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Heparan Sulfate Proteoglycan Specificity During Axon Pathway Formation in the *Drosophila* Embryo

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Abstract

Axon guidance is influenced by the presence of heparan sulfate (HS) proteoglycans (HSPGs) on the surface of axons and growth cones (1–5). Multiple HSPGs, including Syndecans, Glypicans and Perlecan, carry the same carbohydrate polymer backbones, raising the question of how these molecules display functional specificity during nervous system development. Here we use the *Drosophila* central nervous system (CNS) as a model to compare the impact of eliminating Syndecan (Sdc) and/or the Glypican Dally-like (Dlp). We show that Dlp and Sdc share a role in promoting accurate patterns of axon fasciculation in the lateral longitudinal neuropil; however, unlike mutations in *sdc*, which disrupt the ability of the secreted repellent Slit to prevent inappropriate passage of axons across the midline, mutations in *dlp* show neither midline defects nor genetic interactions with Slit and its Roundabout (Robo) receptors at the midline. Dlp mutants do show genetic interactions with Slit and Robo in lateral fascicle formation. In addition, simultaneous loss of Dlp and Sdc demonstrates an important role for Dlp in midline repulsion, reminiscent of the functional overlap between Robo receptors. A comparison of HSPG distribution reveals a pattern that leaves midline proximal axons with relatively little Dlp. Finally, the loss of Dlp alters Slit distribution distal but not proximal to the midline, suggesting that distinct yet overlapping pattern of HSPG expression provides a spatial system that regulates axon guidance decisions.

Keywords

heparan sulfate; proteoglycans; axon guidance; Slit; Robo

INTRODUCTION

For a neuron to reach its proper synaptic target, it must navigate unerringly through a complex extracellular environment, often over long distances. The CNS midline has emerged as a premier model system for studies of axon guidance and the logic with which long and short-range extracellular cues pattern the formation of a complex neuropil (Reviewed in: 6). One of the key forces that shapes axon pathways at the midline is the highly conserved, secreted growth cone repellent Slit. In *Drosophila*, where it was first

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discovered, Slit acts both to regulate the passage of axons across a midline barrier composed of specialized glia that express the repellent, and to determine the lateral positioning of axons in the longitudinal neuropil on either side of the repellent source (Reviewed in: 7). The axonal response to *Drosophila* Slit is mediated by three Roundabout (Robo) family receptors, which are distributed in an overlapping medial to lateral “Robo code” that defines three distinct axonal populations with increasing sensitivity to a presumed gradient of Slit emanating from the midline glia (8, 9).

Recent studies have begun to more carefully examine the mechanisms of Slit/Robo interactions during embryonic CNS development in *Drosophila*. Using gene targeting to express Robo1, 2 and 3 from each others' endogenous genomic loci, repulsion from the CNS midline was found to require specific sequences unique to the Robo1 core protein, whereas lateral positioning of longitudinal axons was found to be dependent on the levels of expression of the three robo genes (10). Other studies have demonstrated that the efficient perception of Slit by Robo-expressing axons requires the carbohydrate polymer heparan sulfate (HS) (1, 4, 5). The amino acids required for heparin binding on both Slit and Robo have been identified (11, 12), and are highly conserved across metazoans, suggesting an evolutionarily conserved role for HS in mediating Slit/Robo interactions.

In vivo, HS chains are carried by several classes of proteoglycans (HSPGs), including Syndecans, Glypicans and Perlecan (Reviewed in: 13, 14). Loss of *Drosophila* Syndecan (Sdc) results in reduced efficiency of the Slit/Robo midline repellent system, as indicated by inappropriate midline crossing of axons and potent genetic interactions with Slit and its receptors (4, 5). This loss of efficient repellent activity in *sdc* mutants is likely due to the dramatic shift observed in Slit distribution as it moves away from its midline source, presumably dependent on the observed binding of Slit to Sdc (4). Vertebrate midline axon guidance is also dependent on HS interactions with Slit, although the relevant HSPGs have not been identified (2). Several observations suggest that axonal HSPGs act to shape the guidance factor landscape distant from the source of diffusible cues; however, the relative specificity of different HSPGs for distinct aspects of axon guidance in vivo is unknown. In addition, despite carrying highly similar heparan sulfate sidechains, the phenotypic analysis of HSPG mutants shows that HSPGs are not functionally redundant, but can have distinct (15, 16) and occasionally opposing (4) phenotypes during development. Given the known binding of Glypicans to Slit (17, 18), the finding that the *Drosophila* Glypican Dally-like (Dlp) can partially substitute for Sdc during midline axon guidance (4) suggests that HSPG specificity relies primarily on differential patterns of expression or localization.

Of course, the development of an accurate model of how HSPGs work with Slit and the Robo family of receptors to facilitate the proper development of the embryonic axon scaffold depends on the analysis of *dlp* mutations and Dlp distribution in the developing CNS. Here we present this analysis, and provide evidence that Dlp and Sdc have distinct functions during CNS development in *Drosophila*; with Dlp playing an important role in the formation of the outer longitudinal fascicles, and Sdc regulating axon guidance at the CNS midline. The functions of Sdc and Dlp are also overlapping, as revealed in our double mutant analysis, and support a model in which Sdc and Dlp cooperate with Slit and Robo not only to facilitate proper guidance at the midline but also to promote the proper formation of the lateral fascicles.

METHODS

Genetic Stocks

*Dlp*¹ is a 504 bp deletion that extends two bp beyond the ATG of the presumed *dlp* initiation codon. *Dlp*² is an EMS allele that has not been sequenced, but lacks all detectable Dlp

protein (16). *Df48*, *ubi-Sara* and *sdc*¹⁰⁶⁰⁸ were described previously (4). *slit*² and *robo*^{GA285} were obtained from the Bloomington Stock Center. *Robo*^{GA285}, *robo3*¹ and *robo*^{GA285}, *robo2*⁸ double mutants were obtained from B. Dickson. All mutations on the second chromosome were balanced over CyO-[actin-lacZ] or CyO-[wg-lacZ], and all mutations on the third chromosome were balanced over Tm6B-[ubx-lacZ].

Immunohistochemistry

Staining was conducted using the following antibodies: Anti-Sdc (1:250) (4), anti-Dlar (1:250) (19), anti-Wrapper (1:5) (20), anti-Slit (1:5) (21), anti-Robo 13C9 (1:5) (22), anti-Engrailed (1:2) (23), anti-Dlp 13G8 (1:4) (24) and anti-Robo3 14C9 (1:4) (9). Anti-Sdc, anti-Dlp, anti-Robo3, anti-Wrapper and anti-Slit immunostaining were conducted on stage 14–16 live-dissected embryos as described for Sdc previously (4). 1D4 immunohistochemistry was performed as previously described using a 1:5 dilution of the antibody (25). After extensive washing in PBS + 0.1% Triton (PBT), embryos were incubated for 1–2 hours in 1:500 dilutions of goat-anti-mouse Alexa488 and goat-anti-rabbit Alexa568 (both from Molecular Probes) or of peroxidase conjugated goat-anti-mouse and goat-anti-rabbit (both from Jackson Immunoresearch). Following fluorescent immunohistochemistry, embryos were washed in PBS and mounted in SlowFade (Molecular Probes). Embryos stained with peroxidase-conjugated antibodies were washed in PBT, reacted with a DAB peroxidase substrate kit (Vector Laboratories) and mounted in 70% glycerol. For quantitative analysis of midline guidance and fasciculation defects, stage 17 FasII-stained embryos were dissected along the dorsal midline, the gut was removed, and the ventral nerve cord was imaged on a Nikon E800 microscope. All embryonic mutant phenotypes were scored blind to genotype.

Statistical Analysis of Genetic Interactions

Student's t-tests were performed in order to determine if a particular population of embryos had a significant change in the frequency of defects. Statistical analyses were conducted either on the frequency of midline guidance defects, lateral fascicle breaks, lateral fascicle thinnings, or on the sum of the latter two categories – termed “lateral fascicle defects.” In all figures, * = $p < 0.05$, ** = $p < .01$ and *** = $p < .001$.

Confocal Microscopy

3-dimensional analysis of antibody staining was conducted using a Nikon E800 microscope and a Bio-Rad Radiance confocal or a Nikon-C1-SI confocal. Optical sections of 0.35 microns were taken across the depth of the filleted embryo and were reassembled using Volocity software from Improvision. Prior to image collection, laser power and gain were decreased in order to prevent saturation of fluorescent signal intensity. All antibodies were pre-mixed to minimize subtle changes in antibody concentration between samples. Identical laserpower, gain, and iris settings were used for the collection of data from all compared embryos. To quantify the levels of Slit and Dlar protein, the distribution of Sdc and Dlp protein, or the position of Robo3-positive fascicles, 100 micron long lines were drawn perpendicular to the CNS midline of an image of a z-Projection as previously described (4). Non-normalized data were plotted according to pixel number and pixel intensity and Student's t-tests were conducted to compare pixel intensity.

RESULTS

We previously showed that Dlp protein accumulates on axons in the embryonic *Drosophila* CNS (4). To establish the specificity of this localization pattern, we stained *dlp* alleles reported to be null for the locus (15, 26). We found no detectable Dlp signal in *dlp*¹ embryonic fillets (Figure 1D; see Experimental Procedures). Sdc localization was unaltered

in this genetic background, showing that there is no compensatory change in HSPG expression (Figure 1E, F). The alleles *dlp*² and *dlp*^{A187} also lack detectable Dlp immunoreactivity (data not shown). Similarly, the *sdc* allele *Df48*, *ubi-Sara* lacks detectable Sdc expression (Figure 1H), and there is no detectable compensatory change in Dlp expression (Figure 1G). Since Dlp has been shown to facilitate the morphogen signaling that establishes appropriate patterns of cell fate in some contexts (e.g. 15, 26, 27), we carefully examined *dlp* alleles for alterations in the fate of midline glia and CNS neurons. Using the midline glial marker Wrapper, and the neural cell fate marker Engrailed, we found no abnormalities in the numbers, positions, or morphology of midline glia or in the numbers or positions of neurons expressing Engrailed (data not shown). While previous studies have found severe developmental defects in Dlp germline clones (28), it appears that maternally supplied *dlp* gene product masks a role for Dlp in zygotic CNS cell fate determination and we have confirmed that zygotic nulls lack any significant cell fate defects that might confound our analysis of axon guidance behavior.

We next examined the pattern of CNS axon pathfinding using the anti-FasciclinII (FasII) antibody (mAb1D4) that conveniently labels ipsilateral fascicles of longitudinal axons on either side of the CNS midline (see Experimental Procedures). In wild type stage 17 embryos, three major parallel FasII-positive axon fascicles extend along the dorsal aspect of the CNS anterior-posterior axis (Figure 1J). In *dlp* mutant embryos, we found breaks in the lateral FasII-positive fascicle and thinnings in which the lateral axon fascicle appears to have fewer axons (Figure 1L). Interestingly, these axon fasciculation phenotypes preferentially affected the axons most distant from the source of Slit. Quantitative analysis showed that such defects were rarely observed in wild type embryos (Figure 1P). A comparison of *dlp* and *sdc* phenotypes (Figure 1K and L) revealed similar axonal fasciculation errors in both HSPG mutants, but at a significantly lower frequency in *sdc* mutant alleles ($p < .001$; Figure 1P). To determine whether Sdc and Dlp were functionally redundant in outer fascicle formation, we attempted to rescue the Dlp mutant phenotype with neural expression of either a Sdc (Figure 1M) or a Dlp transgene (Figure 1N). We found significant rescue following neural expression of a Dlp transgene ($p < .001$) but not a Sdc transgene (Figure 1P; $p > 0.1$). Very weak rescue was also found for a Dlp transgene in the absence of a driver (Figure 1O; $p < .05$) consistent with previous observation that the UAS-Dlp transgene used in this study expresses low levels of Dlp independent of a driver (16).

We examined multiple *dlp* alleles for any evidence of the ectopic midline crossing characteristic of *sdc* and *robo* mutants (e.g. Figure 1K), however we found no significant defects in the restriction of midline axon crossing (Figure 1L). While *sdc* mutant alleles display potent dose-sensitive genetic interactions with mutations in *slit* and all three *robo* genes (*robo*, *robo2* and *robo3*) (4, 5), we found no evidence of transheterozygous genetic interaction between *dlp* and *slit* or the *robos* in regulating midline guidance (Figure 2A). Using the same mutant genotypes, we also examined if the pattern of lateral axon fasciculation in *dlp* or *sdc* heterozygotes showed dose-sensitivity to *slit* or the *robos*. Although no genetic interactions were observed between *sdc* and either *slit* or the *robos*, weak but significant genetic interactions were observed between *dlp* and *slit* (Figure 2G) in which the transheterozygote displayed more frequent lateral fascicle defects than either heterozygote alone ($p < .005$; Figure 2B). A similar genetic interaction was found between *dlp* and *sdc* (Figure 2E; $p < .05$). In addition, a significant genetic interaction was found between *dlp* and *robo2,1* in terms of lateral fascicle breaks ($p < .01$) but not in overall lateral fascicle defects (Figure 2J).

While our comparison of *dlp* and *sdc* single mutants suggests some form of functional specificity, we realized that a degree of functional overlap between the two HSPGs might be masked by the presence of the other in a single mutant background. For example, loss of

Robo2 alone shows only very mild effects on the fidelity of midline crossing compared to loss of Robo; however, simultaneous loss of both Robos results in a dramatic collapse of axon pathways comparable to the loss of Slit (9, 29). Thus, we generated and analyzed a *sdg;dlp* double loss-of-function genotype. The double HSPG mutant showed an axonal phenotype far more severe than either single mutant (Figure 3B–D). Among the double mutant embryos, we found three classes of axonal defect: (1) a *robo*-like phenotype far more severe than *sdg* alone [Figure 3B and C; 60%. n = 320 embryonic segments], (2) an inward axon pathway collapse approaching the severity of mutations in *slit* [Figure 3D; 33%. n = 320], and (3) a less frequent class where axons appear unable to extend from segment to segment [7%. n = 320]. These phenotypes exceed the expressivity found in either single HSPG mutant, and were never observed in wild type controls [0%, n = 380, for all three classes of defect]. Compared to the *dlp* or *sdg* single mutants, the *robo*-like class of double mutant embryos had a significant increase in the frequency of ectopic midline crossing (Figure 3E; $p < .001$).

The severe and highly penetrant midline guidance defects seen in the *sdg; dlp* double mutant embryos could be caused either by an essential role for HSPGs in mediating Slit/Robo signaling, or by dramatic reductions in either Slit or Robo expression in this mutant background. To discriminate between these possibilities, we examined *dlp*, *sdg* and *sdg; dlp* double mutant embryos for Slit, Robo and Robo3 protein expression (Figure 3F–Q). *Dlp* mutants show no defects in the expression or distribution of Slit, Robo or Robo3, consistent with the lack of midline guidance defects in this mutant (Figure 3I–K). *Sdg* mutants lack Slit localization on longitudinal fascicles despite normal levels of expression in midline glia (Figure 3L), as described previously (4). In *sdg* mutants, Robo- and Robo3-positive fascicles cross the CNS midline consistent with our previous study (4), despite the expression of both Slit and both Robos at similar levels to wildtype embryos (Figure 3M & N). Similar to the *sdg* mutant, *sdg; dlp* double mutants lack Slit localization on longitudinal fascicles, but retain normal levels of Slit expression on midline glia (Figure 3O). Robo- and Robo3-positive fascicles are also seen crossing the midline in this mutant (Figure 3P & Q), indicating that the midline guidance defects seen in *sdg; dlp* double mutants are not caused by defects in Slit or Robo expression. These data indicate that the defects seen in *sdg; dlp* double mutants are not the result of dramatic changes in Slit or Robo expression, but rather suggest that in the absence of Sdg, Dlp makes an important contribution to midline repulsion.

Our analysis to this point showed that Dlp and Sdg play overlapping, yet distinct roles in patterning the CNS neuropil, somewhat analogous to the relationship between the different *Drosophila* Robo receptors. But, if the HSPGs are largely overlapping in their functional capacity, what explains the lack of midline crossing errors in *dlp* mutant embryos? One possible explanation could come from differences in the pattern of HSPG localization within the CNS neuropil. Thus, we performed a quantitative comparison of Dlp and Sdg distribution across the medio-lateral axis of the CNS by confocal microscopy (see Experimental Procedures). While Dlp and Sdg overlapped across much of the neuropil, two differences were seen: (1) the peak of axonal Sdg was more medial than that of Dlp, and (2) Dlp levels drop significantly in the axons most medial to the midline (Figure 4A & B). The resulting composite pattern leaves relatively little Dlp on the medial axons and higher levels of Dlp on the most lateral axons. In fact, when we calculated the ratio of Dlp signal to Sdg signal across the medial to lateral neuropil axis, we see a significant gradation in relative signal (Figure 4C). Thus, we find a spatial pattern of axonal HSPGs, albeit more subtle than the Robo code.

While the spatial pattern of HSPG localization could explain the functional specialization of the two HSPGs in midline repulsion, we were concerned that the differential between Dlp

and Sdc, even proximal to the midline, might be too subtle to account for a real difference in the effective concentration of Slit. Since we have shown that loss of Sdc results in a dramatic change in Slit distribution along the medio-lateral axis of the CNS (distant from the source of Slit) (4), we decided to measure the pattern of Slit protein in *dlp* mutants. In contrast to Sdc, loss of Dlp had very little effect on Slit distribution. Proximal to the midline, we found no significant difference in Slit levels between *dlp* and wild type controls (Figure 4D). The only region of the neuropil that appeared sensitive to Dlp for Slit localization is the most lateral neuropil. Notably, this was the very region where we observed most of the axonal defects in *dlp* mutants, and the region in which the ratio of Dlp to Sdc is highest.

DISCUSSION

In this study we provide evidence that Dlp and Sdc have both overlapping and distinct functions in axon guidance and in defining accurate patterns of axonal fasciculation within the lateral CNS neuropil. Dlp regulates the proper formation of the outermost longitudinal fascicle, localizes Slit to this outer fascicle, and provides cell surface HS that, in the absence of Sdc, can facilitate Slit/Robo mediated repulsion from the midline. Sdc, as previously described, cooperates with Slit and Robo to regulate axon guidance at the CNS midline (4, 5), localizes Slit protein to the longitudinal fascicles (4), and regulates the formation of the longitudinal fascicles. The phenotypic differences between Sdc and Dlp, combined with the observation that Sdc and Dlp are unable to completely rescue one another's mutant phenotypes, suggests a degree of functional specialization between Dlp and Sdc. Sdc function is required for axon guidance decisions especially for axons located most proximal to the midline, whereas Dlp function is required for fascicle formation at a distance from the midline. Thus, HSPG expression is necessary to insure high fidelity responses to the presentation of axon guidance information.

Together, our observations suggest a model where Dlp and Sdc work together to shape a long-range repellent signal with multiple roles in axonal patterning. The difference in Dlp and Sdc levels on the most medial axons defines a medial zone most sensitive to the loss of Sdc alone (Zone 1; Figure 4E). The loss of Slit protein and repellent activity in the most lateral axons observed in *dlp* mutants defines a lateral zone where Dlp is essential for accurate axon guidance (Zone 3; Figure 4E). In between these two extremes, the two HSPGs seem more equivalent and functionally redundant (Zone 2; Figure 4E). Of course, Sdc and Dlp cannot be completely equivalent in Zone 2, since loss of Sdc but not Dlp dramatically alters Slit levels in this region. This may mean that Sdc and Dlp bind to Slit with different affinities/kinetics. This could reflect differences in HS polymer modifications or differential contributions from the two core proteins. Alternatively, Sdc and Dlp may show binding specificity for other co-factors in addition to Slit, as suggested by recent work in cell culture (30). While the pattern of HSPGs that we see in the *Drosophila* CNS is not as distinctive as the spatial code of Robo receptors, this may be better suited as a system whose function is to sculpt the extracellular movement of a graded signal.

The fact that not all *sdc;dlp* double null mutants display a phenotype approaching the severity of a *slit* mutant, suggests that HSPGs may not be essential for Slit to elicit some type of response from growth cones. This is consistent with in vitro experiments indicating that HS improves the affinity of Slit-Robo interactions more than 10-fold, but is not absolutely required for Slit-Robo binding (12). However, the observation that growth cone repulsion by Slit is absolutely dependent on cell surface expression of HSPGs (1) suggests one of two possibilities; first, that there is another HSPG expressed in the *Drosophila* CNS that facilitates very limited Slit/Robo interactions in the absence of Sdc and Dlp, or second, that maternal contribution of Sdc and/or Dlp supplies a limited amount of these HSPGs, allowing a small amount of repulsion to occur from the midline. Although Dlp has

substantial maternal contribution, the severe embryonic patterning defects observed in germline clones of Dlp (28) limits our ability to address this question experimentally.

Our studies highlight the fact that guidance factor secretion alone is not sufficient to generate the stable pattern of cues needed for robust and accurate axon patterning within the CNS. Cell surface HSPGs are essential not only for proper guidance at the CNS midline, but also for the proper formation of longitudinal fascicles and the appropriate distribution of Slit at a distance from the midline. Although we do not know why multiple, overlapping HSPGs are required to pattern axon guidance information within the *Drosophila* CNS, it is tempting to speculate that additions or modifications to the pattern of HSPG expression would provide a convenient means to alter CNS architecture over evolutionary time by adding complexity to the long range distribution of guidance information without disrupting the pattern of cells that secrete these cues.

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Figure 1. Dally-like mutants have defects in lateral fascicle formation

A–C: A wild type stage 16 embryo fillet stained with antibodies directed against Dlp (A) and Sdc (B) shows a ladder-like CNS neuropil. Along the anterior-posterior axis, two thick longitudinal bundles of axons run on either side of the midline and stain for both Dlp and Sdc. In each segment, two commissural bundles of axons cross the midline, and two bundles of axons exit the CNS on either side of the neuropil. These commissural fascicles, and those exiting the neuropil also stain for both Dlp and Sdc, which show a largely overlapping pattern of expression (C). D–F: A *dlp* mutant embryo (*dlp¹* shown) stained and imaged as in A–C shows no detectable Dlp staining (D), but a Sdc expression comparable to wildtype (E). G–I: A *sdc* mutant embryo (*Df48,ubi-sara* shown) stained and imaged as in A–C shows Dlp expression comparable to wildtype (G), but no detectable Sdc staining (H). J: A wildtype stage 17 ventral nerve cord is shown stained with anti-FasII (mAb 1D4) revealing the three dorsal axon fascicles on either side of the midline, from medial to intermediate to lateral. K: In *sdc* mutants (*Df48,ubi-sara* shown), we find fasciculation defects such as abnormal thinnings of the most lateral fascicle (arrowheads) plus ectopic midline crossing events (asterisks). L: In *dlp* mutant embryos (*dlp¹* shown), we find frequent gaps in the most lateral axon fascicle (arrows) and abnormal thinnings of this fascicle (arrowheads), but these mutants lack the frequent midline crossing defects found in *sdc* mutants. M–O: Neural expression of a Sdc transgene cannot rescue the Dlp mutant phenotype (M), but neural expression of Dlp (N) or a Dlp transgene without a driver (O) can rescue this phenotype. P: Quantification of the fasciculation defects indicates a significant increase in the frequency of lateral fascicle defects observed between wildtype embryos and either Sdc (*Df48,ubi-sara*) homozygotes ($p < .01$) or Dlp (*Dlp¹/Dlp²*) homozygotes ($p < .001$). The Dlp mutant phenotype is not rescued with neural expression of a Sdc transgene ($p > .1$), but is almost completely rescued with neural expression of a Dlp transgene ($p < .001$). The UAS-Dlp construct alone can partially rescue the Dlp mutant phenotype ($p < .05$), consistent with previous findings that this transgene is expressed at low levels independent of a driver (16). Data shown are mean values $\pm$ standard error of the mean.

Figure 2. HSPG mutations have differential effects on CNS axon guidance and fasciculation

A: Quantification of ectopic midline crossing defects confirm that while embryos homozygous for *sdc* alleles frequently display this phenotype, neither *dlp¹*, *dlp²*, nor *dlp¹/dlp²* (shown) mutants have significant midline guidance errors ($p > .1$; $250 < n < 380$). In addition, unlike the *sdc* mutation *Df48, ubi-Sara* (4), *dlp¹* does not show significant transheterozygous genetic interactions with either *slit²* or a *robo2, robo* double mutant during midline axon guidance ($p > .1$; $300 < n < 660$). **B:** Wild type embryos (W1118) have few defects in lateral fascicle formation, but a quantitative analysis shows that both *dlp* and *sdc* mutants exhibit significantly more frequent defects in the organization of lateral longitudinal fascicles ($p < .01$). For axon fasciculation phenotypes of *sdc* mutants, no significant transheterozygous genetic interactions are observed with components of the Slit/Robo pathway ($p > .1$; $130 < n < 350$), however, *Dlp* mutants display weak but significant genetic interactions with components of the Slit/Robo repulsion system. *Sdc/+; +/Dlp* and *Slit/+; +/Dlp* transheterozygotes have more frequent lateral fascicle defects than either single mutant alone ($p < .05$ and $p < .01$ respectively). *Robo2, 1/+; +/Dlp* also had significantly more lateral fascicle breaks than either single mutant alone ($p < .05$) and a similar but insignificant trend was seen for lateral fascicle thinnings. Data shown are mean $\pm$ standard error of the mean, the blue bar highlights the background frequency of lateral fascicle thinnings seen in W1118 controls. **C–K:** Representative images show lateral fascicle thinnings (arrowheads), lateral fascicle breaks (arrows) in addition to midline guidance defects (asterisks) for each genotype not shown in Figure 1.

Figure 3. Double mutants reveal an overlap between Dlp and Sdc Function in Axon Guidance

A: A wildtype (W1118) stage 17 CNS control stained with mAb 1D4. B–D: Three examples of the most frequent axonal phenotypes observed in *sdc; dlp* double homozygous embryos (*Df48, ubi-Sara; dlp*²) are shown with mAb 1D4. Many double mutants display a *robo*-like phenotype where all segments contain ectopic midline axon crossing (arrows) and some segments contain multiple crossing fascicles (B, C). A significant percentage of double mutant embryos display a phenotype approaching the severity of *slit*, where all FasII-positive axon bundles collapse towards the midline (D). E: Quantification of the midline guidance defects in either *sdc* homozygous mutants lacking a copy of *dlp*, or in *dlp* homozygous mutants lacking a copy of *sdc* do not show significant increases in the frequency of midline guidance defects over the homozygous mutant alone; however, in the *robo*-like *sdc, dlp* double mutant class, a significant increase in the frequency of fascicles crossing the midline ($p < .001$) is revealed. F–Q: Wild type, *dlp* (*dlp*¹/*dlp*²) mutant, *sdc* (*Df48, ubi-Sara*) mutant, or *sdc; dlp* (*Df48, ubi-Sara; dlp*²) double mutant stage 16 embryos were stained for Slit, Robo and Robo3. *Dlp* mutants showed no defects in the expression or distribution of Slit, Robo or Robo3 (I–K) when compared to wild type (F–H). *Sdc* mutants lack Slit localization on longitudinal fascicles despite normal levels of expression in midline glia (L), as described previously (4). In *sdc* mutants, Robo- and Robo3-positive fascicles cross the CNS midline (M, N), despite the expression of these Robos at similar levels to wildtype embryos. Similarly, *sdc; dlp* double mutants lack Slit localization on longitudinal fascicles, but retain the expression of Slit on midline glia (O). Robo and Robo3 are expressed on longitudinal axons in *sdc; dlp* mutants at levels indistinguishable from wildtype; however, these Robo- and Robo3 positive fascicles cross the midline in this mutant (P, Q).

Figure 4. Spatial patterns of CNS HSPGs that shape Slit distribution

A: Laser-scanning confocal microscopy was used to compare the localization of Dlp (green) and Sdc (red) immunofluorescence signal intensity from line scans across the medio-lateral axis of the wild type CNS (see Experimental Procedures). For orientation, the position of midline glia is indicated by the red bar and longitudinal neuropil by the blue bars just beneath the pixel intensity profiles. These data points, and the ones shown in (C) and (D) represent the mean value (box) plus or minus the standard error of the mean (bracket). B: A dual channel confocal projection of a wild type ventral nerve cord used for the analysis in A, suggests that Sdc but not Dlp, is expressed on midline glia. In addition, Sdc expression is more concentrated on the medial aspect of the longitudinal fascicle, while Dlp localizes to more lateral parts of the fascicle. The positions of the midline glia and the longitudinal fascicles are indicated by the relative positions of the red and blue orientation marker bars. The scale bar in panel B indicates four microns. C: A ratiometric analysis of HSPG distribution within the longitudinal neuropil demonstrates a significant difference in the ratio of Dlp:Sdc in the lateral third of the fascicle when compared to the medial third of the fascicle ($p < .001$), with a significantly higher ratio of Dlp:Sdc on the lateral aspect of the fascicle. Grey bars indicate the relative positions of lateral, intermediate and medial axon populations; the midline is to the right. D: To determine the role of Dlp in shaping Slit distribution, we quantified Slit localization across the medio-lateral axis in wild type (black), *dlp* (*dlp*¹; green) and *sdc* (*Df48,ubi-sara*; red) embryo fillets, using a technique described in (4) (note that all of the data points for the *sdc* profile shown here were previously included in (4)). Slit levels are comparable in the midline glia in all three genotypes (above the large red bar). However, in contrast to the major reduction in Slit accumulation in *sdc* mutants, Slit levels on medial axons (proximal to the midline) are within the normal range in *dlp* mutants, but a subtle decrease in Slit levels is detected in the lateral neuropil in *dlp* mutants. E: A model of the *Drosophila* ventral nerve cord (VNC), shows the position of midline glia (MG) relative to the commissural axons (C, lavender) and longitudinal axons (L, light blue) of the CNS neuropil. Below, in an expanded cross-sectional schematic of one side of the

neuropil, the approximate locations of the FasII-positive axon fascicles (orange circles) within the longitudinal neuropil (L, light blue) are shown relative to the positional code of Robo-family receptors (the “Robo code” is shown in three shades of grey: Light grey = Robo alone in the medial axons, medium grey = Robo + Robo3 in the intermediate axons, and dark grey = Robo + Robo3 + Robo2). Below the neuropil, the presumed gradient of Slit and the observed distributions of the HSPGs Dlp and Sdc are summarized relative to the three HSPG zones defined by axonal defects and Slit distribution.