

The Transmembrane Semaphorin Sema I Is Required in *Drosophila* for Embryonic Motor and CNS Axon Guidance

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Summary

The semaphorins comprise a large family of conserved glycoproteins, several members of which have been shown to function in repulsive neuronal growth cone guidance. We show here that *Drosophila* Semaphorin I (Sema I), a transmembrane semaphorin expressed on embryonic motor and CNS axons, is required for correct guidance of motor axons and for the formation of CNS pathways. In mutant embryos lacking Sema I, motor axons stall and fail to defasciculate at specific choice points where normally they would project to their muscle targets. In addition, a specific CNS fascicle fails to form correctly in these embryos. Rescue and ectopic expression experiments show that Sema I is required in neurons to mediate axon guidance decisions. These studies further suggest that like secreted semaphorins, transmembrane semaphorins can function as repulsive guidance cues for specific axon guidance events during neurodevelopment.

Introduction

During development, axons respond to myriad cues to reach their targets. These cues can act over short or long distances and can be localized to neuronal and nonneuronal surfaces. Initially, neuronal growth cones respond to these cues and navigate to the vicinity of their targets in the absence of neuronal activity. Subsequent activity-dependent events lead to the refinement of initial arborizations and formation of mature, functioning synapses (Goodman and Shatz, 1993). Our understanding of the molecular mechanisms underlying these events has advanced markedly in recent years with the identification of several diverse gene families that encode phylogenetically conserved proteins capable of mediating growth cone guidance over short and long distances. Some of these proteins act as attractants, others as repellents, and still others as bifunctional attractants and repellents (Tessier-Lavigne and Goodman, 1996). In order to uncover the mechanisms that govern the elaboration of complex neural circuits, it is essential to understand precisely how particular cues mediate specific growth cone guidance decisions. To this end, we have undertaken a genetic analysis of *Drosophila* Semaphorin I (Sema I), a transmembrane member of the large semaphorin family of glycoproteins that contains several members strongly implicated in mediating neuronal growth cone guidance.

The semaphorins include both secreted and transmembrane proteins that are selectively expressed in unique subsets of neurons in developing invertebrate and vertebrate nervous systems (Kolodkin, 1996; Mark et al., 1997). Both vertebrate and invertebrate secreted semaphorins have been strongly implicated in genetic studies to function as repulsive neuronal guidance cues in vivo, and several secreted vertebrate semaphorins can function as repulsive cues in vitro (Matthes et al., 1995; Adams et al., 1997; Koppel et al., 1997; Taniguchi et al., 1997). Semaphorins are defined by the presence of a large phylogenetically conserved ~500 amino acid semaphorin (sema) domain. Secreted semaphorins have an immunoglobulin (Ig) domain C-terminal to the sema domain, while transmembrane semaphorins can have an Ig, thrombospondin type I repeat, or no identified structural motif C-terminal to their sema domain. Transmembrane semaphorins have relatively short cytoplasmic domains that contain no obvious catalytic domains. The existence of both secreted and transmembrane semaphorins suggests that semaphorins function over long and short distances.

Though a large number of transmembrane semaphorins expressed in invertebrate and vertebrate nervous systems have been described, little is known about the roles these proteins play in growth cone steering decisions. Grasshopper Semaphorin I (G-Sema I, formerly Fasciclin IV), was the first semaphorin to be identified (Kolodkin et al., 1992). G-Sema I is a transmembrane semaphorin containing no obvious structural domains C-terminal to the semaphorin domain and is one of several invertebrate and vertebrate semaphorins with this overall structure (Kolodkin et al., 1992, 1993; Eckhardt et al., 1997; Kikuchi et al., 1997; Zhou et al., 1997). In vivo antibody perturbation experiments show that G-Sema I plays an important role in establishing the axonal trajectory of the well-characterized T11 pioneer neurons (Kolodkin et al., 1992). In addition to peripheral epithelial localization, G-Sema I is also found on grasshopper embryonic CNS axons in a highly selective pattern, suggesting that it is also likely to function in axon guidance events during CNS development.

Sema I is, thus far, the semaphorin in *Drosophila* most closely related to G-Sema I. It is similar in overall structure and shares 60% amino acid identity with G-Sema I over its sema domain. Analysis of *Semal* mRNA distribution during *Drosophila* neurodevelopment showed that like G-Sema I, *Semal* mRNA is expressed in the nervous system (Kolodkin et al., 1993). Though *Semal* appears to be expressed in a much larger subset of neurons in *Drosophila* than is G-Sema I in the related grasshopper nervous system, these results suggested that a genetic analysis of *Semal* should yield important in vivo observations and directly assess the cellular basis of neuronal transmembrane semaphorin function. In the present study, we have characterized the distribution of Sema I protein and have identified a null mutation in the *Semal* locus. We find that Sema I is required for the generation of the precise pattern of embryonic neuromuscular connectivity and appears to function at

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motor neuron pathway choice points in the periphery. Further, *Sema I* is also required for CNS pathfinding events. Finally, rescue and ectopic expression experiments strongly suggest that *Sema I* can function as a repulsive ligand for motor axons that normally require it to navigate peripheral choice points, supporting the idea that both transmembrane and secreted semaphorins can function *in vivo* as repulsive guidance cues.

Results

Sema I is Expressed on Motor and CNS Axons

Previous work showed that *Semal* mRNA is found in a large subset of neurons during *Drosophila* embryonic neurodevelopment (Kolodkin et al., 1993). In order to identify populations of neurons that might require *Sema I* protein during axon pathfinding and target recognition, we performed immunohistochemistry in wild-type embryos using polyclonal antibodies directed against epitopes in the extracellular portion of *Sema I*. These antibodies are specific for *Sema I* protein since they detect no protein in *Semal* homozygous mutant embryos. Antibodies directed against epitopes in the cytoplasmic portion of the *Sema I* protein, both polyclonal and monoclonal, show similar patterns of *Sema I* protein localization during embryogenesis (data not shown). In the nervous system, *Sema I* protein is first detected in stage 11 embryos, just prior to axonogenesis, in a continuous stripe along the midline.

At late stage 16, following the establishment of commissural and longitudinal pathways and also the basic pattern of motor projections in the periphery, robust levels of *Sema I* protein are observed on most CNS commissural and longitudinal pathways, as well as on the intersegmental nerve (ISN) and segmental nerve (SN) roots exiting the CNS (Figures 1A and 1B). *Sema I* protein is observed at low levels in the midline in the ventral portion of CNS, posterior to the posterior commissure and consistent with expression on the ventral unpaired median (VUM) neurons (Figure 1B). In the periphery, *Sema I* is found on all five motor axon branches: ISN, ISNb (formally SNb), ISNd (formally SND), SNa, and SNC (Figures 1C and 6A). Though the resolution of *Sema I* protein localization in the periphery is somewhat diffuse, we were still able to observe *Sema I* protein on several of the pathways that make up these motor axon branches. For example, *Sema I* protein was detected on both dorsal and lateral branches of SNa and on ISNb in branches that include the identified motor axons RP3 and RP5 (Figure 1C). We did not detect *Sema I* protein in peripheral sensory neurons of the dorsal or ventral clusters; however, we did observe very low levels of *Sema I* in the lateral cluster of chordotonal sensory organs. In addition to neuronal localization, *Sema I* was also observed, starting at stage 14, at segment boundaries in the position of the muscle attachment sites of the ventral lateral muscles 12, 13, 6, and 7 (Figures 1A and 1C). We did not detect *Sema I* protein or a maternal contribution of *Semal* RNA in very early wild-type embryos.

Characterization of *Sema I* Loss-of-Function Mutants

To identify mutations in *Semal*, we took advantage of P transposable element enhancer trap lines to find insertions in the *Semal* locus. Two lines, referred to here

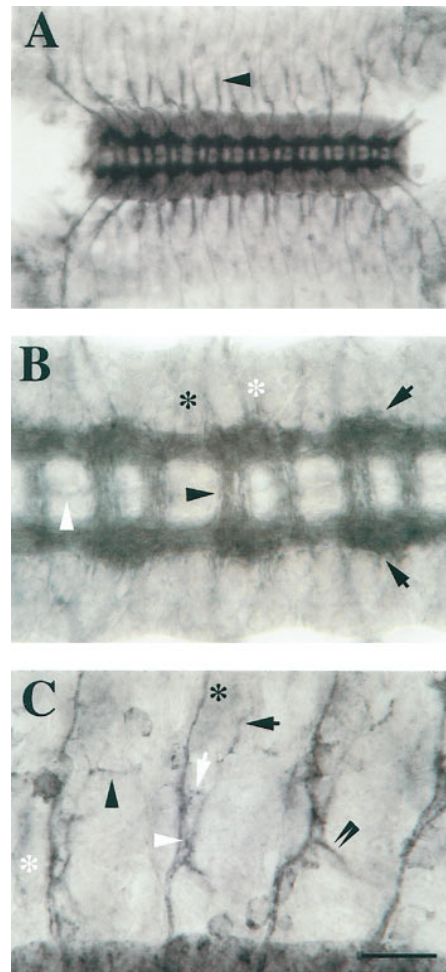


Figure 1. *Sema I* is Expressed on Motor Axons and Most CNS Axons
Sema I protein localization visualized in a filleted late stage 16 wild-type embryo using anti-*Sema I* antiserum.

(A) Low magnification of CNS and PNS. *Sema I* is localized on CNS axons and on motor axons in the periphery. *Sema I* protein is also observed at the segment boundary in the vicinity of the ventral lateral muscle attachment sites (arrowhead).

(B) Higher magnification view of CNS. *Sema I* is expressed on most longitudinal connectives (black arrows) and on anterior and posterior commissural axons (black arrowhead pointing to anterior commissure). Some heterogeneity in *Sema I* CNS commissural axonal localization is observed; this may reflect restriction of *Sema I* to a large subset of CNS axons. *Sema I* can also be seen on the nerve roots of the ISN (black asterisk) and SN (white asterisk) exiting the CNS. Midline staining, located in a deeper focal plane, is in a position consistent with localization on VUM neurons (white arrowhead).

(C) Higher magnification view of PNS. In the periphery, *Sema I* is expressed on motor axon pathways: ISN (black asterisk), SNa (black arrow and arrowhead), ISNb (white arrowhead), SNC (not in this focal plane), and ISNd (double black arrowhead). On the SNa, *Sema I* is observed on both dorsal (black arrow) and lateral (black arrowhead) branches. On the ISNb, *Sema I* is observed on axons of motor neurons RP5 (white arrow) and RP3 (out of this focal plane). *Sema I* is also observed, in a more lateral focal plane, at the segment boundary in the location of the VLM attachment sites (white asterisk). Scale bar, 35 μ m in (A); 10 μ m in (B); and 15 μ m in (C).

as *Semal*^{P1} and *Semal*^{P2}, were identified as potential insertions in *Semal*, because their chromosomal cytological location (29E1–2) is coincident with that of *Semal*, the embryonic expression pattern of β -galactosidase of

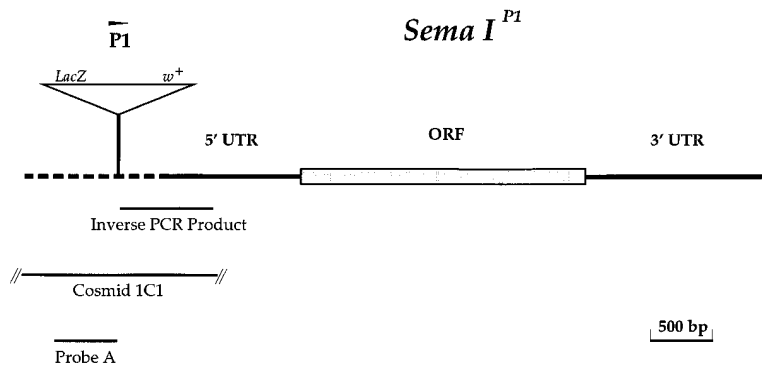


Figure 2. Molecular Map of *SemaI^{P1}*

The insertion that defines the *SemaI^{P1}* allele is located within the 5' UTR of *SemaI*. Shown is the *SemaI* cDNA derived from *Drosophila* embryonic cDNA clones: ORF (shaded box) and 5' and 3' UTRs (solid lines). The dotted line denotes 5' UTR sequences that are also part of the transcribed portion of *SemaI* based on genomic mapping and Northern analysis. The P element *PlacW* is inserted in the *SemaI* 5' UTR in the indicated orientation. Only a portion of the cosmid 1C1 is shown. Inverse PCR performed on *SemaI^{P1}* flies generated an amplification product that contains both P element sequences and sequences from the *SemaI* locus that overlap with the

5' portion of the *SemaI* cDNA. The *SemaI* sequence contained in this inverse PCR fragment is identical to that of genomic DNA obtained from the cosmid 1C1. Further, no RNA splice site consensus sequences were found in the sequenced genomic DNA to either side of the P element (0.8 Kb to left and 0.4 kb to right of P element). Northern blots probed with either Probe A (a 500 bp genomic DNA fragment derived from cosmid 1C1 that lies on the side of the P element upstream from the *SemaI* ORF), the inverse PCR product, or the entire *SemaI* cDNA detected the same alternatively spliced *SemaI* transcripts of 8.5 kb and 7.5 kb in length (data not shown). Taken together, these data indicate that the P element in *SemaI^{P1}* is not inserted in an intron.

SemaI^{P1} is reminiscent of the neuronal distribution of Sema I protein (data not shown), and the insertions fail to complement one another. *SemaI^{P1}* and *SemaI^{P2}* are semi-lethal, exhibiting ~1% and ~10% escaping adults, respectively. These escapers do not show observable morphological defects. The semi-lethality observed in transheterozygotes of *SemaI^{P1}* and a chromosomal deficiency that includes the *SemaI* locus, *Df(2L)N22-5* (Neuman-Silberg and Schubach, 1993), is not significantly different from that seen in *SemaI^{P1}/SemaI^{P1}* homozygotes. However, when *SemaI^{P1}* is transheterozygous to a related deficiency that does not include the *SemaI* locus, *Df(2L)N22-3*, no lethality is observed. Unlike *SemaI^{P1}*, the semi-lethality observed in *SemaI^{P2}* homozygotes was enhanced over *Df(2L)N22-5*. In addition, all embryonic phenotypes observed in *SemaI^{P1}* homozygous embryos are not enhanced when placed over *Df(2L)N22-5*. Mobilization of both P elements resulted in the recovery of completely viable lines that exhibit no embryonic neuronal phenotypes (described below), showing that these elements are affecting a gene responsible for these phenotypes. Lethal excisions of *SemaI^{P1}* were not obtained owing to the presence of a large intron located between this element and the start of the *SemaI* open reading frame (see Experimental Procedures). Therefore, by genetic criteria, *SemaI^{P1}* is a null mutation or a severe loss-of-function allele of *SemaI*, and *SemaI^{P2}* is likely to be a hypomorphic *SemaI* allele.

Additional analyses confirm that *SemaI^{P1}* and *SemaI^{P2}* are indeed *SemaI* mutants. First, molecular analysis revealed that *SemaI^{P1}* is inserted in the 5' untranslated region (UTR) of the *SemaI* transcript (*SemaI^{P2}* is located within a 5 kb region upstream from *SemaI^{P1}*). This was shown by isolating DNA sequences flanking both sides of the P element *SemaI^{P1}* and establishing by sequence analysis the collinearity of this DNA with independently isolated genomic DNA from this region and the 5' UTR from *SemaI* cDNA clones. Northern analysis using both of these flanking sequences as probes detected *SemaI* transcripts identical to those detected using the entire *SemaI* cDNA as a probe (Figure 2 and Experimental Procedures). Second, immunohistochemistry using our

Sema I antibodies in embryos homozygous for *SemaI^{P1}* revealed a complete lack, and in homozygous *SemaI^{P2}* embryos a significant reduction, of detectable Sema I protein (data not shown). Finally, because *SemaI* is expressed in both CNS and motor axons, and because embryonic defects reflecting a loss of this expression were observed in mutant embryos, we ectopically expressed *SemaI* cDNA in all neurons using the *UAS-GAL4* ectopic expression system (Brand and Perrimon, 1993) in order to effect a rescue of both embryonic and adult phenotypes. We found almost complete rescue of the semi-lethality seen in *SemaI^{P1}* and *SemaI^{P2}* mutant flies and a significant rescue of all embryonic neuronal phenotypes observed in *SemaI^{P1}* (see below) and *SemaI^{P2}* (data not shown) mutant embryos. Taken together, these results demonstrate that *SemaI^{P1}* and *SemaI^{P2}* are both alleles of *SemaI*, and that *SemaI^{P1}* is a null or severe loss-of-function allele of *SemaI*.

Sema I is Required for Motor Axon and CNS Pathfinding

The development of the stereotypic pattern of neuromuscular connectivity in embryonic *Drosophila* abdominal segments has provided an excellent system in which to study axon guidance events that include motor axon fasciculation and defasciculation, target region identification, target recognition and initial synapse formation, and the later events of synaptogenesis (Sink and Whittington, 1991; Keshishian et al., 1993; Van Vactor et al., 1993; Bate and Broadie, 1995). Each hemisegment contains 30 identified muscles that are innervated by ~40 motor neurons, several of which are identified. The pathfinding events that bring these motor axons to their targets involve several discrete steps. These motor neurons initially exit the CNS as part of the ISN or SN (see Figure 6A for schematic). They then divide among five major motor neuron branches that target different muscle groups, eventually elaborating synapses upon individual target muscles. This stereotypic pattern of motor axon branches can be easily observed in late stage 16/early stage 17 (stage 16/17) embryos by using the monoclonal antibody (mAb) 1D4, which is directed

Table 1. ISNb Phenotypes (% Total Hemisegments)

Genotype	Total Abnormal [n]	Bypass ^e	Stall ^a	w/o 6/7 Synapse	Aberrant 6/7 Synapse (% Normal) ^d	Aberrant 12 Synapse (% Normal)
<i>Sema1^{P1}/Sema1^{P1}</i>	87 [213]	7	49 ^b	18	35 (47)	15 (29)
<i>Sema1^{P1}/Df(2L)N22-5</i>	86 [101]	5	36	36	24 (40)	26 (33)
<i>Sema1^{P1}/Df(2L)N22-3</i>	6 [71]	0	3	0	2	2
Wild type (CS)	14 [127]	0	0	1	8	6
C155GAL4/+;						
<i>Sema1^{P1}/Sema1^{P1} UAS Sema1</i>	20 [86]	0	6	6	9	6
<i>Sema1^{P1}ScabGAL4/</i>						
<i>Sema1^{P1}UAS Sema1</i>	16[73]	0	5	0	5	7
C155-GAL4/+;+/UAS <i>Sema1</i>	11 [66]	0	3	3	2	3
C155GAL4/C155GAL4;						
<i>Sema1^{P1}UAS Sema1^{EC}/</i>						
<i>Sema1^{P1}UAS Sema1^{EC}</i>	60 [148]	5	45	13	18	6
<i>Sema1^{P1}ScabGAL4/</i>						
<i>Sema1^{P1}UAS Sema1^{EC}</i>	61 [160]	1	26	12	28	12
<i>UAS Sema1/UAS Sema1;</i>						
24BGAL4/24BGAL4	86[176]	24 {15PB;9FB}	13	41	23 (36)	16 (48)
<i>Sema1^{P1}/Sema1^{P1}UAS Sema1;</i>						
24BGAL4/+	100 [127]	58 {3PB;55FB}	39 ^c	87	13 (0)	1 (0)
+/ <i>UAS Sema1; 24BGAL4/+</i>	20 [109]	0	1	6	7	7

Data are presented as % of total hemisegments scored [n] exhibiting indicated phenotype.

^a Stall phenotype presented here is the sum of SNb stalls between muscles 6 and 13 and between muscles 7 and 6.

^b 39% of hemisegments exhibit ISNb stalls between muscles 6 and 13 and 10% between muscles 7 and 6.

^c 17% of hemisegments exhibit ISNb stalls between muscles 6 and 13 and 22% between muscles 7 and 6.

^d For clarity, the % of hemisegments with apparently normal synaptic arborizations is shown in parentheses.

^e Bypass events are broken down into parallel bypass (PB) and fusion bypass (FB) as indicated in brackets {} for two of the genotypes presented.

against the axonal glycoprotein Fasciclin II (Fas II) and serves as a robust marker for all motor axons in the periphery (Van Vactor et al., 1993).

Since Sema I is found on most, if not all, motor axons, we examined *Sema1* mutant embryos for defects in the establishment of neuromuscular connectivity. Homozygous *Sema1^{P1}* mutant embryos show dramatic and highly penetrant pathfinding defects in both the ISNb and SNa pathways. We examined these trajectories in filleted stage 16/17 embryos (the stage used for motor axon and CNS phenotype analysis for all embryos in this study) using mAb 1D4.

In *Sema1^{P1}* mutant embryos, 87% of the ISNb pathways were abnormal (see Table 1). Normally, the ISN, ISNb, and ISNd branches exit the CNS as a single pathway (see Figure 6A for schematic). Just lateral to the CNS, in the vicinity of the ventral oblique muscles (15–17), the motor neurons of the ISNb and ISNd branches defasciculate from the ISN. The ISNd subsequently innervates muscles 15, 16, and 17, while the ISNb continues to extend dorsally. After encountering muscle 28, the ISNb extends along the external surface of ventral lateral muscles (VLMs) 6 and 7 and the internal surface of VLMs 14 and 30. The ISNb then projects along the internal surface of VLMs 13 and 12. Synaptic arborizations are formed by defasciculation of several motor axons from the ISNb (the RP3 motor neuron between muscles 6 and 7, the RP1 and RP4 motor neurons on muscle 13, and the RP5 motor neuron on muscle 12). The ISN continues to extend dorsally and contacts its dorsal target muscles, resulting in the formation of three characteristic arborizations in the dorsal muscle field (Figure 5F).

In 49% of hemisegments in *Sema1^{P1}* mutant embryos the ISNb stalled, failing to extend from the external surface of VLMs 6 and 7 to the internal surface of VLMs 12

and 13. Most of these stalled ISNb branches terminated between muscles 6 and 13 (39% of hemisegments); however, some ISNb pathways stalled at a more ventral position, between muscles 6 and 7 (10% of hemisegments). ISNb stalls were almost always observed to terminate in these discrete locations, and no wandering of aberrant ISNb axons was observed within the ventral muscle field. In a small but significant fraction of hemisegments in *Sema1^{P1}* mutant embryos (7%; $p < 0.001$), ISNb was observed to undergo a fusion bypass with the ISN (Lin and Goodman, 1994). In these fusion bypass events, the ISNb bypassed the ventral muscle field and extended along the ISN at least to the dorsal level of the lateral muscles. Synaptic arborizations between muscles 6 and 7 were also abnormal in ~35% of hemisegments where they were present (Table 1). These abnormal arborizations were substantially smaller and thinner than those normally observed in wild-type embryos. Finally, the ISNd branch was defective, either missing or severely truncated and thinner than normal, in 36% of hemisegments in *Sema1^{P1}* mutants as compared to 9% in wild type (Figure 3B). Therefore, Sema I is required for correct ISNb and ISNd pathfinding and for the formation of specific synaptic arborizations. In the absence of Sema I, ISNb motor neurons often fail to extend from the external to the internal VLM surface, stalling at positions where motor axons normally defasciculate from the ISNb and form synapses on target muscles (Figure 6B). The defects in ISNb pathway formation we observe are likely not to reflect loss of RP motor neurons that contribute to ISNb or an inability of these neurons to extend axons contralaterally across the midline and out of the CNS. Immunohistochemistry using mAb 7G10, which is directed against the axonal glycoprotein Fasciclin III (Patel et al., 1987), revealed no abnormalities in the RP fascicles in *Sema1^{P1}* mutant

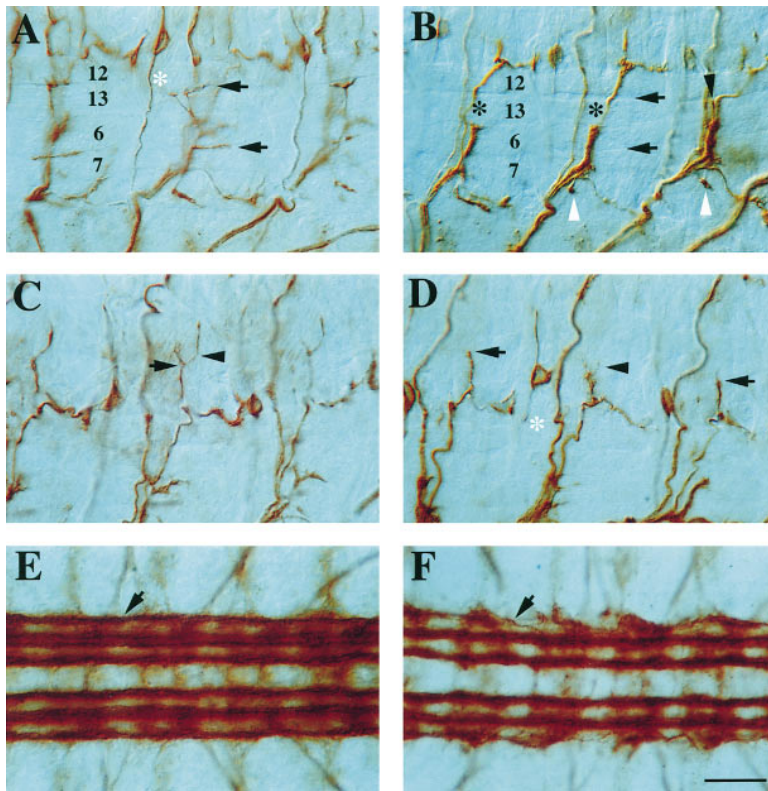


Figure 3. Sema I is Required for Motor and CNS Axon Pathfinding

Abdominal segments of filleted stage 16/17 wild-type embryos ([A], [C] and [E]) and *SemaI^{PI}* ([B], [D], and [F]) embryos stained with mAb 1D4 to reveal ISNb ([A] and [B]), SNa ([C] and [D]) and CNS phenotypes ([E] and [F]).

(A) The ISNb pathway in a wild-type embryo is shown, revealing the synaptic arborization between muscles 6 and 7 elaborated by RP3 (lower arrow) and the synaptic arborization in muscle 12 made by RP5 (upper arrow). The position of the transverse nerve is also indicated (white asterisk). Numbers mark the location of the ventral muscles 12, 13, 6, and 7. (B) In *SemaI^{PI}* mutant embryos, ISNb is most often stalled between muscles 13 and 6 (black asterisks). RP3 and RP5 synaptic arborizations are absent (arrows). An abnormal RP5 synaptic arborization on muscle 12 is observed in one segment of this embryo (black arrowhead). Abnormal ISNb pathways are also observed (white arrowheads).

(C) The SNa pathway in a wild-type embryo is shown. The dorsal SNa branch bifurcates at a characteristic choice point, located between muscles 22 and 23 (arrow). The resulting anterior branch extends dorsally to elaborate a synaptic arborization between muscles 22 and 23. The resulting posterior branch extends as well, then turns dorsally (arrowhead) and forms a synaptic arborization on muscle 24.

(D) In *SemaI^{PI}* mutant embryos, the dorsal SNa branch is most often stalled at the choice point (arrow) where normally it would bifurcate. Occasionally, multiple aberrant filopodia are observed at the termination of the stalled SNa dorsal branch (arrowhead). The transverse nerve often stalls in the region of the VLMs (white asterisk).

(E) The three Fas II-positive CNS longitudinal connectives (from medial to lateral: pCC/MP2, MP1, and the third longitudinal axonal connective) are shown in a wild-type stage 16/17 embryo. Each of these three connectives is continuous and well separated from its neighboring connective (arrow indicates the third longitudinal connective).

(F) In *SemaI^{PI}* mutant embryos, pCC/MP2 and MP1 longitudinal connectives are continuous, well separated from each other, and appear normal. The third longitudinal connective, however, is discontinuous and wavy. Often axons that make up the defective connective are misrouted and contact the neighboring MP1 connective (arrow).

Scale bar, 12 μ m in (A) through (D) and 10 μ m in (E) through (F).

embryos, either within or exiting the CNS, or in RP motor neurons themselves.

In *SemaI^{PI}* mutant embryos, highly penetrant SNa pathway defects were also observed (92% of hemisegments; Figure 3D; Table 2). The SNa and SNa branches constitute the SN branch. After exiting the CNS, the SNa normally defasciculates from the SNa and subsequently innervates ventral external muscles. The SNa then extends dorsally to innervate several lateral muscles (LMs). In the vicinity of the LMs, the SNa contains two major branches. The major dorsal SNa branch extends between muscles 22 and 23 and subsequently bifurcates at a characteristic choice point, extending one process posteriorly across muscle 23 that then extends dorsally along muscle 24 (Figures 3C and 6A) and a second process dorsally that extends between muscles 22 and 23. The major lateral branch of SNa extends posteriorly to contact muscles 5 and 8.

As was observed in *SemaI^{PI}* mutant ISNb pathways, these mutant SNa pathways also exhibited discrete defects that reflect an inability of motor axons in this pathway to extend past specific choice points. These defects primarily affect the dorsal, not the lateral, SNa branch (Figures 3D and 6B). The dorsal branch of the SNa often

stalled at a characteristic location between muscles 22 and 23 (69% of hemisegments; called "S1" in Table 2). This is the choice point where SNa motor axons that innervate muscle 24 normally defasciculate from this dorsal branch, extend posteriorly across muscle 23, and then extend dorsally along muscle 24 (Figure 3D). In some dorsal SNa branches in *SemaI^{PI}* mutant embryos, this choice point was navigated correctly; however, the motor axon that innervates muscle 24 failed to extend dorsally after reaching muscle 24 (19% of hemisegments; called "S2" in Table 2). As was observed in *SemaI^{PI}* mutant ISNb branches, mutant SNa branches were not observed to wander in the vicinity of the lateral muscle field. In addition to these SNa defects, the SNa was observed to be defective in 11% of mutant hemisegments, compared to 1% in wild type. Finally, we observed transverse nerve defects in 20% of hemisegments in *SemaI^{PI}* mutant embryos, in some cases resulting in the establishment of ectopic synapses on VLMs (Figure 3D).

Taken together, these results show that Sema I is required for the generation of the precise pattern of neuromuscular connectivity in the *Drosophila* embryo. This requirement appears to be for the navigation of

Table 2. SNa Phenotypes (% Total Hemisegments)

Genotype	Total Abnormal [n]	Fusion Bypass	w/o Dorsal Branch	Stall S1	Stall S2
<i>Sema1^{P1}/Sema1^{P1}</i>	92 [254]	0	4	69	19
<i>Sema1^{P1}/Df(2L)N22-5</i>	92 [120]	0	2	66	25
<i>Sema1^{P1}/Df(2L)N22-3</i>	7 [95]	0	0	4	3
Wild type (CS)	10 [146]	0	0	7	3
C155GAL4/+;					
<i>Sema1^{P1}/Sema1^{P1} UAS Sema1</i>	34 [97]	0	2	21	11
<i>Sema1^{P1}ScabGAL4/</i>					
<i>Sema1^{P1} UAS Sema1</i>	35 [84]	0	1	17	17
C155GAL4/+;+/ <i> UAS Sema1</i>	11 [73]	0	0	7	4
C155GAL4/C155GAL4;					
<i>Sema1^{P1} UAS Sema1^{EC}/Sema1^{P1} UAS Sema1^{EC}</i>	50 [207]	0	2	32	16
<i>Sema1^{P1}ScabGAL4/</i>					
<i>Sema1^{P1} UAS Sema1^{EC}</i>	48 [186]	0	2	28	17
<i>UAS Sema1/UAS Sema1;</i>					
24BGAL4/24BGAL4	45 [190]	0	5	31	9
<i>Sema1^{P1}/Sema1^{P1} UAS Sema1;</i>					
24BGAL4/+	93 [148]	12	19	48	14
+/ <i>UAS Sema1; 24BGAL4/+</i>	11 [131]	0	0	6	5

Data are presented as % of total hemisegments scored [n] exhibiting indicated phenotype. See text for definitions of SNa stall phenotypes S1 and S2.

specific choice points in motor axon pathways and not a general requirement for process outgrowth. ISN defects were not observed in *Sema1^{P1}* mutants, and the defects observed in the SNa and the ISNb occur at discrete locations along these pathways where motor axons normally defasciculate from their main branch and either extend toward, or elaborate synaptic arborizations on, their target muscles. Analysis of the peripheral sensory neurons with mAb 22C10 (Zipursky et al., 1984) revealed no defects in their mature axonal trajectories, consistent with our inability to detect Sema I protein on these PNS sensory afferents.

In addition to motor pathway defects, *Sema1^{P1}* mutants exhibit highly penetrant and specific CNS defects. mAb 1D4 reveals that Fas II is expressed on a subset of CNS longitudinal axon connectives (Grenningloh et al., 1991; Lin and Goodman, 1994). There are three Fas II-expressing longitudinal connectives: the most medial pCC/MP2, the more lateral MP1, and the most lateral third longitudinal connective (Figure 3E; Hidalgo and

Brand, 1997). In a wild-type embryo these connectives are continuous between segments and evenly separated from each other over the entire length of the CNS. In *Sema1^{P1}* mutant embryos, the pCC/MP2 and MP1 connectives appear normal; however, the third longitudinal connective is abnormal in 31% of hemisegments, as compared to ~1% in wild type. These defective pathways were discontinuous, thin and wavy between segments, and often individual axons were seen to contact the more medial MP1 pathway (Figure 3F). Overall CNS organization, as revealed by the mAb BP102 that illuminates all CNS axons, is not altered in *Sema1^{P1}* mutant embryos. Analysis of *Sema1^{P1}* mutant embryos using mAbs 1D4 and 22C10 at stage 16/17, as well as earlier developmental stages, revealed normal development of several discrete CNS pathways, including the MP1, pCC, RP, and VUM pathways in *Sema1^{P1}* mutants. Connectin-expressing longitudinal pathways (Nose et al., 1992) also appeared normal in *Sema1^{P1}* mutants (data not shown).

All embryonic phenotypes in motor and CNS axons

Table 3. CNS Phenotypes and Adult Lethality as Affected by Ectopic Sema1

Genotype	% Abnormal CNS Hemisegments [n]	Adult Lethality (% Expected Adult Flies)
<i>Sema1^{P1}/Sema1^{P1}</i>	31 [410]	0.8
<i>Sema1^{P1}/Df(2L)N22-5</i>	30 [200]	0.1
<i>Sema1^{P1}/Df(2L)N22-3</i>	0 [60]	96
Wild type (CS)	0.4 [260]	100
C155GAL4/+;		
<i>Sema1^{P1}/Sema1^{P1} UAS Sema1</i>	3 [200]	92
<i>Sema1^{P1}ScabGal4/</i>		
<i>Sema1^{P1} UAS Sema1</i>	1 [190]	0
C155GAL4/+;		
<i>Sema1^{P1}/Sema1^{P1} UAS Sema1^{EC}</i>	ND	25
C155GAL4/+;		
<i>Sema1^{P1} UAS Sema1^{EC}/Sema1^{P1} UAS Sema1^{EC}</i>	17 [365]	2.4
C155GAL4/C155GAL4;		
<i>Sema1^{P1} UAS Sema1^{EC}/Sema1^{P1} UAS Sema1^{EC}</i>	17 [630]	0
<i>Sema1^{P1}ScabGAL4/</i>		
<i>Sema1^{P1} UAS Sema1^{EC}</i>	15 [320]	0

were similar both in mutant embryos transheterozygous for *Sema^{P1}* and the deficiency *Df(2L)N22-5* (which deletes the *Sema1* locus) and in homozygous *Sema^{P1}* embryos (Tables 1–3). Further analysis of *Sema^{P1}* mutants using mAbs 4D9 and 3C10 (Patel et al., 1989), directed against the engrailed and even-skipped proteins, respectively, and also mAbs 1D4, 22C10, and 7G10 showed that the loss of *Sema1* does not grossly affect the differentiation of many of the motor neurons that send axons into the motor pathways requiring Sema I. The differentiation of a large subset of CNS neurons that are also likely to express Sema I appears normal. Finally, muscle development and morphology, in particular the degree of adhesion between neighboring muscles, appears normal in *Sema^{P1}* mutants as revealed by an anti-myosin mAb (data not shown).

Neuronal Expression of Sema I Rescues *Sema^{P1}* Phenotypes

To assess the ability of Sema I to rescue *Sema^{P1}* mutant phenotypes and to confirm that it is required on motor neurons, we used the GAL4 ectopic expression system (Brand and Perrimon, 1993). Transgenic flies carrying a full length *Sema1* cDNA (see Experimental Procedures) under the control of a *GAL4* upstream activation sequence (*UAS*) were crossed to flies that express the *GAL4* transcription factor, resulting in *GAL4*-dependent expression of Sema I. We used the *C155-GAL4* line, which directs *GAL4* expression in all neurons (Lin and Goodman, 1994), to rescue the *Sema^{P1}* mutant phenotypes. The level of *C155*-driven Sema I protein, as assessed immunohistologically, was equal to or higher than that of Sema I observed in wild-type embryos. We observed no embryonic neuronal defects in wild-type embryos that express Sema I under the control of the *C155-GAL4* transgene (Tables 1 and 2).

In *Sema^{P1}* mutant embryos that contain both *C155-GAL4* and *UAS-Sema1* transgenes, we observed a complete rescue of all ISNb phenotypes (Table 1; Figure 4A), a partial but significant rescue of SNa phenotypes (from 92% to 34% abnormal pathways; Table 2; Figure 4B), and an almost complete rescue of the CNS phenotype (Table 3; Figure 4C). In addition to rescue of embryonic neuronal phenotypes, we found that the *C155-GAL4* and *UAS-Sema1* transgenes were able to rescue almost completely the lethality of *Sema^{P1}* homozygotes (Table 3). We used a second *GAL4* line that expresses *GAL4* in all neurons, *Scabrous-GAL4* (Klaes et al., 1994), in rescue experiments and obtained rescue of *Sema^{P1}* embryonic neuronal phenotypes similar to those obtained with *C155-GAL4*. We did not, however, see rescue of adult lethality with this line (Tables 1–3). This may reflect differences in the localization or duration of *Scabrous-GAL4* and *C155-GAL4* expression during development.

Sema I is a transmembrane semaphorin with a small cytoplasmic domain. To address how Sema I functions in neurons to mediate defasciculation events, we performed additional rescue experiments using a modified form of Sema I protein that lacks the transmembrane and cytoplasmic domains (called Sema I^{EC}). The level of *C155*-driven Sema I^{EC}, assessed immunohistologically, was equal to or higher than that of Sema I observed in

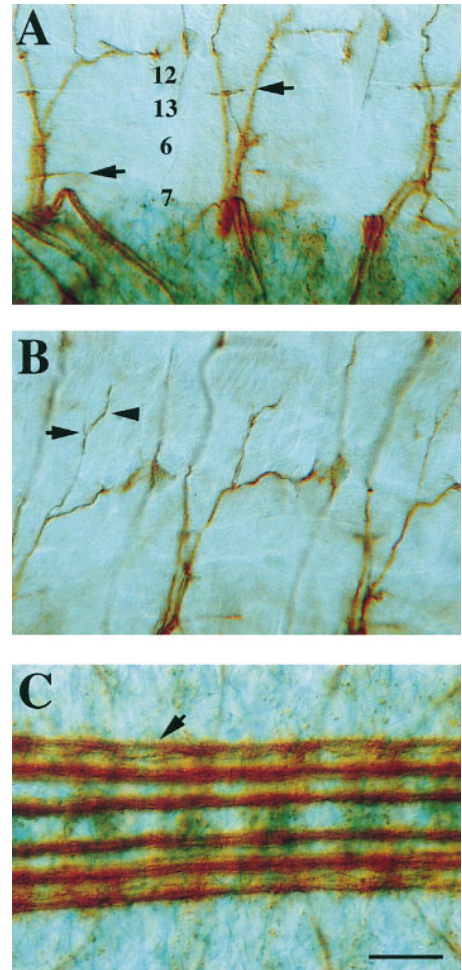


Figure 4. Neuronal Sema I Rescues the Motor Axon and CNS Path-finding Defects in *Sema^{P1}* Mutant Embryos

Sema I was expressed on all neurons using the *GAL4* expression system to rescue *Sema^{P1}* embryonic neuronal phenotypes (see text). Ectopic expression of Sema I in all neurons rescues motor axon (A) and (B) and CNS (C) phenotypes, visualized using mAb 1D4 (blue color is β -galactosidase activity staining, used to identify *Sema^{P1}* mutant embryos).

(A) Rescue of ISNb phenotypes is observed following pan-neuronal *Sema1* expression. Synaptic arborizations between muscles 12 and 13 and between muscles 6 and 7 appear normal (arrows).

(B) *Sema^{P1}* mutant SNa dorsal branches are partially rescued following neuronal *Sema1* expression. The dorsal posterior branch now turns at the bifurcation choice point (arrow) and extends dorsally along muscle 24 (arrowhead).

(C) In the CNS, the third longitudinal connective is continuous and well separated from the MP1 connective following neuronal *Sema1* expression (arrow).

Scale bar, 12 μ m in (A) and (B) and 10 μ m in (C).

wild-type embryos (data not shown). Expression of Sema I^{EC} in all neurons using the *C155-GAL4* driver results in a partial, but significant, rescue of embryonic neuronal *Sema^{P1}* phenotypes and adult lethality. For example, there is a 46% reduction (from 92% to 50%; Table 2; $p < 0.001$) in the fraction of abnormal SNa pathways observed in *Sema^{P1}* mutant embryos when Sema I^{EC} expression is driven by *C155-GAL4*. This reduction is primarily a rescue of the defasciculation defect

at position S1. Similar results are observed using *Scabrous-GAL4* to drive *Sema I^{EC}* in *SemaI^{P1}* mutant embryos (Table 2). Neuronal expression of *Sema I^{EC}* does significantly rescue the ISNb phenotypes observed in *SemaI^{P1}* mutant embryos (from 87% to 60%; $p < 0.001$; Table 1). The partial rescue of *SemaI^{P1}* motor axon pathway phenotypes may reflect a requirement for *Sema I* localization or presentation for ISNb and SNa development that is not completely satisfied by the *Sema I^{EC}* protein in the context of the ectopic expression system used here. In addition to partial rescue of SNa and ISNb defasciculation defects, neuronal expression of *Sema I^{EC}* (either *C155* or *Scabrous-GAL4*-driven) partially but significantly rescues the *SemaI^{P1}* embryonic CNS phenotype, resulting in a 46% reduction (from 31% to 17%; $p < 0.001$) in the fraction of hemisegments with a discontinuous Fas II-positive third longitudinal connective (Table 3). Finally, *C155-GAL4*-driven *Sema I^{EC}* significantly rescues *SemaI^{P1}* adult lethality (to a level of 25% expected adult progeny; Table 3). However, increasing the dosage of *Sema I^{EC}* results in a reduction of this rescue of *SemaI^{P1}* adult lethality, presumably reflecting deleterious effects of panneuronal *Sema I^{EC}* expression.

Taken together, these rescue experiments show that *Sema I* is required in neurons to mediate motor neuron defasciculation events during neurodevelopment. Further, these results strongly suggest that *Sema I* is a ligand for an as yet unidentified receptor on motor axons.

Sema I Can Act as a Repulsive Cue for Motor Axons

To understand better how *Sema I* functions in neurons to mediate axon guidance decisions, we ectopically expressed *Sema I* in all muscles in both wild-type and *SemaI^{P1}* mutant embryos. The *24B-GAL4* line, in combination with the *UAS-SemaI* transgene, was used to ectopically express *Sema I* in all muscles (Luo et al., 1994). Dramatic motor axon defects are observed when *Sema I* is expressed in all muscles in wild-type embryos, and these defects are sensitive to the endogenous dosage of *Sema I*.

The ISNb phenotypes observed in the presence of ectopic expression of *Sema I* protein on all muscles in wild-type embryos are qualitatively similar to those observed in *SemaI^{P1}* mutant embryos alone. First, there is a significant increase, from 0% in wild type to 24% ($p < 0.001$) when *Sema I* is expressed in all muscles in wild-type embryos, in the fraction of hemisegments with a bypass of ISNb with the ISN (Figure 5E; Table 1). These bypass events include both parallel bypass (PB) events, where the ISNb fails to enter the ventral muscle field and extends dorsally in close proximity to the ISN as a separate pathway, and fusion bypass (FB) events, where the ISNb fails to enter the ventral muscle field and extends dorsally along the ISN. Second, there are a significant number of ISNb stall events that are similar to those observed in *SemaI^{P1}* mutant embryos, in that they occur at discrete locations between either muscles 7 and 6 or between muscles 6 and 13. Finally, ISNb pathways with no evidence of synaptic arborizations between muscles 6 and 7, or aberrant synaptic arborizations between

muscles 7 and 6 or on muscle 12, are observed in a large percentage of hemisegments (Table 1).

The occurrence of these motor axon pathfinding defects, which appear to reflect defects in motor axon defasciculation, is critically dependent on the dosage of ectopic *Sema I* expressed on muscles. A single copy of both the *24B-GAL4* driver and *UAS-SemaI* transgenes does not result in the significant ISNb defects observed with two copies of each transgene (Table 1). These events are sensitive, however, to the endogenous dosage of *Sema I* on axons, since a single copy of each transgene in a *SemaI^{P1}* mutant background results in a dramatic enhancement of the ISNb phenotypes observed in *SemaI^{P1}* mutants alone. For example, there is an increase from 7% to 58% in the fraction of hemisegments showing a bypass of ISNb with the ISN (Figures 5A and 5C; Table 1). Unlike the approximately equal numbers of PBs and FBs observed following ectopic *Sema I* expression on muscles in a wild-type background, these bypass events were almost always FBs ($p < 0.001$) (Table 1; Figure 5C).

Effects qualitatively similar to those described above for ISNb were also observed for SNa following ectopic expression of *Sema I* on all muscles both in wild-type and *SemaI^{P1}* mutant embryos. A significant number of S1 stall events was observed when *Sema I* was ectopically expressed on muscles in wild-type embryos (Table 2). SNa defects also include a dramatic enhancement of *SemaI^{P1}* SNa phenotypes when *Sema I* is expressed on all muscles in this genetic background using only a single copy of each transgene. These enhanced defects include SNa fusion bypass events in which SNa fails to enter the ventral muscle field and instead extends dorsally along the ISN (Figure 5B). SNa FBs were never observed in wild-type or *SemaI^{P1}* mutant embryos in the absence of ectopic *Sema I* muscle expression (Table 2; $p < 0.001$). In this genetic background, there is a significant increase in the fraction of hemisegments that exhibit a complete loss of the entire major dorsal SNa branch, and in those pathways that do still have this branch a larger fraction stall at the initial bifurcation of this branch in the lateral muscle group (Figure 5B; Table 2, S1).

Finally, in addition to the enhancement of ISNb and SNa phenotypes observed in *SemaI^{P1}* mutants, ectopic expression of *Sema I* on muscles in this genetic background results in ISN defects. In the vicinity of the dorsal muscles, the ISN normally forms a stereotypic pattern of three arborizations (Desai et al., 1997; Figure 5E). The formation of these arborizations is not disrupted in *SemaI^{P1}* mutants or by ectopic expression of *Sema I* in wild-type embryos using a single copy of each transgene. In *SemaI^{P1}* mutant embryos that express *Sema I* on all muscles, however, the first and second arborizations of ISN were not observed in 29% and 32% of hemisegments, respectively (Figure 5F; $n = 98$). No significant stalling of the ISN was observed in this genetic background. These ISN phenotypes are consistent with the observed ISN expression of *Sema I*.

The motor axon guidance defects produced by ectopically expressing *Sema I* in all muscles are specific to motor axons, do not affect peripheral sensory axon

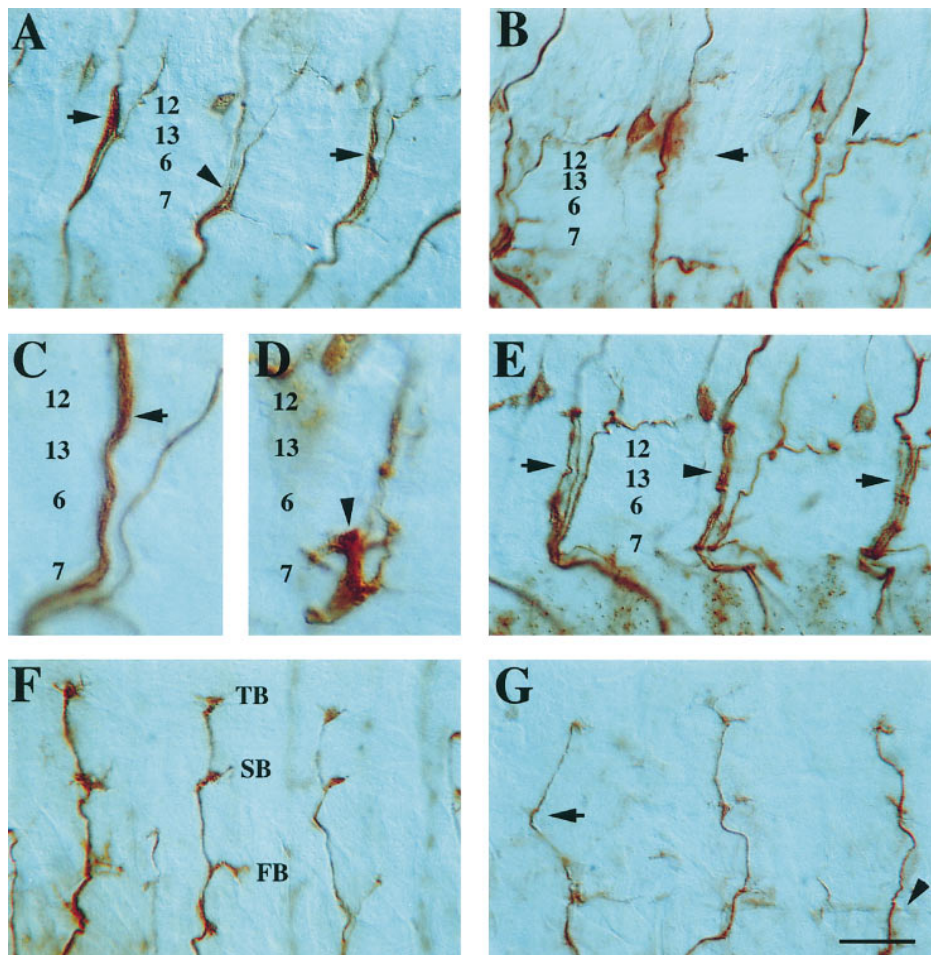


Figure 5. Sema I Can Act as a Repulsive Cue for Motor Axons

Abdominal segments of filleted stage 16/17 *Semal^{P1}* mutant ([A]–[D]) and wild-type (E) embryos that ectopically express Sema I on all muscles reveal ISNb ([A], [C], [D], and [E]), SNa (B) and ISN (G) phenotypes detected using mAb 1D4 immunostaining. (A) Dramatically enhanced *Semal^{P1}* ISNb phenotypes were observed that include failure of ISNb to defasciculate from the ISN, resulting in a fusion bypass with the ISN (arrows). ISNb was also observed to stall ventrally between muscles 6 and 7 (arrowhead). (B) The SNa phenotypes in *Semal^{P1}* embryos that ectopically express *Semal* in all muscles are also enhanced. Often, the entire dorsal SNa branch is missing (arrowhead). Occasionally, the entire SNa is absent from the ventral and lateral muscle fields and appears to have undergone a fusion bypass with the ISN (arrow). (C) A higher magnification view of the ISNb fusion bypass phenotype (arrow) illustrates the thickening of the ISN that accompanies this event. (D) A high magnification view of an ISNb stall between muscles 6 and 7, illustrating the enhancement of the *Semal^{P1}* phenotype caused by ectopic *Semal* on all muscles (arrowhead; compare to Figure 3B). (E) Ectopic expression of Sema I in all muscles in a wild-type background results in ISNb parallel bypass (arrows) and fusion bypass (arrowhead). (F) The three dorsal branches of ISN are easily seen with mAb 1D4 in wild-type embryos that do not express ectopic Sema I (first lateral branch [FB], second lateral branch [SB], and terminal branch [TB] from ventral to dorsal). (G) In *Semal^{P1}* mutant embryos with ectopic expression of Sema I in all muscles, FB (arrowhead) and SB (arrow) often fail to form. Scale bar, 12 μ m in (A), (B), (E), and (F) and 6 μ m in (C) and (D).

pathfinding, and do not appear to be the result of changes in neuronal or muscle cell fate or muscle morphology (assessed as described above for *Semal^{P1}* mutants). The motor axon phenotypes observed by expressing Sema I on all muscles demonstrate an ability of Sema I to prevent motor axons from entering regions of Sema I expression. This results in a failure of motor axon defasciculation at specific choice points and strongly suggests that Sema I can act as a repulsive axon guidance cue.

Discussion

Repulsive cues, acting over either short or long distances, play an essential role in the guidance of axons to their targets during development. Using genetic analysis in *Drosophila*, we have shown that the transmembrane semaphorin Sema I is required in neurons to generate the embryonic pattern of neuromuscular connectivity in the periphery and longitudinal connective formation in the CNS. Our analysis of *Semal* mutants

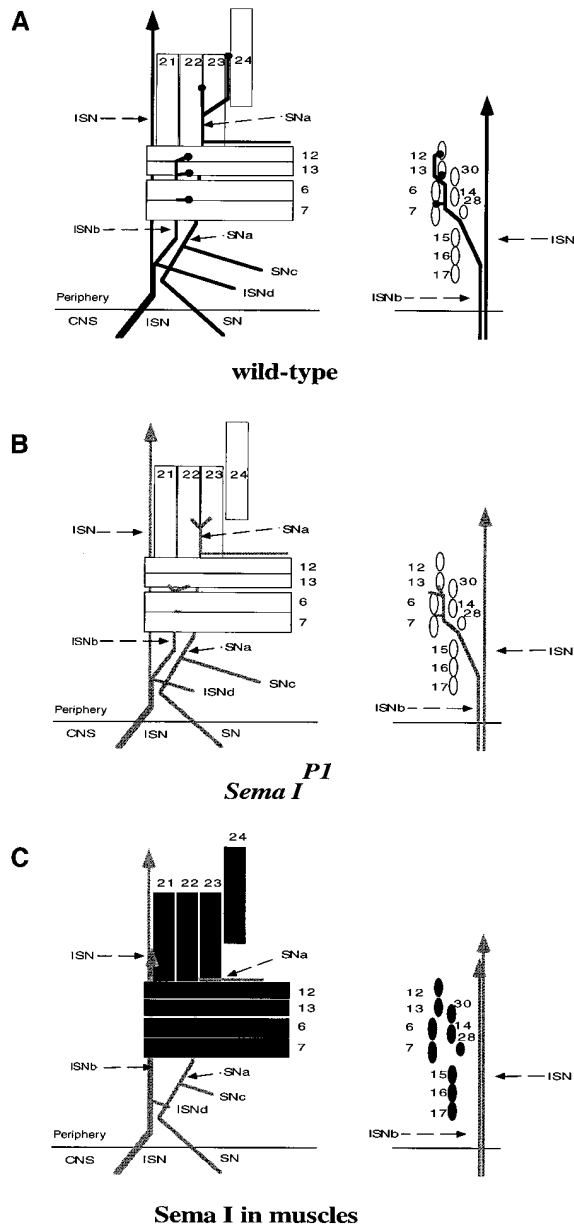


Figure 6. Schematic Representations Summarizing Motor Axon Projections Observed in Wild-Type Embryos, *Sema1^{PI}* Mutant Embryos, and Embryos With Ectopic Expression of Sema I on All Muscles

Schematics of embryonic motor axon branches relevant to this study in lateral and ventral muscles of abdominal segments (A2–A7) in wild-type embryos (A), *Sema1^{PI}* mutant embryos (B), and *Sema1^{PI}* or wild-type embryos with ectopic expression of Sema I on all muscles (C). Representative pathways are shown ([B] and [C]) for the most abundant class of phenotype observed. Diagrams on the left are a lateral view from the internal muscle surface (anterior is to the left), and diagrams on the right are cross sections of the ISNb pathway (internal muscle surface is to the left). Motor axon pathways and muscles in black in (A) and (C) have Sema I on their surfaces. Motor axon pathways in gray ([B] and [C]) are mutant and do not express Sema I. Closed black circles denote synaptic arborizations. (A) SNa and ISNb in wild-type embryos establish characteristic synaptic arborizations in the ventral and lateral muscle fields (see text). The approximate position where the ISNb splits off from the ISN,

suggests that Sema I normally functions to mediate specific axon defasciculation events. We have also demonstrated that motor axons that normally require Sema I for defasciculation events during development are prevented from entering a muscle field where Sema I is ectopically expressed. These results strongly suggest that transmembrane semaphorins, like secreted semaphorins, can mediate repulsive axon guidance decisions during neurodevelopment.

The Role of Sema I in *Drosophila* Neurodevelopment

The establishment of the stereotypic pattern of motor pathways in the PNS requires a series of axon pathfinding events that guide growth cones to sites of synapse formation. Central among these are defasciculation events that occur at discrete locations along these motor pathways. *Sema1* mutants have defects at several of these locations, consistent with motor axons requiring Sema I for choice-point defasciculation. For example, *Sema1* mutants exhibit a low but significant number of ISNb fusion bypass events with the ISN and a large number of ISNb stall events terminating at two locations in the VLMs, where individual motor axons leave the main ISNb branch and elaborate synaptic arborizations. SNa defects in *Sema1* mutants are primarily observed in the dorsal SNa branch where defasciculating motor axons establish separate branches that innervate lateral muscle targets. In *Sema1* mutants, we did not observe SNa or ISNb stall events at positions between defasciculation choice points, nor were motor axons observed to wander far from their normal trajectories. Sema I is localized on most motor and CNS axons, and *Sema1* mutant phenotypes are almost completely rescued by expressing Sema I on all embryonic neurons. Further, a secreted form of Sema I that lacks its transmembrane and cytoplasmic domains is capable of partially rescuing some *Sema1* embryonic neuronal pathfinding defects and adult lethality. Therefore, Sema I is required in neurons to navigate various choice points in motor axon pathfinding and is likely to act as a ligand.

How does Sema I perform this function? We assessed the effect of ectopically expressing Sema I on all muscles during *Drosophila* embryogenesis to see how motor axons respond to this guidance cue. In a wild-type background this has a profound, dosage-sensitive effect on motor axon pathfinding. We observed dramatic motor axon defects that include ISNb bypass events, stalls at discrete locations where synaptic arborizations are normally elaborated, and aberrant synaptic arborization

pathway choice points within the VLMs, and also the location of ISNb synaptic arborizations are indicated in cross-section.

(B) In *Sema1* mutant embryos, ISNb most often stalls between muscles 6 and 13, the location where normally it extends from the external surface of ventral muscle 6 to the internal surface of muscle 13. The dorsal SNa branch most often stalls at the point where normally it would bifurcate. The ISNd is also defective.

(C) In wild-type and *Sema1* mutant embryos that ectopically express Sema I on all muscles, ISNb most often does not enter the ventral muscle field, exhibiting instead a bypass with the ISN. The entire dorsal branch of the SNa is often missing in the *Sema1^{PI}* mutant background.

morphology. These results show that Sema I on muscles can prevent the entry of ISNb axons into the ventral muscle field. Since both ISNb fusion and parallel bypass events are observed, we might predict that endogenous axonal Sema I can still function often in this situation to allow ISNb defasciculation from the ISN. Our observation that lack of endogenous Sema I now renders the ISNb sensitive to a low dose of ectopic Sema I on muscles, which in a wild-type background has no effect, supports this prediction. The bypass events observed in this sensitized background are mostly fusion bypasses, not parallel bypasses, and this provides further support for Sema I's role in mediating defasciculation events during normal motor axon pathfinding. Taken together, all of these data support a model in which neuronal Sema I acts as a ligand for an as yet unidentified receptor on motor axons to mediate repulsive axon-axon interactions at motor pathway defasciculation choice points.

Certain secreted guidance cues, when expressed on all muscles, can also alter motor axon pathfinding in a wild-type background (i.e., Beat, netrin A and netrin B, and Sema II; Matthes et al., 1995; Fambrough and Goodman, 1996; Mitchell et al., 1996) and, like transmembrane Sema I, are presumably able to compete effectively for neuronal receptors with their endogenous counterparts. Though the simplest interpretation of all of these experiments is that the ectopically expressed cues function by directly interacting with neuronal receptors, in the absence of information about receptor identity and distribution, alternative explanations are formally possible. For example, ectopic Sema I expressed on muscles could either bind and inactivate components that normally promote defasciculation or could activate components that can normally promote fasciculation. These models, however, are not supported by the observed defasciculation defects in *SemaI^{P1}* mutants that suggest that Sema I on axons normally promotes defasciculation. Future studies directed toward characterization of a Sema I receptor should clarify our model for Sema I function.

Though the overall penetrance of defective motor axon pathways in *SemaI^{P1}* mutants is very high, the penetrance of specific defects along these pathways is variable. Our results strongly suggest that the *SemaI^{P1}* mutant is a null or very severe loss-of-function *SemaI* allele. Therefore, Sema I function is not absolutely required for motor axon defasciculation events to occur, and it is important to consider Sema I in the context of the complex panoply of motor axon guidance cues that have already been shown to function in these guidance events. Several genes have been identified that affect defasciculation events in motor pathways. These genes, which are expressed on many motor axon branches, include: *FasII*, which encodes an Ig superfamily member that is a homophilic cell adhesion molecule (CAM) (Grenningloh et al., 1991; Lin and Goodman, 1994); *PTP69D* and *PTP99A*, which encode receptor protein tyrosine phosphatases (RPTPs) (Tian et al., 1991; Desai et al., 1996, 1997); and *beaten path (beat)*, which encodes Beat, a novel secreted protein expressed by motor neurons (Fambrough and Goodman, 1996). Mutations in the gene *sidestep (side)*, which has yet to be cloned, also affect defasciculation of the ISNb from the ISN (Sink

and Goodman, 1994, Soc. Neurosci., abstract). The importance of modulating axon-axon adhesive interactions during motor axon pathfinding is underscored by the genetic interactions between *FasII* and *beat*. ISNb axons in *beat* mutants often fail to defasciculate from the ISN; however, this phenotype can be suppressed by hypomorphic *FasII* mutations, suggesting that Beat somehow modulates CAM function in axons to promote defasciculation at choice points (Fambrough and Goodman, 1996).

There are similarities and differences among the motor axon guidance phenotypes we observe in *SemaI* mutants and those seen in mutants for the proteins described above. For example, unlike *beat* or certain RPTP mutant genetic backgrounds, loss of Sema I does not appear to affect ISN pathfinding, since the ISN extends into the vicinity of its dorsal targets in *SemaI^{P1}* mutants, either in the absence or presence of Sema I on muscles. Further, we do not observe in *SemaI^{P1}* mutants the same range of detour and partial bypass phenotypes seen in certain RPTP or Fas II-overexpression mutant genetic backgrounds (Lin and Goodman, 1994; Desai et al., 1996, 1997; Krueger et al., 1996). It remains to be determined whether synergy exists between Sema I and the other proteins expressed on axons that mediate defasciculation decisions. It is clear, however, that regulation of adhesive interactions among motor neurons is crucial for correct choice-point navigation. Sema I may serve to provide an important axonal repulsive component required for regulating defasciculation.

Sema I is not the only semaphorin in *Drosophila* capable of acting as a repulsive cue for motor axons. Sema II, a secreted semaphorin, can act as a selective repellent for the RP3 motor axon when ectopically expressed on ventral abdominal muscles (Matthes et al., 1995). In *SemaII* mutants, embryonic motor axon guidance appears normal. Sema II expression is very high in a single thoracic ventral muscle. Recent observations show that Sema II is also found at low levels in all muscles, and this general expression is important for motor axon guidance (M. Winberg and C.S. Goodman, personal communication). Sema I can act as a repulsive guidance cue for motor axons that require it for pathfinding, which suggests that Sema I-mediated repulsive growth cone guidance normally plays an important role in motor axon pathfinding. Future studies will address whether or not Sema II exerts its repulsive effect on RP3 through a different, or the same, signaling pathway as does Sema I.

Sema I is also required for the formation of the third, most lateral, Fas II-positive connective in the *Drosophila* CNS. The defects in this pathway include abnormal contact of lateral axons with more medial Fas II-expressing connectives and suggest that Sema I may normally function to prevent contact between axons that pioneer this later-forming pathway and connectives that have formed earlier in development. The identities of the neurons that contribute to this connective are not known; however, we observed no defects in numerous earlier-forming CNS pathways in *SemaI^{P1}* mutants. This demonstrates that though Sema I is expressed on most CNS axons from the onset of axonogenesis, it is not generally required for CNS pathway development. Though future experiments are required to directly show that Sema I

can function as a repulsive cue in the CNS, our current study demonstrates a requirement for Sema I in CNS selective fasciculation decisions.

Transmembrane Semaphorins and Neuronal Growth Cone Guidance

Sema I in *Drosophila* is closely related to G-Sema I, the first semaphorin to be identified, which is required for the guidance of the Ti1 afferent pathway in the embryonic grasshopper limb bud (Kolodkin et al., 1992). Antibody blocking experiments show that G-Sema I is required for the Ti1 axons, which do not have G-Sema I on their surface, to make successfully a stereotypic turn on a band of G-Sema I-expressing epithelium. In these experiments, perturbation of G-Sema I results in extensive defasciculation and ectopic branching of the Ti1 axons, showing that G-Sema I on epithelial cells drives fasciculation of these axons. One interpretation of these results, consistent with the aberrant trajectories observed in the antibody blocking studies, is that G-Sema I localized on this band of epithelial cells acts in a repulsive fashion to stall or stop proximally extending growth cones, thereby allowing other guidance cues to direct Ti1 pathway formation. It is interesting to compare these observations in the grasshopper to those in *Drosophila*, as *Drosophila* Sema I on axons appears to drive defasciculation. These results suggest a role dependent on the context of presentation (axonal versus surrounding environment) for these transmembrane semaphorins in regulating the balance between fasciculation and defasciculation of extending axons.

Since Ti1 growth cones have been shown to adhere preferentially to the stripe of G-Sema I-expressing epithelial cells (Condic and Bentley, 1989), and given the caveats inherent in antibody perturbation experiments, it is possible that in the grasshopper G-Sema I serves a permissive or attractive function. Recent experiments have provided evidence that G-Sema I is required in the developing limb bud for initial axonal outgrowth from the subgenual organ (SGO) (Wong et al., 1997). The SGO resides distal to the Ti1 cell bodies and is separated from them by a band of G-Sema I-expressing epithelial cells. Antibody blocking experiments show that proximal extension across this G-Sema I-expressing epithelial band by SGO axons requires G-Sema I. This is consistent with G-Sema I being a permissive cue for SGO axon extension. Our observation that Sema I in *Drosophila* can act as a repulsive cue for motor axons is clearly in contrast to how G-Sema I appears to function in SGO axon guidance. This may simply reflect the bifunctional guidance potential of these related transmembrane semaphorins when mediating axon guidance events involving different classes of neurons. Precedence for bifunctionality of axon guidance cues is best exemplified by the attractive and repulsive functions of netrins (Keynes and Cook, 1996).

Transmembrane semaphorins with the same overall structure as Sema I are selectively expressed in invertebrate and vertebrate nervous systems (Kolodkin et al., 1992, 1993; Eckhardt et al., 1997; Kikuchi et al., 1997; Zhou et al., 1997). For example, the murine semaphorins Sema VIa, Sema VIb, and Sema Z belong to this class

of transmembrane semaphorin and are selectively expressed in subsets of PNS and CNS embryonic and adult neurons. These vertebrate semaphorins are also likely to provide essential local guidance cues that allow complex central pathways to develop in an orderly fashion.

The molecular mechanisms that govern semaphorin signaling to the growth cone cytoskeleton are poorly understood. It has recently been shown in vertebrates that neuropilin-1, one of a family of proteins selectively expressed on subsets of embryonic neurons, is a receptor or a component of a receptor for Sema III/Coll-1 (He and Tessier-Lavigne, 1997; Kolodkin et al., 1997). Neuropilin is a transmembrane protein with a very short novel cytoplasmic domain that contains no obvious catalytic potential. Our results suggest that Sema I, like certain secreted semaphorins, can act as a repulsive guidance cue, and that the extracellular sema domain can mediate in vivo repulsive guidance events. Whether any transmembrane semaphorin requires neuropilin-1 or related proteins to signal growth cone steering events is not known. Further genetic analysis of *Semal* may uncover Sema I signaling components and begin to address these issues.

Experimental Procedures

Molecular Analysis of *Semal*^{P1} and *Semal* cDNA Assembly

A *Semal* cDNA containing the complete *Semal* open reading frame (ORF) was assembled from two partial but overlapping *Semal* cDNA clones (which themselves were originally cloned into the *pCR II* vector [Invitrogen]). One cDNA contained the 5' UTR and the proximal part of the *Semal* ORF (ending at amino acid [aa] 490), while the second contained the distal part of the ORF (starting at aa 148) and the 3' UTR. An EcoRV-Bsm I fragment from the 5' cDNA clone (containing aa 430 to 3' UTR) was ligated to the second cDNA clone (containing 5' UTR to aa 430) that had been restricted with EcoRV and Bsm I. To generate the *UAS-Semal* and *UAS-Semal*^{EC} rescue constructs, a 2.5 kb XbaI-XhoI *Semal* polymerase chain reaction (PCR) amplification product containing the entire *Semal* ORF, or a 1.8 kb EcoRI-XbaI PCR product containing just the Sema I ectodomain (aa 1–612), was cloned into XbaI-XhoI or EcoRI-XbaI sites of the *pUAST* vector and introduced into *w*¹¹¹⁸ background by P element transformation (Brand and Perrimon, 1993).

A cosmid clone (1C1) containing *Semal* genomic sequences was isolated by screening a *Drosophila* (iso-1) genomic cosmid library (Tamkun et al., 1992) using a 2 kb fragment of the *Semal* cDNA. Genomic DNA from *Semal*^{P1} flies was partially restricted using Sau3A and subjected to inverse PCR using specific primers for the P transposable element *P[lacW]* ("P-ry" = GATTGTTGATTAACCTTAGCA TGTCCTG and "P-TR2" = CGACGGGACCACCTTATGTTATTCAT CATG). The resulting 789 bp PCR product was cloned and sequenced, and this genomic DNA was found to be identical to genomic DNA sequence from cosmid 1C1 and to also contain sequences identical to those at the 5' end of the *Semal* cDNA. The location of the P element insertion in *Semal*^{P1} was confirmed by sequencing 1C1 using a primer pointed upstream from the start of *Semal* cDNA sequence. This sequence was identical to that of the inverse PCR product. Additional upstream primers were synthesized to obtain another 805 bp of sequence on the opposite side of the *Semal*^{P1} insertion. The *Semal*^{P1} insertion was shown to interrupt the 5' transcribed but untranslated region of *Semal* mRNA by Northern analysis in which a probe (Probe A; see Figure 2) containing 500 bp of DNA upstream of the *Semal*^{P1} insertion was shown to detect the same *Semal* transcripts as does the entire *Semal* cDNA. Southern analysis using 1C1 and other phage P1 genomic clones from this region show that the ORF of *Semal* spans a genomic region of ~50 kb in length. Further, this analysis shows that the *Semal*^{P1} insertion is on the 5' side of an intron that is at least 10 kb in length. The

splice site for this intron is located at least 1 kb downstream of *SemaI^{P1}* but upstream of the start of translation.

Sema I Antibodies and Immunohistochemistry

Six histidine-tagged bacterial fusion proteins were made to generate rabbit polyclonal antisera or mouse monoclonal antibodies against Sema I. Fusion proteins were constructed by cloning PCR fragments containing either the extracellular semaphorin domain (aa 225–612) or intracellular domain (aa 638–776) of Sema I into *pGE30* vector (Qiagen). Fusion proteins were purified using nickel-chelate affinity chromatography. Immunohistochemistry of *Drosophila* embryos was performed as described (Patel, 1994), using peroxidase-conjugated affinity-purified goat anti-rabbit and anti-mouse IgG purchased from Jackson ImmunoResearch Laboratories. Anti-Sema I antisera was used at a 1:3000 dilution. *SemaI^{P1}* homozygous embryos were identified by performing β -galactosidase activity staining and selecting those embryos with the *SemaI*-specific distribution of β -galactosidase activity but without the pan-neuronal pattern of β -galactosidase activity that is produced by the marked balancer chromosome. Mutant embryos selected in this fashion were processed for subsequent immunohistochemistry using the Vectastain ABC kit (Vector Laboratories) to enhance the sensitivity.

Drosophila Genetics and *SemaI*

Phenotypic Characterization

The two *SemaI* alleles *SemaI^{P1}* and *SemaI^{P2}* were obtained from the Berkeley *Drosophila* Genome Project (#13702 and #03509, respectively). For rescue experiments, a stock with the *UAS-SemaI* inserted on the second chromosome was recombined into a *SemaI^{P1}* or *SemaI^{P2}* background, and this stock was crossed to the GAL4 driver lines *C155*, *Scabrous*, and *24B* for rescue and ectopic expression experiments. All semi-lethal lines were balanced with a marked second chromosome balancer strain (*CyOlacZ*). Staging of embryos was performed according to the pattern of head involution, CNS retraction, and gut morphology (Campos-Ortega and Hartenstein, 1985; Klambt et al., 1991). Embryos showing CNS retraction to abdominal segment A6, stage 16/17, were chosen for scoring all embryonic phenotypes. SNa and ISNb phenotypes were characterized in segments A2–A7, and CNS phenotypes in segments A2–A6. Only dissected embryos were used to evaluate and score phenotypes. Adult lethality was determined by calculating the ratio of observed adult flies of the indicated genotype to expected adults by Mendelian segregation. The number of balancer-containing heterozygotes, used to determine this ratio, was in each cross over 600 flies. The chi-squared test was used to analyze the statistical significance of differences in embryonic and adult phenotypes observed among the genotypes described in this study.

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