Drosophila Plexin B is a Sema-2a receptor required for axon guidance

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Plexin receptors play a crucial role in the transduction of axonal guidance events elicited by semaphorin proteins. In *Drosophila*, Plexin A (PlexA) is a receptor for the transmembrane semaphorin semaphorin-1a (Sema-1a) and is required for motor and central nervous system (CNS) axon guidance in the developing embryonic nervous system. However, it remains unknown how PlexB functions during neural development and which ligands serve to activate this receptor. Here, we show that *plexB*, like *plexA*, is robustly expressed in the developing CNS and is required for motor and CNS axon pathfinding. PlexB and PlexA serve both distinct and shared neuronal guidance functions. We observe a physical association between these two plexin receptors in vivo and find that they can utilize common downstream signaling mechanisms. PlexB does not directly bind to the cytosolic semaphorin signaling component MICAL (molecule that interacts with CasL), but requires MICAL for certain axonal guidance functions. Ligand binding and genetic analyses demonstrate that PlexB is a receptor for the secreted semaphorin Sema-2a, suggesting that secreted and transmembrane semaphorins in *Drosophila* use PlexB and PlexA, respectively, for axon pathfinding during neural development. These results establish roles for PlexB in central and peripheral axon pathfinding, define a functional ligand for PlexB, and implicate common signaling events in plexin-mediated axonal guidance.

KEY WORDS: Plexin, Semaphorin, MICAL, Axon guidance, *Drosophila*

INTRODUCTION

Semaphorins, along with other families of guidance cues, play key roles in neural development (Dickson, 2002). Through both repulsion and attraction, semaphorins guide neuronal growth cones and thereby promote the establishment of neuronal connectivity and circuit formation. By relaying guidance information to the growth cone cytoskeleton of responding neurons, plexin proteins serve as central signaling components of many semaphorin receptor complexes (Kruger et al., 2005). Understanding how neurons integrate a complex palette of guidance cue information through the action of related guidance cue receptors is necessary to reveal the molecular mechanisms underlying the steering of neuronal processes during development and also following nerve injury.

In vertebrates, nine different plexin proteins are known and they are organized into four distinct classes based upon their degree of evolutionary conservation (Plexin A-D); seven of these plexins belong to classes A and B (Tamagnone et al., 1999). In the fruit fly this complexity is not as great as the *Drosophila melanogaster* genome includes only two plexins, one belonging to class A and one to class B (PlexA and PlexB). PlexA functions as a receptor for the transmembrane semaphorins Sema-1a and Sema-1b (Winberg et al., 1998b). In vivo analyses demonstrate that, through the action of PlexA, Sema-1a regulates the defasciculation of motor axon bundles during embryogenesis (Winberg et al., 1998b; Yu et al., 1998). Although gain-of-function (GOF) studies strongly suggest that *Drosophila* PlexB mediates repulsive guidance events in vivo (Hu et al., 2001), and in vitro studies demonstrate that vertebrate plexin-B

proteins mediate growth cone and COS cell collapse (Oinuma et al., 2003; Swiercz et al., 2002), the consequences of removing PlexB function in *Drosophila*, or in vertebrates, have not been determined. It is unclear, therefore, how Plexin B proteins function during neural development.

It is also unclear whether the different classes of plexins play distinct or redundant roles in the establishment of neuronal connectivity. In *Drosophila*, *plexA* and *plexB* are both expressed throughout the nervous system during development, indicating that they are likely to function within the same neuronal classes (Winberg et al., 1998b). When overexpressed in all neurons, both *plexA* and *plexB* can produce similar phenotypes, suggesting that these receptors participate in related signaling events (Hu et al., 2001; Winberg et al., 1998b). Interestingly, vertebrate plexin A1 and plexin B1 both modulate R-Ras activation through their intrinsic GTPase activating protein (GAP) domains, and this is essential for semaphorin-mediated repulsion in vitro (Oinuma et al., 2004; Toyofuku et al., 2005). These data point towards common, or perhaps redundant, signaling mechanisms that may underlie the in vivo functions of A and B class plexin receptors.

By contrast, although A and B class plexins are highly conserved, many differences exist among proteins belonging to these two plexin classes. Plexin A proteins are functional receptors for transmembrane class 1 semaphorins in *Drosophila* and class 6 transmembrane semaphorins in vertebrates. Secreted class 3 semaphorins also signal through class A plexins; however, this requires the assembly of a distinct holo-receptor complex that includes either neuropilin 1 or neuropilin 2, obligate co-receptors that serve to facilitate class 3 semaphorin binding and plexin A activation (Kruger et al., 2005). Plexin B proteins in vertebrates bind to different transmembrane semaphorin ligands, including those from classes 4 and 5; however, no ligand has been identified for *Drosophila* PlexB (Kruger et al., 2005). Differences also exist between the downstream signaling events mediated by A and B class plexins (Negishi et al., 2005). The cytoplasmic domains of *Drosophila* PlexA and PlexB share a high degree of amino acid

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sequence identity (Winberg et al., 1998b), yet they appear to differ with respect to the signaling molecules with which they directly associate. For example, although PlexB directly interacts with the small GTPase Rac, PlexA does not (Driessens et al., 2001; Hu et al., 2001). Likewise MICAL, a large cytosolic oxidoreductase that is crucial for semaphorin-mediated repulsion, associates with PlexA but not PlexB (Terman et al., 2002). Therefore, PlexA and PlexB may also serve non-overlapping roles during neural development.

We examine here the consequences of disrupting PlexB function for *Drosophila* neural development, allowing for a direct comparison between PlexB and PlexA axon guidance functions. We show, by using genetic and biochemical analyses, that PlexB and PlexA serve distinct and overlapping roles in motor and CNS axon guidance. The similarities we observe in PlexA and PlexB functions may be explained by our findings that these receptors can assemble into a heteromultimeric complex, and also that they employ common downstream signaling components to guide axons during development. Finally, we observe that plexin interactions with different semaphorin ligands are likely to contribute the distinct roles PlexA and PlexB serve in establishing neuronal connectivity.

MATERIALS AND METHODS

In situ hybridization
Non-radioactive RNA in situ analysis of whole-mount *Drosophila* embryos was performed as described previously (Terman et al., 2002). Digoxigenin-labeled riboprobes corresponding to the cytoplasmic portions and 3′ UTR of *plexA* and *plexB* were used for the hybridization. No specific hybridization signal was visualized with sense probes.

Drosophila genetics
Culturing of *Drosophila* was performed as described (Terman et al., 2002). All crosses and embryo collections were performed in a humidified incubator maintained at 25°C. The KG00878 line was a generous gift from the P-element Screen/Gene Disruption Project (Bellen et al., 2004). Mutations on the fourth chromosome were identified in embryos by first crossing the lines to either of the following *lacZ*-containing genetic elements: *l(4)P{lacW}IA5/ey^D* (Kronhamn and Rasmuson-Lestander, 1999), *P{LacW}ci^Dplac* (Eaton and Kornberg, 1990), or *P{lacZ}Pax2^Δ122* (Hirth et al., 2003). The desired embryos were selected after an X-gal reaction was performed to detect β-galactosidase activity as described (Yu et al., 1998). All other stocks have been previously described: *plexA^Df(4)C3* (Winberg et al., 1998b), *Df(3R)swp2^MICAL*, *UAS:HA-plexA* (Terman et al., 2002), *UAS:plexB* (Hu et al., 2001), *elav-GAL4* (Yao and White, 1994), *Df(4)M101-62f* (Sousa-Neves et al., 2005), *UAS:Sema-2a* (Winberg et al., 1998b), *24B-GAL4* (Luo et al., 1994).

Alkaline phosphatase-binding assays
The binding of alkaline phosphatase (AP)-tagged ligands to transfected S2R+ cells (Yanagawa et al., 1998) was performed as described (Flanagan and Cheng, 2000). Briefly, S2R+ cells were transfected with cDNAs downstream of UAS sequences, and an Act5C-GAL4 promoter construct using the Effectene transfection reagent (Qiagen, Valencia, CA). AP-ligands were collected from supernatants of transfected HEK 293e cells and concentrated using a Centriprep centrifugal filtering device (Millipore, Billerica, MA). Transfected cells were incubated with AP-tagged ligands for 1 hour at room temperature with mild agitation, washed thoroughly, and then assayed visually by detecting AP activity, or in liquid form by assaying for absorbance at 405 nm and subtracting background levels.

Yeast interactions
Yeast protocols were performed as described previously (Golemis et al., 1994; Terman et al., 2002). Portions of the intracellular domains of *Drosophila* PlexA (PlexA C1 domain, amino acids 1308-1701; PlexA C2 domain, amino acids 1702-1945), and *Drosophila* PlexB (PlexB C1 domain, amino acids 1402-1784; PlexB C2 domain, amino acids 1785-2051) were inserted into the appropriate yeast vectors and the expression of all four constructs was confirmed at the expected size from yeast lysates on western

blots. Activation assays showed that none of the baits could activate transcription independently. Interactions were experimentally examined based on growth and color analyses (Golemis et al., 1994; Terman et al., 2002).

In vivo co-immunoprecipitation
To generate an amino-terminally tagged *myc-plexB* transgene, the amino-terminal fragment of *plexB* was engineered by PCR to remove the *plexB* signal sequence and to include three Myc epitopes (EQLISEEDL) in frame with an upstream ATG start codon and an Ig κ-chain leader sequence from the pSecTagB vector (Invitrogen, Carlsbad, CA). An *NheI/XbaI* fragment including the start codon, signal sequence and *myc-plexB* sequence, was then subcloned into the *XbaI* site in the pUASt vector. Proper orientation of the insert was confirmed using restriction analysis. Transformation of this construct into embryos was performed as described (Terman et al., 2002). Flies containing the *UAS:myc-plexB* transgene were crossed to either *UAS:HA-plexA, elav-GAL4/CyO* or *elav-GAL4/CyO* flies. Embryo lysates were isolated and immunoprecipitated with the 12CA5 anti-HA monoclonal antibody (Roche, Nutley, NJ) as described (Terman et al., 2002). Immunoprecipitates were probed using an HRP-conjugated anti-HA antibody at 1:4000 (3F10; Roche) and an anti-Myc monoclonal antibody at 1:3000 (9E10 mouse ascites; Developmental Studies Hybridoma Bank).

Microscopy and imaging
Images were captured using IPLab software (BD Biosciences, Rockville, MD) with a Qimaging Retiga 2000R CCD camera (Qimaging, Burnaby, BC) on a Zeiss Axioskop 2 upright microscope (Zeiss, Thornwood, NY) with a 63× oil-immersion objective, or using Adobe Photoshop (Adobe, San Jose, CA) with a Qimaging Micropublisher 5.0 RTV digital camera on a Zeiss Stemi SV 11 dissecting microscope. Brightness, contrast and color balance of images were adjusted using Adobe Photoshop.

RESULTS

PlexB is required for motor axon guidance
To determine how PlexB functions in *Drosophila* neural development, we examined a *plexB* loss-of-function (LOF) mutation. *Drosophila plexB* is located on the fourth chromosome at cytolocation 102A1 and comprises 5 exons (Fig. 1A). PlexB shares a similar overall domain structure, and a high degree of amino acid sequence identity over these domains, with PlexA (Winberg et al., 1998b). *plexB*, like *plexA*, is also highly expressed in the embryonic nervous system (Fig. 1B-E) (Winberg et al., 1998b). Gain-of-function (GOF) analyses suggest that these two plexins may function in a similar manner (Hu et al., 2001; Winberg et al., 1998b). To better define the role played by PlexB in establishing neural connectivity, we identified a SUPor-P insertion on the fourth chromosome (KG00878), generated by the P-element Screen/Gene Disruption Project (Bellen et al., 2004), that resides within the first exon, 25 amino acids downstream of the *plexB* open reading frame start codon (Fig. 1A). This insertion line, *plexB^KG00878*, is semi-lethal and yields a small percentage (5.7%) of homozygous adult flies that are unable to mate successfully (Table 1). *plexB^KG00878* fails to complement *Df(4)M101-62f*, a deficiency that includes the *plexB* gene (Sousa-Neves et al., 2005), with respect to this lethality phenotype (Table 1).

We examined how this *plexB* LOF allele affects several well-studied motor and CNS axonal projections in the developing *Drosophila* embryo (Araujo and Tear, 2003; Ayoob et al., 2004; Landgraf et al., 1997; Van Vactor et al., 1993). The anti-fasciclin II (FasII) monoclonal antibody (mAb) 1D4 labels motor and several CNS axon bundles in the *Drosophila* embryonic nervous system (Van Vactor et al., 1993). *Drosophila* motor axon pathways provide a powerful model for identifying and studying genes involved in motor axon pathfinding and fasciculation (Araujo and Tear, 2003). In S*ema-1a* and *plexA* mutants, motor axons that contribute to the
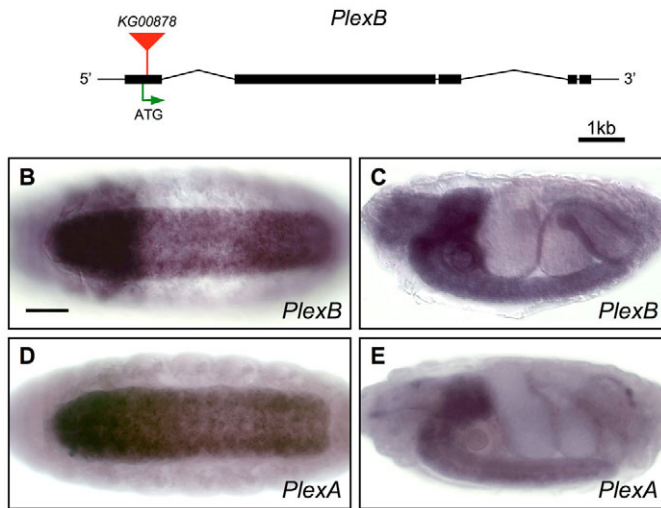
Fig. 1. *Drosophila* Plexin A and Plexin B share similar embryonic expression patterns. (A) A scale representation of the PlexB gene. ATG and the green arrow indicate the position of the open reading frame start codon. The SUPor-P element used for LOF analyses (KG00878) is inserted 25 amino acids downstream of the PlexB start codon and is denoted by the red line and triangle. **(B-E)** Whole-mount wild-type stage 15-16 embryos hybridized with digoxigenin-labeled cRNA probes specific for the intracellular regions of *plexB* (B,C) and *plexA* (D,E). Both plexin transcripts show robust expression within the ventral nerve cord (VNC). Scale bar in B: ~60 μm for B-E.

intersegmental nerve (ISN), the segmental nerve (SN), and a subset of longitudinally projecting CNS axons, fail to defasciculate from one another, revealing a role for these molecules in axon-axon repulsion (Winberg et al., 1998b; Yu et al., 1998). In *plexB*^{KG00878} homozygous embryos, we observe highly penetrant axon guidance defects affecting these same motor axon bundles, and we do not detect defects in overall muscle morphology (data not shown). Furthermore, *plexB*^{KG00878}/*Df(4)M101-62f* double heterozygous embryos reveal phenotypes that are virtually identical to those observed in homozygous *plexB*^{KG00878} embryos, indicating that the *plexB*^{KG00878} allele is a null, or strong hypomorphic, *plexB* allele (Table 2).

Axons in the ISNb nerve bundle normally separate from the main ISN bundle and further defasciculate from one another when they enter the ventral longitudinal muscle field to innervate muscles 12,

Table 1. Lethality rescue of *PlexB*^{KG00878} mutants

Sibling cross or mating	*Homozygous viable progeny (n)
PlexB^{KG00878}/*ci*^D	5.7% (3272)
PlexB^{KG00878}/*ci*^D ×*Df(4)M101-62f*/*ey*^D	0[†] (150)
UAS:PlexB, elav-GAL4/TM3, Sb; PlexB^{KG00878}/*ci*^D	16.8% (2391)
UAS:HA-PlexA, elav-GAL4/CyO;; PlexB^{KG00878}/*ci*^D	7.2%[‡] (2709)

*Homozygous viable progeny scored as progeny not displaying either the wing vein phenotype from the dominant marker cubitous interruptus (*ci*^D) or the reduced eye phenotype from the eyeless dominant marker (*ey*^D). Because the *ci*^D mutation is homozygous lethal, the expected non-*ci*^D progeny from a sibling cross would be 33% if the 'balanced' mutation is viable. *n* represents the total number of offspring scored.
[†]If *PlexB*^{KG00878}/*Df(4)M101-62f* progeny are viable, they would comprise 25% of total progeny, as *ci*^D/*ey*^D animals are viable.
[‡]Statistically different from values for *PlexB*^{KG00878} homozygous mutants. Fisher's exact test using a 2×2 contingency table; $P<0.03$.

13, 6 and 7 (Fig. 2A,G). In *plexA*^{Df(4)C3} homozygous embryos, these ventral muscles lack innervation in most of the segments examined (Fig. 2B,G) (Winberg et al., 1998b). Interestingly, *plexB*^{KG00878} homozygous embryos display a similar, highly penetrant, defect in the innervation of these muscles (Fig. 2C,G; Table 2). To confirm that the defects we observe are due to the loss of PlexB function in *plexB*^{KG00878} embryos, we performed a rescue experiment by providing exogenous *plexB* to homozygous *plexB*^{KG00878} mutants using the UAS/GAL4 system (Brand and Perrimon, 1993). Expression of a *UAS-plexB* transgene in all neurons in a *plexB*^{KG00878} homozygous background, using the *elav-GAL4* transactivator, significantly rescues these ISNb motor axon defects, indicating that *plexB* is required for establishing this motor axon pathway (Fig. 2D; Table 2). Restoring *plexB* to all neurons also rescues the lethality associated with the *plexB*^{KG00878} mutants, demonstrating an essential requirement for neuronal PlexB during development (Table 1). These results define a crucial role for PlexB in establishing the ISNb pathway. Furthermore, ISNb defects in *plexB*^{KG00878} mutants resemble those observed in *plexA*^{Df(4)C3} mutants, suggesting that these plexin receptors have overlapping functions and may share downstream signaling components.

Motor axons in the SNa pathway are also affected in *plexB*^{KG00878} mutant embryos. In wild-type embryos, SNa motor axons navigate dorsally, pass the ventral muscle field innervated by the ISNb motor axons, and then defasciculate from one another to send one bundle of axons posteriorly to innervate muscles 5 and 8 and another dorsally between muscles 22 and 23. One motoneuron, derived from neuroblast clone 3-2 (Landgraf et al., 1997; Schmid et al., 1999), extends an axon that defasciculates from the dorsal-most SNa bundle and makes a characteristic turn en route to forming synaptic arborizations on muscle 24 (Fig. 3A,G). This most distal SNa axon is often unable to separate from the dorsal SNa bundle in *plexA*^{Df(4)C3} homozygous embryos and so fails to innervate muscle 24, resulting in a 'stall' phenotype (Fig. 3B,G) (Winberg et al., 1998b). We observe this SNa stall phenotype in one quarter of all hemisegments in *plexB*^{KG00878} mutants (Fig. 3C″; Table 2). However, the predominant SNa pathfinding phenotype (Fig. 3B,G) we observe in *plexB*^{KG00878} mutants, seen in almost one half of all hemisegments but not observed in *plexA* mutants, is the incorrect anterior projection of the SNa dorsal branch between muscles 21 and 22 instead of more posteriorly between muscles 22 and 23 (Fig. 3C′). We further classified these anteriorly misprojecting SNa motor axons into those that take the wrong path but subsequently make two turns to reach muscle 24 ('double turn'; Fig. 3C′,G; 33.1% of all hemisegments), and those that take the wrong path and are unable to reach their proper target ('lost'; not shown; 14.7% of all hemisegments; see Table 2). When we restore *plexB* to the nervous system of *plexB*^{KG00878} mutants, the overall penetrance of total SNa defects is reduced by over 50% (Fig. 3D; Table 2). Therefore, PlexB is essential for normal SNa motor axon bundle formation and pathfinding. Furthermore, these data demonstrate unique and shared roles for PlexB and PlexA in motor axon guidance.

In addition to serving crucial functions in establishing motoneuron connectivity, both PlexB and PlexA play important roles in CNS axon guidance events. Three longitudinal axon bundles that reside on each side of the midline within the CNS are revealed by the 1D4 anti-FasII mAb (Fig. 4A,G) (Grenningloh et al., 1991). Previous observations show that in *plexA*^{Df(4)C3} and *Sema-1a*^{P1} homozygous embryos, the outermost FasII-positive axon bundle is reduced in thickness and is discontinuous along its entire length (Fig. 4B,G) (Winberg et al., 1998; Yu et al., 1998). However, in contrast to *plexA*^{Df(4)C3} mutants, we observe in *plexB*^{KG00878} mutants that the

Table 2. ISNb and SNa phenotypes of *plexin B* LOF mutants

Genotype	*Abnormal ISNb pathways (*n*) [bypass]	†Abnormal SNa pathways (*n*)	Distinct SNa defect‡		
			dt	stall	lost
Wild type	12.6% (294) [0]	5.5% (293)	1.0%	4.4%	0.0%
PlexB^{KG00878}/+	15.3% (157) [2.0%]	25.9% (155)	2.6%	22.6%	0.6%
PlexB^{KG00878}/*PlexB*^{KG00878}	55.3% (349) [16.0%]	73.6% (341)	33.1%	25.8%	14.7%
PlexB^{KG00878}/*Df(4)M101-62f*	57.4% (129) [10.9%]	76.9% (127)	24.4%	28.3%	22.0%
UAS:PlexB, elav-GAL4/+; PlexB^{KG00878}/*PlexB*^{KG00878}	20.0% (115) [0.9%]	36.3% (124)	10.5%	20.2%	4.8%
UAS:HA-PlexA, elav-GAL4/+;; PlexB^{KG00878}/*PlexB*^{KG00878}	40.9%§ (215) [8.4%]¶	74.2% (213)	36.2%	20.2%	18.3%
PlexA$^{Df(4)C3}$/*PlexA*$^{Df(4)C3}$	65.7% (140) [10.7%]	60.3% (136)	3.7%	50.0%	4.4%
UAS:PlexB, elav-GAL4/+; PlexA$^{Df(4)C3}$/*PlexA*$^{Df(4)C3}$	81.2% (144) [23.6%]	81.0% (137)	2.2%	71.5%	7.3%

*Abnormal ISNb phenotype defined as the failure of ISNb axons from the RP5, V or RP3 neurons to properly innervate ventral longitudinal muscles 12/13 or 6/7. Phenotypes include weak or absent innervations, target bypasses and axon bundle stalling. The number of hemisegments scored for each genotype is listed as *n*. Bypass indicates the percentage of all segments in which the ISNb fails to fully or partially separate away from the ISN.
†Abnormal SNa phenotype defined as the failure of SNa axons to defasciculate, to reach muscle 24 and/or to project along the appropriate route. The number of hemisegments scored for each genotype is listed as *n*.
‡'dt', 'stall' and 'lost' are distinct phenotypes that comprise the total SNa defects. dt (double turn) indicates the percentage of all hemisegments in which the dorsal SNa projects inappropriately between muscles 21 and 22 and ultimately reaches muscle 24. stall defines the population of SNa axons that are unable to reach muscle 24. lost represents the percentage of dorsal SNa projections that fail to reach muscle 24 after inappropriately projecting between muscles 21 and 22.
§Statistically different from values for *PlexB*^{KG00878} homozygous mutants. Fisher's exact test using a 2×2 contingency table; *P*<0.0005.
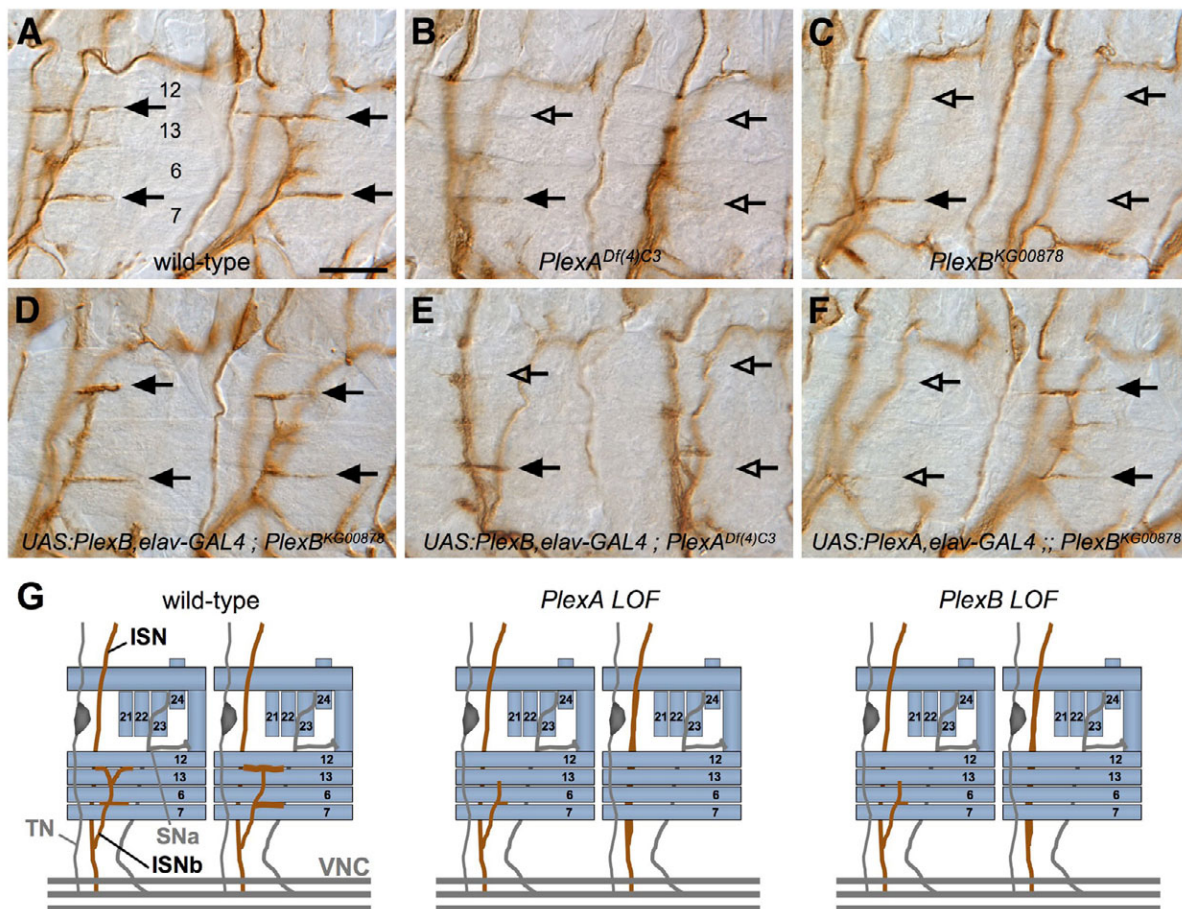¶Statistically different from values for *PlexB*^{KG00878} homozygous mutants; *P*<0.01.

Fig. 2. ISNb motor axon pathfinding defects in *plexB* mutants resemble *plexA* mutants. (A-F) Filleted preparations of late stage 16 embryos stained with the anti-Fasciclin II monoclonal antibody to reveal motor axons of the ISNb. Arrows and open arrows indicate proper and absent innervation, respectively, by axons of the ISNb. Anterior, left; dorsal, up. (**A**) In a wild-type embryo, axons within the ISNb innervate the ventral longitudinal muscles 12, 13, 6 and 7 (arrows). (**B**) ISNb motor axons fail to defasciculate in *plexA*$^{Df(4)C3}$ mutants and often do not innervate their proper muscle targets (open arrows). (**C**) *plexB*^{KG00878} mutant ISNb motor axon pathways, like *plexA*$^{Df(4)C3}$ mutants, also fail to reach their proper muscle targets (open arrows). (**D**) Neuronal expression of *plexB* in a *plexB*^{KG00878} mutant background restores proper neuromuscular connectivity (arrows). (**E**) Neuronal *plexB* expression in a *plexA*$^{Df(4)C3}$ mutant background completely fails to rescue the *plexA*$^{Df(4)C3}$ mutant phenotype (open arrows). (**F**) Neuronal *plexA* expression in a *plexB*^{KG00878} mutant background partially rescues the *plexB*^{KG00878} mutant phenotype (absent, left, and normal, right, innervation are shown). (**G**) Schematics of two adjacent hemisegments illustrating ISNb phenotypes observed in wild type (left), *plexA* mutants (middle) and *plexB* mutants (right). Scale bar in A: 10 μm for A-F.

outermost FasII-positive axon bundle remains intact but the medial FasII-positive axon tract is severely defasciculated along its length in all embryos examined (Fig. 4C,G). This fully penetrant *plexB*^{KG00878} CNS phenotype is rescued by neuronal expression of a *plexB* transgene (Fig. 4D). Therefore, PlexA and PlexB are independently responsible for the formation of two distinct axon bundles within the embryonic CNS. Taken together, these results show that PlexA and PlexB not only function collaboratively to establish axon trajectories, but also serve unique roles in peripheral and central nervous system axon guidance.

plexA partially rescues *plexB*, but *plexB* fails to rescue *plexA*

Our analysis of *plexB*^{KG00878} mutants reveals overlapping and non-overlapping PlexA and PlexB functions. ISNb phenotypes in both mutants are quite similar, whereas CNS defects are distinct. SNa pathway defects in *plexB*^{KG00878} mutants display both unique and shared phenotypes compared with those observed in *plexA*$^{Df(4)C3}$ mutants. These similarities and differences provide an in vivo experimental system with which to address whether members of different classes of plexins serve redundant or unique functions in embryonic axon guidance events. To directly address this issue, we expressed a *plexA* cDNA in all neurons in the *plexB*^{KG00878} homozygous mutant background, and a *plexB* cDNA in all neurons in the *plexA*$^{Df(4)C3}$ homozygous mutant background. In the latter case, the *plexB* transgene was unable to rescue any of the ISNb, SNa or CNS defects we observe in *plexA*$^{Df(4)C3}$ mutants (Fig. 2E, Fig. 3E, Fig. 4E; Table 2). Moreover, the *plexA*$^{Df(4)C3}$ defects affecting the ISNb and SNa are enhanced by 19% and 25%, respectively, when *plexB* is expressed in this genetic background (Table 2).

The *plexA* transgene is, however, partially able to compensate for *plexB* in certain contexts. Neuronal expression of *plexA* significantly rescues the total number of *plexB*^{KG00878} ISNb defasciculation defects by 25%, and the more severe ISNb bypass phenotypes by
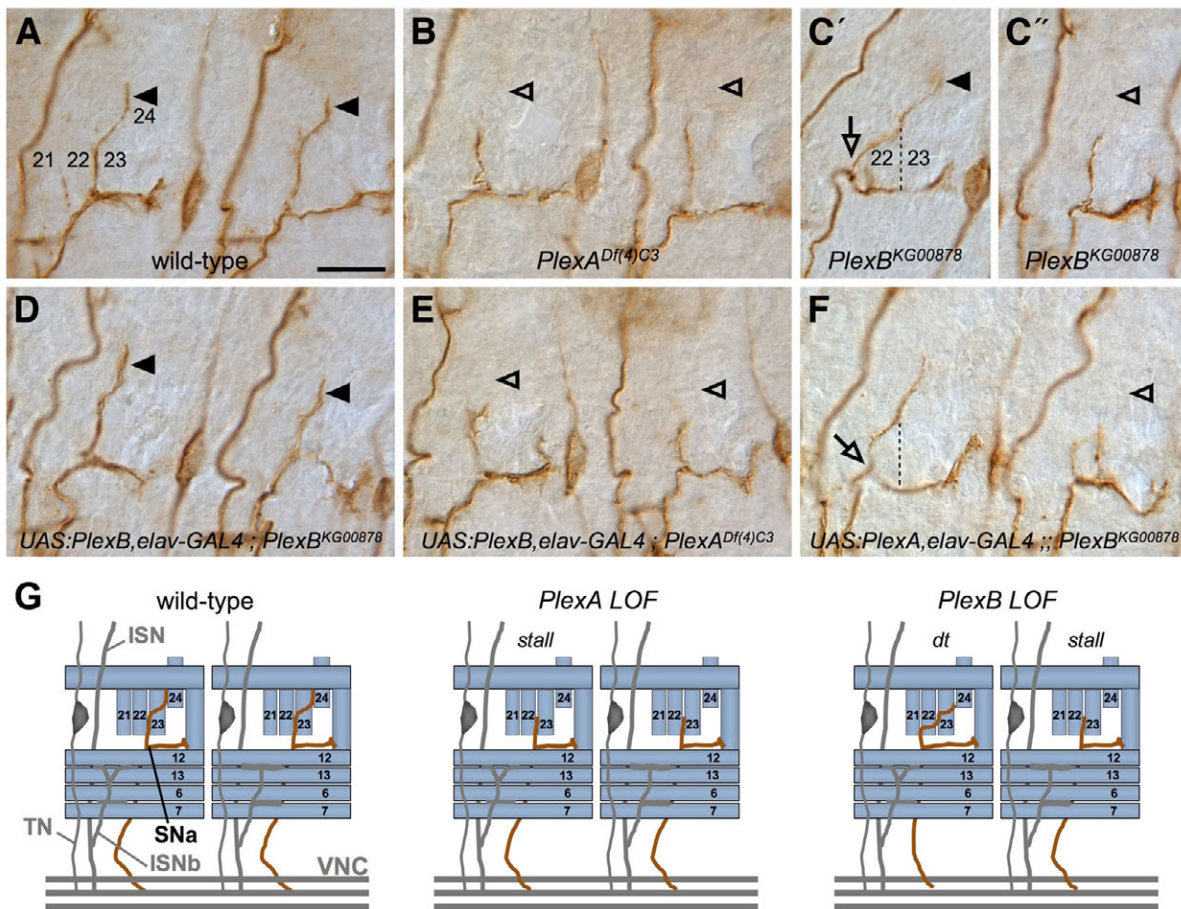

Fig. 3. SNa motor axon pathfinding defects in *plexB* mutants resemble *plexA* mutants and display novel guidance errors. (A-F) Filleted preparations of late stage 16 embryos stained with the anti-Fasciclin II monoclonal antibody to reveal motor axons of the SNa. Anterior, left; dorsal, up. (**A**) In wild-type embryos, the dorsal branch of the SNa projects correctly between muscles 22 and 23 before defasciculating and innervating muscle 24 (arrowhead). (**B**) In *plexA*$^{Df(4)C3}$ mutants, the dorsal-most projecting axon of the SNa fails to reach its proper target, muscle 24 (open arrowhead). (**C′**) *plexB*^{KG00878} mutant SNa pathways show defects in pathfinding choice. This mutant SNa axon bundle projects incorrectly between muscles 21 and 22 (open arrow), and then extends toward its proper target, muscle 24. The dashed line indicates the path that this bundle of SNa axons normally follows. (**C″**) *plexB*^{KG00878} mutants, like *plexA*$^{Df(4)C3}$ mutants, also show a lack of muscle 24 innervation (open arrowhead). (**D**) Neuronal expression of *plexB* in a *plexB*^{KG00878} mutant background restores proper neuromuscular connectivity (arrowheads). (**E**) Neuronal *plexB* expression in a *plexA*$^{Df(4)C3}$ mutant background fails to rescue the *plexA*$^{Df(4)C3}$ mutant phenotype (open arrowheads). (**F**) Neuronal expression of *plexA* in a *plexB*^{KG00878} mutant background fails to rescue the *plexB*^{KG00878} mutant pathfinding and innervation defects (open arrow and open arrowhead, respectively). (**G**) Schematics of two adjacent hemisegments illustrating SNa phenotypes observed in wild type (left), *plexA* mutants (middle) and *plexB* mutants (right). Scale bar in A: 10 μm for A-F.
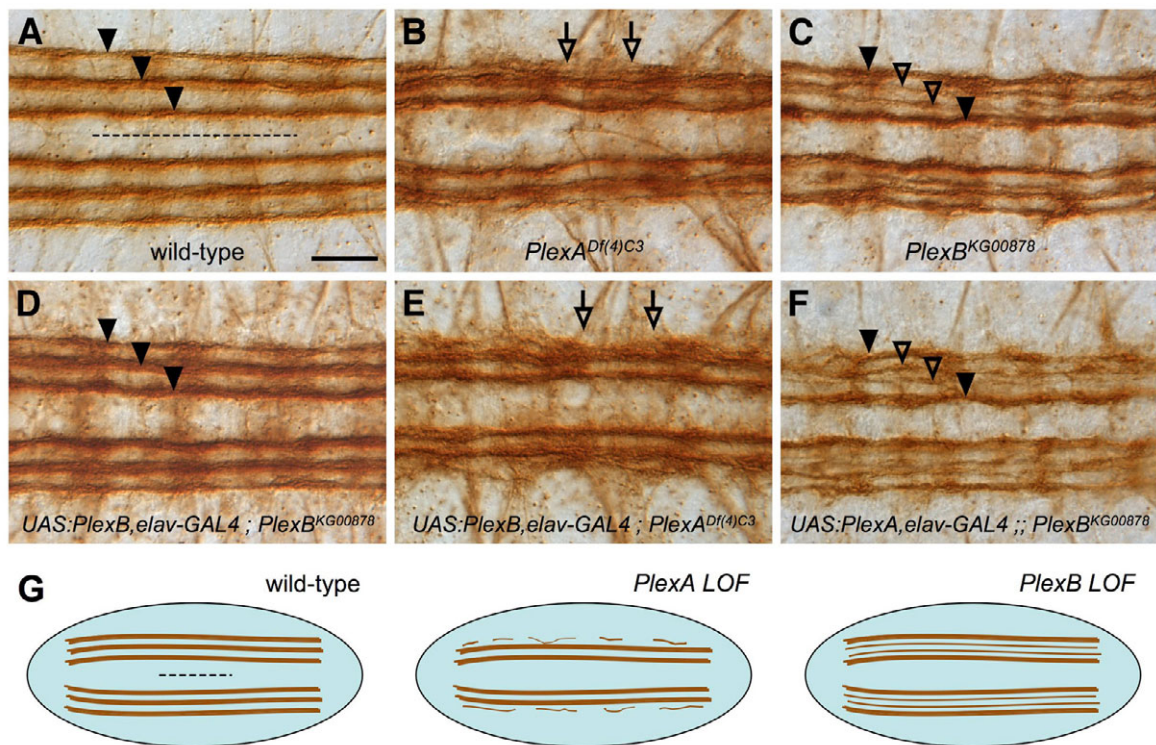
Fig. 4. *plexB* mutants display a novel CNS axon bundling defect. (A-F) Filleted preparations of late stage 16 embryos stained with the anti-Fasciclin II monoclonal antibody to reveal three fascicles of longitudinally projecting axon bundles on both sides of the midline (dashed line in A) within the ventral nerve cord. Anterior, left. (**A**) In a wild-type embryo the three bundles of axons on either side of the midline are tightly fasciculated (arrowheads). (**B**) The outermost bundle of axons in *plexA^Df(4)C3^* mutants is disrupted at several places along the embryo (open arrows). (**C**) The medial bundle of Fasciclin II-positive axons in *plexB^KG00878^* mutants is split apart and appears as two separate fascicles (open arrowheads). (**D**) Neuronal expression of *plexB* in a *plexB^KG00878^* mutant background restores proper bundling of the medial fascicle (arrowheads). (**E**) Neuronal *plexB* expression in a *plexA^Df(4)C3^* mutant background fails to rescue the *plexA^Df(4)C3^* mutant phenotype (open arrows). (**F**) Neuronal *plexA* expression in a *plexB^KG00878^* mutant background fails to rescue the *plexB^KG00878^* mutant phenotype (open arrowheads). (**G**) Schematics illustrating CNS phenotypes observed in wild type (left), *plexA* mutants (middle) and *plexB* mutants (right). Scale bar in A: 10 μm for A-F.

almost 50% (Fig. 2F; Table 2). An interesting trend is also seen in the SNa pathway. *plexA* does not rescue the 'double turn' or 'lost' phenotypes of *plexB^KG00878^* mutants, phenotypes observed only in *plexB^KG00878^* and not *plexA^Df(4)C3^* mutants. *plexA* does, however, reduce the incidence of *plexB^KG00878^* SNa 'stall' phenotypes in *plexB^KG00878^* mutants to the same degree as does *plexB* (a 20% reduction; Table 2). *plexA* also provides a modest reduction in the lethality observed in *plexB^KG00878^* mutants (Table 1). *plexA* expression does not reduce the total fraction of defective SNa pathways and is incapable of rescuing *plexB^KG00878^* CNS phenotypes (Fig. 3F, Fig. 4F; Table 2). Although PlexA is able to partially substitute for PlexB in the ISNb pathway, *plexB* does not resuce any *plexA^Df(4)C3^* phenotypes. These results show that PlexA and PlexB are unable to substitute for each other in rescuing phenotypes not common to both mutants, indicating there are differences, perhaps at the level of activating ligands, which distinguish PlexA from PlexB signaling.

PlexB, like PlexA, signals through MICAL

In our LOF analyses and reciprocal rescue experiments, we observe similar ISNb pathway defects in *plexA^Df(4)C3^* and *plexB^KG00878^* mutants, suggesting a functional redundancy for PlexA and PlexB in ISNb pathfinding. Therefore, we sought to identify other common links between these two receptors to better understand the mechanisms underlying their related functions in motor axon

pathfinding. Previously, genetic interactions between both *Drosophila* plexins and the small GTPase *Rac* were observed in a GOF paradigm; increasing the levels of *plexA* or *plexB* enhances the phenotype produced by the expression of a dominant-negative *Rac* mutant in all neurons (Hu et al., 2001). Although genetic interactions are observed between each plexin and *Rac*, a physical association was observed only between PlexB and Rac (Hu et al., 2001). Conversely MICAL, which is required for PlexA signaling, binds to PlexA but not to PlexB (Terman et al., 2002). We investigated whether PlexB signaling also uses MICAL, even though these two proteins do not directly associate with one another. In a genetic interaction assay employing embryos doubly heterozygous for both *MICAL* and *plexB*, we observe a robust genetic interaction, whereas no appreciable phenotypes are observed separately in either heterozygous background (Fig. 5A,B). Of the 212 hemisegments scored in *Df(3R)swp2^MICAL^/+; plexB^KG00878^/+* transheterozygous embryos, 58.5% showed ISNb defects. This penetrance is equivalent to that seen for these same ISNb defects in *plexB^KG00878^* homozygous mutants (Table 2). These results support the idea that both PlexA and PlexB share similar downstream signaling mechanisms, as both receptors show genetic interactions with *MICAL* in addition to those observed with *Rac*. Interestingly, each plexin does not physically associate with both MICAL and Rac directly. Therefore, a macromolecular complex including both plexins may provide PlexB and PlexA access to both MICAL and Rac.
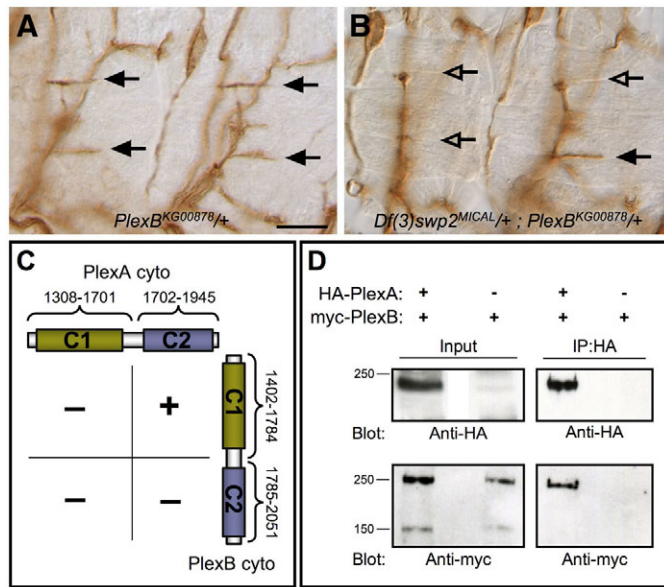
Fig. 5. PlexB genetically interacts with MICAL and forms a complex with PlexA. (A,B) Filleted preparations of late stage 16 embryos stained with the anti-Fasciclin II monoclonal antibody to reveal motor axons of the ISNb. (**A**) ISNb axons of *plexB*[KG00878] heterozygous embryos defasciculate properly and resemble wild-type embryos. (**B**) ISNb axons in embryos double heterozygous for both *plexB* and *MICAL* (*Df(3R)swp2*[MICAL]/+; *plexB*[KG00878]/+) often fail to defasciculate properly and do not innervate their proper muscle targets (open arrows). (**C**) A chart outlining the results from yeast two-hybrid interaction assays with cytoplasmic regions of PlexA and PlexB. Numbers indicate the amino acids of PlexA or PlexB that comprise each fragment. A positive interaction is indicated by a plus sign (+). The C2 region of the PlexA cytoplasmic domain (animo acids 1702-1945) and the C1 region of the PlexB cytoplasmic domain (amino acids 1402-1784) support growth and reporter gene transcription when grown on selective plates, whereas all other combinations between PlexA and PlexB do not. (**D**) Lysates from embryos expressing Myc-PlexB, with or without HA-PlexA, were immunoprecipitated using anti-HA antibodies and blotted with anti-HA or anti-Myc antibodies to detect the presence of HA-PlexA or Myc-PlexB, respectively. HA-PlexA immunoprecipitates from embryo lysates also contain Myc-PlexB. Scale bar in A: 10 μm for A-B.

Neuronal PlexA and PlexB associate in vivo

The ability of PlexA to rescue the PlexB ISNb phenotypes common to both *plexA* and *plexB* mutants, and select transheterozygous interactions between *plexB* and *MICAL*, together indicate that PlexA and PlexB are likely to function in a concerted fashion to establish the ISNb pathway. We investigated, therefore, whether these two plexin receptors physically associate with one another. Using a yeast interaction assay, we assessed interactions between the cytoplasmic domains of PlexA and PlexB. Similar to vertebrate plexin A1 and plexin B1 (Usui et al., 2003), we observed an interaction between the C2 domain of PlexA and the C1 domain of PlexB (Fig. 5C). Next, we investigated whether PlexA and PlexB associate in vivo in neurons. We expressed a hemagglutinin (HA)-tagged version of PlexA (HA-PlexA) and a Myc-tagged version of PlexB (Myc-PlexB) selectively in the nervous system of *Drosophila* embryos using the UAS-GAL4 system. Immunoprecipitation of HA-PlexA using an anti-HA antibody co-immunoprecipitates Myc-PlexB (Fig. 5D). Importantly, we do not detect Myc-PlexB in HA precipitates from flies expressing only Myc-PlexB and not HA-PlexA (Fig. 5D), demonstrating the absence of non-specific anti-HA association with PlexB. These

experiments show that PlexA and PlexB can indeed form heteromultimeric complexes in vivo, suggesting that this association enables access of PlexA and PlexB to both Rac and MICAL.

Plexin B binds to Sema-2a

Although we find similarities in PlexB and PlexA requirements for certain motor axon guidance events, our results also show that each plexin serves non-redundant PNS and CNS guidance functions. PlexA binds to the extracellular (EC) domains of the transmembrane semaphorins Sema-1a and Sema-1b, and genetic experiments provide support for the functional significance of this interaction in the establishment of neuromuscular connectivity (Winberg et al., 1998b). Does PlexB bind to these transmembrane semaphorins, or might PlexB be a receptor for the previously characterized class 2 secreted semaphorin Sema-2a, for which a receptor has yet to be identified (Kolodkin et al., 1993; Matthes et al., 1995; Winberg et al., 1998a)? Most vertebrate secreted semaphorins do not bind to plexins directly but, rather, use neuropilin proteins as ligand-binding components of a semaphorin receptor complex (Kruger et al., 2005). Interestingly, in the developing vasculature the secreted semaphorin Sema3E signals repulsion in a neuropilin-independent manner through a divergent plexin, plexin-D1, demonstrating that secreted semaphorins are indeed able to bind directly to plexin receptors (Gu et al., 2005). As there are no neuropilin orthologs in *Drosophila*, we sought to determine whether the secreted semaphorin Sema-2a binds to PlexB. We performed ligand-binding assays using alkaline phosphatase (AP)-tagged semaphorins and S2R+ cells, a *Drosophila*-derived adherent cell line (Yanagawa et al., 1998), transfected with cDNAs encoding either PlexB or green fluorescent protein (GFP). Sema-2a-AP robustly binds to cells expressing PlexB but not to cells expressing GFP (Fig. 6A). Scatchard analysis reveals a dissociation constant of ~2nM for this association (Fig. 6D). This affinity is similar to that for Sema-1a or Sema-1b binding to PlexA (Winberg et al., 1998b). We observed no significant binding of Sema-1a[EC]-AP, AP-Sema-1b[EC], or AP alone to PlexB- or GFP-expressing cells (Fig. 6B; data not shown). The interactions between Sema-1a or Sema-1b with PlexA in vitro are dependent on divalent metal ions (Winberg et al., 1998b). However, this is not the case for Sema-2a binding to PlexB. Although EDTA reduces S2R+ cell substrate adhesion, it does not block Sema-2a-AP binding to PlexB-expressing cells (Fig. 6C). These results, showing that PlexB binds to Sema-2a but not to class 1 transmembrane semaphorin ligands, reveal a major distinction between PlexA and PlexB. Therefore, the activation of each receptor by different ligands is a likely mechanism underlying the differences we observe between *plexA* and *plexB* SNa and CNS mutant phenotypes.

PlexB is a functional Sema-2a receptor in vivo

To determine whether Sema-2a binding to PlexB is functionally significant, we examined this interaction in vivo using a genetic assay. When *Sema-2a* is expressed in all muscles using the 24B-GAL4 transactivator, it leads to aberrant formation of the transverse nerve (TN), and inhibits the innervation of muscles 6 and 7 by the RP3 motoneuron (Winberg et al., 1998a). Our analysis confirms that the TN phenotype observed in this GOF paradigm is particularly robust. The TN normally forms by the fasciculation of the TN motoneuron axon emanating from the nerve cord with a ventrally projecting axon from the peripherally located lateral bipolar dendritic (LBD) neuron (Bodmer and Jan, 1987; Gorczyca et al., 1994; Schmid et al., 1999; Thor and Thomas, 1997; Winberg et al., 1998a). These two axonal processes extend towards each other over the body wall muscles near the segment boundary and then
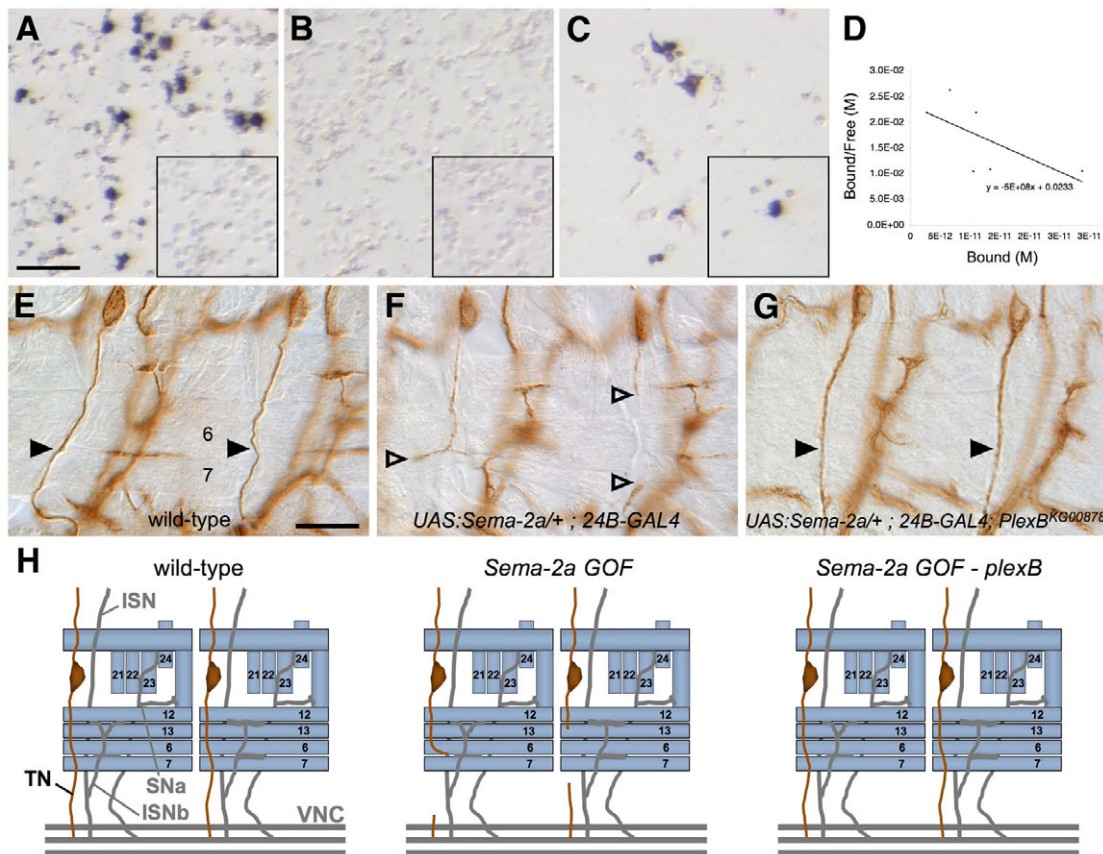
DEVELOPMENT

Fig. 6. PlexB is a Sema-2a receptor. (A-C) Cultured S2R+ cells transfected with cDNAs for either PlexB or GFP to which Sema-2a-AP, Sema-1a[EC]-AP, or AP-Sema-1b[EC] was added. (**A**) AP-tagged Sema-2a binds to the surface of S2R+ cells transfected with PlexB. Sema-2a-AP does not bind to S2R+ cells transfected with GFP (inset). (**B**) Sema-1a[EC]-AP and AP-Sema-1b[EC] (inset) do not bind to S2R+ cells transfected with PlexB. (**C**) Sema-2a-AP binds to PlexB in the presence of 1 mM and 10 mM (inset) EDTA. (**D**) Scatchard analysis of Sema-2a-AP binding to PlexB-expressing S2R+ cells. Data points were normalized to Sema-2a-AP binding to GFP-expressing cS2R+ cells. (E-G) Filleted preparations of late stage 16 embryos stained with the anti-Fasciclin II monoclonal antibody to reveal the transverse nerve (TN). (**E**) In a wild-type embryo, in the vicinity of the ventral longitudinal muscle field two axonal projections come together to form the TN (arrowheads). (**F**) Expression of Sema-2a in all muscles inhibits the TN from forming properly. Axons from the TN motoneuron and LBD neuron, which normally fasciculate and form the TN, stall and are unable to complete the nerve (open arrowheads). (**G**) Using the *plexB*[KG00878] mutant allele to remove one copy of *plexB* from this *Sema-2a* GOF background restores the formation of the TN (arrowheads). (**H**) Schematics of two adjacent hemisegments illustrating the TN phenotypes observed in wild type (left), *Sema-2a* overexpressing embryos (middle), and *Sema-2a* overexpressing embryos with one copy of *plexB* removed (right). Scale bars: in A, 50 μm for A-C; in E, 10 μm for E-G.

fasciculate in the vicinity of muscles 6 and 7 (Fig. 6E,H). When *Sema-2a* is overexpressed in all muscles, we see an appreciable failure of TN formation (27.9% of 233 hemisegments). This shows that ectopically expressing *Sema-2a* in muscles, including in muscles 6 and 7 over which the TN motor and LBD axons meet, repels these axons and keeps them from entering this region, causing them to stall or enter inappropriate areas, and thus preventing TN formation (Fig. 6F). When we remove one copy of *plexB* in this same genetic background, the TN phenotype is greatly reduced in severity (12.2% of 230 hemisegments; *P*<0.00005; Fig. 6G). The requirement for PlexB in order to observe the full penetrance of this *Sema-2a*-dependent GOF phenotype, together with our observation that AP-Sema-2a binds to PlexB expressed on insect cells in vitro, demonstrate that PlexB functions as a Sema-2a receptor.

DISCUSSION
Plexin receptors expressed at the leading edge of navigating axonal growth cones receive and transduce instructive signals encoded by semaphorins. Deciphering how plexins translate external stimuli into

intracellular responses is paramount for understanding how the nervous system is wired. Analysis of neural development in *Drosophila* allows for direct functional comparisons between plexins from distinct classes, as *Drosophila* contains only two plexin proteins. In this study, we have analyzed *plexB* mutants and compared the phenotypes we observe to those found in *plexA* mutants, detecting both similarities and differences in the LOF phenotypes of these two genes. We identify a direct physical interaction between PlexA and PlexB, and demonstrate a convergence of these signaling pathways upon the effector molecule MICAL. We also show that these two receptors bind to semaphorin ligands from different classes. These results demonstrate that plexins from different classes can respond in vivo to distinct semaphorin ligands, but have the capacity to work cooperatively using common downstream signaling molecules.

Drosophila Plexins in axon guidance
In *Drosophila*, PlexA is required for the proper defasciculation of motor and CNS axon bundles (Winberg et al., 1998b). This axon-axon repulsion enables individual axons to overcome the adhesive

forces holding them together, to separate from each other, and to innervate their appropriate targets (Winberg et al., 1998b; Yu et al., 1998; Yu et al., 2000). Here, we examined the role played by PlexB in motor and CNS axon pathfinding during *Drosophila* embryogenesis and found that *plexB* mutants display defects in axon fasciculation that dramatically affect pathfinding. Similar to what has been observed in *plexA* mutants, *plexB* mutants display a failure of ISNb motor axons to initially separate from the main ISN bundle or, at later stages of ISNb pathway formation, to separate from other ISNb axons. Defasciculation errors similar to those observed in *plexA* mutants are also observed for the dorsal branch of the SNa in *plexB* mutants. However, other *plexB* mutant SNa axon bundles display navigation phenotypes not seen in *plexA* mutants. These dorsal SNa axons follow an aberrant trajectory to their target, muscle 24, and as a consequence are often unable to reach this post-synaptic partner. In the CNS, however, PlexB and PlexA play distinct roles. Loss of *plexA* disrupts the contiguity of the outermost bundle of axons, whereas losing *plexB* causes excessive defasciculation of the medial tract. This differential requirement for plexins in medial and lateral FasII-postive CNS axon bundles is strikingly reminiscent of the specific requirements for differential expression of roundabout (Robo) proteins to regulate the formation of the inner, medial and lateral FasII-positive axon tracts (Rajagopalan et al., 2000; Simpson et al., 2000). Determining whether the positioning and consolidation of CNS longitudinal tracts by Robos and plexins are separate or integrated processes will lend insight into how axons respond simultaneously to distinct guidance influences that serve to regulate neuropil organization.

Plexins belonging to different classes can act cooperatively to guide the same neuronal trajectories

Although unique axonal fasciculation and pathfinding defects are observed in *plexA* and *plexB* mutants, ISNb motor axon phenotypes in these mutants are remarkably similar. This suggests that plexins from different classes may function collaboratively to pattern certain neuronal trajectories. *Drosophila* provides a robust experimental model with which to examine this issue. As there are only two *Drosophila* plexins, we performed cross-rescue experiments with *plexA* and *plexB*. Expression of *plexA* in a *plexB* mutant background significantly reduces the severity of *plexA* ISNb defects, although it does not fully rescue these defects. *plexA* expression is, however, unable to rescue the SNa and CNS phenotypes that we observe in *plexB*, but not *plexA*, mutants. In the reciprocal experiment, PlexB cannot replace any PlexA function, either in motor axon pathways or in the CNS. Our immunoprecipitation and genetic interaction experiments provide an explanation for why PlexB cannot substitute for PlexA. When we express epitope-tagged versions of PlexA and PlexB in vivo, immunoprecipitating PlexA brings down PlexB, indicating that these two receptors can associate in a complex in vivo. Furthermore, for ISNb pathway phenotypes, we observe genetic interactions between *plexB* and *MICAL* heterozygotes, strongly supporting a requirement for MICAL in PlexB signaling, although these two proteins do not interact directly. We propose that PlexB gains access to MICAL through its association with PlexA. Because MICAL is a crucial downstream signaling component for plexin-mediated axonal repulsion, PlexA may be able to substitute in a limited fashion for PlexB through its ability to recruit MICAL and mediate repulsion of ISNb axons. However, the inability of PlexB to substitute at all for PlexA may stem from its inability to directly recruit MICAL.

Two other transmembrane proteins play important roles in PlexA-mediated axon guidance events and may facilitate the formation of complexes that contain PlexB and PlexA. The catalytically inactive receptor tyrosine kinase Off-track (Otk), which binds to and functions with PlexA in Sema-1a signaling, is also able to associate with two vertebrate plexins from classes A and B (Winberg et al., 2001). It is unknown whether Otk binds to *Drosophila* PlexB. However, in the *Drosophila* CNS, Otk may function separately with PlexA and PlexB. *Otk* mutants display a disrupted outer Fas-II-positive fascicle, a phenotype specific to *plexA*, and also a defasciculated middle Fas-II-positive axon bundle, a phenotype specific to *plexB* (Winberg et al., 2001). Overexpression of another PlexA signaling component produces phenotypes also seen in *plexB* mutants. Increasing in all neurons the levels of *Gyc76C*, a receptor guanylyl cyclase involved in PlexA signaling, produces an SNa pathfinding defect very similar to the 'double turn' SNa phenotype seen in *plexB* mutants (Ayoob et al., 2004). Future work will reveal whether either of these transmembrane proteins involved in PlexA signaling serve as co-receptors for PlexB ligands and participate in the PlexB signaling cascade.

PlexA and PlexB are receptors for different classes of semaphorin ligands

There are five semaphorins in *Drosophila*. Sema-1a and Sema-1b, two class 1 transmembrane semaphorins, bind to PlexA (Winberg et al., 1998b). We find here that AP-tagged versions of the extracellular domains of these transmembrane semaphorins do not bind to PlexB in vitro. However, we do observe robust binding of AP-tagged Sema-2a, a secreted semaphorin, to insect cells expressing PlexB. Our genetic analysis shows that this interaction is indeed functional, as *plexB* LOF suppresses a *Sema-2a* GOF phenotype. Our data also suggest that there are additional PlexB ligands. *plexB* mutants show more severe and complex phenotypes than do the low-penetrance phenotypes reported for *Sema-2a* mutants (Winberg et al., 1998a). Sema-2b, the other *Drosophila* secreted semaphorin, is a likely candidate PlexB ligand. *Sema-2b* resides at cytolocation 53C4 on chromosome 2 and is only separated from *Sema-2a* by a few genes. Sema-2a and Sema-2b share 70% amino acid identity (84% similarity), and it seems likely this semaphorin duo is a product of a genetic duplication and that these two secreted semaphorins share certain neuronal signaling functions. *Sema-2b* is expressed in a small subset of neurons within the CNS suggesting that, alone, or in combination with Sema-2a, it is responsible for maintaining the medial bundle of longitudinally projecting CNS axons as a tight fascicle (J.C.A., J.R.T. and A.L.K., unpublished) (Kolodkin et al., 1993; Rajagopalan et al., 2000). Consistent with findings for class A and B plexins in vertebrates, we find that PlexA and PlexB in *Drosophila* serve as receptors for different classes of semaphorins. This specificity provides a basis for postulating distinct functions for the two *Drosophila* plexins in motor and CNS axon guidance. Because secreted semaphorins are not tethered to their substrate, as are transmembrane semaphorins, the range over which these cues might act is greater, enabling PlexB to mediate not only axonal defasciculation, but also growth cone steering and surround repulsion.

In addition to being repulsive axon guidance receptors, plexins also interact homophilically (Hartwig et al., 2005; Ohta et al., 1995). Therefore, it is possible that in some instances PlexB might function in a semaphorin ligand-independent manner, perhaps even as an adhesive molecule. Proteolytic processing may also regulate PlexB function. The extracellular domains of B-class plexins contain a protease site, located close to the plasma membrane, that

is cleaved by a subtilisin-like proprotein convertase (Artigiani et al., 2003). In our western blots of Myc-PlexB extracts, we detect, in addition to full-length PlexB at 250 kDa, a smaller protein at 150 kDa (Fig. 5D). The size of this protein is equal to that of the PlexB ectodomain and correlates well with a predicted PlexB protease cleavage product. We also observe these bands in the lysates of S2R+ cells transfected with Myc-PlexB. Conditioned media from these transfected cells contains only the smaller (150 kDa) form of PlexB, presumably the ectodomain released from the membrane and into the media (J.C.A., J.R.T. and A.L.K., unpublished). This proteolytic processing of the PlexB receptor may play a role in the modulation of its activity.

In conclusion, we present evidence that plexin B receptors, like plexin A receptors, are crucial for the generation of neuronal connectivity in vivo. Our results show that A and B class plexins can regulate similar axon guidance events collaboratively, whereas interactions with distinct classes of semaphorin ligands are likely to mediate receptor-specific functions. Further analysis of how these guidance receptors function in *Drosophila* will allow for a better understanding of the complex roles played by plexins during neural development, and will define plexin-mediated convergent and divergent signaling events.

References

Araujo, S. J. and Tear, G. (2003). Axon guidance mechanisms and molecules: lessons from invertebrates. *Nat. Rev. Neurosci.* **4**, 910-922.

Artigiani, S., Barberis, D., Fazzari, P., Longati, P., Angelini, P., van de Loo, J. W., Comoglio, P. M. and Tamagnone, L. (2003). Functional regulation of semaphorin receptors by proprotein convertases. *J. Biol. Chem.* **278**, 10094-10101.

Ayoob, J. C., Yu, H. H., Terman, J. R. and Kolodkin, A. L. (2004). The Drosophila receptor guanylyl cyclase Gyc76C is required for semaphorin-1a-plexin A-mediated axonal repulsion. *J. Neurosci.* **24**, 6639-6649.

Bellen, H. J., Levis, R. W., Liao, G., He, Y., Carlson, J. W., Tsang, G., Evans-Holm, M., Hiesinger, P. R., Schulze, K. L., Rubin, G. M. et al. (2004). The BDGP gene disruption project: single transposon insertions associated with 40% of Drosophila genes. *Genetics* **167**, 761-781.

Bodmer, R. and Jan, Y. N. (1987). Morphological differentiation of the embryonic peripheral neurons in *Drosophila. Roux's Arch. Dev. Biol.* **196**, 69-77.

Brand, A. H. and Perrimon, N. (1993). Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development* **118**, 401-415.

Dickson, B. J. (2002). Molecular mechanisms of axon guidance. *Science* **298**, 1959-1964.

Driessens, M. H., Hu, H., Nobes, C. D., Self, A., Jordens, I., Goodman, C. S. and Hall, A. (2001). Plexin-B semaphorin receptors interact directly with active Rac and regulate the actin cytoskeleton by activating Rho. *Curr. Biol.* **11**, 339-344.

Eaton, S. and Kornberg, T. B. (1990). Repression of ci-D in posterior compartments of Drosophila by engrailed. *Genes Dev.* **4**, 1068-1077.

Flanagan, J. G. and Cheng, H. J. (2000). Alkaline phosphatase fusion proteins for molecular characterization and cloning of receptors and their ligands. *Methods Enzymol.* **327**, 198-210.

Golemis, E. A., Gyuris, J. and Brent, R. (1994). Interaction trap/two hybrid system to identify interacting proteins. In *Current Protocols in Molecular Biology* (ed. Fred M. Ausubel, Roger Brent, Robert E. Kingston, David D. Moore, J. G. Seidman, John A. Smith and Kevin Struhl), pp. 13.14.1-13.14.17. New York: Wiley.

Gorczyca, M. G., Phillis, R. W. and Budnik, V. (1994). The role of tinman, a mesodermal cell fate gene, in axon pathfinding during the development of the transverse nerve in Drosophila. *Development* **120**, 2143-2152.

Grenningloh, G., Rehm, E. J. and Goodman, C. S. (1991). Genetic analysis of growth cone guidance in Drosophila: fasciclin II functions as a neuronal recognition molecule. *Cell* **67**, 45-57.

Gu, C., Yoshida, Y., Livet, J., Reimert, D. V., Mann, F., Merte, J., Henderson, C. E., Jessell, T. M., Kolodkin, A. L. and Ginty, D. D. (2005). Semaphorin 3E and plexin-D1 control vascular pattern independently of neuropilins. *Science* **307**, 265-268.

Hartwig, C., Veske, A., Krejcova, S., Rosenberger, G. and Finckh, U. (2005). Plexin B3 promotes neurite outgrowth, interacts homophilically, and interacts with Rin. *BMC Neurosci.* **6**, 53.

Hirth, F., Kammermeier, L., Frei, E., Walldorf, U., Noll, M. and Reichert, H. (2003). An urbilaterian origin of the tripartite brain: developmental genetic insights from Drosophila. *Development* **130**, 2365-2373.

Hu, H., Marton, T. F. and Goodman, C. S. (2001). Plexin B mediates axon guidance in Drosophila by simultaneously inhibiting active Rac and enhancing RhoA signaling. *Neuron* **32**, 39-51.

Kolodkin, A. L., Matthes, D. J. and Goodman, C. S. (1993). The semaphorin genes encode a family of transmembrane and secreted growth cone guidance molecules. *Cell* **75**, 1389-1399.

Kronhamn, J. and Rasmuson-Lestander, A. (1999). Genetic organization of the ci-M-pan region on chromosome IV in Drosophila melanogaster. *Genome* **42**, 1144-1149.

Kruger, R. P., Aurandt, J. and Guan, K. L. (2005). Semaphorins command cells to move. *Nat. Rev. Mol. Cell. Biol.* **6**, 789-800.

Landgraf, M., Bossing, T., Technau, G. M. and Bate, M. (1997). The origin, location, and projections of the embryonic abdominal motorneurons of Drosophila. *J. Neurosci.* **17**, 9642-9655.

Luo, L., Liao, Y. J., Jan, L. Y. and Jan, Y. N. (1994). Distinct morphogenetic functions of similar small GTPases: Drosophila Drac1 is involved in axonal outgrowth and myoblast fusion. *Genes Dev.* **8**, 1787-1802.

Matthes, D. J., Sink, H., Kolodkin, A. L. and Goodman, C. S. (1995). Semaphorin II can function as a selective inhibitor of specific synaptic arborizations. *Cell* **81**, 631-639.

Negishi, M., Oinuma, I. and Katoh, H. (2005). Plexins: axon guidance and signal transduction. *Cell. Mol. Life Sci.* **62**, 1363-1371.

Ohta, K., Mizutani, A., Kawakami, A., Murakami, Y., Kasuya, Y., Takagi, S., Tanaka, H. and Fujisawa, H. (1995). Plexin: a novel neuronal cell surface molecule that mediates cell adhesion via a homophilic binding mechanism in the presence of calcium ions. *Neuron* **14**, 1189-1199.

Oinuma, I., Katoh, H., Harada, A. and Negishi, M. (2003). Direct interaction of Rnd1 with Plexin-B1 regulates PDZ-RhoGEF-mediated Rho activation by Plexin-B1 and induces cell contraction in COS-7 cells. *J. Biol. Chem.* **278**, 25671-25677.

Oinuma, I., Ishikawa, Y., Katoh, H. and Negishi, M. (2004). The Semaphorin 4D receptor Plexin-B1 is a GTPase activating protein for R-Ras. *Science* **305**, 862-865.

Rajagopalan, S., Vivancos, V., Nicolas, E. and Dickson, B. J. (2000). Selecting a longitudinal pathway: Robo receptors specify the lateral position of axons in the Drosophila CNS. *Cell* **103**, 1033-1045.

Schmid, A., Chiba, A. and Doe, C. Q. (1999). Clonal analysis of Drosophila embryonic neuroblasts: neural cell types, axon projections and muscle targets. *Development* **126**, 4653-4689.

Simpson, J. H., Bland, K. S., Fetter, R. D. and Goodman, C. S. (2000). Short-range and long-range guidance by Slit and its Robo receptors: a combinatorial code of Robo receptors controls lateral position. *Cell* **103**, 1019-1032.

Sousa-Neves, R., Lukacsovich, T., Mizutani, C. M., Locke, J., Podemski, L. and Marsh, J. L. (2005). High-resolution mapping of the Drosophila fourth chromosome using site-directed terminal deficiencies. *Genetics* **170**, 127-138.

Swiercz, J. M., Kuner, R., Behrens, J. and Offermanns, S. (2002). Plexin-B1 directly interacts with PDZ-RhoGEF/LARG to regulate RhoA and growth cone morphology. *Neuron* **35**, 51-63.

Tamagnone, L., Artigiani, S., Chen, H., He, Z., Ming, G. I., Song, H., Chedotal, A., Winberg, M. L., Goodman, C. S., Poo, M. et al. (1999). Plexins are a large family of receptors for transmembrane, secreted, and GPI-anchored semaphorins in vertebrates. *Cell* **99**, 71-80.

Terman, J. R., Mao, T., Pasterkamp, R. J., Yu, H. H. and Kolodkin, A. L. (2002). MICALs, a family of conserved flavoprotein oxidoreductases, function in plexin-mediated axonal repulsion. *Cell* **109**, 887-900.

Thor, S. and Thomas, J. B. (1997). The Drosophila islet gene governs axon pathfinding and neurotransmitter identity. *Neuron* **18**, 397-409.

Toyofuku, T., Yoshida, J., Sugimoto, T., Zhang, H., Kumanogoh, A., Hori, M. and Kikutani, H. (2005). FARP2 triggers signals for Sema3A-mediated axonal repulsion. *Nat. Neurosci.* **8**, 1712-1719.

Usui, H., Taniguchi, M., Yokomizo, T. and Shimizu, T. (2003). Plexin-A1 and plexin-B1 specifically interact at their cytoplasmic domains. *Biochem. Biophys. Res. Commun.* **300**, 927-931.

Van Vactor, D., Sink, H., Fambrough, D., Tsoo, R. and Goodman, C. S. (1993). Genes that control neuromuscular specificity in Drosophila. *Cell* **73**, 1137-1153.

Winberg, M. L., Mitchell, K. J. and Goodman, C. S. (1998a). Genetic analysis of the mechanisms controlling target selection: complementary and combinatorial functions of netrins, semaphorins, and IgCAMs. *Cell* **93**, 581-591.

Winberg, M. L., Noordermeer, J. N., Tamagnone, L., Comoglio, P. M., Spriggs, M. K., Tessier-Lavigne, M. and Goodman, C. S. (1998b). Plexin A is a neuronal semaphorin receptor that controls axon guidance. *Cell* **95**, 903-916.

Winberg, M. L., Tamagnone, L., Bai, J., Comoglio, P. M., Montell, D. and Goodman, C. S. (2001). The transmembrane protein Off-track associates with Plexins and functions downstream of Semaphorin signaling during axon guidance. *Neuron* **32**, 53-62.

Yanagawa, S., Lee, J. S. and Ishimoto, A. (1998). Identification and characterization of a novel line of Drosophila Schneider S2 cells that respond to wingless signaling. *J. Biol. Chem.* **273**, 32353-32359.

Yao, K. M. and White, K. (1994). Neural specificity of elav expression: defining a Drosophila promoter for directing expression to the nervous system. *J. Neurochem.* **63**, 41-51.

Yu, H. H., Araj, H. H., Ralls, S. A. and Kolodkin, A. L. (1998). The transmembrane Semaphorin Sema I is required in Drosophila for embryonic motor and CNS axon guidance. *Neuron* **20**, 207-220.

Yu, H. H., Huang, A. S. and Kolodkin, A. L. (2000). Semaphorin-1a acts in concert with the cell adhesion molecules fasciclin II and connectin to regulate axon fasciculation in Drosophila. *Genetics* **156**, 723-731.