SEMA-1A REVERSE SIGNALING PROMOTES MIDLINE CROSSING IN RESPONSE TO SECRETED SEMAPHORINS

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A DISSERTATION

in

Neuroscience

Presented to the Faculties of the University of Pennsylvania

in

Partial Fulfillment of the Requirements for the

Degree of Doctor of Philosophy

2016

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Dedicated to Lucas Xavier Fleming

You are sure to be my greatest experiment in developmental neuroscience

ACKNOWLEDGMENTS

I would like to thank all the members of the Bashaw lab past and present. Your curious minds inspire good science and make research fun. Greg especially has cultivated an environment where young scientists can grow and follow where their curiosity leads them. I am grateful to all the members of my thesis committee, your constructive criticism and support has made me a better scientist. I also want to acknowledge the members of my graduate group along with the faculty and staff that make it function. I have always felt like our graduate group was willing to help make this an enriching experience.

I am eternally grateful to my family who has made this all possible. Especially, my sisters and our mothers for taking care of Lucas so I could write this! And Mike, I can never thank you enough for everything you do.

ABSTRACT

SEMA-1A REVERSE SIGNALING PROMOTES MIDLINE CROSSING IN RESPONSE TO SECRETED SEMAPHORINS

Melissa Hernandez-Fleming

Greg Bashaw

For the majority of axons, an essential step in proper guidance involves crossing the midline, and failure to do so often results in an inability to coordinate movement. Attraction to the midline depends in part on the highly conserved guidance receptor DCC, or Frazzled in Drosophila, which signals chemoattraction upon binding its ligand, Netrin. DCC mutations in humans are associated with mirror movement disorder, an inability to independently control the right and left sides of the body. Although Frazzled/Netrin signaling is required for many axons to cross the midline, netrin and frazzled/DCC mutants still exhibit significant midline crossing, implicating additional pro-crossing mechanisms. The Drosophila embryonic midline provides an ideal model to investigate nervous system development in vivo as it is genetically tractable and axon guidance cues are highly conserved. To identify additional pro-crossing pathways, we initiated a screen for modulators of midline crossing in a sensitized genetic background wherein Frazzled signaling is partially disrupted. Axon crossing defects in this background are enhanced by mutations in the transmembrane semaphorin, Sema-1a. Mutations in *sema-1a* also dominantly enhance crossing defects in a *netrin* mutant, indicating that Sema-1a functions in a Netrin independent pathway to promote midline crossing. Here we identify the transmembrane Semaphorin, Sema-1a, as a novel regulator of midline crossing in the *Drosophila* CNS. We show that Sema-1a functions as a receptor in response to the secreted Semaphorins, Sema-2a and Sema-2b, to promote midline crossing. In contrast to other examples of reverse signaling where Sema1a triggers repulsion through Plexin binding, in commissural neurons Sema-1a acts independently of Plexins to inhibit Rho and promote attraction to the midline. These findings suggest that Sema-1a reverse signaling can elicit distinct axonal responses depending on differential engagement of ligands and signaling effectors.

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CHAPTER 1: SEMAPHORIN REVERSE SIGNALING: DEVELOPMENT AND BEYOND

1.1 Transmembrane Semaphorins: Key Family Members

Semaphorins are ancient signaling molecules that are highly conserved and have been shown to play important roles in a diverse array of biological processes. First identified by their ability to direct axons at the growth cone (Kolodkin et al., 1992; Luo et al., 1993), they have since been found to influence a wide range of events from organ formation to immune responses (Shi et al., 2000; Toyofuku et al., 2004b; Yazdani and Terman, 2006). In general, semaphorins act to modulate cell shape and mobility, allowing cells to respond to their changing environment. For this reason, there is a wealth of evidence for semaphorins serving vital functions during growth and development. These developmental activities are involved again in many diseases such as cancer and neurodegeneration where cells undergo renewed growth and motility or lack thereof (Pasterkamp and Roman J. Giger, 2009; Rehman and Tamagnone, 2013). Outside of development, semaphorins can regulate the mobility of immune cells and sculpt synapses. Given their involvement in these broad reaching activities it is no doubt that there are a multitude of mechanisms at their disposal.

The semaphorin signaling family, as a whole, is large and varied. Family members take many forms and can be found as secreted, transmembrane, or membrane tethered molecules. All semaphorins share a defining feature called the Sema domain in the extracellular portion of the protein. The Sema domain is

roughly 500 amino acids and forms a seven blade β propeller fold that resembles integrins (Gherardi et al., 2004). This Sema domain is required for binding and signaling (Koppel et al., 1997; Tamagnone et al., 1999). Nearly all semaphorins also contain a cysteine rich domain (CRD), also called the PSI (present in Plexins, Semaphorins and Integrins) domain, immediately C-terminal to the Sema domain. Only some viral semaphorins lack this domain. The PSI domain is essential for dimerization (Klostermann et al., 1998).

Semaphorins are divided into 8 classes based on structural features and species of origin. Classes 2 and 3 are comprised of the secreted variants from invertebrates and vertebrates, respectively. The transmembrane semaphorins constitute the majority of the semaphorin family and include classes 1, 4, 5 and 6. Membrane tethered semaphorins make up the class 7 semaphorins. The final class of semaphorins is virally derived and considered class V semaphorins. This review will focus on only the transmembrane classes.

Semaphorin signaling has traditionally involved receptor complexes that include Plexin receptors as the signal-transducing partner. However, semaphorins have been shown to bind non-Plexin receptors as well. In fact, the majority of secreted semaphorins do not bind directly to Plexin receptors and instead bind a Neuropilin co-receptor (Feiner et al., 1997). Studies show that semaphorins can exert a diversity of cellular responses based on the distinct combination of molecules in their receptor complex.

The overwhelming majority of semaphorin signaling events result in a chemorepulsive output or retraction response. Initial characterization of semaphorins revolved around semaphorin induced growth cone collapse. This response came to typify semaphorins signaling, however, cases of attraction and adhesion are beginning to come to light (Bagnard et al., 1998; Hsieh et al., 2014; Sun et al., 2015; Wolman et al., 2004; Wu et al., 2011).

A growing number of studies have identified transmembrane semaphorins functioning as receptors to mediate cellular responses; this is termed “reverse signaling” while traditional signaling through a Plexin receptor is referred to as “forward signaling”. Although, this bi-directionality has not been identified in all transmembrane semaphorins it is likely to be a function shared by most. This review will focus predominantly on reverse signaling, but will also address forward signaling where it provides particular insight. As it is, most cases of reverse signaling occur in tissues where forward signaling is also known to function. For this reason, it has been difficult to isolate the role of reverse signaling.

Class 1 Semaphorins

The class 1 semaphorins are only found among invertebrates and have the most homology with the class 6 vertebrate semaphorins (Yazdani and Terman, 2006). Class 1 semaphorins are best characterized in *Drosophila*. Their cytoplasmic regions are relatively short (~200 amino acids) and contain no recognizable functional domains (Kolodkin et al., 1993). There is very

strong conservation between class 1 semaphorins amongst *Drosophila* with cytoplasmic regions of high identity across species, but further work is still required in order to attribute any functionality to these conserved regions. Recent work from Jeong, et al., has identified an important binding site located in one of these conserved domains of Sema-1a in *Drosophila*. This binding site was found to bind downstream effectors that regulate the Rho GTPase. Both a positive (RhoGEF) and a negative (RhoGAP) regulator of Rho compete for this cytoplasmic binding region (Jeong et al., 2012). Two other binding motifs have been identified in Sema-1a's cytoplasmic domain a putative Enabled binding motif (LPQP) and a PDZ binding motif (VYL) (Godenschwege et al., 2002). As there are no invertebrate Neuropilins, both transmembrane and secreted semaphorins bind directly to Plexins. However, studies have shown alternative binding partners and Plexin independent functions particularly when signaling in reverse (Jeong et al., 2012; Sweeney et al., 2011). There are a growing number of studies that have documented reverse signaling through *Drosophila* Sema-1a, many of which will be discussed further. There have been no reports of the other *Drosophila* transmembrane Sema-1b functioning in reverse.

Class 4 Semaphorins

The class 4 semaphorins comprise the largest group of transmembrane semaphorins and include seven members, Sema4A-G. They are found only in vertebrates and have been found to function in diverse contexts. Their cytoplasmic domains are more substantial than class 1 semaphorins and a

number of Sema4s have recognizable PDZ interaction motifs. Furthermore, this motif has been shown to promote interactions with PSD-95/SAP90, an essential synaptic scaffold protein of the postsynaptic density in at least 3 different Sema4 proteins. Sema4C contains a PDZ domain binding motif (SSV) on its carboxyl terminal and has been demonstrated to bind multiple proteins with PDZ domains including PSD-95 (Inagaki et al., 2001), Norbin (Ohoka et al., 2001) and SEMCAP1/GIPC (Wang et al., 1999). However, in the case of SEMCAP1/GIPC, this interaction was found to control subcellular distribution. Sema4B (Burkhardt et al., 2005) and Sema4F (Schultze et al., 2001) both require this C-terminal motif to bind PSD-95 in hippocampal neurons. Sema4D does not have a PDZ binding motif, but it has been found to interact with a protein tyrosine phosphatase (CD45) and a serine/threonine kinase in the immune system (Elhabazi et al., 1997; Herold et al., 1996).

The majority of Sema4s have not been associated with Plexin binding and their receptors are unknown for the most part. Sema4D binds PlexinB1 (Tamagnone et al., 1999) and PlexinB2 (Masuda et al., 2004), but it also binds a non-classical receptor, CD72, in the immune system (Kumanogoh et al., 2000). Sema4A binds Neuropillin1 (Delgoffe et al., 2013) in the immune system, but also binds Tim-2 in the lymphocytes (Kumanogoh et al., 2002).

There is evidence that Sema4D can function in a monomeric or homodimeric form and that homodimers are preferentially cleaved (Elhabazi et al., 2001).

Sema4D undergoes processing by ADAM17 and this cleavage is negatively regulated through binding to Lrig2 (Van Erp et al., 2015).

Class 5 Semaphorins

Class 5 semaphorins are found in both vertebrates (Sema5A and Sema5B) and invertebrates (Sema5C). They share a characteristic stretch of seven canonical type 1 thrombospondin repeats (TSRs) in their ectodomain. Class 5 semaphorins are the only class of transmembrane semaphorins that have not been directly implicated in reverse signaling. Sema5A can bind PlexinB3 to induce chemorepulsion or a PlexinB3-Met receptor complex to induce chemoattraction, while other functions have unidentified binding partners (Artigiani et al., 2004). The TSRs of Sema5A are also essential for interactions with heparin sulfate proteoglycans (HSPGs) and chondroitin sulfate proteoglycans (CSPGs), which modify the response Sema5A elicits from attraction to repulsion respectively (Kantor et al., 2004).

Class 6 Semaphorins

Class 6 semaphorins are the second largest group of transmembrane semaphorins with four members Sema6A-D. Sema6s have the longest cytoplasmic domains compared to the other transmembrane semaphorins (~400 amino acids). Their cytoplasmic domains are highly divergent even amongst other members of the same class yet they all contain proline rich motifs that are likely to interact with SH3 domains. Both human and mouse Sema6A can bind EVL (ena/VASP like protein) through their proline rich

zyxin-like domain (Klostermann et al., 2000). The proline rich domain of Sema6B interacts with the SH3 domain of Src (Eckhardt et al., 1997). Sema6D binds the SH3 domain of Abl and Mena through its proline rich zyxin-like domain (Toyofuku et al., 2004b). These cytoplasmic interactions were strong indications that Sema6s could function in reverse and almost all of the Sema6s, save Sema6C, have been demonstrated to do so.

Sema6s bind directly to their Plexin receptors and preferentially bind the PlexinAs. Sema6B has been shown to form homodimers and may undergo posttranslational modifications in some cells (Eckhardt et al., 1997). There is evidence that Sema6D is cleaved *in vivo* (Toyofuku et al., 2004b).

1.2 Differentiation, Migration and Maturation

Myoblast Differentiation

Sema4C Reverse signaling has been implicated in muscle development, although the binding partner for this signaling cascade remains unknown (Ko et al., 2005). Ko and colleagues utilized a culture system of C2C12 murine myoblasts to demonstrate that Sema4C expression is specifically elevated during early stages of mouse skeletal muscle differentiation *in vitro*. This increased Sema4C expression occurs when myoblasts form myotubes and precedes the terminal differentiation markers myoD and myogenin. After myotube formation, Sema4C mRNA and protein levels are reduced. This culture system reveals that Sema4C is required for this terminal differentiation step as myotube formation was inhibited upon Sema4C RNAi knockdown.

Treatment of cultured cells with the Sema4C ectodomain blocks myotube formation; this is likely due to a dominant negative effect, suggesting Sema4C functions cell autonomously. Additionally, Sema4C expression was up-regulated *in vivo* during muscle regeneration after injury implicating a broader role for Sema4C induced differentiation.

Myocardial Cell Migration

One of the first examples of semaphorin reverse signaling documented in vertebrates comes from studies in the chick heart. This is also one of the best examples of forward and reverse signaling functioning together. Heart chamber maturation is a fundamental step in cardiac development and disruptions to this process result in congenital heart defects (Epstein et al., 2015). At this stage of development, the heart is a looped tube with endocardial cells lining the inside of the tube and myocardial cells forming the outer layer. Dynamic interactions between the endocardial and the myocardial cells lead to the formation of two distinct layers within the myocardium of the ventricular segment. An outer compact layer forms and expands through circumferential migration while an inner trabeculated layer is formed through perpendicular migration. This circumferential migration is a result of simultaneous forward and reverse signaling through Sema6D and PlexinA1 since the cells that make up the compact layer express both Sema6D and PlexinA1. RNAi knockdown of either Sema6D, PlexinA1 or both resulted in decreased ventricle size and poor trabeculation (Toyofuku et al., 2004a). It is not clear how these signaling pathways are transduced simultaneously

especially given the fact that cis interactions between Sema6A and PlexinA4 or PlexinA2 have been shown to result in inhibition of signaling (Haklai-Topper et al., 2010; Sun et al., 2013; Suto et al., 2007).

Myocardial cells in the trabeculated layer express only Sema6D and are therefore repelled by the PlexinA1 in the compact layer towards the endocardium. Trabecular defects in the PlexinA1 knockdown embryos can be rescued by the ectodomain of PlexinA1 indicating that this trabeculation process is indeed mediated through the Sema6D binding PlexA1 as a ligand (Toyofuku et al., 2004b). Downstream effectors for this reverse signaling were also identified though a yeast two hybrid screen with multiple clones of Abl-kinase recovered. The cytoplasmic domain of Sema6D contains two consensus motifs for SH3 domain binding (PXXP) through which it can bind Abl. Co-immunoprecipitation experiments show an increase in Abl binding and phosphorylation upon treatment with PlexinA1. These experiments also revealed increased cleavage of Sema6D upon PlexinA1 treatment with an enrichment of Abl associated with the truncated Sema6D fragments suggesting proteolytic processing to downregulate reverse signaling. In the absence of PlexinA1, Sema6D was found to associate with Mena (Mammalian Ena homologue) through a zyxin-like domain. This association with Mena is reduced with PlexinA treatment and results in increased phosphorylation of Mena. The biological relevance of this association is not known, but the negative regulation of Mena binding is thought to increase cell motility (Bear et al., 2000, 2002).

The trabeculated layer and the endocardial cells are separated by a thick mix called cardiac jelly. The ectodomain of Sema6D is cleaved and secreted into the cardiac jelly (Toyofuku et al., 2004b). This accumulation of secreted Sema6D prevents the migration of endocardial cells through forward signaling as endocardial cells express only PlexA1. Repulsive forward signaling in endocardial cells was mediated by a PlexinA receptor complex with Off-Track which is distinct from the forward signaling complex required for earlier cardiac tube bending via VEGF2 and PlexA1 (Toyofuku et al., 2004a).

Sema6D plays multiple roles in cardiac formation first through forward signaling to facilitate the looping of the cardiac tube with a VEGF2/PlexinA1 receptor complex. This step in cardiac development is then followed by a less defined action of simultaneous signaling of both forward and reverse pathways to promote myocardial expansion of the compact layer. At the same time, reverse signaling promoted trabeculation, which in turn prevent migration of endocardial cells. Of note, Sema6D mutant mice do not display any heart defects like they do in the chick embryo. However, there are many instances in chick where expression patterns of Sema/Plexin molecules do not match their most closely related orthologue in mouse.

Oligodendrocyte Maturation

Oligodendrocytes are responsible for the myelination of neurons in the central nervous system. In order to induce myelination oligodendrocytes must mature from proliferative oligodendrocyte precursor cells (OPCs) to immature

and mobile oligodendrocytes and finally to mature oligodendrocytes. The maturation of oligodendrocytes depends upon signals from neurons and astrocytes as well as intrinsic programs. Sema4D was found to be expressed exclusively in oligodendrocytes within the CNS and functions to control apoptosis through an autocrine mechanism (Yamaguchi et al., 2012). However, recent evidence for reverse signaling has been demonstrated for Sema6A.

Sema6A plays an important role in oligodendrocyte maturation; its expression is very high in developing oligodendrocytes with a peak corresponding to myelination. Oligodendrocytes from Sema6A deficient mice fail to myelinate axons. Knock-out mice show a delay in oligodendrocyte maturation both *in vivo* and *in vitro*. However, the canonical binding partners, PlexinA2 and Plexin A4, do not display any oligodendrocyte defects. Oligodendrocyte cultures lacking Sema6A cannot be rescued by treatment with Sema6A ectodomain suggesting a requirement for reverse signaling (Bernard et al., 2012).

B-lymphocyte proliferation

Sema4D is highly expressed in the immune system particularly in T-lymphocytes and B-lymphocytes (Shi et al., 2000). Both Sema3 and Sema4D have been shown to block the migration of dendritic cells as secreted cues, but Sema4D can promote B-lymphocyte proliferation cell autonomously (Delaire et al., 2001; Granziero et al., 2003; Wang et al., 2001). Sema4D

reverse signaling was found to increase proliferation and lifespan in both healthy and leukemic B lymphocytes (Granziero et al., 2003). PlexinB1 binds Sema4D with high affinity and may act as the ligand to trigger B-cell proliferation (Tamagnone et al., 1999). PlexinB1 is expressed by bone marrow stromal cells, follicular dendritic cells and activated T-cells. These cells are abundant in the immune system and would provide ample signaling opportunity for Sema4D B-cells. Sema4D positive B-lymphocytes demonstrate increased proliferation when co-cultured with PlexinB1 expressing cells. Although this appears to be an interaction for healthy B-lymphocyte proliferation it may also mediate proliferation of malignant B-lymphocytes (Granziero et al., 2003).

1.3 Visual System

Photoreceptors

Sema-1a was found to function as a receptor in the development of the *Drosophila* visual system. Sema-1a is expressed on the growth cones of photoreceptor cells (R-cells). While there are 8 different types of R-cells only R-cells 1-6 project to the superficial layer of the optic lobe called the lamina; R-cells 7 and 8 project to the deeper medulla layer. Sema-1a is required for this projection pattern (Cafferty et al., 2006). In the absence of Sema-1a, the photoreceptors (R-cells1-6) fail to recognize their target or elaborate their growth cones at the lamina. This phenotype can be rescued by restoring full-length Sema-1a expression to all neurons. However, no rescue is achieved when a truncated Sema-1a lacking its cytoplasmic domain is expressed. This

indicates that Sema-1a requires its cytoplasmic domain and functions cell autonomously to guide R-cells 1-6 to terminate at the lamina. Furthermore, overexpression studies show that a full-length Sema-1a can induce hyperfasciculation amongst R-cells while the truncated Sema-1a could not (Cafferty et al., 2006). This function was later found to require Sema-1a's canonical binding partner, PlexinA (PlexA). RNAi knockdown and *plexA* mutants phenocopy the Sema-1a loss of function phenotype. Additionally, PlexA overexpression could also induce hyperfasciculation, and this could be suppressed by loss of *sema-1a*. Interestingly, overexpression of PlexA lacking its cytoplasmic domain also induced hyperfasciculation, further supporting the notion that Sema-1a was acting as a receptor for PlexA (Yu et al., 2010). Genetic interactions also indicate that *sema-1a* and *plexA* function in the same pathway and that putative downstream effectors Enabled or Abl are unlikely to contribute. Further studies demonstrated that negative regulation of Rho1 mediates the attractive response between axons since it decreases surface levels of the adhesive molecule FasII. Rho1 activity is negatively regulated by Moesin (Moe). Sema-1a and Moe genetically and physically interact. Activation and phosphorylation of Moe is increased upon Sema-1a reverse signaling. Knockdown or mosaic mutant analysis of both Moe and FasII demonstrate R-cell defects that phenocopy Sema-1a mutants (Hsieh et al., 2014). This was the first demonstration of an attractive output for Sema-1a reverse signaling and a direct contrast to the forward signaling affect on FasII (Yu et al., 2000).

Laminar Neurons

In the *Drosophila* visual system the photoreceptors that terminate in the lamina (R-cells 1-6) also induce the differentiation of their target neurons, the laminar neurons (L1-5). These first order interneurons then project into the deeper layers of the medulla (Clandinin and Zipursky, 2000). Sema-1a reverse signaling is important for directing L3 laminar neurons to the correct layer. L3 mistargeting defects were observed when using a mosaic analysis with a repressible cell marker (MARCM) strategy to analyze single L3 neurons rendered homozygous mutant for Sema-1a in an otherwise heterozygous animal. These mistargeting defects where L3 neurons extended beyond their appropriate layer can be rescued with the expression of full-length Sema-1a. A novel sparse labeling technique demonstrated that Sema-1a expression is enriched on L3 growth cones during L3 targeting. Sema-1a functions cell autonomously to reshape the growth cones and restrict them to a single layer. This growth cone retraction is triggered by PlexA, which acts as a repulsive cue. PlexA is expressed on tangential fibers and forms a repellant barrier (Pecot et al., 2013). N-cadherin (CadN), a classical adhesion molecule, was previously demonstrated to play a cell autonomous role in L3 targeting (Nern et al., 2008). However, genetic interactions between Sema-1a and CadN suggest that Sema-1a reverse signaling functions in a parallel pathway than CadN signaling (Pecot et al., 2013). Furthermore, this function is not limited to L3 neurons, instead Sema-1a repulsion and CadN homophilic adhesion function together to broadly restrict laminar neurons to a general

domain in the outer medulla. Consistent with this, Sema-1a and CadN are expressed on most laminar neurons and L1, L3 and L5 laminar neurons initially project to the same region before refining their termination to distinct layers. Interestingly, L1 neurons were found to relay directional information similar to ON bipolar cells in the vertebrate retina (Borst et al., 2010).

Direction Selective Ganglion Cells

Semaphorin reverse signaling also plays an important role in the mouse visual system. In the vertebrate visual system, visual information is communicated from the photoreceptors to the bipolar cells within the outer plexiform layer where the information is processed laterally through horizontal cells (Wassle, 2004). Bipolar cells then synapse with the primary output neurons, the retinal ganglion cells (RGCs), within the inner plexiform layer, which is stratified by directional selectivity (Wei and Feller, 2011). RGCs exit the retina and form connections in the brain for higher order visual processing. One such region is the accessory optic system (AOS) where image stabilization occurs. The AOS induces eye movements called an optokinetic reflex (OKR) in response to retinal input to stabilize visual input. Retinal inputs from direction-selective retinal ganglion cells (DSGCs), both the on DGCs and a subset of the on-off DSGCs, target to the AOS. When this innervation is disrupted it results in defective eye movements (Masseck and Hoffmann, 2009).

The On DSGCs are the major contributing pathway to the AOS and they express the transmembrane Sema6A. Sema6A mutant mice exhibit defects in the DSGCs axon trajectory to the AOS and OKR behavior. PlexA2 and PlexA4 are known binding partners for Sema6A. Neither Plexin is expressed in the DSGCs, but both are present in the target tissue. A similar AOS phenotype was not identified in single Plexin mutants (for either PlexA2 or PlexA4); however, the double mutants phenocopy Sema6A. Cultured retinal explants also suggest that the Sema6A reverse signaling output is likely attractive rather than repulsive, which is consistent with the expression pattern (Sun et al., 2015).

Unlike in Drosophila, there is no evidence that sema reverse signaling is involved in photoreceptor projections; however, forward signaling has been implicated in layer specificity (Matsuoka et al., 2011, 2012; Sun et al., 2013).

1.4 Olfactory System

Projection Neurons

Sema-1a reverse signaling is involved in establishing the spatial organization in the olfactory system. Sema-1a functions as a receptor in the projection neurons (PNs) to direct both PN dendrites and PN axons to their appropriate targets. PNs send their dendrites to the antennae lobe in order to synapse with olfactory receptor neurons (ORNs) at discrete functional domains called glomeruli. These glomeruli act as organizing centers for segregating ORNs by odor receptor type. Sema-1a expression is distributed throughout the

antenna lobe in a graded fashion with the highest concentration in the dorsolateral region. This pattern is established by the PNs themselves and the PNs with the highest level of Sema-1a target to the most dorsolateral glomeruli. Loss of Sema-1a leads to dendrite and axon mistargeting, which can be rescued cell autonomously with full-length Sema-1a. A Sema-1a transgene without the cytoplasmic domain fails to rescue these defects in either dendrites or axons. Overexpression of Sema-1a in PNs results in a directional shift toward the dorsolateral zone of the antenna lobe (Komiyama et al., 2007). This targeting is achieved through the repulsive action of Sema-1a reverse signaling in PNs in response to an opposing gradient of the secreted Sema-2s. Sema-2a and Sema-2b are expressed in overlapping patterns and function redundantly to repel Sema-1a expressing PNs. Sema-2a and Sema-2b single mutants lack PN defects, yet the double mutants display significant mistargeting errors. This interaction is likely to be indirect since physical interactions between Sema-1a and Sema-2a or Sema-2b could not be demonstrated. However, Sema-1a can bind *in vivo* to tissue overexpressing Sema-2a. The source of the secreted Sema-2s appears to be the degenerating larval ORNs. Complete ablation of these larval ORNs or RNAi knockdown of Sema-2s in larval ORNs both lead to a ventromedial shift in dorsolateral projecting PNs. Furthermore, the PN mistargeting defects in Sema-2a, Sema-2b double mutants could be rescued by overexpression of Sema-2a in the larval ORNs (Sweeney et al., 2011).

1.5 Motor Neuron Development

Motor Axon Defasciculation

Motor axon pathfinding in the *Drosophila* embryo requires both forward and reverse Sema-1a signaling. Sema-1a mutants exhibit motor neuron defasciculation defects, which are only partially rescued with the expression of the ectodomain alone. Pebble (pbl) and RhoGAPp190 (p190) were identified as potential downstream effectors of Sema-1a reverse signaling through their physical association with Sema-1a's cytoplasmic domain. Both Pbl and p190 regulate the small GTPase Rho1, yet they do so in opposing ways. Pbl positively regulates Rho1, while p190 negatively regulates Rho1. Structure function analysis indicates that the two downstream effectors competitively bind to the same region of the cytodomain. *In vitro* assays using Drosophila cells provided insights into the functional response of Sema-1a reverse signaling. In cultured cells, overexpression of Sema-1a or pbl results in a reduction in cell size, which is enhanced when both are overexpressed. However, this reduced cell size effect is lost if Rho1 is knocked down. In contrast, p190 overexpression leads to the opposite effect and cell size is increased. Mutations in *pbl* or RNAi knockdown leads to defasciculation and target recognition defects in motor neurons that are rescued when Pbl is expressed panneurally. Loss of *p190* also disrupts motor neuron pathfinding, but with a distinct premature branching defect that is rescued upon panneural expression. To link these downstream effectors to Sema-1a reverse signaling, the authors examined genetic interactions. Dominant interactions

between *pbl* and *sema-1a* revealed a positive functional relationship while p190 acted to antagonize Sema-1a reverse signaling. Further supporting this functional relationship, combined overexpression of Sema-1a and Pbl in all neurons leads to central nervous system defects only when Sema-1a's cytoplasmic domain is in tact.

Boundary Cap Cell Aggregation

The separation between the central nervous system and the peripheral nervous system is maintained during development through a population of cells called the boundary cap cells. This cluster of cells forms a temporary structure at the dorsal root entry zone (DREZ) and the ventral motor axon exit point (VMEP). When this structure is lost it results in the migration of motor neurons from the spinal cord. Sema6A reverse signaling is required to prevent this motor neuron exit. Sema6A is highly expressed in the boundary cap cells and these cells fail to cluster in the absence of Sema6A. Motor neurons expressing PlexinA1 are not repelled from Sema6A in culture. Instead, the boundary cap cells require Sema6A as a receptor to cluster appropriately by recognizing PlexinA1 on motor neurons as a stop signal (Mauti et al., 2007).

1.6 Midline

Post-crossing

During the development of the chick central nervous system axons from commissural neurons cross the midline at the floor plate and make a rostral

turn. Axons continue to travel rostrally along the longitudinal aspect of the spinal cord. Sema6B reverse signaling is required in commissural neurons in order to make this rostral turn post crossing. When Sema6B is knocked down commissural neurons incorrectly navigate after exiting the floor plate. The majority of axons stalls and fails to turn completely while others turn caudally. This guidance step coincides with the transient expression of Sema6B in dorsal commissural neurons. Knockdown defects can be rescued with a full-length Sema6B that is resistant to knockdown. However, Sema6B without its cytoplasmic domain fails to rescue suggesting Sema6B mediates this post-crossing guidance through reverse signaling. The binding partner for Sema6B within the floor plate was identified as PlexinA2. Cell-binding assays and co-immunoprecipitation experiments demonstrate a physical interaction between the two proteins. Additionally, selective knockdown of PlexinA2 in the floor plate leads to similar post-crossing defects. These axon guidance defects are rescued with both the full length and truncated forms of PlexinA2 injected specifically into the floorplate. Primary cell culture of commissural neurons show enhanced growth on substrate coated with PlexinA2 indicating that Sema6B initiates an outgrowth response to PlexA2. Interestingly, loss of PlexinA2 and PlexinA4 from commissural neurons also results in axon guidance defects in commissural neurons after crossing. This finding is similar to what had been previously demonstrated for forward signaling at the mouse midline (Charoy et al., 2012; Nawabi et al., 2010). Overexpression of PlexinA2 in commissural neurons leads to a premature stalling phenotype

suggesting that forward signaling is required post-crossing, and that it must be negatively regulated pre-crossing. Due to the co-expression of Sema6B and PlexinA2 in the commissural neurons, the authors speculate that Sema6B may negatively regulate forward signaling through *cis* interactions (Andermatt et al., 2014). Futile cis interactions between Sema6s and their Plexin receptors have been previously demonstrated in the mouse retina (Sun et al., 2013)and sensory neurons of the dorsal root ganglia (Haklai-Topper et al., 2010).

1.7 Synaptogenesis

Presynaptic

The Giant Fiber system is *Drosophila* is a well defined circuit that controls the jump-and-flight reflex. This circuit consists of a giant interneuron that sends a single giant axon fiber (GF) from the brain and makes a monosynaptic connection with a motorneuron (TTMn) for the jump muscle (tergotrochanteral muscle:TTM). In Sema-1a mutants this GF often fails to properly navigate to its motor neuron target. However, this guidance step is mediated by forward signaling and the expression of the Sema-1a ectodomain can rescue these defects when expressed either presynaptically in the GF or postsynaptically in TTMn. Surprisingly, the functionality of the synapse appears to be very sensitive to Sema-1a reverse signaling. Overexpression of Sema-1a presynaptically, but not postsynaptically, has a destabilizing effect on the synapse resulting in retraction of the GF terminal. Overexpression of the Sema-1a ectodomain fails to induce this effect. Moreover, this function can be

mapped to a specific motif within the cytoplasmic domain. Deletion of a putative Enabled binding motif (LPQP) abolishes this activity and heterozygosity of Enabled suppresses it; this suggests Enabled may be functioning downstream of this Sema-1a reverse signaling.

CHAPTER 2: SEMA-1A REVERSE SIGNALING PROMOTES MIDLINE CROSSING IN RESPONSE TO SECRETED SEMAPHORINS

2.1 Abstract

Commissural axons must cross the midline to form functional midline circuits. In the invertebrate nerve cord and vertebrate spinal cord, midline crossing is mediated in part by Netrin-dependent chemoattraction. Loss of crossing, however, is incomplete in mutants for Netrin or its receptor Frazzled/DCC, suggesting the existence of additional pathways. We identified the transmembrane Semaphorin, Sema-1a, as a novel regulator of midline crossing in the *Drosophila* CNS. We show that in response to the secreted Semaphorins Sema-2a and Sema-2b, Sema-1a functions as a receptor to promote crossing in parallel to Netrin. In contrast to other examples of reverse signaling where Sema1a triggers repulsion through activation of Rho in response to Plexin binding, in commissural neurons Sema-1a acts independently of Plexins to inhibit Rho and promote attraction to the midline. These findings suggest that Sema-1a reverse signaling can elicit distinct axonal responses depending on differential engagement of ligands and signaling effectors.

2.2 Introduction

The ability to coordinate the right and left sides of the body relies heavily on intricate circuits within the midline. Disruptions to these midline circuits during development, or after injury, often result in an inability to coordinate movement (Engle, 2010). For the majority of midline circuits, appropriate circuit formation requires axons to cross the midline. Netrin and its attractive receptor DCC, or Frazzled (Fra) in *Drosophila*, are highly conserved guidance factors known to promote midline crossing (Harris et al., 1996; Kennedy et al., 1994; Kolodziej et al., 1996; Neuhaus-Follini and Bashaw, 2015a; Serafini et al., 1994). Loss of function mutations in this receptor have been associated with movement disorders in zebrafish, mice and humans(Jain et al., 2014; Rabe Bernhardt et al., 2012; Srour et al., 2010). Despite this strongly conserved role in midline axon guidance, many axons still cross the midline in both *netrinAB* double mutants (hereafter referred to as *netAB*) and *fra* mutants in *Drosophila*, suggesting that there must be additional pathways to promote midline crossing (Kolodziej et al., 1996; Mitchell et al., 1996). Studies in vertebrate systems have yielded a few promising leads for pathways contributing to midline crossing, such as Shh/Boc (Charron et al., 2003), VEGF/Flk1(Ruiz de Almodovar et al., 2010), and Sema/Plexin (Nawabi et al., 2010; Zou et al., 2000). Unfortunately, redundancies in both ligands and receptors have led to ambiguous results when trying to discern molecular mechanisms from mutant phenotypes (Charoy et al., 2012; Delloye-Bourgeois et al., 2014; Hernandez-enriquez et al., 2015; Parra and Zou,

2010; Sloan et al., 2015). In order to identify additional pathways in a more tractable system, we developed a genetic modifier screen where Fra signaling is specifically reduced in a small subset of commissural neurons in the *Drosophila* embryo. By screening a library of chromosomal deletions for enhanced crossing defects, we identified the transmembrane semaphorin, Sema-1a, as novel regulator of midline crossing.

Semaphorin/Plexin signaling is highly conserved and has been shown to play many roles within the nervous system. In vertebrates, the Sema/Plexin family of signaling molecules is large and diverse; while in *Drosophila,* Semas and Plexins constitute a fairly small family. There are five semaphorins identified in *Drosophila* and only two Plexins. Semas are divided into two classes: transmembrane (Sema-1a, Sema-1b and Sema-5c) or secreted (Sema-2a and Sema-2b)(Pasterkamp, 2012). Neither Sema-1b nor Sema-5c show neural expression in the developing CNS, though they are highly enriched in embryonic ectoderm and mesoderm, respectively (Khare et al., 2000). The transmembrane Semas bind Plexin A (PlexA), while Plexin B (PlexB) binds the secreted Semas (Ayoob et al., 2006; Winberg et al., 1998). In the fly, Sema-1a is known to act as a repulsive/de-adhesive signal during motor axon guidance (Jeong et al., 2012; Yu et al., 1998a, 2000). A broader role within the CNS, however, is not surprising since the expression patterns of Sema-1a and PlexA both appear to be pan-neural and the longitudinal connectives within the CNS show defects in both *sema-1a* and *plexA* mutants (Kolodkin et al., 1993; Winberg et al., 1998). In fact, it has been proposed that repulsive Sema-1a/PlexA signaling may act as an

organizing factor at the midline to restrict sensory afferents to more ventral positions of the neuropil (Zlatic et al., 2009). Still, a role for Sema-1a in commissure formation has never been explored. In vertebrates, secreted Semas are important for commissure formation because they repel crossing axons from the floorplate (Jongbloets and Pasterkamp, 2014; Nawabi et al., 2010; Zou et al., 2000). The expression pattern of Sema-1a, however, precludes a similar function in fly. Intriguingly, a growing body of evidence has demonstrated that Sema-1a can signal in both a forward direction as a ligand and in reverse as a receptor itself. Sema-1a reverse signaling can occur through PlexA binding, as observed in the visual system and the giant fiber circuit (Cafferty et al., 2006; Godenschwege et al., 2002; Pecot et al., 2013)or through indirect interactions with other secreted Semas as shown in the olfactory system (Komiyama et al., 2007). In other cases, such as during the guidance of Drosophila motor axons, Sema-1a appears to act independently of Plexin and the ligand is not known (Jeong et al., 2012).

In this study, we find that Sema-1a promotes midline crossing in parallel to Netrin/Frazzled chemoattraction. Sema-1a mediates this function cell autonomously in commissural neurons. A region of Sema-1a's cytodomain previously shown to bind Pebble and RhoGAP190 is required for Sema-1a to promote crossing. In addition, RhoGAP190 and the downregulation of Rho1 are important for midline crossing. Surprisingly, Sema-1a's canonical binding partner, PlexA, does not contribute to Sema-1a's pro-crossing function. Instead, the secreted Sema2s confer signaling cues. Taken together, these data are

consistent with a model where Sema-1a mediates midline crossing through an attractive/adhesive mechanism via RhoGAPp190 in response to secreted semaphorins at the midline.

2.3 Materials and Methods

Genetic Analyses

The following Drosophila mutant alleles were used: *fra*^{3}, *fra*^{4} ,*fra*^{6}, *netAB, egMZ360 (eg-GAL4), slit*^{2}, *robo-1*^{GA285}. The following flies were obtained from the Bloomington Stock Center: s*ema-1a*^{P1}, *plexin A* ^{EY16548}, *plexin B*^{KG00878} , *pbl*^{2}, and *Rho1*^{72F}. The following stocks were kind gifts from A. Kolodkin: *sema-2a*^{B65} *(Wu et al., 2011), sema-2b*^{C4}*(Wu et al., 2011), sema-2ab*^{A15}*(Wu et al., 2011)*, and *p190*^{2} *(Jeong et al., 2012).* The *sema-1a artificial exon* was a kind gift from L. Zipursky. The following transgenes were used: UAS-FraΔC (Garbe et al., 2007), UAS-sema-1aFL, UAS-sema-1aΔ31-60, UAS-sema-1aECFC (*Jeong et al., 2012), UAS-FLP recombinase, UAS-26XLexAopmyrGFP, UAS-mycp190, UAS-Rho*^{N19}*.* GAL4 drivers used were elav-GAL4 and eg-GAL4. All crosses were carried out at 25˚C. Embryos were genotyped using balancer chromosomes carrying lacZ markers or by the presence of epitope-tagged transgenes.

Immunofluorescence and imaging

Dechorionated, formaldehyde-fixed, methanol devitellinized embryos were fluorescently stained as previously described (Kidd et al., 1998). The following primary antibodies were used in this study: mouse anti-1D4/FasII [Developmental Studies Hybridoma Bank (DSHB); 1:100], mouse anti-Beta gal [DSHB; 1:150],

mouse anti-Robo [DSHB; 1:50], mouse anti-Myc [DSHB (9E10); 1:500] rabbit anti-GFP [Invitrogen(#A11122); 1:500], mouse mAb anti-V5 [Serotec; 1:200], Mouse anti-HA [Covance (16B12) 1:250], Alexa647-conjugated goat anti-HRP [1:500, Jackson Immunoresearch (#123-605-021); 1:500]. Cyanine 3-conjugated goat anti-rabbit [Jackson; 1:1000], Alexa488-conjugated goat anti-mouse [Molecular Probes; 1:500] were used as secondary antibodies. Stage selected embryos were filleted to reveal the CNS from the dorsal side and mounted in 70% glycerol/PBS. Images were acquired using a spinning disk confocal system (PerkinElmer) built on a Nikon Ti-U inverted microscope using a Nikon OFN25 60X or 40X objective with a Hamamatsu C10600-10B CCD camera and Yokogawa CSU-10 scanner head with Volocity imaging software. Images were processed using ImageJ.

Phenotypic Quantification

For EW commissural neuron crossing phenotypes, whole-mount or filleted embryos were analyzed at Stages 15 and 16. Eight abdominal segments were analyzed per embryo where possible, and for each embryo, the percentage of non-crossing segments was calculated. A segment was considered non-crossing when both clusters of EW axons (six axons per segment) failed to reach the midline. For quantification of phenotypes using HRP, both posterior and anterior commissures were scored. A commissure was considered absent if it was not continuous or distinguishable from the other commissure in the segment. Commissures were thin/defective if they were substantially thinner than in wild-type (WT) embryos or excessively defasciculated. For statistical analysis,

comparisons were made between genotypes using the Student's t-test.

2.4 Results

A genetic screen identifies Sema-1a as a factor that promotes midline crossing

In order to identify molecules that function to promote midline crossing, we performed a genetic screen using a truncated Fra receptor (Fra∆C) lacking its cytoplasmic domain, that functions as a dominant negative (Garbe et al., 2007). By specifically expressing Fra∆C in a small subset of commissural neurons, the eagle neurons, we were able to establish a highly sensitized background. The eagle neurons are grouped into two clusters per hemisegment, the EGs and EWs. Approximately ten EG neurons project their axons through the anterior commissure, while only three EW neurons project their axons through the posterior commissure (Higashijima et al., 1996)(Figure 2.1A). These neurons can be easily identified and manipulated with eg-Gal4 and have been found to rely on Netrin/Fra chemoattraction. In *netAB* mutants or *fra* mutants, the EW neurons show a marked decrease in midline crossing, while the EG neurons are unaffected (Garbe et al., 2007). These defects can be easily quantified by calculating the fraction of abdominal segments where EW neurons fail to cross the midline. In *fra* mutants the EW axons fail to cross the midline in approximately 34% of abdominal segments, and expressing Fra∆C specifically in the eagle neurons of an otherwise wild-type embryo results in a similar phenotype (Figure 2.1B, C, G). We screened large deficiencies covering a

majority of the second chromosome and identified dominant enhancers of the Fra∆C crossing defects. This approach allowed us to identify even subtle crossing defects in heterozygous embryos and thus circumvent any obstacles like early gene requirement that would normally preclude many genes from examination.

A deficiency on the second chromosome, DF(2L)ED623, enhances the Fra∆C phenotype to 49% (Figure 2.1G). The enhancer activity in this interval was genetically mapped to Sema-1a and a null allele, *sema-1a*^{P1}, is able to fully recapitulate the enhanced EW defects observed with the deletion (Figure 2.1D, G). These crossing defects are dose dependent and are strongly enhanced when both copies of *sema-1a* are removed (Figure 2.1E, G). Furthermore, this severe mutant phenotype can be robustly rescued when full-length Sema-1a (Sema-1aFL) is restored selectively in the eagle neurons (Figure 2.1F, G). In order to validate the effects of *sema-1a* seen in the screen, we analyzed the genetic interaction between *sema-1a* heterozygotes and *fra* hypomorphs. Loss of one copy of *sema-1a* leads to an enhancement of EW neuron crossing defects in multiple hypomorphic backgrounds (Figure 2.3). This result further supports an endogenous role for *sema-1a* in promoting midline crossing.

Sema-1a promotes midline crossing independently of Netrin/Fra chemoattraction

To test whether Sema-1a functions together with, or independently of, Netrin/Fra chemoattraction, we examined genetic interactions between *sema-1a* and *fra* or *NetAB* mutants. The hallmarks of reduced midline crossing can be readily

observed when the entire axon scaffold is stained with anti-HRP antibodies. In wild-type embryos, thick commissures form in the anterior and posterior of each segment (Figure 2.2A). Both *NetAB* and *fra* null mutants display mild crossing defects, which are observed as thin or occasionally missing commissures (Figure 2.2B and 2.3). *Sema-1a* null mutants, however, show no significant crossing defects in either the axon scaffold or the eagle neuron commissural subset (Figure 2.2C and data not shown). If *sema-1a* were functioning in a parallel pathway to promote midline crossing, we would expect the loss of *sema-1a* to enhance the mild crossing defects seen in *fra* and *NetAB* mutants. While embryos heterozygous for both *fra* and *sema-1a* display no defects, the double mutants have a very different phenotype (Figure 2.2D and E). When we examine *sema-1a, fra* double mutants, we see a strong enhancement in crossing defects compared to *fra* single mutants (total defects: *sema-1a, fra* = 92% vs. *fra* = 40%; Figure 2.2E) as well an increase in the number of missing commissures (missing: *sema-1a, fra* =68% vs. *fra*=10%; Figure 2.2E). The majority of *sema-1a, fra* double mutant embryos are nearly commissureless, and these defects can be directly attributed to the loss of s*ema-1a* since double mutants can be robustly rescued with pan-neural expression of Sema-1aFL (total defects: 56%, missing: 25%; Figure 2.2F). Furthermore, this dramatic double mutant phenotype is not specific to *sema-1a, fra* double mutants, as it is nearly identical to the phenotype of *NetAB; sema-1a* double mutants (total defects: *NetAB, sema-1a* = 71% vs. *NetAB* = 25%), again with the strongest increase in the number of missing commissures (missing: *NetAB, sema-1a* = 48% vs. *NetAB* = 6%; Figure 2.3).

To further support the argument that *sema-1a* acts independently of the Netrin/Fra pathway, we analyzed dominant genetic interactions in the eagle commissural neurons. The crossing defects in both *fra* or *NetAB* mutants are significantly increased when a single copy of *sema-1a* is removed (Figure 2.3). These data demonstrate that Sema-1a must function independently of Netrin/Fra chemoattraction. We also explored the possibility that the effect of *sema-1a* on midline crossing could be due to up-regulation of Robo1 repulsion. We found that loss of *sema-1a* did not result in changes in Robo1 protein expression, nor does loss of *sema-1a* show genetic interaction with *slit* or *robo* mutants (Figure 2.4). Taken together, this evidence suggests that Sema-1a acts in parallel to Netrin/Fra and is unlikely to exert its pro-crossing effect through regulation of midline repulsion.

Sema-1a is endogenously expressed in eagle commissural neurons during midline crossing

Previously published expression data suggests that Sema-1a is expressed pan-neurally and that Sema-1a protein can be detected throughout the ventral nerve cord including in axon commissures (Kolodkin et al., 1993; Yu et al., 1998a). Ablation studies have suggested that these Sema-1a positive cells are mostly the motor neurons and Gad positive interneurons (Zlatic et al., 2009). Our initial findings, specifically the pan-neural rescue of the *sema-1a, fra* double mutant, would suggest that Sema-1a is required in neurons to promote midline crossing.

However, it is still unclear in which neurons Sema-1a is acting to promote midline crossing, since it could function in the commissural neurons themselves or in surrounding neurons. To address this question, we wanted to first know if Sema-1a is endogenously expressed in the eagle commissural neurons. Antibody staining and *in situ* hybridization techniques suggested co-localization with eagle neurons, but due to the broad expression of Sema-1a throughout the neuropil we are unable to adequately resolve individual neurons (data not shown). To definitively distinguish endogenous Sema-1a expression in a tissue specific manner, we took advantage of a genetically engineered fly line developed in the Zipursky lab that allows sparse labeling of endogenous Sema-1a (Pecot et al., 2013). Pecot and colleagues generated an artificial exon within the endogenous locus carrying a conditional genetic tracer that allows us to visualize both the cells that express Sema-1a and the Sema-1a protein itself (Pecot et al., 2013). This dual visualization is achieved by the co-expression of a V5-tagged Sema-1a and a LexA transcription factor, which are restricted from expression by a stop cassette flanked by FRT sites (Figure 2.5A). Thus, tissue specific expression of FLP excises the stop cassette, allowing visualization of endogenous Sema-1a expression only in the tissue of interest. Expression of FLP in eagle neurons resulted in an a mosaic expression during the time of midline crossing (Figure 2.5B). This sparse labeling allowed us to capture endogenous *sema-1a* expression at single cell resolution. Assessments across multiple embryos indicate that Sema-1a is indeed endogenously expressed in all eagle neurons including the EW cluster (Figure 2.5C'). Visualization of Sema-1a molecules

using the V5 tag reveals a punctate pattern on cell bodies and strong labeling of the axons during the time when they are crossing the midline (Figure 2.5C").

Sema-1a functions cell autonomously, and its cytoplasmic domain is required for midline crossing

Sema-1a is traditionally thought to act as a ligand for PlexA receptors, yet it has been demonstrated to function in reverse as a receptor itself (Cafferty et al., 2006; Godenschwege et al., 2002; Hsieh et al., 2014; Jeong et al., 2012; Komiyama et al., 2007; Pecot et al., 2013; Sweeney et al., 2011). Given the fact that Sema-1a is expressed in commissural neurons and appears to function in neurons to promote crossing, we wanted to explore if Sema-1a functions as a receptor in this context. To determine if Sema-1a promotes midline crossing through reverse signaling, we tested if Sema-1a's cytoplasmic domain is required cell autonomously in commissural neurons. To address cell autonomy without introducing non-autonomous "follower effects," we used a *sema-1a* mutant expressing the dominant negative Fra receptor (FraΔC) in the eagle neurons only. These embryos display the same level of defects in the eagle neurons as *sema-1a, fra* double mutants, while the rest of the CNS appears largely wild-type. We compared the ability of full-length and two truncated Sema-1a transgenes to rescue crossing defects in this genetic background. These transgenes are targeted to the same genomic locus and are expressed at comparable levels. All three transgenes are capable of rescuing forward signaling yet only the full-length transgene is able to completely rescue reverse signaling (Jeong et al.,

2012). A robust rescue is achieved when the full-length Sema-1a transgene (Sema-1aFL) is restored to eagle neurons in this Fra∆C background, with eagle neuron crossing defects reduced from 98% to 26% (Fig. 4 and Fig. 1). This would suggest a cell autonomous requirement since there is no Sema-1a present to function cell non-autonomously in this background. Furthermore, the truncated Sema-1a transgene (UAS-Sema-1a∆C) completely fails to rescue, suggesting that the cytoplasmic domain is required and that Sema-1a likely mediates midline crossing through reverse signaling. To further determine the region within the cytoplasmic domain that is necessary for midline crossing, we tested a third transgene (Sema-1a∆31-60) carrying a small deletion within the cytoplasmic domain, which removes amino acids 31-60. This cytoplasmic region includes the binding site for downstream effectors of Sema-1a reverse signaling in motor neurons and was demonstrated to physically interact with two opposing regulators of the small GTPase Rho1(Jeong et al., 2012). Expression of this transgene results in a dramatically reduced rescue, implicating this region in midline crossing and further supporting the conclusion that Sema-1a promotes midline crossing through reverse signaling (Figure 2.6). Although Sema-1a∆31-60 does produce a small but significant reduction in crossing defects, it does not rescue crossing nearly as well as the full-length transgene.

These findings in the eagle neurons are consistent with the pan-neural rescue of the *sema-1a, fra* double mutants. When we pan-neurally express these Sema-1a transgenes we get a similar rescue profile where Sema-1a-FL leads to a strong yet partial rescue, Sema-1a∆31-60 produces a blunted rescue, and Sema-1a∆C

completely fails to rescue (Figure 2.7). Notably, Sema-1aΔC does rescue forward signaling in other systems(Godenschwege et al., 2002; Jeong et al., 2012). If forward signaling were contributing to midline crossing directly, then we would expect a partial rescue with the Sema-1aΔC transgenes, yet this is not what we see in any genetic background. These data indicate that Sema-1a promotes midline crossing through reverse signaling since it functions cell autonomously and its cytoplasmic domain is required. The results with the small cytoplasmic deletion also point to specific binding partners that may be important for mediating the downstream pathway involved in Sema-1a dependent midline crossing.

RhoGAPp190 and the negative regulation of Rho1 are required for midline crossing

A recent study identified Pebble RhoGEF (Pbl) and RhoGAPp190 (p190) as potential effectors of Sema-1a reverse signaling in *Drosophila* motor neurons (Jeong et al., 2012). Both proteins bind the cytoplasmic region of Sema-1a, and both mutants display distinct defects in motor axon guidance. To investigate the roles of Pbl and p190 in midline crossing, we examined their genetic interactions with *sema-1a* and *fra*. Pbl and p190 are known to exert opposing effects on the actin cytoskeleton through regulation of the small GTPase, Rho1. Pebble positively regulates Rho1 and is proposed to function in concert with Sema-1a to produce a repulsive/de-adhesive response in motor neurons (Jeong 2012), while RhoGAPp190 acts as a negative regulator of Rho1 and has been demonstrated

to promote adhesion and branch stability (Billuart et al., 2001; Jeong et al., 2012). To investigate if these effectors modulate midline crossing downstream of Sema-1a, we examined whether heterozygosity for *pbl* or *p190* mutations dominantly enhance crossing defects in the sensitized Fra∆C background. Heterozygosity for *p190* does not significantly enhance crossing defects (46%; Figure 2.8). To test this finding further, we examined *p190* zygotic null mutants in this background, and this produces a dramatic increase in crossing defects similar to *sema-1a* nulls in the same background (81%; Figure 2.8). Overexpression of p190 in the eagle neurons reduces the number of defects seen in Fra∆C background to 16% of abdominal segments (Figure 2.8). In contrast, heterozygosity for *pbl* did not result in an enhancement of crossing defects. Instead, it suppressed these defects to 10% (Figure 2.8). We were unable to test *pbl* null mutants since *pbl* is required for cytokinesis, but we were able to evaluate their shared downstream target, *rho1*(Prokopenko et al., 1999). Reductions in *rho1* lead to a similar suppression as *pbl,* where only 21% of eagle neurons fail to cross the midline. Additionally, expression of a dominant negative Rho1 transgene specifically in the eagle neurons similarly suppresses crossing defects to 25%. This result is consistent with the hypothesis that Sema-1a promotes midline crossing through RhoGAPp190 and the down regulation of Rho1.

The secreted semaphorins function to promote midline crossing

In order to better understand the cellular mechanism of Sema-1a-mediated midline crossing, we next sought to determine which, if any, of the known extra-

cellular binding partners of Sema-1a might act as a ligand for reverse signaling in commissural neurons. We would expect that any component of the Sema-1a mediated midline crossing pathway should phenocopy the strong *sema-1a, fra* double mutant phenotype. Importantly, embryos lacking both *fra* and *plexA or plexB* fail to phenocopy *sema-1a, fra* double mutants, and the crossing defects are not significantly different from *fra* mutants alone (Figure 2.9). These results strongly suggest that Plexins are not contributing to Sema1a-dependent midline crossing. In contrast, *fra, sema-2a* double mutants exhibit defects that resemble *sema-1a, fra* double mutants, and total defects are significantly enhanced compared to *fra* single mutants. Although total crossing defects are comparable between the *sema-2a, fra* double mutants and the *sema-1a, fra* double mutants, there is a distinct shift in the profile of these defects. The majority of defects identified in *fra, sema-2a* double mutants are thin/defective commissures while *sema-1a, fra* double mutants primarily exhibit absent commissures (Figure 2.9).

One reason why the *fra, sema-2a* double mutants may fail to fully recapitulate the *sema-1a, fra* double mutants may be because of compensation by the other secreted semaphorin, Sema-2b. Sema-2a and Sema-2b show 70% amino acid identity and have been demonstrated to function redundantly in certain tissues (Sweeney et al., 2011; Wu et al., 2011). The secreted semaphorins are both expressed in the developing nerve cord at the time of commissure formation and both proteins are found to decorate the anterior and posterior commissures (Emerson et al., 2013; Kolodkin et al., 1993; Wu et al., 2011; Zlatic et al., 2009). Sema-2a, however, displays a distinct enrichment at the midline (Kolodkin et al.,

1993; Wu et al., 2011). To test for a contribution of Sema-2b, we generated *fra, sema-2a, sema-2b* triple mutants. However, commissural defects in these triple mutants are not significantly different from those seen in the *fra, sema-2a* double mutants (Figure 2.9). Because it is difficult to capture subtle changes in commissural defects when examining the entire axon scaffold with HRP, we also evaluated *fra, sema-2b* double mutants in eagle neurons. We see a clear enhancement of crossing defects when *sema-2b* is lost (50%) compared to fra single mutants (27%). This enhancement is not as robust as the enhancement seen in *fra, sema-2a* double mutants (75%; Figure 2.10). The *fra, sema2ab* triple mutants display defects similar to the double mutants (58%).

In order to more directly assess if Sema-1a mediates midline crossing in a PlexA or Sema-2 dependent manner, we examined the ability of UAS Sema-1a to rescue *sema-1a*-dependent crossing defects in the absence of either *plexA* or *sema-2a*. If either gene is a required component of the Sema-1a pathway, the ability of UAS Sema-1a to rescue should be suppressed when *plexA or sema-2a* are also mutant. Therefore, we evaluated the degree of rescue when Sema-1a is expressed in a *sema-1a;;plexA* double mutant with Fra∆C in eagle neurons. Sema-1a is still able to rescue crossing in the absence of *plexA,* strongly arguing that Sema-1a mediated midline crossing is PlexA independent. However, Sema-1a is not able to rescue to the same extent when expressed with Fra∆C in the eagle neurons of *sema-1a, sema-2a* double mutants (Figure 2.11). The incomplete suppression is likely due to compensation by Sema-2b. These data

would indicate that Sema-2a, and not PlexA, functions to instruct the Sema-1a mediated midline crossing pathway.

2.5 Discussion

These data demonstrate that Sema-1a represents a novel pathway for promoting midline crossing. We find that Sema-1a not only functions as a receptor to promote midline crossing, but it does so independently of its canonical binding partner PlexA. Our genetic data suggest that the secreted Semas represent components of the Sema-1a ligand in this context. Furthermore, the spatial distribution of these components, as well as the known roles of the downstream effectors, suggest this Sema-1a signaling pathway results in an attractive or adhesive response, rather than the repulsive response that is typically associated with Sema/Plexin signaling. In most systems where Sema-1a reverse signaling has been identified, forward signaling has also been found to function. This bidirectional signaling has made it difficult to divorce the two signaling cascades and determine the distinct mechanism of Sema-1a reverse signaling. We find that specific genetic manipulations in a well defined tissue such as the ventral midline allow us to establish a system where the two pathways can be more clearly separated. In this way, we can begin to define the Sema-1a reverse signaling contribution to midline crossing.

Sema-1a functions in a novel pathway for promoting midline crossing

By using a sensitized background and looking specifically at the tissue of interest, we were able to identify alternative pathways for promoting midline crossing. Sema-1a has never before been associated with midline crossing since the null mutants alone show no commissural defects. Analysis of mutants in the eagle neurons fails to show a significant reduction in crossing when Sema-1a is absent (data not shown). The effect of Sema-1a loss of function is only apparent when the major attractive pathway of Netrin/Fra signaling is removed. We observed this interaction in a number of different backgrounds, first with the Fra dominant negative (FraΔC), as well as with the *fra* and *netAB* mutants, and then most dramatically with the *sema-1a, fra* or *netAB; sema-1a* double mutants. Our lab previously uncovered a netrin-independent role for Fra as well as a role for *robo2* in promoting midline crossing (Evans et al., 2015; Neuhaus-Follini and Bashaw, 2015b; Yang et al., 2009). Both of these pathways appear to function by negatively regulating Robo1 repulsion at the midline. In order to understand how redundant/ convergent these pathways may be, we further explored the interactions between Sema-1a and known midline pathways. Genetic interactions reveal a clear parallel function between Fra/Netrin chemoattraction and Sema-1a. Genetic interactions with *robo1, slit* double heterozygotes suggest that Sema-1a does not function as another anti-repulsive mechanism (Figure 2.4). Additionally, Robo1 protein expression does not appear to be upregulated in *sema-1a* mutants (Figure 2.4). Taken together, our observations indicate that Sema-1a promotes midline crossing through an independent pathway.

Sema-1a mediates midline crossing through reverse signaling in commissural neurons

Reverse signaling through transmembrane semaphorins has been demonstrated in both invertebrates and vertebrates, where the class 6 semaphorins show a particular similarity with *Drosophila* Sema-1a. The role of Sema6D in endocardial cell migration was the first *in vivo* demonstration of reverse signaling in vertebrates (Toyofuku et al., 2004b). More recently, findings of semaphorin reverse signaling in neurons have revealed that class 6 semaphorins may have more axon guidance roles similar to those identified for Sema-1a reverse signaling in *Drosophila*. A recent study in chick by Andermatt and colleagues demonstrated that Sema6B functions as a receptor in post-crossing commissural neurons potentially by promoting an outgrowth response (Andermatt et al., 2014). Evidence of a more instructional role for reverse signaling was found in a subset of On direction-selective ganglion cells (OnDSGCs). Here, Sema6A mediates axonal targeting to the accessory optic system (AOS) through an attractive response to Plexin A2 and A4 (Sun et al., 2015). Although it is clear that the capability of transmembrane semaphorins to signal in reverse and function as axon guidance receptors is highly conserved and that Sema/Plexin signaling participates in midline guidance, it had not been previously known whether Sema reverse signaling contributes directly to midline crossing until now.

RhoGAPp190 mediates Sema-1a reverse signaling to promote midline crossing

In the majority of cases, Sema-1a reverse signaling promotes repulsive guidance in response to Plexins, yet there are attractive signaling outputs and binding partners as well. Two classes of neurons in the visual system, the laminar neurons and the photoreceptors were both found to employ Sema-1a reverse signaling and both bound the canonical binding partner PlexA; however, the laminar neurons exhibit a repulsive response to PlexA, while the photoreceptors show an adhesive response (Cafferty et al., 2006; Hsieh et al., 2014; Pecot et al., 2013). This variable signaling output highlights the need to identify other pathway components that may regulate distinct functional responses.

The discovery of competitive downstream effectors (Pbl and RhoGAPp190) with opposing effects on Rho1 began to explain how Sema-1a reverse signaling could have multiple, and even opposite outputs. This competition introduces one level of regulation and we speculate that there are additional regulators that function to modulate the activity of these effectors and the ultimate axonal response. For instance, Src family kinases, which phosphorylate p190 within the GTP binding domain, leading to inhibition of p190 activity (Billuart et al., 2001; Brouns et al., 2001; Roof et al., 2000) may act to modulate Sema-1a reverse signaling. Indeed, previous findings from our lab analyzing *src* mutations in multiple *frazzled* backgrounds found that src kinases antagonizes midline crossing in a Netrin/Frazzled independent fashion, suggesting *src* is acting on an unidentified parallel pathway for midline crossing (O'Donnell and Bashaw, 2013). A role for

p190 in midline crossing would reconcile these findings while also pointing at interactions that may modulate Sema-1a reverse signaling output.

The cytoplasmic region between amino acids 31-60 of Sema-1a provides the binding site for Pbl and p190, but it also includes part of a putative Enabled (ena) binding site (LPQP). This enabled binding site is required in the giant fiber for Sema-1a reverse signaling (Godenschwege 2002). To test whether the requirement for this region in midline crossing was due to p190 interactions rather than *ena,* we assessed *ena* mutants in the screening background, and found that crossing defects are not enhanced in *ena* mutants (data not shown).

Interestingly, the Sema-1a mediated adhesive response uncovered in the photoreceptors is also dependent on the down regulation of Rho1 (Hsieh et al., 2014). In the photoreceptors, however, the adhesive molecule FasII, which is not expressed in the commissural eagle neurons, ultimately mediated adhesion. Other adhesive molecules like integrins are also unlikely to function downstream of Sema-1a in the context of midline crossing, since they were previously tested in our lab when the p190 inhibitor, Src, was identified as an antagonist to midline crossing (O'Donnell and Bashaw, 2013). The implication of p190 as a downstream effector in the context of Sema-1a mediated midline crossing is intriguing since it represents an alternative output for Sema-1a reverse signaling. While Pbl mediates repulsion/defasciculation and target recognition in the motor neurons, p190 is thought to control fasciculation by antagonizing Pbl activity. p190 has been shown to stabilize branches and promote adhesion in other

systems, but negative regulation of Rho1 may also promote attraction (Billuart et al., 2001; Ng and Luo, 2004; Yuan et al., 2003). With these possibilities in mind, it was unclear what response p190 might be mediating in the commissural neurons. Taken together, our findings point to a Sema-1a mechanism that is neither repulsive nor adhesive, leading us to explore the possibility of an attractive mechanism.

The secreted Sema2s function as attractive/ adhesive ligands for Sema-1a mediated midline crossing

The genetic interactions we tested implicate the secreted Sema-2s as the potential signaling partners for Sema-1a mediated midline crossing. Sweeney et al. clearly demonstrate that the Sema-1a ectodomain selectively binds to tissue where Sema-2a is overexpressed, yet evidence for a direct physical interaction is still lacking (Sweeney et al., 2011). Although this interaction is unlikely to be direct, we show that Sema-1a requires Sema-2a to rescue midline crossing (Figure 2.11). Furthermore, the double mutant phenotypes with *fra* demonstrate that the secreted semaphorins are required for axons to cross the midline (Figure 2.9 and 7). The medial expression of the secreted Sema2s, in particular Sema-2a, suggests that they signal directional information rather than promote permissive adhesion. Sema-2b has indeed been shown to signal attraction in sensory neurons (Wu et al., 2011). We propose a model where the secreted Sema2s act as attractive cues to promote midline crossing as the simplest interpretation of the observed phenotypes.

Future Directions

While we demonstrate a role for Sema-1a reverse signaling in pre-crossing commissural axons, forward signaling is important for the formation of longitudinal tracts post-crossing(Jeong et al., 2012; Terman and Kolodkin, 2004; Yang and Terman, 2012; Yu et al., 1998b). The midline, as an intermediate target, may offer a unique context for the shift between forward and reverse signaling. Further investigation to uncover regulatory components of the Sema-1a reverse signaling pathway would prove illuminating in understanding how these distinct outputs are achieved.

Sema-1a Reverse Signaling Figures

Figure 2.1

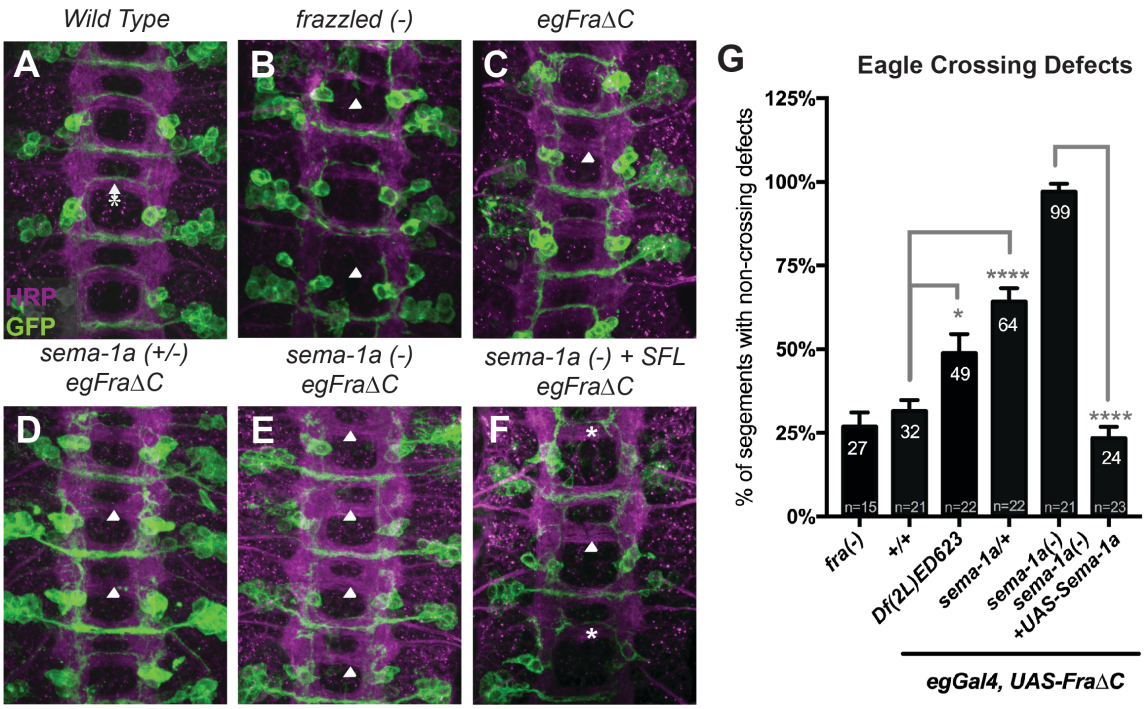


Figure 2.1. Sema-1a is a positive regulator of midline crossing

(A–F) Stage 15–16 embryos of the indicated genotypes carrying eg-GAL4 and UAS-CD8 GFP transgenes, stained with anti-HRP (magenta) and anti-GFP (green) antibodies. Anti-GFP labels cell bodies and axons of the eagle neurons (EG and EW) in these embryos. Arrowheads indicate segments with non-crossing EW axons and asterisks indicate rescued EW crosses. (A) EW neurons cross in the posterior commissure in 100% of segments in wild-type embryos (starred arrowhead). (B) *frazzled* (*fra3/fra3*) mutants show crossing defects in eagle neurons, where EW neurons fail to cross in 27% of segments (arrowheads). (C) Expression of a Frazzled dominant negative receptor (UAS-Fra∆C) selectively in eagle neurons produces a Fra-like phenotype where EW neurons fail to cross in 32% of segments. (D) Heterozygosity of s*ema-1a* dominantly enhances the EW crossing defects in a Fra∆C background to 64%. (E) Complete loss of *sema-1a* leads to further enhancement and EW neurons fail to cross in 99% of segments. (F) EW crossing defects in the *sema-1a* null expressing Fra∆C can be robustly rescued from 99% to 24% when a UAS Sema-1a transgene is expressed in eagle neurons (G) Histogram quantifies EW midline crossing defects in the genotypes shown in (A–F). Data are represented as mean+SEM. n, number of embryos scored for each genotype. Significance was assessed by multiple comparisons using the Student's t-test (****p<0.0001). Brightness and contrast are enhanced on the GFP channel to make eagle neurons more visible over HRP.

Figure 2.2

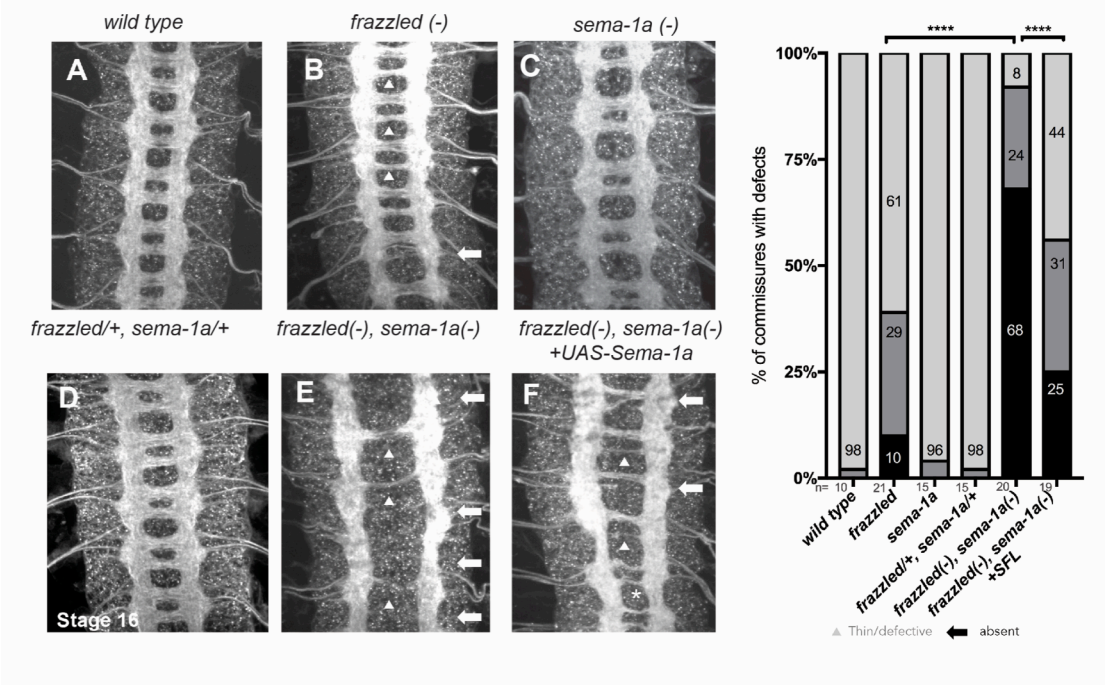
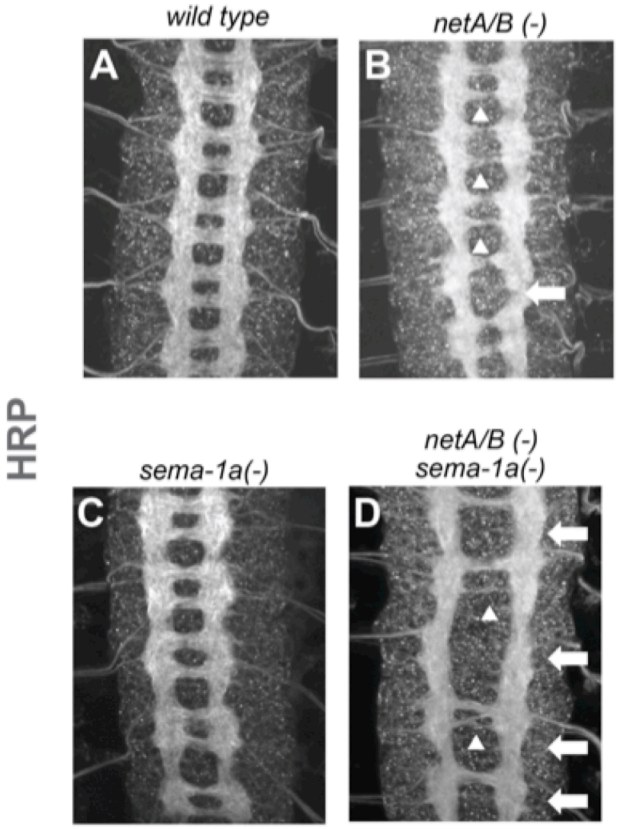
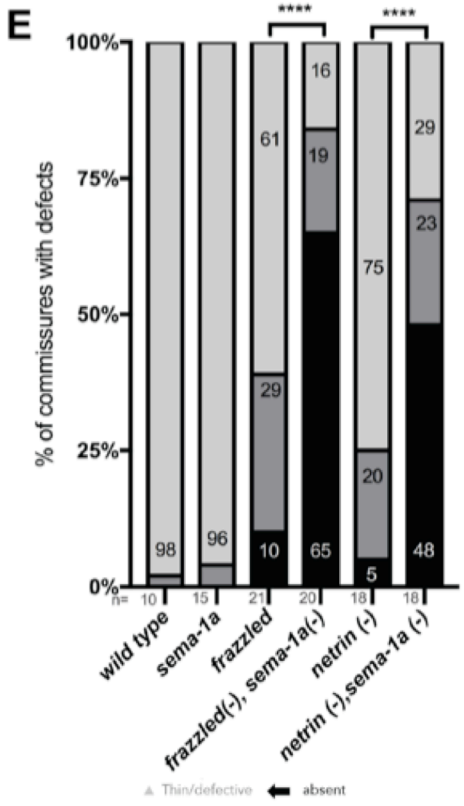

wild type
frazzled (-)
sema-1a (-)
A
B
C
frazzled/+, sema-1a/+
frazzled(-), sema-1a(-)
frazzled(-), sema-1a(-)
+UAS-Sema-1a
D
E
F
Stage 16
% of commissures with defects
100%
75%
50%
25%
0%
****
****
98
10
61
29
96
98
8
24
68
44
31
25
n= 10
21
15
15
20
19
wild type
frazzled
sema-1a
frazzled/+, sema-1a/+
frazzled(-), sema-1a(-)
frazzled(-), sema-1a(-)
+SFL
Thin/defective
absent

Figure 2.2 Sema-1a functions in parallel to *frazzled* to promote midline crossing

(A–F) Stage 16 embryos of the indicated genotypes stained with anti-HRP antibodies. Arrowheads indicate thin/defective commissures, arrows indicate missing commissures and asterisks indicate rescued commissures. (A) Thick anterior and posterior commissures are formed as axons cross the midline in nearly every segment. (B) *frazzled* (*fra3/fra4*) mutants show thin (29%) and occasionally missing commissures (10%). (C) *sema-1a* mutants show no obvious signs of commissural defects. (D) Embryos heterozygous for both s*ema-1a* and *fra* appear wild-type. (E) Loss of *sema-1a* significantly worsens the crossing defects of *Fra* single mutants and *fra, sema-1a* double mutants show a 68% loss of commissures. (F) Pan-neural expression of Sema-1a partially rescues these defects, and reduces missing commissures to 25%. (G) Histogram quantifies commissural defects as absent (black bar), thin/defective (dark gray) or wild-type (light grey) in the genotypes shown in (A–F). Data are represented as mean+SEM. n, number of embryos scored for each genotype. Significance was assessed by multiple comparisons using the Student's t-test (****$p<0.0001$). See also Figure 2.1.

Figure 2.3


wild type
netA/B (-)
A
B
HRP
sema-1a(-)
netA/B (-)
sema-1a(-)
C
D


E
% of commissures with defects
100%
75%
50%
25%
0%
****
****
98
96
10
61
29
65
16
19
5
75
20
48
29
23
n=
10
15
21
20
18
18
wild type
sema-1a
frazzled
frazzled(-), sema-1a(-)
netrin (-)
netrin (-),sema-1a (-)
Thin/defective
absent

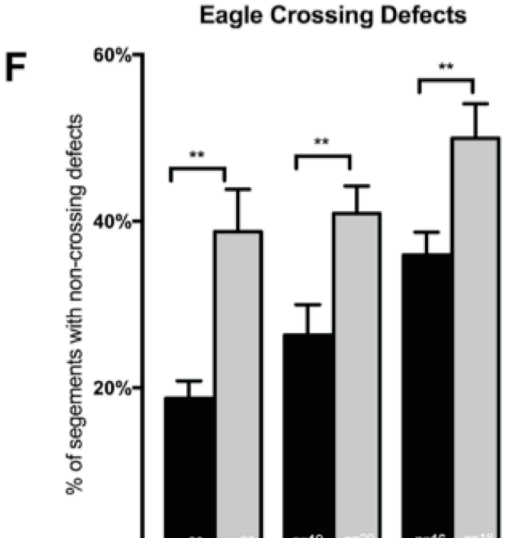

Eagle Crossing Defects
F
% of segements with non-crossing defects
60%
40%
20%
**
**
**

Figure 2.3. Sema-1a dominantly enhances the eagle crossing defects in both *frazzled* and *netrinAB/B* mutants.

(A–D) Stage 16 embryos of the indicated genotypes stained with anti-HRP antibodies. Arrowheads indicate thin/defective commissures, arrows indicate missing commissures and asterisks indicate rescued commissures. (A) Thick anterior and posterior commissures are formed as axons cross the midline in nearly every segment. (B) *netAB* mutants show thin (20%) and missing commissures (5%). (C) Sema-1a mutants show no obvious signs of commissural defects. (D) *NetAB, sema-1a* double mutants show a 48% loss of commissures. (E) Histogram quantifies commissural defects as absent (black bar), thin/defective (dark gray) or wild-type (light grey) in the genotypes shown in (A–D). Scores for *fra* and *fra, sema-1a* double mutants are included as reference. Data are represented as mean+SEM. n, number of embryos scored for each genotype. Significance was assessed by multiple comparisons using the Student's t-test (****p<0.0001). (F) Heterozygosity for *sema-1a* significantly enhances the EW crossing defects in *fra* hypomorphs (fra^3/fra^6) to 38%. Loss of one copy of *sema-1a* also enhances crossing defects in *fra* single mutants (fra^3/fra^4) from 24% to 43%. EW crossing defects in *NetAB* mutants (34%) are also increased when a single copy of *sema-1a* is removed (50%). Data are represented as mean+SEM. n, number of embryos scored for each genotype. Significance was assessed using the Student's t-test (**p<0.01).

Figure 2.4

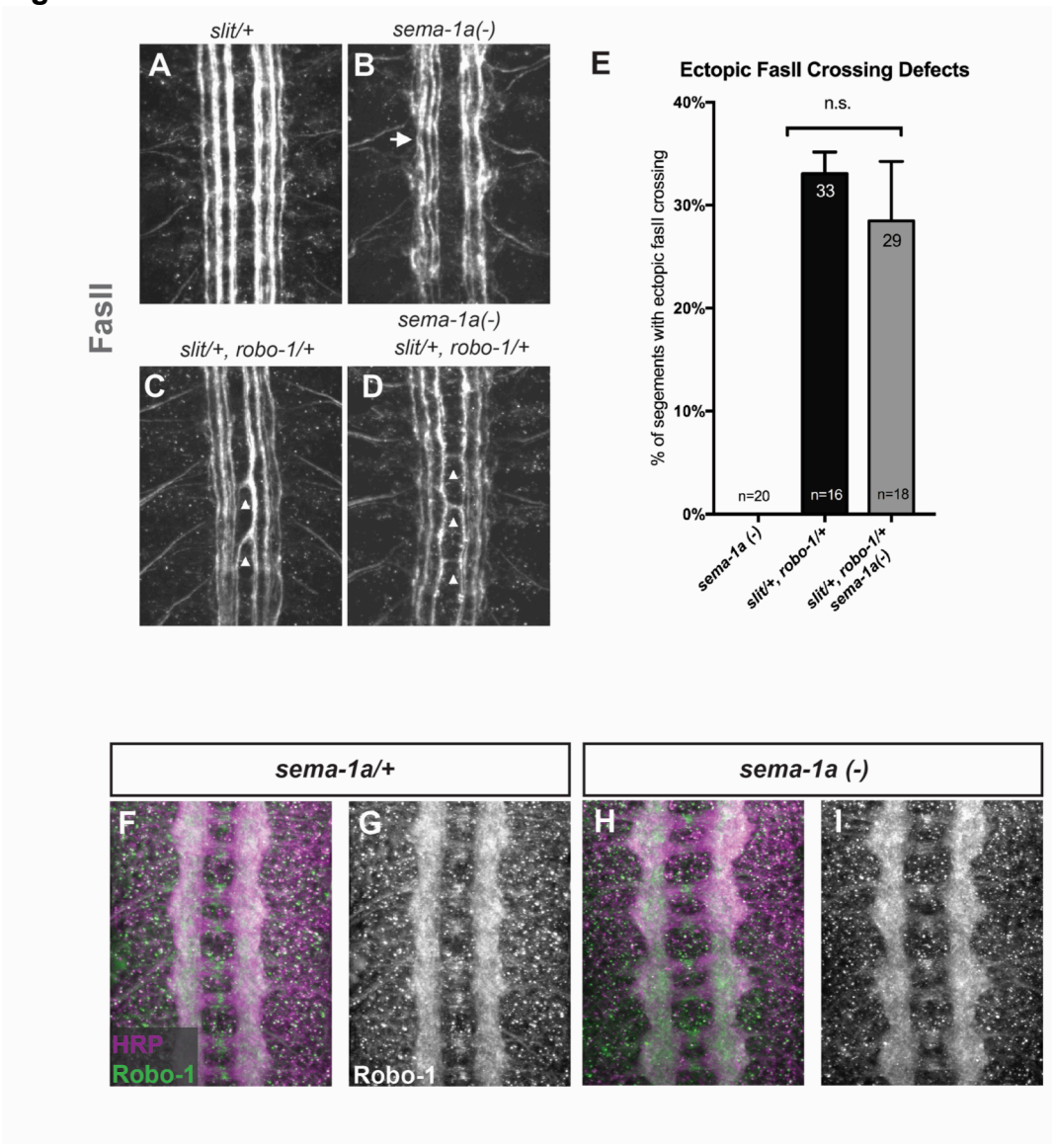

slit/+
sema-1a(-)
A
B
FasII
slit/+, robo-1/+
sema-1a(-)
slit/+, robo-1/+
C
D
E
Ectopic FasII Crossing Defects
n.s.
40%
30%
20%
10%
0%
% of segements with ectopic fasII crossing
33
29
n=20
n=16
n=18
sema-1a (-)
slit/+, robo-1/+
slit/+, robo-1/+ sema-1a(-)
sema-1a/+
sema-1a (-)
F
G
H
I
HRP
Robo-1
Robo-1

Figure 2.4. Loss of sema-1a does not suppress ectopic FasII crossing defects or expand Robo-1 expression.

(A–D) Stage 17 embryos of the indicated genotypes stained with the Anti-FasciclinII (FasII) antibodies. Anti-FasII labels longitudinal tracts of ispilateral axons in these embryos. Arrowheads indicate segments with ectopic crossing of FasII axons. (A) Embryos heterozygous for *slit/+* (or *robo-1*/+ or *sema-1a*/+) display intact longitudinals and FasII positive neurons never cross the midline. (B) sema-1a mutants show longitudinal breaks (arrow) but FasII neurons never cross the midline (C) Embryos heterozygous for *robo-1* and *slit* show ectopic crossing defects (33%) due to reduced repulsion from the midline (D). These ectopic crossing defects are not significantly suppressed when *sema-1a* is also mutant suggesting that Sema-1a does not act as a negative regulator of Robo-1 repulsion. (E) Histogram quantifies ectopic FasII crossing defects in the genotypes shown in (A–D). Data are represented as mean+SEM. n, number of embryos scored for each genotype. Significance was assessed by using the Student's t-test. (F-I) Stage 15–16 embryos of the indicated genotype stained with anti-HRP and anti-Robo-1 antibodies. (F) Embryos heterozygous for *sema-1a* exhibit the normal distribution of Robo-1 protein expression (G) Robo-1 is normally excluded from the commissural segment of axons (H) *sema-1a* mutant embryos do not show any qualitative elevation of Robo-1 protein expression (I) and Robo-1 protein is still restricted from commissural segments in s*ema-1a* mutants, suggesting that Sema-1a does not endogenously function to regulate Robo-1 protein expression at the midline.

Figure 2.5

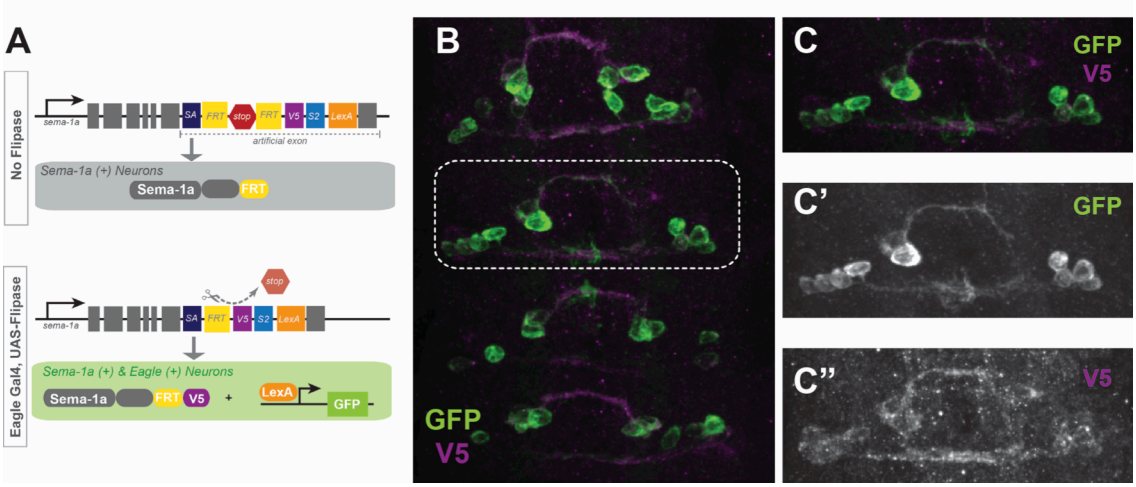

A
No Flipase
sema-1a
SA
FRT
stop
FRT
V5
S2
LexA
artificial exon
Sema-1a (+) Neurons
Sema-1a
FRT
Eagle Gal4, UAS-Flipase
stop
Sema-1a (+) & Eagle (+) Neurons
Sema-1a
FRT
V5
+
LexA
GFP
B
GFP
V5
C
GFP
V5
C'
GFP
C''
V5

Figure 2.5. Sparse labeling of Sema-1a reveals endogenous expression in the commissural eagle neurons

An artificial exon knocked into the endogenous locus for *sema-1a,* developed in the Zipursky lab, allows for tissue specific labeling of endogenous Sema-1a expression. (A) Schematic of sparse labeling paradigm adapted from Pecot, et al. 2013. In the presence of a FLP recombinase, Sema-1a becomes tagged with a V5 epitope and LexA driven membrane bound GFP labels the full extent of the Sema-1a positive cells. (B-C) Early *s*tage 15 embryo carrying the artificial exon, egGal4, UAS-FLP recombinase and LexAop-myrGFP. Embryo is stained with anti-GFP (green) and anti-V5 (magenta) antibodies. (B) Eagle neurons endogenously express Sema-1a during midline crossing. (C) Magnification of the boxed region in B. (C') GFP only staining shows two EW axons crossing the midline (C'') V5 staining reveals that Sema-1a protein is expressed throughout the growing axon.

Figure 2.6

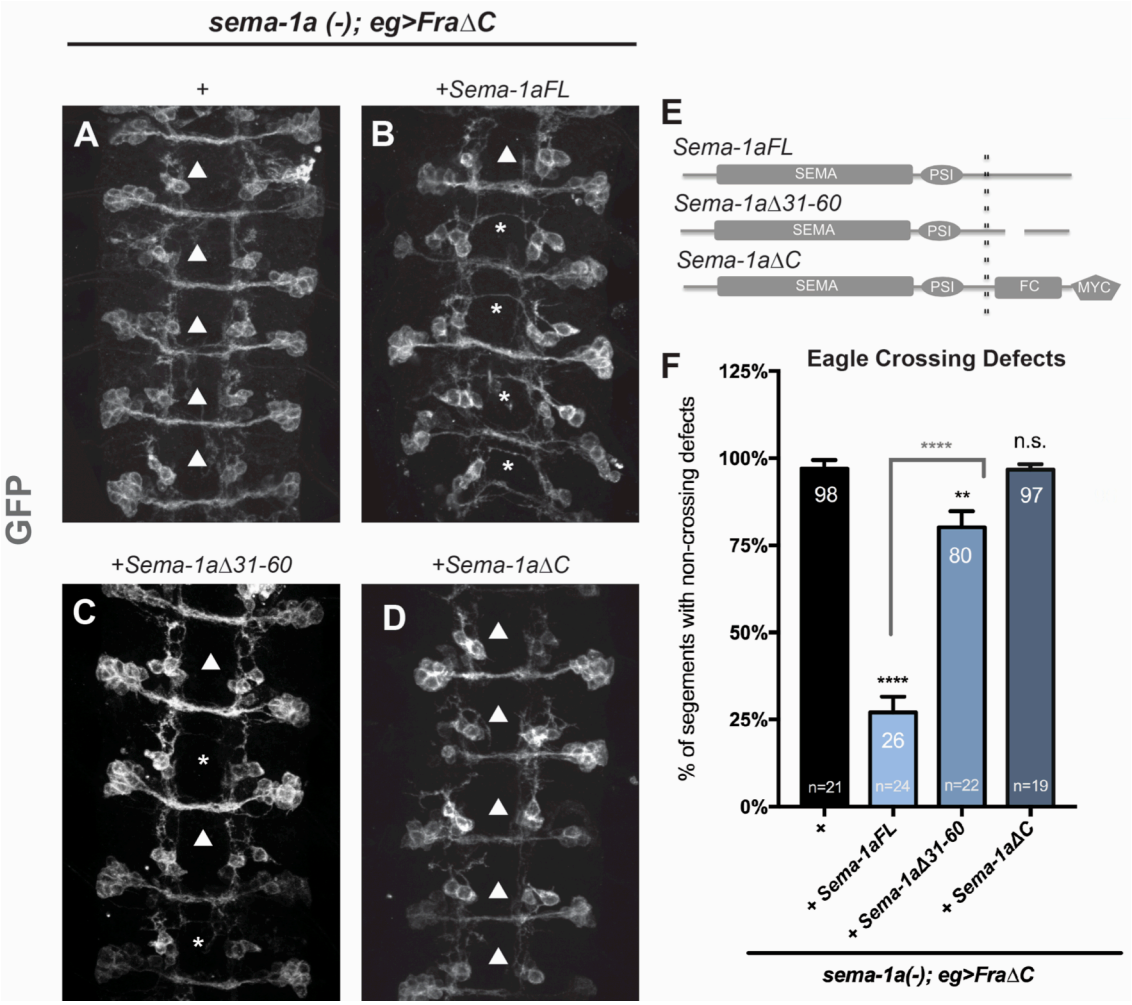

sema-1a (-); eg>FraΔC
+
+Sema-1aFL
+Sema-1aΔ31-60
+Sema-1aΔC
GFP
A
B
C
D
E
Sema-1aFL
SEMA
PSI
Sema-1aΔ31-60
Sema-1aΔC
FC
MYC
F
Eagle Crossing Defects
% of segements with non-crossing defects
125%
100%
75%
50%
25%
0%
98
26
80
97
n=21
n=24
n=22
n=19
****
**
n.s.
+ Sema-1aFL
+ Sema-1aΔ31-60
+ Sema-1aΔC
sema-1a(-); eg>FraΔC

Figure 2.2.6. Sema-1a can rescue midline crossing cell autonomously.

(A–D) Stage 15–16 embryos of the indicated genotypes carrying eg-GAL4, UAS-Fra∆C and UAS-CD8 GFP transgenes, stained with anti-GFP (green) antibodies. Anti-GFP labels cell bodies and axons of the eagle neurons (EG and EW) in these embryos. Arrowheads indicate segments with non-crossing EW axons and asterisks indicate rescued EW crosses. (A) *sema-1a* null embryos expressing Fra∆C show severe crossing defects in EW neurons, which fail to cross the midline in 98% of abdominal, segments (arrowheads). (B) Expression of a full-length Sema-1a transgene in eagle neurons strongly rescues these defects (asterisk), with only 26% non-crossing (arrowheads). (C) In contrast, a Sema-1a transgene lacking a small region of the cytoplasmic domain (from aa31-60) significantly reduced defects to a much lesser extent (80%), suggesting this region is important for promoting midline crossing (D) Expression of a Sema-1a transgene without its cytoplasmic domain does not significantly rescue crossing defects and EW neurons still fail to cross in 97% of segments. (E) Diagram of transgenic rescue constructs (F) Histogram quantifies EW midline crossing defects in the genotypes shown in (A–D). Data are represented as mean+SEM. n, number of embryos scored for each genotype. Significance was assessed by multiple comparisons using the Student's t-test (****$p<0.0001$).

Figure 2.7

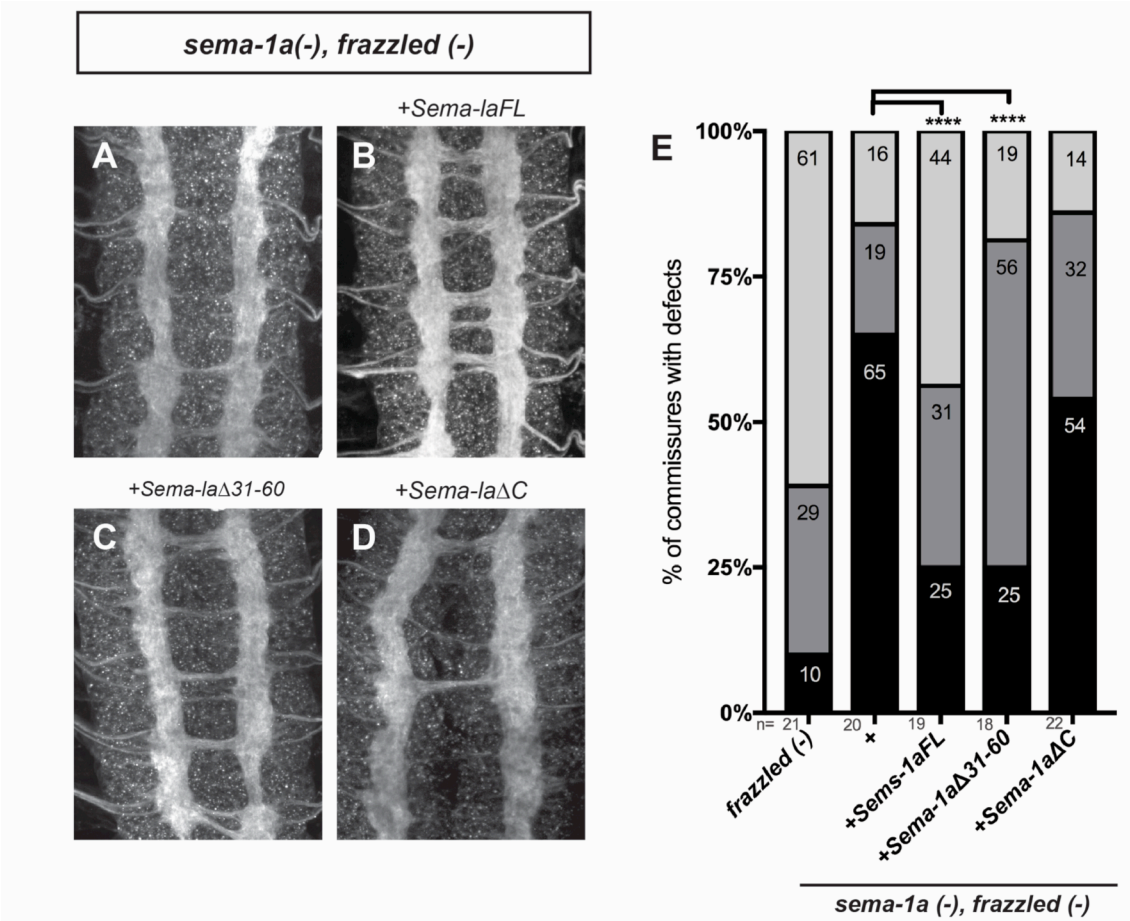

sema-1a(-), frazzled (-)
+Sema-laFL
A
B
+Sema-laΔ31-60
+Sema-laΔC
C
D
E
% of commissures with defects
100%
75%
50%
25%
0%
61
29
10
16
19
65
44
31
25
19
56
25
14
32
54
****
****
n= 21
20
19
18
22
frazzled (-)
+
+Sems-1aFL
+Sema-1aΔ31-60
+Sema-1aΔC
sema-1a (-), frazzled (-)

Figure 2.7. The cytoplasmic domain of Sema-1a is required to promote midline crossing indicating Sema-1a promotes midline crossing through reverse signaling.

(A–F) Stage 16 embryos of the indicated genotypes stained with anti-HRP antibodies. Arrowheads indicate thin/defective commissures, arrows indicate missing commissures and asterisks indicate rescued commissures. (A) Commissure formation is impaired in *sema-1a, fra* double mutants and 65% of commissures are absent. (B) Pan-neural expression of full length Sema-1a with elavGal4 can partially rescue crossing defects in *sema-1a, fra* double mutants reducing the number of missing commissures to 25%. (C) A Sema-1a transgene lacking a small region of the cytoplasmic domain (from aa31-60) does not rescue the midline crossing phenotype as well as wild type, suggesting this region is important for promoting midline crossing. (D) A Sema-1a transgene lacking the cytoplasmic domain fails to rescue the crossing defects and commissure formation is not significantly different from double mutants. (E) Histogram quantifies commissural defects as absent (black bar), thin/defective (dark gray) or wild-type (light grey) in the genotypes shown in (A–F). Data are represented as mean+SEM. n, number of embryos scored for each genotype. Significance was assessed by multiple comparisons using the Student's t-test (****$p<0.0001$).

Figure 2.8

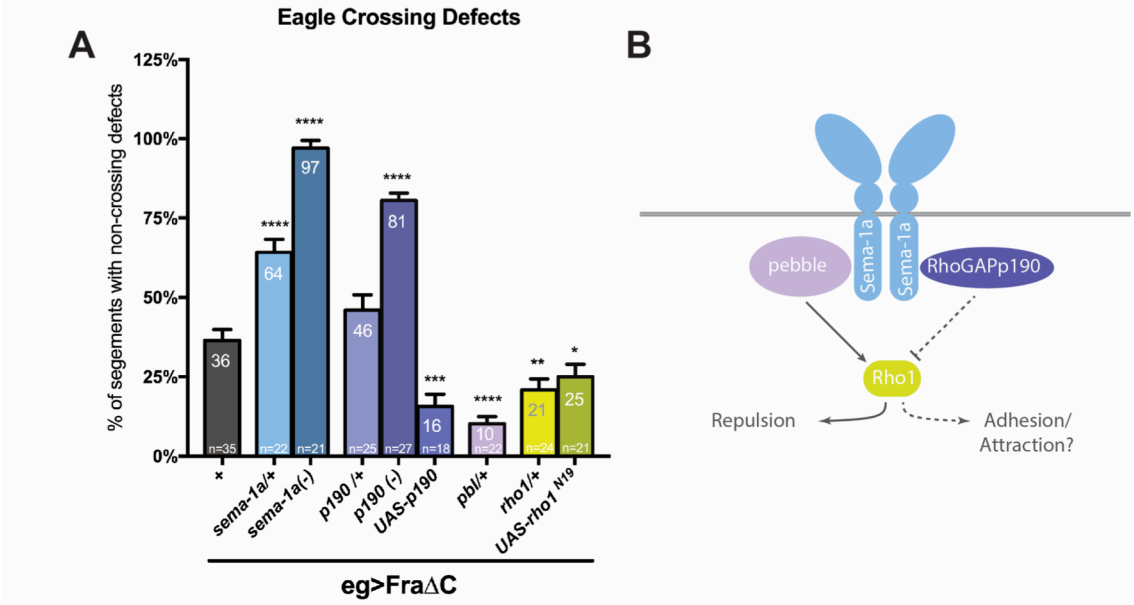

Eagle Crossing Defects
A
% of segements with non-crossing defects
125%
100%
75%
50%
25%
0%
36
64
97
46
81
16
10
21
25
n=35
n=22
n=21
n=25
n=27
n=18
n=22
n=24
n=21
****
****
****
***
****
**
*
+
sema-1a/+
sema-1a(-)
p190 /+
p190 (-)
UAS-p190
pbl/+
rho1/+
UAS-rho1 N19
eg>FraΔC
B
pebble
Sema-1a
Sema-1a
RhoGAPp190
Rho1
Repulsion
Adhesion/
Attraction?

Figure 2.8 *RhoGAPp190*, but not *Pebble*, significantly enhances crossing defects in the Fra∆C background

Histogram quantifies EW midline crossing defects in the Fra∆C screening background. Heterozygosity for *RhoGAPp190* does not show a significant enhancement in crossing defects, however, *RhoGAPp190* nulls do strongly enhance these defects (81%). *pebble* heterozygotes significantly suppressed these defects (10%). In addition, heterozygosity for *rho1* or expression of a *Rho1* dominant negative also suppress the Fra∆C phenotype. Data are represented as mean+SEM. n, number of embryos scored for each genotype. Significance was assessed by multiple comparisons using the Student's t-test (****p<0.0001, ***p<0.001, **p<0.01, *p<0.05). (B) Model of functional responses of Sema-1a reverse signaling through its downstream effectors.

Figure 2.9.

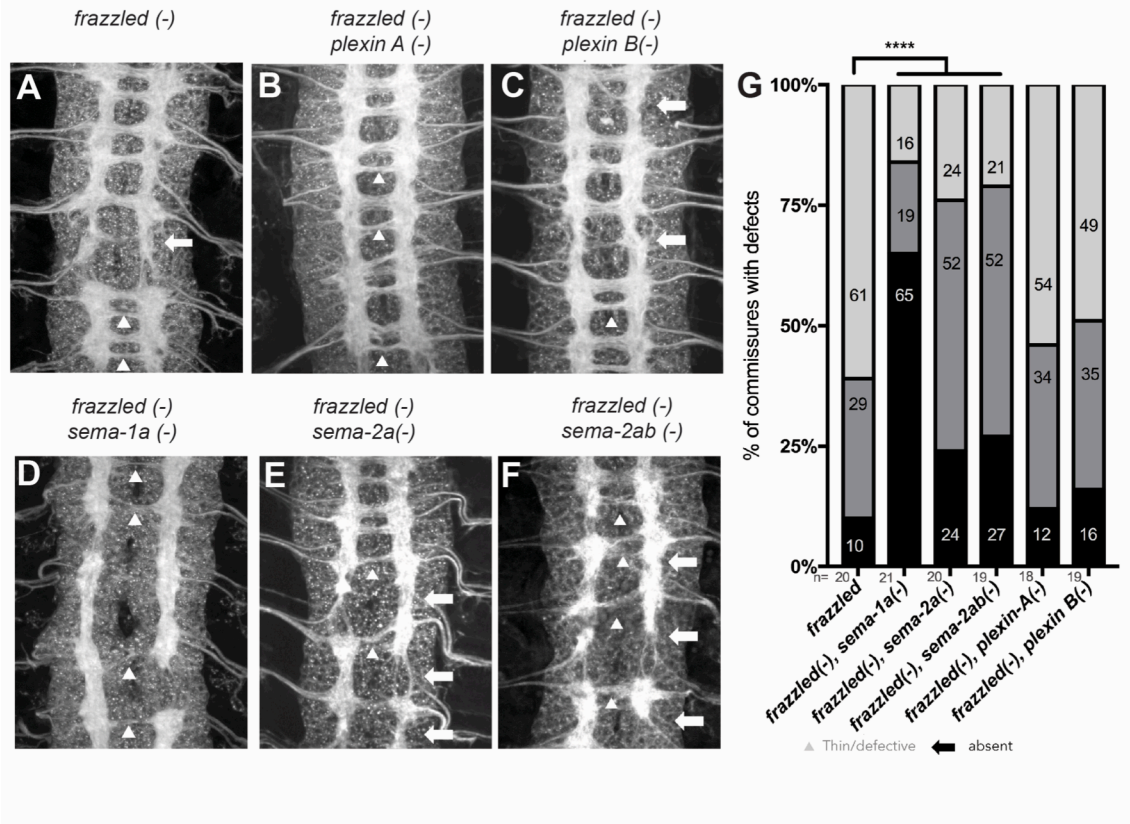

frazzled (-)
frazzled (-)
plexin A (-)
frazzled (-)
plexin B(-)
A
B
C
frazzled (-)
sema-1a (-)
frazzled (-)
sema-2a(-)
frazzled (-)
sema-2ab (-)
D
E
F
G
****
100%
75%
50%
25%
0%
% of commissures with defects
61
29
10
16
19
65
24
52
24
21
52
27
54
34
12
49
35
16
n= 20
21
20
19
18
19
frazzled
frazzled(-), sema-1a(-)
frazzled(-), sema-2a(-)
frazzled(-), sema-2ab(-)
frazzled(-), plexin-A(-)
frazzled(-), plexin B(-)
Thin/defective
absent

Figure 2.9. *Sema-2a* significantly enhances crossing defects in *frazzled* mutants while *plexins* do not

(A–F) Stage 16 embryos of the indicated genotypes stained with anti-HRP antibodies. Arrowheads indicate thin/defective commissures and arrows indicate missing commissures. (A) *fra* (*fra3/fra4*) mutants show thin (10%) and occasionally missing commissures (29%). (B) *fra; plexinA* *(plexA^{EY16548}/plexA^{EY16548})* double mutants resemble *fra* single mutants with 12% absent, 32% thin/defective and 54% wild-type commissures. (C) *fra; plexin B* *(plexB^{KG00878}/plexB^{KG00878})* double mutants also show no significant enhancement of the *fra* single mutants with 16% absent, 35% thin/defective and 49% wild-type commissures. (D) Embryos mutant for s*ema-1a* and *fra* display severe commissural defects. (E) Loss of *sema-2a* significantly worsens the crossing defects of *fra* single mutants with 24% absent, 52% thin/defective and only 24% wild-type commissures. (F) Triple mutants lacking *fra, sema2a* and *sema-2b* are not significantly different from the *fra, sema-2a* double mutants (G) Histogram quantifies commissural defects as absent (black bar), thin/defective (dark gray) or wild-type (light grey) in the genotypes shown in (A–F). Data are represented as mean+SEM. n, number of embryos scored for each genotype. Significance was assessed by multiple comparisons using the Student's t-test(****p<0.0001).

Figure 2.10

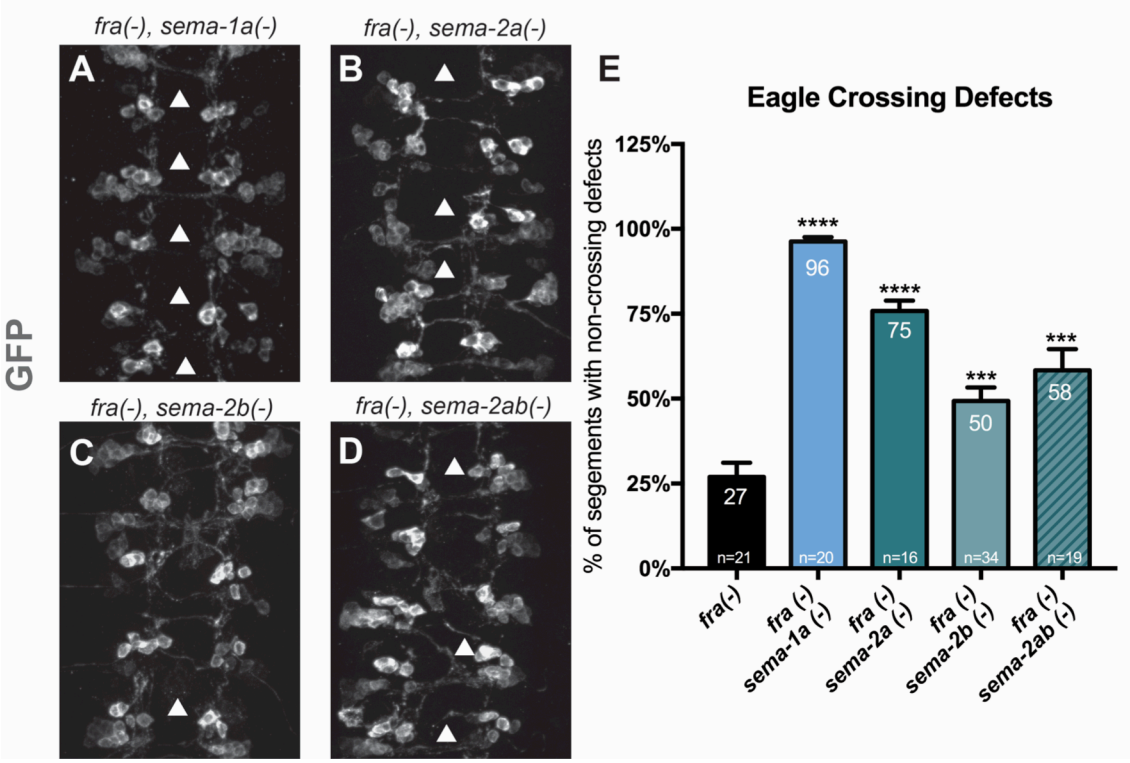

fra(-), sema-1a(-)
fra(-), sema-2a(-)
A
B
GFP
fra(-), sema-2b(-)
fra(-), sema-2ab(-)
C
D
E
Eagle Crossing Defects
% of segements with non-crossing defects
125%
100%
75%
50%
25%
0%
****
96
****
75
***
50
***
58
27
n=21
n=20
n=16
n=34
n=19
fra(-)
fra (-) sema-1a (-)
fra (-) sema-2a (-)
fra (-) sema-2b (-)
fra (-) sema-2ab (-)

Figure 2.10. The secreted *sema-2s* enhance crossing defects in *fra* mutants

(A–D) Stage 15–16 embryos of the indicated genotypes carrying eg-GAL4, and UAS-taumycGFP transgenes, stained with anti-GFP antibodies. Anti-GFP labels cell bodies and axons of the eagle neurons (EG and EW) in these embryos. Arrowheads indicate segments with non-crossing EW axons. (A) *fra, sema-1a* double mutants display strong EW crossing defects (arrowheads 97%). (B) Embryos mutant for both *fra* and *sema-2a* show increased crossing defects (75%) when compared to *fra* single mutants. (C*) fra, sema-2b* double mutants also show a significant increase in EW crossing defects (50%) suggesting *sema-2b* also promotes midline crossing. (D) Triple mutants exhibit elevated EW crossing defects (58%). (F) Histogram quantifies EW midline crossing defects in the genotypes shown in (A–D). Data are represented as mean+SEM. n, number of embryos scored for each genotype. Significance was assessed by multiple comparisons using the Student's t-test (****$p<0.0001$, ***$p<0.001$).

Figure 2.11

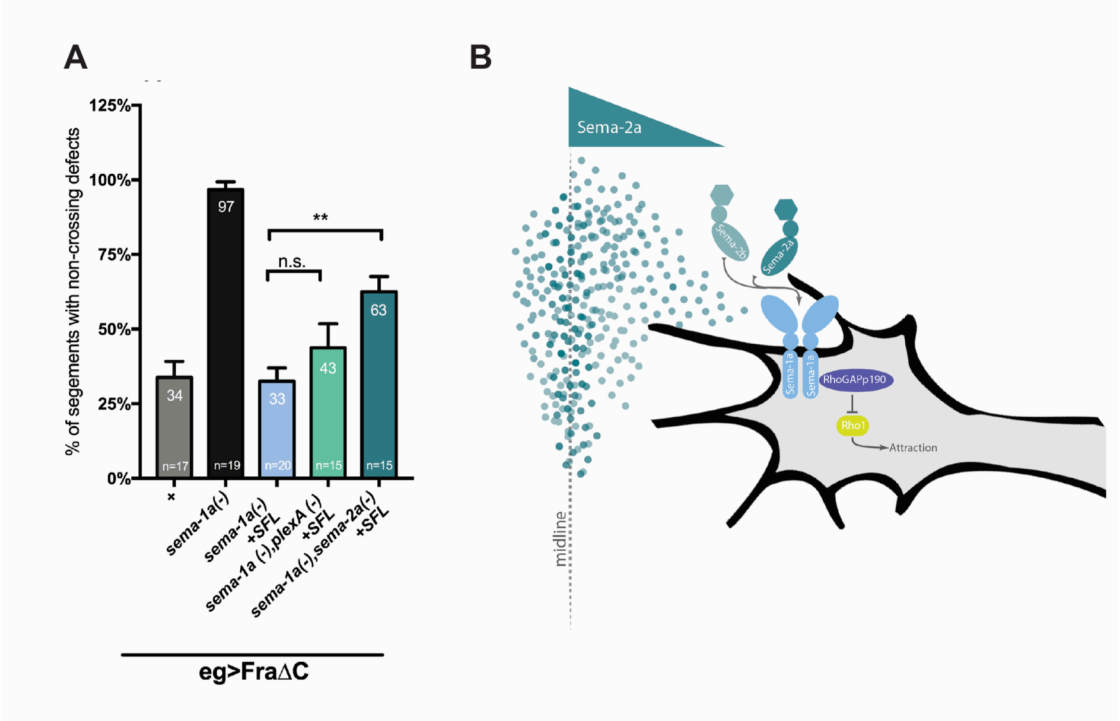

A
B
% of segments with non-crossing defects
125%
100%
75%
50%
25%
0%
97
34
33
43
63
n=17
n=19
n=20
n=15
n=15
**
n.s.
+
sema-1a(-)
sema-1a(-) +SFL
sema-1a (-),plexA (-) +SFL
sema-1a(-),sema-2a(-) +SFL
eg>FraΔC
Sema-2a
midline
Sema-2b
Sema-2a
Sema-1a
Sema-1b
RhoGAPp190
Rho1
Attraction

Figure 2.11 Sema-2a is required for Sema-1a mediated midline crossing

(A) Histogram quantifies EW midline crossing defects in *sema-1a* null mutants carrying the transgenes for egGal4 and UAS-Fra∆C. This background shows strong EW crossing defects (97%) that can be rescue cell autonomously when full length Sema-1a is expressed selectively in eagle neurons (33%). In the absence of *plexA* this rescue is not significantly reduced (43%). However, loss of *sema-2a* significantly suppresses this rescue and embryos still exhibit severe crossing defects (63%) suggesting that *sema-2a* is required for *sema-1a* mediated midline crossing. Data are represented as mean+SEM. n, number of embryos scored for each genotype. Significance was assessed by multiple comparisons using the Student's t-test (**p<0.01). (B) Model of Sema-1a mediated midline crossing.

CHAPTER 3: CONCLUSIONS AND FUTURE DIRECTIONS

3.1 Introduction

This work has demonstrated that Sema-1a reverse signaling is a novel pathway for promoting midline crossing. Yet many questions still remain. First, I will discuss the main conclusions and the broad reaching questions that we hope to work towards answering. Next, I will outline outstanding questions and the experiments underway to address them. With future experiments we hope to shed light on what intrinsic and extrinsic factors regulate the distinct outputs of Sema-1a reverse signaling generated in each biological context. With more information we hope to better understand how similar pathways impinge on each other to promote discreet outputs. As semaphorin signaling has been implicated in a number of diseases this detailed understanding may help develop therapies targeted at specific semaphorin signaling cascades that influence cancer, immune system dysfunction or nerve regeneration.

3.2 Additional Pathways for Midline Crossing

We have found that Sema-1a reverse signaling is independent of Netrin/Frazzled chemoattraction and represents a novel pathway to promote midline crossing. We were able to uncover this alternative pathway through a very targeted approach in a sensitized background. We hope to uncover still more pathways through continued screening; however, this screen is labor intensive and there are limitations to its sensitivity. Sema-1a was an unlikely candidate since *sema-*

1a mutants lacked any overt defects in midline crossing. It is very likely that additional alternative pathways will harbor ever more subtle phenotypes. For such a fundamental aspect of development, it is not surprising that there would be multiple layers of redundancy. It is unclear how many different pathways may be contributing to midline crossing and if they act in discrete cell types. Therefore, it would be beneficial to develop new tools that allow more precise control at these early stages of development. Many of the high resolution sparse labeling techniques used later in development are inadequate for embryonic investigations. Large-scale generation of promoter based Gal4 lines are underway and these will prove to be instrumental in the future. Our screen has identified a number of genes on chromosome two, but an adapted version has been initiated on chromosome three. Hopefully we will be able to fill in the gaps in our knowledge for existing pathways while also discovering new pro-crossing pathways. With this knowledge, we can ultimately work towards understanding how multiple signaling cascades are integrated to mediate axonal responses.

3.3 Sema Reverse Signaling: More common than we think?

Sema-1a, Frazzled double mutants reveal strong commissural defects that would suggest the Sema-1a reverse signaling is broadly used to promote midline crossing; Sema-1a is also expressed by a large population of neurons within the CNS. Taken together, it is tempting to speculate that Sema-1a reverse signaling is acting in all commissural neurons to promote crossing. In contrast, Sema-1a

single mutants display no gross defects in commissure formation. Could this signaling pathway be restricted to a specific subset of pioneering neurons? Ablation studies suggested that Sema-1a positive neurons could be classified into two large subsets: the HB9+ motor neurons and the GAD+ Gabaergic interneurons. Considering the motor neurons utilize Sema-1a forward and reverse signaling later in pathfinding, it would be easy to presume they also use it to cross the midline. It would be interesting to see if there is a commissural defect in Sema-1a mutants when examining either of these selective neuronal subsets. Notably, the eagle interneurons used through out this study are serotonergic interneurons that we empirically determined to be Sema-1a positive, therefore these ablation studies are not exhaustive and likely reflect those neural populations that express the highest levels of Sema-1a. Understanding which cells require Sema-1a reverse signaling for crossing and are sensitive to the loss of Sema-1a would help identify intrinsic properties that allow for Sema-1a reverse signaling and the regulation of its distinct outputs. Furthermore, the ability to target semaphorin reverse signaling specifically will make it easier to address whether this pathway is conserved in the vertebrate midline. The midline may be one of many tissues where Sema-1a reverse signaling is masked in some way. The clearest examples of Sema-1a reverse signaling have been identified with single cell resolution. Taken together, this may suggest that more careful examination will be required to capture the full extent of Sema-1a reverse signaling in other systems.

3.4 Regulating Distinct Forward and Reverse Signaling pathways

We were able to identify a context where Sema-1a forward signaling and reverse signaling we clearly separated. However, it is unclear how this separation is regulated and what changes may occur upon reaching the midline. Vertebrate literature suggests that Sema/Plexin forward signaling is important post-crossing and Plexin is highly regulated to prevent premature signaling; perhaps, this is also true in *Drosophila* (Charoy et al., 2012; Nawabi et al., 2010; Zou et al., 2000). It is possible that Sema-1a reverse signaling occurs independently from forward signaling only because there are other mechanisms for silencing forward signaling until after crossing the midline. A number of negative regulators for Sema-1a forward signaling have already been identified (Terman and Kolodkin, 2004; Yang and Terman, 2012). This might also suggest that Sema-1a reverse signaling might be modified upon crossing, as Plexins may no longer be negatively regulated. As an intermediate target the midline offers an opportunity for changes in responsiveness and it would be very interesting to test further how Sema-1a reverse signaling informs axons to change their responses at the midline. According to findings in the chick spinal cord, semaphorin reverse signaling is also important for post-crossing navigation, through Sema6B and its PlexinA receptors (Plexa2 and PlexA4) (Andermatt et al., 2014). This would imply that forward and reverse signaling may function simultaneously when they produce the same response much like they do during heart chamber development (Toyofuku et al., 2004b) or in *Drosophila* motor neurons (Jeong et

al., 2012). It will be very illuminating if future studies could decipher how forward and reverse signaling pathways are segregated and/or integrated. Some insights are available from the Eph/Ephrin or Tumor Necrosis Factor (TNF) signaling pathways that are also known to function bi-directionally (Davy and Soriano, 2005; Eissner et al., 2004). It would be interesting if any of the mechanisms identified in these bidirectional pathways also apply to Sema/Plexin signaling. Futile *cis* interactions between Sema/Plexin have already been uncovered in the hippocampus (Suto et al., 2007) and dorsal root ganglion(Haklai-Topper et al., 2010). Perhaps subcellular localization to lipid rafts, which has been identified in Eph/Ephrin signaling, will also be important for Sema/Plexin bi-directional signaling.

3.5 Future Directions

Identify components of the ligand and receptor complex

Although semaphorins traditionally bind plexin receptors there are a number of documented cases where semaphorins bind other proteins. We have found that Sema-1a meditates its midline crossing functions through a non-canonical signaling partner, the secreted Sema-2s. This interaction has been previously identified in the olfactory system where it was found to prepattern the olfactory bulb (Sweeney et al., 2011). This binding is likely to be indirect since direct physical interactions could not be demonstrated. The interaction between Sema-1a and the Sema-2s may involve an intermediate protein to act as an adaptor or

co-receptor. It is clear that purified Sema-1a ectodomain can bind to tissue, both neuronal and non-neuronal, when misexpressing secreted or membrane-tethered Sema-2a (Sweeney et al., 2011). The fact that the Sema-1a ectodomain was purified suggests that any intermediate protein is less likely to act as a co-receptor in *cis* and more likely to function in *trans*. Further, the evidence that binding can occur *in vivo* in multiple tissues suggest that the intermediates should be broadly expressed and are not strictly neuronal. To identify potential intermediates we could perform co-immunoprecipitation experiments with embryo lysates from embryos expressing a tagged Sema-1a in eagle neurons. Proteins could be identified though mass spectroscopy and physical interactions validated through cell overlay assays. These proteins would then be tested for genetic interactions in the screening background and with *frazzled* mutants.

In the olfactory system, Sema-1a reverse signaling produces a repulsive response while an attractive response best reconciles our findings. How could the same receptor-ligand pair produce differing outputs? There may be more than one intermediate that can influence the directional output similar to what is found with Sema5A and Heparin Sulfate Proteoglycans (HSPGs) or Chondroitin Sulfate Proteoglycans (CSPGs). Sema5A induces an attractive response when bound to HSPGs while CSPG binding induces a repulsive response (Kantor et al., 2004). In fact, Syndecan, an HSPG found in the *Drosophila* ventral nerve cord, has also been identified in our screen to promote midline crossing. Syndecan is also required for correct photoreceptor targeting and shows defects that resemble Sema-1a in the retina (Rawson et al., 2005). This is compelling

since these defects are attributed to Sema-1a reverse signaling, yet Plexin A is the functional ligand in this context (Cafferty et al., 2006; Hsieh et al., 2014; Yu et al., 2010). Additionally, Perlecan, a secreted HSPG, has been found to facilitate Sema-1a forward signaling in motor neurons (Cho et al., 2012). Initial attempts to analyze *syndecan, frazzled* double mutants revealed no significant enhancement of overall commissural defects (data not shown). However, these embryos we not evaluated specifically in the eagle neurons, which is more sensitive and quantifiable. Considering, our findings with Sema-2b, this might prove more informative and may warrant re-examination. Furthermore, it is possible that there is compensation from other HSPGs so these should also be tested in the screening background and in combination with Syndecan.

Determine factors regulating Sema-1a signaling

Sema-1a reverse signaling has been demonstrated to produce multiple signaling outputs and it is unknown how these conflicting responses are regulated. The ability for Sema-1a to bind and signal through effectors that regulate Rho1 in opposing ways demonstrates how this might be accomplished. However, it is unclear how it is determined which effector is functional at which time. The Src family kinases were previously identified as negative regulators of commissural guidance (O'Donnell and Bashaw, 2013). Interesting this src function is independent of Netrin/Frazzled chemoattraction and thus, must be negatively regulating a parallel pathway for midline crossing. The Sema-1a reverse

signaling pathway we uncovered is a very likely candidate. Src is a highly conserved negative regulator of RhoGAPp190 (Brouns et al., 2001; Roof et al., 2000) . This would be consistent with a model where Src antagonizes midline crossing by negatively regulating Sema-1a reverse signaling through phosphorylation of RhoGAPp190. We have started to test this model genetically by evaluating src mutants in the screening background where they have been found to suppress crossing defects. We have also found that heterozygosity for *src* suppresses defects in *frazzled* hypomorphs, but not when *sema-1a* is mutant. This would indicate that the suppressive effect of *src* loss of function is dependent on Sema-1a. This experiment will be more convincing if repeated with complete loss of *src*. In the future we would like to build upon these observations and test if loss of *src* enhances the ability for Sema-1a to rescue midline crossing. Also, a direct interaction with Src and RhoGAPp190 in the eagle neurons should be demonstrated biochemically. We have demonstrated that overexpression of RhoGAP190 can rescue crossing defects in the screening background, but we would like to test if a better rescue is achieved with a variation of RhoGAPp190 that cannot be phosphorylated by src. In addition, another gene that was identified through the screen, *brain tumor* (*brat*), negatively regulates Src by sequestering *src* mRNA (Marchetti et al., 2014). This further supports the notion that Src antagonizes midline crossing and needs to be negatively regulated in order to promote crossing. Moreover, this type of regulation allows for tight temporal and spatial control, which might explain how Sema-1a reverse signaling might be compartmentalized within a neuron.

We have constructed a model of Sema-1a reverse signaling at the midline that suggests attraction to the midline in response to Sema2s. We cannot rule out adhesion, which is also a common outcome of RhoGAPp190. We would ideally be able to assay the Sema-1a mediated response in a more direct manner. We initially attempted misexpression of Sema-2s from different sources around eagle neurons in order to identify a response within eagle neurons, but this failed to produce any phenotype. This is likely do to the fact that Sema-1a reverse signaling is best targeted when Netrin/Frazzled signaling is also reduced. We would like to repeat these experiments in a *frazzled* heterozygous or hypomorphic background.

In order to discern the necessary source of the Sema-2s, we are currently trying to establish a rescue assay. Expression of UAS-Sema-2a should be able to rescue the crossing defects in the *frazzled, sema-2a* double mutant, at least partially. In this way we hope to determine whether Sema-2a is functioning in a directional or permissive manner. If our model is correct, and Sema-2a functions to promote crossing through attraction to the midline then crossing defects should be rescued when Sema-2a is expressed in the midline glia. However, pan-neural expression should be able to rescue crossing if Sema-2a is functioning as a permissive/non-instructive cue.

Although an *in vivo* assay for functional responses would be ideal, an *in vitro* approach would also provide valuable insights. The combination of *Drosophila's* genetic tools with primary cell culture would allow us to assay functional

responses to purified proteins at specified locations and concentrations. There has been much progress in developing a system to culture primary *Drosophila* neurons (Bai et al., 2009; Küppers-Munther et al., 2004; Prokop and Küppers-munther, 2012). Coupled with the advances in nanofabrication for microfluidic devices and microcontact printing, axonal responses can be assayed directly (Dupin et al., 2013; Lang et al., 2008; Millet and Gillette, 2012; von Philipsborn et al., 2006). To better understand the Sema-1a mechanism for midline crossing in eagle neurons and how reverse signaling changes outputs in different contexts, we would like to establish a primary culture assay. This method would allow us to determine how eagle neurons respond to a direct source of Sema-2a. This is a major undertaking and we are currently still experimenting with culturing conditions.

In conclusion, future studies are needed to further the understanding of the molecular components of the Sema-1a reverse signaling pathway in the context of midline crossing and in other systems. Uncovering the mechanisms leading to such diverse signaling outputs will also advance the knowledge of axon guidance as a whole.

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