



HHS Public Access

Author manuscript

Cell Rep. Author manuscript; available in PMC 2017 January 22.

Published in final edited form as:

Cell Rep. 2017 January 03; 18(1): 174–184. doi:10.1016/j.celrep.2016.12.027.

Sema-1a reverse signaling promotes midline crossing in response to secreted Semaphorins

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SUMMARY

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 an important 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 independently of 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 to promote attraction to the midline. These findings suggest that Sema-1a reverse signaling can elicit distinct axonal responses depending on differential engagement of distinct ligands and signaling effectors.

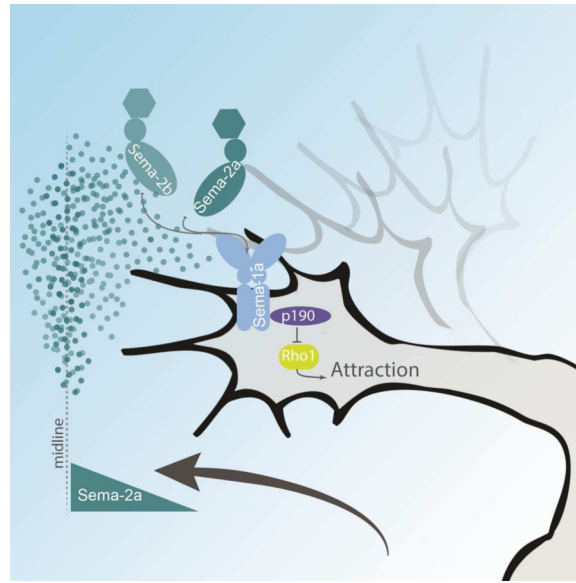
Graphical abstract

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Author contributions

MHF, GJB, Conception and design, Acquisition of data, Analysis and interpretation of data, drafting or revising the article. ER, Acquisition of data, Analysis and interpretation of data.



Keywords

Axon Guidance; Midline; Semaphorin; *Drosophila*; Reverse Signaling; Netrin; DCC; chemoattraction; growth cone

INTRODUCTION

The ability to coordinate the right and left sides of the body relies on neural circuits at the midline. Disruptions to these circuits during development often result in an inability to coordinate movement. 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). Mutations in DCC are associated with movement disorders in zebrafish, mice and humans (Jain et al., 2014; Rabe Bernhardt et al., 2012; Srour et al., 2010). Despite the conserved role of Netrin and Fra/DCC in midline axon guidance, many axons still cross the midline in *netrinAB* double mutants (hereafter referred to as *NetAB*) and *fra* mutants in *Drosophila*, suggesting that additional pathways promote midline crossing (Kolodziej et al., 1996; Mitchell et al., 1996). Studies in vertebrate systems have identified additional factors 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., 2015; 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 (O'Donnell and Bashaw, 2013a) and identified the transmembrane Semaphorin, Sema-1a, as an important

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 (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., 1998; Yu et al., 2000). A 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). Still, a role for Sema-1a in commissure formation has never been explored. In vertebrates, secreted Semas act at the midline to repel crossing axons from the floor plate (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. Sema-1a reverse signaling can be engaged by PlexA binding, as observed in the visual system and the giant fiber circuit, or potentially through indirect interactions with other secreted Semas as suggested in the olfactory system (Cafferty et al., 2006; Godenschwege et al., 2006; Komiyama et al., 2007; Pecot et al., 2013; Sweeney et al., 2011). 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 independently of Netrin/Frazzled chemoattraction. Sema-1a functions cell autonomously in commissural neurons to promote midline crossing. A region of Sema-1a's cytodomain previously shown to bind Pebble and RhoGAPp190 is required for Sema-1a to promote midline crossing, and we find that RhoGAPp190 and the downregulation of Rho1 are likely to be 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 act as 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 Semas.

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 1A). 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). In *fra* mutants the EW axons fail to cross the midline in 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 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 subtle crossing defects in heterozygous embryos, and thus avoid potential complications from early gene requirement.

A deficiency on the second chromosome, DF(2L)ED623, enhances the Fra C phenotype to 49% (Figure 1G). The enhancer activity in this interval was genetically mapped to *Sema-1a* and a null allele, *sema-1a^{PI}*, fully recapitulates the enhanced EW defects (Figure 1D, G). *Sema-1a* mutants alone show no appreciable crossing defects in eagle neurons; however, in the Fra C screening background, eagle neuron crossing defects are dose dependent and are strongly enhanced when both copies of *sema-1a* are removed (Figure 1E, G). Furthermore, this mutant phenotype can be rescued when full-length *Sema-1a* (*Sema-1aFL*) is restored selectively in the eagle neurons (Figure 1F, G). Close examination of the resulting phenotypes demonstrates that eagle neurons continue to grow ipsilaterally and fail to turn towards the midline. Axons do not exit the CNS or misroute to the anterior commissure. This phenotype suggests that *Sema-1a* does not mediate axon growth; rather, *Sema-1a* is required for the medial turn towards the midline. 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 S1). 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. Reduced midline crossing can be readily observed when the entire axon scaffold is stained with anti-HRP antibodies. In wild-type embryos, thick anterior and posterior commissures form in each segment (Figure 2A). Both *NetAB* and *fra* null mutants display mild crossing defects, which are observed as thin or occasionally missing commissures (Figure 2B and Figure S1). *Sema-1a* null mutants, however, show no significant crossing defects in either the axon scaffold or in eagle neurons (Figure 1G and Figure 2C). If *sema-1a* were functioning in an independent 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 exhibit a striking enhancement in crossing defects compared to *fra* single mutants (total defects: *sema-1a, fra* = 92% vs. *fra* = 40%; Figure 2D and E) as well an increase in the number of missing commissures (missing: *sema-1a, fra* = 68% vs. *fra* = 10%; Figure 2D and E). This phenotype suggests a broad role for *Sema-1a* in commissural guidance and analysis of commissural neuron subsets further supports this idea. Crossing defects are seen in POXN and *sema2b-tmyc* neurons, but the defects in eagle neurons are by far the most severe (data

not shown and Figure S2). Additionally, these defects can only be rescued when both *Sema-1a* and *Fra* are co-expressed in *sema-1a, fra* double mutants indicating they function cooperatively to promote midline crossing (Figure S2). The majority of *sema-1a, fra* double mutant embryos are nearly commissureless, and these defects can be directly attributed to the loss of *sema-1a* since the axon scaffold defects in double mutants can be robustly rescued with pan-neural expression of *Sema-1aFL* (total defects: 56%, missing: 25%; Figure 2F). Importantly, this rescue is only partial since the embryos are still mutant for *fra*. 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 S1).

To further support the argument that *sema-1a* acts independently of the Netrin/*Fra* pathway, we analyzed dominant genetic interactions in commissural neurons. The crossing defects in both *fra* or *NetAB* mutants are significantly increased when a single copy of *sema-1a* is removed (Figure S1). 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 S3). Taken together, this evidence suggests that *Sema-1a* acts independently of 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., 1998). 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 first wanted to know if *Sema-1a* is endogenously expressed in eagle neurons. Antibody staining and *in situ* hybridization techniques suggested expression in eagle neurons, but due to the broad expression of *Sema-1a*, 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 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 in 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 3A). Thus, tissue specific expression of FLP recombinase 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 mosaic expression during the time of midline crossing (Figure 3B). 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 3C'). Visualization of *Sema-1a* protein using the V5 tag reveals a punctate pattern on cell bodies and strong labeling of the axons during midline crossing (Figure 3C''). Interestingly, we see no reductions in *Sema-1a* expression post-crossing (data not shown).

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

Given 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. To address cell autonomy without introducing non-autonomous "follower effects," we used a *sema-1a* mutant expressing the dominant negative Fra receptor (Fra^C) in the eagle neurons. 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 rescue reverse signaling (Jeong et al., 2012). When full-length *Sema-1a* (UAS-*Sema-1a*FL) is restored in this background, eagle neuron crossing defects are reduced from 98% to 26% (Figure 4 and Figure 1), suggesting a cell autonomous requirement. 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 (UAS-*Sema-1a*³¹⁻⁶⁰) carrying a small deletion within the cytoplasmic domain, which removes amino acids 31–60. This 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 4). 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 S4). Notably, *Sema-1a*^C 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-1a*^C transgenes, yet this is not what

we see. 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 *Sema1a*-mediated midline crossing

Pebble RhoGEF (*Pbl*) and RhoGAPp190 (*p190*) have been identified as 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 et al., 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 5); however *p190* zygotic null mutants produce a dramatic increase in crossing defects, similar to *sema-1a* nulls in the same background (81%; Figure 5). Like *sema-1a* mutants, *p190* mutants exhibit no eagle axon crossing defects on their own. The enhancement in the screening background is significantly weaker than what is observed in the *sema-1a* nulls, suggesting the possibility that other downstream effectors may also be involved. In contrast, heterozygosity for *pbl* did not result in an enhancement of crossing defects. Instead, it suppressed these defects to 10% (Figure 5). 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 also suppresses crossing defects. Finally, overexpression of *p190* reduces the number of defects seen in *Fra C* background to 16% of abdominal segments (Figure 5). Taken together, these results are consistent with the hypothesis that *Sema-1a* promotes midline crossing through RhoGAPp190 and the down regulation of Rho1.

The secreted Semas 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 6). These results

strongly suggest that Plexins are not contributing to Sema1a-dependent midline crossing. Furthermore, multiple alleles of *plexA* fail to enhance eagle crossing defects in the screening background (Figure S5) or in *frazzled* mutants (data not shown). 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 6).

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 Semas 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 (Figure S5)(Emerson et al., 2013; Kolodkin et al., 1993; Wu et al., 2011). Sema-2a, however, displays a distinct enrichment at the midline (Figure S5) (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 6). 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 strong as the enhancement seen in *fra, sema-2a* double mutants (75%) (Figure S6). The *fra, sema2ab* triple mutants display defects similar to the double mutants (58%). Since the triple mutant fails to recapitulate the *sema-1a, fra* double mutant, it is likely that the secreted Semas are not the only upstream factors in the Sema-1a pathway.

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 in the absence of Sema-2a (Figure 7). These data indicate that Sema-2a, and not PlexA, contributes to the Sema-1a mediated midline crossing pathway.

DISCUSSION

These data demonstrate that Sema-1a represents an important 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 uncouple the two signaling cascades and determine the mechanism of Sema-1a reverse signaling. We find that specific genetic manipulations at 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 promotes midline crossing

Sema-1a has never before been associated with midline crossing since the null mutants alone show no commissural defects. Eagle neurons in *sema-1a* mutants also show no significant reduction in crossing (Figure 1). 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 (Fra^C), 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 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 clearly reveal an independent function between Fra/Netrin chemoattraction and Sema-1a. Genetic interactions with *robo1*, *slit* double heterozygotes suggest that Sema-1a is unlikely to function as another anti-repulsive mechanism (Figure S3). Additionally, Robo1 protein expression does not appear to be upregulated in *sema-1a* mutants (Figure S3). Taken together, our observations indicate that Sema-1a promotes midline crossing independently of known pathways.

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

Reverse signaling through transmembrane Semas has been demonstrated in both invertebrates and vertebrates, where the class 6 Semas 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., 2004). More recently, studies of semaphorin reverse signaling in neurons have revealed that class 6 Semas may have roles in axon guidance. For example, a recent study in chick 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 Semas to signal in reverse and function as axon guidance

receptors is highly conserved, it had not been previously known whether Sema reverse signaling contributes directly to midline crossing.

RhoGAPP190 mediates Sema-1a reverse signaling to promote midline crossing

In the majority of cases, Semaphorin reverse signaling promotes repulsive guidance in response to Plexin receptors, yet there are attractive signaling outputs and binding partners as well. Two classes of neurons in the *Drosophila* 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). The discovery of competitive downstream effectors (Pbl and RhoGAPP190) with opposing effects on Rho1 begins to explain how Sema-1a reverse signaling could have multiple, and even opposite outputs. It seems likely that there are additional factors that modulate the activity of these Pbl and P190 and impact the ultimate axonal response. For instance, Src family kinases lead to inhibition of p190 activity by phosphorylating p190 within the GTP binding domain (Billuart et al., 2001; Brouns et al., 2001; Roof et al., 2000). Indeed, we have found that src kinases antagonize midline crossing in a Netrin/Frazzled independent fashion (O'Donnell and Bashaw, 2013b), suggesting *src* may modulate Sema-1a reverse signaling through its effect on P190.

Interestingly, the Sema-1a mediated adhesive response uncovered in the photoreceptors is also dependent on the down regulation of Rho1 (Hsieh et al., 2014). However, in the context of photoreceptor axon guidance, adhesion is mediated by FasII, which is not expressed in the commissural eagle neurons. 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; O'Donnell et al., 2009). Complete loss of p190 in the screening background leads to defects that are similar to complete loss of sema-1a, yet significantly less severe (Figure 5). This may indicate the contribution of additional downstream effectors. While the cytoplasmic region between amino acids 31–60 of Sema-1a provides the binding site for Pbl and p190, it also includes a putative Enabled (Ena) binding site (LPQP). This Ena binding site is required for Sema-1a reverse signaling in the giant fiber (Godenschwege et al., 2002); however, Ena does not appear to contribute Sema-1a's activity to promote midline crossing (Figure S7). It is likely that additional context specific signaling effectors await discovery.

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 in mediating 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 7). While an interaction between the *Sema-2s* and *Sema-1a* reverse signaling was initially proposed in the olfactory system, we show the first genetic evidence to directly link the *Sema-2s* to a *Sema-1a* function. Furthermore, the double mutant phenotypes with *fra* demonstrate that the secreted Semas are required for axons to cross the midline (Figure 6 and Figure S6). Surprisingly, compound mutants with *fra* also revealed that the removal of *sema-2a* and *sema-2b* together (*fra, sema-2ab*) fails to fully recapitulate the loss of *sema-1a* (*sema-1a, fra*) in the eagle neurons or the CNS as a whole (Figure 6 and Figure S6). This may suggest the possibility of additional upstream signaling components and a potentially more complex function for *Sema-1a* reverse signaling. We used the *Fra* C screen to test all the identified Semas and Plexins (including *Sema-1b* and *Sema-5c*; data not shown and Figure S7) yet failed to uncover additional enhancers, suggesting that additional factors await discovery. We propose a model where the secreted *Sema2s* act as attractive cues to promote midline crossing as the simplest interpretation of the observed phenotypes. The medial expression of the secreted *Sema2s*, in particular *Sema-2a*, suggests that they signal directional information rather than promote permissive adhesion.

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., 1998). 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.

EXPERIMENTAL PROCEDURES

Genetics

The following *Drosophila* mutant alleles were used: *fra*³, *fra*⁴, *fra*⁶, *NetAB*, *egMZ360* (*eg-GAL4*), *slit*², *robo-1*^{GA285}. The following stocks were from Bloomington: *sema-1a*^{P1}, *plexin A*^{EY16548}, *plexin B*^{KG00878}, *pbP*², and *Rho1*^{72F}. The following stocks were gifts from A. Kolodkin: *sema-2a*^{B65}, *sema-2b*^{C4}, *sema-2ab*^{A15}, *p190*² and the *PlexinA* and *Sema2b* Bac transgenes. The *sema-1a* artificial exon was a gift from L. Zipursky. The following transgenes were used: UAS-*Fra* C, UAS-*sema-1a*FL, UAS-*sema-1a* 31–60, UAS-*sema-1a*ECFC, *UAS-FLP Recombinase*, *UAS-26XLexAopmyrGFP*, *UAS-mycp190*, and *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. See Supplemental Table 1 for a complete list of genotypes for all of the figures.

Immunofluorescence and imaging

Dechorionated, formaldehyde-fixed, methanol devitellinized embryos were fluorescently stained as previously described (Bashaw, 2010b). Live-dissected embryos were stained as previously described (Bashaw, 2010a). The following primary antibodies were used: 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], Mouse anti-Sema2a [DSHB 1:10], 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. 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 a detailed description of the quantification methods, please see the supplemental experimental procedures.

Statistical Analysis

All statistical analysis was performed in GraphPad. Comparisons were made between genotypes using ANOVA. For multiple comparisons, significance was assessed by using a Bonferroni correction.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We would like to thank members of the Bashaw lab past and present for their constructive discussions throughout this work. In particular, we thank Jonathan Levin for help conducting the primary screen and Celine Santiago for help constructing Supplemental Figure 5. We thank Alex Kolodkin, Liqun Luo and Larry Zipursky for providing countless reagents.

Funding

MHF was supported by NIH T32 GM 7517-34, T32 HD007516-15, and F31 NSO86340-01. This work was supported by NSF Grant IOS-1355181, and NIH Grants R01NS-046333 and R01NS-054739 to G.J.B.

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Highlights

- Sema-1a functions independently of Netrin to promote midline crossing
- Sema-1a mediates midline crossing through reverse signaling in commissural neurons
- RhoGAPp190 and the negative regulation of Rho1 is required for midline crossing
- The Secreted Sema2s function as attractive or adhesive midline cues

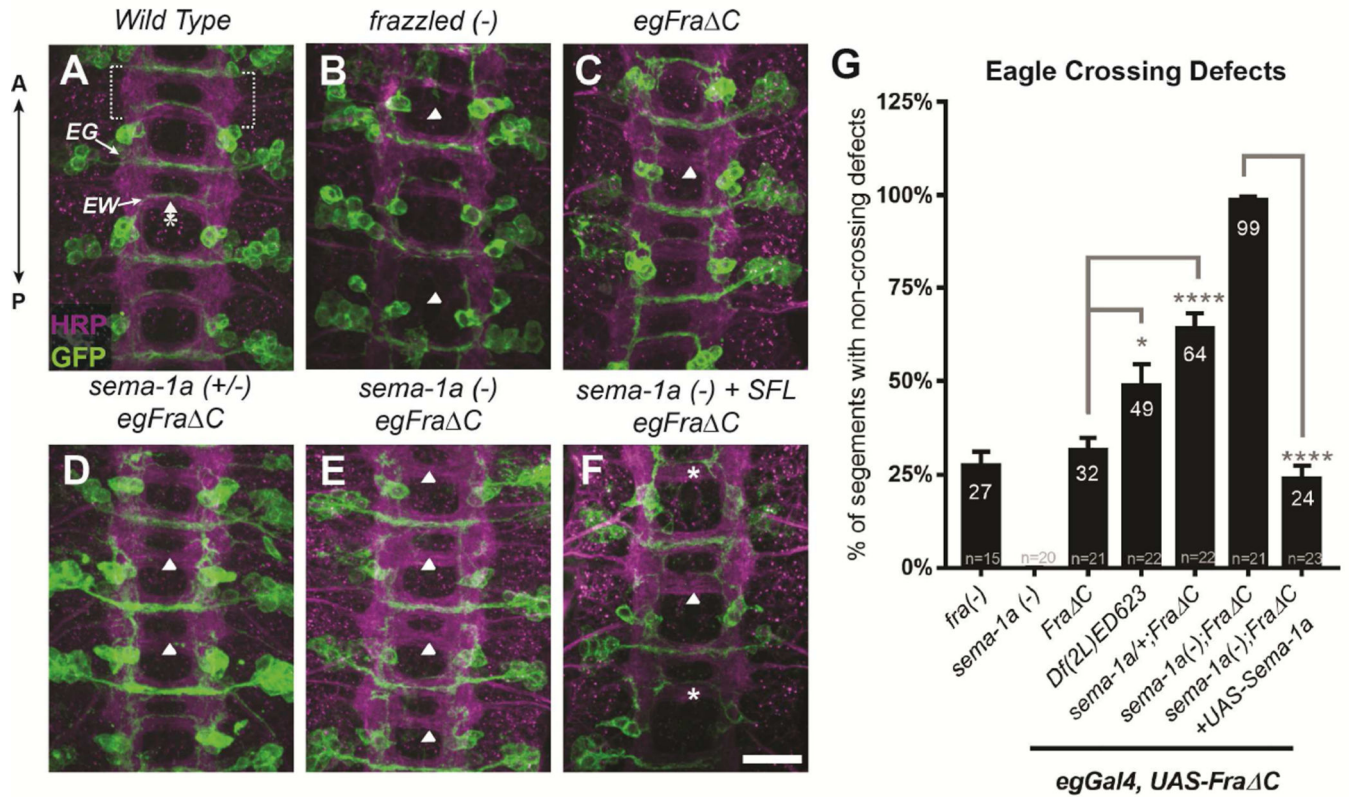


Figure 1. Sema-1a is a positive regulator of midline crossing

(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). Anterior is up in all images. Scale bar represents 15 μ m (F). Dotted lines indicate segment boundaries. EG neurons project through the anterior commissure of each segment while EW neurons project through the posterior commissure. 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) In *fra* mutants EW neurons fail to cross in 27% of segments (arrowheads). (C) Expression of *UAS-Fra C* selectively in eagle neurons produces a *Fra*-like phenotype where EW neurons fail to cross in 32% of segments. (D) Heterozygosity for *sema-1a* dominantly enhances the EW crossing defects in a *Fra C* background to 64%. (E) Complete loss of *sema-1a* further enhances the EW crossing defect to 99% of segments. (F) EW crossing defects in the *sema-1a* null expressing *Fra C* are robustly rescued (99% vs. 24%) when *UAS Sema-1a* is expressed in eagle neurons. (G) Quantification of EW midline crossing defects in the genotypes shown in (B–F) as well as *sema-1a* null mutants. *Df(2L)ED623* is a chromosomal deficiency containing *sema-1a*. Data are presented as mean+SEM. n, number of embryos scored for each genotype. Significance was assessed by multiple comparisons using ANOVA (**** $p < 0.0001$).

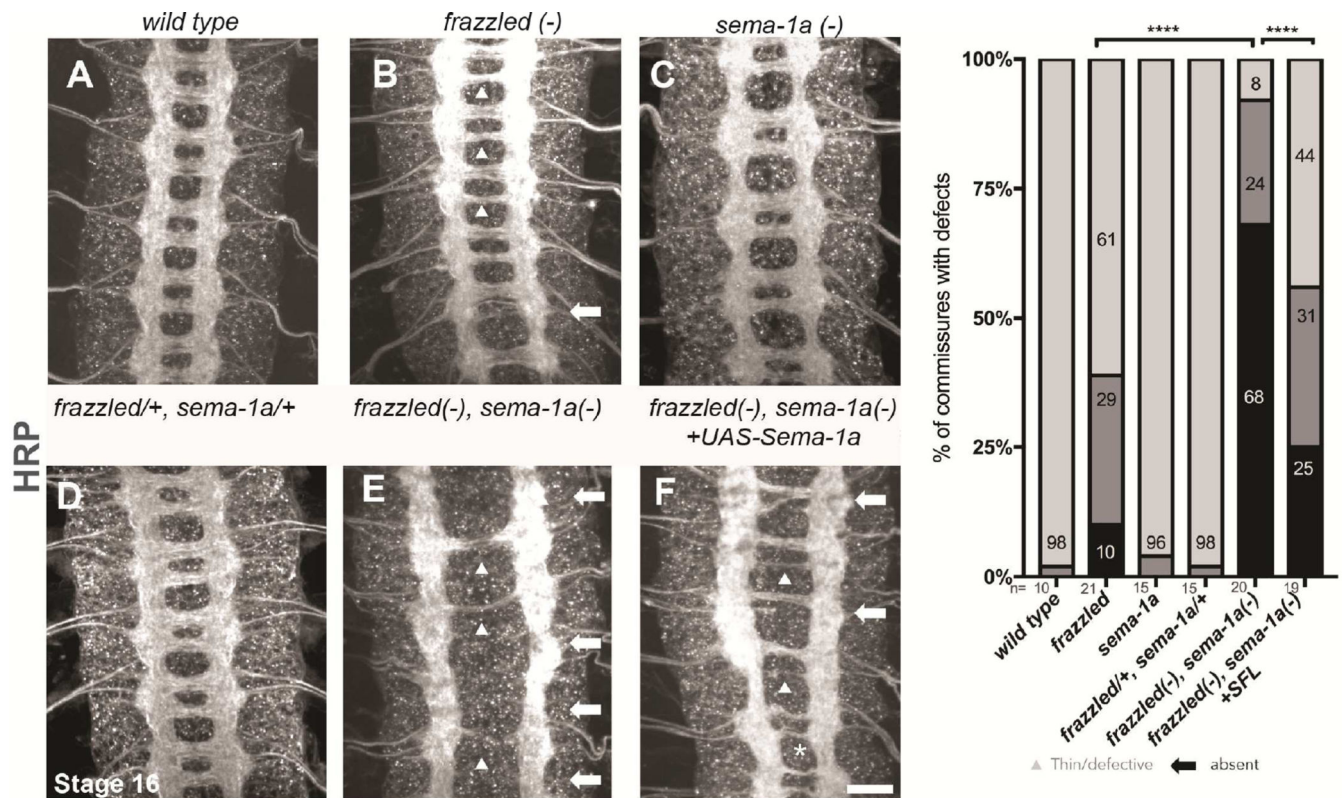


Figure 2. Sema-1a promotes midline crossing independent of *Netrin/fra*
 (A–F) Stage 16 embryos of the indicated genotypes stained with anti-HRP. Arrowheads indicate thin/defective commissures, arrows indicate missing commissures and asterisks indicate rescued commissures. Scale bar represents 15 μ m (F). (A) Thick anterior and posterior commissures are formed as axons cross the midline in 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 *sema-1a* and *fra* appear wild-type. (E) *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) Quantification of 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 ANOVA (****p<0.0001).

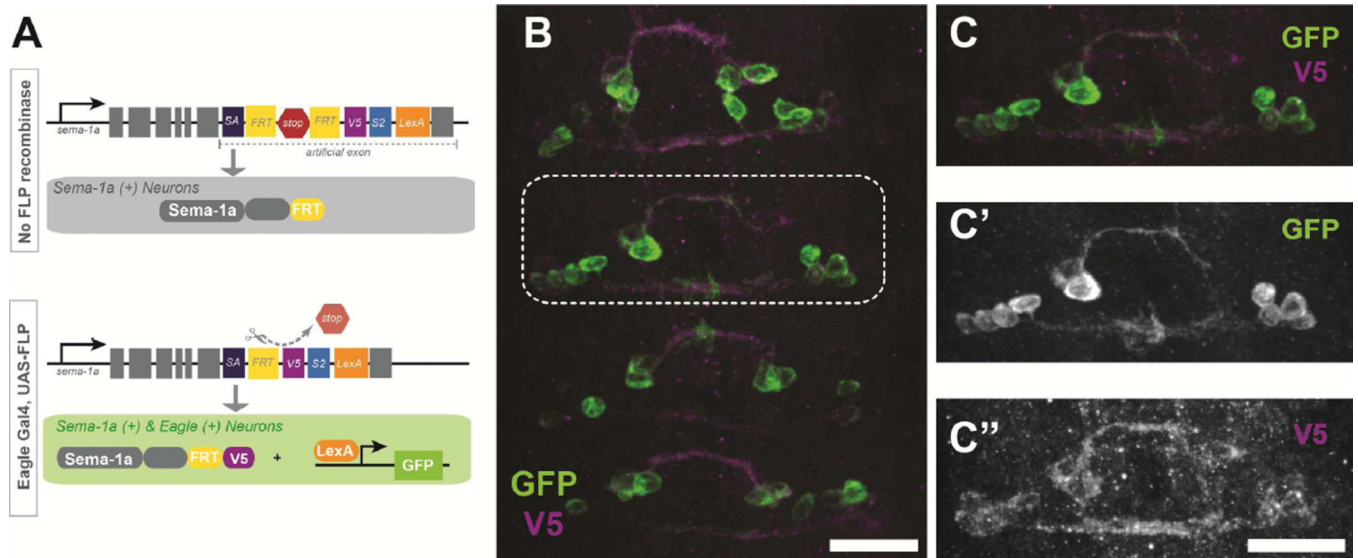


Figure 3. Sema-1a protein is expressed on Eg axons

An artificial exon inserted into the endogenous locus for *sema-1a* allows for tissue specific labeling of endogenous Sema-1a expression. (A) Schematic of sparse labeling strategy adapted from Pecot, et al. 2013. In the presence of FLP recombinase, Sema-1a becomes tagged with a V5 epitope and LexA driven membrane bound GFP labels Sema-1a positive cells. (B–C) An early stage 15 embryo carrying the artificial exon, egGal4, UAS-FLP and LexAop-myrGFP stained with anti-GFP (green) and anti-V5 (magenta) antibodies. (B) Eagle neurons endogenously express Sema-1a during midline crossing. Scale bar represents 15 μ m (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. Scale bar represents 15 μ m

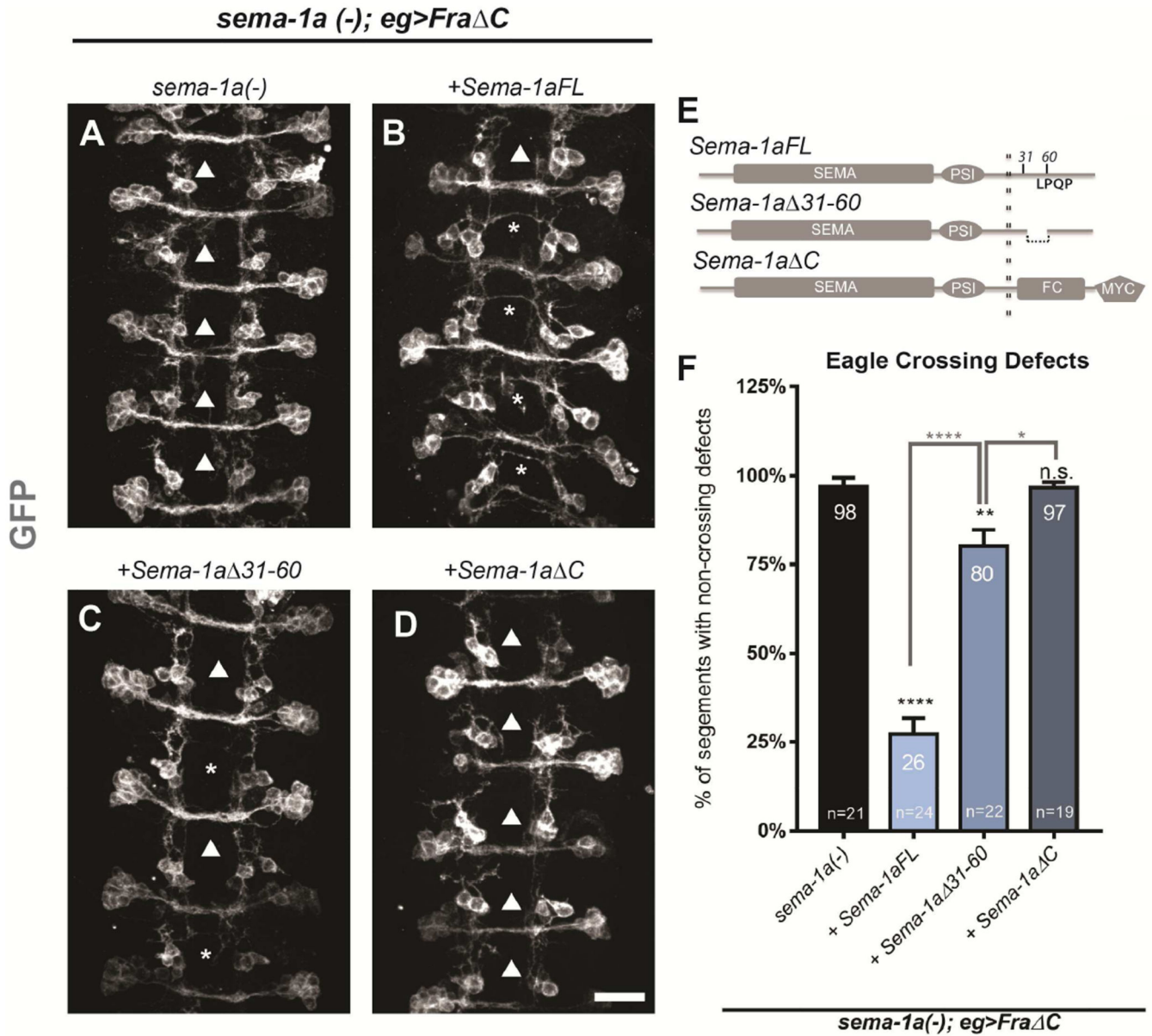


Figure 4. Sema-1a expression rescues midline crossing cell autonomously

(A–D) Stage 15–16 embryos of the indicated genotypes carrying *eg-GAL4*, *UAS-Fra Δ C* and *UAS-CD8 GFP* transgenes, stained with anti-GFP (green) antibodies to reveal cell bodies and axons of the eagle neurons (EG and EW). Arrowheads indicate segments with non-crossing EW axons and asterisks indicate rescued EW crosses. Scale bar represents 15 μ m (D). (A) In *sema-1a* null embryos expressing *Fra Δ C*, EW axons fail to cross the midline in 98% of segments (arrowheads). (B) Expression of a full-length *Sema-1a* transgene 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) rescued the defects to a much lesser extent (80%). (D) Expression of a *Sema-1a* transgene lacking its entire cytoplasmic domain does not significantly rescue crossing defects. (E)

Diagram of transgenic rescue constructs with relevant cytoplasmic regions labeled. (F) Quantification of 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 ANOVA (****p<0.0001).

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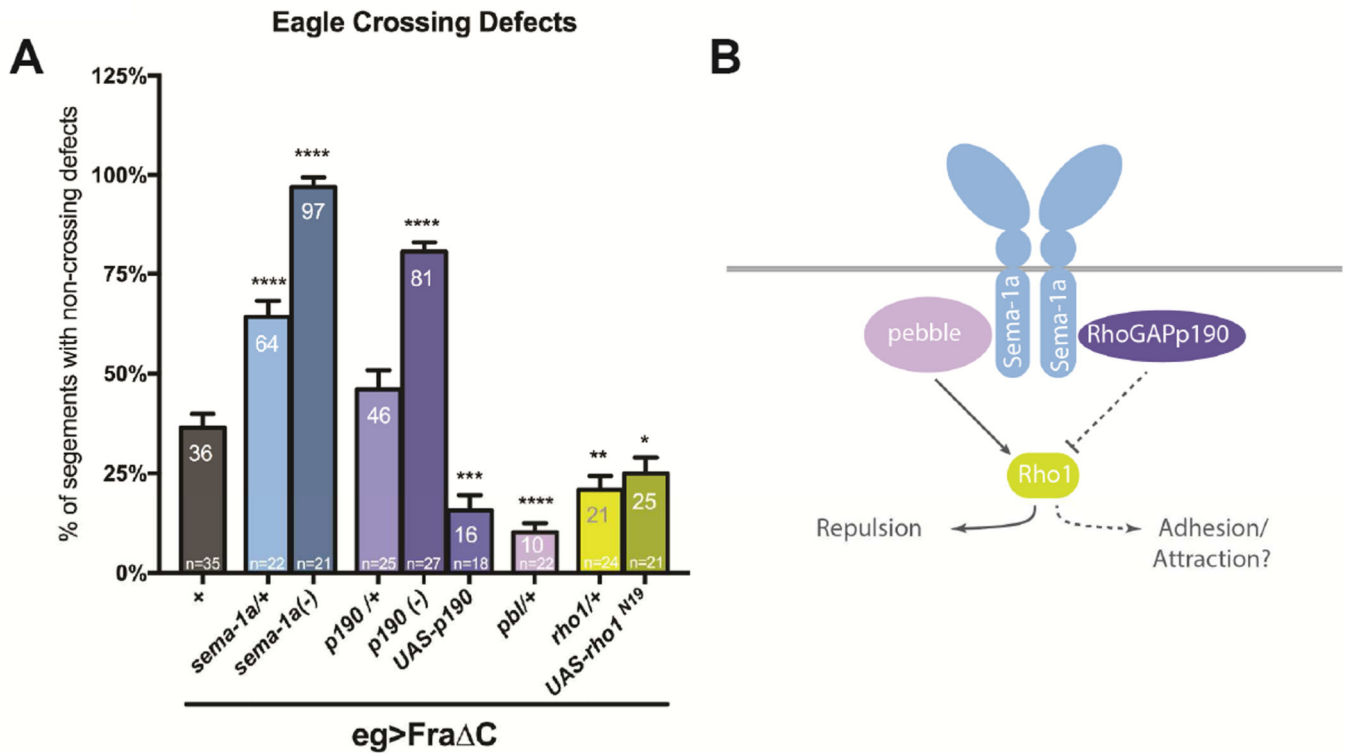


Figure 5. *RhoGAPp190* but not *Pebble* significantly enhances crossing defects in the *Fra C* background

(A) Quantification of 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 suppresses 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 ANOVA (**** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$). (B) Model of functional responses of Sema-1a reverse signaling through its downstream effectors.

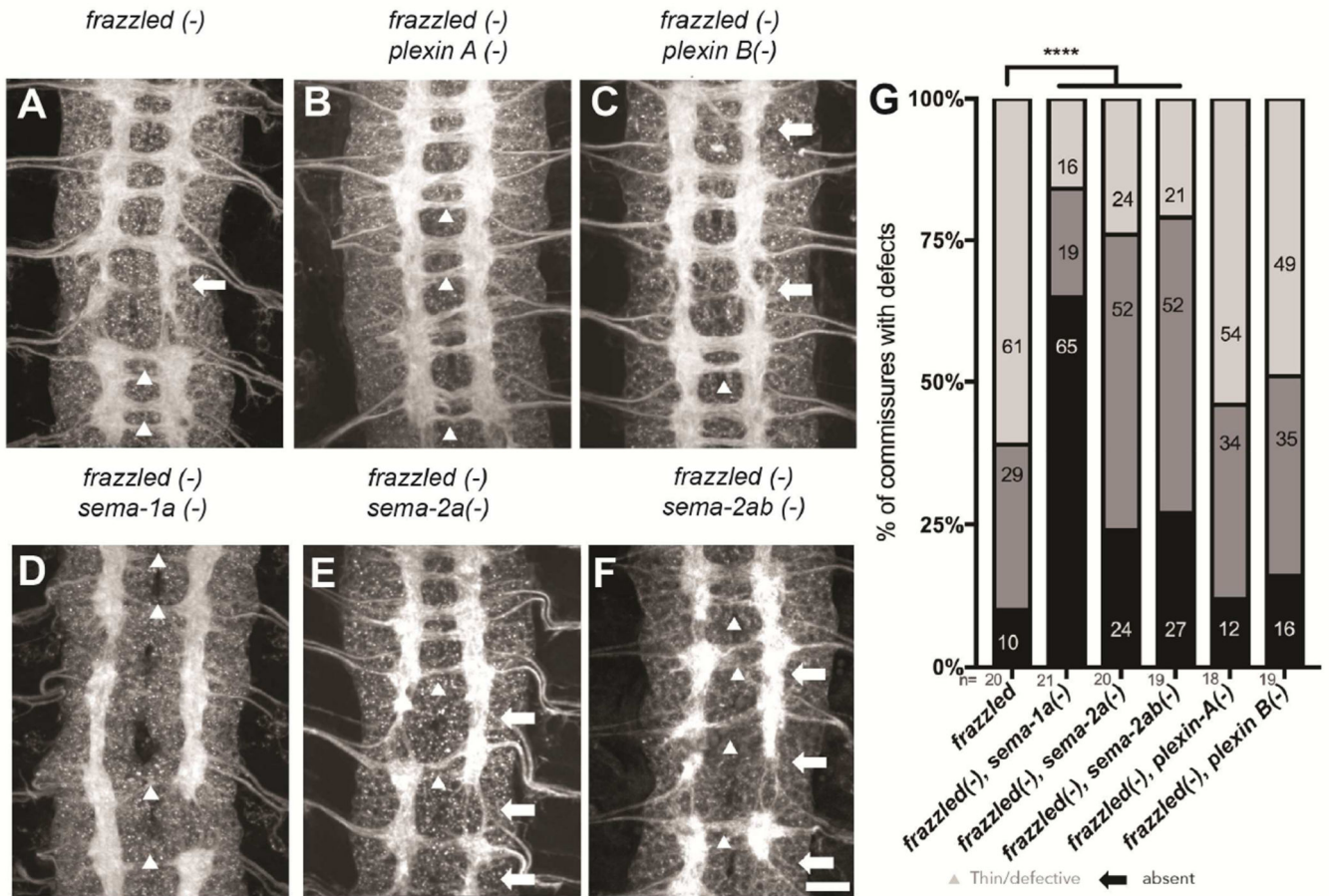


Figure 6. *Sema-2a* significantly enhances crossing defects in *frazzled* mutants while *plexins* do not (A–F) Stage 16 embryos of the indicated genotypes stained with anti-HRP antibodies. Arrowheads indicate thin/defective commissures and arrows indicate missing commissures. Scale bar represents 15µm (F). (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 *sema-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) Quantification of 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 ANOVA (****p<0.0001).

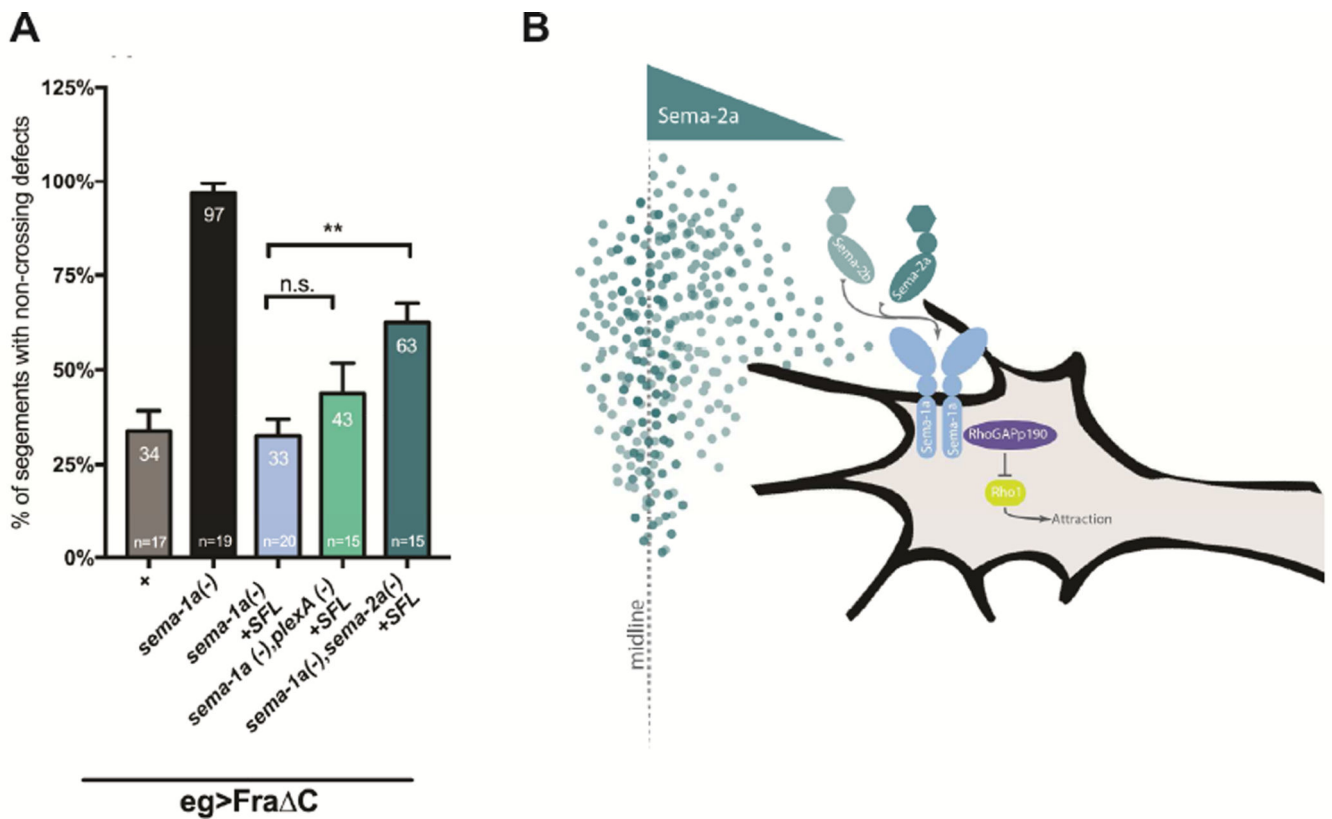


Figure 7. Sema-2a is required for Sema-1a mediated midline crossing

(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 rescued 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 ANOVA (** $p < 0.01$). (B) Model of Sema-1a mediated midline crossing.