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Midline axon guidance in the *Drosophila* embryonic central nervous system

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Abstract

Studies in the fruit fly *Drosophila melanogaster* have provided many fundamental insights into the genetic regulation of neural development, including the identification and characterization of evolutionarily conserved axon guidance pathways and their roles in important guidance decisions. Due to its highly organized and fast-developing embryonic nervous system, relatively small number of neurons, and molecular and genetic tools for identifying, labeling, and manipulating individual neurons or small neuronal subsets, studies of axon guidance in the *Drosophila* embryonic CNS have allowed researchers to dissect these genetic mechanisms with a high degree of precision. In this review, we discuss the major axon guidance pathways that regulate midline crossing of axons and the formation and guidance of longitudinal axon tracts, two processes that contribute to the development of the precise three-dimensional structure of the insect nerve cord. We focus particularly on recent insights into the roles and regulation of canonical midline axon guidance pathways, and on additional factors and pathways that have recently been shown to contribute to axon guidance decisions at and near the midline.

Keywords

Axon guidance; *Drosophila*; midline; Slit; Robo; Netrin; Frazzled

1. Introduction

During embryonic development, the nervous system must construct itself by orchestrating not only the specification of numerous types of neurons, but also the ways in which membrane processes from these neurons (axons and dendrites) connect to each other and to non-neural cells. Though relatively simple by comparison with more sophisticated nervous systems such as those of vertebrates, the larval nervous system in the fruit fly *Drosophila melanogaster* must nonetheless allow the newly-hatched animal to crawl freely while seeking food and avoiding predators and nociceptive stimuli. This requires the same

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underlying neural and muscular processes as more complex behaviors in vertebrate animals, including detecting, processing, and integrating sensory information from a number of sensory modalities (touch, temperature, light, smell) and responding with coordinated muscle contraction [1,2].

Like its eventual adult form, the *Drosophila* larva has a segmented body plan. This extends to the embryonic central nervous system (CNS), which exhibits a segmentally-repeated pattern of bilaterally symmetric neuromeres (or segmental ganglia) in the ventral nerve cord (VNC, analogous to the dorsal spinal cord in vertebrates) (Figure 1A). Each abdominal hemisegment contains the same set of approximately 300 neurons (36 motor neurons plus around 270 uniquely identifiable interneurons) [3,4], each of which make stereotyped and reproducible axon guidance decisions from segment to segment and animal to animal (Figure 1B). This makes it possible to examine the same identifiable neurons facing the same guidance decisions in multiple segments and multiple animals across different genetic backgrounds, which greatly facilitates the quantitative examination of individual developmental outcomes.

The fruit fly has long been an attractive model system for studies in genetics and molecular biology, and the adoption of genetic screening methods for studying developmental biology in the 1970s and 1980s [5] allowed developmental neuroscientists to apply this powerful technique to the study of axon guidance [6,7]. Because of the relatively simple and stereotyped structure of the embryonic nervous system, the fast developmental time, ease of culture, large number of offspring, and powerful genetic tools, many fundamental insights into basic axon guidance mechanisms and evolutionarily conserved molecules and pathways have been achieved using the fly embryonic CNS as a model [8,9]. One of the most fundamental of axon guidance decisions, and one that many axons in the CNS of bilaterian animals must face, is whether or not to cross the midline. This decision has important consequences for integration of sensory cues, information processing, and neuromuscular coordination between the two sides of the body, and misregulation of midline crossing during development can lead to a number of neurological disorders in humans [10–12].

Compared to vertebrate systems, a smaller number of signaling pathways regulate guidance at and around the midline in insects, but these core signaling pathways are evolutionarily conserved in other animals including humans [13]. This has contributed to the relative ease in which genetic studies can be carried out in flies, where loss-of-function phenotypes are often stronger and more straightforward to interpret thanks to a relative lack of genetic redundancy compared to vertebrate genomes. In addition, a number of subset-specific antibodies and genetic tools for labeling subsets of commissural (midline-crossing) or ipsilateral (non-midline-crossing) neurons and their axons (such as *TauMyc* and *GAL4/UAS* transgenic reporter lines) allow precise, reproducible, and quantitative examination of midline crossing and axon pathway formation in the *Drosophila* embryonic CNS (Figure 1).

In this review, we discuss our current understanding of the signaling pathways and molecular mechanisms that regulate axon guidance in the *Drosophila* embryonic CNS, focusing on two key guidance decisions that are essential for assembling the precise three-dimensional structure of the insect ventral nerve cord: midline crossing and longitudinal

pathway formation. We discuss recent insights into how axon guidance receptors signal in response to their respective ligands, ligand-dependent versus -independent guidance decisions and signaling mechanisms, and the regulation of and crosstalk between major attractive and repulsive signaling pathways. A list of guidance pathway components and their proposed roles is compiled in Table 1.

2. Midline attraction

The majority of neuronal axons in the *Drosophila* embryonic CNS are commissural; that is, they cross the midline to innervate synaptic targets on the opposite (contralateral) side of the body [3]. A key early step in axon guidance for commissural neurons is for their axons to orient toward, then grow toward and across the midline. The midline thus represents a key organizing center for embryonic axon guidance, and specialized glial cells located at the midline (known as midline glia) produce both attractive and repulsive signaling cues which promote or inhibit midline crossing of pathfinding axons, respectively [13,14]. Axons approaching, crossing, then leaving the midline must control their responses to various attractive and repulsive cues with a high degree of temporal precision, to ensure that once a commissural axon enters the midline, it will continue across and exit before proceeding on the next leg of its journey, never to re-cross. Non-crossing, or ipsilateral, axons must either ignore attractive cues or respond preferentially to repulsive cues, keeping them on the same side of the body as they originated.

2.1. Netrin-Frazzled/DCC-mediated midline attraction

Midline attraction and midline crossing of axons in *Drosophila* is influenced by a number of molecules and signaling pathways; chief among these is the Netrin-Frazzled/DCC pathway, which is evolutionarily conserved across bilaterian animals and promotes midline crossing of axons in a wide variety of animal groups [15–26]. In *Drosophila*, two functionally redundant Netrin ligands (NetA and NetB) signal through their receptor Frazzled (Fra, a member of the DCC/Deleted in Colorectal Cancer family) to promote midline crossing [23,25,26]. Initial models considered Netrins as long-range chemoattractants, secreted by and diffusing away from midline cells to attract commissural axons toward the midline at a distance. However, this model has been contradicted by evidence from both *Drosophila* and vertebrates, suggesting that Netrins may instead act as short-range or contact-dependent cues. In *Drosophila*, tethering Netrin to the cell membrane does not block its function in commissure formation, suggesting that it does not need to diffuse from the cells producing it at the midline [27]. Even in embryos lacking both Netrin genes, commissural axons are able to orient toward the midline. This indicates that Netrin acts as a short-range permissive cue to promote growth across the midline in *Drosophila*, rather than a long-range chemoattractant to draw commissural axons toward the midline [27]. Also in *Drosophila*, Net and Fra regulate axon-target adhesion in R8 photoreceptor axons, supporting a role for local Net-Fra signaling in contexts outside of the embryonic CNS [28]. Recent data from studies in mouse embryos suggest that mammalian Netrin-1 likewise may not act as a long-range chemoattractant, but rather may promote midline crossing through local contact-dependent adhesion [29–31]. A study of cell death in *Netrin* mutants in *Drosophila* suggests that NetB also acts as a neurotrophic factor, promoting neuronal survival in addition to

midline attraction, and that blocking apoptosis in neurons can rescue midline crossing defects seen in *NetAB* mutants [32].

Abelson tyrosine kinase (Abl) and the guanine nucleotide-exchange factor Trio both influence axon pathfinding at the *Drosophila* embryonic CNS midline, and appear to act at least in part downstream of Netrin and Frazzled [33–35]. Abl and Trio physically interact with the cytoplasmic domain of Fra. An increase in Abl expression in cells increases tyrosine phosphorylation of Fra and Trio. This indicates that Abl, Trio and Fra function together in commissure formation. It can also be resolved that Abl and Trio function downstream of at least one other receptor that has a pro-crossing role in regulating commissure formation [33].

2.2. Netrin-independent transcriptional regulation by Frazzled

In addition to acting as a Netrin receptor to mediate midline attractive signaling, *Drosophila* Fra also functions as a transcriptional activator to regulate expression of *commisureless* (*comm*), a key modulator of axonal responsiveness at the midline [36]. Proteolysis of Fra in response to an uncharacterized but Netrin-independent signal releases the Fra intracellular domain (ICD), which moves to the nucleus and is sufficient to activate *comm* transcription and promote midline crossing. Fra regulation of *comm* expression relies on the conserved P3 motif within the Fra ICD, which functions as a transcriptional activator [37] and has also been implicated in attractive signaling by Fra [38]. The Fra ortholog in mosquito (*Aedes aegypti*) is also required for midline crossing, and promotes transcription of the *comm2* gene, suggesting that Frazzled's dual role in attractive signaling and transcriptional activation is evolutionarily conserved, at least within a subset of insects [22,39]. Vertebrate orthologs of Fra (DCC and Neogenin) can also regulate transcription via nuclear localization of their ICDs [40,41], although the *in vivo* transcriptional targets of these receptors have not yet been identified, and *comm* orthologs do not appear to be present in vertebrates.

2.3. Signaling pathways acting in parallel to Net-Fra to promote midline crossing

Although the Net-Fra pathway appears to be the major midline attractive pathway in *Drosophila*, many commissural axons cross the midline in the absence of Net-Fra signaling [23,25,26], suggesting the presence of additional pathways which can promote midline crossing independently of Net or Fra. Mutations in *Abl* or *trio* enhance midline crossing defects in a *fra* null mutant, indicating that they function in a parallel pathway in addition to any role they might have downstream of *fra* [33]. Similarly, mutations in a number of other genes result in a commissureless or near-commissureless phenotype when introduced into *NetAB* or *fra* mutants; notably, most of these mutations cause little or no detectable defects in midline crossing on their own, suggesting that they may act redundantly to Net-Fra signaling to promote midline crossing (or that their individual mutant phenotypes are too mild to detect) [42–45].

The transmembrane cadherin flamingo (Fmi) can mediate both a homophilic and heterophilic cell–cell interaction, as well as transmit signals on the interior of the cell. The Fmi extracellular domain has been shown to be essential for promoting cell adhesion *in vitro*. Although *fmi* single mutants do not display any midline crossing defects, mutations in

fmi strongly enhance both the *fra* and *NetAB* mutant phenotypes [42]. Restoring expression of Fmi broadly in neurons could rescue this enhancement, but not restoring Fmi in a restricted set of commissural neurons. The intracellular domain of Fmi was also required for rescue, suggesting that Fmi must interact with cytoplasmic components for proper attractive function, perhaps by mediating cell-cell adhesion in commissural neurons [42].

The microtubule-binding protein Mushroom body defect (Mud) is expressed in post-mitotic neurons of the ventral nerve cord throughout embryogenesis. Mud is able to regulate the orientation of microtubule-based spindle fiber formation, and its function in neurons may be to connect information about polarity with actin motor complexes in order to orient microtubule structures within neurons to encourage directed growth [43]. Deletion of *mud* alone has little or no effect on midline crossing, but strongly enhances midline crossing defects of *NetAB* mutants. Mud has been associated with signaling downstream of Frizzled (Fz) [46], and genetic interaction experiments suggest that Fz and Mud may function in the same pathway to promote midline crossing, parallel to Net and Fra, and could cooperate with Fmi [42,43].

The immunoglobulin (Ig) superfamily gene *turtle* (*tutl*, also known as *Dasm1*) encodes multiple transmembrane and secreted Tutl isoforms expressed throughout the developing embryonic CNS, in both neurons and glial cells [47,48]. *tutl* mutants display a number of axon guidance phenotypes, including gaps in the longitudinal connectives, thin or absent commissures, and ectopic midline crossing of axons [47]. Loss of *tutl* dominantly enhances *NetAB* midline crossing phenotypes, indicating that it promotes midline crossing independently of Netrins. No genetic interactions were detected between *tutl* and either *slit* or *robo1*, suggesting that Tutl is unlikely to act as a direct inhibitor of Slit-Robo repulsion to promote midline crossing [47]. Secreted and transmembrane isoforms of Tutl were able to rescue midline crossing defects in *tutl* mutants when expressed in neurons or midline glia, consistent with a non-autonomous role for Tutl in axon guidance, perhaps as a ligand for an unknown cognate receptor [47]. Outside of the CNS, Tutl has been proposed to regulate photoreceptor axon targeting via homophilic cell-cell interactions [49], and to interact with the transmembrane Ig protein Borderless (Bdl) to promote glial ensheathment of photoreceptor axons [50]. Whether these mechanisms might also account for Tutl's influence on midline crossing in the embryonic CNS remains to be determined.

Down syndrome cell adhesion molecule (Dscam) in *Drosophila* is a contact-dependent homophilic cell repulsive molecule with thousands of alternatively spliced isoforms [51–55]. Dscam is involved in axon guidance and targeting, segregation of axon branches, and dendritic patterning [51,56–60]. Dscam has been shown to bind Netrin proteins in an evolutionarily conserved manner, with similar affinity to Net-Fra binding [45,61,62]. It is suggested that Dscam acts *in vivo* as a required receptor facilitating attraction to Net. However, there is a dramatic reduction in number of midline crossing axons in *Dscam/fra* double mutants significantly greater than seen in *net* mutants. This suggests that Dscam most likely participates in Net-dependent and independent pathways, both participating in pro-crossing roles [45]. Dscam also plays a role in longitudinal axon guidance in the fly embryonic CNS, where it appears to cooperate with Robo1 in response to Slit (see section 5.3 below).

Drosophila Hedgehog (Hh) is a classical morphogen that is required for the specification of the midline glia, the cells that commissural axons grow towards [63]. In vertebrates, the Hh ortholog Sonic Hedgehog (Shh) has been identified as a midline-derived chemoattractant for commissural axons that acts in parallel to Netrin to promote midline crossing [64].

Drosophila hh mutants show strong CNS patterning defects, likely due to its role in cell fate specification [65]. Ricolo et al [66] used a temperature-sensitive allele of *hh* to show that removing *hh* function specifically in later embryos (after its patterning role has been fulfilled) resulted in defective commissural axon guidance, suggesting that *hh* also plays a role in midline crossing in the fly embryo. Hh is produced at the *Drosophila* CNS midline at low levels, and the Hh receptors Patched (Ptc) and Smoothed (Smo) are also found localized to commissural axons. Gain of function experiments suggested that Hh can attract commissural axons in *Drosophila*, acting in parallel to the Net-Fra pathway and likely through a non-canonical signaling pathway involving Src42A [66].

Semaphorin (Sema) family proteins are one of the four “classical” axon guidance ligands (along with Netrins, Slits, and Ephrins) [67,68]. Semaphorins are multifunctional axon guidance proteins, in some contexts act as ligands to signal through their canonical Plexin and Neuropilin family receptors, and in other contexts can act as receptors in response to Plexins or other Semaphorins as ligands (reverse signaling) [68]. Mammalian Sema-3A, signaling through Plexin-A1 and Neuropilin-2, has been shown regulate midline crossing in the vertebrate spinal cord, primarily in the context of repulsion of post-commissural axons [69,70]. In *Drosophila*, Semas have mostly been known for their roles in axon guidance outside of the embryonic CNS (including axon and dendrite patterning in the olfactory lobe [71–73], guidance of sensory axons [74], photoreceptor targeting [75], motor axon fasciculation and targeting [76–79]) and in axon fasciculation and longitudinal pathway formation in the CNS [77,79–81]. There are five Sema family members in *Drosophila* (Sema-1a, Sema-1b, Sema-2a, Sema-2b, and Sema-5c), and the transmembrane Semaphorin Sema-1a acts as a repulsive/de-adhesive signal during motor axon guidance, where it acts as a receptor to activate reverse signaling in response to PlexA [75,76,82,83]. Recent evidence reveals that Sema-1a also acts in parallel to Net-Fra to promote midline crossing, acting cell autonomously in response to secreted Sema2 ligands instead of Sema-1a’s canonical binding partner PlexA [84]. Sema-1a promotes midline crossing through an attractive mechanism mediated by RhoGAPp190, in contrast to its repulsive signaling in other contexts which involves the downstream effectors Pebble (Pbl), Varicose (Var), and Cheerio (Cher) [82,83].

3. Midline repulsion

While many individual molecules and a variety of signaling pathways have been identified which promote midline crossing in the fly embryonic CNS, midline repulsion of axons in the *Drosophila* embryonic CNS appears to be exclusively under control of the midline repellent ligand Slit and its Roundabout (Robo) family receptors. Like the Net-Fra pathway, the Slit-Robo pathway is evolutionarily conserved across bilaterian animals and appears to have a universal role in regulating midline crossing of axons [85–96]. *Drosophila slit* mutants suffer a complete absence of midline repulsion, causing all CNS axons to collapse at the midline [97,98]; this severe effect is phenocopied in embryos lacking the two midline repulsive Slit receptors *robo1* and *robo2* [99,100]. Members of the Robo receptor family have an

evolutionarily conserved “5+3” protein structure, shared by all three *Drosophila* Robos: five immunoglobulin-like (Ig) domains, three fibronectin type III (Fn) repeats, a transmembrane domain and two to four conserved cytoplasmic motifs (CC0, CC1, CC2, and CC3) [85,86,101]. *Drosophila* Robo1 has all four cytoplasmic motifs, while Robo2 and Robo3 only have CC0 and CC1 [99,100,102,103]. Despite the conserved protein structure, these receptors differ from each other in localization and function. All three are localized to longitudinal axon segments and absent from commissures, but they differ in their localization to discrete medial-lateral regions within the neuropile. The innermost medial region contains only Robo1, the intermediate region combines Robo1 and Robo3, and all three receptors are present in the outermost lateral region [102,103]. Robo1 and Robo2 both function in midline repulsion [99,100]. However, while this appears to be Robo1’s sole function in the embryonic CNS, Robo2 also promotes midline crossing and contributes to lateral positioning of axon pathways in the embryonic nerve cord [44,102–104]. Robo3 appears to have no role in midline repulsion, and instead is dedicated to positioning of longitudinal axon pathways and sensory axon terminals in the embryonic nerve cord [44,102,103,105].

3.1 Slit is secreted at the midline and binds to the Ig1 domain of all Robo family members

Since Slit and Robo were identified as a ligand-receptor pair in 1999, a series of genetic interaction and *in vitro* biochemical studies have pinpointed the binding site to the Ig1 domain of Robo1 and the second leucine-rich repeat (LRR D2) of Slit [87,88,98,106–111]. However, it was only recently formally demonstrated that Slit binding to Robo1’s Ig1 domain is required *in vivo* for Robo1’s midline repulsive function [112].

Slit is produced and secreted by midline glia, and is detectable on the surface of both midline cells and longitudinal axons which express the Slit-binding Robo receptors [81,98,113]. The secretion of Slit is dependent upon a glycosylation event mediated by *Mummy* (*mmy*), a gene that encodes the only known uridine diphosphate-*N*-acetylglucosamine (UDP-GlcNAc) diphosphorylase in *Drosophila*. In *mmy* mutants, where glycosylation of Slit cannot be completed, Slit is only found at the VNC midline and is completely absent on longitudinal and commissural axon tracts. However, this only interferes with the ligand’s ability to be secreted, and not its ability to bind Robo1 [114]. Slit and Robo are known to interact with heparin, which can form a ternary complex with Slit and Robo and influence their affinity for each other [111,115,116]. Disruption of genes encoding heparin sulfate proteoglycans (HSPGs) in *Drosophila* can also lead to ectopic midline crossing of axons, and the HSPG Syndecan (*Sdc*) can influence the extracellular distribution of Slit in the embryonic nerve cord [113,117,118].

3.2 Early Robo1 inhibition allows axons to initially cross midline

Many axons need to cross the midline in order to innervate the contralateral side of the body and carry out proper motor functions. This necessitates precise temporal regulation of Slit-Robo repulsion. In *Drosophila* pre-crossing commissural axons, Commissureless (*Comm*) protein limits the amount of Robo1 on the growth cone surface by endosomal sorting [119–121]. In *comm* mutants, Robo1 is constitutively trafficked to the surface of growth cones, preventing normally commissural axons from crossing the midline and producing the

can regulate *Drosophila* Robo1 like its vertebrate counterpart has yet to be determined, if the need for such regulation even exists due to the presence of Comm.

The vertebrate proline rich and Gla domain gene PRRG4 shares some sequence similarity with *Drosophila comm* (in particular the functionally critical GLPSYDEAL motif [121]), and misexpression of PRRG4 in *Drosophila* neurons caused ectopic midline crossing [127]. In cultured cells, PRRG4 was able to prevent surface localization of mammalian Robo1, similar to Comm's effect on *Drosophila* Robo1 [121,127]. Whether PRRG4 might be a true (if cryptic) ortholog of Comm, or instead an independently evolved protein with functional analogy to Comm is unclear.

3.3 Factors that stabilize Robo1 on axons and enhance midline repulsion

In contrast to the factors described above which negatively regulate Robo1's surface expression or activity, others have been identified which enhance Robo1's midline repulsive activity by promoting or stabilizing Robo1 levels at the cell surface. While Mummy (Mmy) has not been shown to directly glycosylate Robo1, *mmy* mutant stage 12–14 embryos show significantly reduced amounts of Robo1 protein present on axons. Observations of Robo2 and Robo3 protein levels in *mmy* mutants yielded similar results. This data indicates that Mmy acts in an indirect manner to regulate and maintain the abundance of all three Robo receptors via an unknown, slit-independent mechanism. Ectopic midline crossing and longitudinal pathway defects are observed in *mmy* mutant embryos, suggesting that Mmy may influence multiple Robo-dependent axon guidance decisions [114].

Another effector that influences Robo1 signaling is Canoe (Cno). During early stages of embryogenesis, Cno is expressed in the ipsilateral axons, while in later stages Cno is expressed in commissural axons that have already crossed the midline once. The expression pattern alone indicates a role in midline repulsion – preventing early ipsilateral axons from crossing and late commissural axons from re-crossing the midline ectopically – and is mechanistically reminiscent of vertebrate RabGDI. *cno* mutant embryos display a variety of axon guidance defects, including axon stalling, ectopic midline crossing, and defasciculation, consistent with multiple roles for Cno in early axon guidance events. Genetic interaction and *in vitro* experiments suggest that Cno forms a complex with Robo1 *in vivo* which is required for the receptor's localization and midline repulsive function [132].

Outside of the embryonic VNC, the receptor tyrosine phosphatase RPTP69D directly binds to Robo3 to increase surface protein levels and enhance axonal response to Slit and thus its function in axon growth in the adult brain [133]. RPTP10D and RPTP69D have previously been shown to be important for embryonic neural development by interacting with the Slit-Robo pathway [134]. It will be interesting to see if RPTP69D or other RPTPs can similarly regulate surface levels of Robo proteins in embryonic neurons.

3.4 Robo1 signaling downstream of Slit binding

According to the current model, after the receptor binds Slit at the midline, Robo1 must undergo two processes to activate its midline repulsive function: cleavage and clathrin-dependent endocytosis. First, the metalloprotease Kuzbanian (*kuz*) cleaves Robo1 near the transmembrane domain, effectively shedding the receptor's ectodomain [135]. The exact site

of *kuz* cleavage remains unknown as the enzyme's substrate specificity is not well characterized, but the cleavage site must be located at some point between the first Fn repeat and the transmembrane domain as Coleman et al. were able to create an uncleavable form of Robo1 by switching Robo1's three Fn repeats and juxtamembrane region with the corresponding regions of Frazzled (*fra*) [135]. This shedding event causes a conformational change in the receptor allowing downstream cytoplasmic domains to interact with Son of Sevenless (Sos) via the Dreadlocks (Dock) adaptor protein. Following cleavage, Robo1 is endocytosed and trafficked from early to late endosomes. Genetic interaction studies suggest that this endocytic event contributes to receptor activation by positively regulating midline repulsion [136]. This is accomplished through a network of downstream effectors recruited to the receptor's CC2 or CC3 motifs after slit stimulation. Abelson (Abl), Enabled (Ena), Dock and Cno directly bind to the cytoplasmic domain of Robo1 [101,132,137–139]. Slit-dependent Robo1 endocytosis to the early endosome is essential for recruitment of Sos to this complex, which interacts with Dock and regulates local Rac activation via its DH RhoGEF domain [136,138].

Robo2 appears to act similarly to Robo1 to signal midline repulsion in response to Slit; it is co-expressed with Robo1 in ipsilateral pioneer neurons during early stages of CNS development, and axons inappropriately cross the midline in *robo2* mutants (although to a far lesser extent than in *robo1* mutants) [99,100]. Despite this, very little is known about the signaling mechanisms or cytoplasmic effectors of Robo2 in the context of midline repulsion. Robo2 lacks the CC2 and CC3 motifs that mediate interactions with known downstream components of Robo1 signaling, suggesting that it may signal through a distinct set of downstream factors to mediate midline repulsion. Similarly, it is not yet known if endocytosis and/or proteolytic processing are required for midline repulsive signaling by Robo2.

3.5 Functional relevance of Robo ectodomain elements

While all three *Drosophila* Robo receptors share the conserved 5 Ig + 3 Fn ectodomain structure that is characteristic of Robo family proteins, our current understanding of the individual roles each of these domains play in midline repulsion *in vivo* is limited. *In vitro* studies of both insect and vertebrate Robo receptors have established Ig1's role in binding Slit ligands [109–111], and recent *in vivo* studies have confirmed the functional importance of *Drosophila* Robo1 Ig1 in Slit-dependent midline repulsion in the fly embryonic CNS [112,140]. Despite their evolutionary conservation, the four remaining Ig domains in *Drosophila* Robo1 (Ig2–Ig5) are individually dispensable for Slit binding and midline repulsive signaling by Robo1 in embryonic neurons. Ig2–Ig5 are also not required for proper expression, axonal localization, or commissural clearance of Robo1 protein *in vivo*, and endosomal sorting by Comm is unaffected in Robo1 variants lacking any of the five individual Ig domains [140]. Current studies in our lab indicate that each of the three Fn domains (Fn1–Fn3) are also individually dispensable for Slit binding and midline repulsive activity, but have revealed a requirement for Fn3 for commissural clearance and downregulation by Comm (H.E.B., unpublished).

Although nearly all Robo receptors share the same ectodomain structure, there are examples of Robo family proteins that lack various domains: in the silkworm *Bombyx mori*, two orthologs of Robo1 (BmRobo1a and BmRobo1b) lack the Ig5 and Fn1 domains [93], and the vertebrate Robo4/Magic Roundabout receptor is missing Ig3–Ig5 and Fn1 [141]. Although it retains all eight ectodomain elements, the mammalian Robo3/Rig-1 protein is not a Slit receptor; it has acquired sequence changes in Ig1 that prevent Slit binding, and instead binds the novel ligand NELL2 in an Fn-dependent manner [142,143]. Notably, Robo3/Rig-1 orthologs from non-mammalian vertebrate species (zebrafish, *Xenopus*, and chick) retain the ability to bind Slit, suggesting that a “signaling switch” in Robo3 occurred at some point in the mammalian lineage [143].

Drosophila Robo2 is a multi-functional receptor, able to promote midline crossing in some neurons while inhibiting crossing in others [44,125]. Robo2 also regulates the formation and lateral position of longitudinal axon pathways (see section 5.2 below) [44,102–104]. Gain-of-function studies suggest that the Ig1 domain of Robo2 (which, like in Robo1, is necessary for Slit binding) is required for its midline repulsive activity, but at least partially dispensable for its pro-crossing role. Instead, Ig2 is critical for promotion of midline crossing by Robo2 [125]. The Ig1 and Ig3 domains of Robo2 have both been implicated in Robo2’s role in lateral positioning, and Ig3 in particular appears to influence the multimerization of Robo2 [104].

4. Choice of commissure

In each segment of the *Drosophila* embryonic ventral nerve cord, commissural axons cross the midline in the anterior commissure (AC) or posterior commissure (PC) [3]. Choice of commissure is a stereotypical decision, such that the same identifiable neuron will project its axon into the same commissure in each segment (Figure 1). Whether an axon crosses the midline in the AC or PC is regulated by the secreted ligand Wnt5 and its receptor Derailed (Drl). Wnt5 is expressed in neurons located adjacent to the PC [144], and repels Drl-expressing axons which cross the midline in the AC [145]. Drl expression is tightly regulated, and is only detectable on axons as they are crossing the midline [146]. In *wnt5* or *drl* mutants, axons that normally project through the AC mis-project into the PC or wander between the AC and PC as they cross the midline [144,146], while misexpression of Wnt5 or Drl can induce PC axons to switch to the AC [144,146]. It is unclear whether the PC represents a “default” pathway for commissural axons, or whether additional signaling pathways specifically instruct axons to cross via the AC in the absence of Wnt5-Drl signaling.

Drl is a member of the “related to tyrosine kinases” (RYK) family of receptors, and signals axon repulsion independently of its atypical tyrosine kinase domain [147]. Instead, the Wnt5-Drl signaling pathway involves the non-receptor tyrosine kinases Src42A and Src64B through a mechanism that includes Wnt5-induced homodimerization of Drl [148,149]. In a recent study, Long et al. [150] used a series of chimeric receptors to demonstrate that the cytoplasmic domains of three known repulsive axon guidance receptors in *Drosophila* (Robo1, Drl, and Unc5) can act equivalently to repel axons in three distinct axon guidance contexts (midline crossing, commissure choice, and CNS exit). This intriguing result

suggests that these three receptors could signal through a common cytoplasmic signaling pathway which may involve the downstream effector Trio [150].

5. Longitudinal axon guidance

5.1. Post-crossing guidance of longitudinal axons

After crossing the midline, commissural axons gain sensitivity to Slit by expressing Robo1 on their growth cones, preventing them from re-crossing. Once on the contralateral side of the midline, many commissural axons make an anterior or posterior turn and extend along longitudinal axon pathways at specific dorsal-ventral and medial-lateral positions within the neuropile [3,81,151]. These pathways initially form as pioneer axons from adjacent segments grow towards each other, then contact and grow along each other to form continuous longitudinal axon bundles (or fascicles) during the early stages of axon guidance [152]. As neural development progresses, later-developing axons select from and fasciculate with these pathways as they extend towards their ultimate synaptic targets. It appears that repulsive signaling via the Slit-Robo pathway is required for the maintenance of ipsilateral (and presumably post-crossing commissural) axon pathways, via a mechanism that involves the cytoskeletal protein beta-spectrin and may be distinct from the repulsive signaling that occurs during axon pathfinding [153].

5.2. Robo receptors regulate medial-lateral position of longitudinal axon pathways

The dorsal-ventral and medial-lateral positions of longitudinal axon pathways in the *Drosophila* embryonic CNS are patterned by orthogonal gradients of Slit (medial-lateral) and Sema (dorsal-ventral) ligands [81]; the same cues also regulate the positions of neuronal dendrites and sensory axon terminals within the same three-dimensional neuropile [105,154]. In addition to their canonical role in Slit-dependent midline repulsion, Robo receptors in *Drosophila* also play an important role in specifying the formation and medial-lateral positioning of longitudinal axon pathways [44,102–104]. While Robo1 appears dedicated to midline repulsion, Robo2 is required for the formation of longitudinal pathways in the lateral region of the neuropile, while Robo3 promotes pathway formation in the intermediate region [102,103]. While both Robo1 and Robo2 can substitute for Robo3 to specify intermediate pathways, neither Robo1 nor Robo3 can rescue Robo2's role in lateral pathway formation, suggesting that Robo2 acts through a distinct mechanism [44]. Notably, Robo2 and Robo3 orthologs are not conserved outside of insects, and a single ancestral Robo2/3 receptor appears to combine the activities of both Robo2 and Robo3 in the flour beetle *Tribolium castaneum* [90]. *Tribolium* Robo2/3 can substitute for *Drosophila* Robo3 to promote intermediate pathway formation, suggesting that the underlying mechanism is conserved and pre-dates the gene duplication event that produced *robo2* and *robo3* in dipteran insects [155]. Chimeric receptor experiments indicate that the ectodomain of Robo2 (in particular, the Ig1 and Ig3 domains) is important for specifying its lateral positioning activity [44,104], yet the mechanism(s) underlying Robo2's and Robo3's control of lateral position in *Drosophila* remain poorly characterized; while initial models posited that this activity was in response to a midline-derived Slit gradient [102,103], no direct evidence has shown that Slit binding by either Robo2 or Robo3 is required for their respective roles. Similarly, while vertebrate Robo1 and Robo2 receptors influence the positions of

longitudinal axon tracts in the hindbrain and spinal cord, it is not clear whether they do so through the same mechanism(s) as *Drosophila* Robo2 and Robo3 [88,156–158].

5.3. Semaphorins and their Plexin receptors pattern the dorsal-ventral axis and promote longitudinal pathway formation

Drosophila Plexin receptors (PlexA and PlexB) are required for proper formation and fasciculation of lateral (PlexA) and intermediate (PlexB) longitudinal pathways in the embryonic nerve cord [77,79], and the PlexA ligand Sema-1a is also required for the proper formation of lateral axon pathways [76] in addition to its role in promoting midline crossing via reverse signaling in response to Sema-2s [84]. Both Sema-1a and Sema-2a exhibit restricted expression patterns along the dorsal-ventral axis in the developing neuropile, with Sema-1a highest in dorsolateral regions and Sema-2a forming a gradient with the highest in the central region and lower levels in dorsal and ventral regions [81]. This Sema expression restricts Plexin-expressing sensory axon terminals to specific regions within the neuropile, presumably the same regions that their post-synaptic dendritic targets are located [81]. Although the two secreted Semaphorins in *Drosophila* (Sema-2a and Sema-2b) are both ligands of PlexB, they serve distinct guidance functions in the embryonic CNS [80]. PlexB appears to integrate Sema-2a repulsion and Sema-2b attraction to synergistically manage construction of specific CNS longitudinal projections and select sensory afferent innervation within that same CNS region. Manipulations of the PlexB receptor signaling results in larval sensory-dependent behavioral deficits. This suggests that in conjunction with longer range Slit, semaphorins ensure the accuracy of CNS interneuron projection organization and sensory afferent targeting in the CNS [80].

5.4. Crossing the segment boundary

Longitudinal axon pathways ensure connectivity between adjacent segments, which is critical for neuromuscular coordination along the anterior-posterior axis and the patterns of peristaltic muscle contraction that characterize the movement of the *Drosophila* larva [159]. A number of signaling pathways that regulate midline crossing also contribute to growth across the segment boundary. Hiramoto and colleagues [160,161] suggested a model whereby Fra acts to re-localize Netrin proteins secreted by midline cells to longitudinal axons, where it can act to guide longitudinal axons anteriorly towards the next segment [160]. To prevent these axons from then turning towards the midline source of Netrin, Slit and Robo act to suppress responsiveness to Netrin via a G protein-dependent signaling pathway [161]. Kuzina and colleagues proposed that this Netrin redistribution mechanism depends on a meshwork of neuronal projections at the segment boundary, whose production is stimulated by Notch signaling [162]. Recent evidence suggests that Dscam is also involved in promoting axon growth across segment boundaries, where it acts in a complex with Robo1 to bind a proteolytically processed form of Slit (SlitN) and modulate the activity of the Robo-Slit complex to change the canonical activity of the complex from repulsion to outgrowth promotion [56]. Dscam had previously been shown to act as a Slit receptor to promote axon collateral formation in mechanosensory axons in the adult fly, in concert with RPTP69D [163].

6. Summary and conclusions

Thanks to its relatively simple and highly organized embryonic nervous system and the highly stereotyped nature of individual axon guidance decisions in identifiable and easily labeled and manipulated subsets of neurons, the fruit fly *Drosophila melanogaster* is a powerful system for studying basic questions in developmental neuroscience. Genetic studies of axon guidance in *Drosophila* over the last two and a half decades have revealed that a seemingly simple binary decision, whether or not to cross the midline, is regulated by a complex and intricate underlying network involving multiple signaling pathways and regulatory and crosstalk mechanisms. Thanks to the broad evolutionary conservation of these pathways and many of their individual components, studies of midline axon guidance in the fruit fly have provided powerful insights into general developmental principles that have a high degree of relevance for understanding development in disease in other animal groups, including humans.

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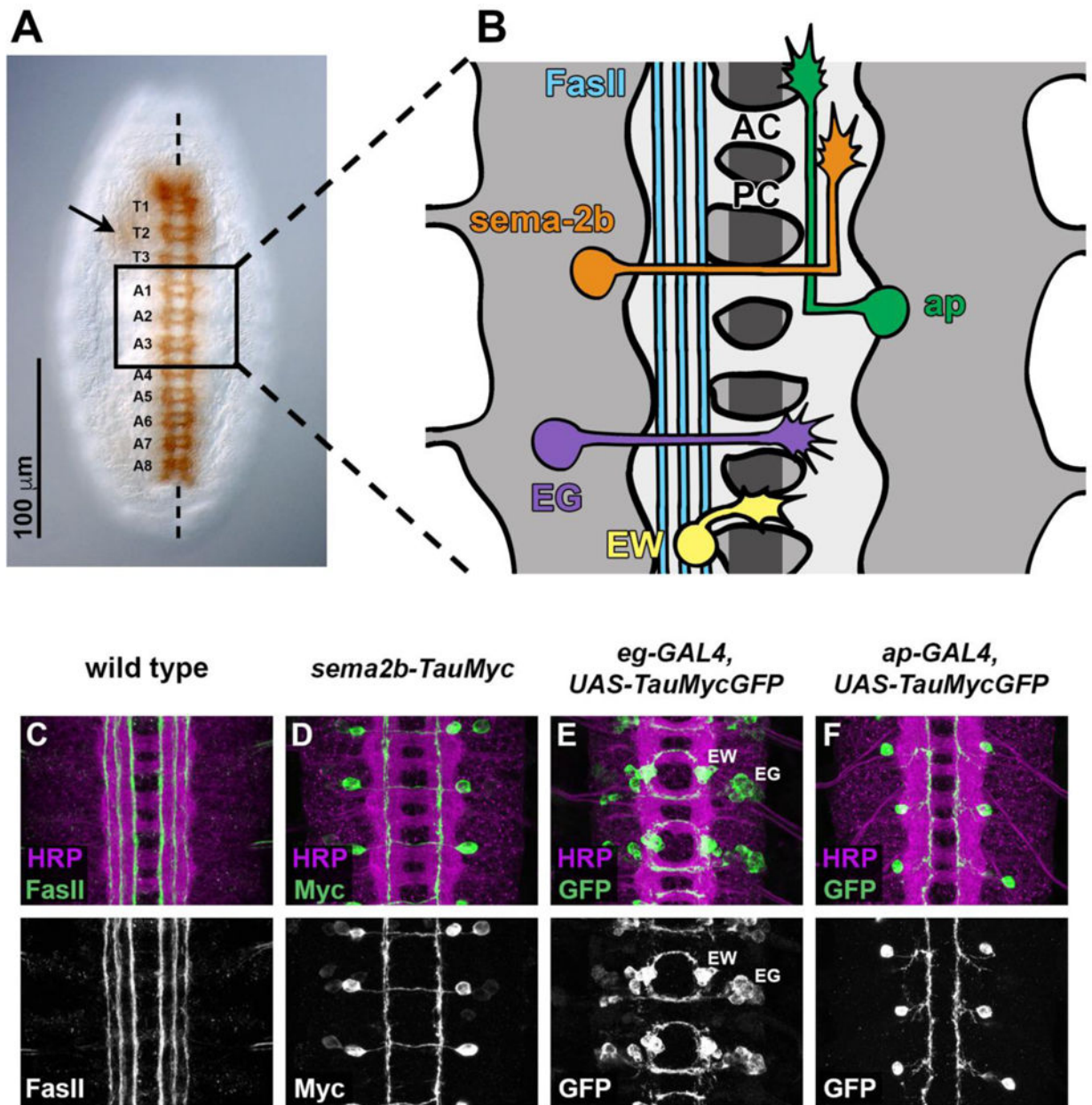


Figure 1. Structure of the *Drosophila* embryonic CNS, and identifiable subsets of neurons
 (A) Ventral view of a mature *Drosophila* embryo, stained with the monoclonal antibody BP102, which labels all of the CNS axons. The segmentally-repeated pattern of ganglia in the ventral nerve cord is apparent. T1–T3 indicate thoracic segments; A1–A8 indicate abdominal segments. Arrow indicates position of the brain, which is located more dorsally and out of the focal plane. Dashed vertical line indicates the midline.
 (B) Schematic of three adjacent segments from the abdominal ventral nerve cord, illustrating identifiable neuronal subsets and their axon projection patterns. Anterior (AC) and posterior (PC) commissures are labeled in the top segment. FasII-positive axon pathways (blue) consist of ipsilateral and post-crossing commissural axons, and form in discrete medial, intermediate, and lateral zones within the neuropile. A subset of commissural neurons is

labeled by the *sema2b-TauMyc* reporter transgene (orange); their axons cross the midline in the anterior commissure and form a longitudinal pathway in the intermediate region. Two subsets of commissural neurons and their axons are labeled by the *eg-GAL4* reporter transgene: the EG axons (purple) cross the midline in the anterior commissure and the EW axons (yellow) cross the midline in the posterior commissure. A subset of ipsilateral neurons is labeled by the *ap-GAL4* reporter transgene (green); their axons project towards the midline, then turn without crossing and project anteriorly in the medial region of the neuropile.

(C–F) Confocal micrographs of wild type (A) or transgenic (C–F) embryos stained with the indicated antibodies. Anti-HRP (magenta) labels all CNS axons; anti-FasII (green in C) labels FasII-positive longitudinal pathways; anti-Myc (green in D) or anti-GFP (green in E,F) labels the cell bodies and axons of transgenically-labeled neurons carrying each of the transgenes described above.

Table 1

Components of axon guidance pathways involved in midline crossing, midline repulsion, commissure choice, and guidance of longitudinal axons in the *Drosophila* embryonic CNS.

Protein	Role(s)	Pathway or cognate ligand/ receptor	References
Ligands		Cognate receptor(s)	
Amalgam (Ama)	midline crossing	Nrt	Liebl et al. [164]
Hedgehog (Hh)	midline crossing	Ptc	Ricolo et al. [66]
Netrin A (NetA)	midline crossing, longitudinal axon guidance	Fra, other (unknown)	Mitchell et al. [26], Harris et al. [25], Hiramoto et al. [160]
Netrin B (NetB)	midline crossing, longitudinal axon guidance	Fra, Dscam	Mitchell et al. [26], Harris et al. [25], Hiramoto et al. [160], Andrews et al. [45]
Sema-1a	lateral pathway formation, D/V patterning	PlexA	Yu et al. [76], Zlatic et al. [81]
Sema-2a	intermediate pathway formation, midline crossing, D/V patterning	PlexB, Sema-1a	Wu et al. [80], Hernandez-Fleming et al. [84], Zlatic et al. [81]
Sema-2b	intermediate pathway formation, midline crossing	PlexB, Sema-1a	Wu et al. [80], Hernandez-Fleming et al. [84], Zlatic et al. [81]
Slit	midline repulsion, longitudinal axon growth, longitudinal pathway formation	Robo1, Robo2, Robo3, Dscam	Kidd et al. [98], Brose et al. [87], Hiramoto et al. [161], Alavi et al. [56]
Turtle (Tutl)	midline crossing	unknown	Bodily et al [48], Al-Anzi et al [47]
Wnt5	commissure choice	Drl	Yoshikawa et al [144]
Receptors		Cognate ligand(s)	
Derailed (Drl)	commissure choice	Wnt5	Callahan et al [145]
Down syndrome cell adhesion molecule (Dscam)	midline crossing, longitudinal axon growth	SlitN, NetB	Andrews et al. [45], Alavi et al. [56]
Flamingo (Fmi)	midline crossing	unknown	Organisti et al. [42]
Frazzled (Fra)	midline crossing, comm transcription, longitudinal axon guidance (Net redistribution)	NetA, NetB, other (unknown)	Kolodziej et al. [23], Yang et al. [36], Hiramoto et al. [160]
Neurotactin (Nrt)	midline crossing	Ama	Liebl et al. [164]
Patched (Ptc)	midline crossing	Hh	Ricolo et al. [66]
Plexin A (PlexA)	lateral pathway formation, D/V patterning	Sema-1a	Winberg et al. [77], Zlatic et al. [81]
Plexin B (PlexB)	intermediate pathway formation, D/V patterning	Sema-2a, Sema-2b	Ayoob et al. [79], Zlatic et al. [81]
Robo1	midline repulsion, longitudinal axon growth	Slit	Kidd et al. [86], Hiramoto et al. [161], Alavi et al. [56]
Robo2	midline repulsion, lateral pathway formation	Slit	Simpson et al. [99], Simpson et al. [102], Rajagopalan et al. [100], Rajagopalan et al. [103]
Robo3	intermediate pathway formation	Slit	Simpson et al. [102], Rajagopalan et al. [103]

Protein	Role(s)	Pathway or cognate ligand/ receptor	References
Sema-1a	midline crossing	Sema-2a, Sema-2b	Hernandez-Fleming et al. [84]
Downstream effectors and pathway regulators		Associated pathway(s)	
Abelson tyrosine kinase (Abl)	midline crossing	Net-Fra, Slit-Robo, Ama-Nrt	Bashaw et al. [101], Forsthoefel et al. [33], O'Donnell et al. [35]
Beta-spectrin	midline repulsion	Slit-Robo	Garbe et al. [153]
Calmodulin (CaM)	midline crossing	unknown	Hsouna et al. [165]
Canoe (Cno)	midline repulsion	Slit-Robo	Slovakova et al. [132]
Commissureless (Comm)	midline crossing	Slit-Robo, Fra (Net-independent)	Kidd et al. [119], Keleman et al. [120,121], Gilestro et al. [122], Yang et al. [36]
Dally-like protein (Dlp)	midline repulsion	Slit-Robo	Johnson et al. [118]
Dreadlocks (Dock)	midline repulsion	Slit-Robo	Fan et al. [137]
Enabled (Ena)	midline crossing, midline repulsion	Net-Fra, Slit-Robo	Bashaw et al. [101], Forsthoefel et al. [33]
GEF64C	midline crossing	unknown	Bashaw et al. [166]
Kuzbanian (Kuz)	midline repulsion	Slit-Robo	Coleman et al. [135]
Mummy (Mmy)	midline repulsion	Slit-Robo	Manavalan et al. [114]
Mushroom body defect (Mud)	midline crossing	Frizzled	Cate et al. [43]
Myosin II	midline crossing	Net-Fra	Dorsten et al. [167]
Neurexin IV (Nrx IV)	midline repulsion	Slit-Robo	Banerjee et al. [168]
Pak	midline repulsion	Slit-Robo	Fan et al. [137]
Presenilin (Psn)	midline attraction	Fra (Net-independent)	Neuhaus-Follini et al. [37]
RhoGAPp190		Sema2-Sema1a	Hernandez-Fleming et al. [84]
Robo2	midline crossing	Slit-Robo	Spitzweck et al. [44], Evans et al. [104,125]
RPTP10D	midline repulsion	Slit-Robo	Sun et al. [134]
RPTP69D	midline repulsion	Slit-Robo	Sun et al. [134]
Son of sevenless (Sos)	midline repulsion	Slit-Robo	Yang et al. [138]
Src42A	midline crossing, inhibition of midline crossing, commissure choice	Hh, Wnt5-Drl, other (unknown)	Ricolo et al. [66], Wouda et al [149], O'Donnell et al. [169]
Src64B	inhibition of midline crossing, commissure choice	Wnt5-Drl, unknown	Wouda et al [149], O'Donnell et al. [169]
Syndecan (Sdc)	midline crossing	Slit-Robo	Johnson et al. [118], Steigemann et al. [117]
Trio	midline crossing	Net-Fra, other (unknown)	Forsthoefel et al. [33]
Vilse/CrGAP	midline repulsion	Slit-Robo	Hu et al. [170], Lundstrom et al. [171]