Embryonic axon guidance: insights from Drosophila and other insects
Timothy A Evans

During embryonic development, growing axons are guided by cellular signaling pathways that control a series of individual axon guidance decisions. In Drosophila, two major pathways (Netrin-Frazzled/DCC and Slit-Robo) regulate axon guidance in the embryonic ventral nerve cord, including the critical decision of whether or not to cross the midline. Studies in the fruit fly have revealed a complex picture of precise regulation and cross-talk between these pathways. In addition, Robo receptors in Drosophila have diversified their activities to regulate additional axon guidance decisions in the developing embryo. Here, I discuss recent advances in understanding roles and regulation of the Net-Fra and Slit-Robo signaling pathways in Drosophila, and examine the evolutionary conservation of these signaling mechanisms across insects and other arthropods.

Address
Department of Biological Sciences, University of Arkansas, Fayetteville, AR 72701, United States

Corresponding author: Evans, Timothy A (evanst@uark.edu)

Introduction
Axon guidance, the process by which developing neuronal axons follow a series of directional cues as they grow toward their final synaptic targets, is an essential aspect of nervous system development. In many animals with bilateral symmetry, the embryonic midline is a source of important cues that regulate axon guidance decisions during development of the central nervous system (CNS) [1,2].

The regulation of midline crossing (i.e. controlling where, when, and which axons cross the midline) and the choice of longitudinal pathway are essential to establishing this precisely organized orthogonal array of axon tracts.

Much of our understanding of the molecular and genetic regulation of axon guidance in the insect CNS has come from studies in Drosophila. Of particular importance are two opposing signaling pathways that regulate the decision of whether or not to cross the midline: the attractive Netrin-Frazzled/DCC pathway, and the repulsive Slit-Robo pathway. Since their initial characterization two decades ago, we have developed an increasingly detailed understanding of the roles and regulation of the Net-Fra and Slit-Robo signaling pathways in Drosophila, and a complex picture has emerged of both functional diversification within pathways and cross-regulation between pathways (Figure 1).

In many respects Drosophila is highly evolutionarily derived, and may not serve as an ideal representative of the majority of insects. It is therefore imperative to consider whether insights gained from studies in the fly are applicable to other species. As the number of available insect genome sequences increases [3], and techniques for gene engineering and manipulation become more feasible across a wider variety of insect species [4], there is increasing opportunity for comparative studies of the molecular biology of axon guidance within this large and diverse assemblage of species.

In this review, I discuss recent insights into the mechanisms regulating midline axon guidance in Drosophila, and comparative studies of axon guidance pathways in flies and other insects that illuminate the conservation and divergence of signaling mechanisms within insects. I focus on ligands and receptors of the Net-Fra and Slit-Robo pathway, with emphasis on the functional diversity of insect Robo receptors, especially Drosophila Robo2.

Classical midline axon guidance pathways in Drosophila
Genetic screens in Drosophila identified genes with essential roles in promoting or inhibiting midline crossing of axons in the embryonic CNS [5]. Chief among these was the secreted ligand slit, produced by glial cells located at the CNS midline, and robo1, a Slit receptor expressed on the surface of pathfinding axons [6,7]. Mutations in slit or robo1 reduce midline repulsion, leading to ectopic midline crossing. A second Robo family gene (robo2) acts
Axon guidance pathways in Drosophila and Tribolium. Top, three consecutive segments from the embryonic ventral nerve cords of Drosophila (left) and Tribolium (right) are shown. Fluorescently labeled antibodies label all axons (anti-HRP, magenta) or a subset of longitudinal axon pathways (anti-Fasl, green). Within each segmental ganglion, axons cross the midline in the anterior (A) and posterior (P) commissures. Fasl-positive axon pathways form in distinct medial (M), intermediate (I), and lateral (L) zones in both insects (arrows). Bottom, genetic regulation of midline crossing and longitudinal pathway formation. In Drosophila, Fra promotes midline attraction in response to NetA/B [14–16], while Robo1 and Robo2 promote midline repulsion in response to Slit [7–9]. Robo1 repulsion is negatively regulated by Comm and Robo2 [11,21]. Tribolium does not possess a Comm ortholog, and it has retained the ancestral receptor Robo2/3. TcSlit, TcRobo1, and TcRobo2/3 all promote midline repulsion in Tribolium [41**]. Drosophila Robo2 and Robo3 promote the formation of lateral and intermediate longitudinal axon pathways, respectively [24,25]. These two roles are both performed by TcRobo2/3 in beetles [41**]. Predicted or unconfirmed roles are indicated with gray dashed arrows.

regulated, turning on just as commissural axons are crossing the midline, then extinguished rapidly thereafter [13]. This provides a short window in which commissural axons are free to ignore the normally repulsive Slit-expressing midline and cross to the other side, whereupon surface Robo1 expression is re-established to prevent re-crossing.

The major signaling pathway promoting midline attraction in Drosophila is the Netrin-Frazzled/DCC pathway. Netrin ligands (encoded by the functionally redundant NetA and NetB genes in Drosophila) are produced by midline cells and signal through the DCC family receptor Frazzled (Fra) to attract axons toward and across the midline [14–16]. In NetAB double mutant or fra mutant embryos, midline crossing is reduced and commissures are thin or absent. However, many axons successfully cross the midline even in the complete absence of attractive Net-Fra signaling, suggesting additional pathways that can promote midline crossing independently of Netrin and Frazzled. In addition to mediating Netrin-dependent midline attraction, Fra also acts independently of Netrin to promote comm transcription in pre-crossing commissural axons [17].

**Additional factors promote midline crossing in Drosophila**

The observation that many axons are able to cross the midline even in the complete absence of Net-Fra attractive signaling has motivated researchers to search for additional pathways that might act redundantly to Net-Fra to promote midline crossing. Indeed, a number of mutations produce a commissureless or nearly commissureless phenotype when introduced into a NetAB or fra mutant background. These include mutations in the Down Syndrome Cell Adhesion Molecule (Dscam) gene [18], the atypical cadherin gene flamingo (fmi) [19], and the Robo family receptor robo2 [20,21]. Notably, mutations in each of these genes do not produce detectable defects in midline crossing on their own; it is only when NetAB or fra are also removed that the defects become apparent. In addition, a recent report showed that the Hedgehog (Hh) morphogen is produced at the Drosophila CNS midline and is able to promote ectopic midline crossing of axons when misexpressed [22], perhaps echoing Sonic Hedgehog’s (Shh) role in attracting axons toward the midline in the vertebrate spinal cord [23]. In the case of Robo2, enhancement of the fra phenotype reflects a role for Robo2 in antagonizing canonical Slit-Robo1 repulsion [21]. It remains to be determined precisely how these other factors might act alongside Net-Fra to promote midline crossing.

**Functional diversity of Robo receptors: midline crossing and beyond**

In addition to signaling midline repulsion in response to Slit, Robo family receptors in Drosophila regulate other axon guidance outcomes in the embryonic CNS
Axon guidance roles of the *Drosophila* Robo2 receptor. Left, schematic of the Robo2 protein highlighting the functional roles of individual protein domains. Robo2 is a transmembrane protein with five immunoglobulin-like (Ig) domains and three fibronectin-like (Fn) domains in its extracellular portion, and two short conserved cytoplasmic (CC) motifs. Ig1 is the canonical Slit-binding domain, which functions in midline repulsion and lateral positioning of longitudinal axons (along with Ig3) [26]. Ig2 is required for Robo2’s non-autonomous role in promoting midline crossing of commissural axons [21]. The Robo2 cytoplasmic domain specifies its role in motor axon guidance [27]. Right, schematic of the *Drosophila* embryonic nerve cord, illustrating the four distinct axon guidance decisions that are regulated by Robo2. Robo2 acts in early ipsilateral pioneer axons to prevent midline crossing in response to Slit (purple) [9,9], while promoting midline crossing of commissural axons by inhibiting Slit-Robo1 repulsion in trans (orange) [21]. Robo2 is required for guidance of motor axons in the ISNb motor nerve, which exit the nerve cord to innervate ventral body wall muscles (yellow) [27]. Later in embryonic development, Robo2 is required for formation of longitudinal axon pathways in the lateral region of the neuropile (green) [24,25]. *Drosophila* Robo2’s midline repulsion and lateral positioning roles are shared by *Tribolium* Robo2/3 [41**], but it is unknown whether its other roles are evolutionarily conserved.

(Figure 2). Robo2 and Robo3 cooperate to specify the medial-lateral position of longitudinal axon pathways [20,24–26], and Robo2 is required for proper guidance of ventrally-projecting motor axons in the ISNb motor nerve [27]. Robo2 also acts non-autonomously in midline cells to promote midline crossing of commissural axons through inhibitory interactions with Robo1 [21**]. It appears that some of the divergent roles of the three *Drosophila* Robos depend merely on differences in expression pattern (e.g., robo2-dependent midline repulsion defects are rescued when robo1 is expressed in its place; likewise, both robo1 and robo2 can substitute for robo3 to promote longitudinal pathway formation in intermediate regions of the neuropile [20]), while others reflect intrinsic differences in receptor activities (Robo2’s roles in promoting midline crossing [20,21**] and motor axon guidance [27] depend on unique structural features within its Ig2 domain and cytoplasmic domain, respectively, and neither robo1 nor robo3 can substitute for robo2 to promote lateral axon pathway formation [20]). Apart from midline repulsion, it is unknown if any of the other roles of *Drosophila* Robos are Slit-dependent, although the Slit-binding Ig1 domain of Robo2 appears at least partially dispensable for Robo2’s role in promoting midline crossing [21**].

Mechanisms of receptor signaling: recent insights from *Drosophila*

Although Slit and Robo1 were identified as a ligand–receptor pair over 17 years ago [7], it was only recently demonstrated that the ability to bind Slit is necessary for Robo1’s midline repellant activity in vivo, as *Drosophila* embryos expressing a Slit-binding-deficient version of Robo1 phenocopy robo1 null mutants [28]. In the context of midline repulsion, both regulated proteolytic cleavage (via the ADAM family metalloprotease Kuzbanian) and clathrin-dependent endocytosis appear to be required for Robo1 activation in response to Slit [29,30**]. It will be interesting to learn whether the various roles of the other Robo receptors in *Drosophila* (Robo2 and Robo3) also depend on receptor proteolysis and/or endocytosis, and whether this signaling mechanism is conserved in Robo1 orthologs in other species. This latter possibility is supported by the observation that cytoplasmic sequences necessary for endocytosis in *Drosophila* Robo1 are conserved in human Robo1 [30**].

Regulated proteolytic cleavage also plays a role in Frazzled/DCC signaling in *Drosophila*: the Fra intracellular domain (ICD) is released from the receptor in response to an as-yet- unidentified signal and translocates into the nucleus to activate expression of comm in pre-crossing commissural axons [31**]. This mechanism of receptor cleavage and transcriptional regulation is reminiscent of the canonical Notch pathway, and indeed production of the Fra ICD fragment, like that of Notch, depends on the activity of the gamma-secretase gene presenilin [31**,32]. Notably, vertebrate orthologs of Fra (DCC and Neogenin) can also undergo gamma-secretase-dependent cleavage and their ICD fragments can act as nuclear transcriptional activators, but vertebrates appear to lack a comm gene and the transcriptional targets of these vertebrate receptors have not yet been identified [33,34].
Comparative studies of Net-Fra signaling in insects and other arthropods

While much of the work of the past two decades has focused on characterizing axon guidance pathways in *Drosophila*, in recent years a number of researchers have turned to investigating whether the genes and mechanisms that regulate axon guidance in the *Drosophila* embryonic CNS are conserved in other insects. Ligands and receptors of the Net-Fra and Slit-Robo pathways are present across insects and other arthropods [35–37], and expression and functional analyses support evolutionary conservation of their canonical roles in midline attraction and repulsion, respectively.

Netrin ligands are expressed at the midline of the embryonic CNS in a number of insect and non-insect arthropod species, including *Drosophila melanogaster* [14,15], the mosquito *Aedes aegypti* [38], the flour beetle *Tribolium castaneum* [38], the branchiopod crustacean *Artemia franciscana* [39] and *Triops longicaudatus* [38], the amphipod crustacean *Parhyale hawaiensis* [38], the terrestrial isopod crustacean *Porcellio laevis* [38], and the spider *Cupiennius salei* [40]. Although functional studies of Netrins in most of these species are lacking, RNAi-based knockdown of *C. salei netrin* caused a reduction in axons crossing the midline in spider embryos, supporting an evolutionarily conserved role for Netrins in midline attraction across the arthropod clade [40].

The attractive Netrin receptor Frazzled/DCC is likewise present in the genomes of insects and other arthropods, but few studies have examined the expression pattern or function of Fra orthologs in arthropods other than *Drosophila*. Thus, it is not yet clear how broadly *Drosophila* Fra’s dual roles in promoting Netrin-dependent attraction and Netrin-independent activation of *comm* transcription might be conserved across arthropod species. It is likely that the Netrin-dependent attractive signaling role of Fra will be more broadly conserved than its role in regulating *comm* transcription, as *comm* does not appear to be conserved even within insects. For example, orthologs of the three *Drosophila* *comm* genes are not detectable in the *Tribolium castaneum* genome sequence [41**,42]. Sarro and colleagues [43**] identified genes orthologous to *Drosophila* *comm2* in the mosquitoes *Culex quinquefasciatus* and *Aedes aegypti* and reported that siRNA-mediated knockdown of *A. aegypti fra* or *comm2* produced a commissureless phenotype, and that *fra* knockdown resulted in a loss of *comm2* expression in *A. aegypti*, suggesting that *bra*’s role in activating *comm* (or *comm2*) transcription may be conserved, at least within dipterans [43**,44]. The severity of the *fra* knockdown phenotype in *A. aegypti* may indicate that the redundant pathways which promote midline crossing in the absence of Net-Fra signaling in *Drosophila* (i.e. *robo2*, *Dscam*, and *flamingo*), do not have equivalent roles in mosquitoes [44].

Comparative studies of Slit-Robo signaling in insects

The evolutionary history of Robo family receptors in insects is more complex, involving multiple instances of gene duplication, neofunctionalization and/or subfunctionalization, producing the three *Drosophila* Robos with their distinct suite of axon guidance roles. *Drosophila* *robo2* and *robo3* are the products of a recent gene duplication [24,25], and exist as distinct genes only within dipteran insects. Other insect orders have instead retained the ancestral *robo2/3* gene, which itself was separated from *robo1* via gene duplication sometime early in insect evolution, as non-insect genomes do not contain an identifiable *robo2/3* ortholog [41**]. This evolutionary history is particularly interesting considering the multifunctional roles of the *Drosophila* Robo2 protein (Figure 2).

In the flour beetle *Tribolium castaneum*, the two Robo orthologs (TcRobo1 and TcRobo2/3) cooperate to signal midline repulsion in response to *Tribolium* Slit (TcSlit), similarly to *Drosophila* Robo1 and Robo2 [41**]. TcRobo2/3 also specifies the positions of longitudinal pathways in the intermediate and lateral regions of the neuropile, thus combining the roles of *Drosophila* Robo2 and Robo3 in this context [41**]. This is especially intriguing given that in *Drosophila*, Robo3 is unable to substitute for Robo2 to specify lateral pathway formation, while Robo2 can rescue Robo3’s role in intermediate pathway formation but is not normally expressed in the right neurons to do so [20]. This suggests a combination of subfunctionalization events wherein Robo2 lost a portion of its ancestral expression pattern (in intermediate regions of the neuropile), while Robo3 lost an ancestral protein function (the ability to specify lateral pathways). Expression analyses in *Tribolium* embryos and cross-species gene replacement experiments in *Drosophila* and *Tribolium* will be necessary to test this hypothesis.

A recent series of studies in the silkworm *Bombyx mori* suggests that its single *slit* ortholog (*Bmslit*) and three *robo* genes (*Bmrobo1a*, *Bmrobo1b*, and *Bmrobo2/3*) are also involved in midline axon guidance, although it is difficult to disentangle effects on midline crossing from medial-lateral positioning (i.e. distance from the midline) due to a lack of specific markers for normally non-crossing axons [45**,46**,47**]. Somewhat puzzlingly, given the partial redundancy of *Drosophila* *robo1* and *robo2* and *Tribolium* *Robo1* and *Robo2/3*, knocking down either *Bmrobo1a* or *Bmrobo1b* individually (these two genes represent a lepidopteran-specific duplication of the insect *robo1* gene) produced identical phenotypes [45**], which also appear indistinguishable from the effects of knocking down *Bmrobo2/3* [46**] or *Bmslit* [47**]. A closer examination of individual and combinatorial knockdowns in this species (or CRISPR-based gene knockouts [48,49]), ideally with an ipsilateral axon marker like anti-FasII, is clearly warranted.
Robo2’s role in promoting midline crossing has not yet been examined in any insects other than *Drosophila*. Although no apparent decreases in midline crossing were observed in *Triboletium* or *Bombyx* embryos under *TriRobo2/3* or *Bmrobo2/3* knockdown conditions, respectively [41**-46**], this is perhaps not surprising given that *Drosophila* Robo2’s pro-crossing activity only becomes apparent when Net-Fra signaling is also disrupted (i.e. in *NetAB,robo2* or *fra,robo2* compound mutants) [20,21**]. Thus, determining whether Robo2 orthologs in dipterans or Robo2/3 orthologs in other insects share *Drosophila* Robo2’s pro-crossing activity will likely require simultaneous knockdown or CRISPR-based knockout [50] of *robo2* or *robo2/3* along with *Net* or *fra*. The observation that *fra* knockdown in mosquito produces more severe defects in midline crossing than *Drosophila* fra mutants [44] suggests that Robo2’s pro-crossing role may not be shared in other insects.

**Conclusions**

We have derived much insight into the genetic regulation of axon guidance from studies in *Drosophila*, but it is clear that not all of the components and signaling mechanisms are conserved in other insects. Comparative studies in other genetically tractable insects such as *Aedes*, *Bombyx*, and *Triboletium* have the potential to place those insights into a larger evolutionary context, define conserved versus divergent signaling mechanisms, and potentially identify novel factors and signaling mechanisms that are not present in *Drosophila*. Deciphering the evolution of axon guidance mechanisms in insect embryos may lead to further insights into understanding the evolution of the insect nervous system.

**Acknowledgements**

This work was supported by funding provided to T.A.E. by the University of Arkansas.

**References and recommended reading**

Papers of particular interest, published within the period of review, have been highlighted as:

- of special interest
- of outstanding interest


This study dissected the mechanism by which *Drosophila* *Robo2* promotes midline crossing of commissural axons. Biochemical, gene expression, and structure/function evidence suggests that *Robo2* protein expressed in midline cells can promote midline crossing non-autonomously by inhibiting canonical *Slit-Robo1* repulsion through Ig2-dependent interactions with Robo1 in trans.


30. Chance RK, Bashaw GJ: Slit-dependent endocytic trafficking of the Robo receptor is required for son of sevenreaper and midline axon repulsion. PLoS Genet 2015, 11:e1005402. This paper provides intriguing insight into the mechanism of Slit-Robo repulsive signaling in drosophila. Subsequent to Slit activation, endocytosis of the Robo receptor and trafficking into specific endosomal compartments is necessary for the recruitment and activation of downstream signaling factors required for midline repulsion.

31. Neuhaus-Follini A, Bashaw GJ: The intracellular domain of the frazzled/DCC receptor is a transcription factor required for commissural axon guidance. Neuron 2015, 87:751-763. This study reports a novel role for the intracellular domain of drosophila Frazzled as a nuclear-localized transcriptional activator. In response to an unknown (Netrin-independent) activating signal, the Fra receptor undergoes proteolytic cleavage to produce an intracellular domain fragment which can translocate to the nucleus and activate transcription of comm. This mechanism represents a novel paradigm for guidance receptor signaling that could be conserved in other axon guidance pathways.


41. Evans TA, Bashaw GJ: Slit/Robo-mediated axon guidance in Tribolium and Drosophila: divergent genetic programs build insect nervous systems. Dev Biol 2012, 363:265-278. This study is the first investigation of Slit-Robo signaling in an insect other than Drosophila. RNAi-based knockdowns in Tribolium and transgenic experiments in Drosophila suggest that the regulation of Robo-dependent axon guidance decisions has undergone evolutionary change within insects.


43. Sarro J, Andrews E, Sun L, Behura SK, Tan JC, Zeng E, Severson DW, Duman-Scheel M: Requirement for commissureless2 function during dipteran insect nerve cord development. Dev Dyn 2013 http://dx.doi.org/10.1002/dvdy.24089. This paper, the first functional study of a commissureless gene outside of Drosophila, reports that the comm2 gene is necessary for midline crossing in the mosquito Aedes aegypti. In addition, the authors show that knocking down the frizzled gene in Aedes leads to a reduction in comm2 expression, suggesting that Frp-dependent activation of commn/comm2 transcription is conserved within dipterans.


47. Yu Q, Li X-T, Liu C, Cui W-Z, Mu Z-M, Zhao X, Liu Q-X: Evolutionarily conserved repulsive guidance role of slit in the silkworm Bombyx mori. PLoS ONE 2014, 9:e109377. This study along with Refs. [45**,46**] reports the characterization of Slit and its Robo receptors in the silkworm Bombyx mori. Knockdown of each of the slit and robo genes produce defects in midline axon guidance. Notably, both Robo1 orthologs (BmRobo1a and BmRobo1b) have lost the IgS and Fn1 structural domains, which are otherwise highly conserved in insect and most vertebrate Robo receptors.

