Cell, Vol. 95, 903–916, December 23, 1998, Copyright ©1998 by Cell Press

Plexin A Is a Neuronal Semaphorin Receptor that Controls Axon Guidance

Margaret L. Winberg,* Jasprina N. Noordermeer,* Luca Tamagnone,† Paolo M. Comoglio,† Melanie K. Spriggs,‡ Marc Tessier-Lavigne,§ and Corey S. Goodman*‖

*Howard Hughes Medical Institute
Department of Molecular and Cell Biology
University of California
Berkeley, California 94720

†Department of Molecular Oncology
Institute for Cancer Research
University of Torino Medical School
I-10060 Candiolo, Torino
Italy

‡Department of Molecular Biology
Immunex Corporation
51 University Street
Seattle, Washington 98101

§Howard Hughes Medical Institute
Department of Anatomy and
Department of Biochemistry and Biophysics
University of California
San Francisco, California 94143

Summary

The Semaphorins comprise a large family of secreted and transmembrane proteins, some of which function as repellents during axon guidance. Semaphorins fall into seven subclasses. Neuropilins are neuronal receptors for class III Semaphorins. In the immune system, VESPR, a member of the Plexin family, is a receptor for a viral-encoded Semaphorin. Here, we identify two *Drosophila* Plexins, both of which are expressed in the developing nervous system. We present evidence that Plexin A is a neuronal receptor for class I Semaphorins (Sema 1a and Sema 1b) and show that Plexin A controls motor and CNS axon guidance. Plexins, which themselves contain complete Semaphorin domains, may be both the ancestors of classical Semaphorins and binding partners for Semaphorins.

Introduction

Neuronal growth cones are guided by a variety of attractive and repulsive molecules, functioning at short or long range (Tessier-Lavigne and Goodman, 1996). One family of secreted and transmembrane axon guidance molecules is the Semaphorins, which are defined by having a conserved ~500 amino acid extracellular Sema domain (Kolodkin et al., 1993). Since the discovery of the prototypic Semaphorins in insects (Kolodkin et al., 1992, 1993), chick (Luo et al., 1993), and human (Kolodkin et al., 1993), a large number of Semaphorins, representing seven subclasses, have been discovered in animals from diverse phyla. To date, the family includes ~25 members in mammals. Many appear to function as chemorepellents or inhibitors, influencing steering, fasciculation, branching, and synapse formation (Kolodkin et al., 1992; Luo et al., 1993; Matthes et al., 1995; Messersmith et al., 1995; Puschel et al., 1995; Winberg et al., 1998). Semaphorins can also function as attractants (Wong et al., 1997; Bagnard et al., 1998).

Neuropilins have been shown to function as receptors (or components of receptor complexes) for class III secreted Semaphorins (Chen et al., 1997; He and Tessier-Lavigne, 1997; Kolodkin et al., 1997). Neuropilin-1 (Takagi et al., 1991), originally discovered in *Xenopus*, is expressed by specific layers in the optic tectum (Takagi et al., 1987). Though it can mediate homotypic adhesion, recent studies revealed that Neuropilin-1 also binds with high affinity many class III Semaphorins, including Sema III, Sema E, and Sema IV (Chen et al., 1997; He and Tessier-Lavigne, 1997; Kolodkin et al., 1997; Takahashi et al., 1998). Antibodies to Neuropilin-1 block the ability of Sema III to repel sensory axons and to induce growth cone collapse. Studies using mutant mice also indicate that Neuropilin-1 is a Sema III receptor (Kitsukawa et al., 1997). The closely related protein Neuropilin-2 (Chen et al., 1997; Kolodkin et al., 1997) does not bind Sema III, but it does appear to be a receptor component for other class III Semaphorins, including Sema A, Sema E, and Sema IV (Chen et al., 1997; Takahashi et al., 1998).

What are the receptors for other Semaphorins? The genomes of several viruses, including the poxviruses vaccinia and variola (Kolodkin et al., 1993) and a herpesvirus (Ensser and Fleckenstein, 1995), harbor open reading frames encoding secreted Semaphorins that might modulate immune function (Kolodkin et al., 1993). One such viral Semaphorin, vaccinia A39R, was used to affinity purify a novel cellular receptor from a human B cell line (Comeau et al., 1998). This virus-encoded Semaphorin protein receptor (VESPR) is a novel transmembrane protein that is a divergent member of the Plexin family. VESPR binds A39R with high affinity and herpesvirus AHV Sema with lower affinity. Human Sema K1 is a class VII Semaphorin that may be the cellular counterpart of AHV Sema; Sema K1 does not bind either Neuropilin (Xu et al., 1998).

Like Neuropilins, Plexins were identified through a screen for antigens expressed in the optic tectum (Takagi et al., 1987). *Xenopus* Plexin-1 was shown to contain an ectodomain related to the c-*met* protooncogene and to mediate calcium-dependent homophilic binding (Ohta et al., 1995). Neuropilin-1 and Plexin-1 are expressed on different groups of olfactory axons that independently segregate, leading to the speculation that these two proteins help organize axon projections (Satoda et al., 1995). Plexin-1 was subsequently cloned in mice and is the prototype of a family of proteins (Kameyama et al., 1996).

Plexins in human were cloned independently, based on their homology to the ectodomain of the oncogene MET (Maestrini et al., 1996). Called the SEX family of genes, several members (SEX, OCT, and NOV) are expressed predominantly in the brain, giving rise to the

‖To whom correspondence should be addressed.

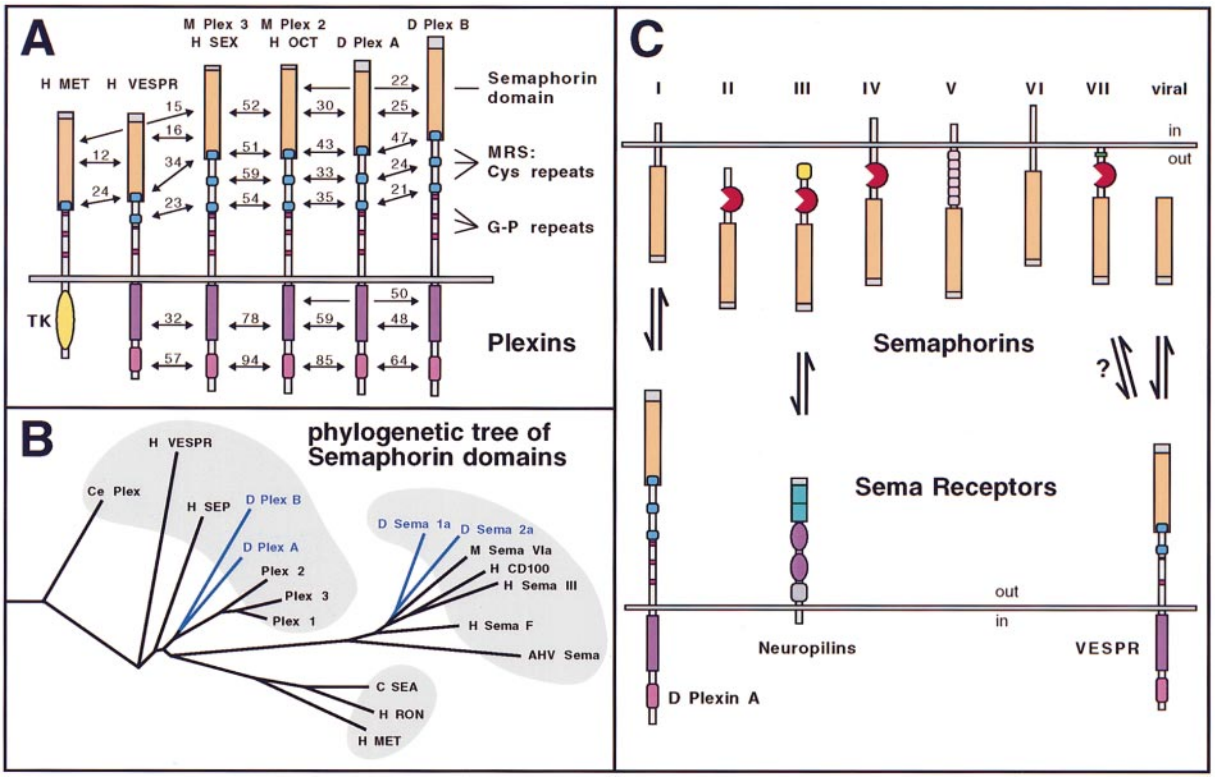

Figure 1. Plexins and Related Proteins

(A) Domain structure of Plexins, with percent amino acid identity between domains. MRS, MET-related sequence; G-P, glycine-proline-rich repeat; TK, tyrosine kinase domain.

(B) Relationships between Semaphorin domains of Plexins, Semaphorins (representing 7 subclasses), and MET-like receptors determined by CLUSTAL analysis; fly proteins are in blue.

(C) Semaphorin subclasses and Semaphorin receptors: VESPR (Comeau et al., 1998), Neuropilins (Chen et al., 1997; He and Tessier-Lavigne, 1997; Kolodkin et al., 1997), Plex A (this study). Orange, Sema domains; red, Ig domains; pink, Thrombospondin repeats. Neuropilins are structurally unrelated to Plexins.

suggestion that they represent novel neuronal receptors for unknown ligands.

Given that a divergent member of the Plexin/SEX family is a receptor for the viral Semaphorin A39R and that most Plexins are expressed in the developing nervous system, we wondered whether Plexins are neuronal Semaphorin receptors and whether they function to control axon guidance. To investigate this possibility, we have cloned and characterized the genes encoding two Plexins in *Drosophila*.

Here, we present genetic and biochemical evidence that Plexin A (Plex A) in *Drosophila* is a receptor for the transmembrane class I Semaphorins Sema 1a and 1b, and we show that Plex A controls important aspects of axon guidance. (Because we and others find two class I Semaphorins and two class II Semaphorins in *Drosophila*, we and our colleagues [A. Kolodkin and S. Baumgartner, personal communication] have renamed the original Sema I as Sema 1a, the new class I Semaphorin as Sema 1b, the original Sema II as Sema 2a, and the new class II Semaphorin as Sema 2b.) Previous studies (Yu et al., 1998) showed that mutations in *Sema1a* display specific defects in motor and CNS axon guidance. We show that mutations in *PlexA* display the same phenotypes and that the two loci interact genetically in a fashion consistent with their functioning in the same signaling pathway. Along with selective binding results, these data show that Plexins do indeed function as neuronal Semaphorin receptors and that Plexins control important aspects of axon guidance.

Results

Identification of Two *Drosophila* Plexins

Candidate partial cDNAs for *Drosophila* Plexins were found in a screen for transmembrane and secreted cDNAs (Kopczynski et al., 1996, 1998). Additional cDNA clones were identified in a public database of expressed sequence tags. These clones represent transcripts from two genes that we named *PlexA* and *PlexB*. Probes derived from these cDNAs were used to isolate additional clones that span the complete open reading frames (ORF). Conceptual translation predicts that each is a large type 1 membrane glycoprotein; the Plex A ORF is 1945 amino acids long with 16 potential N-linked glycosylation sites, and the Plex B ORF is 2051 amino acids with 15 sites.

These two fly proteins are similar to previously described Plexins (Ohta et al., 1995; Kameyama et al., 1996; Maestrini et al., 1996) with homology extending the length of each protein (Figures 1 and 2). The extracellular domains include the motif of three cysteine-rich repeats (called MET-related sequences [MRS]) (Maestrini et al.,

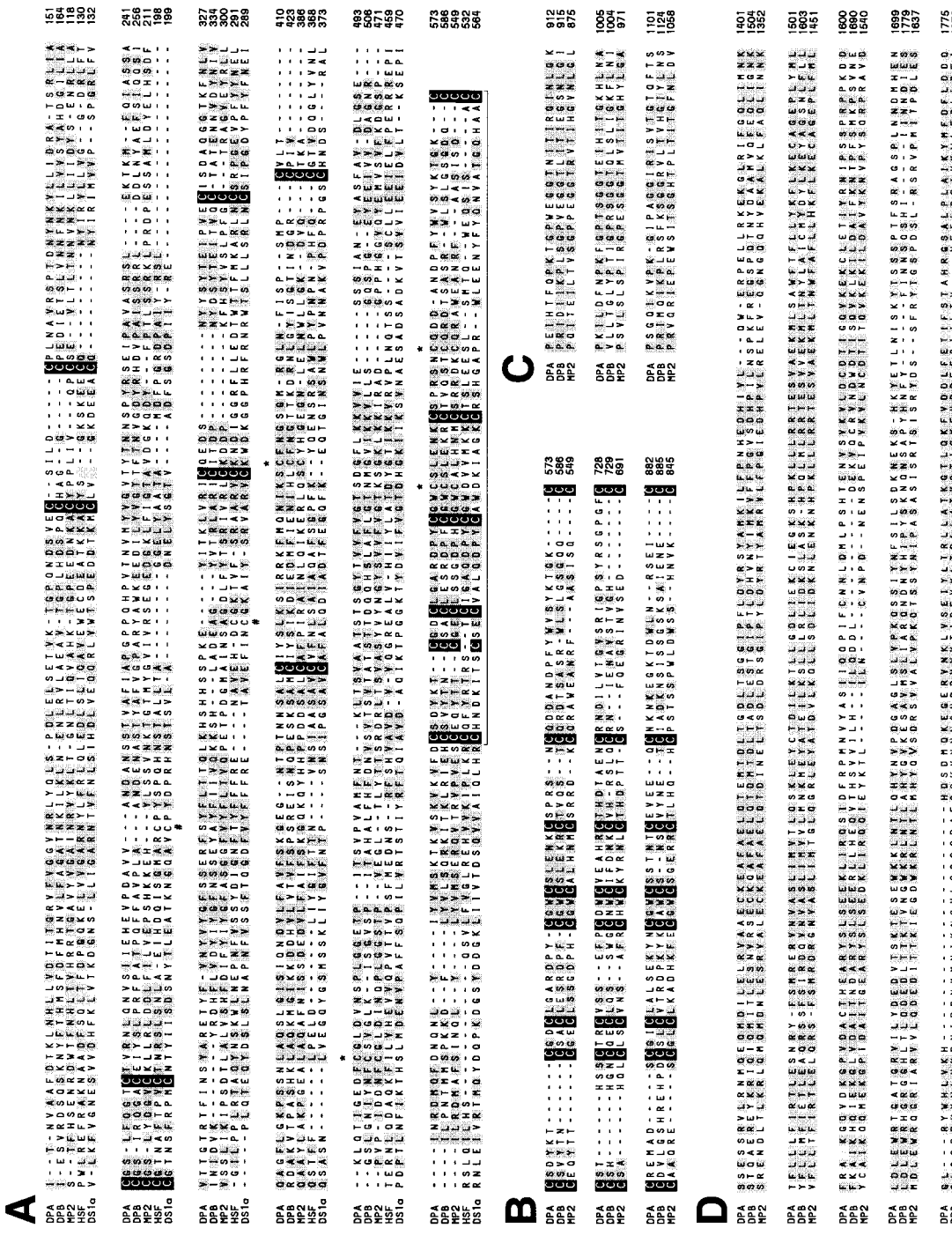

Figure 2. Protein Alignments of Conserved Domains

(A) Sema domains of Plexins and Semaphorins. *Drosophila* Plexins A and B (DPA and DPB) compared with mouse Plexin 2 (MP2), human Sema F (HSF), and *Drosophila* Sema 1a (DS1a); identical residues are shaded. Cysteines conserved between Plexins and Semaphorins are in reverse color. Two additional cysteines are conserved among Semaphorins (#), and four more are conserved among Plexins (*). The first MRS repeat is underlined. Amino acid numbers are taken from the proprotein sequence.

(B) Three MET-related sequence (MRS) repeats are found in *Drosophila* as well as in vertebrate Plexins.

(C) Plexins contain three extracellular glycine-proline-rich (G-P) repeats.

(D) The intracellular domains of Plexins are highly conserved.

1996) in the middle of the ectodomain (Figures 1A and 2B). The membrane-proximal regions of the Plexin ectodomains contain another type of repeat that is rich in glycine and proline residues (G-P repeats) (Figures 1A and 2C). Three G-P repeats are spaced ~50 amino acids apart; the intervening sequences are not notably similar except that each contains two conserved cysteines. All of these features of the ectodomains are shared among

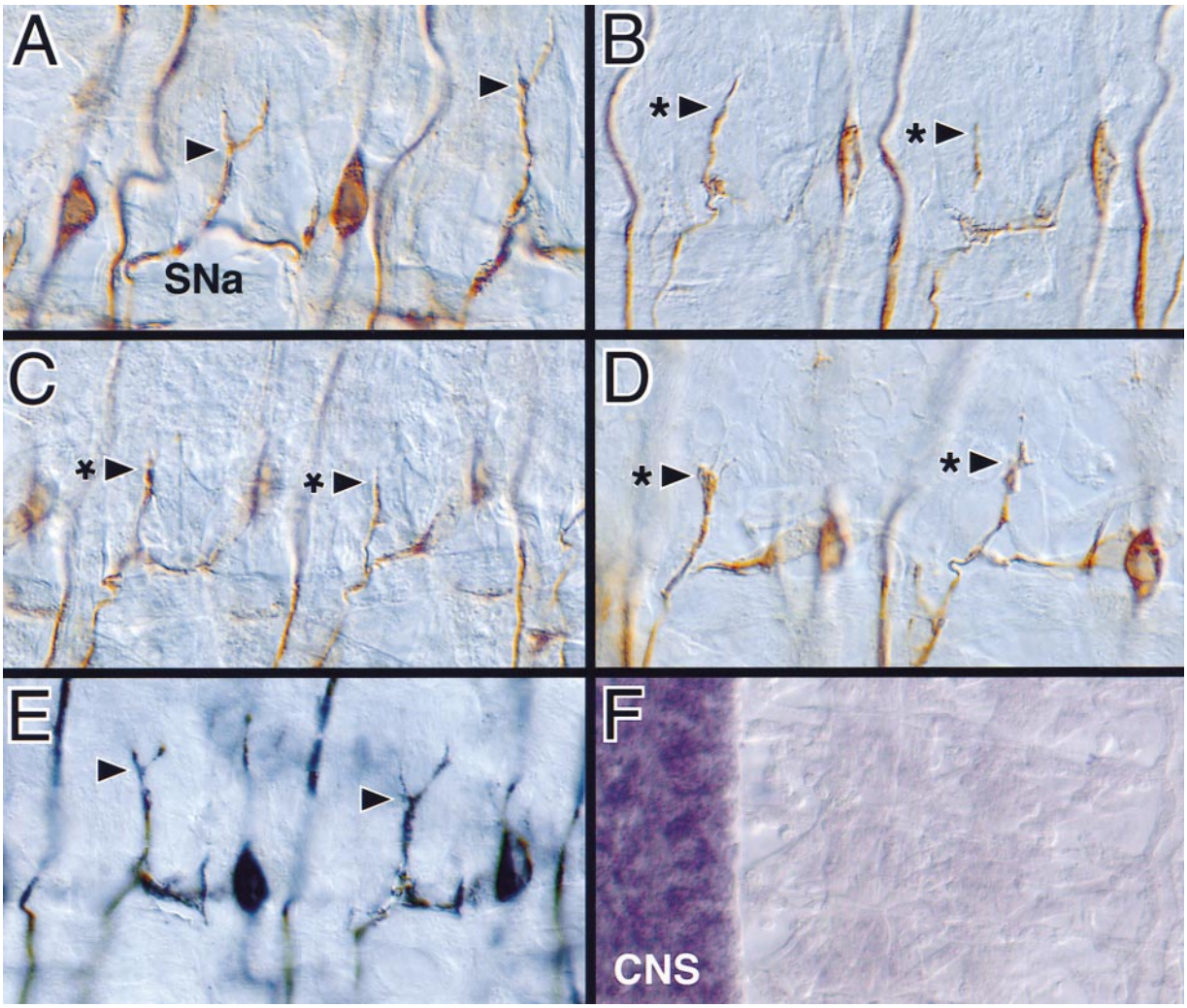

Figure 3. Abnormal Projection of the SNa in *PlexA* and *Sema1a* Mutants

(A–E) Photomicrographs of filleted late stage 16 embryos stained with mAb 1D4 to reveal motor projections, focusing on the lateral muscle area. Two segments per panel; anterior is left and dorsal is up.

(A) Wild type. The SNa is bifurcated normally at choice point 2 (arrowheads).

(B) *Sema1a* mutant. The dorsal SNa branch is not bifurcated (* arrowheads).

(C) *PlexA* deficiency mutant. The dorsal SNa is similarly abnormal (* arrowheads).

(D) *Sema1a*/+; *PlexA*/+ transheterozygote. The dorsal SNa branch is stalled at choice point 2 (* arrowheads).

(E) Rescue of the SNa in a *PlexA* deficiency mutant by restoring neuronal expression of Plex A with *elav-GAL4* and *UAS-PlexA*. The dorsal branch bifurcates normally at choice point 2 (arrowheads).

(F) *PlexA* mRNA is expressed broadly at high levels in the CNS (left). Muscles and other peripheral tissues express much lower levels (right). Anterior is up; ventral midline is at left edge.

Drosophila and vertebrate Plexins. They are also shared with MET-like tyrosine kinase receptors and VESPR, except that in these cases, there are different numbers of repeats (Figure 1A).

The intracellular region of vertebrate Plexins has been divided into two blocks of strong homology, separated by a variable linker (Maestrini et al., 1996). Both *Drosophila* proteins conform to this pattern; indeed, the area of highest amino acid identity between the fruit fly and vertebrate Plexins is within these intracellular domains (Figures 1A and 2D).

Plexins Contain Semaphorin Domains

A region of ~100 amino acids overlapping with the first of the cysteine (MRS) repeats was shown (Comeau et al., 1998) to resemble sequences at the C terminus of the Sema domain found in all Semaphorins (Kolodkin et al., 1993). Upon further examination we find that the ectodomains of all Plexins contain a complete ~500 amino acid Sema domain, with 14 of the 16 conserved cysteines and many short peptide sequences that are characteristic of the Semaphorin family (Figure 2A). The MET-related receptors also contain complete (albeit divergent) Sema domains.

The Sema domains of Semaphorins typically share 25%–30% amino acid identity between pairs of Semaphorins. The Sema domains of Plexins share 25%–30% amino acid identity between pairs of Plexins. Between Plexins and Semaphorins, the Sema domains share only 15%–20% amino acid identity. The Sema domains of

Table 1. Genetic Interactions between Loss-of-Function Mutations in *Sema1a* and *PlexA*

Genotype	ISNb Bypass	ISNb Abnormal Innervation of Muscles			SNa Abnormal Branching	TN Ectopic Contacts	CNS Breaks in Outer Fascicle
		6/7	13	12			
Wild type	0% (n = 105)	4.8 (105)	11.4 (105)	1.9 (105)	19.0 (105)	4.8 (104)	1.8 (108)
Sema1a$^{-/+}$	0.7 (138)	7.2 (138)	13.0 (138)	4.3 (138)	25.1 (135)	5.9 (134)	4.9 (144)
PlexA Df$^{-/+}$	1.8 (108)	4.6 (108)	6.5 (108)	2.8 (108)	19.0 (105)	6.7 (105)	3.7 (108)
Sema1a$^{-/-}$	11.8 (102)	52.0 (102)	41.2 (102)	58.9 (102)	67.0 (100)	4.4 (90)	50.9 (108)
PlexA Df$^{-/-}$	19.9 (161)	43.5 (161)	32.9 (161)	55.9 (161)	74.7 (158)	36.5 (156)	49.4 (162)
Sema1$^{-/+}$; *PlexA Df*$^{-/+}$	8.6 (151)	56.3 (151)	25.8 (151)	37.7 (151)	72.1 (154)	6.0 (149)	20.5 (156)
PlexA Df$^{-/-}$; PlexA rescue (*elav-GAL4*; *UAS-PlexA*)	1.4 (72)	9.7 (72)	12.5 (72)	6.9 (72)	30.5 (72)	2.8 (71)	9.7 (72)
PlexA Df$^{-/-}$; *FasII*$^{-/+}$	1.5 (67)	28.3 (67)	11.9 (67)	23.9 (67)	66.7 (63)	6.3 (63)	2.8 (72)

MET receptors share only about 10% amino acid identity with Semaphorins and 15%–20% identity with Plexins. Examination of phylogenetic relationships among the Sema domains of Plexins, Semaphorins, and MET-related receptors shows that each of the three groups of proteins clusters separately (Figure 1B).

Plex A and Plex B Are Expressed in the Nervous System

Embryonic expression of Plex A and Plex B was examined by Northern analysis and by mRNA in situ hybridization. *PlexA* probes detect a single transcript of about 7.5 kb; *PlexB* probes detect two transcripts, approximately 8 and 12 kb (data not shown). *PlexA* and *PlexB* mRNAs show largely similar in situ localization: both are maternally deposited and are broadly distributed during early embryogenesis. Beginning with germband retraction, both transcripts show a reduction in general expression, while remaining highly expressed in the CNS. After embryonic stage 15, during the period in which many motor axons are reaching their targets, *PlexA* and *PlexB* transcripts are largely confined to the CNS, where they are expressed by many neurons. Although transcripts are found in myoblasts, expression does not persist in muscle fibers (Figure 3F).

PlexA Loss-of-Function Mutant Phenotypes Resemble Those of *Sema1a*

Cytological mapping localized the *PlexA* gene to band 102E1-2 on the fourth chromosome. The *PlexA* transcript is absent from embryos homozygous for a small genetic deficiency that uncovers the region, *Df(4)C3* [102D6-F], but it is not affected by the overlapping deficiency, *Df(4)G* [102E6-F]. These strains provide an entry to the genetic analysis of *PlexA* function. If Plexins are indeed Semaphorin receptors, then lesions in the *PlexA* gene might be expected to show similar axon guidance defects as are displayed by embryos mutant for either *Sema1a* (Yu et al., 1998) or *Sema2a* (Winberg et al., 1998).

In embryos lacking Plex A, we found axon guidance defects both in the CNS and in the projections of motor nerves to their muscle targets in the periphery. These embryos did not show any morphological abnormalities, muscle defects, or cell fate changes. Embryos from the *Df(4)G* strain (that does not remove *PlexA*) displayed normal axon guidance, indicating that the observed phenotypes are closely linked with the *PlexA* gene.

To test whether axon guidance phenotypes associated with the deficiency are actually due to the lack of Plex A, we restored expression using a transgenic construct, *UAS-PlexA*, under the transcriptional control of the neuron-specific driver, *elav-GAL4* (Luo et al., 1994). This combination rescues motor and CNS axon guidance defects in homozygous deficiency embryos (Table 1; Figures 3E, 4G, and 6C). Some segments are not completely restored to wild type, but rather than displaying loss-of-function phenotypes, these segments display gain-of-function phenotypes for Plex A (see below). Thus, we conclude that the aberrations seen in the deficiency strain do indeed result from the lack of Plex A. Moreover, neuron-specific replacement of Plex A is sufficient to rescue the observed guidance phenotypes.

PlexA-deficient embryos show axon guidance phenotypes that markedly resemble defects seen in *Sema1a* loss-of-function mutants (Yu et al., 1998). Examples are seen in the "b" branch of the intersegmental nerve (the ISNb), which innervates ventral muscles. ISNb axons normally exit the CNS as part of the ISN; they defasciculate from and exit the ISN at ISNb choice point 1, entering the ventral muscle region as a fasciculated bundle (Figures 4A and 5A). At ISNb choice point 2, a single axon leaves the ISNb to innervate muscle fibers 6 and 7. At ISNb choice point 3, certain growth cones extend further dorsally to innervate muscle 12, while others stop and innervate muscle 13.

In the absence of Plex A, ISNb growth cones often fail to defasciculate from one another at any or all three of the ISNb choice points. Occasionally they fail to exit the ISN and thus bypass the ventral muscles (Figure 4E); in some cases they innervate their ventral muscle targets via small projections made directly from the main branch of the ISN (data not shown). More often, they exit the ISN but then fail to defasciculate from each other at choice points 2 and/or 3, leading to a thickened, stalled nerve branch and failure to innervate muscles 6 and 7 and/or 12 (Figures 4C and 4E; 5B and 5C; Table 1).

In addition to the ISNb, the segmental nerve (SN) is also frequently abnormal, with defects resembling those of *Sema1a* mutants (Yu et al., 1998). In wild-type embryos, the SN exits the CNS, and its main branch, the SNa, extends past the ventral muscle domain to the lateral muscle region. The SNa then divides into a lateral and a dorsal branch at SNa choice point 1, and then further dorsally, bifurcates again at SNa choice point 2 (Figure 3A). In *PlexA*-deficient embryos examined at late stage 16, axons at the second choice point failed to defasciculate from one another in roughly 70% of segments and instead extended dorsally as a single branch (Figure 3C and Table 1).

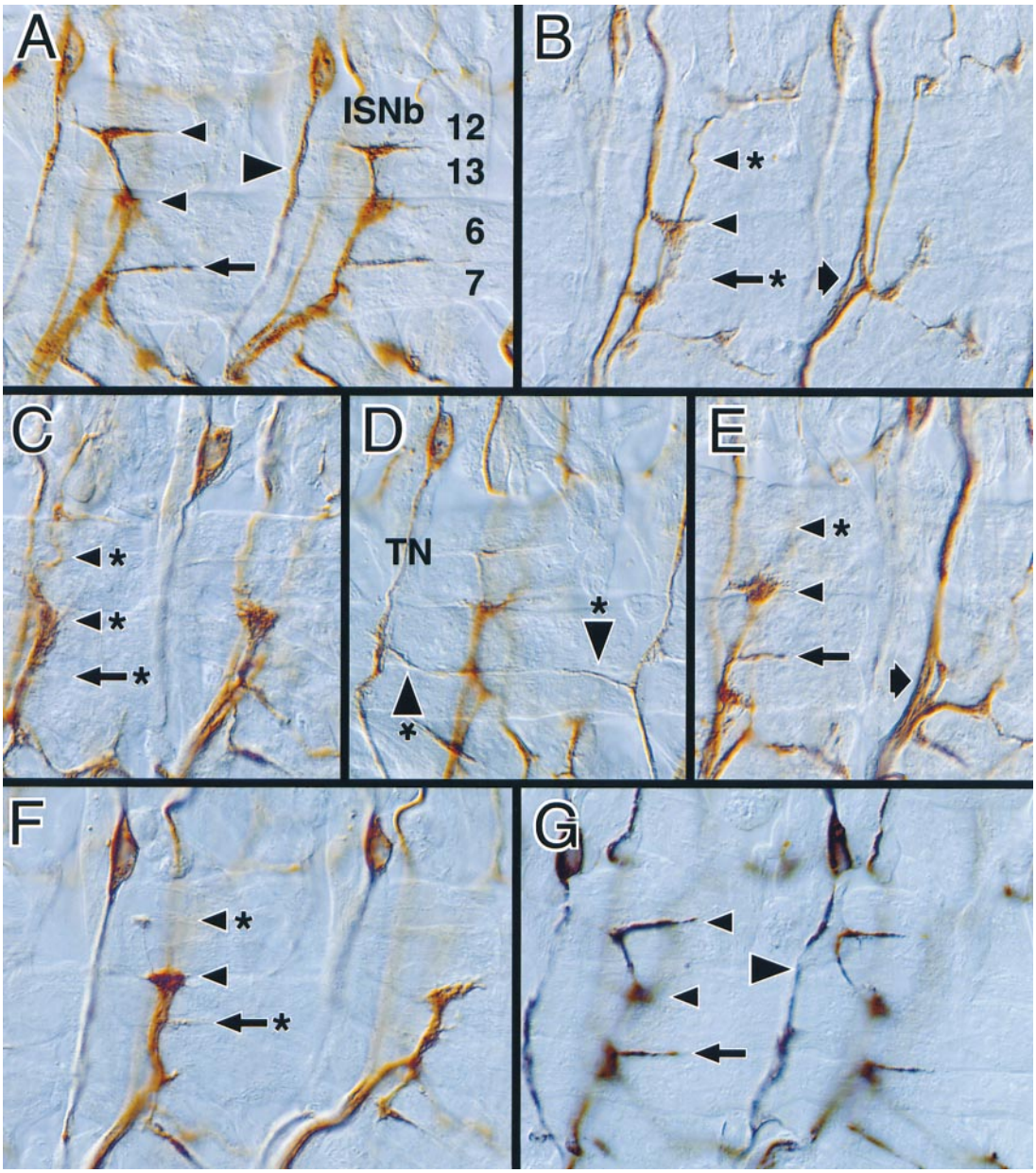

Figure 4. Abnormal Projection of the ISNb in *PlexA* and *Sema1a* Mutants

Late stage 16 embryos stained with mAb 1D4, focusing on the ventral muscle region.

(A) Wild type. The ISNb extends into the ventral domain and forms three major branches, one in the cleft between muscle fibers 6 and 7 (arrow), and one each at the ventral edges of muscle 13 and of muscle 12 (arrowheads). The transverse nerve (TN) runs along the segment border without innervating the ventral muscles (large arrowhead).

(B) *Sema1a* mutant. At left, the ISNb is abnormally stalled at the edge of muscle 13 (arrowhead), fails to reach muscle 12 (* arrowhead), and fails to extend into the muscle 6/7 cleft (* arrow). At right, the ISNb has not separated fully from the major ISN projection (fat arrow).

(C, D, and E) *PlexA* deficiency mutants. (C) The ISNb fails to innervate muscle 12 and shows abnormal morphology at muscle 13 (* arrowheads). Innervation at the muscle 6/7 cleft has failed (* arrow). (D) The TN projects abnormally onto ventral muscles 6 and 7 (* large arrowheads). (E) At left, muscle 12 is not innervated (* arrowhead), but the muscle 6/7 cleft is innervated normally (arrow). At right, the ISNb is not separated fully from the major ISN projection (fat arrow).

(F) *Sema1a*/+; *PlexA*/+ transheterozygote. Innervation at muscles 6, 7, and 12 is weak (* arrow, * arrowhead) or absent (at right). Arrowhead indicates innervation at muscle 13.

(G) Rescue of the ISNb and TN in a *PlexA* deficiency mutant by restoring neuronal expression of Plex A. Normal innervation at muscles 12 and 13 (arrowheads), and the muscle 6/7 cleft (arrow). The TN projects normally past the ventral muscles (large arrowhead).

Projections within the CNS are also abnormal in both *Sema1a* and *PlexA* mutants. Three major longitudinal axon fascicles on each side of the CNS are detected with the 1D4 mAb against Fas II. In wild-type embryos these tracts are evenly spaced and show fairly uniform thickness. In embryos lacking Plex A, the outermost

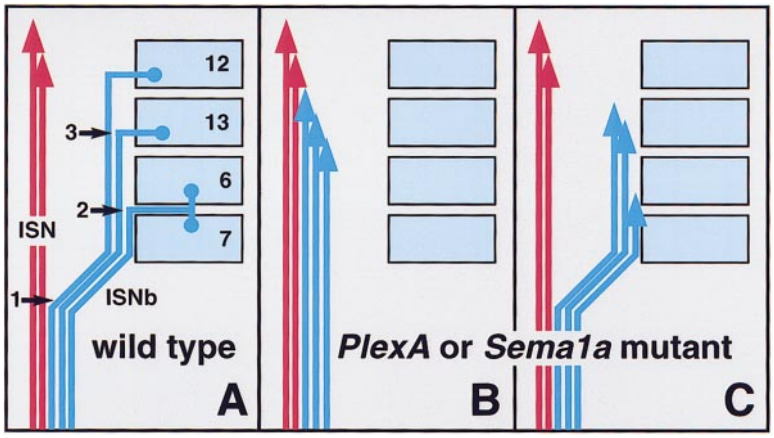

Figure 5. ISNb Phenotypes in *Sema1a* and *PlexA* Mutants

(A) Three choice points for the ISNb are indicated by arrows. At choice point 1, ISNb axons (blue) defasciculate from the ISN (red). At choice points 2 and 3, axons within the ISNb defasciculate from each other, allowing individual axons to innervate target muscles 12 or 13 or 6 and 7.

(B) In the mutants, the ISNb may fail to separate from the ISN at choice point 1 and bypass the ventral muscles.

(C) More often in the mutants, the ISNb leaves the ISN and enters the ventral muscle domain but then fails at one or both choice points in this region.

longitudinal Fas II–positive fascicle is often disrupted, being thinner in some segments, discontinuous in others, and sometimes fused with the middle Fas II–positive fascicle (Figure 6B).

This analysis of phenotypes in *PlexA Df* mutant embryos (and their rescue by a *PlexA* transgene) clearly indicates an important role for Plex A in axon guidance. The phenotypes for *PlexA* in the projections of the ISNb, the SNa, and within the CNS are strikingly similar, both qualitatively and quantitatively, to those reported for null mutations of *Sema1a* (Yu et al., 1998) (Table 1). The high degree of phenotypic correspondence strongly suggests that these two genes encode components of the same pathway. Because Sema 1a has been described as a repulsive ligand for growth cone guidance, we propose that Plex A functions as a Sema 1a receptor for these guidance events.

An Additional Role for Plex A

A further loss-of-function phenotype for *PlexA* is seen in the projection of the transverse nerve (TN). This nerve is composed of two parts, a peripheral neuron that extends an axon toward the CNS, and a pair of central neurons that project outward. These two projections normally extend toward one another along a shared mesodermal substrate, meeting and fasciculating near muscle 7. In *PlexA*-deficient embryos, growth cones from the TN extend ectopic projections onto ventral muscles in 36.5% of segments, compared with 2.8% in the *PlexA* rescue background (Figure 4D). This phenotype is not seen in *Sema1a* mutants, raising the possibility that Plex A may also interact with one or more additional ligands.

PlexA and *Sema1a* Loss-of-Function Mutations Interact Genetically

One way to check the hypothesis that Plex A functions as a Sema 1a receptor is to test for dominant genetic interactions between the two genes. In most cases, reducing gene dosage by one copy (thus reducing protein by 50%) has little phenotypic effect. However, simultaneously reducing the dose of two genes whose protein products function together may sufficiently impair their combined function such that phenotypes appear. Such a "transheterozygous" phenotype has been demonstrated for various ligand–receptor pairs in *Drosophila* (e.g., Delta and Notch; Artavanis-Tsakonas et al., 1995).

We examined embryos heterozygous for either or both *Sema1a* and *PlexA* and found significant enhancement in embryos in which both were heterozygous (Table 1). Each of the phenotypes described above for the ISNb, SNa, and CNS is recapitulated in the double heterozygotes. For example, removing one copy of either *Sema1a* or *PlexA* permits nearly wild-type levels of ventral muscle innervation by ISNb neurons. Removing both copies of either gene leads to abnormal innervation in most segments. Removing one copy each of *Sema1a* and *PlexA* causes the same repertoire of defects in a similar proportion of segments as the single homozygous mutants (Figure 4F and Table 1). Likewise, the rate of defasciculation failures in the dorsal branch of the SNa is almost the same in the transheterozygous combination as it is in the *Sema1a* or *PlexA* homozygous mutants alone, roughly 70% (Figure 3D). The fraction of affected segments within the CNS is smaller in the transheterozygotes (20% compared to 50% in *Sema1a* or *PlexA*) but still much more than would be expected from simple addition ($<$10%). These results strongly suggest that Sema 1a and Plex A are in the same pathway and further suggest a direct physical interaction between the two proteins.

As a negative control, we made the double heterozygous combination between the *PlexA* deficiency and mutations in the *beaten path* (*beat*) gene (Fambrough and Goodman, 1996). Beat has been shown to function as a motor axon antiadhesion molecule; *beat* homozygous mutant embryos show highly penetrant ISNb and ISNd bypass phenotypes and often fail to innervate muscles in the ventral domain. Nevertheless, mutations in *beat* do not show dominant interactions with either *Sema1a* or *PlexA* ($<$6% of segments abnormal, n = 81).

PlexA Loss-of-Function Suppresses *Sema1a* Gain-of-Function Phenotypes

Sema 1a is expressed by neurons and is required for appropriate defasciculation. Loss-of-function analysis for this gene does not indicate whether Sema 1a functions as a ligand or as a receptor. However, misexpressing Sema 1a on muscles repels motor axons, demonstrating that Sema 1a is able to act as a target-derived

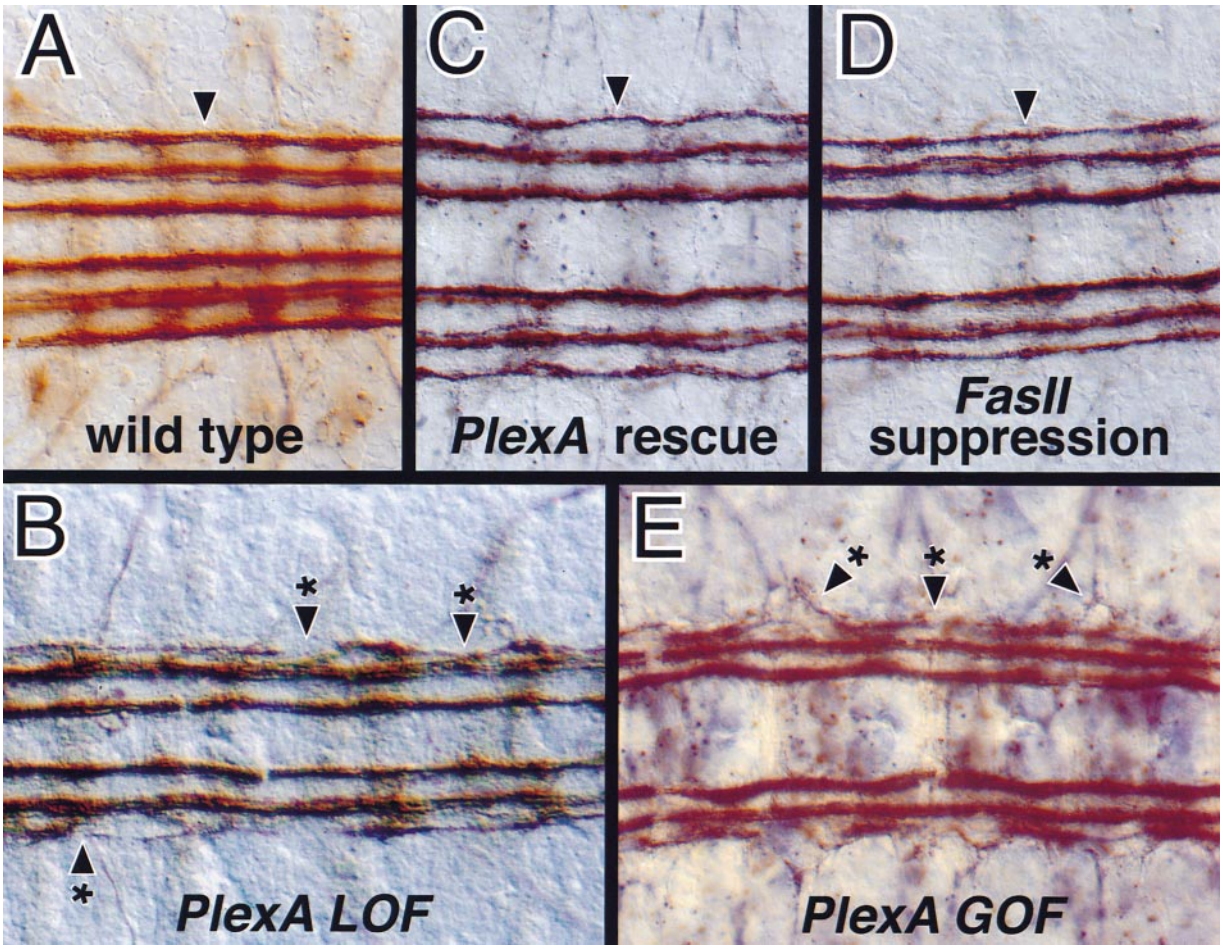

Figure 6. CNS Fasciculation Defects in *PlexA* Loss- or Gain-of-Function Mutants

Stage 17 embryos stained with mAb 1D4 and focusing on the ventral midline; anterior is left. (B and C) are also stained for *PlexA* 5′UTR mRNA to identify deficiency homozygotes.

(A) Wild type shows three major longitudinal connectives on each side of the CNS.

(B) *PlexA* deficiency mutant. The outermost connective is discontinuous, either stalling or fusing with the adjacent connective.

(C) Rescue of the CNS phenotype in a *PlexA* deficiency mutant by restoring neuronal expression of Plex A.

(D) Suppression of the CNS phenotype in a *PlexA* deficiency mutant by reducing gene dosage of *FasII* (*FasII*^{e76} heterozygote).

(E) Neuronal overexpression of Plex A in an otherwise wild-type genetic background. All the major fascicles show occasional breaks. Axons from the outermost connective extend abnormally away from the rest of the CNS.

repulsion cue (Yu et al., 1998). If Plex A is the receptor for Sema 1a, then reducing Plex A expression levels in the presence of ectopic Sema 1a ligand should suppress the severity of the gain-of-function repulsion phenotypes.

We used two different GAL4 enhancer trap lines (Lin and Goodman, 1994) to misexpress Sema 1a on muscle subsets. The first, *H94-GAL4*, is highly expressed by muscle fibers 6 and 13, and moderately by muscle 12 (genetic rescue data suggest that it is also expressed by some motor neurons at a very low level, although this has never been directly visualized [Davis et al., 1997]). Using *H94-GAL4* to drive *UAS-Sema1a* in these muscles disrupts their innervation by ISNb axons, with the strongest effect seen at muscle 13. A second line, *F63-GAL4*, was used to drive *UAS-Sema1a* specifically in muscles 6 and 7, thereby inhibiting innervation at the muscle 6/7 cleft (F63 is not expressed by motor neurons). For both GAL4 lines, the inhibitory effect of muscle-derived Sema 1a is suppressed in *PlexA Df* heterozygotes (Table 2). This dominant suppression of the *Sema1a* gain-of-function suggests that neuronally expressed Plex A acts downstream of Sema 1a in mediating repulsion.

Ectopic expression also provided a means to test for interactions between Plex A and Sema 1b, another *Drosophila* protein similar to Sema 1a (Yu et al., 1997). We used *H94-GAL4* and *F63-GAL4* to test whether Sema 1b can also act as a muscle-derived repellent and found that it was equally capable in repelling motor axons as was Sema 1a (Table 2). Likewise, reducing the gene dose of *PlexA* suppresses to a similar extent the guidance defects caused by misexpression of Sema 1b. Based on the similarity of structure and sequence, as well as

Table 2. Genetic Suppression of *Sema1a* and *Sema1b* Gain-of-Function by *PlexA* Partial Loss-of-Function

A. Ectopic Expression of Semaphorins on Muscles 6, 13, and 12

Genotype	Abnormal Innervation of Muscle 13
H94-GAL4/+	19.1% (n = 94)
UAS-Sema1a/+; *H94-GAL4*/+	52.7 (127)
UAS-Sema1a/+; *H94-GAL4*/+; *PlexA* $Df^{-/+}$	20.7 (106)
UAS-Sema1b/+; *H94-GAL4*/+	52.5 (120)
UAS-Sema1b/+; *H94-GAL4*/+; *PlexA* $Df^{-/+}$	22.8 (79)

B. Ectopic Expression of Semaphorins on Muscles 6 and 7

Genotype	Missing or Weak Innervation at the Muscle 6/7 Cleft
F63-GAL4/+	3.7% (n = 135)
UAS-Sema1a/+; *F63-GAL4*/+	40.3 (154)
UAS-Sema1a/+; *F63-GAL4*/+; *PlexA* $Df^{-/+}$	21.8 (142)
UAS-Sema1b/+; *F63-GAL4*/+	47.7 (86)
UAS-Sema1b/+; *F63-GAL4*/+; *PlexA* $Df^{-/+}$	29.8 (94)

parallel gain-of-function phenotypes and suppression, we propose that Sema 1b may serve as an additional ligand for Plex A.

Reducing the Level of Fas II Suppresses *PlexA* Phenotypes

Given the model that Plex A mediates repulsive guidance and thus drives defasciculation, we asked whether removal of the major motor axon cell adhesion molecule, Fasciclin II, would genetically suppress *PlexA* mutant phenotypes. In agreement with the model, all of the ISNb motor axon *PlexA* phenotypes (but not the SNa mutant phenotypes) and the CNS *PlexA* phenotypes are partially suppressed when one copy of the *FasII* gene is removed (Figure 6D and Table 1).

Overexpression of Plex A Disrupts Axon Guidance

We also examined defects arising from overexpression of Plex A. Driving high levels of *UAS-PlexA* in all neurons using *elav-GAL4* leads to axon guidance phenotypes in all parts of the motor projection and also within the CNS (Figures 6E and 7). In most cases, Plex A overexpression results in phenotypes opposite of those seen in the *PlexA* loss-of-function. For example, in 66% of segments, Plex A overexpression caused the dorsal SNa to split prematurely into multiple projections (n = 70) (Figure 7A). Similarly, the dorsal extension of the ISN defasciculated inappropriately in 25% of segments (Figure 7B). The TN, which made exuberant contacts onto ventral muscles in the deficiency embryos, stalled in 26% of segments with Plex A overexpression, resulting in the two halves of the nerve failing to meet (Figure 7D). These defects are readily interpreted as resulting from increased sensitivity to repulsive cues.

For the ISNb, the Plex A overexpression phenotype is equally dramatic but less easily interpreted. ISNb axons often fail to reach their muscle targets; however, there is no apparent increase in axon–axon repulsion or defasciculation. Instead, the nerve displays a thickened clumped structure, with stalls at choice points 2 and 3. Thus, for this nerve, the gain-of-function looks remarkably similar to the loss-of-function phenotype (compare Figure 7C to Figures 4C and 4E).

Within the CNS, all three Fas II–positive axon fascicles are disrupted by Plex A overexpression (Figure 6E). The longitudinal tracts are less tightly fasciculated than normal and not always continuous between segments. The outermost Fas II–positive fascicle, which in the loss-of-function embryos stalls and sometimes fuses with the neighboring fascicle, in Plex A–overexpressing embryos defasciculates and extends away from the CNS.

Plex A Binds Sema 1a and Sema 1b In Vitro

Genetic analysis provides strong evidence that Plex A is a necessary component of the Sema 1a signaling pathway and is likely to function as a Sema 1a receptor. Moreover, it suggests that Plex A may have additional ligands, including Sema 1b. We tested these possibilities in a heterologous expression system using alkaline phosphatase (AP) fusion proteins (Cheng et al., 1995; He and Tessier-Lavigne, 1997) in binding assays on membranes from COS cells transiently transfected with a full-length Plex A construct (Figure 8A).

Both AP-Sema 1a and AP-Sema 1b bound to Plex A–expressing membranes at significantly higher levels than to mock-transfected membranes or to membranes containing another *Drosophila* repulsive axon guidance receptor, Robo 1 (K. Bland and C. S. G., unpublished data) (Kidd et al., 1998). We did not observe specific binding between Plex A and other Semaphorins, including Sema III-AP (He and Tessier-Lavigne, 1997), AP-Sema E (Chen et al., 1997), and AP-Sema B (A. Chedotal and C. S. G., unpublished data), nor between Plex A and AP-Beat (Q. Lin and C. S. G., unpublished data). By Scatchard analysis, we estimate the K_d for AP-Sema 1a binding to Plex A at 2.2×10^{-9} M, and for AP-Sema 1b, 2.5×10^{-9} M (data not shown). These values are comparable with previous studies of Sema A39R-VESPR (Comeau et al., 1998) or Sema-Neuropilin binding affinities (Chen et al., 1997; He and Tessier-Lavigne, 1997; Kolodkin et al., 1997).

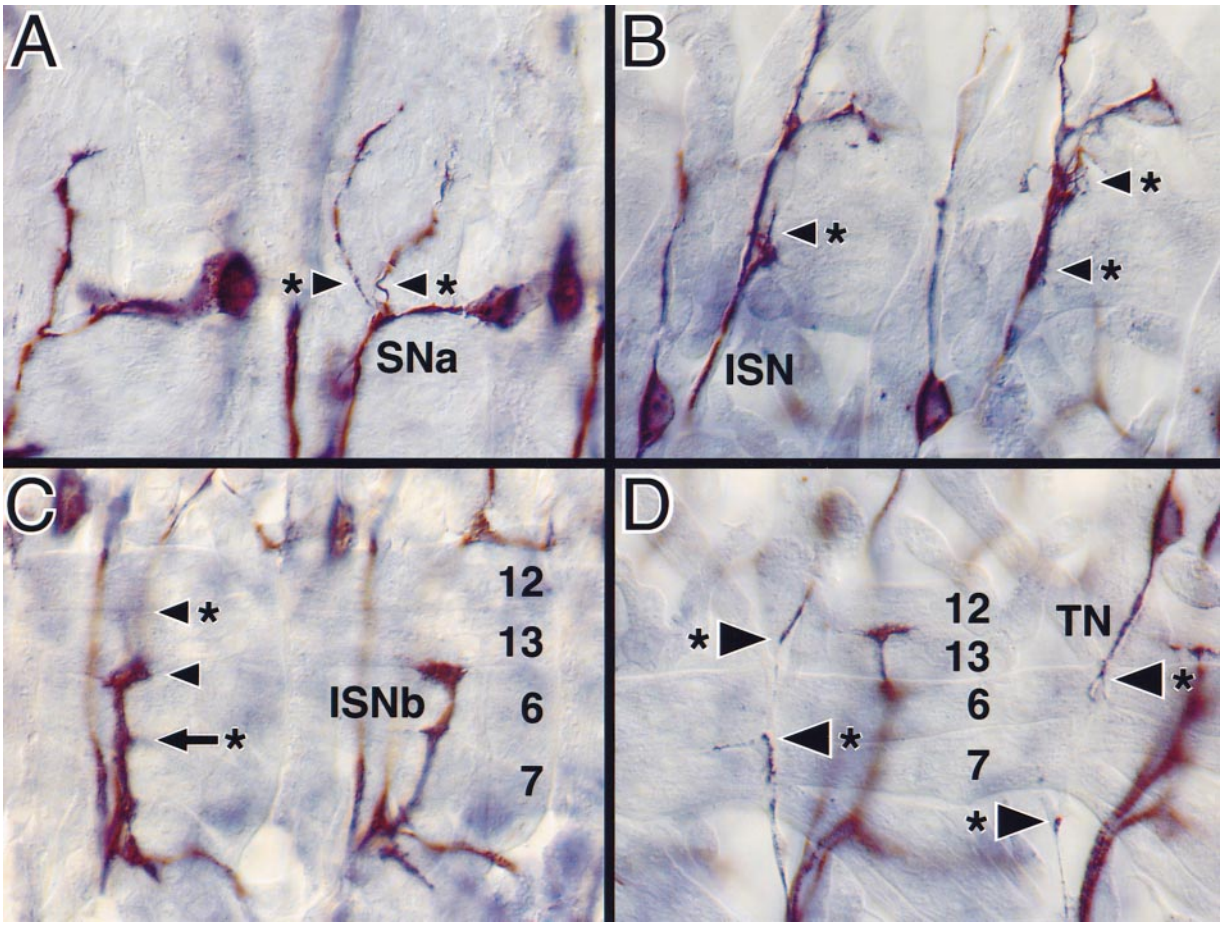

Figure 7. Abnormal Motor Projections Due to Plexin A Overexpression

Late stage 16 embryos, with one copy each of *elav-GAL4* and *UAS-PlexA* transgenes to give neuronal overexpression, stained with mAb 1D4 and *PlexA* in situ probes.

(A) The dorsal branch of the SNa is defasciculated prematurely at choice point 1 (* arrowheads, compare with Figure 3A).

(B) The dorsal extension of the ISN (near muscle 4) is poorly fasciculated and makes abnormal substrate contacts.

(C) The ISNb fails to innervate ventral muscles 6/7 (* arrow) and 12 (* arrowhead) and shows abnormal morphology at muscle 13 (arrowhead).

(D) The two halves of the TN fail to extend toward one another. In most cases the axons stall; less often they project ectopically onto ventral muscles (lower left * arrowhead).

Lastly, we asked whether Sema–Plexin binding, like Plexin homophilic interaction in *Xenopus*, requires the presence of divalent cations (Ohta et al., 1995). Binding was eliminated by the inclusion of either EGTA or EDTA (10 mM) in the ligand supernatant, or by the omission of divalent cations from the wash buffer (Figure 8B). Binding was preserved in the presence of either Mg^{2+} or Ca^{2+}. In contrast, binding of Semaphorins to Neuropilins does not appear to require calcium or magnesium (He and Tessier-Lavigne, 1997; Kolodkin et al., 1997).

Discussion

In this paper we present genetic and biochemical evidence that Plex A in *Drosophila* is a receptor for the transmembrane class I Semaphorins Sema 1a and 1b. We also show that Plex A controls important aspects of axon guidance, in part through its interaction with Sema 1a.

The genetic analysis is most complete for an interaction of Plex A with Sema 1a. Previous studies (Yu et al., 1998) showed that mutations in *Sema1a* (Kolodkin et al., 1992, 1993) lead to specific defects in motor and CNS axon guidance. We find that a deficiency removing the *PlexA* gene displays the same phenotypes and that these phenotypes can be rescued by a *PlexA* transgene. Moreover, the two loci interact in a fashion that suggests they function in the same signaling pathway: embryos that are heterozygous for mutations in both genes (*Sema1a* and *PlexA*) display the same axon guidance defects as seen in either homozygous mutant alone. This analysis is complemented by dominant suppression experiments, in which removing one copy of *PlexA* partially suppresses the gain-of-function phenotype generated by misexpressing Sema 1a. These data provide compelling genetic evidence for a direct interaction between the products of the *PlexA* and *Sema1a* genes, with *PlexA* acting downstream of *Sema1a*.

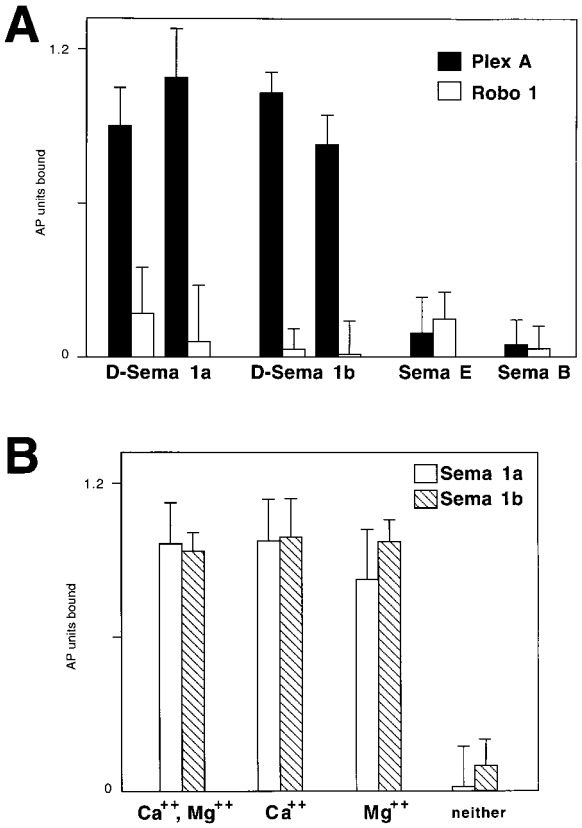

Figure 8. Type I Semaphorins Bind Plexin A

AP-bearing ligands were adjusted to a final concentration of 2 nM and bound to COS cell membranes. Specific binding was obtained by subtracting values for untransfected cells from those expressing Plex A or Robo 1.

(A) D Sema 1a and D Sema 1b specifically bind Plex A.

(B) Binding requires divalent cations. Ligand-containing supernatants and Plex A–expressing membranes were treated identically, except that final washes were performed in the presence or absence of 1 mM $MgCl_2$ and 5 mM $CaCl_2$.

Further genetic evidence suggests interactions between Plex A and another class I Semaphorin, Sema 1b. As with Sema 1a, mutations in *PlexA* also partially suppress the misexpression gain-of-function phenotype of Sema 1b. The notion that Plex A might interact with other Semaphorins is consistent with the observation that the *PlexA* deficiency displays certain phenotypes not seen in the *Sema1a* mutant, and that the *PlexA* gain-of-function cannot entirely be explained by its interactions with Sema 1a.

Our biochemical analysis is based on heterologous expression in vitro. Using Semaphorin fusion proteins in binding assays to membranes from PlexA-expressing COS cells, we find that Plex A membranes can bind *Drosophila* Sema 1a and Sema 1b (but not mammalian Sema III, Sema E, or Sema D), supporting the idea that this Plexin interacts with multiple ligands.

Taken together, these data support the model that Plex A in *Drosophila* functions as an axon guidance receptor for multiple Semaphorins. We speculate that vertebrate Plexins may function as neuronal receptors for a number of different classes of Semaphorins, and that many members of the Plexin family are likely to play important roles in controlling axon guidance. *Drosophila* appears to have two Plexins and at least five Semaphorins, whereas mammals appear to have around seven Plexins and about 25 Semaphorins. Promiscuous binding of Plexins to Semaphorins suggests both a high degree of complexity and potential functional redundancy in the interactions between the two families.

Semaphorins and Plexins Control Axon Defasciculation

Semaphorins and their Plexin receptors are likely to mediate many different aspects of axon guidance and target recognition, particularly in a repulsive or inhibitory fashion. The analysis presented here points to one major role for Semaphorins and Plexins in the regulation of axon fasciculation. Axon fasciculation versus defasciculation appears to be determined by a balance of attractive and repulsive forces on the axons relative to their surrounding environment (Tessier-Lavigne and Goodman, 1996).

During the development of the projection of motor axons in *Drosophila*, the Ig CAM Fas II is normally expressed at high levels on motor axons and plays a role in mediating fasciculation (Lin and Goodman, 1994; Lin et al., 1994). When the levels of Fas II on the axons are increased transgenically, the ISNb axons fail to defasciculate at their first choice point and instead continue extending dorsally along the ISN. The *beat* gene (Van Vactor et al., 1993; Fambrough and Goodman, 1996) encodes a candidate negative regulator of Fas II function. Loss-of-function *beat* mutations display ISNb defasciculation phenotypes similar to those observed when Fas II levels are increased. *beat* encodes a novel secreted protein expressed by motoneurons and appears to function as an antiadhesive protein (i.e., as a negative regulator of axon fasciculation). Genetically removing Fas II suppresses the *beat* mutant phenotype and returns the motor axon projection back to nearly wild type (Fambrough and Goodman, 1996).

Both Sema 1a and Plex A are expressed on most if not all motor axons. These two proteins appear to help establish a basal level of axon–axon repulsion. For much of their journey, motor axons are fasciculated to other motor axons; we presume that the levels of attraction (mediated in part by Fas II) are sufficient to overcome the Sema 1a/Plex A–mediated repulsion. However, signals (such as the release of Beat [Fambrough and Goodman, 1996]) at key choice points may shift the relative balance by decreasing axon–axon attraction locally. Decreased local attraction would allow axons to selectively defasciculate even though Sema 1a/Plex A repulsion remains the same.

The model that Plex A is a Semaphorin receptor that mediates repulsive guidance and thus drives defasciculation is supported by genetic interactions with *FasII*. We found that removal of one copy of *FasII* partially suppresses many of the defasciculation failures (both by peripheral motor and CNS axons) observed in *PlexA* mutant embryos. Thus, decreasing axon–axon attraction can compensate for a decrease in axon–axon repulsion as seen in a *PlexA* mutant embryo.

Plexins as Large Transmembrane Semaphorins

Comeau et al. (1998) noted that VESPR and other Plexins share a ~100 amino acid region of homology with Semaphorins near the C terminus of the Sema domain. Upon further sequence analysis, we find that Plexins and their relatives, the MET-related tyrosine kinase receptors (including MET, RON, and SEA), all contain a complete ~500 amino acid Sema domain near the N terminus of their ectodomains. Thus, Plexins may be considered as large transmembrane Semaphorins (Figures 1A, 1B, and 2A).

We used the CLUSTAL algorithm to examine the relationships among the Sema domains of Plexins, Semaphorins, and MET-related receptors (Figure 1B). Each of the three groups of proteins clusters separately. The structure of the tree suggests a phylogenetic model in which Plexins may have been the ancestral molecules from which Semaphorins and MET-related receptors evolved; from this perspective, Semaphorins may be considered as specialized Plexins.

Our data are consistent with a model in which Semaphorins are ligands and Plexins are receptors mediating repulsive axon guidance. Such a model may capture only part of the complexity of how these molecules function. The finding that Plexins can bind Semaphorins, combined with previous studies showing that Plexins can bind Plexins (Ohta et al., 1995), raises questions of whether some Semaphorins might bind one another and whether class I Semaphorins might also function as receptors. In the immune system, CD100, a class IV Semaphorin, promotes B cell aggregation and also plays a role in T cell activation (Hall et al., 1996; Herold et al., 1996). Some of its functions are consistent with CD100 functioning as a receptor as well as a ligand.

Evolution of Semaphorins and Semaphorin Receptors

To date, searching the genomes of the nematode *C. elegans* and the fruit fly *Drosophila* has not revealed homologs for Neuropilins, the receptors (or receptor subunits) for class III Semaphorins (He and Tessier-Lavigne, 1997; Kolodkin et al., 1997). Nematodes and fruit flies also appear to lack class III Semaphorins; worms have classes I and II (nematode genome sequence), while flies have classes I, II, and V (our data, and A. Kolodkin and S. Baumgartner, personal communication). Both these invertebrate genomes also appear to lack MET tyrosine kinase receptor homologs, but they do contain genes encoding Plexins. Thus, Plexins appear to be the oldest Semaphorin-binding proteins. It may be that during chordate evolution, a new class of Semaphorins (type III) evolved, and with them, a new class of Semaphorin receptors (the Neuropilins).

Experimental Procedures

Cloning and Molecular Analysis

Partial cDNAs for *Drosophila* Plexins were found in a screen of transmembrane and secreted cDNAs (Kopczynski et al., 1998). Additional ESTs were identified using BLAST searches (Altschul et al., 1997) starting from *Xenopus* Plexin-1. Full-ORF clones were found by hybridization to the LD embryonic cDNA library (Berkeley *Drosophila* Genome Project/HHMI EST Project). Sequences were analyzed with LaserGene software (DNASTAR). A sequence similar to *Sema1a* (Yu et al., 1997) was used to identify EST clone LD14102 as a full-length clone for *Sema1b*. Sema 1b has appeared previously as D Sema III (Baumgartner et al., 1996, Abstracts of the GSA *Drosophila* Meeting).

Cytology and Genetics

PlexA and *PlexB* were mapped by chromosomal in situ to 102E1-2 and 101F, respectively. Deficiency strains for the area (S. Flister and W. Gehring, personal communication) were tested for *PlexA* mRNA. *Df(4)C3* [102D6-F] deletes *PlexA*; *Df(4)G* [102E2-F] and *Df(4)O* [102D] do not affect *PlexA* transcript expression. Other stocks were as described: *Sema1a* and *UAS-Sema1a* (Yu et al., 1998), *F63-GAL4* and *H94-GAL4* (Winberg et al., 1998), and *elav-GAL4* (Luo et al., 1994).

Immunohistochemistry

Embryos were prepared using standard protocols. Monoclonal antibody 1D4 (anti-Fas II) was used at 1:5 (Van Vactor et al., 1993). Digoxigenin-labeled antisense probes from coding or 5′UTR regions were used to identify *PlexA* homozygous or overexpressing embryos (Kopczynski et al., 1996).

Expression Constructs

PlexA was cloned into pSecTagB (InVitrogen) using an internal EcoRI site and the vector-derived signal sequence; 38 residues from the N terminus of the mature polypeptide are replaced by 26 amino acids from the vector. Cloning sites were introduced by PCR into Sema 1a and Sema 1b immediately downstream of the signal peptide and upstream of the transmembrane region; fragments were cloned into pSecTagB with the alkaline phosphatase coding sequence upstream. The modified *PlexA* cDNA and the *Sema1b* cDNA (LD14102) were cloned into pUAST (Brand and Perrimon, 1993) and injected into fly embryos for germline transformation.

Binding Analysis

COS cells were transfected using Lipofectin (Life Technologies). Ligand-expressing cells were incubated for 2 days in serum-free DMEM; supernatants were collected, concentrated, and supplemented with sodium azide and 2% serum. Plex A–expressing or control cells were harvested 3 days after transfection in HBS (20 mM HEPES [pH 7.2], 150 mM NaCl, 5 mM $CaCl_2$, 1 mM $MgCl_2$); crude membranes were prepared (Roberts, 1986) and distributed at 0.4 mg/ml into microtiter plates for ELISA assay. Robo 1 expression was confirmed by anti-Robo antibody (Kidd et al., 1998). Supernatants were incubated 90 min at room temperature to give equilibrium binding; after washing, bound AP was measured by colorimetric assay.

Acknowledgments

We are grateful to H. Yu and A. Kolodkin for Sema 1a reagents, A. Kolodkin and S. Baumgartner for discussions of Semaphorin sequences and nomenclature, S. Flister and W. Gehring for unpublished deficiency stocks, K. Bland, A. Chedotal, Q. Lin, and J. Rehm for expression constructs, and L. Hong, D. Harvey, and G. M. Rubin for the LD cDNA library. We thank T. Laverty for cytological analysis, S. Artigiani for helpful discussions and contributions, R. Smith, W. Chen, and T. Wu for technical assistance, and D. Parnas for valuable discussions. Supported by National Institutes of Health grant HD21294 (C. S. G.) and the Armenise-Harvard Foundation for Advanced Scientific Research (P. M. C.). C. S. G. and M. T.-L. are Investigators and M. L. W. and J. N. N. are Postdoctoral Fellows with the Howard Hughes Medical Institute.

Received October 27, 1998; revised November 17, 1998.

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GenBank Accession Numbers

GenBank accession numbers for Plex A and Plex B are AF106932 and AF106933, respectively.