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Semaphorin II Can Function as a Selective Inhibitor of Specific Synaptic Arborizations

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Summary

Previous studies showed that grasshopper semaphorin I, a transmembrane semaphorin, functions in vivo to steer a pair of growth cones, prevent defasciculation, and inhibit branching; and that chick collapsin, a secreted semaphorin, can function in vitro to cause growth cone collapse. Semaphorin II, a secreted semaphorin in Drosophila, is transiently expressed by a single large muscle during motoneuron outgrowth and synapse formation. To test the in vivo function of semaphorin II, we created transgenic Drosophila that generate ectopic semaphorin II expression by muscles that normally do not express it. The results show that semaphorin II can function in vivo as a selective target-derived signal that inhibits the formation of specific synaptic terminal arbors.

Introduction

The formation of specific synaptic connections is generated in part by the molecular mechanisms controlling growth cone guidance and target recognition (reviewed by Goodman and Shatz, 1993; Goodman, 1994). Pathway and target recognition is mediated by a range of mechanisms, including contact-mediated attraction, chemoattraction, contact-mediated repulsion, and chemorepulsion. Members of a variety of gene families have been implicated to function in vivo as signals for pathfinding and targeting, including, for example, members of the immunoglobulin (Grenningloh et al., 1991; Ramos et al., 1993; Lin et al., 1994; Chiba et al., 1995), leucine-rich repeat (Nose et al., 1992, 1994), netrin/UNC-6 (Hedgecock et al., 1990; Ishii et al., 1992; Hamelin et al., 1993; Serafini et al., 1994; Kennedy et al., 1994), and semaphorin (Kolodkin et al., 1992, 1993; Luo et al., 1993) gene families.

The semaphorins are a family of cell surface and secreted proteins that are conserved from insects to humans. Semaphorins are ~750 amino acids in length (including signal sequence) and are defined by a conserved ~500 amino acid extracellular semaphorin domain containing 14–16 cysteines, many blocks of conserved residues, and no obvious repeats (Kolodkin et al., 1992, 1993; Luo et al., 1993). The transmembrane semaphorins have an additional ~80 amino acid stretch, a transmembrane domain, and a 80–110 amino acid cytoplasmic domain. The secreted semaphorins have an additional ~20 amino acid stretch, a single immunoglobulin domain, and a 70–120 amino acid C-terminal region. In addition, two semaphorins are encoded in viral genomes (Kolodkin et al., 1993).

Two complementary sets of results suggest that semaphorins can function as signals that guide growth cones. First, semaphorin I (Kolodkin et al., 1992), a transmembrane semaphorin in insects, functions in the grasshopper limb bud to stall and then steer a pair of growth cones as they encounter epithelial cells expressing it. Semaphorin I also prevents the axons that encounter it from defasciculating and inhibits branching. Second, collapsin, a secreted semaphorin in chick, in vitro can cause the collapse of growth cones from dorsal root ganglion neurons (Luo et al., 1993).

Semaphorin II is a secreted semaphorin in Drosophila that is dynamically expressed during embryonic development by a subset of neurons in the central nervous system (CNS) and by a single large thoracic muscle in the periphery (Kolodkin et al., 1993). Its transient expression by a specific muscle during motoneuron outgrowth and synapse formation suggests that, among its potential roles, semaphorin II might function as a secreted target-derived signal that regulates the formation of synaptic connections.

The generation of neuromuscular specificity in Drosophila provides an ideal system for testing the in vivo function of such a putative guidance and targeting molecule because much is already known about the cellular environment, pathfinding, and targeting events that underlie its development (e.g., Sink and Whitington, 1991; Van Vactor et al., 1993; Keshishian et al., 1993; Broadie et al., 1993). All motoneuron growth cones and axons express fasciclin II during axon outgrowth and synapse formation (Van Vactor et al., 1993) and thus can be visualized using the 1D4 monoclonal antibody (MAb) that recognizes fasciclin II (G. Helt and C. S. G., unpublished data). Moreover, some embryonic motoneurons (e.g., RP3 and RP1) can be penetrated with microelectrodes and filled with dye to reveal their growth cones and terminals (Sink and Whitington, 1991).

To test the in vivo function of semaphorin II during growth cone guidance and target recognition, we created transgenic Drosophila that express semaphorin II by muscles that normally do not express it. The results show that semaphorin II can function as an inhibitory signal during target recognition. Semaphorin II inhibits two identified motoneuron growth cones (RP3 and DC1) from forming normal synaptic terminal arborizations on their target muscles, while two other growth cones (RP1 and RP4) appear unresponsive to contact with the protein.

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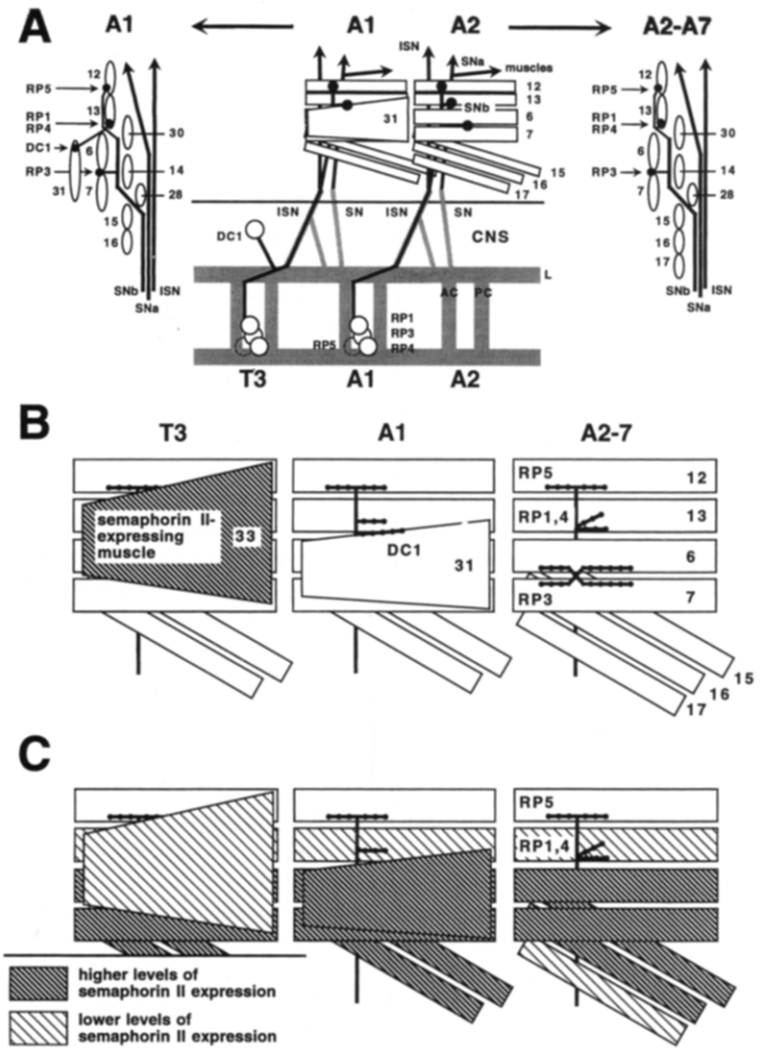


Figure 1. Motoneuron Projections in Relation to Semaphorin II Expression in Wild-Type and *Toll–small* Transgenic Embryos

Schematic diagrams of the ventral muscles in the T3, A1, and A2–A7 segments of the Drosophila embryo, focusing on those muscles innervated by SNb, and the identity, synapses, axon trajectories, and cell body locations of identified SNb motoneurons. SN, segmental nerve; ISN, intersegmental nerve; RP1, RP3, RP4, and RP5, identified motoneurons in the RP cluster; DC1, identified motoneuron innervating muscle 31 in segment A1 and not a member of the RP cluster; numbers refer to muscle identities; L, longitudinal connectives; AC and PC, anterior and posterior commissures.

(A) The embryonic motoneuron branches (with details of the SNb) and ventral muscles in hemisegments of abdominal segments A2–A7, which have four internal longitudinal ventral muscles (7, 6, 13, and 12), and of segment A1, which has an extra internal longitudinal ventral muscle (31), are shown schematically. Diagram in the middle is a view from the internal surface (dorsal is up; anterior is to left). Diagrams at left and right are cross-sectional views (dorsal is up; inner surface is to left, outer surface to the right).

(B) Enlarged view of the ventral muscles and their innervation as visible from an internal view, showing muscle 33 in segment T3, which expresses high levels of semaphorin II in wild-type embryos.

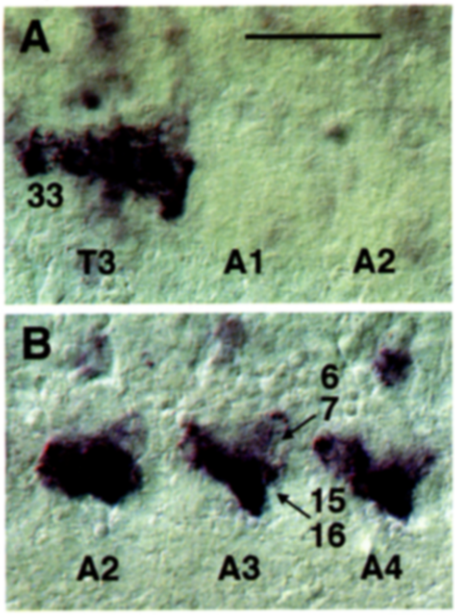
(C) Ectopic expression of semaphorin II in *Toll–small* embryos. Note high levels of semaphorin II expression by muscles 15, 16, 7, 6, and 31 and low levels by muscles 17, 12, and 33, as driven by the *Toll–small* transgene (in the embryo, this is superimposed on the normal high level of expression by muscle 33). Diagram shows resulting pattern of SNb branching and innervation in A1 and A2 segments as viewed from internal surface; note absence of normal pattern of innervation of muscles 7, 6, and 31. See text for further details.

Results

Semaphorin II is normally expressed by ventral muscle 33 in thoracic segment T3 (Hooper, 1986) (Figures 1B and 2A). On either side in each larval abdominal segment from A2 to A7 in Drosophila, there are a total of 30 muscles (1–30), including four prominent internal ventral longitudinal muscles (from ventral to dorsal): 7, 6, 13, and 12 (Figures 1A and 2B). The ventral muscles in the first abdominal segment (A1) are largely identical to those found in A2–A7, except for one additional muscle, 31 (Hooper, 1986), just internal to muscles 7, 6, and 13 (A1 is also missing two muscles, 17 and 25; Bate, 1993). The pattern of ventral muscles in segment T3 displays a number of differences from abdominal segments, among them being muscle 33 just internal to muscles 7, 6, 13, and 12. Larval muscle 33 stretches across the other thoracic segments to attach to the mouth parts (Hooper, 1986; Bate, 1993). All of these internal ventral muscles are innervated by branches of segmental nerve b (SNb), including muscle 33 in segment T3 (Figure 1A).

At late stage 16/early stage 17, the synaptic arborizations on muscles 7, 6, 13, and 12 in A1–A7 and on muscle 31 in A1 are all easily visualized with MAb 1D4 (anti-fasciclin II) (Figures 3A and 3C) and are accessible to 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI) backfills in dissected embryos (data not shown). Most of the motoneurons that innervate these muscles can be readily identified in the CNS, penetrated with microelectrodes, and filled with the intracellular dye Lucifer yellow (LY) (Figures 4A and 4B). In these ways, we can study the innervation of these muscles in wild-type, mutant, and transgenic embryos.

The RP3 growth cone synapses on muscles 7 and 6 in the cleft between these two muscles; RP1 and RP4 growth cones synapse on the proximal edge of muscle 13, and



RP5 synapses on muscle 12 (see Figures 1A and 1B; Figures 4A and 4B) (Sink and Whitington, 1991; Van Vactor et al., 1993). In the present study, we used DiI backfills from the synapse to show that muscle 31 in segment A1 is innervated at early stage 17 on its dorsal edge by at least one motoneuron, called DC1, which has an ipsilateral cell body lateral to the longitudinal connectives (see Figure 1A).

Figure 2. Muscle Expression of Semaphorin II RNA in Wild-Type and *Toll–semaII* Transgenic Embryos

Whole-mount dissections of early stage 16 Drosophila embryos (anterior to left, dorsal up) showing expression of *semaII* RNA in wild-type (A) and *Toll–semaII* transgenic (B) animals as revealed by digoxygenin whole-mount in situ hybridization. (A) The *semaII* RNA is transiently expressed at high levels in muscle 33 in segment T3 in wild-type embryos. (B) In *Toll–semaII* embryos, *semaII* RNA is expressed at high levels in muscles 15, 16, 7, and 6 (and at lower levels in other muscles; see Figures 1 and 5 and text for details). Scale bar, 30 μm.

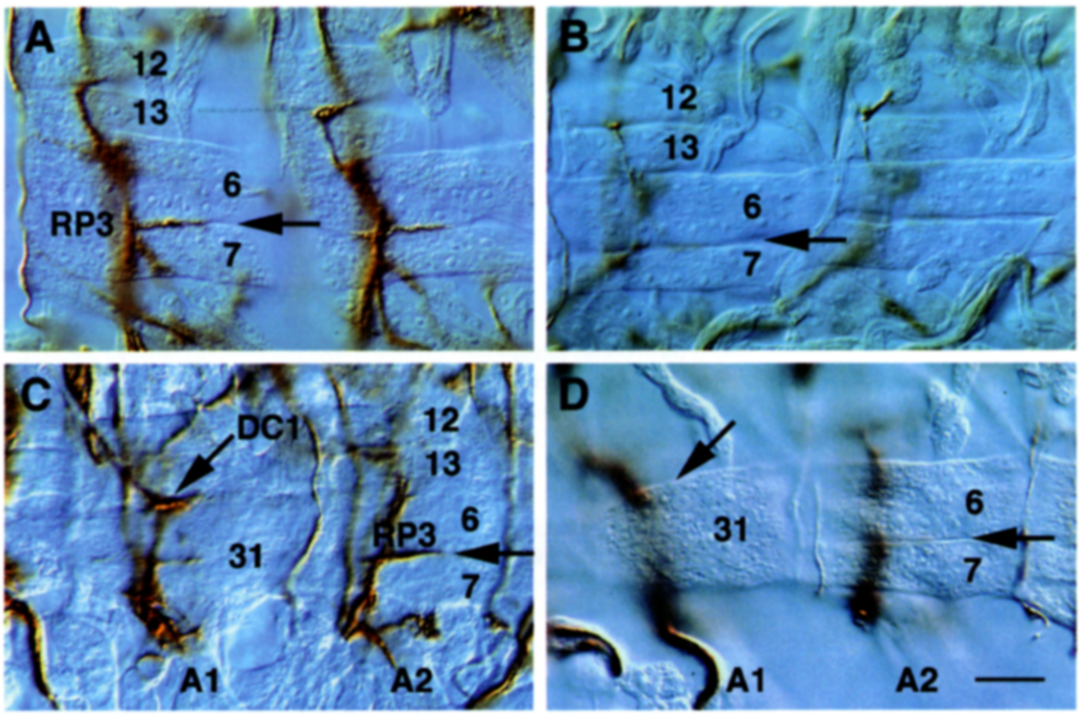


Figure 3. Ectopic Semaphorin II Expression Alters Muscle Innervation

Photomicrographs of two abdominal segments in late stage 16 filleted wild-type (A and C) and *Toll–semaII* (B and D) embryos as stained with MAb 1D4 (anti–fasciclin II) and horseradish peroxidase immunohistochemistry.

(A and B) Pairs of abdominal segments A2–A7. The focus is on the cleft between muscles 7 and 6 (arrow), where in wild type (A) the RP3 growth cone has transformed into a synaptic terminal arbor. Note the absence of the normal terminal arbor in the *Toll–semaII* transgenic embryo (B).

(C and D) Pairs of abdominal segments A1 and A2. The focus is on the dorsal edge of muscle 31 in segment A1 (left arrow in left segment), where in wild type (C) the DC1 growth cone has transformed into a synaptic terminal arbor, and on the cleft between muscles 7 and 6 (right arrow in right segment), where in wild type (C) the RP3 growth cone has transformed into a synaptic terminal arbor. Note the absence of the normal terminal arbor on muscle 31 in segment A1 and on muscles 7 and 6 in segment A2 in the *Toll–semaII* transgenic embryo (D). Numbers refer to muscle identities. Scale bar, 10 μm.

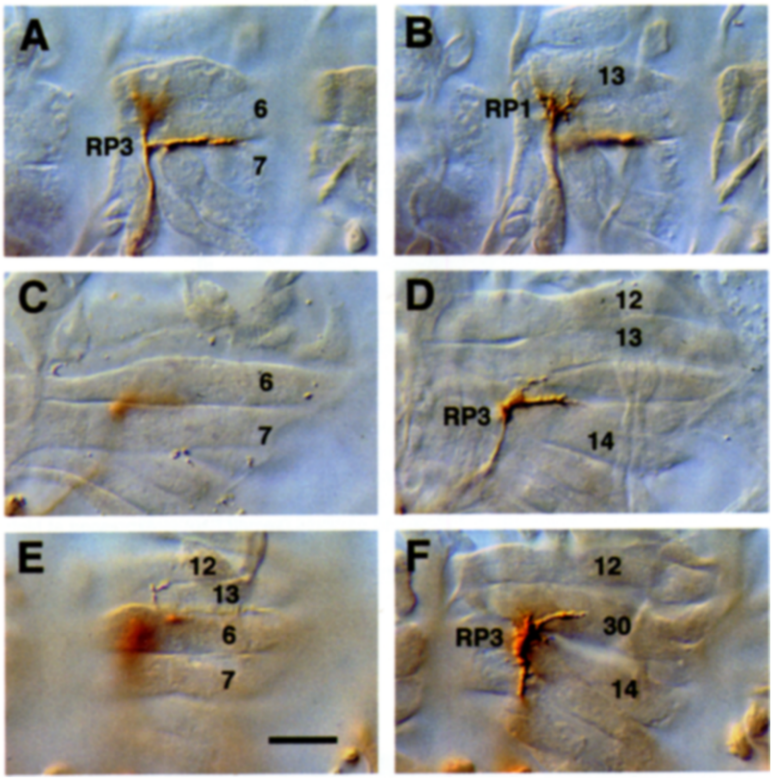


Figure 4. Ectopic Semaphorin II Expression Alters RP3 Development

Photomicrographs of late stage 16 filleted embryos in which the cell bodies of the RP3 (and RP1 in [A] and [B]) motoneurons have been penetrated with microelectrodes and filled with the dye LY, followed by horseradish peroxidase immunocytochemistry using an anti-LY antibody. The left–right pairs shown in (A) and (B), (C) and (D), and (E) and (F) are two different focal planes of the same preparations. The left focal plane in each pair is on the cleft between muscles 7 and 6, where in wild-type embryos the RP3 growth cone would have transformed into a synaptic terminal arbor. The right focal plane in each pair is external to muscles 7 and 6 and just internal to muscles 14 and 30. This is the region where the RP3 growth cone is often located in *Toll–semaII* transgenic embryos at this stage.

(A and B) An example of wild-type morphology of the RP3 (A) and RP1 (B) terminal arbors in a *Toll–semaII* transgenic embryo; wild-type morphology was observed in about 30% of RP3 motoneurons filled with dye in these transgenic embryos. Note that this is an example from a double fill of both neurons.

(C and D; E and F) Two examples of abnormal RP3 growth cones in *Toll–semaII* transgenic embryos. Instead of forming a normal synaptic terminal arborization in the cleft between muscles 7 and 6 (C and E), RP3 instead is still a growth cone in the region around muscle 14. See text for further details. Scale bar, 10 μm.

In contrast with the relative ease of studying the internal ventral muscles in segments A1–A7, the innervation of muscle 33 in segment T3 is very difficult to study because it is innervated on its external face on the opposite side from the surface exposed in dissected embryos. Thus, we do not know the identity of which motoneuron(s) innervates it in wild-type, in mutant, or in transgenic embryos.

During early to mid–stage 16, SNb growth cones make extensive filopodial contact with both their target muscles and adjacent muscles. By late stage 16, these growth cones retract their filopodia on adjacent muscles and form functional synapses on their appropriate target muscles, a process that is complete by early stage 17. Thus, our analysis of growth cone behavior is focused on late stage 16/early stage 17 when in wild-type embryos the normal pattern of synaptic innervation is already present.

We first examined embryos homozygous for loss-of-function mutations in the semaphorin II gene, *semaII* (Kolodkin et al., 1993). Semaphorin II is normally expressed by muscle 33 in segment T3. Using 1D4 staining, no dramatic loss-of-function phenotype is seen in the pattern of SNb branching around muscle 33 in segment T3 in *semaII* mutant embryos. This observation is reminiscent of previous studies in this same system that showed that the ectopic expression of connectin generates a much stronger and more penetrant phenotype (the gain of function) than does simply removing the protein (the loss of function) (Nose et al., 1994). With the connectin experiments (Nose et al., 1992, 1994) as our paradigm, we next examined the gain-of-function phenotype by ectopically expressing semaphorin II by other ventral muscles whose innervation is well known.

We used P element–mediated transformation to generate ectopic expression of semaphorin II by a different subset of ventral embryonic muscles during motoneuron pathfinding and targeting. We used a heterologous enhancer (from the *Toll* gene) to ectopically express semaphorin II in all segments by some of the ventral muscles that normally do not express it, but that are adjacent to the normally semaphorin II–expressing muscle 33 in segment T3 (see Figures 1C and 2B).

Toll is expressed on a subset of embryonic muscles (Nose et al., 1992). A 6.5 kb fragment in the 5′ flanking region of the *Toll* gene was previously shown to function as an enhancer element that can drive expression of a *lacZ* reporter gene in a subset of muscles (Wharton and Crews, 1993). At stage 16, *lacZ* is driven at high levels in muscles 28, 14–16, 7, and 6 and at lower levels in muscles 30, 17, 13, and some lateral muscles (Nose et al., 1992); we also observe *lacZ* at a high level in muscle 31 in A1 and at a lower level in muscle 33 in T3.

This 6.5 kb fragment of the *Toll* gene was previously used to ectopically express connectin (Nose et al., 1994), and here it was used ectopically to express semaphorin II by these ventral muscles (see Figures 1C and 2B). The construct contains the 6.5 kb *Toll* enhancer, the *hsp70* promoter, a *semaII* cDNA with the entire open reading frame, and the SV40 polyadenylation site, all inserted into

Table 1. Defects in Innervation of Ventral Muscles by SNb Motoneurons in Different Genotypes with P Elements Driving Ectopic Semaphorin II Expression

Genotype[a]	P Element Copy Number	Late Stage 16			Mid-Stage 17		
		Muscles 6/7 (A2–A7) %	Muscle 31 (A1) %	Muscle 13 (A2–A7) %	Muscles 6/7 (A2–A7) %	Muscle 31 (A1) %	Muscle 13 (A2–A7) %
Wild type	0	3 (59)[b]	0 (9)	0 (70)	0 (40)	0 (8)	0 (48)
687/687	2	64 (123)	50 (8)	0 (44)	27 (132)	80 (15)	0 (95)
685/685	2	68 (104)	61 (13)	0 (104)	29 (41)	60 (5)	N (40)
78/78	2	53 (184)	57 (21)	0 (141)	ND	ND	ND
685/685; 78/78	4	74 (59)	60 (10)	0 (59)	ND	ND	ND

[a] For details on generation of specific P element genotypes, see Experimental Procedures.
[b] Percent is of muscle innervation absent. Numbers shown in parentheses are number of segments examined.
ND, not determined.

a P element vector. Thirteen independent *Toll–semall* transformant lines were isolated, three of which are analyzed here (Table 1).

As expected, *semall* mRNA is ectopically expressed by the normally Toll-positive ventral muscle fibers in *Toll–semall* embryos (see Figure 2B). The ectopic muscle expression is first detected at stages 11–12, peaks at stages 14–15, and declines during stage 16. It is strongest in muscles 28, 14–16, 7, and 6. By late stage 16, ectopic *semall* mRNA expression can no longer be detected. The 6.5 kb *Toll* enhancer drives expression of a reporter *lacZ* gene in some other tissues known to express Toll, including the dorsal vessel (Wharton and Crews, 1993). Ectopic *semall* mRNA expression persists into stage 17 in the dorsal vessel, thus allowing for the unambiguous identification of embryos carrying the transgene at this stage.

No gross defects are seen in the CNS or in the periphery in the *Toll–semall* embryos. The development of the muscles that ectopically express semaphorin II appears normal in *Toll–semall* embryos, as indicated both by their morphology (as visualized with Nomarski optics) and their expression of various other surface markers (fasciclin III and fasciclin II) during stages 15–17. The timing and morphology of the differentiation of the muscle pioneers, muscle insertions, and myoblast fusions to the pioneers are normal in the semaphorin II–expressing muscles in these embryos. These observations suggest that the ectopic expression of semaphorin II does not alter the differentiation of ventral muscles per se.

Although the muscles appear normal in *Toll–semall* embryos, we observe abnormalities in the development of certain branches of the SNb motor nerve that normally innervate muscles 7 and 6 in all abdominal segments and muscle 31 in A1. We observe the same phenotypes in all three *Toll–semall* lines examined (Table 1). The effects of ectopic semaphorin II expression are specific to SNb and SNd motoneurons. The other three motor nerves (the intersegmental nerve, SNa, and SNc) retain their normal morphology and branching in *Toll–semall* embryos. The SNd motoneurons, which normally innervate muscles 15–17 (which ectopically express semaphorin II in *Toll–semall* embryos), display less severe abnormalities at similar frequencies to the SNb in these embryos.

The innervation of muscles 7 and 6 by the RP3 motoneuron is dramatically altered in *Toll–semall* embryos (see Figure 3B; Table 1); innervation is used here as an anatomical description of the normal terminal synaptic arborization in the cleft between muscles 7 and 6. In *Toll–semall* embryos, muscles 7 and 6 appear uninnervated in 53%–68% of segments at late stage 16 (depending upon which line is examined; see Table 1). A prominent feature of the abnormal SNb projection at this stage is the large growth cone processes emanating from the SNb axons just beyond muscle 14 and near to or in contact with muscle 30. During mid–stage 17, several hours after ectopic semaphorin II expression has disappeared, muscles 7 and 6 remain uninnervated in 27%–29% of segments. In contrast, in wild-type embryos of the same genetic background but lacking the *Toll–semall* transgene, muscles 7 and 6 are innervated in >97% of segments at late stage 16 and 100% of segments at mid–stage 17.

Intracellular dye fills of the RP3 motoneuron (Sink and Whitington, 1991) in *Toll–semall* embryos (line 685/685) at late stage 16/early stage 17 confirm this result: 11 of 16 RP3 axons (69%) were abnormal. Instead of forming the normal synaptic terminal arborization in the cleft between muscles 7 and 6 (Figures 4C and 4E), these RP3 growth cones are instead just external to muscles 7 and 6, near muscle 14 and often near muscle 30 as well (Figures 4A and 4B, Figures 4C and 4D, and Figures 4E and 4F show different focal planes of the same preparations). These results are summarized in Figure 5B.

As a control, the innervation of muscle 13 by both RP1 and RP4 appears unperturbed in 100% of segments in *Toll–semall* embryos (Table 1). Muscle 13 is likely to express low levels of semaphorin II in *Toll–semall* embryos. In addition, to reach muscle 13, the motoneurons that innervate it (RP1 and RP4) normally extend between muscles that express very high levels of semaphorin II in *Toll–semall* embryos (e.g., in the region between muscles 15, 28, 14, 7, and 6) (see Figures 1 and 5). This axon trajectory appears normal in *Toll–semall* embryos. Moreover, intracellular dye fills of RP1 in *Toll–semall* embryos at late stage 16/early stage 17 reveal that 7 of 7 RP1 axons follow their normal trajectory to innervate the inner surface of muscle 13 (Figure 5B).

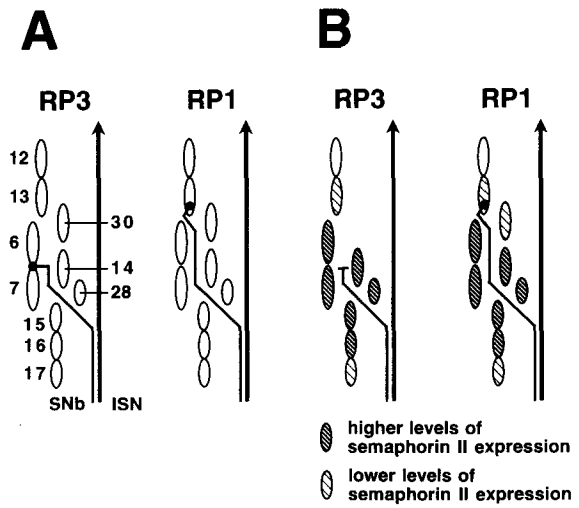


Figure 5. Behavior of RP3 and RP1 Growth Cones in Response to Ectopic Semaphorin II Expression

(A) Schematic diagrams showing cross-sectional views of wild-type pattern of RP3 and RP1 motoneuron axon trajectories and synaptic connections. In late stage 16 wild-type embryos, RP3 and RP1 growth cones have reached their targets and transformed into synaptic terminal arbors. The numbers are muscle identities. External is to the right. The intersegmental nerve (ISN) innervates dorsal muscles, while SNb, which contains the RP3 and RP1 axons, innervates ventral muscles. The RP3 motoneuron innervates muscles 7 and 6; RP1 innervates muscle 13.

(B) In *Toll–semaII* embryos, semaphorin II is ectopically expressed at high levels by muscles 15, 16, 28, 14, 7, and 6 and at much lower levels by muscles 17 and 13. The RP3 motoneuron shows dramatic abnormalities, typically entering the ventral muscle field at the normal location and extending to the region near muscle 14, but (so long as semaphorin II is expressed) failing to innervate muscles 7 and 6. RP1 appears wild type in these embryos.

We also examined the innervation of muscle 31 in segment A1 in *Toll–semaII* embryos. Muscle 31 expresses high levels of semaphorin II in these embryos. The DC1 motoneuron, which innervates muscle 31, normally follows the same trajectory as the RP1 motoneuron innervating muscle 13 (see Figure 1A). Instead of innervating the inner surface of muscle 13, DC1 extends inward to innervate the dorsal surface of muscle 31 (see Figures 1 and 3C). In *Toll–semaII* embryos, DC1 is abnormal. In wild-type embryos, muscle 31 is innervated in 100% of A1 segments at late stage 16 (Table 1). However, in *Toll–semaII* embryos, DC1 does not innervate muscle 31 in 60%–80% of segments at mid–stage 17 (see Figure 3D), depending upon the line examined (Table 1; because there is only one A1 segment per embryo, the total number of A1 segments examined is much lower than A2–A7, and thus the differences between different lines and stages are not significant).

The phenotypes observed with MAb 1D4 staining (see Figure 3) do not qualitatively change in embryos from three different transgenic lines carrying independent insertions that drive different levels of expression and, as a result, different levels of phenotypic penetrance (Table 1). The phenotypes also do not qualitatively change in embryos carrying four copies of the transgene as compared with two copies (Table 1). The penetrance of the muscle 6/7 phenotype increases slightly (from 53%–68% in the two parental two-copy lines to 74% in the combined four-copy line), but no new phenotypes are observed in embryos carrying an increased copy number. Most important, in all lines and all dosages, the RP3 growth cone enters the ventral muscle region but stalls near muscle 14 and fails to form its synaptic terminal arborization on its normal target muscles 7 and 6. All that changes is the penetrance of this phenotype.

Discussion

The present study shows that semaphorin II in Drosophila can function in vivo as a target-derived signal that inhibits the formation of synaptic terminal arbors. In contrast with connectin (Nose et al., 1994), netrin-1 (Colamarino and Tessier-Lavigne, 1995 [this issue of *Cell*]), and semaphorin III (Messersmith et al., 1995), which can function as repellents for growth cone pathfinding, semaphorin II appears to be able to function in vivo in a qualitatively different fashion as an inhibitor of synapse formation during target recognition.

Semaphorin II is transiently expressed by a single large ventral muscle in segment T3 during motoneuron outgrowth. We tested the in vivo function of semaphorin II by creating transgenic Drosophila that generate ectopic semaphorin II expression by other ventral muscles in thoracic and abdominal segments that normally do not express it. The results lead to three major conclusions.

First, when semaphorin II is ectopically expressed by many ventral muscles along the SNb pathway, the RP1 and RP4 growth cones are unperturbed in their pathfinding toward their target, muscle 13 (which does express semaphorin II, but at considerably lower levels than by the targets of RP3, muscles 7 and 6). These growth cones pathfind right between muscles 15, 28, 14, 7, and 6, which express high levels of semaphorin II. Thus, the pathfinding of these growth cones (and perhaps their targeting as well) appears unresponsive to semaphorin II. Moreover, there is nothing intrinsically nonpermissive about the SNb pathway in the *Toll–semaII* embryos.

Second, when semaphorin II is ectopically expressed at high levels by muscles 7 and 6 (the normal targets for RP3), the RP3 growth cone is inhibited from forming a normal synaptic terminal arborization with its two target muscles. Similarly, when semaphorin II is ectopically expressed by muscle 31 in segment A1, DC1 is also inhibited from forming its normal synaptic arborization. These results indicate that semaphorin II can function as a target-derived inhibitor.

Third, the RP3 growth cone, while inhibited from forming its synaptic arborization, is not prevented from extending into the region of muscles expressing high levels of semaphorin II. It extends within several microns of muscles 15, 28, 14, 7, and 6 and is often located in close proximity to muscle 14. All of these muscles express high levels of semaphorin II. This result indicates that for certain neurons, semaphorin II can inhibit the growth cone from form-

ing a synaptic arborization, without repelling growth into the region where the protein is expressed.

Taken together, these results suggest that the RP3 and DC1 growth cones express a semaphorin II receptor on their surface that confers an inhibitory response to this signal. The RP1 and RP4 growth cones either do not express this receptor or, alternatively, express the receptor but are nevertheless unresponsive to semaphorin II. As discussed below, this putative semaphorin II receptor on the RP3 and DC1 growth cones can prevent growth cone branching and exploration and inhibit them from forming synaptic arborizations, but it does not appear to deter growth cone pathfinding.

The Guidance Functions of Semaphorin II versus Connectin

Insight thus far into how semaphorin II and connection function during pathfinding and targeting has come largely from gain-of-function experiments using ectopic expression. The RP1 growth cone clearly behaves quite differently when the two guidance signals are ectopically expressed by the same muscles. In the semaphorin II experiments, RP1 appears unresponsive, extending along its normal trajectory and innervating muscle 13 in a normal fashion. In contrast, in the connectin experiments (Nose et al., 1994), RP1 changes its trajectory and takes a detour to reach muscle 13, thus avoiding the ectopic connectin-expressing muscles. These results suggest that the RP1 growth cone expresses a functional receptor for connectin that confers a repulsive role during pathfinding, but not for semaphorin II.

In one respect, the RP3 growth cone behaves similarly in both experiments, being inhibited from forming a synaptic arborization on its normal targets (muscles 7 and 6) when they ectopically express either molecule. These results indicate that RP3 expresses receptors for both signals. However, there is also one interesting difference in the RP3 growth cone behavior in the two experiments. When connectin is ectopically expressed, RP3 does not enter the ventral muscle field in the normal location, but rather either stalls at this location or detours around the connectin-expressing muscles. Thus, connectin appears to repel RP3 pathfinding. In contrast, when semaphorin II is ectopically expressed at the same time by the same muscles, RP3 behaves quite differently. RP3 enters the ventral muscle field in the normal location. RP3 only stalls when it nears its semaphorin II–expressing target muscles.

It is possible that the qualitative differences in RP3 behavior simply reflects quantitative differences in the levels of expression of the two guidance signals. Arguing in favor of real qualitative differences in responsiveness, however, are the observations on penetrance and dosage. In each experiment, RP3 was examined in multiple independent transgenic lines with independent insertions and with different copy number of insertions (e.g., homozygous versus heterozygous) driving different levels of expression. Nevertheless, the only differences observed were in the penetrance of the RP3 phenotype, not in the quality of the phenotype. Moreover, these different lines drive expression of both connectin protein and *semaII* RNA that appears to be within the normal range of biological expression, suggesting that these qualitative differences are biologically significant. These results suggest that connectin functions as a repellent for RP3 pathfinding and targeting, while semaphorin II functions in a qualitatively different fashion to inhibit the formation of RP3 synaptic arborizations on muscles 7 and 6.

This qualitative difference in RP3 behavior when exposed to connectin versus semaphorin II could be explained by differences in either the timing or the quality of receptor-mediated response. In the first model, RP3 is equally inhibited by both semaphorin II and connectin, but simply expresses functional semaphorin II–repulsive receptors at a later time during its journey toward its target muscles. In the second model, the quality of the response to the two signals is different, connectin equally repelling the RP3 growth cone during both pathfinding and targeting, with semaphorin II inhibiting RP3 during targeting while not repelling its axon growth.

These results suggest that neuromuscular specificity is controlled by a combination of attraction versus inhibition, repulsion, or both, that these signals can either be secreted or cell surface, that different motoneuron growth cones express different combinations of receptors, and that these receptor systems can function in either pathfinding or targeting or in both events. In such a model, different types of inhibitory and repulsive molecules play different roles in establishing the final pattern of axon projections and synaptic connections. In this way, each motoneuron has its own particular response profile in terms of both its pathfinding and targeting preferences.

Inhibitory and Repulsive Functions of Semaphorins

These observations on semaphorin II function in the Drosophila embryo are similar in certain respects to previous results on semaphorin I function in the grasshopper limb bud (Kolodkin et al., 1992). Semaphorin I functions to stall and then steer the pair of Ti1 growth cones as they encounter a stripe of epithelial cells expressing it. The expression of semaphorin I on epithelial cells prevents the Ti1 axons that encounter it from defasciculating and inhibits branching. However, although having a potent affect on their steering, fasciculation, and branching, the Ti1 growth cones are still able to grow on the semaphorin I–expressing cells (see discussion in Kolodkin et al., 1993). Similarly, as shown here in Drosophila, RP3 enters into the region of muscles expressing semaphorin II, but it fails to form synaptic arborizations on its targets.

An interesting comparison can also be made between the in vivo results reported here and previously (Kolodkin et al., 1992) for semaphorin I and semaphorin II function in insects and the in vitro results reported previously for retinal ganglion growth cones in the rat (Roskies and O'Leary, 1994). In an in vitro stripe assay, temporal retinal axons can extend across alternating membranes from the topographically correct rostral superior colliculus and the incorrect caudal superior colliculus of embryonic rats. They are not repelled by the incorrect membranes, but rather preferentially branch on the correct membranes and do not branch on the wrong ones. This branching prefer-

ence is due to a molecule in the caudal superior colliculus that inhibits branching of temporal retinal axons. These results lead to the suggestion that in certain contexts, some guidance molecules may inhibit axon branching without repelling axon growth (Roskies and O'Leary, 1994).

For the Drosophila RP3 growth cone in vivo, semaphorin II does not appear to repel axon growth, but rather does behave as a potent inhibitor of synapse formation. For mammalian sensory axons in vitro, semaphorin III can function as a selective chemorepellent (Messersmith et al., 1995). Whether these differences in inhibition versus repulsion are biologically significant or simply reflect the different types of in vivo and in vitro assays used remains to be determined. In the developing organism, semaphorins appear capable of inhibiting branching (semaphorin I in grasshopper), influencing steering decisions (semaphorin I in grasshopper and semaphorin III in mammals), preventing axons from entering certain target regions (semaphorin II in Drosophila and perhaps semaphorin III in mammals), or inhibiting the formation of synaptic terminal arborizations (semaphorin II in Drosophila). It is possible that the same molecule may play different roles in different contexts within the same developing organism. In this regard, it will be important in the future to identify the receptor(s) for semaphorins and begin to elucidate their signal transduction mechanisms in these different contexts.

The Loss of Function versus the Gain of Function

One finding in the present study is that the ectopic expression of semaphorin II generates a much stronger and more penetrant phenotype (the gain of function) than does simply removing the protein (the loss of function). A similar conclusion was reached in the previous analysis of connectin function (Nose et al., 1994) and in a recent analysis of fasciclin III function in this same system (Chiba et al., 1995). This striking trend, in which the ectopic expression of three different targeting molecules yields stronger phenotypes than the loss of function, must reflect some inherent property in the way targeting systems are built. For example, the identity of a target may be specified not by a single molecule, but rather by a combination of different molecules, each of which is interpreted as either attractive or repulsive by particular growth cones depending upon the combination of receptors they express. If this is correct, then the introduction of one of these components in a novel location might have a more disruptive effect on the final outcome than would removal of any one component.

Based on our analysis of connectin function (Nose et al., 1994), we previously suggested that some guidance molecules may be in part refractory to loss-of-function genetic analysis in which gene functions are removed one at a time. Rather, molecular genetic methods that rely on the ectopic expression of these molecules may be required to reveal their function more clearly. Previous systematic genetic screens for mutations with highly penetrant phenotypes that perturb specific aspects of neuromuscular specificity in the Drosophila embryo did not recover mutations in either the *semaII*, *connectin*, or *fasIII* genes (Van Vactor et al., 1993; Sink and Goodman, 1994, Soc. Neurosci., abstract). And yet all three of these genes appear to encode important targeting signals in this system (Nose et al., 1994; Chiba et al., 1995; this paper).

Experimental Procedures

Expression Construct and Germline Transformation

pCaSpeR2/17 (Nose et al., 1994), a transformation vector that contains the *white*$^{+}$ (*w*$^{+}$) gene, *hsp70* promoter, and SV40 polyadenylation site, was cut between the promoter and polyadenylation site. A 2.6 kb Asp-718–XbaI fragment of Drosophila *semaII* containing the entire open reading frame and 400 bp of 5′ and 230 bp of 3′ untranslated sequence was inserted into the cut vector. A 6.5 kb NotI fragment of the *TolI* enhancer region (Wharton and Crews, 1993) was then inserted 5′ to the *hsp70* promoter in the *pCaSpeR–semaII* construct to form the expression construct *pCaSpeR–TolI–semaII*. Each newly formed junction was sequenced with *pCaSpeR*-specific primers.

The *pCaSpeR–TolI–semaII* construct was introduced into *w*^{1118} embryos by P element–mediated transformation (Spradling and Rubin, 1982) with the helper plasmid pUChspΔ2-3. We established 13 independent transformants lines, including 457, 483, and 685, each with an insert on chromosome 2, and 78 and 687, each with an insert on chromosome 3. Most have near wild-type levels of *semaII* mRNA expression in the *TolI* muscle pattern (highest in muscles 14–16; less in 17, 7, and 6; and lower still in 5, 22–24, and 31) as compared with the normal expression in muscle 33 in segment T3.

Genetics

Loss-of-function analysis was done using homozygous line 3021rec14, a recombined derivative of P1.0 (Kolodkin et al., 1993) that has a *rosy* P element inserted in codon 33 of the *semaII* open reading frame. This recombinant line is homozygously semiviable and fertile (M. Winberg and A. L. K., unpublished data), although the adults behave abnormally in visual orientation tests and hold their wings in an abnormal posture (Kolodkin et al., 1993). Transgenic lines 685;78, 457;78, and 483;78 each have four *pCaSpeR–TolI–semaII* inserts. They were established by standard genetic crosses.

Immunocytochemistry

Embryos were dechorionated, fixed, and devitellinized as described in Patel et al. (1987). Embryos were stained with the following antibodies using standard protocols: MAb 1D4 (anti–fasciclin II; G. Helt and C. S. G., unpublished data; see Van Vactor et al., 1993), MAb 22C10 (Fujita et al., 1982), and anti-β-galactosidase (gift from R. Holmgren). Embryos were dissected as described elsewhere (Van Vactor et al., 1993; Kolodkin et al., 1993).

Staging of embryos was according to Campos-Ortega and Hartenstein (1985) and Van Vactor et al. (1993), with particular attention to head involution, CNS condensation, gut morphology, and appearance of MAb 1D4 staining of the lateral longitudinal axon tracts. Muscle identity and nomenclature are from Crossley (1978), Hooper (1986), and Bate (1993). In situ hybridization was performed as described by Kolodkin et al. (1993).

Intracellular Dye Fills

Individual RP motoneurons (RP1 and RP3) were visualized with Nomarski optics in unfixed, dissected late stage 16 embryos, identified according to their cell body positions between the axon commissures, penetrated with microelectrodes, iontophoretically injected with LY as described previously (Sink and Whitington, 1991), and processed using anti-LY antibody (Molecular Probes) (Taghert et al., 1982).

Motoneuron DC1 was identified by iontophoretic application of DiI (0.4% in ethanol) to the axon of the lateral surface of muscle 31 in segment A1. After 15 min, the embryo was fixed for 15 min in 4% formalin–citrate phosphate buffer, rinsed extensively with PBS for 30 min, and then photo-oxidized with DAB (1 mg/ml) under a rhodamine filter set until brown reaction product was seen in the backfilled neuron.

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