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Slit/Robo-mediated axon guidance in *Tribolium* and *Drosophila*: divergent genetic programs build insect nervous systems

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Abstract

As the complexity of animal nervous systems has increased during evolution, developmental control of neuronal connectivity has become increasingly refined. How has functional diversification within related axon guidance molecules contributed to the evolution of nervous systems? To address this question, we explore the evolution of functional diversity within the Roundabout (Robo) family of axon guidance receptors. In *Drosophila*, Robo and Robo2 promote midline repulsion, while Robo2 and Robo3 specify the position of longitudinal axon pathways. The Robo family has expanded by gene duplication in insects; *robo2* and *robo3* exist as distinct genes only within dipterans, while other insects, like the flour beetle *Tribolium castaneum*, retain an ancestral *robo2/3* gene. Both Robos from *Tribolium* can mediate midline repulsion in *Drosophila*, but unlike the fly Robos cannot be down-regulated by Commissureless. The overall architecture and arrangement of longitudinal pathways are remarkably conserved in *Tribolium*, despite it having only two Robos. Loss of *TcSlit* causes midline collapse of axons in the beetle, a phenotype recapitulated by simultaneous knockdown of both Robos. Single gene knockdowns reveal that beetle Robos have specialized axon guidance functions: *TcRobo* is dedicated to midline repulsion, while *TcRobo2/3* also regulates longitudinal pathway formation. *TcRobo2/3* knockdown reproduces aspects of both *Drosophila robo2* and *robo3* mutants, suggesting that *TcRobo2/3* has two functions that in *Drosophila* are divided between Robo2 and Robo3. The ability of *Tribolium* to organize longitudinal axons into three discrete medial-lateral zones with only two Robo receptors demonstrates that beetle and fly achieve equivalent developmental outcomes using divergent genetic programs.

Keywords

Axon Guidance; Midline; Repulsion; Roundabout; Slit; *Drosophila*; *Tribolium*

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Introduction

During development, neural circuits are formed as axons and dendrites extending from neuronal cell bodies follow a series of short- and long-range guidance cues toward their ultimate synaptic partners (Dickson, 2002; Tessier-Lavigne and Goodman, 1996). The concentration of neurites into a neuropile structure in modern animal nervous systems allows economical and efficient assembly and function of nerve pathways, and requires precise and coordinated control of multiple guidance decisions as axons and dendrites navigate an increasingly complex environment (Landgraf et al., 2003; Mauss et al., 2009; Zlatic et al., 2009). To facilitate this increase in complexity over evolutionary time, existing guidance pathways have been redeployed in diverse contexts, and novel activities have arisen as the repertoire of guidance molecules has expanded.

The midline repellent Slit and its cognate Roundabout (Robo) receptors constitute an ancient and widely conserved axon guidance pathway which regulates midline crossing in the nervous systems of bilaterally symmetric animals (Dickson and Gilestro, 2006; Evans and Bashaw, 2010a). In addition to this canonical midline repulsive role, Slit and its Robo receptors also control diverse axon guidance decisions in insects and vertebrates (Chen et al., 2008; Farmer et al., 2008; Jaworski et al., 2010; Kasthuber et al., 2009; Long et al., 2004; Sabatier et al., 2004). In the *Drosophila* embryonic CNS, the three fly Robo receptors exhibit specialized guidance functions. Robo and Robo2 cooperate to promote Slit-dependent midline repulsion, while Robo2 and Robo3 direct the formation of longitudinal pathways within distinct regions of the neuropile (Evans and Bashaw, 2010b; Rajagopalan et al., 2000a; Rajagopalan et al., 2000b; Simpson et al., 2000a; Simpson et al., 2000b; Spitzweck et al., 2010). Alone among the *Drosophila* Robos, Robo2 can also promote midline crossing in addition to its conventional role in midline repulsion (Evans and Bashaw, 2010b; Simpson et al., 2000b; Spitzweck et al., 2010).

The divergent activities of the three *Drosophila* Robo receptors depend in part on differential expression patterns, and in part on functional distinctions between the receptors themselves (Evans and Bashaw, 2010b; Spitzweck et al., 2010). While *robo* and *robo2* are both required for Slit-dependent midline repulsion, *robo2* cannot functionally replace *robo* in this context. Similarly, neither *robo* nor *robo3* can substitute for *robo2* to promote the formation of axon pathways in the lateral regions of the neuropile. *robo3*'s role in longitudinal pathway formation in intermediate regions, however, does not depend on unique features of Robo3 as both *robo* and *robo2* can substitute for *robo3* in this context (Spitzweck et al., 2010). A complementary series of domain swapping studies from our lab and our colleagues in the Dickson lab recently demonstrated that Robo's role in midline repulsion depends on unique sequences within its cytoplasmic region, while individual extracellular immunoglobulin-like (Ig) domains specify Robo2's singular roles in lateral position and midline crossing (Evans and Bashaw, 2010b; Spitzweck et al., 2010).

How do related receptors acquire divergent axon guidance activities, and how has this contributed to the evolution of nervous systems? The study of conservation and divergence of developmental genetic mechanisms has provided enormous insight into the evolutionary history of animals, from establishing body axes to formation and patterning of the nervous

system. Studies of neural differentiation and the initial formation of axon pathways in arthropods and related animals have provided a glimpse into the variety of neurodevelopmental mechanisms at work in this group (Duman-Scheel et al., 2007; Fischer and Scholtz, 2010; Linne and Stollewerk, 2011; Mayer and Whittington, 2009; Simanton et al., 2009; Stollewerk and Eriksson, 2010; Ungerer et al., 2011). Intriguingly, a recent analysis of axon pathway formation in onychophorans (worm-like relatives of arthropods with unsegmented bodies and unjointed legs) suggested that increasingly precise control of axon guidance may have been crucial to the evolution of the modern arthropod nervous system by facilitating intersegmental coordination between limb and body muscles (Mayer and Whittington, 2009; Whittington and Mayer, 2011). Illuminating the processes by which axon guidance mechanisms have changed over time may provide valuable insight into the evolution of this diverse and highly successful group of animals. While researchers have begun to investigate the function of conserved axon guidance genes in arthropods other than *Drosophila* (Clemons et al., 2011; Haugen et al., 2011; Linne and Stollewerk, 2011), a direct examination of the evolutionary diversification of axon guidance receptor function has not been attempted.

Here we describe the role of Slit/Robo signaling in midline repulsion and lateral positioning of longitudinal pathways in the beetle *Tribolium castaneum*. Unlike *Drosophila*, *Tribolium* retains an ancestral set of insect Robo receptors, allowing us to ask how Robo axon guidance functions have changed during evolution. We find that Slit/Robo-mediated midline repulsion is well conserved in *Tribolium*, but that Robo receptor control of longitudinal pathway formation differs from *Drosophila*. In *Tribolium*, a single ancestral Robo2/3 receptor controls distinct aspects of longitudinal pathway formation that in *Drosophila* are subdivided between Robo2 and Robo3. We also report that orthologs of Robo2/3 are not found outside insects, perhaps suggesting that expansion and functional diversification of the Robo family has contributed to the evolution of nervous system connectivity in insects and their relatives.

Materials and Methods

Tribolium culture and embryo collection

Tribolium castaneum were obtained from Carolina Biological (Burlington, NC) and cultured at 25°C on unbleached flour plus 5% dried yeast. For embryo collections, adults were incubated in flour for 24 hours, then removed using a 850 µm sieve and the embryos were either fixed immediately or allowed to develop for an additional 1–5 days at 25°C. Eggs were harvested using a 300 µm sieve and dechorionated with 50% bleach for 2 min, then rinsed thoroughly with water. Embryos were fixed in glass vials containing 10 ml of heptane and 10 ml of 8% formaldehyde for 30–45 min at room temperature with vigorous shaking, after which the fixative was removed and replaced with an equivalent volume of methanol. Embryos were devitellinized by 2–5 rounds of sonication for 5 seconds each using a Branson Sonifier 250 at 10% output. Devitellinized embryos and fragments (those that sank to the bottom of the methanol phase) were collected after each round of sonication. Embryos were stored at –20°C in 100% methanol.

Immunohistochemistry

Anti-TcFasII antibody was produced by GenScript (Piscataway NJ). Rabbits were immunized with a TcFasII-specific peptide (CKQRNSQDLSKSDRF) conjugated to KLH. Polyclonal antiserum was affinity-purified using the peptide antigen and used at 1:1000 dilution. Other antibodies used were: mouse MAb 1D4/anti-FasII (Developmental Studies Hybridoma Bank [DSHB]; 1:100), mouse anti- β gal (DSHB; 1:150), FITC-conjugated goat anti-HRP (Jackson ImmunoResearch; 1:50), Cy3 goat anti-rabbit (Jackson; 1:500), and Cy3 goat anti-mouse (Jackson; 1:1000). Antibody staining was performed as in Patel, 1994. Nerve cords were dissected and mounted in 70% glycerol/PBS. Confocal stacks were collected using a Leica Confocal TCS SL microscope and processed by NIH ImageJ and Adobe Photoshop software.

Phylogenetic analysis

Robo orthologs were identified by searching animal genome sequences by tblastn using *Drosophila* protein sequences. Intron-exon structures were manually annotated from the genomic sequence using the *Drosophila* sequences as references. Predicted *Tribolium* Slit and Robo sequences were confirmed by sequencing cDNA clones. *Tribolium* Robo sequences were deposited in the GenBank database. Accession numbers: JN634958 (TcRobo) and JN634959 (TcRobo2/3). Protein alignments of receptor ectodomains and resulting neighbor joining trees were generated using ClustalX. Trees were drawn using DrawTree from the PHYLIP software package.

Molecular biology

Total RNA was extracted from 0–6 day old *Tribolium* embryos using TRIzol (Invitrogen) and cDNA was synthesized using SuperScript (Invitrogen). Beetle genes were amplified by PCR using the following primers and cloned into TOPO vector (Invitrogen): *TcSlit* (5'-ATGCTAGCCTCCGGTGTCCAGAGGATGC-3' and 5'-GCCTCGAGTCAGTAGCATTTTTTCGTACACGC-3'), *TcRobo* (5'-TAGCTAGCCCGCGGATCACCGAGCACCCC-3' and 5'-CGGGTACCTCACTTCCCGCACCTTTTCACCTGG-3'), *TcRobo2/3* (5'-TAGCTAGCCCTCGAATCACCGAACACCCTGTAG-3' and 5'-GCGGTACCTCACAACATATTGCCAGGCTCACTCC-3'). *Drosophila* and *Tribolium* Robo coding sequences (beginning with the first immunoglobulin [Ig1] domain) were subcloned into a pUAST vector including identical heterologous 5'UTR and signal sequences (derived from the *Drosophila wingless* gene) and an N-terminal 3 \times HA tag.

Double-stranded RNA (dsRNA) was synthesized with MEGAscript RNAi kit (Ambion) using ~800 bp PCR product templates. Forward and reverse PCR primers both included T7 promoter sequences at their 5' ends; gene-specific sequences were as follows: *TcSlit* (5'-CATCTGGCTGATGAGCTGTTCAACGG-3' and 5'-GTACACGCGCATTTGCGCACGATGTC-3'), *TcRobo* (5'-CAAGGCCCTTATTCCAACCAAGTGCC-3' and 5'-TCACTTCCCGCACCTTTTCACCTGGC-3'), *TcRobo2/3* (5'-CCGTAAGAGTGCGCAGCAATGACACGC-3' and 5'-TCACAACATATTGCCAGGCTCACTCC-3').

Genetics

The following *Drosophila* stocks were used: (1) *w¹¹¹⁸*, (2) *slit²/CyO, wg-lacZ*, (3) *slit^{E158}/CyO, wg-lacZ*, *robo^{GA285}/CyO, wg-lacZ*, (4) *robo2^{x33}/CyO, wg-lacZ*, (5) *robo2^{x123}/CyO, wg-lacZ*, (6) *robo3¹/CyO, wg-lacZ*, (7) *robo^{GA285}, robo2^{x33}/CyO, wg-lacZ*, (8) *robo^{GA285}, robo2^{x123}/CyO, wg-lacZ*, (9) *elav-GAL4*, (10) *robo^{GA285}/CyO, wg-lacZ; elav-GAL4*, (11) *eg-GAL4, UAS-TauMycGFP/TM3, tub-GAL80*, (12) *robo^{GA285}, UAS-TauMycGFP/CyO, tub-GAL80; eg-GAL4*, (13) *UAS-Comm/CyO, tub-GAL80; elav-GAL4*. Transgenic flies were generated by BestGene Inc (Chino Hills, CA) using Φ C31-directed site-specific integration into the same landing site for all constructs (at cytological position 86FB) to ensure comparable expression levels between lines. All crosses were carried out at 25°C.

Parental RNAi

Female *Tribolium* pupae were collected just before eclosion and glued by the posterior abdomen to a glass microscope slide using rubber cement. Approximately 0.1–0.2 ul of dsRNA (0.25–1.0 ug/ul concentration) was injected laterally into the ventral abdomen, and slides were placed upside down in flour until adult beetles eclosed. Injected females were mated to untreated males and egg collections began one week after mating.

Results

Expansion of the Robo family by gene duplication in insects

In *Drosophila*, three Robo receptors (Robo, Robo2, and Robo3) each control a unique set of axon guidance decisions in the embryonic CNS (Rajagopalan et al., 2000a; Rajagopalan et al., 2000b; Simpson et al., 2000a; Simpson et al., 2000b). This functional diversity depends in part on differential expression patterns and in part on structural differences between the three receptors (Evans and Bashaw, 2010b; Spitzweck et al., 2010). The close proximity and genomic organization of *robo2* and *robo3* suggest that these two genes are the product of a recent gene duplication, and raises the possibility that their divergent axon guidance functions are a recent evolutionary development. To gain insight into the evolutionary history of the Robo family, we examined the distribution of Robo orthologs in the sequenced genomes of insects and other animal groups.

Orthologs of all three *D. melanogaster* Robos can be found in each of the twelve sequenced *Drosophila* genomes (Clark et al., 2007), and in the mosquitoes *Anopheles gambiae* (Holt et al., 2002) and *Aedes aegypti* (Nene et al., 2007). In addition, a fourth Robo gene in mosquitoes appears to be the result of a duplication of *robo* in this group. Thus each of the three *Drosophila* Robos are conserved throughout the Diptera. In contrast, the genomes of representatives from four other insect orders (Lepidoptera: *Bombyx mori* (International Silkworm Genomics Consortium, 2008); Coleoptera: *Tribolium castaneum* (Richards et al., 2008); Hemiptera: *Acyrtosiphon pisum* (International Aphid Genomics Consortium, 2010); Phthiraptera: *Pediculus humanus* (Kirkness et al., 2010)) each encode only two Robo genes: one an ortholog of *Drosophila* Robo and the other equally similar to *Drosophila* Robo2 and Robo3 (Figure 1). We refer to this ancestral Robo2/Robo3 receptor as Robo2/3. Thus the gene duplication event that produced *robo2* and *robo3* occurred in a common ancestor of

flies and mosquitoes, while the split between *robo* and *robo2/3* was a more ancient event (Figure 1A).

A single Robo receptor has been described in the nematode *Caenorhabditis elegans* (known as SAX-3) (Zallen et al., 1998), raising the possibility that Robo and Robo2/3 may exist as distinct genes only within arthropods. We searched available non-insect arthropod genomes for Robo receptor orthologs. The genome of the branchiopod crustacean *Daphnia pulex* (Colbourne et al., 2011; Colbourne et al., 2005) does not encode distinct orthologs of Robo and Robo2/3; the three *Daphnia* Robo receptors are more similar to each other than to any insect or vertebrate Robos, indicating that they are also the products of independent gene duplication (Figure 1B). Similarly, the preliminary assembly of the *Ixodes scapularis* (deer tick) genome (<http://iscapularis.vectorbase.org>) encodes four predicted Robo-like sequences, also apparently reflecting *robo* gene duplication in this lineage (not shown). These results suggest that the ancestral arthropod, like nematodes, possessed a single Robo receptor that was duplicated independently in three major arthropod groups (insects, crustaceans, and chelicerates). Within the insects, *robo2* and *robo3* exist as separate genes only in dipterans.

When were the distinct axon guidance functions associated with the three *Drosophila* Robos acquired? What role did *robo* gene duplication and functional diversification play in the evolution of the nervous system in insects and other animal groups? Do divergent guidance receptor complements promote similar or distinct guidance outcomes in related animals? To address these questions, we set out to examine the functions of Robo receptors in the beetle *Tribolium castaneum*, an experimentally tractable insect that retains an ancestral set of Robo receptors.

Tribolium* Robos can mediate midline repulsion in *Drosophila

Our first step was to ask whether one or both of the two *Tribolium* Robos could mediate midline repulsion in response to Slit. To this end, we cloned both *Tribolium* Robos via RT-PCR from beetle embryos and introduced them into *Drosophila* using the GAL4/UAS system (Brand and Perrimon, 1993). We used Φ C31-directed site-specific integration to insert all of our UAS transgenes into the same genomic landing site to ensure comparable mRNA expression levels between lines. Using an N-terminal epitope tag, we confirmed that both beetle receptors could be stably expressed in *Drosophila* embryonic neurons, and exhibited axonal localization patterns similar to those observed for *Drosophila* Robos under equivalent ectopic expression conditions (Figure 2A–D). In an otherwise wild type background, misexpression of TcRobo or TcRobo2/3 either pan-neurally (with *elav-GAL4*; Figure 2) or in a subset of commissural neurons (EW neurons, with *eg-GAL4*; Figure S3) in the embryonic CNS prevented commissural axons from crossing the midline, indicating that both *Tribolium* Robos can trigger midline repulsion in the *Drosophila* embryonic CNS. This phenotype was similar to that caused by high level misexpression of *Drosophila* Robo or Robo2, although the *Tribolium* Robos appeared much more potent at mediating midline repulsion (Figure 2A'–D'). Importantly, this appeared to be a specific effect on axon guidance, as we observed no apparent defects in neuronal survival or axon outgrowth in these embryos.

To test whether one or both beetle Robos could substitute for fly Robo in the context of midline repulsion, we performed a similar set of experiments in a *robo* mutant background. Both TcRobo and TcRobo2/3 were able to prevent FasII axons from crossing the midline in the absence of *robo* (Figure 3). Indeed, loss of *robo* had little effect on the commissureless phenotype caused by pan-neural misexpression of TcRobo or TcRobo2/3, as nearly all segments were commissureless in these embryos. Similarly, both TcRobo and TcRobo2/3 were able to prevent commissural EW axons from crossing the midline in nearly 100% of segments in a *robo* mutant background (Figure S4). This set of experiments confirmed that the *Tribolium* Robos are able to respond to *Drosophila* Slit and activate downstream signaling components in *Drosophila* neurons in the absence of any contribution from endogenous Robo.

***Tribolium* Robos are insensitive to *Drosophila* Comm**

In the course of characterizing our *UAS-TcRobo* and *UAS-TcRobo2/3* transgenic lines, we were struck by the potency of these receptors in mediating midline repulsion in *Drosophila* embryos. Both TcRobo and TcRobo2/3 transgenics consistently displayed stronger midline repulsion activity than any of the transgenic lines we analyzed for the three *Drosophila* Robos. We reasoned that the *Tribolium* Robos might not be subject to the same regulatory mechanisms as the *Drosophila* Robos when expressed in fly neurons. Commissureless (Comm) is a *Drosophila* protein that directs endosomal sorting and degradation of Robo in pre-crossing fly commissural neurons, thus preventing premature Slit sensitivity while axons are crossing the midline (Keleman et al., 2002; Keleman et al., 2005). To test whether Comm could downregulate TcRobo or TcRobo2/3 in the fly embryonic CNS, we expressed HA-tagged forms of fly and beetle Robos in *Drosophila* embryonic neurons using *elav-GAL4*, in the presence or absence of *UAS-Comm* (Figure 2E). While misexpression of Comm strongly reduced the expression levels of both *Drosophila* Robo and Robo2, reflecting Comm-dependent trafficking and endosomal degradation, the levels of TcRobo and TcRobo2/3 were not affected by the presence of Comm (Figure 2E). These results suggest that *Drosophila* Comm is unable to negatively regulate *Tribolium* Robos in fly neurons. Notably, we have been unable to detect orthologs of any of the three *Drosophila* Comm genes in the *Tribolium* genome, perhaps indicating that the *Drosophila* system of Comm-dependent Robo regulation is not conserved in *Tribolium*. Having determined that *Tribolium* Robos can function as midline repellent receptors in the *Drosophila* CNS, we next set out to examine the roles of TcRobo and TcRobo2/3 in axon guidance in the beetle embryonic CNS.

Conservation of CNS architecture in flies and beetles

As axon guidance in the beetle embryonic CNS has not previously been described, we first compared the pattern of axon tracts in wild type *Tribolium* and *Drosophila* embryos. To examine axon guidance in *Tribolium*, we used an antibody against horseradish peroxidase (HRP), which recognizes a pan-neural epitope in insects (Haase et al., 2001; Snow et al., 1987). In hopes of developing a more informative marker for quantifying midline crossing phenotypes and examining the formation of longitudinal pathways, we also generated a polyclonal antibody against the *Tribolium* ortholog of Fasciclin II (TcFasII), a neural cell

adhesion molecule which labels individual longitudinal axon tracts in the *Drosophila* and grasshopper embryonic CNS (Bastiani et al., 1987; Grenningloh et al., 1991).

As in *Drosophila* and grasshopper, anti-HRP staining of *Tribolium* embryos revealed a ladder-like arrangement of segmental ganglia in the ventral nerve cord, with two commissures per segment and segmental (SN) and intersegmental (ISN) nerve roots exiting the CNS (Figure 4A,B). Both anti-HRP and anti-TcFasII antibodies labeled motor axons as they extend into the periphery and innervate body wall and limb muscles. A number of distinct FasII-positive longitudinal pathways were visible in the beetle embryonic CNS, localized (as in the fly) into discrete medial (M), intermediate (I), and lateral (L) regions (Figure 4C,D). Optical cross-sections of fly and beetle embryonic nerve cords revealed that corresponding individual FasII pathways can be identified at similar dorsoventral and mediolateral locations in late-stage embryos of both species (Figure 4C',D'). While some commissural segments of axons are FasII-positive at earlier stages, as in *Drosophila*, in older beetle embryos no FasII-positive axon segments can be found extending across the midline. Thus in *Tribolium*, as in *Drosophila*, FasII is an informative marker for assaying midline repulsion, longitudinal pathway formation, and motor axon guidance. The high degree of similarity in number and position of longitudinal axon pathways is particularly remarkable given that three Robo receptors are required to establish this pattern in *Drosophila*, while *Tribolium* has only two Robos. How do *Tribolium* Slit and its two Robo receptors contribute to midline repulsion and longitudinal pathway formation in the beetle embryonic CNS?

Slit-Robo signaling regulates midline crossing in *Tribolium*

In *Drosophila*, Slit is produced at the midline of the embryonic CNS and prevents Robo-expressing axons from crossing the midline (Kidd et al., 1999). Both *robo* and *robo2* contribute to *slit*-dependent midline repulsion in flies, and *robo, robo2* double mutants phenocopy the midline collapse phenotype of *slit* mutants (Rajagopalan et al., 2000a; Simpson et al., 2000b). To investigate the contributions of Slit and Robos to midline repulsion in *Tribolium*, we used parental RNAi (Bucher et al., 2002) to knock down these genes during embryogenesis and assayed axon guidance in the embryonic CNS using anti-HRP and anti-TcFasII antibodies.

RNAi-mediated knockdown of *TcSlit* produced a severe midline collapse phenotype reflecting a loss of midline repulsion similar to *Drosophila slit* mutants: distinct longitudinal tracts do not form properly, and the normally well separated bilateral connectives fuse as axons enter the midline and do not leave (Figure 5C,G). The *TcSlit* knockdown phenotype appears slightly less severe than in *Drosophila slit* null mutants, as midline gaps between the connectives can still be seen, encircled by FasII-positive axons as they cross and re-cross the midline. This difference likely reflects an incomplete knockout of *TcSlit* function under RNAi conditions.

To complement our RNAi-based knockdown approach, we searched among the collection of recessive lethal *piggyBac* transposon insertions generated by the GEKU consortium for potential loss-of-function alleles of *TcSlit*, *TcRobo*, or *TcRobo2/3* (Trauner et al., 2009). While no insertions mapped to the *TcRobo* or *TcRobo2/3* loci, one line (designated G07615)

was reported to carry an insertion within the sixth intron of the *TcSlit* gene. We found that embryos from heterozygous G07615 parents exhibited strong ectopic crossing phenotypes similar to, but less severe than, the *TcSlit* RNAi phenotype (Figure 5B). A similar range of phenotypes can be seen when comparing hypomorphic and null alleles of *Drosophila slit* (Figure 5F,G) (Kidd et al., 1999). We conclude that the G07615 transposon insertion is a hypomorphic allele of *TcSlit*, and thus refer to this allele as *TcSlit*^{G07615}.

In *Drosophila*, *robo* and *robo2* single mutants both exhibit ectopic midline crossing, but simultaneous removal of both genes is necessary to produce a *slit*-like midline collapse phenotype (Figure 5H) (Rajagopalan et al., 2000a; Simpson et al., 2000b). Similarly, individual knockdown of *TcRobo* or *TcRobo2/3* did not reproduce the *TcSlit* midline collapse phenotype (see below), suggesting that neither of these receptors is solely responsible for mediating Slit-dependent midline repulsion. We therefore asked whether simultaneous knockdown of both *TcRobo* and *TcRobo2/3* would recapitulate the *TcSlit* phenotype. Indeed, embryos from mothers injected with a mix of *TcRobo* and *TcRobo2/3* dsRNAs displayed strong midline collapse phenotypes virtually identical to those caused by *TcSlit* RNAi (Figure 5D). Thus in *Tribolium*, as in *Drosophila*, two Robo receptors cooperate to mediate Slit-dependent midline repulsion in the embryonic CNS.

***Tribolium* Robo receptors have specialized roles in axon guidance**

Drosophila Robo receptors perform specialized roles in axon guidance: while both Robo and Robo2 contribute to midline repulsion, Robo2 (but not Robo) cooperates with Robo3 to specify the lateral position of longitudinal axon pathways in the embryonic CNS (Evans and Bashaw, 2010b; Rajagopalan et al., 2000a; Rajagopalan et al., 2000b; Simpson et al., 2000a; Simpson et al., 2000b; Spitzweck et al., 2010). Notably, while Robo2 can substitute for Robo3 to promote formation of axon pathways in the intermediate region of the neuropile, Robo3 cannot functionally replace Robo2 to promote formation of lateral pathways (Spitzweck et al., 2010). Thus the divergent functions of Robo2 and Robo3 cannot be accounted for solely by differences in expression, and must also reflect intrinsic functional differences in the receptors themselves. How do these different activities relate to those of the ancestral Robo2/3 receptor? To determine whether *Tribolium* Robos exhibit similar functional specialization, we examined the effects of knocking down *TcRobo* or *TcRobo2/3* individually on axon guidance in the *Tribolium* embryonic CNS.

TcRobo knockdown produced an ectopic midline crossing phenotype very similar to *Drosophila robo* mutants: thickened commissures and reduced connectives were revealed by anti-HRP staining, and medial FasII-positive pathways ectopically crossed the CNS midline in over 90% of examined segments (Figure 6A,C; Table 1). Notably, as in *Drosophila robo* mutants, intermediate and lateral FasII-positive axon pathways appeared unaffected in *TcRobo* knockdown embryos, indicating that *TcRobo*, like *Drosophila robo*, is dedicated to midline repulsion and does not contribute to longitudinal pathway formation.

In contrast, the overall organization of the axon scaffold appeared normal in anti-HRP stained *TcRobo2/3* knockdown embryos, and we did not observe any instances of ectopic midline crossing of FasII-positive axons. Instead, anti-TcFasII staining revealed defects in longitudinal pathway formation in these embryos (Figure 6B). The normally distinct FasII

pathways were disorganized in *TcRobo2/3* knockdown embryos, in some cases forming in inappropriate positions or crossing between zones to fuse with other pathways (Figure 6B'; Table 1).

To more thoroughly characterize this phenotype, we used higher magnification imaging and optical cross sections to examine the formation of individual longitudinal pathways (Figure 7). In wild type embryos, two major FasII-positive pathways form in the lateral region of the neuropile: one dorsal and one ventral (Figure 7A). In *TcRobo2/3* knockdown embryos, the dorsal lateral pathway was missing in nearly 70% of hemisegments. In a further 11% of hemisegments, this pathway could be seen shifting medially to join one of the intermediate pathways (Figure 7B; Table 1). Neither of these phenotypes were observed in wild type or *TcRobo* knockdown embryos. The ventral lateral pathway was unaffected by the loss of *TcRobo2/3* (Figure 7B'').

Knockdown of *TcRobo2/3* also altered the position of intermediate FasII pathways. In wild type embryos, a group of 3–4 FasII-positive pathways form within the intermediate region of the neuropile, well separated from the two major medial pathways (Figure 7A). In *TcRobo2/3* knockdown embryos, these intermediate pathways formed closer to the midline, where they mingled with and were often indistinguishable from the normally medial pathways (Figure 7B). Loss of *TcRobo2/3* in beetle embryos therefore results in axon guidance defects that combine aspects of *Drosophila robo2* and *robo3* mutant phenotypes: loss of lateral axon pathways and medial shifting of intermediate pathways are characteristic of *robo2* and *robo3* mutants, respectively (Figure 6D,E). It is unclear whether the *robo2* loss of function phenotype is specific to the dorsal lateral pathway in *Drosophila*, as the ventral lateral pathway is less robust in flies than in beetles and we were sometimes unable to detect it even in wild type *Drosophila* embryos (not shown).

Discussion

Modern animals sense and respond to their environment in highly diverse and sophisticated ways. As the complexity of animal nervous systems has increased, developmental control of neuronal connectivity has become increasingly refined. How have axon guidance molecule repertoires changed over evolutionary time? How has functional diversity been acquired within related groups of guidance molecules, and how has this contributed to the evolution of nervous system connectivity?

Here we describe Slit/Robo-mediated axon guidance in the beetle *Tribolium castaneum* and report on the evolution of the Roundabout (Robo) family of axon guidance receptors in insects. We conclude that the functional diversity exhibited by *Drosophila* Robo receptors was present early in insect evolution, and that the combined role of Robo2 and Robo3 in specifying lateral position of longitudinal pathways in *Drosophila* is an ancestral function performed by a single Robo2/3 receptor in other insects. Robo2/3 orthologs are not found outside insects, suggesting a role for expansion and functional diversification of the Robo family in the evolution of nervous system connectivity.

Slit/Robo-mediated axon guidance in *Tribolium*

Much of our current knowledge of how the Slit/Robo pathway controls axon guidance decisions has come from studies in *Drosophila*, *C. elegans*, and mouse. To gain broader insight into Slit/Robo signaling in insects, we now extend this analysis to a second experimentally tractable insect, the flour beetle *Tribolium castaneum*. To our knowledge, this work represents the first investigation of axon guidance in the *Tribolium* embryonic CNS. While there is a high degree of conservation in Slit/Robo-mediated guidance between these two insects, we also note some important differences. First and most obvious, *Tribolium* has only two Robo receptors (TcRobo and TcRobo2/3), while *Drosophila* has three (Robo, Robo2, and Robo3). Despite this divergent complement of axon guidance receptors, the architecture of the embryonic CNS in *Drosophila* and *Tribolium*, including the number and position of individual axon pathways, is remarkably similar.

Secondly, our results also suggest that the regulation of Slit/Robo signaling differs between *Drosophila* and *Tribolium*. In flies, Commissureless (Comm) is essential for preventing premature response to Slit by preventing Robo receptor molecules from reaching the growth cone surface in pre-crossing commissural axons (Keleman et al., 2002; Keleman et al., 2005). *Tribolium* Robos appear insensitive to regulation by Comm when expressed in *Drosophila* neurons, and the *Tribolium* genome does not appear to encode a Comm ortholog. How, then, is Slit responsiveness suppressed in pre-crossing commissural axons in *Tribolium*? A close examination of Robo receptor expression (at both mRNA and protein levels) and localization in different classes of *Tribolium* neurons may provide insight into the regulation of Slit/Robo signaling in beetles. Novel negative regulators of Slit/Robo signaling in *Tribolium* could be uncovered by forward genetic screens to identify genes required for midline crossing, similar to the screen that identified *Drosophila comm* (Seeger et al., 1993).

Functional specialization of *Tribolium* Robos

Our findings demonstrate that at least two of the axon guidance functions performed by *Drosophila* Robo receptors—midline repulsion and lateral position—are shared by Robos in the beetle *Tribolium*. As in *Drosophila*, TcRobo (the ortholog of *Drosophila* Robo) is dedicated to midline repulsion, and does not appear to play a role in lateral positioning of longitudinal axon tracts. Similarly, TcRobo2/3 (orthologous to the ancestor of *Drosophila* Robo2 and Robo3) controls longitudinal pathway formation and also contributes to midline repulsion. Unlike in *Drosophila robo2* mutants, however, we could not detect ectopic midline crossing in *TcRobo2/3* knockdown embryos. While this may indicate that our RNAi-based approach only achieves a partial reduction of *TcRobo2/3* function, the much more severe midline collapse phenotype seen in *TcRobo+TcRobo2/3* double knockdowns compared to *TcRobo* single knockdowns suggests that we are indeed strongly reducing or eliminating *TcRobo2/3*'s midline repulsive activity (even at one half the dose of the single knockdown). Indeed, we did not observe an increase in severity of *TcRobo2/3*'s lateral positioning phenotype, or an appearance of ectopic midline crossing, when we increased the concentration of *TcRobo2/3* dsRNA nearly ten-fold (to 5 μ g/ μ l; not shown). We therefore interpret the lack of ectopic midline crossing in *TcRobo2/3* single knockdowns as evidence

that *TcRobo2/3*'s role in midline repulsion is largely or completely redundant with that of *TcRobo*.

Our finding that *TcRobo2/3* performs a dual role in positioning both intermediate and lateral pathways indicates that this activity predates the divergence of *Robo2* and *Robo3*, and that these two receptors in flies now subdivide a role that is performed by a single ancestral receptor in other insects. Thus *Robo* receptor control of lateral position of longitudinal pathways is likely to be widespread among insects. As *Robo2/3* appears to be unique to insects, when did this activity arise? We can envision two possibilities: 1) lateral positioning activity was present in the ancestral *Robo* receptor, and retained by *Robo2/3* after the gene duplication event that separated it from *Robo*; 2) *Robo2/3* gained lateral positioning activity after it separated from *Robo*. Examining the roles of *Robo* and *Robo2/3* in axon guidance in additional insects, or related animals that lack a distinct *Robo2/3* receptor (e.g. crustaceans) may distinguish between these possibilities.

The third, and least well understood, function of *Robos* in axon guidance in the *Drosophila* embryonic CNS is *Robo2*'s role in promoting midline crossing. Commissural axons fail to cross the midline when *robo2* and the attractive ligand *netrin* or its receptor *frazzled/DCC* (*fra*) are removed in combination, and pan-neural misexpression of *Robo2* can cause ectopic midline crossing (Evans and Bashaw, 2010b; Simpson et al., 2000b; Spitzweck et al., 2010). This activity is restricted to *Robo2*, as neither *Robo* nor *Robo3* produces the same effect in gain of function assays, and neither *robo* nor *robo3* can rescue the pro-crossing function of *robo2* in *fra, robo2^{robo1}* or *fra, robo2^{robo3}* mutants (Evans and Bashaw, 2010b; Spitzweck et al., 2010). We did not detect any defects in commissure formation after *TcRobo2/3* knockdown in *Tribolium* embryos, and pan-neural misexpression of *TcRobo2/3* did not produce ectopic midline crossing in *Drosophila* embryos. Two direct ways to test whether *TcRobo2/3* shares *Drosophila* *Robo2*'s pro-midline crossing activity would be to examine the effect of simultaneously knocking down *TcFra* and *TcRobo2/3* on commissure formation in the *Tribolium* embryonic CNS, or by replacing *Drosophila robo2* with *TcRobo2/3* and asking whether it can promote commissure formation in a *fra* mutant background.

Conservation of neural architecture in insects with divergent sets of axon guidance receptors

The coordinated targeting of axons and dendrites to specific regions within the three-dimensional network of neurites in the CNS is necessary for the proper assembly of motor and sensory circuits. In the *Drosophila* embryonic ventral nerve cord, the *Slit/Robo* and *Semaphorin (Sema)/Plexin* signaling pathways regulate mediolateral and dorsoventral positioning of neurites, respectively (Wu et al., 2011; Zlatic et al., 2003; Zlatic et al., 2009). *Robo2* is required for the formation of longitudinal axon pathways in the lateral region of the neuropile, while *Robo3* directs axons to join pathways in intermediate regions (Rajagopalan et al., 2000b; Simpson et al., 2000a).

Do the same mechanisms regulate longitudinal pathway choice in other animals? We generated an antibody against the *Tribolium* ortholog of *FasII* (*TcFasII*) in order to determine the number and location of *FasII*-positive axon pathways in the beetle embryonic CNS. Surprisingly, we found that both the number and position of *FasII*-positive

longitudinal pathways are conserved in the fly and beetle embryonic CNS, despite the fact that only two Robo receptors exist in *Tribolium*. Thus, in these two insect species, divergent genetic programs lead to equivalent developmental outcomes. Further, *Drosophila*'s system of three Robo receptors specifying three medial-lateral zones in the neuropile is not typical of insects, as most insects (like *Tribolium*) have only two Robos.

Gene replacement experiments in *Drosophila* demonstrate that Robo2 and Robo3 are not functionally equivalent (Spitzweck et al., 2010), yet a single Robo2/3 receptor performs both roles in *Tribolium*. How is this achieved? Our data suggest that the ancestor of Robo2 and Robo3 was able to specify both intermediate and lateral pathway formation. Subsequent to the gene duplication that produced *robo2* and *robo3* in dipterans, Robo3 apparently lost the ability to promote lateral pathway formation. In contrast, Robo2 retains the ability to specify both intermediate and lateral pathways, but its expression was lost in neurons whose axons join the intermediate pathways. This model makes a number of testable predictions: TcRobo2/3 should be expressed on axons found in the intermediate and lateral zones of the axon scaffold in *Tribolium* embryos, similar to Robo3 expression in *Drosophila*; TcRobo2/3 should be able to substitute for both *robo3* and *robo2* to promote intermediate and lateral pathway formation in *Drosophila*; and *Drosophila robo2*, but not *robo3*, should be able to substitute for TcRobo2/3 to promote lateral pathway formation in *Tribolium*.

One possibility is that TcRobo2/3 expression in the neurons that make up the intermediate and lateral pathways may be differentially regulated, such that TcRobo2/3 is expressed at different levels or at different times in these two neuronal classes, and that these expression differences might be important for the sequential formation of distinct axon pathways. We note that embryogenesis in *Tribolium* takes around six times as long as *Drosophila* at 25°C, perhaps allowing temporal regulation of gene expression to play a more important role in specifying distinct guidance outcomes than in the rapidly developing fly embryo. Direct examination of the expression patterns of both beetle Robos will provide insight into how the regulation of Robo expression differs between *Drosophila* and *Tribolium*, and may also explain why the lateral position of dorsal and ventral lateral pathways exhibit a differential requirement for TcRobo2/3 activity.

Expansion of axon guidance molecule families by gene duplication

Our phylogenetic analysis indicates that the Robo family has expanded by gene duplication independently in a number of animal groups, including vertebrates, crustaceans, chelicerates and insects. In contrast, nematodes have retained a single Robo receptor. Notably, expansion of the Robo family by gene duplication does not appear to correlate with whole genome duplication events in insects, and does not appear to be common among insect axon guidance genes. We found only a single ortholog of Slit in each of the analyzed insect and nematode genomes; similarly, the number of Plexin (two) and Frazzled/DCC (one) receptors is the same in all insects and *C. elegans*.

The pattern of distribution of Robo orthologs together with sequence comparisons therefore suggest that a single Robo receptor was present in the last common ancestor of protostomes and deuterostomes. This ancestral Robo was almost certainly involved in Slit-dependent midline repulsion, but it is unclear whether it would have possessed any of the other known

Robo axon guidance functions. An intriguing possibility is that additional activities were acquired by Robo paralogs subsequent to gene duplication in the lineages leading to vertebrates and insects, and that this functional diversification may have contributed to increases in nervous system complexity in these groups.

Robo receptors and the evolution of the arthropod nervous system

What molecular mechanisms have facilitated increases in nervous system complexity over evolutionary time? The duplication and functional diversification of axon guidance pathway components is one possible mechanism providing increasingly precise control of axon guidance. This appears to be the case with the Robo family of axon guidance receptors, whose members exhibit functional diversity in a number of animal groups with complex nervous systems. Importantly, one key aspect of nervous system patterning and circuit formation in insects, specifying mediolateral targeting of neurites in the CNS neuropile, is controlled by Robo orthologs that appear to be restricted to insects (Robo2/3 and its descendants, Robo2 and Robo3). Correspondingly, insect nervous systems display a level of precision in axon guidance that is not present in close relatives outside the arthropods (Whittington and Mayer, 2011). Elucidating the axon guidance roles of Robo receptors in additional insects and other animals (for example, crustaceans and onychophorans) should provide further insight into the origins of functional diversity in this important family of axon guidance molecules, and the role they have played in the evolution of animal nervous systems.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- > The Robo receptor family has expanded by gene duplication in insects.
- > Slit and Robos have highly conserved roles in midline repulsion in flies and beetles.
- > Insects build similar nervous systems using different numbers of Robos receptors.
- > TcRobo2/3 combines lateral positioning functions of DmRobo2 and DmRobo3.
- > Flies and beetles employ distinct mechanisms to regulate Robo receptor activity.

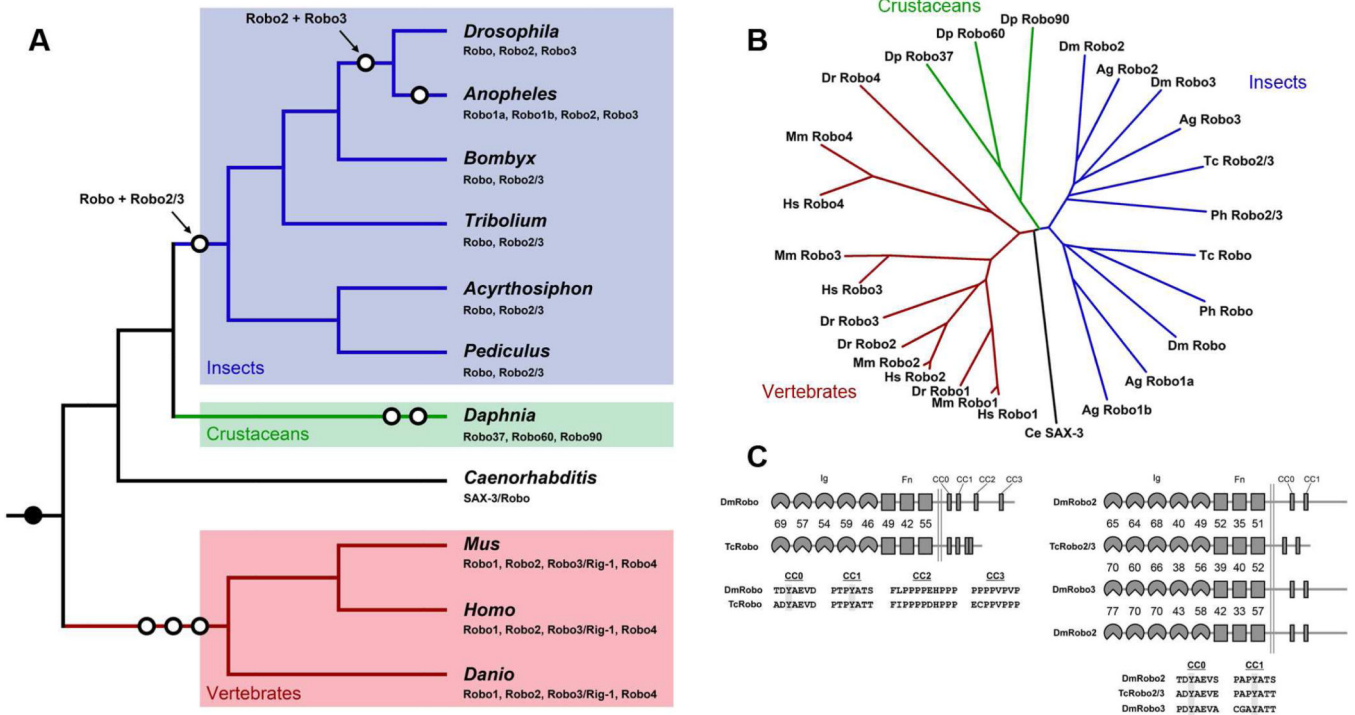


Figure 1. Expansion of the Robo family by gene duplication

A. Schematic phylogeny showing the evolutionary relationship between selected animals, and the complement of Robo receptors present in each animal’s genome. Predicted gene duplication events are represented by open circles. Filled black circle represents the appearance of the ancestral Robo receptor. The Robo family has expanded via gene duplication in insects (blue), crustaceans (green), and vertebrates (red). *Daphnia* Robos are given numerical designation based on the genomic contig in which they are located. Branch lengths are not to scale.

B. Phylogenetic analysis of Robo family members using extracellular (Ig+Fn) protein sequences. Insect (blue), crustacean (green), and vertebrate (red) receptors cluster together, reflecting their independent derivation from a single common ancestral receptor. Note also that Robo2 and Robo3 orthologs in fly (Dm) and mosquito (Ag) are more similar to each other than to Robo2/3 from beetle (Tc) and louse (Ph). A single Robo receptor (SAX-3) has been retained in nematodes. Abbreviations: Dm, *Drosophila melanogaster*; Ag, *Anopheles gambiae*; Tc, *Tribolium castaneum*; Ph, *Pediculus humanus*; Dp, *Daphnia pulex*; Ce, *Caenorhabditis elegans*; Hs, *Homo sapiens*; Mm, *Mus musculus*; Dr, *Danio rerio*.

C. Schematic comparison of *Drosophila* and *Tribolium* Robo receptors showing percent sequence identity between individual protein domains, and highlighting the conserved cytoplasmic (CC) motifs. Lengths of cytodomains are roughly to scale. TcRobo retains all four CC motifs despite its much smaller cytodomain. Note that *Drosophila* Robo2 and Robo3 are more similar to each other than to *Tribolium* Robo2/3.

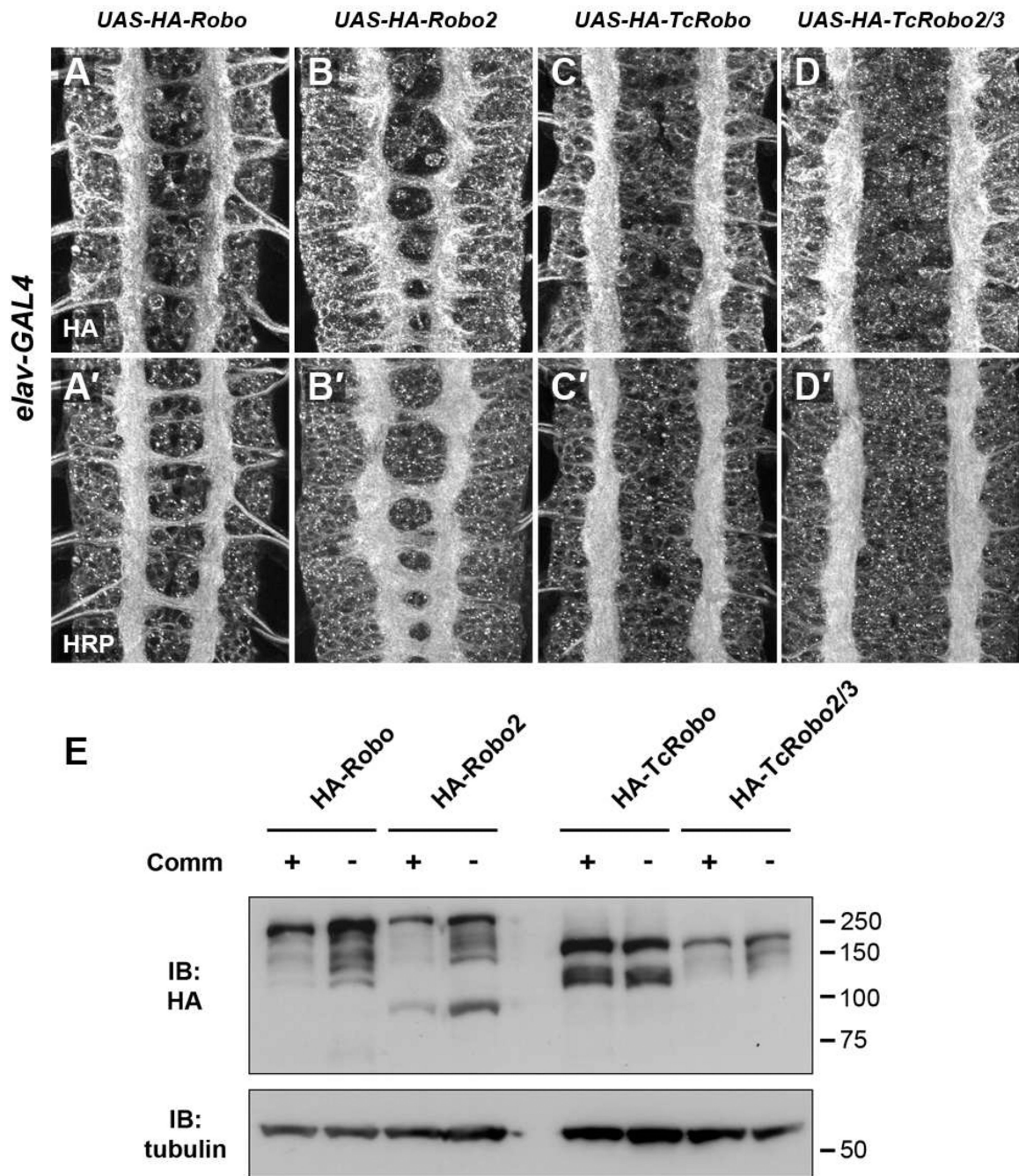


Figure 2. *Tribolium* Robos are potent midline repellent receptors in *Drosophila*

A–D. Stage 16–17 *Drosophila* embryos carrying *elav-GAL4* and the indicated *UAS-Robo* transgenes. In this and all subsequent figures, all *UAS-Robo* transgenes have identical 5'UTR, signal sequence and 3×HA N-terminal tags, and are inserted into the same genomic location (at cytological position 86FB). Anti-HA staining shows that Robo receptors from fly and beetle localize to axons when expressed pan-neurally in the *Drosophila* embryonic CNS (A–D). *Tribolium* Robos are much more effective at preventing axons from crossing

the midline than *Drosophila* Robos, producing a commissureless phenotype as revealed by anti-HRP staining (A'-D').

E. Western blot of total lysates from 0–24 hour embryos carrying *elav-GAL4* and the indicated *UAS-Robo* transgenes, with or without *UAS-Comm*. Lysates were subjected to SDS-PAGE and immunoblotted with anti-HA to detect the levels of HA-tagged receptors, and anti-tubulin as a loading control. Levels of HA-tagged *Drosophila* Robo and Robo2 are strongly reduced in the presence of exogenous Comm, while the levels of HA-tagged TcRobo and TcRobo2/3 were similar in the presence or absence of exogenous Comm.

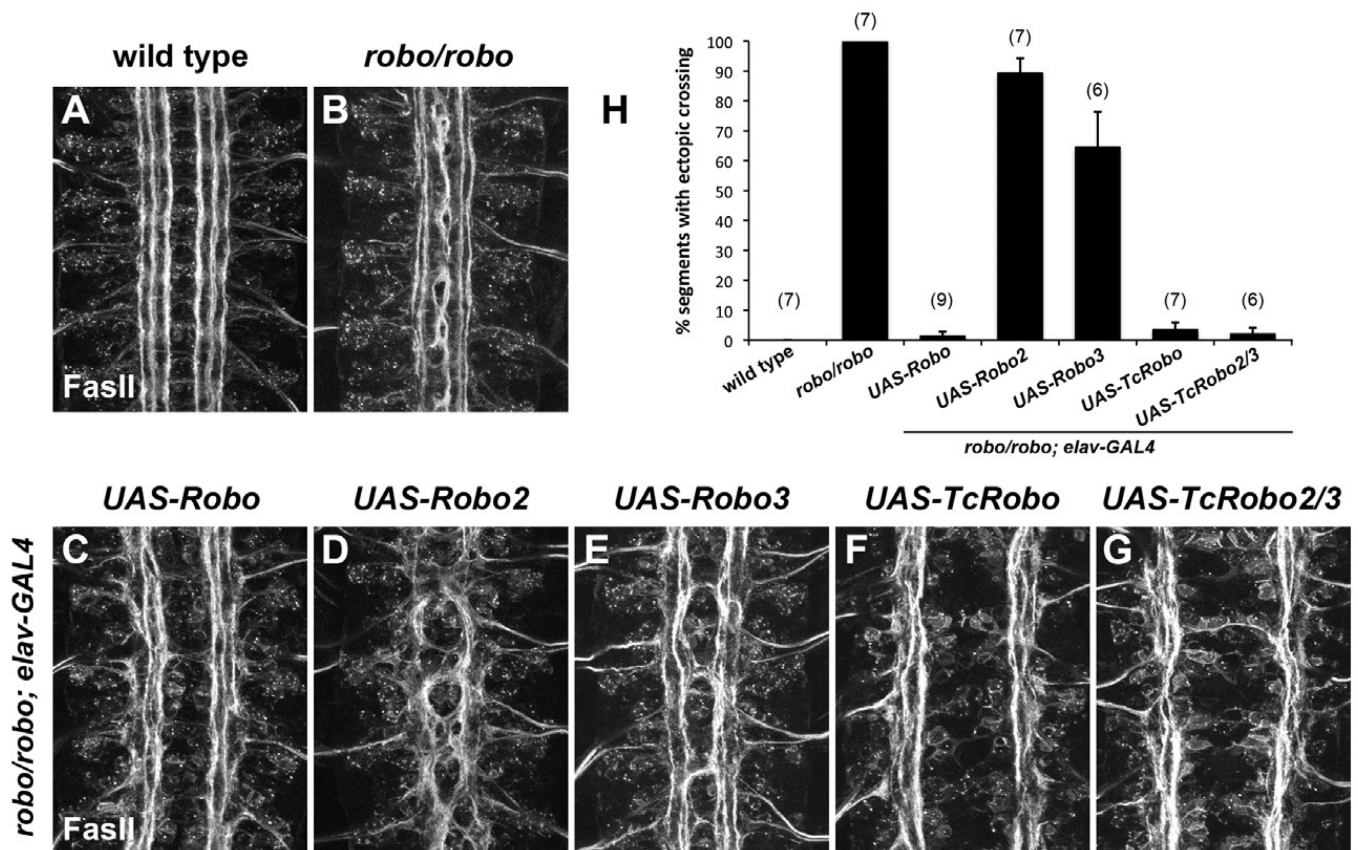


Figure 3. *Tribolium* Robos can mediate midline repulsion in *Drosophila* in the absence of *robo*
A–G. Stage 16–17 *Drosophila* embryos stained with mAb 1D4 (anti-FasII).

A. Wild type embryo. FasII-positive axon pathways do not cross the midline.

B. In *robo* mutant embryos, axons from the medial FasII pathways cross the midline in every segment.

C–G. *robo* mutant embryos carrying *elav-GAL4* and indicated *UAS-Robo* transgenes.
C. Pan-neural expression of *Drosophila* Robo fully rescues the *robo* loss of function phenotype: FasII-positive axons no longer cross the midline.

D,E. Expression of *Drosophila* Robo2 (**D**) or Robo3 (**E**) does not prevent ectopic midline crossing in a *robo* mutant background.

F,G. Expression of *Tribolium* Robo (**D**) or *Tribolium* Robo2/3 (**E**) is sufficient to prevent ectopic midline crossing in the absence of endogenous *robo*.

H. Quantification of ectopic midline crossing (average percent segments with ectopic FasII crossing per embryo) in the genetic backgrounds shown in **A–G**. Error bars represent s.e.m. Number of embryos scored for each genotype is indicated in parentheses.

Drosophila

Tribolium

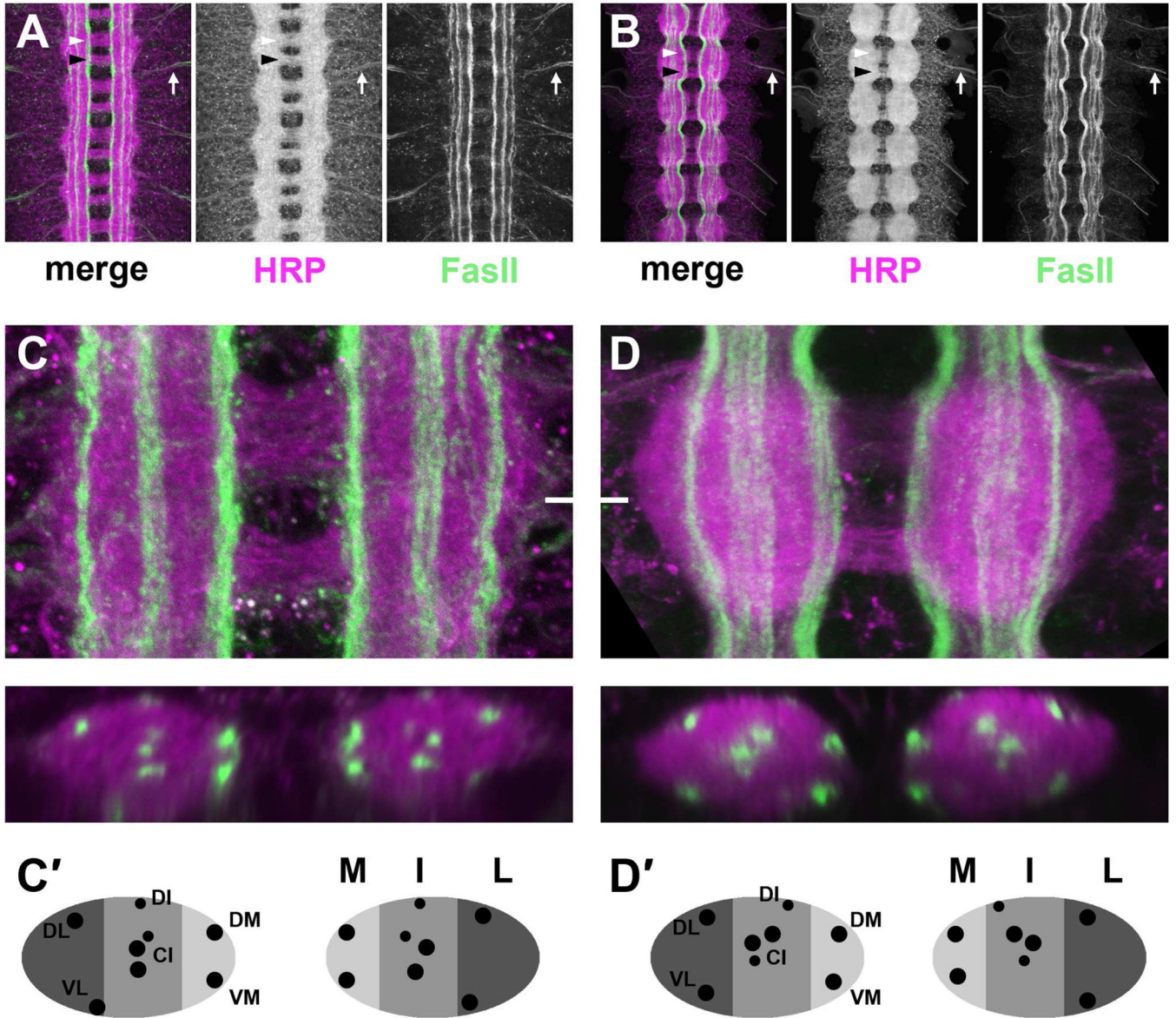


Figure 4. Conservation of CNS architecture in *Drosophila* and *Tribolium*

A–B. Dissected ventral nerve cords from a stage 16–17 *Drosophila* embryo (A) and 4–5 day old *Tribolium* embryo (B) stained with antibodies against HRP (magenta; detects all axons in the CNS) and the longitudinal pathway marker FasciclinII (FasII; green). Both insects exhibit a ladder-like arrangement of axon pathways in the CNS consisting of bilateral longitudinal connectives and two commissures per segment. Anterior and posterior commissures in a single segment are indicated by white and black arrowheads, respectively. Individual FasII-positive axon pathways are visible in both species, and they do not cross the midline. Peripheral nerves are labeled by both anti-HRP and anti-FasII in each species as they exit the CNS (arrows).

C–D. Single abdominal segments of *Drosophila* (**C**) and *Tribolium* (**D**) embryos stained as in **A–B**. Maximum confocal projections through the entire neuropile are shown above; optical cross sections taken at the level of the white hash mark are shown below.

(C',D'). Schematic representation of cross sections shown in **C,D** denoting medial (M, light gray), intermediate (I, medium gray) and lateral (L, dark gray) regions of the neuropile and showing relative positions of individual longitudinal pathways (black dots).

C–C'. FasII-positive pathways form in three distinct zones within the *Drosophila* neuropile. At least eight distinct FasII pathways can be seen in cross section: the dorsal medial (DM) and ventral medial (VM) pathways form within the medial (M) zone; three central intermediate (CI) and one dorsal intermediate (DI) pathway form within the intermediate (I) zone; the dorsal lateral (DL) and ventral lateral (VL) pathways form within the lateral (L) zone. Pathway nomenclature is after Landgraf et al 2003.

D–D'. Similar arrangement of axon pathways in the *Tribolium* embryonic CNS. Distinct FasII-positive pathways are visible at medial, intermediate, and lateral positions. The same number of individual pathways can be seen in cross section at equivalent dorsoventral and mediolateral locations to those in *Drosophila*. In this and all subsequent figures, *Tribolium* embryos are shown scaled to 75% original size for easier comparison with the smaller *Drosophila* embryos.

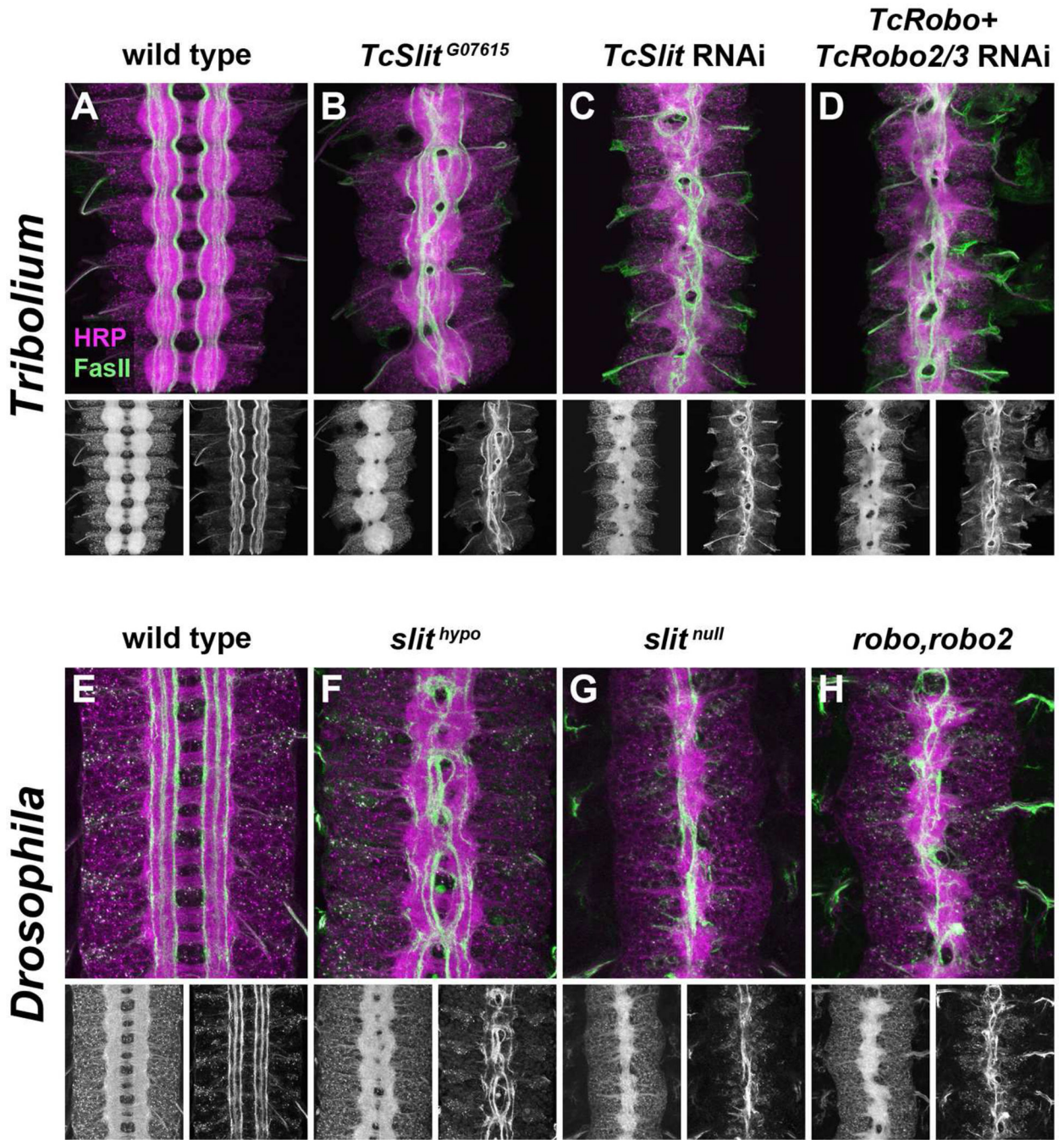


Figure 5. Slit/Robo signaling mediates midline repulsion in *Tribolium*

A–D. 4–5 day old *Tribolium* embryos stained with anti-HRP (magenta) and anti-TcFasII (green). Small panels below show individual HRP (left) and FasII (right) channels.

A. Wild type *Tribolium* embryo. The ladder-like axon scaffold is formed by segmentally repeated pairs of commissures and bilateral longitudinal connectives. Distinct FasII-positive pathways are visible, and they do not cross the midline.

- B.** In *TcSlit*^{G07615} embryos, commissures are fused and FasII-positive axons linger at the midline, similar to the phenotype seen in *Drosophila* hypomorphic *slit* mutants (**F**). Some longitudinal pathways still form relatively normally in these embryos.
- C.** Embryos from mothers injected with dsRNA targeting *TcSlit* display a strong midline collapse phenotype. The width of the axon scaffold is reduced and longitudinal pathways do not form properly, as many FasII-positive axons enter the midline and fail to leave.
- D.** Embryos in which *TcRobo* and *TcRobo2/3* are targeted in combination phenocopy the *TcSlit* knockdown phenotype.
- E–H.** Stage 16–17 *Drosophila* embryos stained with anti-HRP (magenta) and mAb 1D4 (anti-FasII; green).
- E.** In wild type *Drosophila* embryos, the longitudinal connectives are well separated and connected by two commissures per segment. Three distinct FasII-positive pathways can be seen on either side of the midline; they do not cross the midline.
- F.** In hypomorphic *slit* mutants, the connectives are closer together, commissures thicken and begin to fuse, and FasII-positive axons cross the midline in every segment.
- G.** In *slit* null mutants, all axons collapse at the midline, reflecting a complete absence of midline repulsion.
- H.** Double mutant *robo, robo2* embryos display a *slit*-like midline collapse phenotype.

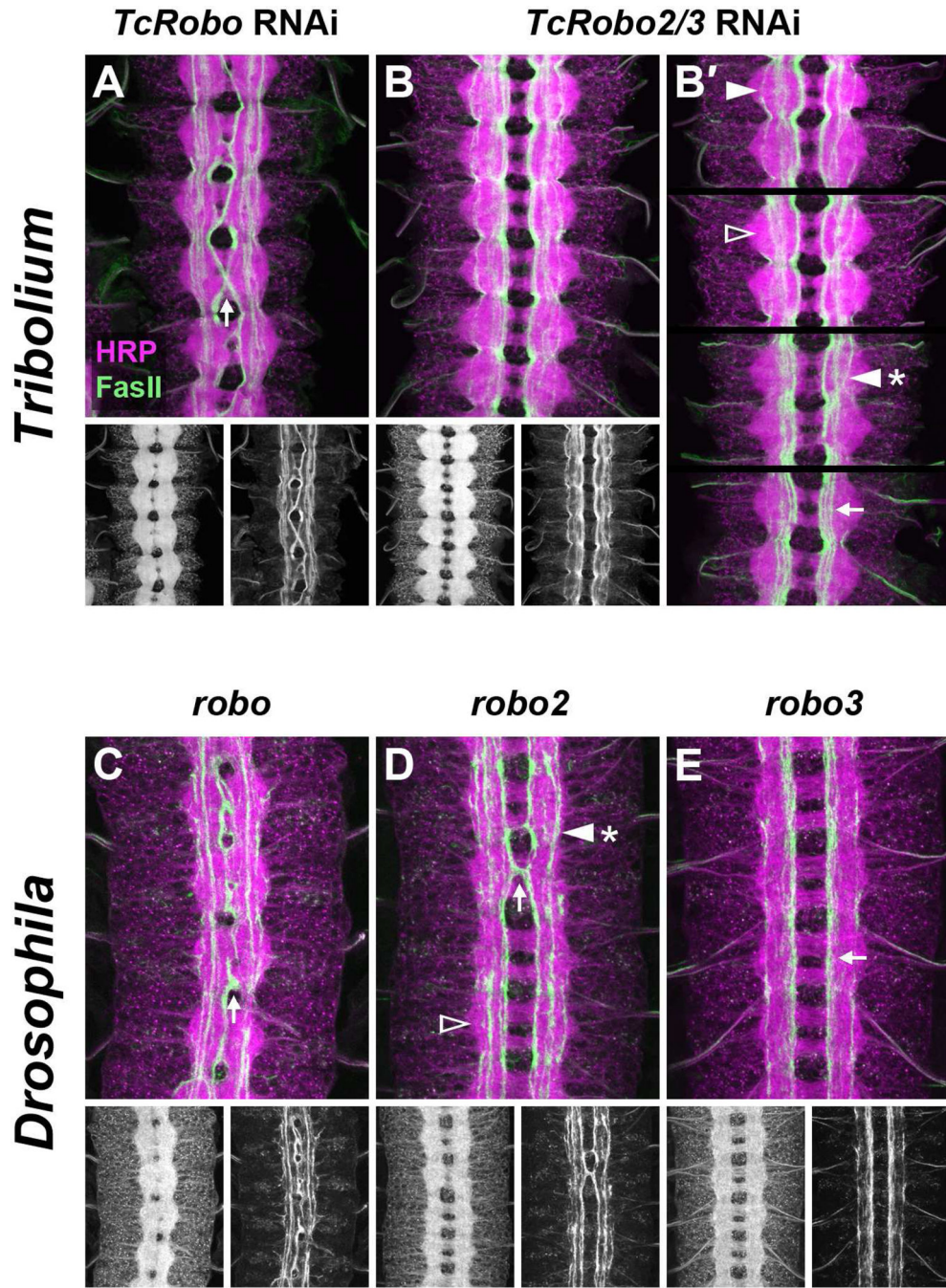


Figure 6. *Tribolium* Robo receptors exhibit specialized axon guidance functions

Tribolium (A,B) and *Drosophila* (C–E) embryos prepared as in Figure 5.

A. *TcRobo* knockdown embryo. Commissures are thickened and FasII axons from medial pathways ectopically cross the midline (arrow). Intermediate and lateral longitudinal pathways are formed relatively normally.

B. *TcRobo2/3* knockdown embryo. The axon scaffold has an overall normal appearance, and no FasII-positive axons cross the midline. Longitudinal pathways are disorganized and often fail to form at their normally stereotyped positions.

B'. Partial confocal projections from four different *TcRobo2/3* knockdown embryos displaying longitudinal pathway defects: missing lateral pathways (empty arrowhead); FasII-positive axons wandering between longitudinal pathways (arrowhead with asterisk); intermediate and lateral pathways compressed into medial zone (arrow). In a minority of segments, intermediate and lateral pathways form relatively normally (solid arrowhead). Projections were made from 8–15 0.2 μm confocal sections and include all dorsal pathways in their entirety while excluding the majority of ventral pathways.

C. *robo* mutant embryo. As in *TcRobo* knockdown embryos (**A**), thickened commissures and ectopic midline crossing of FasII axons can be seen (arrow).

D. *robo2* mutant embryo. Although the axon scaffold appears relatively normal, medial FasII axons can occasionally be seen crossing the midline (arrow). In addition, lateral FasII pathways are often absent (empty arrowhead) or fuse with intermediate pathways (arrowhead with asterisk).

E. *robo3* mutant embryo. The axon scaffold appears normal, and no ectopic crossing is detectable. Intermediate FasII pathways shift towards the midline and fuse with medial pathways (arrow).

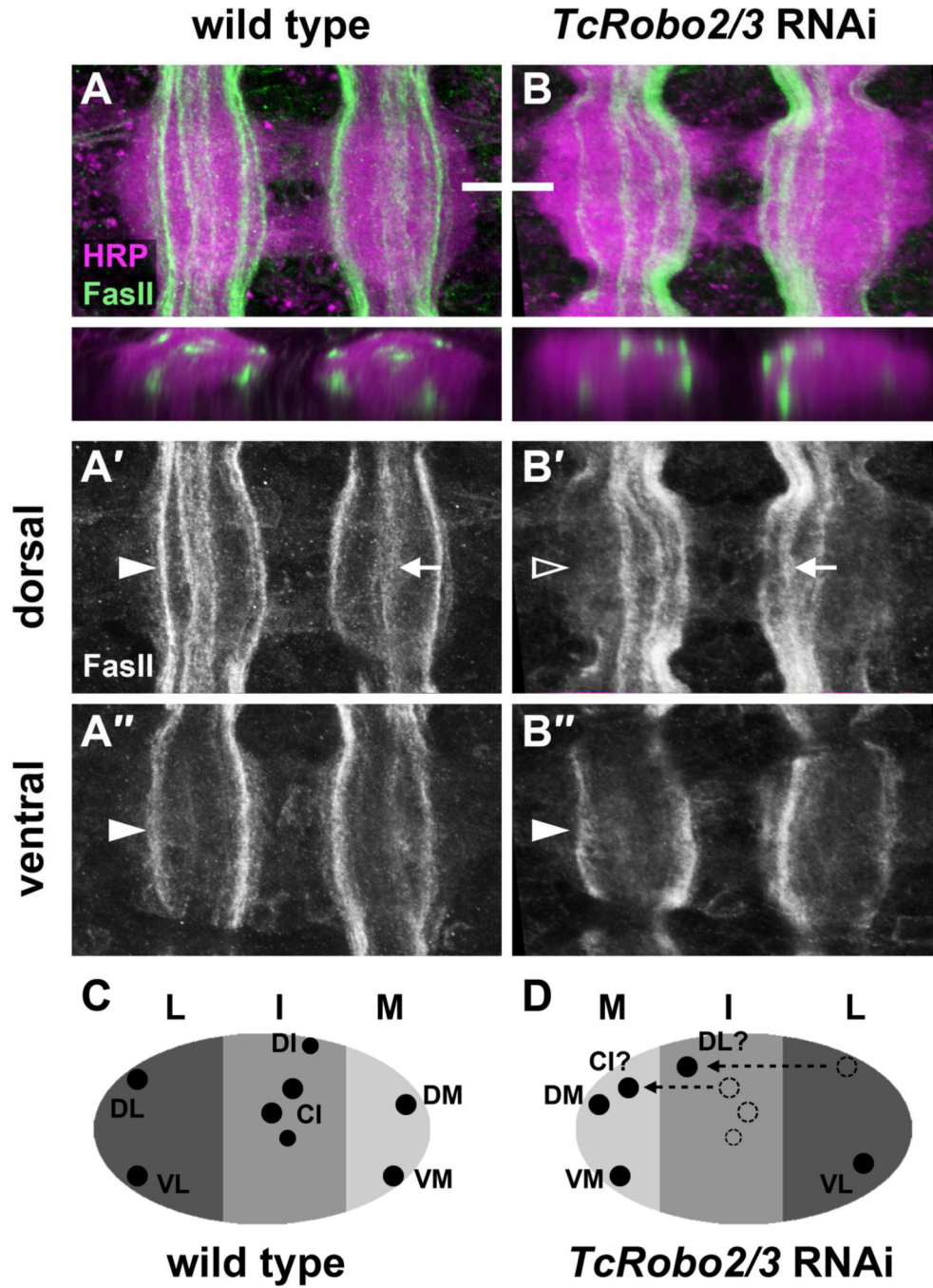


Figure 7. *TcRobo2/3* controls multiple aspects of longitudinal pathway formation

A,B. High-magnification views of single abdominal segments from wild type (**A**) and *TcRobo2/3* knockdown (**B**) embryos. Maximum confocal projections through the entire neuropile are shown above; optical cross-sections taken at the level of the white hash mark are shown below.

A',B'. Partial confocal projections showing only the dorsal-most FasII pathways.

A'',B''. Partial confocal projections showing only the ventral-most FasII pathways.

In wild type embryos, a major FasII pathway forms in the dorsal lateral region of the neuropile (solid arrowhead in **A'**). In *TcRobo2/3* knockdown embryos, this pathway is missing in the majority of segments (empty arrowhead in **B'**). The ventral lateral FasII pathway appears unaffected by the loss of *TcRobo2/3* (solid arrowhead in **A''**, **B''**). In addition, the 3–4 FasII pathways that normally form in the intermediate region (arrow in **A**, **A'**) are shifted medially in *TcRobo2/3* knockdown embryos (arrow in **B**, **B'**).

C,D. Schematic comparison of longitudinal pathways in wild type (**C**) and *TcRobo2/3* knockdown (**D**) embryos. Loss of *TcRobo2/3* results in the absence (dashed circles) or medial shifting (dashed arrows) of both lateral and intermediate pathways. In the absence of more restricted molecular markers, the identity of ectopic pathways forming within the medial zone cannot be determined unless axons can be detected shifting from more lateral pathways. Medial (DM and VM) and ventral lateral (VL) pathways appear unaffected by loss of *TcRobo2/3*. See Figure 4 legend for longitudinal pathway nomenclature.

Table 1Quantification of *Tribolium* Robo knockdown phenotypes.

genetic background	FasII-positive axons at midline ^a	medial shift of intermediate pathway ^b	dorsal lateral pathway defects ^b	
			medial shift ^c	missing
wild type	0.0 (n=48/8)	0.0 (n=93/8)	0.0 (n=95/8)	0.0 (n=95/8)
<i>TcRobo</i> RNAi	91.9 (n=74/11)	1.5 (n=131/11)	0.8 (n=119/11)	0.0 (n=119/11)
<i>TcRobo2/3</i> RNAi	0.0 (n=51/10)	83.3 (n=102/10)	11.0 (n=100/10)	69.0 (n=100/10)

4–5 day old embryos were stained with anti-HRP and anti-TcFasII and abdominal segments were scored for defects in midline repulsion and longitudinal pathway formation. Percentage of segments or hemisegments displaying each phenotype are shown.

^a n = number of segments/number of embryos scored.

^b n = number of hemisegments/number of embryos scored.

^c hemisegments were scored as “medial shift” if axons could be seen transitioning from lateral to intermediate pathways within the hemisegment.