

Slit Is the Midline Repellent for the Robo Receptor in *Drosophila*

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Summary

Previous studies suggested that Roundabout (Robo) is a repulsive guidance receptor on growth cones that binds to an unknown midline ligand. Here we present genetic evidence that Slit is the midline Robo ligand; a companion paper presents biochemical evidence that Slit binds Robo. Slit is a large extracellular matrix protein expressed by midline glia. In *slit* mutants, growth cones enter the midline but never leave it; they abnormally continue to express high levels of Robo while at the midline. *slit* and *robo* display dosage-sensitive genetic interactions, indicating that they function in the same pathway. *slit* is also required for migration of muscle precursors away from the midline. Slit appears to function as a short-range repellent controlling axon crossing of the midline and as a long-range chemorepellent controlling mesoderm migration away from the midline.

Introduction

The *roundabout (robo)* and *commissureless (comm)* genes in *Drosophila* were identified in a large-scale mutant screen for genes that control the decision by axons to cross or not to cross the CNS midline (Seeger et al., 1993). In *robo* mutant embryos, too many axons cross and recross the midline. *robo* encodes an axon guidance receptor of the immunoglobulin superfamily (Kidd et al., 1998a; Brose et al., 1999 [this issue of *Cell*]) that is highly conserved in fruit flies, nematodes (Zallen et al., 1998), and mammals (Kidd et al., 1998a). For those axons that never cross the midline, Robo is expressed at high levels on their growth cones from the outset. For the majority of commissural axons that do cross the midline (but only once), Robo is expressed at high levels on their growth cones after they cross the midline. Transgenic rescue experiments reveal that Robo can function cell autonomously, further supporting the hypothesis that Robo is a growth cone guidance receptor.

comm mutant embryos display the opposite phenotype in that no axons cross the midline. Comm is a novel transmembrane protein (Tear et al., 1996). The *robo; comm* double-mutant phenotype is identical to *robo* alone (Seeger et al., 1993), suggesting that in the absence of Robo, Comm is no longer required to allow axons to cross. Overexpression of Comm (i.e., the *comm* gain of function) leads to a phenotype nearly identical to the *robo* loss of function (Kidd et al., 1998b). Taken

together, these results suggest that Comm regulates Robo function by either controlling Robo levels or Robo signaling. Further analysis revealed that Comm controls Robo expression; increasing Comm leads to a reduction of Robo protein.

These studies led to the model that Robo is a repulsive guidance receptor for an unknown midline ligand and that Comm downregulates the levels of the Robo receptor on commissural axons (Kidd et al., 1998a, 1998b). We argued that this midline repellent is likely to function in a short-range fashion, since growth cones that express high levels of Robo do not necessarily extend away from the midline, but rather extend longitudinally close to the midline. One candidate ligand might be one of the two *Drosophila* Netrins that are expressed by midline glial cells (Harris et al., 1996; Mitchell et al., 1996). However, our unpublished genetic analysis led us to believe that the Netrins are not Robo ligands. What, then, is the midline Robo ligand?

We were also interested in answering a more general question. If the midline, with its expression of Netrins, is such an attractive place, with mirror-symmetric commissural axons from both sides extending toward and entering the midline, why do growth cones ever leave the midline? Why don't these growth cones fasciculate with their contralateral homolog and extend longitudinally along the midline? In a *robo* mutant, axons freely cross and recross the midline, but they do not stay at the midline. However, when the midline cells are genetically deleted in *single-minded (sim)* mutants, all axons converge on the midline and do not leave it, forming a single large fused longitudinal tract at the midline (Crews et al., 1988; Thomas et al., 1988). This phenotype suggests that the midline cells normally express two repellent activities, one that controls crossing (and prevents recrossing) of the midline, and another that assures that axons do not stay at the midline. One possibility is that the same molecule might serve both functions. We hoped that the identification of the Robo ligand might shed some light on this issue.

In principle, if there is a one-to-one relationship between the Robo ligand and the Robo receptor, then we might expect the gene encoding the ligand to have the same mutant phenotype as *robo*. But in our large-scale mutant screen, we screened most if not all of the genome, and although we recovered eight independent alleles of *robo*, we found no other gene whose mutant phenotype is identical to *robo*. The likely explanation is that either there are two ligands for the one Robo receptor (in which case each ligand might have a weaker mutant phenotype than *robo*), or alternatively, there is one ligand but two receptors (in which case the ligand might be expected to have a stronger mutant phenotype than *robo*). We already knew that there was a second Robo receptor expressed in the developing CNS (Kidd et al., 1998a; J. Simpson et al., unpublished results), making the second alternative seem more likely.

In this paper we present genetic evidence that Slit is a Robo ligand. Slit is a large extracellular matrix protein expressed by midline glia (Rothberg et al., 1988, 1990).

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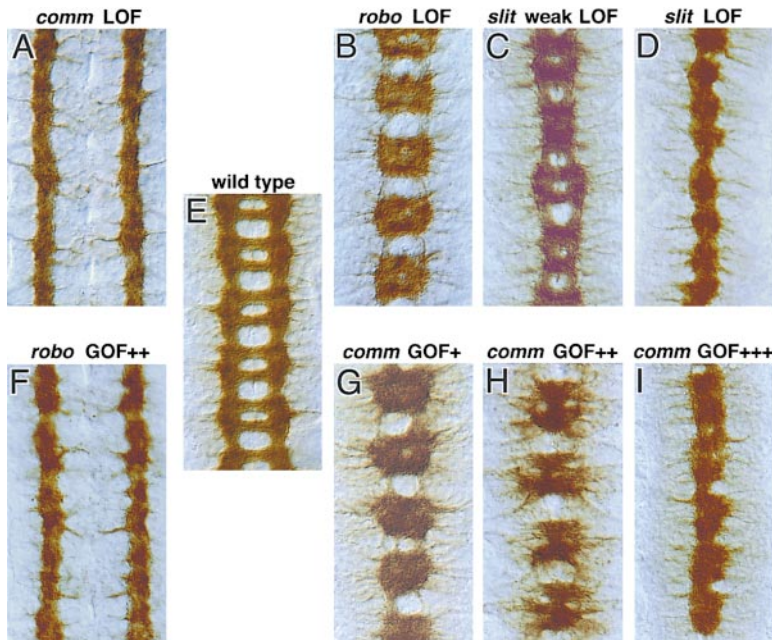


Figure 1. The CNS Axon Scaffold in Wild-Type and Mutant Embryos

Photomicrographs of the CNS in stage 16 embryos stained with mAb BP102 that labels all longitudinal and commissural axons. (A–D) loss-of-function (LOF) mutants, (E) wild type, and (F–I) gain-of-function (GOF) mutants.

(A) *comm*^{Δ39} null LOF allele in which no axons cross the midline.

(B) *robo*¹ null LOF allele in which too many axons cross and recross the midline with the consequence that the commissures are thick and fuzzy and the longitudinals are thinner.

(C) *slit*^{E-158} hypomorphic LOF allele in which the phenotype of segments ranges from that of a *robo* loss-of-function allele to a *slit* loss-of-function allele.

(D) *slit*² null LOF allele in which axons enter but fail to leave the midline and instead run along it in one longitudinal tract.

(E) Wild-type embryo showing the normal pattern of two commissures in each segment.

(F) Embryo with two copies of *elav-GAL4* and *UAS-robo* (i.e., the *robo* GOF) in which no axons cross the midline, phenocopying the *comm* LOF mutant phenotype.

(G–I) Increasing dosage of the transgenes *UAS-comm* and *sca-GAL4* (reflected by the

number of + signs). (G) A phenotype resembling the *robo* LOF in which the commissures are thicker and the longitudinals thinner. (H) A more severe phenotype in which the longitudinals have not formed and many axons remain at the midline between the RP neuron cell bodies (the clear circles on each side of the midline). (I) The most severe *comm* GOF phenotype resembles a *slit* LOF mutant in which all axons run along the midline in a single longitudinal tract.

In *robo* mutant embryos, growth cones that normally do not cross the midline now do so. In *slit* mutant embryos, these same growth cones enter the midline but never leave it. Moreover, they continue to express high levels of Robo even while extending along the midline. *slit* and *robo* display dosage-sensitive genetic interactions, indicating that they are likely to function in the same pathway. *slit* is also required for migration of muscle precursors away from the midline. Slit appears to function as a short-range repellent, controlling axon crossing of the midline. However, the muscle phenotype suggests that Slit also functions as a long-range chemorepellent, controlling mesoderm migration away from the midline. In a companion paper (Brose et al., 1999), we and our colleagues show direct binding between Slit and Robo in *Drosophila* and then go on to present data on the sequence, Robo binding, expression, and function of three mammalian Slits.

Results

High-Level Overexpression of Robo and Comm Generates Opposite Phenotypes

We previously reported that panneuronal transgenic overexpression of *robo* does not give a mutant phenotype due to posttranslational regulation of Robo protein (Kidd et al., 1998a). However, we find that if the copy number of the *UAS-robo* transgene is increased, a *robo* gain-of-function phenotype is generated that is nearly identical to the *comm* loss-of-function phenotype in which axons do not cross the midline. This result further confirms the proposed role of Robo as a repulsive receptor that prevents axons from crossing the CNS midline (Figure 1F). We previously reported that overexpression of

comm produces the complementary *robo*-like phenotype in which axons freely cross and recross the midline (Figure 1G; Kidd et al., 1998b). This phenotype appears to be generated by Comm's ability to negatively regulate Robo protein levels; increasing levels of Comm lead to decreasing levels of Robo. If the copy number of the *comm* transgene is increased, a more severe phenotype results (Figure 1H). The strongest *comm* gain-of-function phenotype has axons entering the midline but not leaving it, leading to a collapse of the CNS axon scaffold onto the midline (Figure 1I). Even in the most extreme *comm* gain-of-function phenotypes, the midline cells are still present as assayed by a monoclonal antibody against Wrapper, a protein expressed specifically by midline glia (data not shown; Noordermeer et al., 1998). The strongest *comm* gain-of-function phenotype is highly reminiscent of the *slit* loss-of-function phenotype (Figure 1D) (Rothberg et al., 1990). The similarity between the *comm* gain-of-function phenotype and the *slit* loss-of-function phenotype led us to evaluate Slit as a candidate ligand for Robo.

Axon Guidance Defects in *slit* Mutant Embryos

slit mutations were first isolated in a screen for mutations affecting the pattern of the larval cuticle (Nüsslein-Volhard et al., 1984) and were found to have defects in CNS formation and head involution. Null alleles of *slit* show a characteristic collapse of the CNS axon scaffold (Figure 1D) (Rothberg et al., 1990). We find that hypomorphic *slit* alleles show a less complete midline collapse of the CNS axon scaffold, with the CNS in some segments resembling a *robo* mutant (Figure 1C). The striking phenotype of *slit* mutant embryos is similar to *sim* mutants. When the *slit* mutant was first characterized, the limited

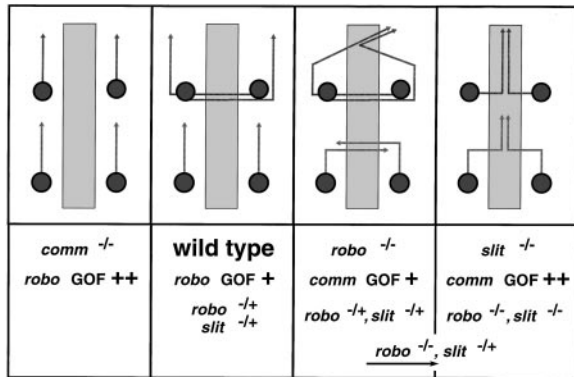


Figure 3. Behavior of Identified Axons in Different Genotypes

Schematic diagram showing the behavior of a pair of interneurons whose axons cross the midline once (SP1; top axons and cell bodies) and a pair of interneurons whose axons project ipsilaterally and do not cross the midline (pCC; bottom axons and cell bodies), in different genotypes. The midline is represented by a gray box. In wild type, the commissural axons grow across the midline before extending longitudinally and never cross the midline again, while the longitudinal axons grow longitudinally from the outset. The same behavior is seen in *robo* or *slit* heterozygotes. In *comm* loss-of-function mutants, the commissural neurons do not cross the midline. The same phenotype is seen when *robo* is overexpressed at high levels (*robo* GOF ++). In *robo* loss-of-function mutants, the commissural axons cross the midline as in wild type, but instead of extending longitudinally, they recross the midline. The longitudinal axons also aberrantly cross the midline. In each case, the axons do not remain at the midline, but extend to one side or the other. The same phenotype is seen when *comm* is overexpressed in all neurons (*comm* GOF +). When *robo* and *slit* are transheterozygous, similar axonal behavior can be seen in a subset of segments. In *slit* loss-of-function mutants, both commissural and longitudinal axons grow toward the midline but then fail to leave and instead grow along it. The same behavior can be seen in *robo slit* double mutants and when *comm* is overexpressed at very high levels (*comm* GOF ++). When one copy of *slit* is removed in an embryo homozygous for *robo*, a subset of segments have axons that fail to leave the midline.

(Figure 2C). Interestingly, this axon behavior looks very similar to the wild-type behavior of the axons from the RP motoneurons whose cell bodies lie equally close to the midline but whose axons normally extend across the midline and fasciculate with their contralateral homologs before extending toward a nerve root and exiting the CNS.

Commissural axons such as SP1 are also unable to leave the midline (as visualized with anti-Connectin antibody; data not shown). In addition, some neuronal cell bodies appear to be closer to the midline than in wild-type embryos, suggesting that *slit* has a role in controlling cell migrations as well as axon guidance. The behavior of axons in the *slit* mutant and other genotypes is summarized in Figure 3.

Genetic Interactions between *robo* and *slit*

The axon guidance defects in *slit* mutant embryos are initially similar to those observed in *robo* mutants in that axons freely extend toward and enter the midline. However, with time the *slit* phenotype becomes more severe because axons do not leave the midline. The *slit* guidance phenotypes are consistent with the hypothesis that Slit is the Robo ligand. To further test this model,

Table 1. Transheterozygous Interactions of *robo* and *slit*

Genotype	Severity of Defects			No. Segments	Defects (%)
	+	++	+++		
<i>slit</i> ^{1/+}	0	0	0	99	0
<i>slit</i> ^{2/+}	1	0	0	121	1
<i>robo</i> ^{1/+}	0	0	0	121	0
<i>robo</i> ^{2/+}	1	0	0	121	1
<i>slit</i> ¹ +/+ <i>robo</i> ¹	6	7	18	110	28
<i>slit</i> ¹ +/+ <i>robo</i> ²	12	9	8	110	26
<i>slit</i> ² +/+ <i>robo</i> ¹	14	17	17	132	36
<i>slit</i> ² +/+ <i>robo</i> ²	8	19	25	132	39

Stage 16/17 embryos in which the six Fas II-positive longitudinal fascicles (three on each side) were scored for axons crossing the midline. Defects were subdivided according to whether the group of axons crossing the midline were less thick (+), the same size (++), or of greater thickness (+++) than a wild-type Fas II-positive fascicle. The abdominal and thoracic segments were scored.

we looked for genetic interactions between these two genes. Dosage-sensitive genetic interactions between two loci are a good indicator that the two gene products are functionally related. We examined the CNS of embryos transheterozygous for *slit* and *robo*, that is, carrying one mutant and one wild-type copy of each gene. We examined whether the Fas II (i.e., staining with the 1D4 mAb) positive fascicles abnormally crossed the midline (particularly the most medial pCC pathway). In either *slit* or *robo* heterozygotes, we observed few guidance defects in these pathways (Table 1; Figure 2F). However, depending upon the combination of alleles used, 26%–39% of the segments examined in embryos transheterozygous for *slit* and *robo* had Fas II-positive axons inappropriately crossing the midline (Table 1; Figure 2E). Such a dosage-dependent, transheterozygous phenotype is a strong indication that Slit and Robo function in the same pathway.

We also generated chromosomes doubly mutant for *slit* and *robo*. The genetic distance between the two loci predicted recovery of the double mutant chromosomes at a frequency of 1 in 8; when null alleles of both *slit* and *robo* were used instead, the recovery rate was 1 in 35, indicating that removal of one copy of each locus decreases viability.

In a late stage wild-type embryo, the cell bodies of the RP neurons are readily visible between the two commissures (Figure 1E). In *robo* mutants, typically one or both RP cell bodies are obscured by the increased number of axons abnormally crossing in the commissures. However, the longitudinal part of the scaffold always remains outside (lateral) of the RP cell bodies. In *slit* mutants, this is not the case (Figures 1C and 1D). We tested the effect of removing one copy of *slit* on the *robo* phenotype. When the spacing of the longitudinal axons was examined, *slit* was found to dominantly enhance the *robo* phenotype, as judged by the presence of segments displaying greater medial constrictions than are ever seen in *robo* mutants alone (Figure 4B). In some instances, an RP cell body can be seen lateral to the axon scaffold.

If Slit is the Robo ligand, then the double *robo slit* mutant phenotype would be predicted to resemble that of a *slit* mutant alone (due to *slit* having the more severe

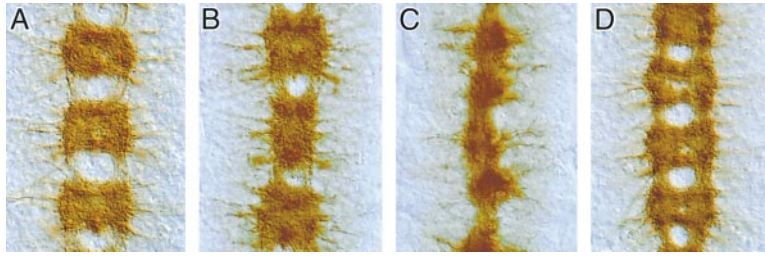


Figure 4. Genetic Interactions between *robo* and *slit*

mAb BP102 staining reveals the CNS axon scaffold in combinations of *slit* and *robo* mutants.

(A) A *robo*⁵ embryo showing the characteristic thickening of the commissures and thinning of the longitudinals. Note the consistent width of the axon scaffold in each segment.

(B) Dominant enhancement of the *robo* phenotype by removing one copy of *slit*. An embryo heterozygous for *slit*¹ and homozygous

for *robo*⁵ showing a lateral compression of the axon scaffold in the middle segment. This phenotype is never seen in *robo* embryos.

(C) An embryo doubly homozygous for *slit*¹ and *robo*⁵. The CNS axon scaffold has collapsed onto the midline and is identical to that seen for *slit* mutants alone.

(D) Rescue of the *slit* phenotype in a *slit*² embryo with a *UAS-slit* transgene driven by a *slit*-*GAL4* transgene. Commissures are present, although slightly thicker and fuzzier than wild type. The longitudinals are also present although not quite as thick as wild type.

phenotype). Embryos homozygous for a recombinant chromosome carrying null alleles of both *slit* and *robo* were found to resemble the *slit* null phenotype (Figure 4C).

Robo and Slit Expression

The commissureless phenotype produced by high-level overexpression of Robo (Figure 1F) suggests that Robo responds to a repulsive cue at the CNS midline. Slit is a large extracellular matrix protein secreted by the midline glia (Figure 5A; Rothberg et al., 1990). Slit was reported to be transferred to axons (albeit at a low level; Rothberg et al., 1990). The mAb we are using displays only a very low level of axon staining, making an analysis of putative transfer in *robo* mutant embryos inconclusive. Robo is primarily localized to growth cones of the longitudinal portion of the axon scaffold (Figure 5B; Kidd et al., 1998a). These expression patterns are consistent with Slit being the repulsive ligand for Robo because Robo-positive axons avoid areas of high Slit expression. We stained *slit* embryos with anti-Robo mAb 13C9 and found that Robo-positive growth cones are now present at the midline (Figure 5C). Staining of the mature CNS in *slit* mutants reveals that Robo protein levels are unaffected (unlike in *comm* gain-of-function embryos), and thus Robo is expressed at high levels along the midline (Figure 5D). In wild-type embryos, Slit and Robo both localize to the muscle attachment sites in complementary dorsoventral gradients, further suggesting the possibility of a functional relationship (data not shown).

Cloning of a Full-Length Open Reading Frame *slit* cDNA

We isolated a *slit* cDNA encoding the complete open reading frame (ORF) from the LD 0–22 hr embryonic library (EST Project, G. Rubin lab). We sequenced the ORF and identified an additional leucine-rich repeat (LRR) that is absent from the cDNA previously published (Rothberg et al., 1990). This additional LRR is between the second and third repeats in the first set of tandem LRR arrays. This LRR is present in vertebrate homologs of *slit* (Brose et al., 1999). In addition to the extra LRR, we identified eight amino acid differences. All of the substitutions are in LRR regions, but none occur in highly conserved residues of the motifs.

Ectopic Expression of Slit

We used the GAL4-UAS system to misexpress *slit* in several tissues (Brand and Perrimon, 1993). We initially showed that expression of *slit* at the midline can rescue a *slit* phenotype. Using the *slit* promoter as the GAL4 driver in a homozygous *slit* mutant background, we achieved partial rescue of the CNS axon phenotype (Figure 4D) in which the commissures and longitudinal tracts are all present and separated from each other. The typical delay of 1.5 hr in expression introduced by using the GAL4 system is probably responsible for the incomplete rescue. Next we examined the effect of high-level overexpression of *slit* in all postmitotic neurons (*elav* promoter). The resulting phenotype resembles the *robo* loss-of-function phenotype (Figure 6A). However, when individual axon fascicles are examined, we observe that the *slit* overexpression phenotype is stronger than the *robo* loss-of-function phenotype. Staining with the 1D4 mAb shows that in addition to aberrant midline crossing by axons in the innermost pCC pathway as seen in *robo* mutants, the medial and lateral pathways are also disrupted, sometimes crossing the midline. The same results were obtained using a different panneural promoter (*scabrous*). These results suggest that when Slit is panneurally expressed throughout the CNS, growth cones are impaired in their ability to respond to Slit at the midline.

A similar effect is seen when Netrins are expressed panneurally in that the panneural overexpression phenotype resembles the loss-of-function phenotype (Harris et al., 1996; Mitchell et al., 1996). In both cases (Slit and Netrins), these results support the notion that the localized distribution of the guidance signal is of crucial importance and that approximating an even distribution throughout the CNS is equivalent to no expression at all.

We overexpressed *slit* at the CNS midline using two different promoters (*slit* and *sim*) but did not observe a consistent phenotype. We suspect that this is due to the commissural axons being highly efficient at down-regulating Slit receptors on their surface to allow midline crossing (Kidd et al., 1998a, 1998b). In addition, we have preliminary evidence suggesting that levels of Slit protein at the midline are tightly regulated.

Finally, we expressed Slit on muscles (using 24B-GAL4) and examined the guidance and connectivity of

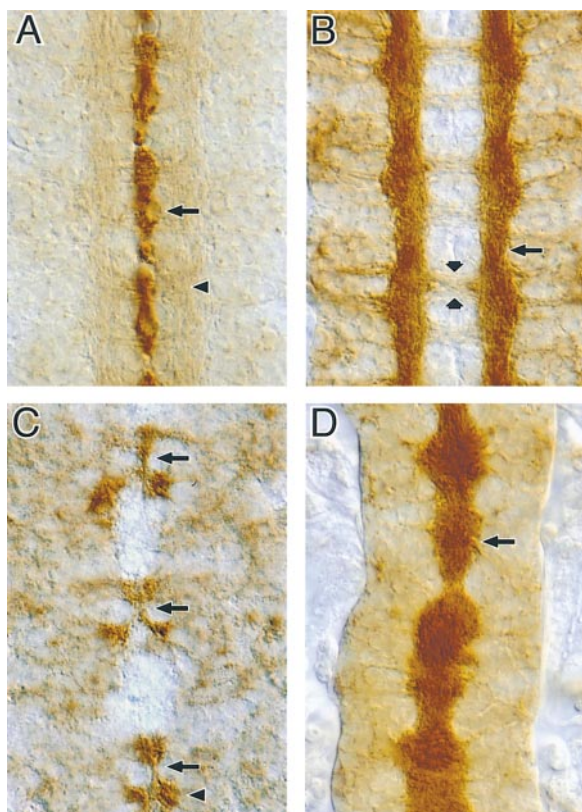


Figure 5. Expression of Slit and Robo

Photomicrographs of the CNS axon scaffold stained with anti-Slit mAb C555.4 (A) and anti-Robo mAb 13C9 (B–D) in wild-type embryos (A and B), a young *slit*² embryo (C), and an old *slit*² embryo (D).

(A) Stage 16 wild-type embryo stained with mAb C555.4 to show Slit expression. The highest level of Slit expression occurs around the midline glia (arrow) that extend below the plane of focus shown here. A faint level of Slit staining is observed around CNS axons lateral to the midline (arrowhead).

(B) Stage 16 wild-type embryo stained with mAb 13C9 to show Robo expression. Robo expression is highest on the longitudinal tracts of the CNS axon scaffold (arrow). A very low level of Robo staining can be seen on the commissural axons (short arrows). Robo staining can also be seen in the neuronal cell bodies on either side of the longitudinal axon tracts but not in cells at or adjacent to the midline. (C) mAb 13C9 Robo staining in a stage 13 *slit*² embryo. The Robo-positive pCC growth cones can be seen growing along the midline (arrows). In wild type, the Robo-positive pCC growth cones would be found growing along the lateral edge of the midline but not entering the midline. The location of the cell body of the pCC neuron (underneath Robo-positive growth cones) is indicated by an arrowhead.

(D) Stage 16 *slit*² embryo stained with anti-Robo mAb 13C9 showing high levels of Robo expression throughout the CNS axon scaffold (arrow) that has characteristically collapsed onto the midline.

motor axons. The ISNb motor axons normally innervate muscles 6, 7, 12, and 13. When their muscle targets abnormally express Slit, their innervation is greatly perturbed. Most of these motor growth cones stall in the vicinity of these muscles and fail to innervate them (61%, $n = 106$; Figure 6B). This lack of innervation is reminiscent of what is observed when the chemorepellent Semaphorin II is ectopically expressed by the same muscles (Winberg et al., 1998a). We examined the morphology of muscles 6, 7, 12, and 13 ectopically expressing Slit with mAb FMM5 (anti-muscle myosin) and found

them to be normal in attachment sites, size, and position relative to each other and to the epidermis ($n = 110$). The motor axon phenotype was not suppressed by removal of *robo* activity, providing further evidence that there is more than one Slit receptor. Robo2 is a potential candidate for mediating the motor axon response to ectopic expression of Slit.

Slit Is Required for Correct Muscle Migration and Patterning Near the Midline

After gastrulation in *Drosophila*, many myoblasts migrate laterally at least five to six cell body diameters away from the ventral midline. This migration occurs over the dorsal surface of the neuroepithelium. Later, some ventral body wall muscles extend back toward the midline ventrally under the developing CNS, normally attaching to the epidermis underneath the CNS at some distance from the midline (Figure 7A). In contrast, in *slit* mutant embryos many developing muscles are found near and at the midline, stretching across the midline dorsally over the CNS (Figure 7C). This defect is not seen in *robo* embryos, although very rarely a single muscle can be seen extending inappropriately dorsally across the CNS (Figure 7B), suggesting that Robo participates in this process in conjunction with at least one other receptor (possibly Robo2). However, in *robo* mutant embryos the ventral muscles are frequently found attached closer to the midline than in wild type, suggesting that Robo may in part prevent muscles from extending too close to the midline. When *slit* mutant embryos are rescued by *slit-GAL4* driving *UAS-slit*, the ventral muscle pattern is restored to near wild type, confirming that Slit expression at the midline is required for migration of muscle precursors away from the midline.

Discussion

We previously reported that Robo appears to function as a repulsive axon guidance receptor on growth cones that responds to a putative midline ligand (Kidd et al., 1998a, 1998b). In the present paper, we present genetic evidence that suggests that Slit is the midline Robo ligand (summarized in Figure 3). In a companion paper, we and our colleagues present biochemical evidence showing that Slit binds to Robo (Brose et al., 1999).

In the original large-scale mutant screen for genes controlling midline axon guidance (Seeger et al., 1993), 8 alleles were recovered of *robo*, 2 alleles of *comm*, and 13 alleles of *slit*. At the time, because *slit* had such a similar axon phenotype to *sim*, which controls midline cell fate and survival (Crews et al., 1988; Thomas et al., 1988), and because of the lack of good midline markers, there was some uncertainty as to whether *slit* also controlled midline cell fate and survival (Rothberg et al., 1990). As a result, we focused our initial attention on *robo* and *comm*, two genes that clearly control midline axon guidance. Nevertheless, there was always the lingering possibility that Slit might directly control axon guidance: Slit is a large extracellular matrix protein expressed almost exclusively by midline cells, some Slit protein is found on axons, and the *slit* mutant displays a striking axon pathway phenotype (Rothberg et al.,

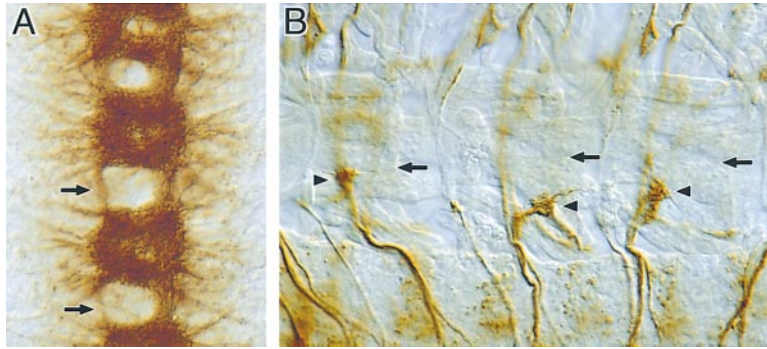


Figure 6. Ectopic Expression of Slit

Photomicrographs of the CNS axon scaffold stained with mAb BP102 (A) and the ISNb motoneuron projection to muscles 6 and 7 stained with mAb 1D4 (B) in embryos ectopically expressing *slit* in neurons (A) or muscles (B).

(A) Panneuronal expression of *slit* by the *elav-GAL4* promoter. The CNS commissures are thicker and more fuzzy when compared to wild type (Figure 1E). The longitudinal tracts are thinner (arrows), and the overall appearance is that of the *robo* loss-of-function phenotype, suggesting that the ectopic Slit interferes with the ability of the growth cones to distinguish Slit at the midline.

(B) Ectopic expression of *slit* in muscles with the *24B-GAL4* promoter. In the three segments shown, the ISNb motoneuron (RP3) that normally innervates the cleft between muscles 6 and 7 (arrows) has stalled either at the cleft (leftmost arrowhead) or upon encountering the muscles (middle and rightmost arrowheads), suggesting that the muscles are now repulsive to the motoneurons.

1990). With the advent of better markers for midline cells, Sonnenfeld and Jacobs (1994) showed that midline cell fate and differentiation are relatively normal in *slit* mutant embryos, thus suggesting that Slit might indeed control axon guidance.

The key result that led us to the insight that Slit is likely to be the Robo ligand came from a further analysis of *Comm*. We previously reported that overexpression of *Comm* produces a *robo*-like phenotype in which axons freely cross and recross the midline (Kidd et al., 1998b). In the present paper, we report that if the copy number of the *comm* transgene is increased, a more severe phenotype results in which axons enter the midline but fail to leave it, leading to a midline collapse of the CNS axon scaffold. The strongest *comm* gain-of-function phenotype is highly reminiscent of the *slit* loss-of-function phenotype and led us to evaluate Slit as a candidate Robo ligand.

The genetic analysis presented here provides strong support for the notion that Slit is the midline Robo ligand. One way to test the hypothesis that two proteins directly interact in a ligand-receptor fashion is to test for dominant genetic interactions between the genes encoding them. In most cases, reducing gene dosage by one copy (thus reducing protein by 50%) has little phenotypic effect. However, simultaneously reducing the dose of two genes whose protein products function together may sufficiently impair their combined function such that phenotypes appear. Such a "transheterozygous" phenotype has been demonstrated for various ligand-receptor pairs in *Drosophila*, including Delta and Notch (Artavanis-Tsakonas et al., 1995), and more recently, in the field of axon guidance, the transmembrane Semaphorins and their Plexin A receptor (Winberg et al., 1998b).

In either *slit* or *robo* heterozygous mutants alone, we observed few midline guidance defects. However, depending upon the combination of alleles used, in embryos that are transheterozygous for both *slit* and *robo* (i.e., carrying one mutant and one wild-type copy of each gene), 26%–39% of segments have midline axon guidance defects. This transheterozygous genetic interaction is a good indicator that Slit and Robo function in a common pathway.

Transgenic experiments reveal that Robo can function in a cell-autonomous fashion consistent with the role of

a receptor (Kidd et al., 1998a), whereas ectopic expression experiments reported here show that Slit can function in a non-cell autonomous fashion consistent with the role of a ligand (e.g., muscle expression repelling motor axons). Taken together with the transheterozygous genetic interaction, these data strongly suggest that Slit is the midline ligand for the Robo receptor in *Drosophila*. In a companion paper (Brose et al., 1999), we and our colleagues present biochemical data supporting the same conclusion: Slit-AP binds COS cells expressing Robo, AP-Robo ectodomain binds cells expressing Slit, and AP-Robo binds Slit attached to protein A-sepharose beads (Brose et al., 1999).

Slit Must Have More Than One Receptor

Given the conclusion that Slit is the Robo ligand, the fact that the *slit* mutant phenotype is stronger than the *robo* phenotype suggests that Slit must have more than one receptor controlling midline guidance in *Drosophila*. In *robo* mutants, axons freely cross and recross the midline, while in *slit* mutants they enter the midline but do not leave it. Clearly, in the absence of Robo some other growth cone receptor must respond to Slit and assure that growth cones do not linger at the midline, even though it still allows them to cross the midline. A good candidate for a second Slit receptor is Robo2, a closely related receptor that is also expressed by developing CNS neurons (J. Simpson et al., unpublished results). Since the *comm* single-dose gain of function generates a *robo*-like phenotype by downregulating Robo protein, and since the *comm* double-dose gain of function generates a *slit*-like phenotype, it follows that *Comm* is likely to also control the level of expression of Robo2 or whatever other Slit receptor controls midline guidance.

This model leads to two clear predictions, both of which should be relatively straightforward to test in the future. First, we would predict that the double mutant combination of *robo* and *robo2* should generate a phenotype that resembles *slit*. Second, we would predict that *Comm* also regulates Robo2. Preliminary evidence shows that overexpression of Robo2 can produce a *comm*-like phenotype (J. Simpson and C. S. G., unpublished results). Moreover, when expressed in tissue culture, Robo2 binds Slit (K. S. B. et al., unpublished results). Both of these results lend support to this model.

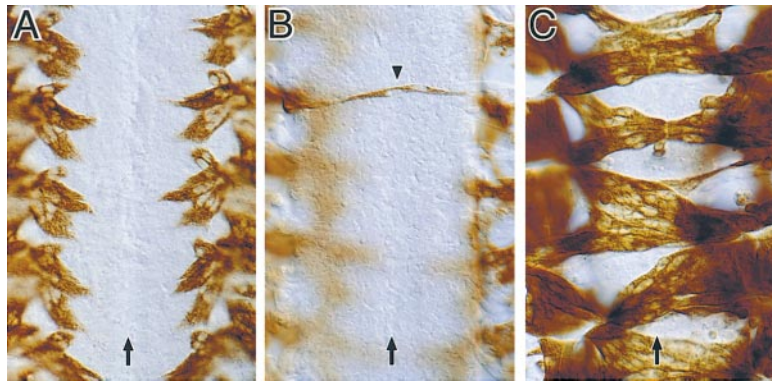


Figure 7. Muscle Phenotypes in *slit* and *robo* Embryos

Embryonic muscles stained with the monoclonal antibody FMM5 that recognizes *Drosophila* muscle myosin. The ventral midline is indicated by an arrow in all panels.

(A) Wild-type embryo in which the ventral muscles anchor to the epidermis underneath the CNS (which is above the plane of focus) and some distance from the midline.

(B) *robo*¹ mutant embryo in which a single muscle inappropriately crosses the midline (arrowhead). The plane of focus is just above the dorsal (axonal) surface of the CNS; the ventral muscles can be seen out of focus underneath the CNS. Some of the muscles are anchoring closer to the midline than in wild type, a phenotype frequently seen in *robo* embryos.

(C) *slit*² mutant embryo in which the ventral muscles now extend over the dorsal surface of the CNS.

Slit Can Function as Both a Short-Range and Long-Range Repellent

The primary location of Slit expression in *Drosophila* is at the ventral midline of the developing CNS. Midline Slit expression begins during gastrulation. Slit is also expressed at certain attachment sites of muscle to epidermis and by cardioblasts of the dorsal tube. Slit is a large extracellular matrix protein, and consistent with its size and location, most Slit protein is detected immediately adjacent to the midline glia that make it. However, in the more mature embryonic CNS in *Drosophila*, some Slit protein (as detected with antibodies against a carboxy-terminal fragment) is observed at a distance from the midline, and in particular associated with the surface of axons. Immunoelectron microscopy confirmed this localization of Slit protein on lateral axons even though there is no evidence that any cells in the CNS other than midline glia make and secrete Slit (Rothberg et al., 1990). On the one hand, the companion paper (Brose et al., 1999) shows that Slit binds to Laminin, which might retain it in the extracellular matrix and help assure its localization as a short-range signal. On the other hand, that paper also shows that Slit is proteolytically processed, raising the question of whether certain fragments of Slit can diffuse for a longer distance than the whole protein. Furthermore, a third companion paper (Wang et al., 1999 [this issue of *Cell*]) shows that the N-terminal proteolytic fragment of Slit2 in mammals can have a different long-range function as a positive regulator of sensory axon growth and branching. These data suggest that Slits are likely to be multifunctional guidance molecules.

The axon guidance defects seen in *robo* mutant embryos in *Drosophila* suggest that the primary function of Slit in controlling Robo-mediated guidance is as a short-range repellent. Growth cones that express high levels of Robo do not extend away from the midline, but rather they avoid entering and crossing the midline. For example, the pCC growth cone expresses high levels of Robo, and it extends anteriorly near the edge of the midline. In a *robo* mutant, the pCC growth cone freely crosses and recrosses the midline; in a *slit* mutant,

the pCC growth cone enters the midline and does not leave it. Although it is possible that Slit might also function as a long-range chemorepellent during axon guidance in *Drosophila*, causing some growth cones to extend some distance away from the midline, at present the strongest genetic evidence in *Drosophila* is for a short-range function.

This is in contrast to its function during mesoderm migration and muscle formation. After gastrulation in *Drosophila*, many myoblasts migrate laterally away from the ventral midline. The ventral body wall muscles normally attach to the epidermis underneath the CNS but stay some distance from and do not cross the midline. In contrast, in *slit* mutant embryos, many developing muscles are found near the midline, stretching across the midline dorsally over the CNS. The *slit* mutant muscle defects are nearly identical to those seen in *sim* mutant embryos in which the midline cells are missing (Lewis and Crews, 1994). In contrast, in *slit* mutants, the midline cells are present but do not secrete Slit into the extracellular environment.

Lewis and Crews (1994) used genetic analysis of *sim* to show that after gastrulation the midline cells are required for the migration of muscle precursor cells away from the midline. Many of these mesodermal cells normally migrate at least five to six cell body diameters away from the midline. In the *sim* mutant, the precursors do not migrate away from the midline, presumably due to the absence of a midline-derived long-range chemorepellent. Moreover, in the *sim* mutant the muscle precursors that extend ventrally toward the midline are not prevented from crossing the midline, presumably due to the absence of a midline-derived short-range repellent. Rather, when these misplaced muscle precursor cells undergo myogenesis, they form abnormal contacts with each other that freely extend across the dorsal midline of the CNS. We found that *slit* mutant embryos display the exact same midline mesoderm phenotypes as do *sim* mutant embryos. This suggests that Slit is both the long-range chemorepellent controlling mesoderm migration away from the midline and the short-range repellent preventing muscles from crossing the midline.

The Robo receptor appears to play only a minor role in the ability of Slit to direct the long-range migration of muscle precursors away from the midline. Either Robo2 or some other Slit receptor must function as the major muscle receptor for Slit-mediated long-range chemorepulsion.

Why Do Growth Cones Leave the Midline?

At the outset we asked the question, if commissural growth cones are so attracted to Netrin, if the highest concentration of Netrin is at the midline, and if when growth cones arrive at the midline they meet their homologs from the other side for which they have a high affinity, why do these growth cones ever leave the midline? Although we do not yet fully understand the mechanism, the answer to this question has something to do with the qualitatively different ways in which growth cones respond to Slit. For growth cones near the midline that do not cross it, Slit forms a strong repulsive barrier. But for growth cones that do cross the midline, Slit cannot be such a strong repellent, rather functioning in a more subtle fashion, somehow preventing them from lingering at the midline and driving them across.

In the absence of Slit, growth cones enter the midline but do not leave it, extending in a single fused longitudinal tract at the midline. Thus, Slit must be part of the anti-linger mechanism. One thing is certain: the ability of Slit to form a repulsive barrier requires the Robo receptor. Any growth cone that expresses high levels of Robo cannot cross the midline. So in a *robo* mutant, growth cones freely cross and recross the midline, but they do not stay at the midline. Two inferences follow from these observations. First, there must be at least one additional Slit receptor that controls midline guidance, and at present Robo2 is the best candidate. Second, because Slit appears to have two different functions (one as a midline repulsive barrier and the second as a midline anti-linger signal), it follows that either Robo2 signals differently from Robo, or alternatively, that the low levels of Robo2 alone (or Robo2 and Robo together) on growth cones crossing the midline give rise to a qualitatively different response as compared to high levels of Robo. Whether we are dealing with two qualitatively different negative responses, or alternatively, quantitative differences in a common repulsive mechanism, is not yet clear. Teasing this mystery apart in the future should shed some light on how growth cones make stereotyped and divergent decisions at complex choice points.

Slits Are Likely to Have Other Functions

In the *Drosophila* embryo, Slit and Robo colocalize at muscle attachment sites. We do not yet know whether this coordinated expression of ligand and receptor represents an attractive or a repellent function. In vertebrates, experimental evidence supports the conservation of the repulsive function for Slits at the midline (Brose et al., 1999). However, other experiments show that a fragment of Slit2 functions to promote axon branching and elongation of sensory axons (Wang et al., 1999). Additional experiments will be required to

determine whether other Slits can also function as positive regulators of axon growth and/or branching.

Experimental Procedures

Genetic Stocks

slit¹ and *slit²* are both null alleles (Nüsslein-Volhard et al., 1984; Rothberg et al., 1988); *slit^{E-158}* is a hypomorphic allele created by insertion of a P element into the 5' region of the gene (Rothberg et al., 1990). *slit¹* and *slit^{E-158}* were obtained from S. Artavanis-Tsakonas. *slit²* was obtained from the Bloomington Stock Center. The *slit¹ robo⁵*, *slit^{E-158} robo⁴*, and *slit^{E-158} robo⁵* chromosomes were generated by recombination. A *twist* allele on the *slit¹* chromosome was removed during this process. *UAS-robo*, *UAS-comm*, and *sca-GAL4* are Goodman laboratory stocks (Kidd et al., 1998a, 1998b). *elav-GAL4* was obtained from A. DiAntonio. *slit-GAL4* was obtained from C. Klambt (Scholz et al., 1997).

RNA Localization and Protein Immunocytochemistry

RNA localization was performed as described in Tear et al. (1996). Immunocytochemistry was performed as described in Kidd et al. (1998a). The anti-Slit mAb C555.4c was used at a dilution of 1:50 in PBS with 0.1% saponin. The anti-muscle myosin mAb FMM5 was used at a dilution of 1:10 (Kiehart and Feghali, 1986).

Transformation Construct

A cDNA containing the *slit* ORF was isolated from the LD cDNA library (EST Project, G. Rubin lab). The ORF was sequenced and found to have an additional LRR between the second and third LRRs in the first set of tandem arrays. The 5' AsnI overhang of an AsnI-DraI fragment containing the ORF was filled in with the Klenow fragment of *E. coli* DNA Polymerase I, and the fragment was cloned into the EcoRV site of pcDNA3 (Invitrogen). An Asp718-XbaI fragment containing the *slit* ORF was dropped out of the pcDNA3 construct and cloned into the Asp718-XbaI sites of pUAST (Brand and Perrimon, 1993). Five transformant lines were generated and mapped using standard techniques.

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GenBank Accession Number

The GenBank accession number for the alternatively spliced form of Slit reported in this paper is AF126540.