Axon repulsion from the midline of the *Drosophila* CNS requires *slit* function

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Accepted 3 March; published on WWW 4 May 1999

SUMMARY

Guidance of axons towards or away from the midline of the central nervous system during *Drosophila* embryogenesis reflects a balance of attractive and repulsive cues originating from the midline. Here we demonstrate that Slit, a protein secreted by the midline glial cells provides a repulsive cue for the growth cones of axons and muscle cells. Embryos lacking *slit* function show a medial collapse of lateral axon tracts and ectopic midline crossing of ventral muscles. Transgene expression of *slit* in the midline restores axon patterning. Ectopic expression of *slit* inhibits formation of axon tracts at locations of high Slit production and misdirects axon tracts towards the midline. *slit* interacts genetically with *roundabout*, which encodes a putative receptor for growth cone repulsion.

Key words: *slit*, *roundabout* (*robo*), Midline growth cone, Glia, Axon guidance, *Drosophila melanogaster*

INTRODUCTION

The navigation of axonal growth cones through the developing nervous system is guided by short and long range signals in the extracellular environment. These signals act as attractive or repulsive cues depending upon whether they facilitate growth cone extension or inhibit it (Goodman, 1996). A major feature of axon guidance in the developing CNS is the establishment of a contralateral projection, or conversely, avoidance of the midline of the CNS. In vertebrates, guidance of axons towards or away from the midline is communicated by attractive and repulsive signals originating from the floorplate (Bernhardt et al., 1992; Colamarino and Tessier-Lavigne, 1995). The midline glia (MG) cells of the *Drosophila* nervous system appear to communicate similar information (Planagan and van Vactor, 1998; Kidd et al., 1998a; Mitchell et al., 1996).

Molecular and genetic studies in *Drosophila* are revealing how midline cells direct the formation of contralateral projections. A number of mutations that disrupt midline axon guidance have been isolated in large screens for axon tract malformations in *Drosophila* (Hummel et al., 1999; Seeger et al., 1993). The diversity of mutant phenotypes observed suggest that multiple mechanisms regulate midline guidance. Among these mutants, the phenotypes of embryos mutant for the *commissureless* (*comm*) or *netrin* genes are distinctive, as they lack most or all commissural axons (Harris et al., 1996; Mitchell et al., 1996). This mutant phenotype suggests a function for these genes in directing commissural axons to the midline. In contrast, mutation of the *roundabout* (*robo*) or *karussell* genes results in an increase in commissural axon number, generated by re-crossing of decussated axons (Hummel et al., 1999; Tear et al., 1996). This suggests that *robo* and *karussell* act to prevent axons from crossing the midline. The phenotype of over-expression of *comm* and *robo* genes results in the midline.
tracts, and errors in ipsilateral or contralateral axon projection. Cells of the midline, in particular the MG, are present in slit mutant embryos, however they are ventrally displaced within the CNS (Sonnenfeld and Jacobs, 1994). Here we demonstrate that reductions in slit function result in ectopic projections of axons and ventral muscles over the ventral midline. Normal and ectopic expression of a slit transgene correlates with repulsion of axon extension. We describe a strong genetic interaction of slit with mutations in robo, suggesting further that Slit may act as a ligand for Robo, mediating axon repulsion from commissural projection.

MATERIALS AND METHODS

Genetic stocks
slit2 (formerly slit2G107), was isolated by Nüsslein-Volhard (Nüsslein-Volhard 1984) robo1 was isolated on a background deficient for fasciclin III and fasciclin I described by Seeger (Seeger et al., 1993). A wild-type genetic background was restored prior to study. A homozygous viable insert of P[sim-GAL4] obtained from S. Crews (generated by J. Nambu), P[eng-GAL4] (T. Bössing), P[scab-GAL4] (C. Goodman) and P[slit1.0-GAL4] (C. Klämbt) were used in the over-expression and rescue experiments. slit2, P[sim-GAL4] and other 2nd chromosome recombinants were generated and then screened using P[UAS-tau-lacZ], BP102 and mAb 1D4 (anti-Fasciclin II). All mutant stocks were isogenised and maintained on a yw mutant background balanced with CyO-P[eng-lacZ].

Immunocytochemistry
Immunocytochemistry was adapted from Patel (Patel, 1994). Embryos were collected at 22°C (or 29°C for some GAL4 experiments) and fixed at 16 hour intervals. BP102, mAb 1D4, α-sim or mAb Cl.427 (provided by N. Patel, C. Goodman, S. Crews and R. White, respectively) were diluted 1:4 in phosphate-buffered saline (PBS) with 0.1% Triton X-100 and incubated at room temperature for 6 hours followed by 2 hours incubation in goat anti-mouse conjugated with HRP (Jackson Immunological) at a 1:1000 dilution. Reactions with most embryos were carried out in the presence of 0.03% cobalt chloride. Nerve cords were dissected in methyl salicylate prior to mounting in DPX (Sigma 31761-6) and visualised on a Zeiss Axioskop microscope.

Fluorescence microscopy
Manually devitellinised embryos were dissected on glass in PBS and fixed for 10 minutes in 4% paraformaldehyde. The dissections were washed in buffer and incubated for 30 minutes in rhodamine-labelled phalloidin (Verheyen and Cooley, 1994) (Molecular Probes R415) and mounted in glycerol with p-phenylene d-amine (Sigma P-6001) as an anti-bleaching agent. Projections were made from confocal images collected with a Zeiss 310.

Electron microscopy
Dechorionated embryos were fixed in heptane equilibrated with 25% glutaraldehyde (Fluka) in 0.1 M sodium cacodylate. Embryos were manually devitellinised in 4% paraformaldehyde and 2.5% glutaraldehyde in cacodylate buffer, post-fixed in 1% osmium tetroxide, and stained before embedding in uranyl acetate (Jacobs and Goodman, 1989). Lead stained 0.1 mm thick sections were examined on a JEOL 1200EXII microscope.

Germline transformation
A 4.2 kb fragment of slit cDNA was engineered by ligation of a C-terminal PCR fragment (primers 5’ CTT CAG AGC TCT GCC ACA ATG GCC 3’ and 5’ CCC TTG AAG ACG CTT CTA CCC ACG 3’) and N-terminal PCR fragment (primers 5’ TGC TTA CTA GTT CCG

RESULTS
Slit is required for midline guidance
Previous studies of slit function characterise the mutant CNS phenotype as a midline fusion of all axon tracts, and a ventral displacement of midline cells (Rothenberg et al., 1988, 1990; Sonnenfeld and Jacobs, 1994). These studies did not establish whether the axon tract phenotype reflects a deficiency in axon guidance, or a secondary effect of disrupting midline cytoarchitecture. We have re-examined axon tract architecture of all CNS axons (labeled with BP102 antibody) and subsets of longitudinal tract fascicles (with antibodies to Fasciclin II) in embryos homozygous for a hypomorphic and a null allele of slit (Fig. 1). In contrast to the ladder-like axon tract organisation of a wild-type CNS (Fig. 1A,G), all axons are displaced towards a single midline tract in a slit amorph, slit2 (Fig. 1C). During stage 12/0, Fasciclin II immunolabeling identifies pioneers of the longitudinal tracts, the pCC, vMP2 and MP1 axons (Greningloh et al., 1991; Jacobs and Goodman, 1989; Fig. 1D). MP pioneers are medi ally displaced in stage 12/0 slit2 mutant embryos, and the growth cone of the more lateral pCC neuron extends towards the midline (Fig. 1F).

Are contralaterally projecting axons also trapped in the midline axon fascicle labeled with BP102 antibody, or are contralateral axon projections still possible in slit amorphs? Ultrastructural examination of the midline of the CNS reveals that contralaterally projecting axons persist in slit mutant embryos despite midline fusion of longitudinal tracts. A cross section of a wild-type nerve cord at the posterior limit of the anterior commissure reveals bundles of commissural axons ensheathed by processes of the MG. Longitudinal axon tracts are located within the developing neuropil of each neuromere (Fig. 2A). In contrast, at an equivalent position in the nerve cord of a slit mutant, the longitudinal tracts are fused at the dorsal midline of the CNS in a single bundle. Underneath the longitudinal tract, fascicles of contralaterally projecting axons are prominent (Fig. 2B). MG are displaced to the ventral limit of the neuropil and still maintain contact with commissural axons. In locations anterior and posterior to the commissural axons, the neuropil of slit mutant nerve cords contains many more growth cone filopodia, and fewer axons than wild type (data not shown).

Longitudinal tract axons in slit2 mutants appear to re-cross the midline as they project anteriorly or posteriorly (Fig. 1I) in a manner comparable to robo mutants (Fig. 5A). The ventral displacement of midline cells clearly contributes to the midline fusion phenotype. The position of midline cells of...
mesectodermal origin can be followed by assessing the expression pattern of Single-minded, a transcription factor required for determination of the *Drosophila* mesectoderm. Mesectodermal cells (MECs) demonstrate stereotyped positioning in the midline of the wild-type nervous system (Fig. 3A). In contrast, most MECs line the ventral midline in *slit* amorphs (Fig. 3B). *Drosophila* mutant for hypomorphic alleles of *slit*, such as *slit*532, have a nearly normal pattern of MEC distribution (Fig. 3C). Nevertheless, axon trajectories in the CNS continue to show misdirection towards the midline. The commissural tracts of *slit* hypomorphs are thickened, and show poor definition of anterior and posterior commissures (Fig. 1B). The thinning of the longitudinal tracts is comparable to that seen in a *slit* amorph. During stage 12/0, pioneers of the longitudinal tracts show misdirection towards the midline. The molecular and genetic structure of *slit* has been previously described (Rothenberg et al., 1988, 1990). We have generated a complete cDNA from clones isolated in this earlier study, and generated transgenic *Drosophila* carrying P[UAS-slit]. To verify that this construct generates functional Slit protein, we then employed the UAS-GAL4 expression system to direct expression of *slit* in midline cells with P[slit1.0-GAL4]. Midline expression of P[UAS-slit] in embryos mutant for *slit*532 generates a partial rescue of the amorphic phenotype, to one resembling a *slit* or *robo* hypomorph. Midline structures, including commissural tracts, have been partially restored, however, errant Fasciclin II-expressing axons continue to cross the midline (Fig. 4A, arrow in D). The same level of midline expression of the *slit* transgene with P[UAS-sim] in *slit*2 mutant

pattern in *slit* mutants after labeling dissected embryos with fluorescently tagged phalloidin, which binds F-actin. In wild type, the most medial muscles, the ventral oblique, avoid the ventral midline and insert underneath the lateral edge of the nerve cord. In embryos mutant for *slit*, the ventral oblique muscles do not insert below the cord. Instead, they cross the dorsal surface of the cord to insert contralaterally with the ventral longitudinal muscles (Fig. 1J,K). Fewer muscles cross the dorsal surface of the cord in embryos mutant for hypomorphic alleles of *slit* (data not shown). These data suggest that the ventral oblique muscles may also respond to a midline repulsive signal missing in *slit* mutants.

**Does slit act as a repulsive signal?**

If Slit provides a signal that repels the growth cones of neurons and muscles, then ectopic expression of *slit* during development should misdirect their growth. The molecular and genetic structure of *slit* has been previously described (Rothenberg et al., 1988, 1990). We have generated a complete cDNA from clones isolated in this earlier study, and generated transgenic *Drosophila* carrying P[UAS-slit]. To verify that this construct generates functional Slit protein, we then employed the UAS-GAL4 expression system to direct expression of *slit* in midline cells with P[sim1-GAL4] and P[slit1.0-GAL4]. Midline expression of P[UAS-slit] in embryos mutant for *slit*532 generates a partial rescue of the amorphic phenotype, to one resembling a *slit* or *robo* hypomorph. Midline structures, including commissural tracts, have been partially restored, however, errant Fasciclin II-expressing axons continue to cross the midline (Fig. 4A, arrow in D). The same level of midline expression of the *slit* transgene with P[UAS-sim] in *slit*2 mutant

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**Fig. 1.** Axon tracts and ventral muscle are medially misplaced in embryos lacking *slit* function. Wild type (A,D,G), *slit*2 (C,F,I) and *slit*532 (B,E,H) nerve cords are labeled with BP102 (A–C) and anti-Fasciclin II (D–I) antibodies. Wild-type embryos have a ladder-like scaffold of axon tracts in the ventral nerve cord (A). Arrows in A, B and C indicate the separation between anterior and posterior commissures; arrowheads identify the intersegmental portion of the longitudinal tract. Embryos homozygous for a null allele of *slit*, *slit*2, show a complete fusion of axon tracts at the midline (C). Embryos homozygous for a hypomorphic allele of *slit*, *slit*532 (B), have a medial narrowing of the axon tract scaffold, and poor definition of the anterior and posterior commissures of each segment (arrow). In late embryogenesis, three well defined longitudinal fascicles expressing FasII are seen in wild type (G). Fusion of FasII-expressing axons into a single medial fascicle is evident in a *slit* amorph (I). In a *slit* hypomorph, Fas II-expressing fascicles are medially displaced, and may cross over the midline (H). The major longitudinal tracts are established during stage 12/0, when Fasciclin II labeling identifies the anterior projection of pCC (arrowhead in D,E,F). Both axons and neuronal somas (MPs identified by arrow) are medially displaced by stage 12 in *slit* amorphs (F). Early medial displacement is significantly less in a *slit* hypomorph (E), however, ectopic midline crossing of Fasciclin II-labeled axons are evident (arrow). Muscles and axon tracts may also be visualised with fluorescently tagged phalloidin, which binds f-actin (J,K). In a wild-type dissected embryo (J), the ventral oblique muscles insert in the ectoderm underneath the lateral edge of the nerve cord (arrowhead). In a dissected embryo homozygous for *slit*2 (K), the ventral oblique muscles cross over the dorsal surface of the cord (arrowhead), to insert together with the ventral longitudinal muscles. Anterior is at top in all panels.
embryos was not sufficient to restore MEC cytoarchitecture, in spite of significant restoration of longitudinal tract structure (Fig. 3D). This further indicates a role for slit in axon guidance independent of its requirement to establish MEC cytoarchitecture.

If P[UAS-slit] expression in the midline is superimposed upon wild-type expression, fewer than normal numbers of axons cross the commissural tracts (Fig. 4C). Midline over-expression also results in thicker longitudinal tracts within segments, and a thinning of intersegmental connections. A more restricted subset of midline and longitudinal fascicles can be visualised with an antibody to Connectin, which labels the SP1 projection. In wild type, the SP1 neuron migrates medially to within one cell diameter of the midline as its axon extends across the midline to contact its contralateral partner, and then turns to extend anteriorly (Fig. 4G). When midline cells produce greater than normal levels of Slit, some commissural axons take circuitous routes across the midline, and SP1 cell position is more irregular, suggesting that medial movement of both growth cones and a neuron soma are affected by levels of Slit protein (Fig. 4H). Midline over-expression with a more effective midline enhancer (P[slit1.0-GAL4], Fig. 4B,E), leads to defasciculation of commissural tracts, some missing commissures and ectopic lateral axon extension.

To further assess the repulsive property of Slit during axon guidance, we directed ectopic P[UAS-slit] expression in embryos. Transgene expression with P[engrailed-GAL4] is restricted to the posterior compartment of the segment (just anterior to the anterior commissure). slit expression here results in breaks in longitudinal tracts, including the MP fascicle (arrow, Fig. 4F) and the later forming SP1 fascicle (arrow, Fig. 4I). Furthermore, redirected Fasciclin II-labeled axons now inappropriately cross the midline (arrowhead, Fig. 4F). Broad expression in the neuroectoderm with P[scabrous-GAL4] decreased the number of axons traversing the segment boundary in the longitudinal tracts, thinning and delaying development of Fasciclin II-expressing fascicles (data not shown).

**Slit function interacts with robo**

The axon tract phenotype of hypomorphs of slit, and partially rescued function of slit amorphs resembles that of amorphic alleles of robo, suggesting at least one component of slit function also requires robo function. If the functions of slit and robo are inter-dependent, for instance, both functioning in rate limiting steps in signaling midline repulsion, then mutant alleles of these two genes should demonstrate a phenotypic interaction (Kidd et al., 1999). Fasciclin II-labeled axon fascicles show deviation towards the midline in embryos heterozygous for either robo1 or slit2, in less than 5% of segments examined, indicating that these alleles lack a penetrant semi-dominant phenotype (Fig. 5B,C). An interaction between these genes is clearly evident when axon tract organisation in embryos heterozygous for both slit2 and robo1 are examined (Fig. 5D). Although these embryos have one functional copy of each gene, the combined effect of a reduction of expression of both slit and robo is reduced midline repulsion of Fasciclin II-expressing axons, resulting in a weak robo-like phenotype. In slit2/robo1 embryos, 74% of all...
segments examined (n=175 segments in 20 embryos) show mild to severe perturbation of FasII-expressing axon fascicle guidance near the midline. To further assess whether the genetic interaction between robo and slit reflects a rate limiting function, we examined transheterozygotes of roboI with a hypomorph of slit (slitE158), believed to express lower levels of Slit because of a transposon insertion 105 bp upstream of the transcription initiation site (Rothberg et al., 1990). We detected midline deviations or crossovers in 32% of all segments examined in roboI/slitE158 transheterozygotes (n=221 segments in 28 embryos).

DISCUSSION

Our analysis of the slit mutant phenotype and the consequences of transgene expression indicate that Slit acts to repel the growth cones of axons and ventral muscles away from the ventral midline. Reduced slit function of a slit hypomorph results in ectopic projections of longitudinal tract axons across the midline. Removal of all slit function results further in the medial fusion of longitudinal tracts, large numbers of decussating axons, ventral displacement of midline cells and mis-projection of muscles across the dorsal surface of the nervous system. Ectopic expression of slit not only inhibits axon tract formation and cell migration close to the point of secretion, but also results in the misdirection of axonogenesis to midline domains where Fasciclin II expression is normally excluded. This suggests that slit-mediated repulsion is dose dependent, and may clearly communicate a gradient of positional cues relative to the CNS midline. Differing levels of robo expression generate a comparable phenotype (Kidd et al., 1998a), suggesting, in addition to the demonstrated genetic interaction between robo and slit, that Robo and Slit proteins function in the same pathway (Kidd et al., 1999).

Slit is a large glycoprotein secreted by the MG, which can be detected at low levels on longitudinal tract axons (Rothberg et al., 1990). Robo is a transmembrane receptor expressed on CNS growth cones, at highest levels in the longitudinal tracts (Kidd et al., 1998a). It is possible that Slit in the extracellular matrix is required to make a repulsive signal available to Robo. Alternatively, Slit may represent the repulsive signal generated at the midline, which binds to Robo on growth cones and directs them to avoid the commissural tracts. Establishment of a commissural projection must therefore proceed by suppression of the repulsive signal. This may proceed by removal of Robo from growth cones, which is found at relatively low levels on commissural axons (Kidd et al., 1998a).

Vertebrate homologues for the receptor of the midline repulsive signal, Robo, have been identified, and are expressed by neurons which must cross or avoid the floorplate (Kidd et al., 1998a). Putative human and rat homologues of slit have
expressing axon fascicles in muscle cells during their insertion in the ectoderm. Second, we have shown that elevated levels of slit expression in a normal pattern, or ectopic expression in the posterior domain of each segment inhibits the establishment of axon tracts in those territories.

Slit transcript has been identified in the MG and the mature dorsal vessel, but not in muscle (Rothberg et al., 1990; R. A. B., J. R. J., unpublished). However, Slit antigenicity has been detected in muscle insertions. It is possible that some if not all of the Slit detected here is originally secreted by the MG, and is bound by a receptor at muscle insertions. The unexpected role for slit in muscle patterning recollects Michael Bate’s seminal characterisation of embryonic muscle patterning in which he observes ‘the first [muscle] precursors appear over the CNS, and it is only later by a lateral migration that they come to span the epidermis. The CNS, like the epidermis, might be capable of specifying an overlying pattern of muscle precursors’ (Bate, 1990). Our observations suggest that secretion of Slit from the midline contributes to this pattern.

The authors thank C. Smith and G. Zhao for technical assistance and G. Boulianne and A. Campos for comments on the manuscript. We are grateful for Drosophila stocks provided by C. Goodman, C. Klämbt, S. Crews and the Indiana fly stock centre. Molecular and antibody reagents were provided by S. Artavanis-Tsakonas, G. Boulianne, C. Goodman, R. White, S. Crews and G. Tear. This work was supported by the MRC of Canada.

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