Regulation of Axon Guidance by Slit and Netrin Signaling in the Drosophila Ventral Nerve Cord

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ABSTRACT

Netrin and Slit signaling systems play opposing roles during the positioning of longitudinal tracts along the midline in the ventral nerve cord of Drosophila embryo. It has been hypothesized that a gradient of Slit from the midline interacts with three different Robo receptors to specify the axon tract positioning. However, no such gradient has been detected. Moreover, overexpression of Slit at the midline has no effect on the positioning of these lateral tracts. In this article, we show that Slit is present outside of the midline along the longitudinal and commissural tracts. Slit from the midline, in a Robo-independent manner, is initially taken up by the commissural axon tracts when they cross the midline and is transported along the commissural tracts into the longitudinal connectives. These results are not consistent with a Sli gradient model. We also find that sli mRNA is maternally deposited and embryos that are genetically null for sli can have weaker guidance defects. Moreover, in robo or robo3 mutants, embryos with normal axon tracts are found and such robo embryos reach pupal stages and die, while robo3 mutant embryos develop into normal individuals and produce eggs. Interestingly, embryos from robo3 homozygous individuals fail to develop but have axon tracts ranging from normal to various defects: robo3 phenotype, robo phenotype, and sli-like phenotype, suggesting a more complex functional role for these genes than what has been proposed. Finally, our previous results indicated that netrin phenotype is epistatic to sli or robo phenotypes. However, it seems likely that this previously reported epistatic relationship might be due to the partial penetrance of the sli, robo, robo3 (or robo2) phenotypes. Our results argue that double mutant epistasis is most definitive only if the penetrance of the phenotypes of the mutants involved is complete.

In the Drosophila ventral nerve cord, there are ~20 longitudinal axon tracts on either side of the midline. The signaling system from the midline mediated by Slit (Sli) and its receptor Roundabout (Robo) appears to prevent these tracts from projecting toward the midline (Kidd et al. 1999). Previous results indicate that there are at least three robo genes in Drosophila. It has been postulated that a gradient of Slit emanating from the midline interacts with Robo receptors in a combinatorial manner to specify the lateral positioning of axon tracts in the longitudinal pathways (Rajagopalan et al. 2000; Simpson et al. 2000). In the Sli gradient scenario, the lowest level of Sli interacts with Robo and Robo2 to specify the lateral-most tracts, an intermediate level interacts with Robo and Robo3 to specify the intermediate tracts, and a high level interacts with Robo to specify the medial tracts (Rajagopalan et al. 2000; Simpson et al. 2000). However, a gradient of Sli extending from the midline has not been detected by antibody staining. More importantly, overexpression of sli at the midline does not alter the lateral positioning of longitudinal tracts (Kidd et al. 1999; our unpublished results), arguing against the gradient model. Consistent with this result, ectopic expression of Sli in front of the growth cones of Robo-expressing neurons does not alter the course of the projections of these neurons (Bhat 2005). The only phenotype that we can observe with a gain-of-function sli is the robo-like phenotype when sli is ectopically expressed everywhere (Kidd et al. 1999; our unpublished data), though the molecular basis for this phenotype has not been determined.

There is an additional signaling system emanating from the midline in Drosophila, which is the Netrin (Net) and its receptor Frazzled (Fra) system. This Net-Fra system appears to function as a midline attractant for the commissural tracts (Harris et al. 1996; Kolodziej et al. 1996; Mitchell et al. 1996). More recently, we have shown that loss of function for Net or Fra activity results in the positioning of longitudinal tracts shifting farther away from the midline compared to wild type (Bhat 2005). Moreover, overexpression of Net at the midline causes a collapsing of the longitudinal tracts at the midline (Bhat 2005).

While there is no evidence of a gradient of Sli emanating from the midline, here we show that there is presence of Sli in the commissural and longitudinal
axon tracts. We show this presence of Sli in axon tracts using two different Sli antibodies: one previously raised against the C-terminal part of Sli and the other that we raised against the N-terminal portion of the protein. We further show that the presence of Sli in commissures and connectives is not due to an uptake of Sli from a gradient diffusing from the midline; instead, it is taken up initially from the midline by commissural tracts when they cross the midline. Furthermore, this transport of Sli is also independent of Robo since embryos lacking the robo gene still have Sli in commissural and longitudinal tracts.

Previous results indicate that the product of commissureless downregulates Robo to allow commissural axons to cross the midline; however, in comm mutants, longitudinal tracts often cross the midline. Our results suggest that this is due to sequestration of Sli by the excess amount of Robo at the commissural tracts. Finally, our previous results indicated that the net phenotype is epistatic to the sli or robo phenotypes (Bhat 2005). This epistasis result, taken together with the failure of ectopic Sli to repel Robo-positive growth cones, as well as results obtained with net loss-of-function and gain-of-function analyses, led to the proposal that the major function of Sli-Robo signaling is to neutralize Net-Fra attractant signaling during the positioning of longitudinal tracts (Bhat 2005). However, our more recent data indicates that this epistatic relationship between net and sli or robo mutants may be due to embryos that are escapers for sli, robo, robo3 (or robo2, etc.) phenotypes. Such escaper embryos can be negative for the gene activity yet can have the net phenotype. Since we did not observe a wild-type positioning of longitudinal tracts in these double mutants, we must consider three possibilities: (1) Net has a direct attractant role on longitudinal tracts; (2) it also partially neutralizes the Sli-Robo signaling; and (3) there is an additional attractant system at the midline. Our results also argue that double mutant analysis to determine epistatic relationship between two mutants is most definitive only if the phenotypes of both mutants are fully penetrant. Our results also suggest that having a balancer in the parents from which the embryos are derived can alter the penetrance and the severity of a mutant phenotype.

MATERIALS AND METHODS

Fly strains and genetics: For the analysis of sli, we used the genetically null allele, sli2. For the analysis of net, we used the previously described deficiency that eliminates the two net genes, Df(1)RK2/FM7a. This deficiency also deletes several other complementation groups; however, the specific defects described here are attributed to loss of netA and netB genes (see Harris et al. 1996). For the analysis of robo, we used robo4, a genetically null amorph (the molecular nature of the mutation is not known), and for the analysis of robo3, we used the previously described loss-of-function allele robo3; the molecular acceptor site of the sixth intron (Rajagopalan et al. 2000). For the analysis of fri, we used fri2 and friG4057. The molecular nature of the fri mutation is a tryptophan to stop codon change at amino acid position 1028 (W1028Stop). The nature of the mutation in friG4057 is not known. For the analysis of abelson tyrosine kinase (abk), we used abk1. For the second chromosome balancer, we used the GFP-Cyo; w1118; In(2LR)Gla, wgGla1/CyO, P[GAL4-tevi2]2.2; P[UA5–2xEGFP]AIH2.2. Homozygous embryos were identified either using the GFP-marked balancer or by lack of a positive staining with an antibody.

Generation and purification of the Sli N-terminal antibody: The most hydrophilic 142 aa (470–611 aa) of the N-terminal part of Sli protein was selected for antibody production. The ~430-bp product was amplified using forward primer 5′-TGCATATGAATCCCATAGACGAGTG-3′ (1659–1677 bp of mRNA-A) containing a NdeI restriction site, which provides the methionine start codon, and the reverse primer 5′-TGAAGCTTCTCCATAGACGAGTGTC-3′ (2062–2084 bp of mRNA-A) containing a SacI restriction site and a stop codon. This fragment was cloned into the expression vector pET28a (Novagen). 6xHis-SliN fusion protein was expressed in the Escherichia coli strain BL21-CodonPlus (Stratagene) and purified on a Ni-NTA agarose column (Qiagen) under native conditions. About 1 ml of the 6xHis-SliN fusion protein (1 mg/ml) was used for rabbit immunization according to the standard protocol (Covance Immunological Services). The obtained polyclonal serum was affinity purified on the AminoLink Plus coupling gel (Pierce) with immobilized 6xHis-SliN fusion protein.

Immunohistochemistry and RNA in situ: Immunohistochemistry was performed using the standard protocols. The dilution of anti-Sli-N antibody used for embryo staining was 1:4000; for Fas II, the dilution was 1:5; BP102 was 1:6; Robo was 1:3; and monoclonal anti-Sli (C555.6D) was 1:10. For whole mount RNA in situ staining, the standard procedure was used with digoxigenin-labeled anti-sense Sli probe. AP-conjugated anti-digoxigenin (1:2000) was visualized according to manufacturer’s protocols; 1:20,000) was visualized according to manufacturer’s protocols using the ECL kit (Pierce). For the monoclonal Sli-C, we used 1:100 dilution.

Determination of sli maternal deposition—RT–PCR: All embryo collections were done at 22°, 14 hr virgins were aged for several days and then left to lay eggs for 24 hr. Total RNA from the dechorionated embryos was isolated using Trizol reagent (Invitrogen, Carlsbad, CA) and 1 μg of total RNA was used for the first-strand cDNA synthesis in 20-μl reaction using Superscript III reverse transcriptase (Invitrogen). sli mRNA from wild type and sli7 mutants was amplified using forward primer 5′-AATCCCATAGACGAGTGTGCG-3′ (1659–1677 bp of mRNA-A; exon 9) and reverse primer 5′-GGTCAACGACTTGAGGTCGGC-3′ (2756–2774 bp of mRNA-A; exon 15), which gives a single product (1134 bp) in wild-type embryos and two products (1188 bp and 1413 bp) in sli7 mutants. As an internal control for various cDNA samples, the 335-bp product of the constitutively expressed mRNA for ribosomal protein 49 (rp49; Rpl32 in FlyBase) was amplified using forward primer 5′-GCTAGCTGTGCACAAATG-3′ and reverse primer 5′-GAATCTTAAGCTACTCGTTCGTC-3′.
The following collections of wild-type embryos were used for RT–PCR analysis: 0–4 hr, 4–8 hr, 8–12 hr, and 12–24 hr. Embryos that are 0–1 hr, 1–2 hr, 2–3 hr, and 3–4 hr old from wild-type females crossed to slt/Bal females were analyzed by RT–PCR to determine the zygotic expression of slt mRNA.

**Mapping the slt mutation in the slt** mutant: The molecular basis of slt allele was previously reported as A → T conversion at the position 3321 bp (mRNA-A variant), which results in premature termination of Slit translation (K → Stop) at position 1024 aa (Battye et al. 2001). To confirm this mutation in slt chromosome, we amplified and sequenced the ~450 bp of exon 16 covering the mutation site using forward primer: 5′-CCATGCCTTGACTACGAAATC-3′ (3092–3111 bp of mRNA-A) and reverse primer 5′-CGATAGTCTGCTGACACGAG-3′ (3538–3556 bp of mRNA-A). slt homozygous embryos were distinguished from their counterparts using the twist-GFP marked balancer chromosome. Since we did not find the previously described mutation and that slt mutant embryos were negative for Sli protein with anti-Sli-C monoclonal, in order to determine the mutation in slt chromosome we first estimated the molecular weight of the truncated Sli protein. Using our Sli-N antibody, we performed western blot analysis on slt embryos. Extracts from 15–24-hr-aged embryos of slt homozygotes and w1118 (wild-type control) were analyzed in individual lanes. In slt homozygotes, instead of the two wild-type Sli bands, we observed a band of ~75 kDa size. We amplified and sequenced the exons and introns from exon 11 to 14 of the slt gene from slt mutants; this region should cover the region of the Sli protein from 61 to 88 kDa. Forward primer 5′-CCTGCTGACTTTAATCCG-3′ (intron 10; 11,197–11,214 bp of slt gene) and reverse primer 5′-AGCAAGTGCAGAAGA-3′ (3092–3111 bp of slt gene) were used to amplify exons 10, 11, and 12 and intron 11. Forward primer 5′-AAGAAGCTTTATCCAAGCC-3′ (intron 12; 12,822–11,842 bp of slt gene) were used to amplify exons 11 and 12 and intron 12. Forward primer 5′-TAATTGCTGGTCTGCATTTGC-3′ (intron 13; 12,340–13,260 bp of slt gene) and reverse primer 5′-TTAATTGCTGGTCTGCATTTGC-3′ (intron 14; 13,869–13,889 bp of slt gene) were used to amplify exons 13 and 14 and intron 12. The sequencing of PCR products from three independent reactions revealed the single base pair substitution G → A in the position of 11,460 bp of the slt gene, which corresponds to the first base pair of intron 11. A transition of G to A in the 5′ splice donor site of intron 11 is expected to result in an aberrant splicing. To confirm this, we amplified this region from slt chromosome using RT–PCR. The RT–PCR from wild-type embryos using forward primer 5′-AATCCCATAGAGACGAGTG-3′ (1069–1677 bp of mRNA-A; exon 9) and reverse primer 5′-GCTAACACCGAGGTC-3′ (2756–3174 bp of mRNA-A; exon 15) gives a single product of size 1134 bp. However, RT–PCR from slt homozygous embryos gives two products, which are different from the wild-type product. Sequencing of these two products confirmed the aberrant splicing of intron 11. The 1413-bp-long band represents the slt mRNA form in which intron 11 is not spliced. The second slt mRNA that gives a 1188-bp-long band is due to the use of an alternative splice donor site in intron 11 located 54 bp downstream from the correct splice site in intron 11. In both slt mRNA forms, the stop codon is located immediately following the mutation and thus, Sli translation is prematurely terminated at aa position 625 (Sli-A variant) and gives the product of ~75 kDa size.

**RESULTS**

Slit is present outside of the midline in the commissural and longitudinal tracts: While previous work showed that Sli is expressed in the midline glial cells, we found that the commissures and longitudinal connectives also have low levels of Sli (Figure 1b). The presence of Sli at very low levels can be detected as early as stage 12 (~10.5 hr of development) in places where the growing commissural tracts intersect the nascent longitudinal tracts (data not shown). The source of this Sli must be the midline since within the nervous cord, the slt gene is not zygotically transcribed outside of the midline (Figure 1a). In slt2 mutant allele, which is genetically a null allele (Battye et al. 2001), the mutant protein is not recognized by the monoclonal antibody raised against the C-terminal portion of the protein; in these embryos, the Sli staining of the commissural and longitudinal tracts was absent (Figure 1c). These results therefore indicate that the source of Sli in the connectives and commissures is unlikely the background.

It is still possible that this extra-midline staining of Sli is an artifact associated with this particular monoclonal antibody. Therefore, we raised a polyclonal antibody against the N-terminal portion of Sli (Figure 1, e–g). When embryos were examined with this antibody, the commissural tracts and the connectives were also positive for Sli (Figure 1, e and f). These results therefore argue that the extra-midline staining of Sli is not an artifact but is due to the presence of Sli in these tracts.

As suggested by previous work (Krn et al. 1998), Sli appears to be processed into an N-terminal part and a C-terminal part and exists along with the unprocessed full-length Sli. With Sli-N antibody, as shown in Figure 1g, we observed the unprocessed ~180 kDa band and the processed N-terminal part (~135 kDa; we observed two to three additional bands with this antibody but these are either degradation products or nonspecific bands). With the Sli-C antibody, we observed the unprocessed Sli and the C-terminal portion of Sli (Figure 1h).

We reasoned that the presence of Sli in axon tracts of commissures and connectives can be utilized to examine the Sli gradient model. If Sli is present in a diffusible gradient extending from the midline (Rajagopalan et al. 2000; Simpson et al. 2000), the presence of Sli in connectives and commissures is likely to be due to the uptake of Sli from the surrounding environment, resulting in the accumulation of Sli in these tracts. Alternatively, Sli in connectives and commissures might be due to a delivery of locally secreted Sli via the commissural tracts given that these tracts are projected across the Sli-expressing midline glial cells.

We sought to distinguish between these two possibilities as follows. If Sli is transported via commissural tracts in embryos that lack most of their commissural tracts, as in loss-of-function commissureless (comm) mutant embryos (see Figure 2A; note that a few axons are still projected across the midline in comm mutant embryos, marked by arrows), the longitudinal tracts will have no or reduced levels of Sli. If the Sli in longitudinal connectives is due to accumulation of Sli from the surrounding environment, comm mutants will still have high levels of Sli in their connectives, as in wild type. When comm embryos were stained with Sli antibody, we...
since Sli stains all axon bundles in the nerve cord. Note that the commissural and connectives staining of Sli is not discrete as Fas II.

Western blotting analysis of wild-type embryo extract with anti-Sli-N; arrows indicate the unprocessed Sli and the processed N-terminal portion of Sli. This antibody does not recognize the processed C-terminal portion of Sli. (h) Western blotting analysis of wild-type embryo extract with anti-Sli-C; arrows indicate the unprocessed Sli and the processed C-terminal portion of Sli. This antibody does not recognize the processed C-terminal portion of Sli. (a) Whole mount RNA in situ of wild-type embryo with sli probe. sli is transcribed only in the midline cells in the nerve cord. (b) Wild-type embryo stained with anti-Sli-C antibody. Sli protein is present at very high levels in the midline, but it is also present at lower levels in the commissural tracts and longitudinal connectives. (c) sli2 mutant embryo stained for Sli. This is a protein null allele. (d) Wild-type embryo stained with Mab BP102 (BP). BP102 stains both the commissures and the connectives, revealing a ladder-like structure of the nerve cord. (e and f) Wild-type embryos stained with anti-Sli-N antibody. Sli protein is present at high levels in the midline, but it is also present at lower levels in the commissural tracts and longitudinal connectives. (g) Western blotting analysis of wild-type embryo extract with anti-Sli-N; arrows indicate the unprocessed Sli and the processed N-terminal portion of Sli. This antibody does not recognize the processed N-terminal portion of Sli. Note that the commissural and connectives staining of Sli is not discrete as Fas II since Sli stains all axon bundles in the nerve cord (~20), whereas Fas II stains only 3–4 bundles.

observed regions along the longitudinal connectives that lack Sli even when these regions are next to Sli-expressing midline cells (Figure 2, B and C, arrowheads). Whenever there is Sli staining in a stretch of longitudinal connectives, the source can be traced through the few commissural tracts to the midline (Figure 2, B and C, arrows), indicating that Sli is transported away from the source via these tracts.

We next determined if the transport of Sli from the midline is Robo-dependent. We examined embryos homozygous for a deficiency that eliminates the robo gene with anti-Sli-C. In these embryos, we found that the commissural and longitudinal tracts had Sli as in wild type (Figure 2D). This indicates that the presence of Sli in these tracts is not Robo-dependent. This does not appear to be a redundancy problem with Robo2 or Robo3 since these Robo are reported to have narrower domains of expression (Robo3 is reported to be in the lateral and intermediate tracts; Robo2 is only in the lateral tracts) compared to Robo, which is in all longitudinal tracts (Rajagopalan et al. 2000; Simpson et al. 2000). Besides, if there is such a redundancy, we would have seen a narrower distribution of Sli in longitudinal connectives, which was not the case. Moreover, Robo receptors are downregulated in commissural tracts (Kidd et al. 1998; Myat et al. 2002) and thus unavailable for binding of Sli in commissures. Therefore, an unknown gene product must be involved in this transport of Sli via commissural tracts.

Longitudinal tracts inappropriately cross the midline in commissureless mutants: Given the above results, we sought to determine if the Fas II-positive tracts were misplaced in comm mutants. We found that the medial longitudinal tracts often crossed the midline in embryos that are loss of function for comm (Figure 3D; one or two segments in ~9% of embryos, n = 200 embryos; see also Fritz and Vanberkum, 2000). In comm mutants, the loss of Comm protein from the commissural tracts causes elevated levels of Robo in these tracts (Kidd et al. 1998; Myat et al. 2002). It has been proposed that this elevated level of Robo leads to a repulsive interaction with midline Sli and as a result these commissural tracts fail to cross the midline, generating a commissureless phenotype (Kidd et al. 1999). However, the inappropriate midline crossing of longitudinal tracts was unexpected for comm mutants. One possibility is that the excessive amount of Robo (due to deregulation of Robo) titrates out Sli, and as a result the longitudinal tracts are able to cross the midline. To test this possibility, we halved the dosage of sli in comm background (sli2/++; comm/comm) and examined the longitudinal tracts in these embryos with Fas II antibody. As shown in Figure 3, E and F, this enhanced the midline crossing phenotype in comm mutants significantly in both the number of segments as
As shown in Figure 4, we observed a mixture of phenotypes, ranging from robo phenotype (Figure 4e) to a fra phenotype (Figure 4, f–h) and an intermediate phenotype (Figure 4i), although the robo phenotype was predominant. The double mutants were picked by the lack of their staining for GFP since the mutant chromosome was balanced with a GFP-marked CyO.

Because of the predominance of embryos with a robo phenotype among robo, fra double mutants, it appears that robo phenotype is epistatic to the fra phenotype. This conclusion is inconsistent with our previous result that Sli neutralizes attraction mediated by Net and the effect of Sli on axon guidance is via Net (Bhat 2005). Therefore, we re-examined robo; net double mutant embryos with Robo and Fas II antibodies (we used a deficiency that eliminates the robo gene). We have previously found that having balancers in the parental cross from which the embryos are derived for staining can often cause significant variability in terms of the penetrance and the actual defect, and this was especially true for axon guidance and RP2 lineage defects. Therefore, we make an attempt to avoid having a balancer in the parental cross as much as possible and use antibody staining or PCR to identify homozygous mutant embryos (as opposed to using LacZ or GFP balancers). Thus, we eliminated the balancer chromosomes from the parents from which the embryos were collected and used the lack of Robo staining and the axon guidance phenotype to identify the double mutants (we could not use this same strategy when examining robo, fra double mutants since the robo mutant allele we used was not protein-null). As shown in Figure 5C, we observed embryos that are Robo-negative but with a net phenotype (~2.75% of the total embryos were of this type; expected was ~6.25% or 1/16 embryos; n = 400). This number is less than expected but the same as we observed previously. It should be noted that often double mutants are found in ratios less than expected due to trans-heterozygous interaction between two mutants or caused by a background effect.

Given these above results, we entertained two possibilities. First, attraction of longitudinal tracts by Net is not mediated via Fra; thus, it is possible to have differing epistasis results between robo; net and robo, fra. Second, robo mutant phenotype is partially penetrant and such embryos in combination with net can be Robo-negative yet have a net phenotype. To test this possibility, we balanced the robo' mutant chromosome with a GFP-marked CyO balancer and selected GFP-negative embryos (n = 572) and allowed these embryos to develop: we observed 48% or 275 of the robo' homozygous embryos hatching into first instar larvae, and 3% or 17 of the embryos developing into pupal stage, but none of these pupae eclosed into adults (on occasions, we have seen as much as 7% of pupae that are robo' homozygotes).

We also stained GFP-negative robo embryos with Fas II antibody. As shown in Figure 6, we observed robo homozygous embryos with more or less normal longitudinal

**Figure 2.** Sli from the midline is delivered to the longitudinal tracts via commissural tracts. (A) comm mutant embryo stained with BP102. Note the absence of commissures; however, there are still axon tracts that project toward the midline (arrows). (B and C) comm mutant embryos stained with anti-Sli. Note the absence of Sli in the commissures adjacent to Sli expressing midline cells (arrowheads). The low levels of Sli in tracts elsewhere appear to be delivered via the few remaining commissural tracts (arrows; outline of these commissural tracts is visible by Nomarski optics). (D) robo deficiency embryo showing Sli in both the commissures and connectives.
axon projections (cf. Figure 6d). This was observed in 3% of embryos \((n = 106)\). These results argue that a significant number of embryos with guidance defects would make it to pupal stages. These results also indicate that the \(\text{robo}; \text{net}\) double mutants that have the \(\text{net}\) phenotype could be embryos that are “escapers” for the \(\text{robo}\) phenotype. We have not examined if the escapers are due to a maternal deposition or redundancy with the other Robo receptors.

A significant number of escapers were found for \(\text{robo}3\) loss-of-function mutation: We next examined if the loss-of-function effects of \(\text{robo}-3\) are also partially penetrant, which would explain again the presence of \(\text{robo}3; \text{net}\) double mutants with a \(\text{net}\) phenotype (Bhat

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**Figure 3.**—Longitudinal axon guidance defects in \(\text{comm}\) mutants and its enhancement by reducing the dosage of sli. Approximately 15-hr-old embryos are stained with Fas II antibody. Anterior end is at top, midline is marked by vertical lines.

**Figure 4.**—Double mutant analysis between \(\text{robo}\) and \(\text{fra}\). Embryos are stained with Fas II antibody. Anterior end is at top, midline is marked by vertical lines. (a) Wild-type embryo showing the Fas II positive longitudinal tracts. (b) \(\text{robo}\) mutant embryo; the intermediate and medial tracts are collapsed at the midline. (c) \(\text{net}\) mutant embryo; the tracts are positioned farther apart. (d) \(\text{fra}\) mutant embryo; tracts are also positioned farther apart. (e-i) Embryos double mutant for \(\text{robo}\) and \(\text{fra}\). Phenotypes varying from \(\text{robo}\)-like to \(\text{fra}\)-like are observed. The results were similar with two different alleles of \(\text{fra}\). The frequency 18% includes phenotypes represented in f–h.
We transferred the robo3 mutant allele into the GFP-marked CyO balancer background and followed the development of the GFP-negative robo3 homozygous mutant embryos (n = 200). We found that 46% or 92/200 such embryos hatched to larvae; 29.5% or 59/200 embryos reached the pupal stage; and 27.5% or 55/200 embryos eclosed into functional normal adults. Consistent with this, when robo3 homozygous embryos from robo3/CyO-GFP parents were stained with Fas II, we observed embryos that are either with robo3 phenotype (61%, n = 31 embryos) or with normal Fas II-positive longitudinal tracts (39%, n = 31). It is most likely that these embryos that develop into viable normal adults have normal axon pathfinding, and in combination with net, such embryos will give a net phenotype.

To explore this issue further, we collected embryos from these robo3 homozygous parents and stained these embryos with Fas II. We found that these parents produced embryos that are completely normal (Figure 7B) but also embryos that have the robo3 mutant phenotype (Figure 7, C and D), a sli-like phenotype (Figure 7E), and a robo-like phenotype (Figure 7F). The sli-like or the robo-like phenotypes were not observed when the embryos were collected and stained from robo3 heterozygous parents but were only among embryos derived from robo3 homozygous parents. This result argues that the axon guidance regulated by Robo3 (or the other two Robo receptors) involves a much more complicated pathway(s) than what has been proposed previously (Rajagopalan et al. 2000; Simpson et al. 2000). An alternative explanation is that there are modifier mutations on the robo3 chromosome, or elsewhere within in the robo3 strain, altering the phenotype. However, if such modifiers exist, we should see a similar variability due to the modifier effect among embryos derived from robo3 heterozygous parents (robo3/+ × robo3/+) which was not the case, and more importantly, the phenotypes in embryos derived from robo3 homozygous parents (robo3/robo3 × robo3/robo3) have very specific phenotypes: robo-like, robo3, sli-like, and, of course, wild type. Yet another possibility is that a Sli pathway-specific parental trans-suppressor exists on the CyO balancer (a balancer-mediated effect); this possibility is consistent with our finding that a robo3 homozygous female can produce robo3 mutant embryos that reach all the way to adulthood if that female parent is mated with a heterozygous male. We have lost the robo2 mutant line and therefore have not re-examined robo2 to determine if the same above results also hold true for robo2, but it seems highly likely that a similar scenario exists for robo2 as well.

**Epistatic relationship between sli and fra:** We next examined the axon guidance in sli; fra double mutant embryos with Fas II staining. As shown in Figure 8, we observed embryos with the sli phenotype (Figure 8A; 88%, n = 400) but also embryos that have a phenotype similar to hypomorphic sli (Figure 8B; 3%) and embryos that are closer to fra phenotype (Figure 8C; 9%). Given these results, we re-examined the previous results by...
generating net; sli double mutants again and double staining these embryos with Sli and BP102. We avoided having a balancer in the parental cross from which the embryos were derived. As shown in Figure 9, we observed double mutant embryos that were Sli-negative but had the net phenotype (Figure 9, C, D, and F). While we expect 6.25% (or 1/16 embryos) will be double mutants for both the chromosomes, we observed 1.9% of embryos (n = 675) that were Sli-negative but with net phenotype.

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phenotype is also partially penetrant and such embryos in combination with net can be Sli-negative but have a net phenotype. To test this possibility, we balanced the sli2 mutant chromosome with CyO balancer marked with GFP and stained GFP-negative embryos with Fas II antibody. As shown in Figure 9H, we observed embryos that are GFP-negative but had a weaker sli phenotype. This was observed in 0.7% of the GFP-negative embryos (n = 300). This number is much less than what we observed when the sli; net (or sli, fra) double mutant combinations were examined. One likely explanation is that the embryos in the sli; net double mutant combination are derived from parents that have no balancers and therefore the partial penetrance of the defects/the variability is much greater compared to embryos from balanced parents. Additionally, genetic background in the combination might alter the frequency. It is also possible that in some embryos, net and sli interact and net may be epistatic to sli.

**Maternal deposition of sli:** We sought to determine if there is any maternal deposition of the sli gene products. We performed RT–PCR of unfertilized embryos for sli and found that there is maternal deposition of sli mRNA to embryos (Figure 10A); however, there was no maternal deposition of Sli protein (data not shown). To determine how long this maternal deposition lasts, we used sli2 embryos. It has been previously reported that the sli2 mutant chromosome carries a stop codon in exon 16 at aa position 1024 (Battye et al. 2001). We thought that it is possible to make use of this change in the sequence to determine the dynamics of maternal deposition/initiation of zygotic transcription of sli. However, we did not find a stop codon in the reported position upon sequencing of the sli gene in sli2 chromosome. Therefore, we decided to examine the location of the mutation in the sli2 mutant. To narrow down the possible mutation in the chromosome, we performed Western blotting analysis of the sli2 homozygous mutant embryos using our newly generated antibody against the N terminus of the Sli protein (Figure 10B). As shown in Figure 10B, sli2 mutant embryos produced a truncated protein of ~75 kDa (the predicted size for isoform A is 69 kDa; often the SDS–PAGE can give a molecular weight greater than the predicted one). We sequenced the corresponding region (from exons 11 to 14 and introns 11 and 12) and found that there is 1 change in the splice site in sli2 mutant: the first base of intron 11 corresponds to the normal splicing of sli mRNA. RT–PCR analysis of wild-type and sli2 mutant embryos produced a truncated transcript in unfertilized embryos (Unf) as well as in fertilized embryos from 0–24 hr of development. The expression of rp49 was used as an internal control (bottom). 

**Figure 10.—Molecular characterization of sli.** (A) Maternal deposition of sli mRNA. RT–PCR analysis of wild-type embryos showing the presence of sli transcript in unfertilized embryos (Unf) as well as in fertilized embryos from 0–24 hr of development. The expression of rp49 was used as an internal control (bottom). (B) Western blot analysis of wild-type and sli2 homozygous embryos with anti-Sli-N antibody. In wild type, the processed and unprocessed Sli can be observed, whereas a truncated Sli2 protein was detected as a single band of ~75 kDa in sli2 mutant embryos (arrowhead). (C) Schematic showing the normal splicing of sli in wild type (top diagram) and the aberrant splicing in sli2 embryos creating a termination of the Sli ORF (the two bottom diagrams; see text for details). (D) Detection of aberrant sli2 transcripts using RT–PCR analysis of sli2 embryos. Intron 11 does not undergo correct splicing in sli2 mutants and only two defective splicing variants (asterisk) are present in sli2 mutant embryos. (E) Zygotic expression of sli mRNA. RT–PCR analysis of embryos from wild-type females crossed to sli2/Bal males reveals that sli2 transcript is present in embryos by 2–3 hr of age.
splice donor. These results are consistent with our above conclusion that the mutation generated a product that has either the entire intron 11 or a part of the intron 11 (when the downstream splice donor site is used).

Using this information, we examined when the zygotic activation of sli is initiated. RT–PCR of embryonic sli mRNA derived from sliT/Cyo males and wild-type females (for the mutant bands) shows that the zygotic expression can be seen in 2–3-hr-old embryos (Figure 10E), much earlier than what has been previously thought.

**Loss of function for abelson tyrosine kinase has axon guidance defects:** We also examined embryos that are mutant for the gene abelson tyrosine kinase (abl), whose product is thought to function downstream of Robo. It has been suggested that Abl phosphorylates Robo, whereas Enabled (Ena), a member of the proteins known to associate with the cytoskeleton, antagonizes Abl, allowing the hypophosphorylated Robo to mediate axon repulsion (Bashaw et al. 2000). According to this hypothesis, loss of function for abl should have no consequence on axon guidance, as was reported (Gertler et al. 1989; Bashaw et al. 2000). However, a subsequent article reported that abl loss-of-function mutants have axon guidance defects: the longitudinal tracts inappropriately cross the midline in abl mutants (Wills et al. 2002). We obtained abl mutant lines from the Goodman lab and examined the embryos derived from parents that had the balancer and also embryos from parents that did not have the balancer. We observed strong axon guidance defects in longitudinal tracts in embryos derived from both sets of parents (Figure 11, A–D). Interestingly, embryos derived from parents that had the balancer contained two classes of mutant embryos. In one class, the longitudinal tracts were overall collapsed toward the midline with medial tracts crossing the midline on an average of seven hemisegments per embryo with a lot more variability between embryos (n = 7 embryos; Figure 11A; we calculate as hemisegments because often tracts from only one side cross the midline) and a second class where no such collapsing of the tracts was observed but these embryos still had the medial tracts crossing the midline at slightly a lower frequency (Figure 11B; average five hemisegments per embryo). Embryos from the parents that had no balancers, however, had only one class of embryos: there was no collapsing of the tracts toward the midline, but the medial tracts were crossing the midline (Figure 11, C and D). The abnormal midline crossing of the medial tracts in these embryos was higher with an average of nine hemisegmental crossings per embryo. In addition to the midline crossing of medial tracts in these embryos, we also observed an inappropriate outward projection of lateral tracts in these embryos (Figure 11, C and D; arrowheads; average three to four projections per embryo). These results indicate that the events downstream of Robo involving Abl and Ena, as proposed, are probably incorrect and remain poorly understood.

**Figure 11.—Axon guidance defects in abl mutants.** Embryos are stained with Fas II antibody. Anterior end is at top, midline is marked by vertical lines. (A–D) abl homozygous embryos. Note the outward projection of longitudinal tracts (arrowheads).

**DISCUSSION**

Our results presented in this article show that the Slit/Robo signaling during axon guidance is much more complicated than previously thought. For example, it has been proposed that the Slit protein is secreted from the midline and a differing concentration of it interacts with Robo receptors to specify the positioning of longitudinal tracts. We found that Slit is located also in the commissural and longitudinal connectives and is transported initially via the growing commissural tracts. Furthermore, we found that a portion of embryos that are mutant for the genetically null allele of robo have more or less normal axon projections and the individuals develop until late pupal stages. A significant number of embryos mutant for robo3 have normal projection pattern and go on to become viable adults. These robo3 homozygous individuals produce embryos, but these embryos exhibit normal axon projections to embryos with varying axon guidance defects: robo3, slit-like, and robo-like. All the embryos from robo3 homozygotes die as late embryo. Moreover, we also found escapers among sli embryos as well, although in this case we did not find embryos that have a completely normal projection pattern from a genetically null allele of sli. This escaper phenomenon in these mutants that has not been reported previously appears to be the likely reason for the different epistatic results obtained between sli/robo and net compared to sli/robo and fra.

In addition to these results, we find that sli mRNA is maternally deposited and the zygotic transcription for sli begins as early as 2–3 hr of development. We also report that the mutation in the sli gene in sliT allele is a change in the splice-donor site, which is different from the mutational change reported previously. Finally, on
the basis of these results, we propose the following: (1) the characterization of a mutant/mutation for a gene must include information on whether or not the defects in question are fully or partially penetrant; (2) any characterization of the double mutant phenotypes between two mutants with opposing phenotypes must include information on the penetrance or variability of the defects; and (3) all the phenotypes must be verified, whenever possible, by staining embryos derived from parents that do not carry a balancer.

**Slit is transported from the midline to the connectives via commissural tracts:** It has been proposed that Sli is secreted from the midline and it exists as a diffusible gradient extending away from the midline (Rajagopalan et al. 2000; Simpson et al. 2000). While no such gradient has been detected, we observed staining of connectives and commissures with at least two different antibodies, one the existing monoclonal and the other a polyclonal that we generated (see Figure 1). Our use of commissureless mutants indeed shows that the extra-midline Sli on the tracts was transported by the commissural tracts and was not taken up by the tracts from the surrounding “environment.” Our results also show that this transport is not via binding to Robo since we observed the staining of tracts for Sli in a deficiency that eliminates Robo. Whether other Robo receptors, whose known expression pattern does not automatically suggest the possibility of a complementation for the loss of Robo, substitute for the loss of Robo is not known. If Robo receptors are not involved, some other Sli transporter protein present on axon tracts must take up the Sli from the midline glial cells and transport it across.

The major question, however, concerning the presence of Sli in tracts is its functional significance: Does this Sli participate in any guidance process? This extra-midline Sli obviously is not altering the projection paths, although it is expected to encounter all three Robo receptors. One possibility is that this Sli is in an inactive form and only midline Sli is functional. Perhaps we can also speculate that this is a mechanism to reduce Sli at the midline. It seems like there is a fine balance for the amount of Sli at the midline as indicated by our results with comm. Loss of function for comm causes a few tracts crossing the midline; however, reducing the dosage of sli in comm mutant background can dramatically enhance this phenotype (Figure 3). In comm mutant background, the Robo receptor is upregulated in the commissural tracts. It seems likely that this excess Robo that Sli have to deal with titrates Sli out, reducing its level. Reducing the dosage of sli therefore would naturally enhance the phenotype. One result that is somewhat inconsistent with these findings is that overexpression of Sli at the midline using UAS-sli × single-minded-GAL4 combination enhances the midline expression of Sli as well as the intensity of Sli staining in the axon tracts; however, it does not cause any phenotype. We think that further progress on this issue requires identification of the Sli-transporter and functional dissection of the Sli protein itself.

**Which of the guidance signaling systems is epistatic, Net-Fra or Slit-Robo?** We find a significant number of escaper embryos reaching to adulthood and producing embryos in the robo^3 mutant. A significant number of the mutant embryos had normal longitudinal axon tracts. Intriguingly, a significant number of homozygous mutant embryos derived from homozygous parents had more severe phenotype than what has been reported for this mutant. That is, embryos with a sli-like phenotype as well as embryos with a robo^2-like phenotype were observed. On the other hand, homozygous embryos derived from robo^3 heterozygotes showed either normal axon guidance or the robo^3 phenotype. One possibility is that there is a partially penetrant maternal effect of robo^3 mutation.

An important conclusion one can draw from these results on robo^3 is that analysis of double mutants for epistasis between robo^3 and any other mutation that has an opposing phenotype (such as netrin) is basically meaningless since those robo^3 mutant embryos that have normal phenotype in combination with net mutant will have net phenotype and the argument for epistasis relationship will then have to be based on frequency data. If the frequency of “normal” mutant embryos is high, the conclusion based on such epistasis results is not reliable.

We also found escaper embryos reaching the late pupal stages from a genetically null allele of robo. One difference between robo^3 and robo is that the number of such embryos in robo^1 is much less than the number observed for robo^3. Nonetheless, such embryos are sufficient to confuse, at the least, double mutant analysis between robo and mutants such as netrin, especially considering that these mutations are on different chromosomes and both the single mutant embryos will also be present in the collection. This problem, however, still exists even if the double mutant analysis involves a recombinant chromosome between two mutations. This is indicated by our finding that embryos that are double mutants for robo and fra can have a fra phenotype; this is also consistent with the observation that robo; netrin embryos can have a netrin phenotype.

Why are there robo mutant embryos that have more or less normal longitudinal axon tracts? One obvious possibility is that it has redundancy with other Robo receptors. The problem with this interpretation is that other Robo receptors are not expressed in all tracts (Rajagopalan et al. 2000; Simpson et al. 2000). On the other hand, we observe robo^3 homozygous embryos that are sli-like or robo-like; therefore, these other Robo receptors are likely to be expressed, perhaps at low levels in all tracts.

The epistasis results are somewhat clearer with our detailed analysis of the sli^2 mutant, a genetically null allele of sli, and the analysis of sli and sli, fra double mutants. However, the interpretation of the epistasis
results between slt, fra or slt; net still depends on the frequency data since we found that slt mutant embryos also give rise to embryos that have milder phenotype. That is, while we found no slt mutant embryos that have normal phenotype, we did find embryos that are similar to phenotypes found in slt hypomorphic alleles. Whether or not a double mutant between this type of embryo and net or fra will have a net/fra phenotype is left to conjecture; we do observe a reasonable number of embryos that have a net phenotype but lack Sli. However, it must be noted that slt; net mutants showing the net phenotype is greater than one would predict from the analysis of the slt single mutant. This may be due to absence of balancers in the parents from which the double mutant embryos are derived, as well as the genetic combination. Nonetheless, because the number of slt mutant embryos showing the milder phenotype is very low, it is likely that slt phenotype is epistatic to net phenotype.

Another important finding is that slt mRNA is maternally deposited to developing embryos, although this deposition appears to be exhausted at the latest by 5 hr of development, well before the requirements of Sli for axon guidance. It may be that Sli is required in neuroblasts or other early developmental processes.

If the Sli-Robo signaling is epistatic to the Net-Fra signaling in the guidance of longitudinal tracts, the most one can suggest are the following two possibilities: (1) these two signaling pathways are independent systems, one repellent and the other attractant; and (2) Net-Fra signaling inhibits the Sli-Robo signaling. Since our previous results indicate that Net-signaling has an attractant effect on longitudinal tracts (Bhat 2005), this will be an additional role for Net-signaling during the positioning of these tracts. With the available data, it is not possible to distinguish with complete certainty whether we can exclude the second possibility. One can argue that if the two systems independently exert influence on the pathfinding of longitudinal tracts, double mutants between the two would have a normal phenotype. In the absence of a normal phenotype in the double mutant, it must be that the second scenario is correct. The problem with this argument is that the repellent force exerted by Sli signaling on growth cones need not be equal to the attractant force by Net-signaling; the total attractant force can be mediated by Net signaling as well as a second attractant pathway. In this scenario, although embryos are lacking both Sli and Net function, the second attractant signaling is still intact and is sufficient to attract growth cones to the midline. What is this attractant signaling that attracts the longitudinal tracts to the midline? We have not identified such an attractant system as yet. We expect that loss of function for such a system will have the longitudinal tracts phenotype similar to the loss of function for the net or fra mutants. Finally, our results with abl indicate that the proposed pathway downstream of Robo involving Abl and Ena is unlikely to be correct (see Bashaw et al. 2000), and therefore, the events downstream of Robo remain obscure.

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