The extracellular matrix proteoglycan perlecan facilitates transmembrane semaphorin-mediated repulsive guidance

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The *Drosophila* transmembrane semaphorin-1a (Sema-1a) is a repulsive guidance cue that uses the Plexin A (PlexA) receptor during neural development. Sema-1a is required in axons to facilitate motor axon defasciculation at guidance choice points. We found that mutations in the *trol* gene strongly suppress Sema-1a-mediated repulsive axon guidance. *trol* encodes the phylogenetically conserved secreted heparan sulfate proteoglycan (HSPG) perlecan, a component of the extracellular matrix. Motor axon guidance defects in *perlecan* mutants resemble those observed in *Sema-1a*- and *PlexA*-null mutant embryos, and *perlecan* mutants genetically interact with *PlexA* and *Sema-1a*. Perlecan protein is found in both the CNS and the periphery, with higher expression levels in close proximity to motor axon trajectories and pathway choice points. Restoring perlecan to mutant motor neurons rescues *perlecan* axon guidance defects. Perlecan augments the reduction in phospho-focal adhesion kinase (phospho-FAK) levels that result from treating insect cells in vitro with Sema-1a, and genetic interactions among *integrin*, *Sema-1a*, and *FAK* in vivo support an antagonistic relationship between Sema-1a and integrin signaling. Therefore, perlecan is required for Sema-1a–PlexA-mediated repulsive guidance, revealing roles for extracellular matrix proteoglycans in modulating transmembrane guidance cue signaling during neural development.

[**Keywords:** axon guidance; semaphorin; perlecan; *Drosophila*; extracellular matrix; proteoglycan]

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Correct nervous system function depends on precise, yet complex, connections among neuronal processes and their targets (Tessier-Lavigne and Goodman 1996). Transmembrane and secreted guidance cues, working as either attractants or repellents, guide extending axons as they establish their trajectories. Although several families of guidance cues have been characterized (Kolodkin and Tessier-Lavigne 2011), it is still unclear how the complexity of neural connectivity arises through the action of a limited number of cues. One way is through modulation of guidance cue signaling by extrinsic factors and also intracellular signaling components (Van Vactor et al. 2006; Bashaw and Klein 2010), although precisely how this modulation occurs is only incompletely understood. *Drosophila* embryonic neural development provides an excellent model system to identify these modulators and determine how they function during the establishment of neuronal connectivity.

The extracellular environment, where axons and dendrites encounter guidance cues, contains a myriad of ex-

tracellular matrix (ECM) components with the potential to influence guidance cue function (Van Vactor et al. 2006). Detailed anatomical studies show that growth cones make direct contact with the ECM in vivo (Roberts and Taylor 1982), and ECM ligands and their receptors, such as laminins and integrins, respectively, regulate axon pathfinding in vivo (García-Alonso et al. 1996; Hoang and Chiba 1998; Myers et al. 2011).

Guidance cue receptors are distributed both uniformly and in a compartmentalized fashion along navigating axons (Katsuki et al. 2009; Winckler and Mellman 2010). However, it is at specific choice points where steering responses to guidance cues must occur in order for precise axon trajectories to be established (Raper and Mason 2010). The distribution of ECM components at select locations and at specific times during development can provide such information for secreted guidance cues. However, modulation of neuronal transmembrane guidance cue ligand–receptor interactions in vivo by ECM components remains to be investigated.

Proteoglycans consist of a core protein that is covalently modified with different classes of glycosaminoglycan (GAG) side chains: heparan sulfate (HS) and chondroitin sulfate (CS) (Van Vactor et al. 2006). Although initially

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considered as only structural ECM components, proteoglycans are now known as modulators of ligand–receptor signaling events in numerous developmental contexts (Van Vactor et al. 2006). Analysis of mice lacking an HS chain biosynthetic enzyme in the nervous system demonstrates that HSs play an essential role in axon guidance at the CNS midline (Inatani et al. 2003). Importantly, the proteoglycan syndecan, a transmembrane HS proteoglycan (HSPG) expressed on neurons, modulates signaling by the secreted guidance cue slit. Syndecan is also a ligand for the LAR receptor protein tyrosine phosphatase, regulating axonal pathfinding and synapse formation (Johnson et al. 2004; Steigemann et al. 2004; Fox and Zinn 2005).

Semaphorin proteins are phylogenetically conserved and include both secreted and transmembrane guidance cues (Tran et al. 2007). Semaphorins are also modulated by GAGs since HSPGs and CS proteoglycans (CSPGs) are required by the murine class 5 semaphorin Sema5A for axon guidance functions (Kantor et al. 2004). Moreover, the growth cone-collapsing activity of a vertebrate-secreted semaphorin, Sema3A, is enhanced by the addition of heparin (De Wit et al. 2005). However, *in vivo* evidence demonstrating that a specific proteoglycan can modulate semaphorin neuronal signaling is lacking.

Here, we show that the secreted HSPG perlecan is required for embryonic motor axon guidance in *Drosophila*. *perlecan*- and *semaphorin-1a* (*Sema-1a*)-null mutants exhibit similar motor axon guidance phenotypes, and we found that perlecan modulates Sema-1a repulsive signaling *in vivo*. Therefore, a secreted ECM protein modulates transmembrane guidance cue signaling during nervous system assembly and plays a key role in the establishment of neural connectivity during development.

Results

A *Sema-1a* gain-of-function (GOF) genetic screen on the X chromosome identifies *perlecan*, a secreted HSPG, as a facilitator of *Sema-1a*–Plexin A (*PlexA*) signaling

In each segment of the *Drosophila* embryonic ventral nerve cord, commissural axons cross the CNS midline, forming one anterior and one posterior tract (Fig. 1A; Seeger et al. 1993). Sema-1a, a transmembrane semaphorin, can serve as a repellent required for CNS axon tract formation (Yu et al. 1998). Ectopic expression of Sema-1a in CNS midline glial cells in a *Sema-1a*-null mutant background (referred to here as “PUP” [*Sema-1a*^{P1}, *UAS-Sema-1a*/+, *P52-Gal4*/+]), provides a sensitized genetic background in which most posterior commissural axons do not cross the midline: a robust *Sema-1a* GOF phenotype (Fig. 1B; Ayoob et al. 2004). In *Drosophila*, PlexA is a Sema-1a receptor (Winberg et al. 1998), and when a single *PlexA*-null allele was introduced into the PUP background, we observed significant suppression (Fig. 1C; Ayoob et al. 2004). Conversely, introduction of a genomic construct containing a bacterial artificial chromosome (BAC) that includes the entire *PlexA* locus (*PlexA*-BAC) into the PUP background leads to dramatic

Figure 1. *Sema-1a* GOF screen on the X chromosome identifies perlecan as a facilitator of Sema-1a–PlexA signaling. (A–H) Commissural axons stained with BP102 (green) and anti-Sema-1a antibody (red). Anterior is to the left. Bar, 20 μ m. (A) In wild-type embryos, each segment contains anterior (A) and posterior (P) commissures. (B) Embryos misexpressing Sema-1a (red) in midline glia in a *Sema-1a*^{P1} mutant background (PUP) lack posterior (P) commissures, producing the *Sema-1a* GOF phenotype. (C) Removing one copy of *PlexA* suppresses the *Sema-1a* GOF phenotype. (D) Introducing the entire *PlexA* locus (*PlexA*-BAC) exhibits enhancement. (E) *Df 209*, a large deficiency that includes *perlecan*, shows suppression. (F) *perlecan*-null mutants suppress the *Sema-1a* GOF phenotype. (G) Ectopic expression of perlecan results in enhancement. (H) Ectopic expression of perlecan in a *PlexA* mutant results in no *Sema-1a* GOF phenotype. (I) Summary of *Sema-1a* GOF phenotypes measured in t-values (see the Materials and Methods for detailed scoring method). Positive and negative t-values indicate suppression and enhancement, respectively. P-values < 0.0001 indicate significant suppression or enhancement (see the Materials and Methods for cutoff calculation methods).

enhancement (Fig. 1D). Here, we extended our use of the PUP background (Ayoob et al. 2004) to screen for novel components of the Sema-1a–PlexA signaling pathway on the X chromosome (see the Materials and Methods).

Heterozygosity for a *PlexA*-null mutant in the PUP background exhibited the strongest suppression (Supplemental

Fig. 1A, red curve), whereas one copy of the *PlexA*-BAC showed the strongest enhancement (Supplemental Fig. 1A, green curve); control crosses resulted in neither suppression nor enhancement (Supplemental Fig. 1A, light-blue curve). We defined cutoffs that allowed us to determine which large deficiency lines significantly suppress or enhance the *Sema-1a* GOF phenotype (see the Supplemental Material). We crossed smaller deficiency lines, or candidate mutants, falling within the larger deficiencies back into the *PUP* background to identify genes responsible for suppression.

Among candidate genes (Supplemental Fig. 1A,B), we found that two different alleles of *trol* (*trol8* and *Pcan^{null}*) significantly suppress the *Sema-1a* GOF phenotype (Fig. 1E,F,I; Supplemental Fig. 1A [orange curves], B). We found that *Df209*, which includes *trol*, shows milder *PUP* suppression than any of the *trol* alleles (Fig. 1E,I), suggesting that other genes contained in *Df209* may negatively regulate *Sema-1a* signaling. *Sema-1a* ectopic expression levels on midline glia in the *PUP* background were not perturbed by loss of *trol* (Fig. 1B,E,F). Furthermore, mid-

line glia do not exhibit aberrant morphology in *trol* mutants (Supplemental Fig. 1C,D).

The *trol* gene in *Drosophila* encodes a secreted HSPG called perlecan, an evolutionarily conserved ECM component (Friedrich et al. 2000; Voigt et al. 2002). Although *Drosophila* HSPGs modulate slit signaling (Johnson et al. 2004), none has been shown to modulate secreted or transmembrane semaphorin function in vivo. *trol* suppression of the *PUP* phenotype suggests that perlecan facilitates *Sema-1a*-PlexA signaling. Therefore, we crossed line *EP1160*, which harbors a P element that includes UAS sequences and is located just upstream of the “RG” *perlecan* isoform transcriptional start site (Fig. 4A), into the *PUP* background. We observed robust enhancement (Fig. 1G,I), confirming that perlecan facilitates *Sema-1a* repulsive signaling. This enhancement is completely absent in *EP1160*; *PUP* embryos that lack *PlexA* (Fig. 1H). Taken together, these data show that perlecan can facilitate *Sema-1a*-mediated axon repulsion in vivo.

Increased levels of perlecan are found in the vicinity of motor axon trajectories and pathway choice points

Perlecan is expressed within the ventral nerve cord and in the embryonic body wall, and anti-perlecan staining is absent in *perlecan*-null mutants (Fig. 2A,B; Friedrich et al. 2000). Perlecan is highly expressed in the dorsal median cells, in close proximity to the medial and intermediate fasciclin-II⁺ (FasII⁺) longitudinal connectives (Fig. 2C),

Figure 2. Perlecan is expressed in the periphery, with increased levels in the vicinity of motor axon trajectories and pathway choice points. (A–F') Dorsal views of filleted stage 16 embryos. (A–C, E–F') Embryos are stained with anti-perlecan (magenta) and anti-FasII mAb 1D4 (green). (A, A') Perlecan expression in wild-type embryos. Bar, 60 μ m. (B) Lack of anti-perlecan staining in *trol8* mutants. Bar, 60 μ m. (C) In wild-type embryos, perlecan CNS expression is in close proximity to the medial and intermediate FasII⁺ longitudinal connectives. Bar, 20 μ m. (D) In *HB9-Gal4* animals ectopically expressing CD8-GFP, perlecan (magenta) is colocalized with GFP where the cell bodies of Islet⁺ RP1/3/4/5 motoneurons reside (yellow arrow head). Bar, 20 μ m. (E, E') Perlecan is highly expressed in muscle attachment sites (white arrowhead), and elevated expression of perlecan is detected along the ISNb (green) motor axonal trajectory (yellow arrowheads). Bar, 10 μ m. (F, F') In *Sema-1a^{P1}* mutant embryos, much weaker perlecan expression is observed along the ISNb axonal trajectory (white arrowheads). Bar, 10 μ m. (G–I) In each panel, on the left is a single ISNb pathway stained with 1D4 antibody (green) and labeled to show the three choice point areas (CP1–3) where axons defasciculate (white translucent circles). At the right of each panel is shown the fluorescence intensity of perlecan expression, based on staining with anti-perlecan, in a heat map format. Bar, 5 μ m. (G) Elevated perlecan expression is detected at the bifurcation points CP1 and CP3: adjacent to CP3 and at the bifurcation point in CP1 (H), and at the bifurcation point in CP1 and before bifurcation in CP3 (I). (J) The SNa pathway stained with 1D4 (green) and anti-perlecan antibody (heat diagram depicts staining intensity). (K) Correlation of perlecan localization with choice point areas (CP1–3) along the ISNb and SNa pathways. (*) $P < 0.01$.

and also in CNS regions where commissures cross the midline (Supplemental Fig. 2A). We performed immunohistochemistry (IHC) for perlecan in *HB9-Gal4*, *CD8-GFP* embryos. In these embryos, CD8-GFP is expressed in subsets of motor neurons, including the *Islet⁺* RP1/3/4/5 motoneurons that innervate the ventral lateral muscle field via the intersegmental nerve b (ISNb) (Odden et al. 2002). Interestingly, perlecan partially colocalizes with these motoneuron cell bodies (Fig. 2D, yellow arrowheads; Supplemental Fig. 2B–B’).

Motor nerves exiting the CNS in each hemisegment include the intersegmental nerve (ISN) and segmental nerve (SN) (Fig. 3G; Landgraf et al. 1997). ISNb motor axons defasciculate from the main ISN bundle and then navigate along ventral lateral muscles 6, 7, 12, and 13 (Figs. 2G, 3G). There are three major ISNb pathway choice points, and they are numbered, medial to lateral, CP1, CP2, and CP3; they are located between muscles 6 and 7, 13 and 6, and 12 and 13, respectively (Fig. 2G). At CP1, RP3 neurons defasciculate between muscles 6 and 7. At CP2, RP1 and RP4 axons defasciculate, and the remaining RP5 axons extend dorsally between muscles 13 and 12, extending processes anteriorly and posteriorly (Figs. 2G, 3G). Axons in the dorsal branch of the SNa pathway innervate muscles 22, 23, and 24 (Figs. 2J, 3G; Landgraf et al. 1997).

Although perlecan is expressed in several regions of the ventral–lateral muscle field, elevated levels of perlecan are observed along motor axon trajectories in close proximity to the ISNb pathway (Fig. 2E–E’, yellow arrowheads). It is not clear whether perlecan is secreted from muscle, glia, or neurons. However, the pattern of elevated perlecan expression along the ISNb trajectory is less robust in *Sema-1a* mutants, where ISNb motor axons are severely stunted and often do not reach their final targets (Fig. 2F–F’, white arrowheads).

To investigate perlecan distribution along the ISNb, we quantitatively assessed perlecan protein distribution using mAb 1D4 to illuminate motor neurons and anti-perlecan. This allowed us to evaluate the correlation between perlecan localization and motor axon choice points. Elevated perlecan levels are detected at CP1 in almost all embryos (Fig. 2G–I,K). This is the branch point where motor axon defasciculation is dramatically compromised in the absence of *Sema-1A*–*PlexA* signaling (Winberg et al. 1998; Yu et al. 1998). At CP2, we observed a reduced correlation, but at CP3, perlecan is found in close proximity to this choice point in most embryos; however, the CP3 perlecan location does not always correlate precisely with the ISNb bifurcation point (Fig. 2G–I,K). We also observed a significant correlation between perlecan expression and SNa choice points: strong corre-

Figure 3. Perlecan is required for *Sema-1a*-mediated motor axon defasciculation. (A–F) Three hemisegments stained with 1D4 antibody. Anterior is at the left, and lateral is at the top. Bar, 20 μ m. (A) In wild-type embryos, ISNb axons navigate dorsally and defasciculate at muscles 6/7 (black arrows) and at muscles 12/13, sending axons both anteriorly and posteriorly (black arrowheads). SNa axons defasciculate between muscles 22/23, grow dorsally, and innervate muscle 24 (black arrowheads). (B) In *Sema-1a^{P1}* mutants, axons comprising the ISNb pathway often fail to defasciculate at muscles 6/7 (red arrows) and 12/13 (red notches); in severe cases, axons stall between muscles 6/13 (white notch). Axons within the SNa pathway fail to defasciculate between muscles 22/23 (red arrowheads). (C,D) In *perlecan* mutants, ISNb axons fail to defasciculate (red notches) at muscles 12/13 and often lack innervation at muscles 6/7 (red arrows); some axons stall between muscles 6/13 (white notch). SNa axons also fail to reach their proper targets (red arrowheads). (E,F) *perlecan* genetically interacts with *Sema-1a* and *PlexA*. Axons within the ISNb fail to defasciculate at muscles 6/7 (red arrows) and 12/13 (red notches), and SNa axons also fail to reach their targets (red arrowheads) in embryos transheterozygous for *trol8^{-/-};Sema-1a^{-/-}* and *trol8^{-/-};PlexA^{-/-}*. (G,H) Schematic diagram of the ISNb and SNa pathways. (I) Quantitative summary of axon guidance defects in each genotype. (*) $P < 0.01$.

lation at CP1 and CP3, and weaker correlation at CP2 (Fig. 2J,K). These data show that perlecan localization is concentrated at motor axon choice points, and so perlecan is well-positioned to facilitate Sema-1a-mediated motor axon defasciculation events.

Perlecan is required for motor axon defasciculation during embryonic development

Sema-1a is expressed along most, if not all, motor axon bundles, and in *Sema-1a^{P1}* mutant embryos, many ISNb axon bundles stall at CP2, unable to send remaining ISNb motor axons dorsally, with some failing to defasciculate at CP3 (Fig. 3B,I; Yu et al. 1998). *PlexA* mutants exhibit similar motor axon guidance phenotypes (Fig. 3I; Winberg et al. 1998). To ask whether perlecan is required for motor axon guidance, we examined two different *trol* alleles (Voigt et al. 2002; Park et al. 2003), both of which significantly suppress the *Sema-1a* GOF *PUP* phenotype

(Fig. 1I). Indeed, both *Pcan^{null}* and *trol8* mutants display motor axon guidance defects similar to those seen in *Sema-1a^{P1}* and *PlexA* mutants, although with somewhat lower penetrance (*Pcan^{null}*, 59.9% total ISNb defects; *trol8*, 69.1%; *Sema-1a^{P1}*, 95.5%; and *PlexA*, 70.8%) (Fig. 3C,D,H,I; Table 1). Most of the ISNb guidance defects observed in *trol* mutants include RP5 and RP3 defasciculation failures (data not shown). The *perlecan* phenotypes that we observed occur in those regions of the ISNb trajectory where we found that perlecan protein expression is enriched: at CP1 and CP3. We did not observe any differences in glial cell nucleus number or positioning (Supplemental Fig. 3C,D) or in glial ensheathment of ISNb motor axons (Supplemental Fig. 3A,B) in *perlecan* mutants. Moreover, overall muscle organization in *perlecan* mutants is indistinguishable from wild type (Supplemental Fig. 3E,F).

To ask whether perlecan functions in Sema-1a–PlexA repulsive signaling, we looked for genetic interactions in

Table 1. Summary of motor axon guidance phenotypes

Genotype	Abnormal ISNb pathway ^{a-d}	Abnormal SNa pathway ^c	Hemisegments scored ^f (x, y)
Controls			
Wild type (w1118)	18.9	10.6	133, 144
<i>Sema-1a^{P1}/+</i>	22.2	18.7	108, 107
<i>PlexA^{df(4)c3}/+</i>	27.1	27.7	96, 94
<i>PlexB^{KG00878}/+</i>	12.6	24.4	101, 98
<i>trol⁸/+</i>	20.7	13.1	102, 105
<i>Pcan^{null}/+</i>	22.1	10.8	77, 74
<i>Sdc/+</i>	8.0	10.0	50, 50
Loss of function			
<i>Sema-1a^{P1}/Sema-1a^{P1}</i>	95.5	95.2	110, 104
<i>PlexA^{df(4)c3}/PlexA^{df(4)c3}</i>	70.8	74.2	96, 97
<i>trol⁸/Y</i>	69.1	41.1	97, 95
<i>Pcan^{null}/Y</i>	59.9	38.7	279, 266
<i>PlexB^{KG00878}/PlexB^{KG00878}</i>	53.3	32.5	199, 194
Rescue			
<i>Pcan^{null}/Y;;UAS-Pcan-RG/+; elav-gal4/+</i>	27.5	20.2	102, 99
<i>Pcan^{null}/Y;;UAS-Pcan-RG/+; repo-gal4/+</i>	43.9	25.8	98, 97
<i>Pcan^{null}/Y;;UAS-Pcan-RG/+; twi-gal4/+</i>	57.6	46.3	85, 80
<i>Pcan^{null}/Y;;UAS-Pcan-RG/+; repo-gal4/+; elav-gal4/+</i>	25.7	21.2	101, 99
Genetic interactions			
<i>trol⁸/+;Sema-1a^{P1}/+</i>	50.5	44.8	97, 96
<i>trol⁸/+;;PlexA^{df(4)c3}/+</i>	60.2	52.4	108, 103
<i>trol⁸/+;;PlexB^{KG00878}/+</i>	28.0	19.0	100, 100
<i>Pcan^{null}/+;Sema-1a^{P1}/+</i>	51.0	41.8	153, 146
<i>Pcan^{null}/+;;PlexA^{df(4)c3}/+</i>	51.5	40	97, 95
<i>Sdc/+; Sema-1a^{P1}/+</i>	25.6	25.9	86, 81
Gain of function			
<i>UAS-dally/+; elav-gal4/+</i>	53.8	25.5	104, 102
<i>UAS-Sema-1a/+;5053-gal4/+</i>	21.3 [95.7, 0, 0]	N/A	108
<i>UAS-Pcan/Y;;5053-gal4/+</i>	18.1 [94.1, 0, 0]	N/A	94
<i>UAS-Pcan/Y;UAS-Sema-1a/+;5053-gal4/+</i>	61.9 [68.6, 12.8, 14.0]	N/A	139

^aISNb defects (percentage hemisegments): The axons fail to separate from the ISN, stall between 6/13, or lack defasciculation at 6/7 or 12/13.

^bPercentage of ISNb axons lacking defasciculation at 12/13.

^cPercentage of ISNb axons making bulb figure.

^dPercentage of ISNb axons bifurcating aberrantly.

^eSNa defects (percentage hemisegments): The dorsal branch fails to separate from the lateral, makes detour, or lacks defasciculation between 22/23 or 23/24.

^f(x, y) Number of hemisegments scored for ISNb (x) and SNa (y).

multiple transheterozygous genotypes. Compared with *Sema-1a*^{P1/+}, *PlexA*^{+/+}, *Pcan*^{null/+}, or *trol8*^{+/+} single heterozygote embryos, all of which exhibit few to no motor axon guidance defects, *Pcan*^{null/+}; *Sema-1a*^{P1/+}, *trol8*^{+/+}; *Sema-1a*^{P1/+}, *Pcan*^{null/+}; *PlexA*^{+/+} and *trol8*^{+/+}; *PlexA*^{+/+} transheterozygous embryos all show significant ISNb and SNa motor axon guidance defects (Fig. 3E,F,I; Table 1). A *PlexB*-null mutation, which removes the secreted *Sema-2a* and *Sema-2b* receptor (Ayoob et al. 2006; Wu et al. 2011), did not exhibit genetic interactions with *trol8* (Fig. 3I). Taken together, these *perlecan* loss-of-function (LOF) and genetic interaction data suggest that perlecan modulates transmembrane *Sema-1a*-mediated axon-axon repulsion.

Cell type-specific rescue of perlecan mutants

We next cloned the full-length *perlecan* cDNA (see the Materials and Methods) and examined in which cell types perlecan is required for motor neuron guidance. We cloned the two alternatively spliced transcripts, called “RG” and “RD,” that are located ~5 kb and ~13 kb, respectively, downstream from the *EP1160* insertion site (Fig. 4A, RG [red] and RD [green]). Expression of both the RG and RD cDNAs strongly enhances the *Sema-1a* GOF phenotype (Fig. 4B–E). Therefore, both *perlecan* cDNAs are sufficient for facilitating *Sema-1a* signaling despite different signal sequences and the ~200-amino-acid sequence difference at the N termini of the perlecan proteins encoded by RG and RD.

We next expressed the RG perlecan isoform in all neurons and detected robust pan-neuronal perlecan protein distribution when expression was driven by *elav-Gal4* in a *perlecan* mutant (Fig. 4F). Neuronal perlecan expression significantly rescued the motor axon guidance phenotypes observed in *Pcan*^{null} mutants (59% ISNb defects in *Pcan*^{null/Y} embryos rescued to 28% in *Pcan*^{null/Y}; *elav-gal4*; *UAS-Pcan-RG/+* embryos) (Fig. 4G,L). In contrast, robust expression of perlecan in all glial cells, including in glial processes that ensheath both ISNb and SNa pathways (Fig. 4H), only very modestly rescues *Pcan*^{null} mutant motor axon defects (59% ISNb defects in *Pcan*^{null/Y} embryos to 44% in *Pcan*^{null/Y}; *repo-gal4*; *UAS-Pcan-RG/+* embryos) (Fig. 4H,I). Furthermore, we observed no rescue of *Pcan*^{null} phenotypes when perlecan was ectopically expressed in mesodermal cells (58% defects observed in *Pcan*^{null/Y}; *twi-gal4*; *UAS-Pcan-RG/+* embryos). This is somewhat surprising, since perlecan, a secreted HSPG expressed under control of the *twi-Gal4* driver, is robustly expressed in the vicinity of developing motor axon trajectories (Fig. 4J). These results suggest that spatial regulation of perlecan expression is important for motor axon guidance at trajectory choice points. Furthermore, expression in motor axons and, to a much lesser extent, in glia apparently better mimics the wild-type perlecan distribution as compared with mesodermal expression.

Perlecan enhancement of CNS and motor axon *Sema-1a*–*PlexA* signaling

We next used a different *PlexA* GOF paradigm whereby neuronal expression of *PlexA*, driven by *elav-Gal4*, causes

Figure 4. Cell type-specific rescue of *perlecan* mutants with functional *perlecan* cDNA. (A) Schematic representation of the *perlecan* genomic organization. The 5' exons of the RD, RG, and RF isoforms are shown in green, red, and blue, respectively. (B–D) Anterior is to the left, and posterior to the right. Bar, 20 μ m. (B) PUP background embryos. (C,D) Both RG and RD *perlecan* isoforms significantly enhance the PUP *Sema-1a* GOF phenotype. (E) Summary of B–D. Embryos stained with anti-perlecan antibody (F,H,I) and with 1D4 antibody (G,I,K) are shown. Bars, 15 μ m. (F) Perlecan is detected along the axonal trajectories and at choice points of the ISNb pathway in *Pcan*^{null} mutants rescued with a pan-neuronal driver. Bar, 8 μ m. (G) *Pcan*^{null} mutants rescued with a pan-neuronal driver. (H) Pan-glial rescue in *Pcan*^{null} shows peripheral glial expression of perlecan. (I) ISNb axons lacking defasciculation at muscles 6/7 (black arrows) in pan-glial rescue animals. (J) Perlecan muscle expression in mesodermal rescue of *Pcan*^{null} mutants. (K) ISNb pathways lack defasciculation at muscles 6/7 and 12/13 (black arrows and arrowheads, respectively) in mesodermal perlecan rescue animals. (L) Summary of cell type-specific rescue experiments. (*) $P < 0.01$.

CNS medial longitudinal tracts that normally never cross the CNS midline to aberrantly cross at a low, but significant, frequency (Fig. 5A,B; Ayoub et al. 2004). Simultaneously overexpressing PlexA and perlecan in all neurons significantly enhances this aberrant CNS midline crossing phenotype (Fig. 5C,D), in line with our previous observations showing that neuronal perlecan is capable of enhancing PlexA signaling following *Sema-1a* GOF in CNS midline glia (Fig. 1C).

We further addressed whether perlecan facilitates *Sema-1a*–PlexA forward signaling in motor axons by providing an ectopic source of *Sema-1a* in *trans* using the *5053A-Gal4* driver (Ritzenthaler et al. 2000) to express *Sema-1a*, perlecan, or both proteins only in the ventral longitudinal muscle 12 (Fig. 5E). Overexpressing *Sema-1a* or perlecan in muscle 12 using one copy of either trans-

gene alone did not significantly affect RP5 motor axon targeting to muscle 12 or defasciculation at the final choice point between muscles 12 and 13 (Fig. 5F,G). However, simultaneous overexpression of *Sema-1a* and perlecan from muscle 12 resulted in a significant increase in RP5 defasciculation defects (Fig. 5H) and targeting defects (Fig. 5H',H''), leading to premature motor axon bifurcation. These phenotypes most likely result from enhanced *Sema-1a*-mediated repulsion in *trans* augmented by perlecan coexpressed in muscle 12. Taken together, these GOF experiments show that perlecan can facilitate PlexA signaling in CNS interneurons and *Sema-1a* signaling in *trans* to motor axons.

Specificity of HSPG-mediated modulation of *Sema-1a* signaling

Several genes in *Drosophila* encode HSPGs, including the transmembrane HSPG *syndecan*, the GPI-linked HSPGs *dally* and *dally-like*, and the secreted HSPG *perlecan* (Van Vactor et al. 2006). In invertebrates, *syndecan* has been studied extensively in both *Drosophila* and *Caenorhabditis elegans* neural development and plays key roles in repulsive axon guidance mediated by slit–Robo signaling and in synaptogenesis at the neuromuscular junction (Johnson et al. 2004; Steigemann et al. 2004; Fox and Zinn 2005; Rawson et al. 2005). In *Drosophila*, *dally-like* has been implicated in visual system and CNS axon guidance (Johnson et al. 2004; Rawson et al. 2005). Both *syndecan* and *dally-like* are expressed on *Drosophila* embryonic CNS axons and are found in both the anterior and posterior commissures of each segment (Johnson et al. 2004). However, the expression of *dally* has not been presented.

We found that neither *syndecan* nor *dally-like* mutants suppressed or enhanced the PUP *Sema-1a* GOF phenotype (Fig. 6A). Furthermore, embryos transheterozygous for *Sema-1a* and *syndecan* mutant alleles do not show significant genetic interactions affecting motor axon guidance (Fig. 6B). In contrast, *dally* mutants significantly enhanced the PUP *Sema-1a* GOF phenotype, suggesting that *dally* may modulate *Sema-1a* signaling but, unlike perlecan, in a negative fashion. We ectopically expressed *dally* in all embryonic neurons in a wild-type background and observed an increased level of motor axon defasciculation defects in the ISNb pathway, reminiscent of mild *Sema-1a* LOF phenotypes (Fig. 6B). This suggests that *dally* and perlecan may act in opposition with respect to modulation of *Sema-1a* signaling.

We also considered whether perlecan modulates other axon guidance signaling pathways, particularly since perlecan CNS expression partially overlaps with the midline guidance cues slit and netrins A and B and also their receptors (Kolodziej et al. 1996; Mitchell et al. 1996; Johnson et al. 2004). *perlecan* mutants do not phenocopy the embryonic CNS midline crossing and recrossing of FasII⁺ longitudinal connectives characteristic of *Robo*-null mutants (Fig. 2B). In addition, we found that *perlecan* mutants do not exhibit any of the CNS or peripheral nervous system (PNS) guidance errors observed in *frazzled* mutants (Kolodziej et al. 1996). These data suggest that perlecan

Figure 5. Perlecan facilitation of *Sema-1a*–PlexA signaling in the CNS and in motor axons. (A–C) Embryos stained with 1D4 antibody. Bar, 20 μ m. (A) Longitudinal axonal tracts never cross the midline in embryos ectopically expressing perlecan in all neurons. (B) Pan-neuronal expression of PlexA results in rare, but significant, abnormal CNS midline crossing (red arrowhead). (C) Overexpressing perlecan with PlexA in the nervous system significantly increases the frequency of abnormal midline crossing (red arrowheads). (D) Average number of midline crossings per embryo. (*) $P < 0.01$. (E) Schematic diagram of the ISNb axon pathway and RP5 axon bundles encountering muscle 12. (F–H'') Embryos stained with 1D4 antibody. Anterior is to the left, and lateral is to the top. Bar, 20 μ m. (F,G) Embryos ectopically expressing either *Sema-1a* or perlecan from muscle 12 in *trans* show few ISNb RP5 motor axon innervation defects. (H–H'') Simultaneous overexpression of *Sema-1a* and perlecan from muscle 12 results in a significant increase in RP5 defasciculation defects at the most dorsal choice point (CP3) and also targeting defects, resulting in premature bifurcations. (I) Perlecan facilitates ectopic *Sema-1a* expression from muscle 12. (*) $P < 0.01$.

Figure 6. Specificity of HSPG-mediated Sema-1a signaling modulation and HS involvement in Sema-1a signaling. (A) Summary of *t*-values for *PUP Sema-1a* GOF assays with various HSPG mutants. *Dlp*¹ (*dally-like*) or *Sdc* (*syndecan*) mutants do not suppress or enhance the *Sema-1a* GOF phenotype. However, *dally*⁸⁰ (*dally*) mutants enhance the *Sema-1a* GOF phenotype. Suppressing (*) and enhancing (**) fly lines with $P < 0.0001$; (NS) not significant, $P > 0.0001$. (B) Summary of hemisegments with motor neuron pathfinding defects in both ISNb and SNa pathways for different genotypes. *syndecan* and *Sema-1a*^{P1} mutants do not genetically interact. Pan-neuronal expression of *dally* results in increased levels of ISNb pathway innervation defects compared with wild type. (*) $P < 0.01$. (C) PlexA and Sema-1a, but not dGIPC, bind to heparin beads. S2R⁺ cells were transfected with the cDNAs indicated above each lane. Lanes 1–4 and 9–11 show Western blots from cell lysates, and lanes 5–8 and 12–14 display initial lysates that were subsequently incubated with heparin beads and then subjected to pull-down using these beads. Lanes 1–8 are blotted with anti-myc antibody to detect PlexA, Sema-1a, and dGIPC, and lanes 9–14 are blotted with anti-Sema-1a to detect Sema-1a. (WB) Western blot; (IP) immunoprecipitation. This experiment was performed twice. (D) Summary of *t*-values for *Sema-1a* GOF assays with HS biosynthetic and modifying enzymes. *sgl* and *sfl* mutants robustly enhance the *Sema-1a* GOF phenotype. (*) $P < 0.0001$; (NS) $P > 0.0001$.

does not play a major role in modulating endogenous embryonic netrin- or slit-mediated axon guidance, exhibiting specificity with respect to its role in regulating neuronal guidance cues.

Perlecan HS chain involvement in Sema-1a signaling facilitation

To investigate HS chain contribution to perlecan-mediated Sema-1a signaling, we first tested physical interactions between HS and Sema-1a or PlexA. Both Sema-1a and PlexA, but not the cytosolic control protein dGIPC, bound robustly to heparin-agarose beads (Fig. 6C, Sema-1a [cf. lanes 1 and 5, and lanes 9 and 12], PlexA [cf. lanes 2 and 6], and dGIPC [cf. lanes 4 and 8]). Moreover, cells coexpressing Sema-1a and PlexA showed similar levels of each protein binding to heparin beads, suggesting that HS does not negatively affect the association between this

ligand and its receptor or vice versa (Fig. 6D, cf. lanes 3 and 7, and lanes 10 and 13). Overall, these interactions suggest that HS chains in perlecan or *dally* may bind to Sema-1a or PlexA directly.

Next, we assessed mutants that affect HS chain biosynthesis in the *PUP* background. Proteins with homology with UDP-glucose dehydrogenase, N-deacetylase/N-sulfotransferase, and EXT are encoded in *Drosophila* by *sugarless* (*sgl*), *sulfateless* (*sfl*), and *EXT* genes, respectively (Botv is required for HS chain initiation, and Ttv and Sotv are required for HS chain elongation) (Van Vactor et al. 2006). *Sgl* is required for the formation of glucuronic acid, which in turn is required for biosynthesis of HS, CS, and dermatan sulfate (Lin and Perrimon 1999). When HS chains are elongated, the enzyme N-deacetylase/N-sulfotransferase, which in *Drosophila* is encoded by *sfl*, removes acetyl groups from GlcNAc residues and substitutes them with sulfate groups (Kamimura et al. 2011).

Mutations in any one of the *EXT* genes affect HSPG biosynthesis in vivo, demonstrating that they are not functionally redundant (Takei et al. 2004). We introduced all five single mutants and also the *ttv*; *sotv* double-mutant combination into the *PUP* background. Interestingly, *sfl* and *sgl* mutations exhibited strong dominant enhancement of the *Sema-1a* GOF phenotype; however, none of the *EXT* mutants exhibited suppression or enhancement (Fig. 6D). The strong enhancement shown by *sfl* and *sgl* mutants suggests that HSs, presumably on perlecan or dally, act to inhibit *Sema-1a* signaling. In addition, *sgl* mutants exhibit stronger enhancement than *sfl* mutants (*sgl* *t*-value = -10.5 ; *sfl* *t*-value = -7.1), raising the possibility that both HSs and CSs are involved in negatively regulating *Sema-1a* signaling. However, relatively stronger maternal effects could explain the lack of suppression or enhancement in *EXT* mutants. Given our biochemical and genetic data, HSs on perlecan or dally could bind to *Sema-1a* and PlexA directly and negatively regulate *Sema-1a* signaling in vivo.

Perlecan enhances *Sema-1a*-mediated focal adhesion kinase (FAK) dephosphorylation

During axon pathfinding, integrins function by selectively stabilizing contacts with permissive ECM substrates, including laminin and fibronectin (Robles and Gomez 2006). Since guidance cue-directed axon pathfinding likely includes regulation of integrin-based adhesion (Myers et al. 2011), cross-talk between semaphorin and integrin signaling has the potential to result in inhibition of integrin-mediated adhesion to the ECM, and vice versa (Serini et al. 2003; Oinuma et al. 2006; Tran et al. 2007).

Integrin receptors bind ECM components and often signal cytoskeletal changes through activation of FAK (Ivankovic-Dikic et al. 2000), and increased levels of FAK phosphorylation are often observed at sites of integrin activation and clustering (Kornberg et al. 1992). In *Drosophila*, phosphorylated FAK is absent from muscle attachment sites in integrin mutants, and *FAK* genetically interacts with *integrin* mutations (Grabbe et al. 2004). Moreover, changes in FAK phosphorylation levels have been observed upon semaphorin treatment (Pasterkamp et al. 2003; Oinuma et al. 2006; Schlomann et al. 2009). Therefore, we asked whether FAK phosphorylation levels might reflect cross-talk between *Sema-1a* and integrin signaling. We observed a dramatic decrease in phospho-FAK levels when the soluble extracellular domain of *Sema-1a*, at a concentration of ~ 35 nM, was used to treat *Drosophila* S2R⁺ cells in vitro for 30 min (Fig. 7A,B; lanes 1,5). However, this decrease was significantly attenuated when the cells were pretreated with *PlexA* dsRNA, strongly suggesting that this effect on FAK phosphorylation is specific to the *Sema-1a*–PlexA signaling pathway (Fig. 7A,B, lanes 5–7). *PlexA* is expressed in S2R⁺ cells, and we observed that the effect of *PlexA* dsRNA on PlexA protein levels is robust (Fig. 7A,B, lanes 2,3,6,7). Consistent with our in vivo genetic analyses, overexpressing perlecan in S2R⁺ cells enhanced *Sema-1a*-mediated dephosphorylation of FAK by $\sim 40\%$ ($P = 0.00095$) (Fig. 7A,B,

lanes 5,8). Interestingly, overexpression of perlecan alone dramatically reduced phospho-FAK levels (Fig. 7A,B, lanes 1,4). However, S2R⁺ cells overexpressing perlecan and *PlexA* dsRNA showed no reduction of phospho-FAK levels, suggesting that perlecan-mediated reduction of phospho-FAK levels is dependent on PlexA signaling (Fig. 7C).

Since phospho-FAK levels are reduced upon *Sema-1a* treatment in vitro, we investigated whether *Sema-1a* and *FAK* genetically interact in vivo. We did not observe any axon pathfinding defects in *Fak56^{CG1}* mutants (data not shown). However, robust genetic interactions were observed between *FAK* and *integrin* in the developing wing disc when FAK was overexpressed in *integrin* heterozygotes (Grabbe et al. 2004). Therefore, we compared *Sema-1a^{P1}/+* heterozygous embryos to either wild-type or *Sema-1a^{P1}/+* heterozygous embryos in which FAK was overexpressed in the nervous system. We observed that neuronal overexpression of *FAK* alone results in the formation of severe gaps at high penetrance in the lateral-most FasII⁺ CNS longitudinal fascicles and also aberrant CNS midline crossing of axons from the medial-most FasII⁺ longitudinal connectives; these phenotypes are reminiscent of CNS defects observed following *integrin* LOF (Fig. 7F; Supplemental Fig. 4A,A',C; Hoang and Chiba 1998). Given that FAK is an integrin effector and *FAK* GOF leads to *integrin* LOF phenotypes, *FAK* GOF may function in a dominant-negative fashion (Hoang and Chiba 1998). Interestingly, removing one copy of *Sema-1a* in this *FAK* GOF background significantly suppresses these CNS longitudinal tract defects, consistent with our in vitro observation that *Sema-1a* treatment down-regulates FAK phosphorylation and so appears to function antagonistically with respect to FAK activation. These results demonstrate a robust genetic interaction between *Sema-1a* and *FAK* GOF in vivo (Fig. 7F; Supplemental Fig. 4B,B',C). One interpretation of these results is that endogenous *Sema-1a* signaling lowers phospho-FAK levels, but when FAK is overexpressed, non-phospho-FAK levels increase relative to phospho-FAK, thus leading to inhibition of integrin signaling. However, when one copy of *Sema-1a* is removed, relative levels of phospho-FAK increase, thereby restoring integrin function. These results suggest that the ability of *Sema-1a* to regulate phospho-FAK protein levels is important for axon guidance.

Sema-1a–PlexA modulation of FAK phosphorylation in vitro and also genetic interactions between *Sema-1a* and *FAK* in vivo suggest that there exists cross-talk between *Sema-1a* and integrin signaling in vivo. Therefore, we asked whether mutations in genes encoding *Drosophila* integrin receptor components—*if*, *mew*, and *mys* (α PS2, α PS1, and β PS subunits, respectively) (Bokel and Brown 2002)—modify the *PUP Sema-1a* GOF phenotype. Interestingly, we found that a mutation in *if* significantly enhanced the *PUP Sema-1a* GOF phenotype, and a mutation in *mys* exhibited a more modest, but significant, level of enhancement; however, *mew* mutants did not exhibit any enhancement or suppression (Fig. 7E). The reason why *if* but not *mew* shows *PUP* enhancement may be due to the specificity of the α PS2 subunit interaction

Figure 7. Perlecan enhances Sema-1a-mediated FAK dephosphorylation. (A, lanes 1,5) Thirty-minute treatment with 35 nM Sema-1a-AP induces a decrease in endogenous phospho-FAK Y397 levels in S2R⁺ cells. (Lanes 5–7) This decrease was significantly attenuated when the S2R⁺ cells were pretreated with *PlexA* dsRNA. Perlecan overexpression alone decreases phospho-FAK level (lanes 1,4), and together with Sema-1a treatment, phospho-FAK levels are further reduced (lanes 5,8). (B) Quantification of multiple experiments shown in A. (*) $P < 0.01$. (C, lanes 3,4) The reduction of phospho-FAK levels in S2R⁺ cells overexpressing perlecan is attenuated when cells are pretreated with *PlexA* dsRNA. (D) Quantification of multiple experiments shown in C. (*) $P < 0.01$. (E) *if* mutants significantly enhance the *Sema-1a* GOF phenotype. (*) $P < 0.0001$. (F) *Sema-1a* and FAK GOF genetically interact. Removing one copy of *Sema-1a* suppresses FAK GOF CNS longitudinal tract phenotypes. The graph on the left shows the percentage of embryos exhibiting the lateral-most FasII⁺ longitudinal fascicle disruption broken down by severity, and the graph on the right presents the medial-most FasII⁺ fascicle crossing broken down by the number of crossings.

for Sema-1a signaling. The lower level of enhancement by *mys*, as compared with *if*, may be due to redundancy with β Int- ν , a second *Drosophila* β subunit (Nagaosa et al. 2011). Nevertheless, these results show that integrin and Sema-1a signaling act antagonistically in the context of CNS neuronal Sema-1a-mediated repulsive axon guidance.

Discussion

Our results provide support for the ability of ECM components to modulate axon guidance cue signaling. We show that transmembrane semaphorin signaling is modulated by the secreted HSPG perlecan, demonstrating that *perlecan* GOF and LOF mutants enhance and suppress, respectively, a robust Sema-1a GOF phenotype. We

also observed that perlecan is required for endogenous repulsive Sema-1a–PlexA signaling in motor axons. Perlecan expression is elevated at motor axon defasciculation choice points, and neuronal expression of *perlecan* rescues guidance defects seen in *perlecan* LOF mutants. Our genetic analyses show that mutations in genes encoding other HSPGs, including *syndecan* and *dally-like*, do not affect Sema-1a neuronal functions. By assessing phospho-FAK levels in vitro, we found that perlecan can enhance Sema-1a-mediated down-regulation of phospho-FAK protein levels, and in vivo we observed that perlecan facilitates Sema-1a antagonistic modulation of integrin signaling. Therefore, neuronal growth cone responses to the transmembrane guidance cue Sema-1a require facilitation by the ECM component perlecan, underscoring

the importance of specific HSPG–guidance cue interactions in the assembly of neural circuits during development.

Perlecan modulates transmembrane semaphorin signaling at motor axon defasciculation choice points

The ECM includes collagens, glycoproteins, and proteoglycans that are produced by developing neurons and glia (Myers et al. 2011). Proteoglycans include versican, aggrecan, neurocan, and perlecan (Milev et al. 1998); however, with the exception of versican, our understanding of their *in vivo* axon guidance functions is incomplete (Dutt et al. 2011). In contrast, studies in mice using *EXT* mutants show that HSs are critical for slit-mediated midline axon guidance *in vivo* and also for Netrin/DCC signaling in spinal commissural axon guidance (Inatani et al. 2003; Matsumoto et al. 2007). In these *EXT* mutants, however, both transmembrane and GPI-linked HSPGs are affected, and it is not clear which HSPGs are important for these guidance events. Furthermore, the transmembrane semaphorin Sema5A can be modulated *in vitro* by both HSPGs and CSPGs (Kantor et al. 2004); however, the proteoglycans involved are also not known. Our present study provides insight into how a specific ECM proteoglycan modulates axonal responses to a well-characterized transmembrane guidance cue *in vivo*.

We found a strong correlation between perlecan localization and motor axon defasciculation choice points. This suggests that perlecan is localized at higher levels where increased Sema-1a–PlexA signaling is required. Our cell type-specific genetic rescue experiments, in combination with analysis of perlecan expression, suggest that perlecan is secreted by developing motor axons, which in turn require perlecan for choice point navigation. This likely occurs at specific interstitial locations along motor axon trajectories defined by glial cell ensheathment of motor nerves (Stork et al. 2008).

HSPG specificity and the requirement for HS chains in Sema-1a signaling

HSPGs in both invertebrates and vertebrates play key roles in axon guidance and synaptogenesis (Fox and Zinn 2005; Van Vactor et al. 2006). For example, in both *Drosophila* and *C. elegans*, syndecan regulates slit–Robo-mediated midline guidance events (Hu 2001; Johnson et al. 2004; Steigemann et al. 2004; Rhiner et al. 2005), and in *Drosophila*, *dally-like* can compensate for the loss of *syndecan* (Johnson et al. 2004). In contrast, during visual system assembly and synaptic development, there is only limited rescue of *syndecan* mutant defects with *dally-like* transgenes and vice versa; this suggests that *dally-like* and *syndecan* can serve distinct functions (Johnson et al. 2006). *syndecan* and *dally-like* mutants did not suppress or enhance the *PUP Sema-1a* GOF phenotype, whereas *dally* mutants were capable of enhancement. This likely reflects specificity conferred by *perlecan* and *dally* for critical interactions with Sema-1a signaling components.

Slit–Robo interactions are enhanced by HS, and HS is required for slit-mediated repulsion *in vitro* (Hu 2001).

Furthermore, increased murine retinal axon guidance defects were observed when the dosage of *Ext1* was lowered in a *slit2* mutant (Inatani et al. 2003). In *C. elegans*, guidance cue signaling activity critically depends on different HS modifications (Bulow et al. 2008). To genetically assess perlecan HS contribution in Sema-1a signaling, we tested several HS biosynthetic mutants in the *PUP Sema-1a* GOF assay, and, surprisingly, both *sgl* and *sfl* mutants exhibited robust enhancement. One interpretation of these results is that HS chains on perlecan may interfere with the ability of the perlecan protein core to interact physically with Sema-1a or PlexA, and this would imply that the perlecan protein core is essential for facilitation of Sema-1a signaling. Alternatively, HS may serve auto-inhibitory functions with respect to perlecan. Alternatively, HS chains on *dally* may be critical for *dally*-mediated signaling, since we observed that *dally*, *sgl*, and *sfl* mutants all enhance the *Sema-1a* GOF phenotype, and *dally* is a likely substrate of Sfl and Sgl (Lin and Perrimon 1999). These scenarios are not mutually exclusive. A key experiment is to mutate all of the potential HS sites on perlecan and test *in vivo* how this affects Sema-1a function.

Perlecan enhancement of Sema-1a-mediated FAK dephosphorylation in vitro highlights interactions between semaphorin and integrin signaling in vivo

Extending motor axons are tightly bundled through the action of cell adhesion molecules but also adhere to the ECM as they navigate specific choice points. Our data support the idea that over most of a developing motor axon trajectory, lower perlecan levels lead to reduced activation of Sema-1a–PlexA signaling. However, when motor axons arrive at specific choice points, Sema-1a–PlexA signaling is enhanced by perlecan, concomitantly down-regulating integrin-mediated adhesive interactions via FAK dephosphorylation and leading to motor axon defasciculation.

We found that mutations in integrin subunit genes significantly enhance the *PUP Sema-1a* GOF phenotype, thereby placing Sema-1a and integrin signaling in an antagonistic relationship, consistent with previous *in vitro* observations (Oinuma et al. 2006). Moreover, the robust FAK dephosphorylation that we observed following Sema-1a treatment in S2R⁺ cells supports the idea that semaphorin and integrin signaling interact antagonistically. The mechanism underlying Sema-1a facilitation of FAK dephosphorylation is not known, and it remains to be determined whether this occurs through direct Sema-1a–PlexA interactions with integrin receptors or indirectly via plexin downstream signaling events. It is also possible that perlecan confers stronger binding affinity of Sema-1a to PlexA, thereby promoting the formation of an active semaphorin signaling complex at the expense of integrin signaling.

We examined *Fak56^{CG1}* mutant embryos and did not observe motor axon guidance defects (data not shown), consistent with the apparent normal development of *Fak56^{CG1}* mutants (Grabbe et al. 2004). One possibility is that FAK may not be essential owing to compensation

by other protein tyrosine kinases. Nevertheless, the robust genetic interactions that we observed in CNS longitudinal tracts between *Sema-1a* LOF and *FAK* GOF suggest that FAK may function as an integrin effector such that Sema-1a and integrin signaling converge on FAK. Upon Sema-1a signaling, FAK phosphorylation levels decrease, and this may inhibit integrin signaling. Our results also suggest that perlecan further inhibits integrin signaling by facilitating the Sema-1a signaling pathway.

In conclusion, we found that perlecan, a secreted ECM HSPG, is a critical component of Sema-1a-mediated repulsive guidance in vivo. Furthermore, Sema-1a signaling apparently acts to antagonize integrin signaling. The regulated spatial localization of ECM components with the capacity to modulate specific guidance cue signaling pathways provides a robust mechanism for imparting localized responses to these cues, thereby facilitating the assembly of neural circuits during development.

Materials and methods

Drosophila strains

All mutant stocks have been previously described: *PlexA*^{Df(4)C3} (Winberg et al. 1998), *Pcan*^{null} (Voigt et al. 2002), *trol*⁸ (Park et al. 2003), *EP1160* (Voigt et al. 2002), *PlexB*^{K^{G00878}} (Ayoob et al. 2006), *dally*⁸⁰, *dlp*¹ (Kirkpatrick et al. 2004), *Sdc*¹⁰⁶⁰⁸ (Johnson et al. 2004), *sfl*⁰³⁸⁴⁴, *sgl*⁰⁸³¹⁰, *botv*⁵¹⁰, *sotv*³²⁶ (Takei et al. 2004), and *ttv*^{00681b} (Bellaiche et al. 1998). *elav-Gal4* was used for pan-neuronal labeling, *5053A-Gal4* (Swan et al. 2004) was used for muscle 12 labeling, *repo-Gal4* for was used for glia labeling, *twi-Gal4* was used for mesodermal expression labeling, and *Hb9-Gal4* (Broihier and Skeath 2002) was used for neuronal labeling. For overexpression studies, we used *UAS-Sema-1a* (Yu et al. 1998), *UAS-HA-PlexA* (Winberg et al. 1998), *UAS-Pcan-RG*, *UAS-Pcan-RD*, *UAS-dally* (Bloomington Stock Center, 5397), *UAS-CD8GFP* (Bloomington Stock Center, 5130), *UAS-FAK*, and *PlexA-BAC*.

Immunohistochemical analyses

The antibodies used were anti-Sema-1a mAb (1:3000) (Yu et al. 1998), anti-perlecan (1:3000) (Friedrich et al. 2000), BP102 mAb (Seeger et al. 1993), anti-Fas II mAb 1D4 (Vactor et al. 1993), rabbit anti-GFP (1:1000; Molecular Probes), anti-Myc mAb 9E10 (1:1000; Sigma), anti-repo (1:10; Developmental Studies Hybridoma Bank, Iowa University), anti-wrapper (1:10) (Noordermeer et al. 1998), anti-MHC, HRP-conjugated goat anti-mouse and anti-rabbit IgG/M (1:500; Jackson ImmunoResearch), Alexa488 or Alexa546-conjugated goat anti-mouse IgG, and Alexa647-conjugated goat anti-rabbit IgG (1:500; Molecular Probes). Images were acquired using a Zeiss LSM 510 confocal microscope.

Ligand production

The extracellular portion of Sema-1a was cloned into the APTag-5 vector (GenHunter) to generate a C-terminally AP-tagged fusion protein. The construct was then transfected into 293T cells with Lipofectamine 2000 (0.5 µg DNA/1 µL Lipofectamine). Three days after transfection, supernatants were collected and concentrated (Amicon Ultra 15). Ligand concentrations were measured by quantifying AP activity.

dsRNA preparation

To generate PlexA dsRNA, PlexA cDNA and primers containing T7 RNA polymerase-binding sites and gene-specific sequences to produce ~500-base-pair (bp) fragments containing T7 promoters at the 5' ends were used. To minimize off-target effects, the "off-target sequence search tool" at the *Drosophila* RNAi Screening Center (DRSC) Web site was used. PCR products were purified and used as templates to produce RNA using the Roche T7 polymerase kit. RNA was NaOAc-precipitated, resuspended in water, and stored at -80°C. Control dsRNA was generated using the same method except using PlexB cDNA.

S2R⁺ cell culture and treatment

S2R⁺ cells were grown in Schneider's medium (Invitrogen) with 10% heat-inactivated fetal bovine serum (FBS) at 25°C in culture flasks. Cells (10⁵) in 1 mL of medium were added to each well of a 12-well plate, and 8 h later, dsRNA (30 µg) was added to each well. The cells were then incubated for 24 h at 25°C before transfecting control or perlecan cDNA with Effectene reagent (Qiagen). Cells were grown for 1 d at 25°C, and additional dsRNA (30 µg) was added to each well. The next day, cells were washed briefly with PBS and treated with Sema-1a-AP or AP for 15 or 30 min depending on experimental purposes. Heparinase III (0.2 µg/µL; Sigma) was used for 30 min before ligand treatment, when necessary. Cells were lysed in 100 µL of lysis buffer (0.5% NP-40, 50 mM Tris 8.0, 150 mM NaCl, protease inhibitor, tyrosine phosphatase inhibitor) for 15 min. Phospho-FAK rabbit mAb Y397 (1:500; Invitrogen) was used for Western blotting.

Heparin-binding assay

Heparin beads (Sigma H6508) were washed with Hanks' balanced salt solution (HBSS) and then blocked in HABH (0.5 mg/mL BSA, 20 mM HEPES at pH 7.0) overnight at 4°C. The beads were washed twice with 20 mM HEPES (pH 7.0) and 150 mM NaCl. 5xmyc-Sema1a, PlexA-5xmyc (±Sema1a), or 5xmyc-dGIPC transfected cell lysates were incubated with the pretreated heparin beads overnight at 4°C. The lysates were washed with 20 mM HEPES (pH 7.0) and 150 mM NaCl seven times.

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