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

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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 repellant 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 (Flanagan and van Vactor, 1992; 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 commisureless (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 produce the complementary phenotype, further supporting this interpretation (Kidd et al., 1998a,b, 1999).

The comm, netrin and robo genes have been cloned and characterized. Homologues for some of these genes have been identified in other model organisms, suggesting a conserved mechanism regulating midline guidance in the CNS. Noteworthy are homologues to C. elegans UNC-6, vertebrate Netrin-1 and its fly homologue, Netrin-A, expressed by the floorplate and midline glia (MG) respectively (Kennedy et al., 1994; Mitchell et al., 1996; Serafini et al., 1994). Netrins are secreted proteins with laminin-like motifs that can attract DCC- or Frazzled-expressing growth cones (Chan et al., 1996; Keino-Masu et al., 1996; Koloedziej et al., 1996). Homologues of C. elegans sax-3, the vertebrate and Drosophila robo genes, encode a transmembrane receptor with multiple immunoglobulin and fibronectin repeats, and is also expressed on growth cones (Kidd et al., 1998a; Zallen et al., 1998). Like Frazzled, it appears that Robo responds to a signal produced by the midline, except that Robo signaling apparently communicates a repulsive signal. Another group has recently identified Drosophila Slit as the repellent ligand for Robo (Kidd et al., 1999), and in collaboration with a second group, has shown that in Drosophila and mammals, Slit orthologs bind Robo (Brose et al., 1999).

A logical candidate for a repulsive signal for midline guidance would be a secreted molecule, produced by the MG, which, when absent, resulted in a loss of midline axon guidance. These criteria are met by slit, a gene encoding a large glycoprotein secreted by the MG, which can be detected on the surface of the MG as well as upon the axon tracts of the CNS. The 200 kDa Slit protein is composed of leucine rich repeats, EGF repeats and a domain similar to the G domain of laminin and agrin (Rothberg et al., 1988, 1990). A loss of slit function results in medial fusion of longitudinal and commissural axon...
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 slitP1017), 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 Axioshot 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-labeled 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 μm 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’TCT CAG AGC TCT GCC ACA ATG GCC 3’ and 5’CCC TTG AAG AGC CGT CTA CCC ACC AGC 3’) and N-terminal PCR fragment (primers 5’TTC TTA CTA GTT CCG CAC TTG CGC 3’ and 5’AGC AGC AGC CCG GCC GCC GCT ACT 3’) to an MluI- and Sfil-digested cDNA clone (Rotheberg et al., 1990) which was then inserted into the KpnI and XbaI sites of pUAST (Brand and Perrimon, 1993) with 3 bp of both 3’ and 5’ slit UTR. Transformant lines were generated and mapped by standard procedures. P[sim-GAL4], slit2/P[sim-GAL4], slit2;P[UAS-slit]/P[UAS-slit] was generated to rescue the slit2 phenotype. Over-expression was produced by crosses generating P[sim-GAL4]/P[sim-GAL4]; P[UAS-slit/P[UAS-slit] and similarly using P[slit1.0-GAL4], P[scab-GAL4] and P[eng-GAL4] lines. Embryos were assayed by staining with mAbs BP102 and 1D4. Transcription of transgenic slit was monitored with an antisense mRNA in situ probe generated from a 600 bp region at the N-terminal of slit cDNA, and synthesis of protein was confirmed with Slit antibody (provided by D. Hartley).

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 (Rotheberg 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,I). During stage 12/0, Fasciclin II immunolabeling identifies pioneers of the longitudinal tracts, the pCC, vMP2 and MP1 axons (Grenningloh et al., 1991; Jacobs and Goodman, 1989; Fig. 1D). MP pioneers are medially 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 dorsolateral 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...
slit is required for midline axon guidance

The ventral muscle formed by muscle cells extending across the dorsal surface of the cord. We have further characterised the ventral muscle by characterising the ventral longitudinal tracts show misdirection and crossing of the midline (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 poor definition of anterior and posterior commissures; arrowheads identify the intersegmental portion of the longitudinal tract. Embryos homozygous for a null allele of slit, show a complete fusion of axon tracts at the midline (C). Embryos homozygous for a hypomorphic allele of slit, 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 FasII 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 FasII-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 slit2 (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.

**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[slit 2], has a nearly normal pattern of 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 slit532, 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 and crossing of the midline, resulting at later stages in a phenotype reminiscent of robo amorphs (Fig. 1E,H). This suggests that disorders in midline axon guidance in slit mutants cannot be solely attributed to changes in MEC position.

We have also noted that the nerve cord of slit mutants is often kinked. Upon closer examination, kinks appear to be formed by muscle cells extending across the dorsal surface of the cord. We have further characterised the ventral muscle 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.
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 that 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 \textit{robo} and \textit{slit} reflects a rate limiting function, we examined transheterozygotes of \textit{robo} \(^1\) with a hypomorph of \textit{slit} \((\textit{slit}^{E158})\), 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 \textit{robo} \(^1\)/\textit{slit} \(^{E158}\) transheterozygotes \((n=221\) segments in 28 embryos).

**DISCUSSION**

Our analysis of the \textit{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 \textit{slit} function of a \textit{slit} hypomorph results in ectopic projections of longitudinal tract axons across the midline. Removal of all \textit{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 \textit{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 \textit{slit}-mediated repulsion is dose dependent, and may clearly communicate a gradient of positional cues relative to the CNS midline. Differing levels of \textit{robo} expression generate a comparable phenotype (Kidd et al., 1998a), suggesting, in addition to the demonstrated genetic interaction between \textit{robo} and \textit{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 \textit{slit} have
expressing axon fascicles in robo1 homozygotes identifies axons crossing and re-crossing the midline (arrowhead). Embryos heterozygous for robo1 (B) and sli2 (C) are similar in appearance to wild type, indicating no semi-dominant effect of these alleles. (D) Embryos doubly heterozygous for robo and sli (robo1/sli2) demonstrate midline crossing and disruption of FasII-expressing longitudinal axon fascicles (arrowhead).

Fig. 5. slit interacts genetically with robo. (A) Labeling of FasII-expressing axon fascicles in robo1 homozygotes identifies axons crossing and re-crossing the midline (arrowhead). Embryos heterozygous for robo1 (B) and sli2 (C) are similar in appearance to wild type, indicating no semi-dominant effect of these alleles. (D) Embryos doubly heterozygous for robo and sli (robo1/sli2) demonstrate midline crossing and disruption of FasII-expressing peripheral axon fascicles (arrowhead).

also been described (Holmes et al., 1998; Itoh et al., 1998), suggesting that the mechanism of midline guidance has been conserved.

If Slit and Robo function in the same pathway, why does a null mutation in slit generate a more severe phenotype? Two possible reasons are evident. First, it is possible that repulsion mediated by Slit is transduced through a Robo-independent pathway. The karussell mutation, for instance, which generates a similar phenotype may function in a Robo-independent repulsion from the midline (Hummel et al., 1999).

Secondly, the similarity of the slit hypomorph (slit532) phenotype to that of robo1 suggests that Slit may have a dual function: a role in repulsion from the midline, attenuated in slit532 mutants, and a function in establishing midline cytoarchitecture, partly intact in slit532 mutants. We have previously documented the survival, and ventral displacement of all midline lineages in slit mutants, implicating the collapse of midline cytoarchitecture as a cause of the midline axon tract fusion phenotype (Sonnenfeld and Jacobs, 1994). The ventral displacement of midline cells precedes axonogenesis, indicating that cell position is not a secondary effect of midline axon tract fusion (R. A. B., J. R. J., unpublished). Levels of slit transgene expression sufficient to rescue axon guidance result in partial restoration of midline cytoarchitecture, suggesting higher levels or a different pattern of Slit is required for establishing MEC cytoarchitecture. Embryos mutant for slit532 have a nearly wild-type midline cytoarchitecture, sufficient to prevent midline fusion of axon tracts. However, mis-projection of Fasciclin II-expressing axons across the midline still persists in many of these embryos, further suggesting independence of the midline fusion and axon guidance phenotypes. Molecular characterisation of this mutant allele may reveal which structural domains of Slit contribute to axon guidance.

We have demonstrated a function for slit in repressing growth cone extension in two fashions that are independent of cytoarchitectural changes at the midline. First, we demonstrate that slit is required to repel medial extension of migrating 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.

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