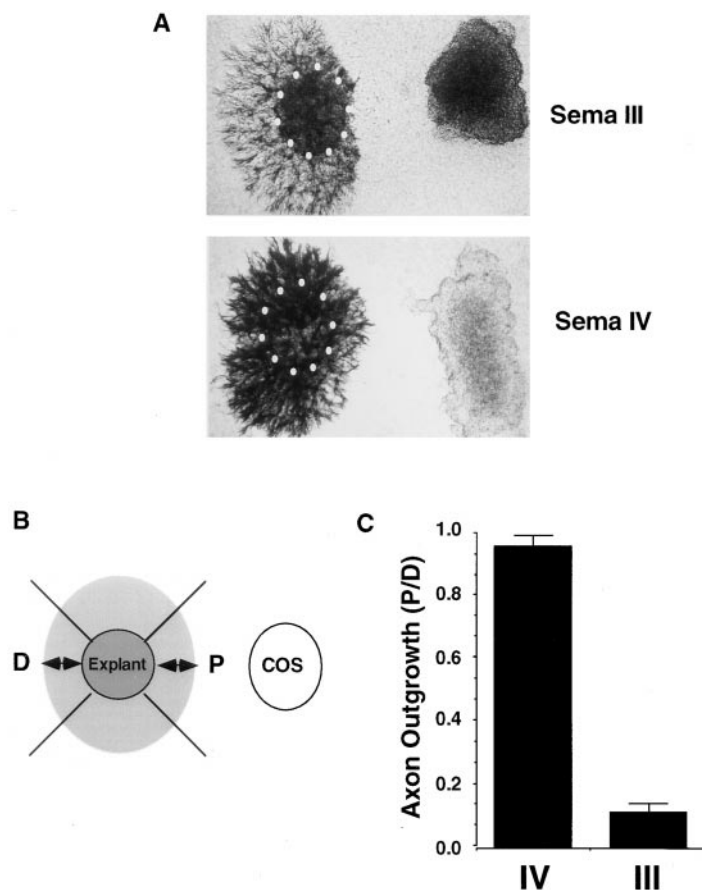


Figure 1. Axons of NGF-Dependent DRG Are Repelled by Sema III but Not by Sema IV

(A) DRG explants were cocultured with COS cells expressing either Sema III or Sema IV and grown for 48 hr.

(B) Schematic diagram of DRG explants and COS cells. The length of axonal projections on the proximal (P) and distal (D) sides of the neuronal explant was measured and used to calculate the P/D value presented in (C). Shown are the means \pm SEM of 32 (Sema IV) and 14 (Sema III) explants ($p < 0.001$).

of expression, consistent with a model in which this ligand-receptor complex participates in the development of axonal trajectories of postganglionic sympathetic neurons and other neuropilin-2-expressing neuronal populations. Lastly, we have determined that the neuropilin-1 CUB and coagulation factor domains, but not the MAM domain, mediate semaphorin binding, while multiple extracellular domains appear to contribute to neuropilin homo- and heteromultimerization. Therefore, neuropilin-1 and neuropilin-2 are functional receptors for distinct secreted semaphorins. We propose a model to explain functional specificity of semaphorin-neuropilin interactions.

Results

Sema III, but Not Sema IV, Repels E15 DRG Neurons

Both Sema III and Sema IV bind to neuropilin-1, which is expressed in E15 DRG neurons. We therefore asked whether neuropilin-1 can function as a receptor for both of these secreted semaphorins in DRG neurons. For these experiments, an *in vitro* paradigm was used in which aggregates of COS cells secreting either Sema III or Sema IV were cocultured with nerve growth factor-(NGF-) dependent sensory neurons emanating from a DRG explant taken from E15 rat embryos. After 2 days of growth, the repulsive activity of the semaphorins toward neuropilin-1-expressing DRG neurons was assessed.

The extent of repulsion was quantified by measuring the length of axons extending proximal (P) and distal (D) relative to the COS cell aggregate. These data are presented as P/D values (Figure 1). As previously described (He and Tessier-Lavigne, 1997; Kolodkin et al., 1997), NGF-dependent DRG neurons were strongly repelled by Sema III, and antibodies directed against neuropilin-1 significantly attenuated this repulsive effect (data not shown). In sharp contrast, NGF-dependent DRG neurons were not repelled by Sema IV. Thus, although Sema IV can bind to neuropilin-1, it is not repulsive for neuropilin-1-expressing DRG neurons.

Neuropilin-1 and Neuropilin-2 Are Expressed in Sympathetic Neurons

In situ hybridization experiments have indicated that *neuropilin-2* is expressed in several populations of neurons, and any or all of these neuropilin-2-expressing neurons may be responsive to Sema IV. To identify populations of neurons that express neuropilin-2 protein, rabbit polyclonal antibodies directed against the MAM domain of rat neuropilin-2 were generated and characterized. Previously, antibodies specific for neuropilin-1 had been generated and characterized (Kolodkin et al., 1997). Anti-neuropilin-1 (α -Npn-1) and anti-neuropilin-2 (α -Npn-2) antibodies do not cross-react (Figure 2A), and α -Npn-2 detected a single band on immunoblots of tissue extracts of superior cervical ganglia (SCG) taken

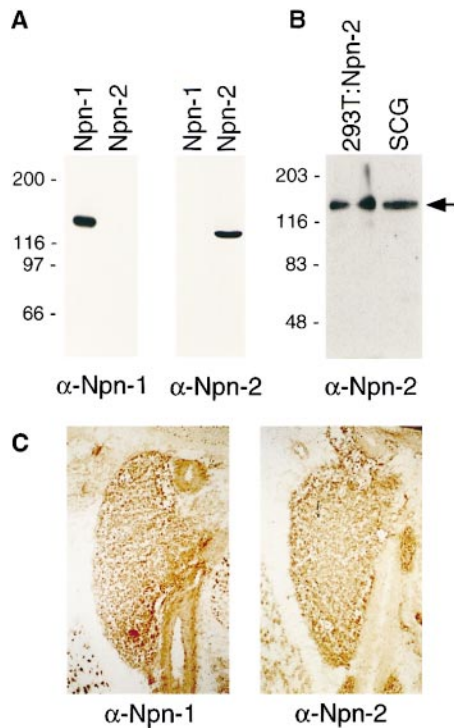


Figure 2. Neuropilin-1 and Neuropilin-2 Are Expressed in Sympathetic Neurons

(A) Immunoblot analysis of whole-cell extracts prepared from 293T cells that were transfected with expression vectors encoding neuropilin-1 or neuropilin-2, respectively. Equal amounts of extract were loaded in each lane, and blots were probed with either α -neuropilin-1 (α -Npn-1) or α -neuropilin-2 (α -Npn-2).

(B) Lysates from 293T cells expressing neuropilin-2 and from rat E18 SCG were resolved by SDS-PAGE and immunoblotted with α -Npn-2. A band of \sim 130 kD, corresponding to the molecular weight of neuropilin-2, is recognized in both the 293T cell extracts and SCG lysates.

(C) SCGs immunostained with either α -Npn-1 or α -Npn-2.

from E18 rat embryos (Figure 2B). Furthermore, neuropilin-1 and neuropilin-2 proteins are expressed in most, if not all, neurons within the SCG at E18 as determined by immunohistochemistry (Figure 2C). Thus, α -Npn-2 specifically recognizes neuropilin-2, α -Npn-1 and α -Npn-2 do not cross-react, and both proteins are expressed in developing postganglionic sympathetic neurons.

Neuropilin-2 Is Necessary for the Chemorepulsive Activity of Sema IV, but Not Sema III, Toward Sympathetic Neurons

Since neuropilin-2 binds to Sema IV with high affinity, and since neuropilin-2 protein is expressed in sympathetic neurons within the SCG (Figure 2C), we next tested whether Sema IV can repel the axons of sympathetic neurons. To assess whether Sema III and/or Sema IV repel the axons of sympathetic neurons, we used an assay in which SCG explants taken from E18 rat embryos were cocultured with cell aggregates secreting either Sema III or Sema IV. Both Sema III and Sema IV were found to be potent repellents of the axons of sympathetic neurons (Figure 3A).

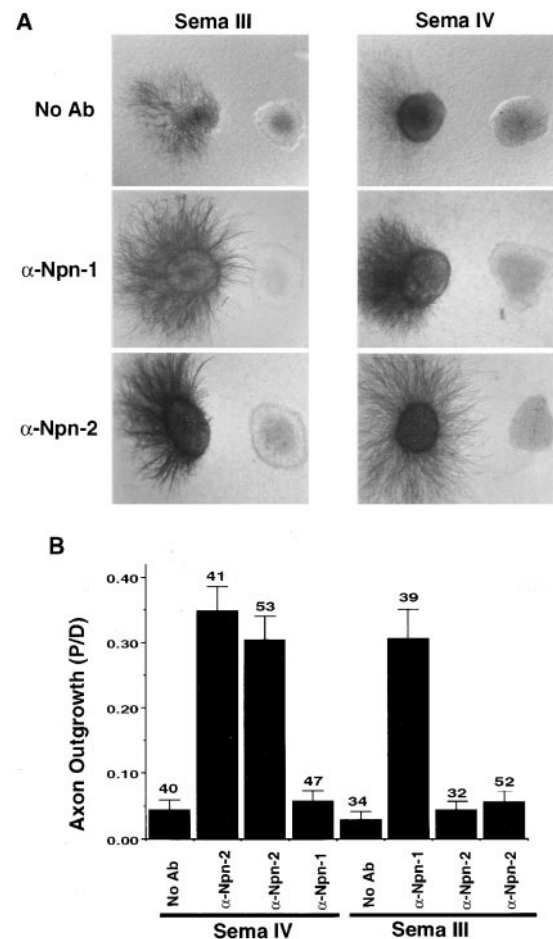


Figure 3. Neuropilin-2, but Not Neuropilin-1, Is Necessary for Sema IV Repulsion of SCG Axons

(A) SCG explants were cocultured with COS cells expressing either Sema III (left panels) or Sema IV (right panels) and grown for 72–84 hr in the absence of antibodies or in the presence of either α -Npn-1 or α -Npn-2 IgG (100 μ g/ml).

(B) Quantitation of the effects of α -Npn-1 and α -Npn-2 on repulsive activities of Sema III and Sema IV. Two different antibodies directed against the Npn-2 MAM domain were used in these experiments. Anti-neuropilin-1 antibodies blocked the repulsive effects of Sema III but not Sema IV. In contrast, both antibodies directed against neuropilin-2 blocked the repulsive effects of Sema IV but not Sema III ($p < 0.001$).

Since Sema IV repels axons of neuropilin-2-expressing sympathetic neurons, but not axons from neuropilin-1-expressing DRG neurons, we hypothesized that neuropilin-2 is the functional receptor for Sema IV. Likewise, since neuropilin-2 does not bind Sema III with high affinity, it is unlikely that neuropilin-2 is a functional receptor for Sema III in sympathetic neurons. To directly test these ideas, α -Npn-1 and α -Npn-2 were used in SCG coculture experiments to assess the requirement for these neuropilins during Sema III- and Sema IV-mediated repulsive guidance of sympathetic neurons. Anti-Npn-1, but not α -Npn-2, blocked the repulsive effects of Sema III toward SCG neurons (Figure 3). These results are consistent with the previous studies that demonstrated that neuropilin-1 is a receptor for Sema III in

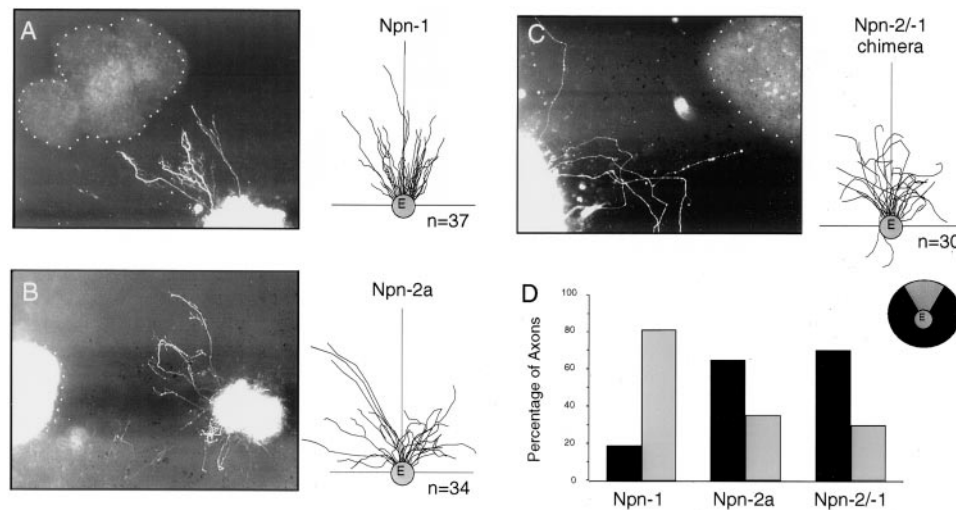


Figure 4. Expression of Neuropilin-2 or a Neuropilin-2/Neuropilin-1 Chimera (Npn-2/-1) Confers Sema IV Responsiveness to DRG Neurons
Expression vectors encoding either neuropilin-2(a17), neuropilin-1, or Npn-2/-1 were cotransfected with a GFP expression vector into NGF-dependent sensory neurons in explants of DRG by particle-mediated gene transfer. Explants were then cocultured in a collagen gel with Sema IV-expressing 293T cells to assess turning responses of DRG axons as visualized by fluorescence microscopy.
(A) Axons from neuropilin-1-transfected DRG neurons that initially project toward the Sema IV-expressing 293T cells do not show altered trajectories and extend directly toward the 293T cell aggregate. Shown is a representative DRG and a camera lucida representation of all axons from transfected neurons used for quantitation. White dots outline the 293T cell aggregate.
(B) Neuropilin-2-transfected DRG neurons extend axons that steer away from the Sema IV-expressing 293T cell aggregate.
(C) Npn-2/-1-transfected DRG neurons also extend axons that steer away from the Sema IV source.
(D) Quantification of DRG particle-mediated gene transfer experiments. Steering was quantified by determining the number of axons from transfected neurons that, at the end of the growth period, were found within (gray) or steering out of (black) a 60° sector centered on the point of origin of the initial trajectory. Chi square analysis shows a significant difference ($p < 0.0001$) between the fraction of axons that steer away from the Sema IV-expressing cell aggregate when comparing neurons expressing neuropilin-1 to neurons expressing either neuropilin-2a or Npn-2/-1.

NGF-dependent DRG neurons. In contrast, α -Npn-2 significantly attenuated repulsion of sympathetic neuron axons by Sema IV (Figure 3). A similar inhibitory effect on Sema IV-mediated repulsion of sympathetic neurons was seen with two different antibodies directed against the neuropilin-2 MAM domain. Moreover, α -Npn-1 did not block the repulsive effects of Sema IV toward SCG neurons (Figures 3A and 3B). Preimmune IgG at equal concentrations had no effect on Sema IV repulsive activity (data not shown). Together with the observation that Sema IV binds neuropilin-2 with high affinity, these results show that neuropilin-2, but not neuropilin-1, is required for the repulsive activity of Sema IV.

Neuropilin-2 Mediates the Repulsive Effects of Sema IV

If neuropilin-2 is a functional receptor for Sema IV, then expression of neuropilin-2 in neurons that normally do not express this neuropilin should confer upon these neurons the ability to respond to Sema IV. As mentioned above, NGF-dependent DRG sensory neurons do not express neuropilin-2, nor do they respond to Sema IV. We utilized particle-mediated gene transfer to introduce an expression vector encoding the neuropilin-2(a17) isoform along with an expression vector encoding green fluorescent protein (GFP) into NGF-dependent sensory neurons in explants of DRG. Then, the transfected DRG explants were cocultured with aggregates of Sema IV-secreting 293T cells and grown in a collagen gel matrix.

After 2 days of growth, the cultures were visualized under phase contrast and fluorescence microscopy to identify axons of GFP-positive sensory neurons emanating from the DRG explants. Neurons cotransfected with expression vectors encoding GFP and neuropilin-2 expressed both proteins (data not shown). As expected, axons of untransfected DRG neurons as well as those expressing GFP alone were not repelled by Sema IV (data not shown) and neither were axons of DRG neurons transfected with neuropilin-1 (Figures 4A and 4D). In dramatic contrast, 293T cells expressing Sema IV potently repelled the axons of DRG neurons cotransfected with GFP and neuropilin-2 expression vectors (Figures 4B and 4D). These results indicate that neuropilin-2 can mediate the repulsive effects of Sema IV. Taken together with the observations that Sema IV binds to neuropilin-2 with high affinity and that neuropilin-2 is necessary for the repulsive effects of Sema IV toward axons on sympathetic neurons (Figure 3), we conclude that neuropilin-2 is a functional receptor for the secreted semaphorin Sema IV.

Sema IV and Neuropilin-2 Are Expressed in Complementary Fashion in the Rat Embryo

Since neuropilin-2 is a functional receptor for Sema IV, *semaIV* and *neuropilin-2* should be expressed in a complementary fashion during neurodevelopment. Expression of *semaIV* was determined by in situ hybridization

to rat embryos and in several instances nicely fulfills this prediction. Below, we describe neuronal and non-neuronal patterns of expression of *semaIV*.

Olfactory System

In the E18 rat olfactory system, *semaIV* is strongly expressed in the cells dispersed throughout the main olfactory epithelium (OE) (Figures 5A and 6D), with somewhat stronger expression observed in the basal OE. In the nasal cavity, expression of *semaIV* is found in the respiratory epithelium, forming a continuum with the more dispersed expression in the more dorsally located OE. The vomeronasal organ (VNO) displays strong *semaIV* expression on the concave aspect of the sensory epithelium (Figure 5A). In contrast, *neuropilin-2* is expressed in the basal V2R neurons of the VNO (Figure 5B) (Chen et al., 1997), suggesting that *Sema IV* may act as a chemorepellent to direct the initial trajectories of these VNO neurons. In the embryonic olfactory bulb (OB), moderate *semaIV* expression is observed in the lateral mitral cell layer, increasing slightly toward the medial portion of the OB (Figure 5A). No *semaIV* expression is observed in the accessory olfactory bulb. Again, this is in contrast to *neuropilin-2* expression in the main OB, which is found in an increasing medial-to-lateral gradient in the mitral cell layer and in the accessory OB (Figure 5B) (Chen et al., 1997). *neuropilin-2* is also expressed in a subset of olfactory receptor neurons (ORNs), suggesting that expression of *semaIV* in the mitral cell layer of the OB prevents ORNs from overshooting their targets in the glomerular neuropil.

Forebrain and Midbrain

In the thalamus at E18, *semaIV* is expressed in the thalamic ventricular zone bordering the medial habenula (Figure 5C). This expression is complementary to the expression of *neuropilin-2* in the more laterally located neurons of the medial habenula (Figure 5D) (Chen et al., 1997), which project through the habenulo-interpeduncular tract to the midbrain. In the telencephalon at E18, expression of *semaIV* is observed in the cortical plate of the cingulate cortex and the neocortex (Figure 6C). No cortical expression is observed in the intermediate zone or the ventricular zone. This is complementary to *neuropilin-2* cortical expression, which is observed in the marginal zone and lower intermediate zone of the developing cortex but is absent from the cortical plate (Chen et al., 1997). The differentiating hippocampus, including the dentate gyrus, shows moderate *semaIV* expression (data not shown), whereas *neuropilin-2* is not expressed in the dentate gyrus but is expressed in the CA1 and CA3 fields (Chen et al., 1997). In the basal ganglia, *semaIV* expression is associated with the differentiating striatum and the globus pallidum. In the ventricles of the brain, maximal expression is associated with cells in the ependymal layer of the choroid plexus. In the midbrain (data not shown), strongest *semaIV* expression is observed in superficial and deep layers of the superior colliculus and in the inferior colliculus. More rostrally, *semaIV* expression extends into the pretectum. The ventricular zone of the aqueduct does not express *semaIV*. Caudally, *semaIV* expression continues into the differentiating cerebellar mantle zone. Midsagittal sections of the pons and medulla show moderate levels of expression of *semaIV* that are more pronounced in the

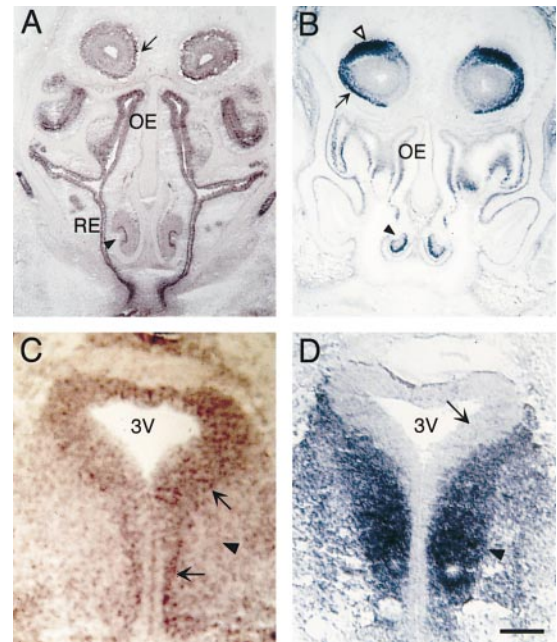


Figure 5. *semaIV* and *neuropilin-2* Are Expressed in Complementary Patterns during CNS Development

(A–D) Coronal sections taken from E18 rat embryos showing *semaIV* (A and C), *neuropilin-2*, (B and D), RNA distribution in the olfactory system (A and B), and thalamus (C and D).

(A) *semaIV* is expressed in the main OE, the respiratory epithelium (RE), the mitral cell layer (with increasing expression toward the medial portion of the OB) (arrow), and the VNO (closed triangle) on the concave aspect of the sensory epithelium.

(B) *neuropilin-2* is expressed in the basal OE of the VNO (closed triangle), in an increasing medial-to-lateral gradient in the mitral cell layer of the OB (arrow), in the accessory OB (open triangle), and in a subset of ORNs in the main OE.

(C) In the thalamus, *semaIV* is expressed in the thalamic ventricular zone bordering the medial habenula and the habenulo-interpeduncular tract (arrows) but not in the neurons of the medial habenula (closed triangle). Abbreviation: 3V, third ventricle.

(D) Thalamic *neuropilin-2* expression is complementary to *semaIV* expression—absent from the thalamic ventricular zone (arrows) but present in the medial habenula (closed triangle). Scale bar, 500 μ m (A and B) and 100 μ m (C and D).

dorsal one-third and become somewhat diminished ventrally. Finally, some neurons in several cranial nerve ganglia, including a subset of neurons located in the trigeminal ganglion, express high levels of *semaIV*.

Spinal Cord

Transverse sections of E14.5 embryos display *semaIV* expression in the spinal mantle zone, a subset of DRG neurons, and in mesenchymal tissue bordering the vertebral body and the neural arch (Figure 6A). Within the spinal cord, *semaIV* is expressed in a high dorsal to low ventral gradient and in a thin stripe of cells that extends ventrally at the lateral border of the spinal ventricular zone. No expression was observed in the basal plate or the floor plate. This is in marked contrast to the expression of *neuropilin-2* at this stage, which is found in the floor plate, the basal plate, and in thin stripes of cells lateral to the ventricular zone but is absent from the DRG and the dorsal horn (Chen et al., 1997; Kolodkin et al., 1997). A similar pattern of *semaIV* expression was

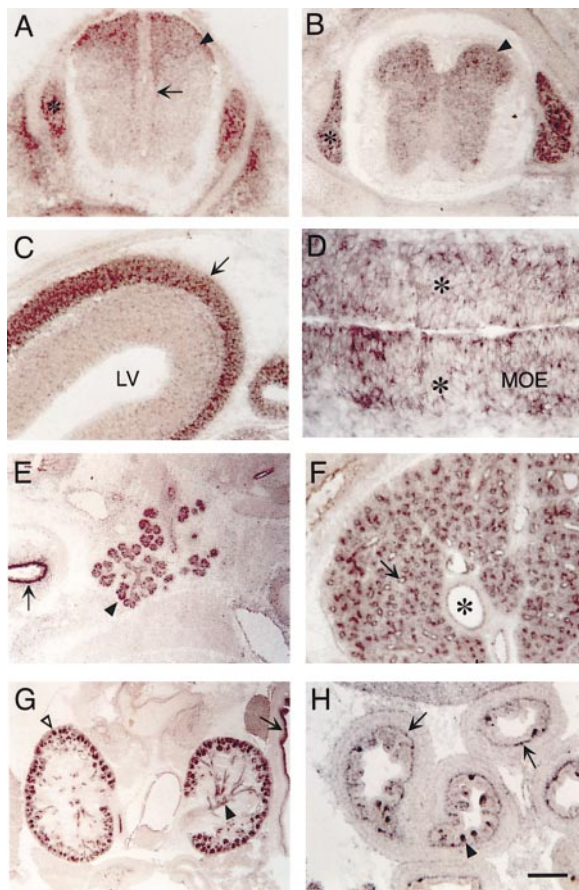


Figure 6. *semalIV* Is Expressed in a Variety of Neuronal and Nonneuronal Tissues during Development

Localization of *semalIV* transcripts at E14.5 (A) and E18 (B–H) was visualized by RNA in situ hybridization.

(A) Cross section of the E14.5 spinal cord shows *semalIV* expression in a high dorsal-to-low ventral gradient within the mantle zone (closed triangle), a thin stripe of cells that extends ventrally at the border of the mantle zone and the ventricular zone (arrow) and in a subset of DRG neurons (asterisk).

(B) Cross section through the E18 spinal cord shows *semalIV* expression in the dorsal horn (closed triangle), a subset of DRG neurons (asterisk), and lack of expression in the cells bordering the ventricular zone.

(C) Coronal section through the cingulate cortex at E18 shows expression of *semalIV* in the cortical plate (arrow). Abbreviation: LV, lateral ventricle; midline is to the right).

(D) High-power magnification of a coronal section through the main olfactory epithelium (MOE) at E18 showing *semalIV* expression that is somewhat stronger in the basal portion of the OE (asterisk).

(E) Parasagittal section at E18 showing *semalIV* expression in the dermis of the skin of the transition region of the mandibula and the neck (arrow) and distinct groups of cells within the submandibular gland (closed triangle).

(F) *semalIV* is expressed in cells lining the luminal side of peripheral pulmonary vessel branches (asterisk) and in the epithelial lining of terminal bronchioles (arrow).

(G) Transverse section showing *semalIV* expression in the kidney in glomeruli dispersed throughout the cortical region (open triangle) and in the medullar region along the collecting ducts (closed triangle). *semalIV* can also be seen in the wall of the stomach (arrow).

(H) Transverse section showing *semalIV* expression in cells located in between the epithelial lining and the developing smooth muscle in the wall of the intestine (arrows). *semalIV* is also expressed in groups of apically located cells in the mucosal folds of the intestine (closed triangle). Scale bar, 250 μ m (A, C, and H), 500 μ m (B, E, F, and G), and 100 μ m (D).

observed at E18 (Figure 6B). Expression of *semalIV* in the most dorsal portion of the alar plate, however, was less pronounced than at E14.5, and labeling in the spinal mantle zone becomes more restricted to the dorsal horn. At E18, the cells at the border between the ventricular zone and the mantle zone no longer express *semalIV*. At E18, DRG display intense *semalIV* expression in what appear to be discrete subpopulations of neurons. The SCG and other sympathetic chain ganglia, which strongly express *neuropilin-2*, do not express *semalIV*.

Eye

Sema IV is expressed in many structures of the developing eye, including the differentiating retina, lens, and cornea (data not shown). Moderate expression is observed in the differentiating retina at E18, with more discrete expression in cells of the outer layer. Expression of *semalIV* was not observed in the ganglion cell layer. Retinal *semalIV* expression is strongest in the anterior portion of the inner neuroblast layer closest to the iris. *semalIV* expression in the lens is restricted to the anterior portion of the capsule. In the cornea, punctate expression is found in small, uniformly distributed cells. Prominent expression is found in the Harderian gland within the orbit.

Gastrointestinal Tract

In the wall of the stomach and the small and large intestines, *semalIV* is strongly expressed by cells located in between the epithelial lining and the developing smooth muscle (Figure 6H). *Neuropilin-2* protein is found in the mesentery, muscular, and submucosal plexuses, which innervate the smooth muscle adjacent to the *semalIV*-expressing epithelium in the intestine (data not shown). *semalIV* is also expressed in groups of apically located cells in the mucosal folds of the intestine. Particularly strong *semalIV* expression was found in the wall of the distal part of the anal canal, as well as in the urogenital tract along the ureter and in the wall of the bladder.

Lung and Kidney

In the primordium of the lung at E15, *semalIV* is expressed in cells lining the lumen of segmental bronchi (data not shown). At E18, strong expression is found in cells lining the luminal side of peripheral pulmonary vessel branches (Figure 6F). Terminal bronchioles also display strong expression in epithelial linings throughout the substance of the future lung. In the developing kidney at E15, intense and localized expression originating from primitive glomeruli is observed in the outer (future cortical) region (data not shown). At E18, maximal *semalIV* expression was observed in glomeruli dispersed throughout the cortical region (Figure 6G). In the medullar region, expression is found along the collecting ducts, extending into the proximal part of the ureter.

Additional Nonneuronal Tissues

semalIV is strongly expressed in cells lining the oropharyngeal cavity, including the dorsal aspect of the tongue, the epithelium in the tooth anlagen, the larynx, and along the luminal side of the esophagus. Upper and lower lips display strong expression in the dermis of the skin and also in the primordium of the follicles of the vibrissae. The entire body of the embryo is lined by a strong expression in the dermis of the skin (Figure 6E). The submandibular gland (Figure 6E) and the thyroid gland show strong *semalIV* expression. Weak expression is seen in the thymus.

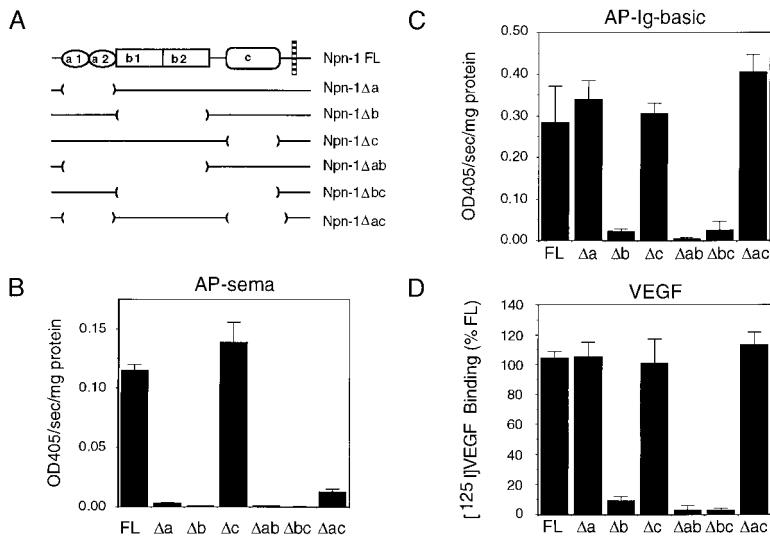


Figure 7. The Neuropilin a and b Domains Mediate Sema III and VEGF Binding

(A) Schematic representation of the neuropilin-1 (Npn-1 FL) and the neuropilin-1 deletion mutants employed in this study. (B) Quantitation of binding of AP-sema to Npn-1 FL and the deletion mutants. (C) Quantitation of binding of AP-Ig-basic to Npn-1 FL and the deletion mutants. (D) Quantitation of binding of [¹²⁵I]VEGF to Npn-1 FL and the Npn-1 deletion mutants. Shown are the means ± SD from four separate binding experiments.

Sema III Binds to the CUB and Coagulation Factor Domains of Neuropilin-1

The sema domains of the chicken semaphorins Coll-1 and Coll-3, and by extension presumably all of the secreted semaphorins, contain a 70 amino acid sequence near their N termini that imparts functional specificity/selectivity to these molecules (Koppel et al., 1997). In contrast, the Ig-basic domains appear to be relatively nonselective and may function to enhance the binding of these ligands to neuropilins or to restrict their distribution in the extracellular environment (Feiner et al., 1997; Koppel et al., 1997). Interestingly, two domains of Sema III, the sema domain and the C-terminal Ig-basic domain, can independently bind to neuropilin-1 (Feiner et al., 1997; He and Tessier-Lavigne, 1997). To better understand the mechanism of semaphorin functional selectivity at the level of receptor-ligand interactions, it was necessary to first delineate the domains of neuropilins that interact with each of these binding domains of the semaphorin ligand. This was accomplished by examining binding of the Sema III sema domain and the Sema III Ig-basic domain to neuropilin-1 and neuropilin-1 variants lacking one or more domains.

Deletion of the coding determinants of the CUB domains (a1 and a2) of neuropilin-1 was done by inverse PCR, giving rise to Npn-1Δa (Figure 6A). In a similar manner, the b1 and b2 coagulation factor domains were removed to give Npn-1Δb, and the MAM domain was deleted to give Npn-1Δc. Pairwise deletions were further constructed to generate Npn-1Δab, Npn-1Δbc and Npn-1Δac. Two ligands were generated for these binding analyses. One ligand consists of the sema domain of human Sema III fused to the Fc portion of human IgG to impart a dimerized state to this molecule (AP-sema). The second ligand consists of the Ig domain and basic tail of the rat Sem D (96% identical to human Sema III) fused at its C terminus to an Fc domain (AP-Ig-basic). Each of these ligands is fused at its N terminus to an alkaline phosphatase (AP) moiety to allow its binding to be detected by a standard AP assay.

Neuropilin-1 deletion constructs were transiently expressed in COS cells. Two days later, cells were incubated with AP-tagged ligand (12–14 nM) for 1 hr. Cells

were then washed extensively, lysed, and bound AP activity was determined (Figures 6B and 6C). To control for potential differences in expression of the neuropilin deletions, the amount of bound AP-tagged ligand was normalized for cell surface expression of neuropilin-1 or the neuropilin-1 deletion constructs. Neither AP-sema nor AP-Ig-basic bound significantly to untransfected COS cells, nor does an AP-Fc fusion protein bind to neuropilin-1-expressing COS cells (data not shown). Deletion of either the a or b domains, singly or in combination with any other domain, led to the nearly complete loss of binding of AP-sema (Figure 6B). Moreover, a small 13 amino acid insertion into the N terminus of the neuropilin-1 a1 domain led to a 75% reduction of binding of AP-sema (data not shown). The binding of AP-Ig-basic, in contrast to that of AP-sema, was not reduced to any significant degree by deletion of the a domains. However, deletion of the b domains (singly or in combination with a or c domains) abolished AP-Ig-basic ligand binding, as was seen for AP-sema (Figure 6C). The b domains are not only necessary for the binding of the AP-Ig-basic ligand but appear to be sufficient, since the b domain alone (Npn-1Δac) conferred nearly wild-type levels of AP-Ig-basic binding. Binding of either AP-sema or AP-Ig-basic domain ligands to neuropilin-1 was unaffected by deletion of the MAM (c) domain.

In addition to being a receptor for secreted semaphorins, neuropilin-1 has been identified as a coreceptor on endothelial cells for vascular endothelial growth factor (VEGF) (Soker et al., 1998). To identify structural features of neuropilin-1 that mediate the binding of VEGF, [¹²⁵I]VEGF binding experiments were performed on the neuropilin-1 deletion mutants (Figure 6D). As seen for the AP-Ig-basic ligand, the b domains of neuropilin-1 are necessary and sufficient for the binding of [¹²⁵I]VEGF; deletions lacking either the neuropilin-1 a domains, the c domain, or both the a and c domains exhibited normal levels of [¹²⁵I]VEGF binding. Consistent with the idea that the Sema III Ig-basic domain and VEGF bind to the same or a similar site within neuropilin-1, the AP-Ig-basic ligand competitively inhibited the binding of [¹²⁵I] VEGF to neuropilin-1 (data not shown). Interestingly, neither the AP-Ig-basic ligand nor VEGF can promote the collapse

of growth cones of neuropilin-1-expressing NGF-dependent DRG neurons, suggesting that engagement of the neuropilin-1 b domains is not sufficient to evoke a functional response (data not shown). These binding data reveal that semaphorin ligands contact both the a and b domains of neuropilin-1. In contrast, the Sema III Ig-basic domain and VEGF appear to associate solely through the neuropilin-1 b domains. The neuropilin-1 MAM domain does not function as a binding site for either the sema domain or the Ig-basic domain of Sema III or VEGF.

Neuropilins Form Homo- and Heteromultimers

The MAM domain of neuropilin-1 does not appear to be involved to any significant degree in the binding of Sema III. MAM domains are present in diverse cell surface proteins, including PTP μ , PTP κ , and PCP-2 protein tyrosine phosphatases (PTPs) as well as the metalloendopeptidase meprin. The MAM domains of the phosphatases and meprin appear to contribute to homophilic interactions (Zondag et al., 1995; Marchand et al., 1996). The presence of a MAM domain in neuropilin-1 suggests that neuropilin-1 might exist in a dimeric or multimeric state in the absence of ligand binding. To assess this possibility, a coimmunoprecipitation assay was employed. Two different C-terminal epitope-tagged forms of neuropilin-1, Npn-1-myc and Npn-1-HA, were coexpressed in 293T cells, and lysates of these cells were subjected to nondenaturing immunoprecipitation (IP) with antibodies directed against the myc-epitope of Npn-1-myc. Analysis of the precipitated protein complex by immunoblotting with an anti-HA antibody revealed the presence of Npn-1-HA, indicating that HA and myc forms of the protein associate (Figure 8A). In addition to forming homomultimers, Npn-1 has the capacity to heteromultimerize with Npn-2 (Figure 8A). Further coprecipitation experiments were undertaken to analyze the domain requirements for multimerization. Coprecipitation of myc- and HA-tagged neuropilin-1 deletion mutants lacking the a and b domains revealed that the neuropilin-1 MAM domain, together with the transmembrane and intracellular domains, is sufficient to mediate multimerization (Figure 8C). The MAM domain, however, is not necessary for Npn-1 to homomultimerize, as indicated by the coprecipitation of myc- and HA-tagged Npn-1 Δ c mutants (Figure 8D). The importance of the transmembrane and intracellular domains for multimerization was assessed in coprecipitation experiments utilizing a glycosylphosphatidylinositol- (GPI-) linked neuropilin-1 molecule that lacks these domains but is attached to the cell surface through the GPI linkage of chick axonin-1 (Lierheimer et al., 1997). This cell-attached form of the neuropilin-1 ectodomain is in fact capable of heteromultimerizing with neuropilin-2 (Figure 8B); thus, the transmembrane and intracellular domains are not required for multimerization. The MAM domain alone is not sufficient to mediate multimerization, since the Npn-1-GPI protein does not interact with myc-tagged Npn-1 Δ ab (data not shown). Taken together, these results indicate that multiple domains can contribute to neuropilin multimerization.

The Neuropilin a and b Domains Confer Functional Specificity to Semaphorin Ligands

Neuropilin-1 and neuropilin-2 are functional receptors for Sema III and Sema IV, respectively. Since the neuropilin a and b domains mediate semaphorin ligand binding, we reasoned that a chimeric neuropilin molecule consisting of the neuropilin-2 ligand-binding domains (the a and b domains) fused to the neuropilin-1 c domain, transmembrane, and intracellular domains (Npn-2/-1) should confer Sema IV responsiveness when expressed in neurons that normally do not respond to Sema IV. To verify that Npn-2/-1 binds Sema IV but not Sema III, binding analyses were performed as described above. The sema domain of Sema IV bound to Npn-2/-1, but the sema domain of Sema III did not (data not shown). To test the idea that the neuropilin-2 a and b domains impart functional responsiveness to Sema IV, Npn-2/-1 and GFP were coexpressed in the axons of NGF-dependent sensory neurons emanating from DRG explants grown in coculture with an aggregate of Sema IV-secreting COS cells. As seen for neurons expressing wild-type neuropilin-2, axons of neurons expressing Npn-2/-1 grew away from the source of Sema IV (Figures 4C and 4D). As described above, neurons expressing GFP alone or neuropilin-1 did not respond to Sema IV. These experiments indicate that binding of Sema IV to the neuropilin-2 a and b domains, but not to the neuropilin-1 a and b domains, is necessary for a functional steering response. Thus, the a and b ligand-binding domains of neuropilins, not the c, transmembrane, or cytosolic domains, mediate the functional specificity of Sema III and Sema IV repulsive guidance events.

Discussion

We show here that neuropilin-2 and not neuropilin-1 is a functional receptor for Sema IV and that *semaIV* and *neuropilin-2* are expressed in strikingly complementary patterns during neurodevelopment. We have used this information to initiate a functional analysis of the interactions between secreted semaphorins and neuropilins. Our results define extracellular neuropilin domains that are essential for ligand binding and function. Further, they suggest that homo- and heteromultimeric interactions between neuropilins may regulate semaphorin function. These observations begin to establish how a large family of related repellents, many of which have overlapping expression patterns and neuropilin binding profiles, can impart specificity to repulsive axon guidance decisions during neurodevelopment through their interactions with neuropilins.

Neuropilin-2 Is a Sema IV Receptor

The observations that neuropilin-2 binds to Sema IV with high affinity (Chen et al., 1997) and that Sema IV is not repulsive toward NGF-dependent DRG neurons that express neuropilin-1, prompted us to ask whether neuropilin-2 functions as a receptor for Sema IV. Indeed, E18 sympathetic neurons, which express both *neuropilin-1* and *neuropilin-2*, are strongly repelled by Sema IV. Antibodies directed against the MAM domain of neuropilin-2 greatly attenuated the repulsive action of Sema

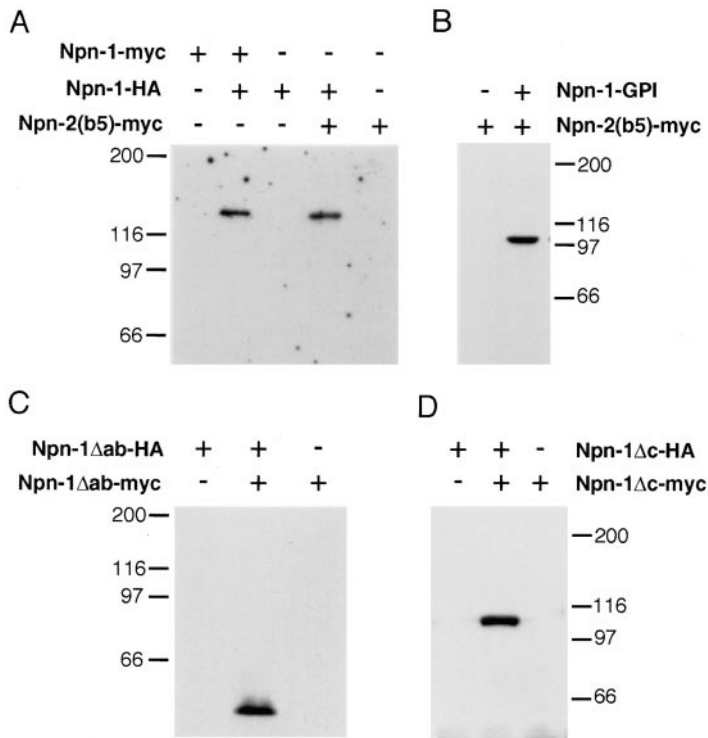


Figure 8. Neuropilin-1 Forms Homomultimers and Heteromultimers with Neuropilin-2

(A) Neuropilin-1 forms homomultimers and heteromultimers with neuropilin-2(b5). Npn-1-HA was coexpressed in 293T cells with either Npn-1-myc or Npn-2(b5)-myc. 293T cell lysates were subjected to IP with a monoclonal antibody directed against the myc-epitope (9E10), and the immune complexes were subjected to immunoblotting with a monoclonal antibody directed against the HA epitope.

(B) The transmembrane and intracellular domains of Npn-1 are not necessary for neuropilin heteromultimerization. Npn-2(b5)-myc was expressed alone or coexpressed with Npn-1-GPI, which lacks the neuropilin-1 transmembrane and intracellular domains. Cell extracts were subjected to IP and immunoblotting with α -Npn-1.

(C) The neuropilin-1 a and b domains are not necessary for homomultimerization. Npn-1Δab-myc and Npn-1Δab-HA were expressed individually or coexpressed in 293T cells, lysates were subjected to myc IP, and immune complexes were immunoblotted with anti-HA.

(D) The neuropilin-1 c domain is not necessary for homomultimerization. Npn-1Δc-myc and Npn-1Δc-HA were expressed alone or together, and cell extracts were subjected to IP and analysis as in (C). Immune complexes were subjected to immunoblot analysis using the HA antibody.

IV on SCG neurons, whereas function-blocking anti-neuropilin-1 antibodies did not. Moreover, expression of neuropilin-2 in NGF-dependent DRG neurons conferred upon those neurons the ability to respond to Sema IV. These results, in addition to establishing that Sema IV can function as a neuronal chemorepellent, suggest that Sema IV acts through its interactions with neuropilin-2 and not neuropilin-1, even though Sema IV is able to bind to neuropilin-1 with high affinity. Whether neuropilin-2 serves as a functional receptor for other semaphorins remains to be determined. Another candidate ligand for neuropilin-2 is Sema E. SCG neurons are repelled by Sema E, and Sema E can bind to neuropilin-2 with high affinity (Adams et al., 1997; Chen et al., 1997). It remains to be determined if neuropilin-2 is necessary and/or sufficient to mediate the repulsive effects of Sema E or whether, in light of our demonstration that neuropilins 1 and 2 can form heteromultimers when expressed in cultured cells, both neuropilins are required for this response. In addition, since our studies have used the neuropilin-2(a)17 isoform, it will be of interest to learn whether there are isoform-specific functional differences among the several *neuropilin-2* splice variants identified thus far.

semaIV and *neuropilin-2* Show Complementary Expression during Neurodevelopment

We observed striking complementarity between the expression of *semaIV* and its receptor *neuropilin-2* during development, reminiscent of the similar complementarity (although in different regions) observed between the expression of *semaIII* and *neuropilin-1* (Satoda et al., 1995; Kobayashi et al., 1997; reviewed by Kolodkin and

Ginty, 1997). *semaIV* and *neuropilin-2* are often expressed in patterns that suggest that Sema IV serves to define regions that are repulsive to specific classes of axons during pathfinding in neurodevelopment.

In the developing olfactory system, *semaIV* is expressed in regions apical to the VNR neurons of the VNO. Equally strong expression of *neuropilin-2* is observed in the V2R neurons of the basal VNO. The V2R neurons initially project basally into the accessory olfactory nerve, away from the region of *semaIV* expression in the VNO (reviewed by Bargmann, 1997), commensurate with the idea that Sema IV serves to direct the initial projections of these neurons. The accessory OB, unlike the main OB, does not express high levels of *semaIV*, and this lack of *semaIV* expression may serve to help segregate VNO projections to the accessory OB from OE projections to the main olfactory bulb. We and others (Chen et al., 1997) note that *neuropilin-2* is strongly expressed by both VNO neurons and their target region in the accessory OB. It is possible that homophilic neuropilin-2 interactions may serve to help establish appropriate connectivity between the VNO and the accessory olfactory bulb and may even function to regulate fasciculation of accessory olfactory neurons. *semaIV* is expressed in the main OE, with stronger expression observed in the basal OE, and since subsets of main ORNs also express *neuropilin-2*, Sema IV may play a role as well in directing the initial outgrowth of ORNs to the olfactory nerve. In addition, *semaIV* is expressed in the main OB in the periglomerular, mitral, and tufted cells and could prevent the main ORNs from overshooting their glomerular targets in the main OB.

Thalamic *semaIV* expression also supports a role for

Sema IV in directing repulsive guidance events during axon pathfinding. *semaIV* expression in the thalamic ventricular zone bordering the medial habenula suggests that Sema IV may provide a repulsive barrier for the more laterally located neurons of the medial habenula that project caudally and ventrally through the habenulo-interpeduncular tract on their way to their target in the interpeduncular nucleus at the base of the midbrain.

In addition to these examples of *semaIV* expression in regions that are avoided by *neuropilin-2*-expressing neurons, additional *semaIV* expression patterns are suggestive of similar events. For example, in the developing spinal cord, *semaIV* expression in a decreasing dorsal-to-ventral gradient may provide repulsive signals for several classes of *neuropilin-2*-expressing neurons. These include motor neurons, whose initial lateral axonal outgrowth or later dorsal or medial dendritic sprouting may be regulated by Sema IV. Sema IV in the developing spinal cord may also influence the migration of *neuropilin-2*-expressing cells that are observed at E14.5 in thin stripes lateral to the ventricular zone. Since *neuropilin-2* is also expressed in commissural neurons and DRG sensory neurons at E11.5 in the mouse (Chen et al., 1997), early *semaIV* expression may influence the ventral trajectories of these neurons as well. The formation of sympathetic chain ganglia, which express both *neuropilin-1* and *neuropilin-2*, also may be affected by Sema IV in closely flanking mesenchymal tissue bordering the vertebral body and rib arches. Taken together, these *semaIV* and *neuropilin-2* expression data strongly suggest that this receptor–ligand pair defines specific repulsive axon guidance events in vivo. Also of interest is the similarity in the expression observed for *neuropilin-1* and *semaIV*, which includes coexpression in several neuronal tissues, including the dorsal horn of the spinal cord, DRG, the cortical plate, and the trigeminal ganglia (Kawakami et al., 1995; this study). Though Sema IV can bind to neuropilin-1 in vitro, this interaction does not lead to an alteration in neuronal guidance. It is possible, however, that this interaction may serve to negatively regulate neuropilin-1 function and thereby render these populations of neurons less sensitive to Sema III during portions of their trajectories.

In the periphery, *semaIV* displays discrete and strong expression in numerous organ systems that receive sympathetic innervation, including the eye, salivary glands, skin, blood vessels, heart, lung, and the gastrointestinal and urogenital tract. Neurons in the superior cervical ganglion, postganglionic neurons in the sympathetic chain, and distinct peripheral plexuses express *neuropilin-2* mRNA (Chen et al., 1997) and protein (data not shown), suggesting that axonal branches of sympathetic neurons form their terminal arborization patterns following Sema IV–mediated chemorepulsive guidance events. In several organ systems, the distribution of *semaIV* is adjacent to developing smooth muscles, and *semaIV* is also found restricted to subsets of cells in exocrine glands; these organs and glands all receive sympathetic innervation. For example, in the intestine, *semaIV* is expressed in cells bordering the inner circular smooth muscle, and this may influence the refinement of the mesentery, mucosal, or submucosal plexuses, all

of which express neuropilin-2 protein (data not shown). This further supports the notion that Sema IV acts in vivo as a chemorepulsive cue for *neuropilin-2*-expressing fibers. Similar to *neuropilin-1*-expressing sensory and motor neurons, which are guided in part by Sema III in the periphery (Taniguchi et al., 1997), projections of sympathetic neurons are often bordered by *semaIV*-expressing mesenchymal tissue, which may enhance axon fasciculation and prevent branching in nontarget tissues. A detailed examination of the distribution of autonomic neurons that express *neuropilin-2*, both in wild-type and *neuropilin-2* mutant mice, will better clarify the role played by Sema IV and neuropilin-2 in the innervation of these organs. In addition, since nonneuronal *neuropilin-2* expression has been observed in these sympathetic target tissues (Chen et al., 1997; this study), Sema IV and neuropilin-2 may also play important roles in cell migration and organogenesis.

Extracellular Neuropilin Domains Function Differentially to Mediate Ligand Binding and Neuropilin Multimerization

The neuropilin extracellular domains are complex, consisting of two CUB domains, two coagulation factor domains, and one MAM domain. All three of these domains have been implicated in mediating protein–protein interactions; however, it is essential to learn exactly how they contribute to forming binding sites for secreted semaphorins, with the ultimate goal being to understand how neuropilins help transduce a repulsive steering signal to responsive neuronal growth cones. One important clue comes from our demonstration that the CUB and coagulation factor domains are the semaphorin ligand-binding domains. Additional insight comes from experiments demonstrating that a chimeric neuropilin domain consisting of the CUB and coagulation factor domains from neuropilin-2 fused to the MAM, transmembrane, and cytoplasmic domains of neuropilin-1 confers a functional response to Sema IV in neurons growing from DRG explants in culture—neurons that normally do not express *neuropilin-2* and are not responsive to Sema IV. Since Sema IV binds to both neuropilin-1 and neuropilin-2, these results suggest that Sema IV functional specificity is the result of the distinct nature of its interactions with the CUB and coagulation factor domains of the two neuropilins. Sema IV binding to the CUB and coagulation factor domains of neuropilin-2 results in receptor activation and signal propagation, while its association with the analogous domains in neuropilin-1 does not result in receptor activation and signal propagation. *semaIV* is expressed in or around several populations of *neuropilin-1*-expressing neurons, including DRG neurons. It is tempting to speculate that the nonproductive nature of the Sema IV–neuropilin-1 interaction may be critical for guiding neuropilin-1-expressing neurons toward their appropriate targets.

The MAM domain of neuropilin-1 is not necessary for the binding of either the Sema III sema domain or the Ig-basic domain. What function might the MAM domain play? To address this issue, we asked whether, like the MAM domain of meprin (and possibly those of RPTP κ and RPTP μ), neuropilin MAM domains contribute to receptor multimerization. While the MAM domain, in

conjunction with the transmembrane and intracellular domains, appears to be sufficient to mediate multimerization, modified neuropilin-1 proteins lacking either the MAM domain or the transmembrane and intracellular domains retain the capacity for multimerization. One potential explanation for these data is that multiple domains, including the MAM domain, contribute to receptor multimerization. While the simplest interpretation of these results is that neuropilins exist in multimeric complexes in the absence of ligand, future analyses will be required to directly assess whether cell surface-localized neuropilins exhibit ligand-dependent multimerization. Lastly, we have provided evidence that neuropilins can form heteromultimers. This observation raises the interesting possibility that neuropilin-1/2 multimers are present on the cell surface of semaphorin-responsive neurons and that heteromultimers participate in semaphorin signaling and repulsive axon guidance.

Though several questions remain with respect to how secreted semaphorins and neuropilins contribute to the propagation of a repulsive growth cone guidance signal, based on our results, we can begin to develop a model for this signaling event. A secreted semaphorin dimer (Klostermann et al., 1998; Koppel and Raper, 1998) binds to both the CUB and coagulation factor domains of a neuropilin dimer present on the cell surface of a growth cone. High-affinity binding of the Ig-basic region of the semaphorin to the coagulation factor domains is not sufficient to propagate a signal, but it may facilitate binding of the sema domain to its contact sites within the CUB and coagulation factor domains. The association between the sema domain and its binding site within neuropilin-1 is critical for triggering a functional response. In addition, it is the nature of the interaction between the secreted semaphorin ligand and the CUB and coagulation factor domains within neuropilins that determines whether a semaphorin-neuropilin interaction leads to the generation of a repulsive growth cone steering response.

Neuropilin-1 not only mediates Sema III function but also serves as a coreceptor for VEGF, enhancing the ability of VEGF to activate the receptor tyrosine kinase KDR (Soker et al., 1998). We have found that the coagulation factor domains of neuropilin-1 are both necessary and sufficient for binding the Ig-basic domain of Sema III and VEGF and that Sema III can effectively compete VEGF binding to neuropilin-1. It is therefore possible that VEGF, in addition to promoting angiogenesis and vascularization, may also impede repulsive semaphorin guidance signals in the periphery, so as to promote associations between neurons and blood vessels.

Our data do not address whether or not the cytoplasmic neuropilin domains are required for signaling, nor do they rule out or rule in the possibility that an additional associated signaling component completes a functional ternary receptor complex. Results of experiments with a chimeric neuropilin receptor do rule out the possibility that the MAM domain imparts functional specificity. Further experiments will be needed to address the necessity and/or sufficiency of the neuropilin intracellular domains, transmembrane domains, and multiple extracellular domains as mediators of semaphorin-neuropilin signal transduction.

Experimental Procedures

Superior Cervical Ganglia Explant Cultures and Antibody Inhibition Experiments

SCG were dissected from E18 rat embryos, and DRG were dissected from E14.5 rat embryos. Ganglia were cultured with transiently transfected aggregates of Sema III-AP-expressing 293EBNA cells or Sema IV-expressing 293T cells (Chen et al., 1997) in a collagen matrix (Kolodkin et al., 1997). For 293T cell transfections, 4 μ g DNA were transfected per one million cells. Cocultures were incubated for 72–84 (SCG) or ~48 (DRG) hr in culture medium (25% F12 media, 69% Opti-MEM media, 0.04 M glucose, 2 mM glutamine, 0.5% heat-inactivated fetal calf serum, and 100 ng/ml NGF). For some experiments, media were supplemented with either anti-neuropilin-1, anti-neuropilin-2, or preimmune IgG (100 μ g/ml). SCG explants, DRG or cell aggregates, were placed ~700 μ m apart. Explants were stained with 2H3 monoclonal anti-neurofilament antibody (Collaborative Research). For quantitation of repulsion, neurite outgrowth was measured from the outer border of each SCG to the growth cone of the longest axon that projected along the axis of the cell aggregate and the center of the explant (Messersmith et al., 1995; Kolodkin et al., 1997). A proximal-to-distal outgrowth ratio was obtained by measuring the proximal and distal axon lengths and taking their ratio. Measurements of fixed, stained cocultures were determined blind by two independent observers. SCG and DRG explants with <200 μ m outgrowth on the distal side were not scored. Statistical analysis was performed using analysis of variance.

Neuropilin Antisera Production, Purification, and Immunoblot Analysis

Neuropilin-1 antibodies were previously described (Kolodkin and Ginty, 1997). Antibodies directed against neuropilin-2 were produced by immunizing rabbits with a six histidine-tagged neuropilin protein that was produced in the BL21 strain of *E. coli*. The bacterial expression construct was made by inserting into the XhoI and HindIII sites of the pTrcHisA vector (Invitrogen), a PCR-amplified cDNA fragment encoding amino acids 591–798 of rat neuropilin-2. Expressed protein was purified by nickel-chelate affinity chromatography. IgG was purified by protein A-sepharose chromatography. The antibody was affinity purified with the neuropilin-2 fusion protein conjugated to Affi-gel 15 (BioRad). Immunoblot analysis was performed as described (Ginty et al., 1994) using extracts of E18 SCG and HEK 293T cells transiently transfected with expression vectors encoding neuropilin-1 or neuropilin-2, respectively.

Immunohistochemistry

E18 rat embryos were fixed for several hours in ice-cold PBS containing 4% paraformaldehyde and cryoprotected overnight at 4°C in a solution of PBS containing 15% sucrose. The following day, embryos were transferred to a solution of PBS containing 30% sucrose for 24 hr. Immunohistochemistry of cryosections (20 μ m) using the IgG fraction of neuropilin-1 immune or neuropilin-2 immune sera was performed as described (Kolodkin et al., 1997).

Repulsion Assay of Transfected DRG Neurons

DRG were dissected from the cervical and upper thoracic levels of E14.5 rat embryos, cut into halves, and grown on glass coverslips coated with polylysine (50 mg/ml) and laminin (20 mg/ml). Culture conditions were identical to the ones described previously (Kolodkin et al., 1997). The next morning, cultures were cotransfected by the Helios Gene Gun System (Bio-Rad), with expression vectors encoding either neuropilin-1, neuropilin-2(a17), or the Npn-2/-1 chimera (in pcDNA 1.1) and GFP (in pEGFP-N1, Clontech) in a 3:1 ratio. As microcarrier for plasmid DNA transfection, we used gold beads with a diameter of 1.6 μ m (Bio-Rad). Plasmid DNA (150 μ g/25 mg microbeads) was surface coated by using the Helios Gene Gun Kit. Briefly, 25 mg sonicated gold beads in 100 μ l 100 mM spermidine was mixed with 150 μ g DNA (in 100 μ l water) and precipitated on the beads by adding dropwise 100 μ l 1 M CaCl₂. After 10 min at room temperature, the beads were spun down in a table top centrifuge, washed three times with ethanol, and recovered in a final volume of 3 ml in 0.5% polyvinyl pyrrolidone in ethanol. Before coating the tubing of the cartridge, the beads were briefly sonicated. For the

gene transfer, DRG attached to glass coverslips were transferred to a new culture dish, shot with the gene gun, and rapidly covered with prewarmed culture medium. Four hours after the gene transfer, aggregates of 293T cells, transiently transfected to express Sema IV, were added to the DRG cultures and subsequently embedded in a collagen matrix. Forty-eight hours after transfection, the cultures were scored by visualizing the fibers with 4× and 10× Nikon Plan Fluor objectives using a CCD camera (Dage, MTI) attached to the side port of a Nikon Eclipse TE300 inverted microscope. Images were captured and analyzed with IPLab Spectrum 3.1.1 image acquisition software. For our analyses, axons were selected whose initial trajectory was toward the proximal side of the DRG toward the aggregate. Data are presented for all axons as superimposed camera lucida drawings. Steering was quantified by determining the number of axons that, at the end of the growth period, were steering out of a 60° sector centered on the point of origin of the initial trajectory.

In Situ Hybridization

Nonradioactive, digoxigenin- (DIG-11-UTP-) labeled cRNA probes with either sense or antisense orientation were synthesized by *in vitro* transcription by using either T3 or T7 RNA polymerases (Boehringer Mannheim). A rat *semaIV* cDNA was isolated from a λZAP (Stratagene) phage library made from E14 spinal cord and DRG (A. K. and D. G., unpublished data), cloned into pBluescript, and sequenced on both strands. Analysis of this sequence revealed that this clone contains most of the *semaIV* ORF 700 bp of 3' UTR; however, the 5'-300 bp are non-*semaIV* sequences resulting from a cloning artifact. Antisense probes were generated with T3 RNA polymerase by linearizing the rat *semaIV* cDNA in the 5'-most portion of the *semaIV* ORF with Age I (nucleotides 697–2995). Sense probes were synthesized with T3 RNA polymerase by using a PCR-amplified fragment, including nucleotides 527–2995 of rat *semaIV* cDNA. All probes for *in situ* analysis contained only *semaIV* sequences. Cryosections (20 μm) of E15 and E18 rat embryos were processed for *in situ* hybridization as described (Kolodkin et al., 1997).

Neuropilin and Semaphorin Ligand Constructs

neuropilin-1 deletion constructs for ligand-binding assays were created by inverse PCR using the ExSite kit (Stratagene). Full length Npn-1 in the expression vector pMT21 served as a template for PCR reactions using oligo pairs that flanked the CUB (a1/a2), coagulation factor (b1/b2), and MAM (c) domains. The oligonucleotides contained a Sal I restriction site at their 5' ends and were designed such that upon annealing, the 3' end of the oligos were directed away from each other. Following amplification, inverse PCR products were digested with Sal I and circularized by ligation yielding Npn-1 mutant constructs: Npn-1Δa (T30-F258), Npn-1Δb (S283-M578), Npn-1Δc (G647-C809), Npn-1Δab (T30-M578), and Npn-1Δbc (S283-C809). In the cases of Npn-1Δa, Npn-1Δc, and Npn-1Δbc, the deleted amino acids were replaced with VE encoded by the Sal I restriction site. In the cases of Npn-1Δb and Npn-1Δab, the deleted amino acids were replaced with VEQVE. To generate the Npn-1Δac construct, a pair of c domain-flanking oligos were utilized in conjunction with Npn-1Δa template to perform inverse PCR, after which the product was blunt end ligated. The deleted c domain (G647-E834) was replaced by VEQ. All cloning sites were sequenced, and the expression of correctly sized Npn-1 mutants was confirmed by immunoblotting.

Myc-tagged Npn-1 constructs for the multimerization experiments were created by PCR amplification of the Npn-1 full length, Npn-1Δab, and Npn-1Δc open-reading frames, excluding the stop codon, with oligos containing terminal restriction sites (Bam HI on the 5' end, Xma I and Xba I on the 3' end). Following amplification, the PCR product was restricted with BamHI and Xba I and cloned into the Bam HI and NheI sites of the pCOS-derived expression plasmid, pCOS-myc, thereby placing the Npn-1 sequences upstream of, and in-frame with, the vector-encoded myc-epitope sequence. To generate HA-tagged Npn-1 forms, PCR amplification of Npn-1Δa, Npn-1Δab, and Npn-1Δa was performed as for the myc constructs, but the amplified products were instead cloned into the BamHI and Spe I sites that flank the myc-epitope sequence in pCOS-myc, thereby removing it. The resulting plasmids were digested with

Xma I, a unique site introduced by the 3' oligo during the PCR amplification, and were ligated with a pair of oligos containing the HA-epitope sequence. To create HA-tagged full length Npn-1 and Npn-1Δc, the a domains were reintroduced into the Npn-1Δa and Npn-1Δac constructs. A 950 bp EcoRI-Pst I fragment containing the signal sequence and a domains of Npn-1 was used to replace the signal sequences of the Npn-1Δa and Npn-1Δac constructs. To make a GPI-linked neuropilin-1 ectodomain construct, the entire ectodomain of neuropilin-1 (M1–P855), was amplified by PCR. Primers were designed to introduce a Hind III site at the 5' end and an EcoRI site at the 3' end. The GPI anchorage of chicken axonin-1 (Lierheimer et al., 1997) was fused to the coding region of the neuropilin-1 ectodomain via its EcoRI site. The chimeric Npn-2/-1 construct consists of the N-terminal portion of the ectodomain of neuropilin-2 (M1–Y645) fused at the junction of the MAM domain by a BspEI site to the C-terminal portion of neuropilin-1 (G641-stop).

Deletion mutants of Sema III used for binding studies were N-terminally tagged with human AP and contained the dimerization motif of the constant region (Fc) of human IgG attached at the C terminus. All ligand constructs were generated by PCR using Tth-polymerase and cycling conditions as recommended by the manufacturer. Ligand constructs were assembled in the expression vector pCDNA 1.1 (Invitrogen) and verified by DNA sequencing. The mutant harboring only the sema domain of Sema III covers amino acids R31–C566 of the coding region and is fused by a Sal I site to the Fc portion of human IgG. The Ig-basic fragment of Sema III consists of the coding region C-terminal to the sema domain (S567–V770) and is joined via XhoI/SalI sites to the human Fc fragment. As a negative control, we generated a construct in which the AP moiety was directly fused to the Fc fragment. All ligand constructs used for binding studies contain a C-terminal myc flag for characterization of the corresponding recombinant proteins.

Ligand Preparation

AP-tagged ligands were produced in HEK 293T cells. DNA was introduced into cells by Lipofectamine (GIBCO) or CaPO₄ coprecipitation. Conditioned medium was harvested 48 hr posttransfection and concentrated in Centri-prep 30 devices (Amicon). Ligand concentration was determined as described (Flanagan and Leder, 1990) assuming a specific activity of 2000 U/mg. VEGF was obtained from Sigma, and [¹²⁵I]VEGF was obtained from Amersham.

Binding Assays

Npn-1 full length and deletion constructs were expressed in COS cells following transfection by Lipofectamine. Forty-eight hours posttransfection, cells were washed with PBS and incubated with ligand for 1 hr at room temperature. Cells were washed six times with HBHA (Cheng and Flanagan, 1994) and lysed in 10 mM Tris and 1% Triton X-100 (pH 8.0). Triton X-100 insoluble proteins were removed by centrifugation at 10,000 × g. AP activity present in the soluble fraction was determined by a soluble AP assay (Flanagan and Leder, 1990). To control for differences in the amount of cellular material in each lysate, bound AP activity was normalized by the amount of total soluble protein in the lysate, as determined by a bicinchoninic acid (BCA) protein assay (Pierce). To control for differences in the expression of various deletion constructs on the surface of the cells, the relative surface expression of Npn-1 was determined. Cells transfected in parallel with those used for the ligand-binding assays were washed in Dulbecco's Modified Eagle Medium (DMEM) and incubated with a saturating concentration of anti-Npn-1 antibodies (anti-[a] domain or anti-[b2/c] domains) diluted in DMEM/5% FBS for 1.5 hr at 4°C. Cells were washed six times in HBHA, then incubated in a saturating concentration of AP-conjugated goat anti-rabbit IgG for 1.5 hr at 4°C. Cells were washed and lysed, and the bound AP activity was determined as described above for ligand binding. Bound AP activity was again normalized for soluble protein concentration in the lysates. The expression of the Npn-1 deletions relative to full length Npn-1, as reflected by antibody binding, was used to adjust the AP activity values obtained in the ligand-binding assay. [¹²⁵I]VEGF-binding assays were performed as described above for ligand binding, except that binding values were not normalized for expression of neuropilin constructs.

IP and Immunoblotting

HEK 293T cells were transfected with Npn-1 constructs by Lipofectamine. Forty-eight hours posttransfection, cells were lysed in IP buffer (50 mM HEPES, 100 mM NaCl, 1 mM EDTA, 50 mM NaF, 10 mM sodium pyrophosphate, 1% Triton X-100, 0.5 mM PMSF, 0.3 μ M aprotinin, 10 μ M leupeptin, and 1 μ M pepstatin, [pH 7.5]). Triton X-100 insoluble proteins were removed by centrifugation at 10,000 \times g for 10 min at 4°C. Concentration of total soluble proteins was determined by BCA assay (Pierce). IPs were routinely performed with 0.5 mg of protein in a volume of 0.5 ml to which 1.5 μ l of anti-myc (9E10) ascites was added. Immune complexes were recovered with a 10-fold binding excess of protein G-sepharose (Gammabind, Pharmacia). Following washing in IP buffer, proteins were eluted in Laemmli sample buffer. Whole-cell lysates were prepared by mixing of the IP buffer lysates with 4 \times Laemmli sample buffer. All protein samples were heated to 100°C for 5 min. Proteins were electrophoresed through standard SDS polyacrylamide gels and transferred to polyvinylidene difluoride (PVDF) membranes. Membranes were blocked in TBS containing 0.5% nonfat dried milk and 0.1% Tween-20. Anti-HA monoclonal antibody (Boehringer) was used as a primary antibody at a concentration of 0.4 μ g/ml; anti-Npn-1 (Kolodkin et al., 1997) polyclonal rabbit IgG was used at 0.5 μ g/ml. For secondary antibody binding, peroxidase-conjugated sheep anti-mouse or donkey anti-rabbit Ig (Amersham) was used at a dilution of 1:5000. Bound secondary antibody was detected with a chemiluminescent peroxidase substrate (Super Signal, Pierce).

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Note Added in Proof

While this manuscript was in review, a paper appeared that includes data showing that Sema IV can function independent of neuropilin-1 as a repulsive guidance cue (Chédotal et al., 1998), and another paper appeared that includes data showing that neuropilin-2 can function as a receptor for Sema A and Sema E (Takahashi et al., 1998).

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