Development/Plasticity/Repair

Independent Functions of Slit–Robo Repulsion and Netrin–Frazzled Attraction Regulate Axon Crossing at the Midline in *Drosophila*

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Slit and Netrin and their respective neuronal receptors play critical roles in patterning axonal connections in the developing nervous system by regulating the decision of whether or not to cross the midline. Studies of both invertebrate and vertebrate systems support the idea that Netrin, secreted by midline cells, signals through DCC (Deleted in Colorectal Carcinoma)/UNC40/Frazzled receptors to attract commissural axons toward and across the midline, whereas Slit signals through Robo family receptors to prevent commissural axons from recrossing the midline, as well as to prevent ipsilateral axons from ever crossing. Recent evidence from both *Xenopus* neuronal cell culture and *Drosophila* genetics have suggested that these signals may interact more directly in a hierarchical relationship, such that one response extinguishes the other. Here we present loss- and gain-of-function genetic evidence showing that the influence of Slit and Netrin on midline axon crossing is dictated by both independent and interdependent signaling functions of the Robo and Frazzled (Fra) receptors. Our results are not consistent with the proposal based on genetic analysis in *Drosophila* that the sole function of Slit and Robo during midline guidance is to repress Netrin attraction.

Key words: **axon guidance; midline; repulsion; attraction; Slit; Robo; Netrin; DCC/Frazzled**

Introduction

Slit and Netrin and their receptors, Robo/Sax3 and Deleted in Colorectal Cancer (DCC)/Frazzled/UNC40, play critical and conserved roles in patterning axonal connections during development of the *Caenorhabditis elegans*, *Drosophila*, and vertebrate nervous systems (Dickson, 2001; Yu and Bargmann, 2001; Garbe and Bashaw, 2004). Studies of these systems support the idea that Netrin–DCC signaling predominantly mediates axon attraction, whereas Slit–Robo signaling mediates repulsion. In the *Drosophila* ventral nerve cord and vertebrate spinal cord, Slit and Netrin are secreted by midline cells (Serafini et al., 1994; Mitchell et al., 1996). In these contexts, Netrin mediates axon attraction across the midline, whereas Slit prevents inappropriate crossing (Serafini et al., 1996; Kidd et al., 1998a; Brose et al., 1999; Kidd et al., 1999; Long et al., 2004).

Two general proposals for how these signals are coordinated during midline guidance have emerged. Classically, it has been proposed that guidance decisions at intermediate targets, such as the midline, are shaped by the balance of attractive and repulsive cues to which the growth cone is exposed (Tessier-Lavigne and Goodman, 1996). An alternative, although not mutually exclusive, idea is that attraction and repulsion are sometimes more intimately linked to ensure robust responses and to prevent conflicting signals from confusing the growth cone. In this case, activation of one receptor directly inhibits the function of another. For example, in cultured *Xenopus* spinal neurons, Slit silences the attractive function of Netrin through induction of a direct receptor–receptor interaction between Robo and DCC (Stein and Tessier-Lavigne, 2001). These findings have raised questions about how much of Slit–Robo function is attributable to axon repulsion as opposed to the inhibition of attraction. Indeed, two recent reports in *Drosophila* have suggested that the major (or in one case the only) function of Slit and Robo during midline guidance is to inhibit responses to Netrin (Bhat, 2005; Hiramoto and Hiromi, 2006). For example, double mutants between *slit* and *Netrin* (the ligands) or *robo* and *fra* (the receptors) were reported to result in phenotypes that were indistinguishable from single *Netrin* and *fra* mutants, leading to the argument that the only function of Slit–Robo signaling is to inhibit Netrin–Fra attraction (Bhat, 2005).

Here, we have examined how Slit repulsion and Netrin attraction are integrated during midline guidance in an effort to resolve whether Slit–Robo signaling acts to repel axons at the midline or whether instead it is solely required to inhibit Netrin-mediated attraction. Analysis of multiple double-mutant combinations of the attractive and repulsive ligands and receptors directly contradicts the previous findings of Bhat (Bhat, 2005). Instead, we observe a combination of both phenotypes: some axons fail to cross the midline, whereas others abnormally cross. Reintroducing ei-

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ther Robo or Fra into the double mutants restores the phenotype to that observed in single mutants. In addition, we demonstrate that postcrossing axons recross the midline in *robo* mutants and that they do so independently of *Netrin–fra* function, suggesting that if silencing attraction is required to prevent axon recrossing in the *Drosophila* CNS, Netrin cannot be the sole arbiter of the attraction.

Materials and Methods

Genetics and molecular biology. The following stocks were used for this study: (1) fra^3/CyWgβGal, (2) fra^4/CyWgβGal, (3) $robo^{Ga285}$/CyWgβGal, (4) *fra3*, $robo^{Ga285}$/CyWgβGal, (5) fra^4, $robo^{Ga285}$/CyWgβGal, (6) *Df NetrinA, B (NP5)/FM7*β_{actin}, (7) $slit^2$/CyWgβGal, (8) *UAS-TauMycGFP/CyTubulinGal80; eagleGal4*, (9) fra^4, *UAS-TauMycGFP/CyTubulinGal80; eagleGal4*, (10) $robo^{Ga285}$, *UAS-TauMycGFP/CyTubulinGal80; eagleGal4*, (11) $robo^{Ga285}$, *UAS-TauMycGFP/CyWgβGal*, (12) $robo^{Ga285}$, *apterousGal4/CyWgβGal*, (13) fra^4, $robo^{Ga285}$, *apterousGal4/CyWgβGal*, (14) fra^3, $robo^{Ga285}$, *UAS-TauMycGFP/CyWgβGal*, (15) fra^4, $robo^{Ga285}$/CyWgβGal; *elavGal4*, (16) fra^3, $robo^{Ga285}$/CyWgβGal; *UAS-RoboMyc#4*, (17) fra^3, $robo^{Ga285}$/CyWgβGal; *UAS-FraMycII*, (18) fra^3, $robo^{Z14}$/CyWgβGal, (19) fra^4, $robo^{Z14}$/CyWgβGal, (20) *UAS-RoFra*$^{2X\ insert}$, (21) *NetA,B/FM7*β_{actin}. We took advantage of the *β-galactosidase* expression associated with the balancer chromosomes together with antibody or mRNA *in situ* analysis to unambiguously genotype mutant embryos.

To generate *UASFramyc*, an *Eco*RI site was included 5′ of the *fra* coding sequence with the following primer: CAA ATA GAA TTC GCA ATC GGC GAT TGG CGG. A *Bam*HI site was also included at the 3′ of the *fra* coding sequence to eliminate the STOP codon and clone *fra* in frame with a 6-myc tag followed by a STOP codon into a pUAST vector (sequence at the junction EFEC/SRIRAGS). *UASFraMyc* transgenic flies were generated according to standard procedures.

Antibody generation and immunohistochemistry. The following primary antibodies were used: (1) mouse (Ms) anti-1D4/FasII [Developmental Studies Hybridoma Bank (DSHB); 1:100], (2) Ms-monoclonal antibody (MAb) BP102 (1:100; DSHB), (3) rabbit (Rb)-anti-Myc (1:500; Sigma-Aldrich, St. Louis, MO), (4) Ms-anti-Slit (1:25; C555.6D; DSHB), (5) Ms-anti-Sex Lethal (1:1000; M18; DSHB), (6) Ms-anti-βgal (40–1a; 1:250; DSHB), (7) Rb-anti-GFP (1:500; Invitrogen, Eugene, OR), (8) Ms-anti-Robo (1:50; DSHB), (9) Rb-anti-HRP (1:2000; MP Biomedicals, Solon, OH). A mouse monoclonal antibody was generated against the Fra protein (amino acids 440–902) as described previously and used at 1:50. The following secondary antibodies were used: (1) Alexa Fluor 488 goat anti-Rb (1:500; Invitrogen), (2) cyanine 3 (Cy3) goat anti-Ms (1:1000; Jackson ImmunoResearch, West Grove, PA). Embryos were fixed and stained as described previously (Kidd et al., 1998a). Stacks of images were obtained using a Leica (Nussloch, Germany) DMIRE2 confocal and a 63× oil-immersion objective. A maximum projection of the stacks was generated with NIH Image/ImageJ software.

Fluorescence mRNA in situ. Embryo collection and *in situ* hybridization were performed as described previously with digoxigenin-labeled probes (Tear et al., 1996). The *NetrinA* and *NetrinB in situ* probes were PCR amplified and transcribed from full-length cDNAs cloned into pBluescript. Hybridized probes were detected with anti-digoxigenin–HRP (Roche Diagnostics, Indianapolis, IN) and cyanine 5-labeled tyramide (see Fig. 2) or fluorescein-labeled tyramide (supplemental Fig. 2, available at www.jneurosci.org as supplemental material) (TSA Fluorescence System; PerkinElmer Life Sciences, Waltham, MA) was used as a substrate. After *in situ* hybridization, embryos were immunostained with anti-SlitC and anti-HRP as shown in Figure 2 or anti-Myc and anti-FasII as shown in supplemental Figure 2 (available at www.jneurosci.org as supplemental material). Stacks of images were obtained and processed as described above.

Results

To investigate the relationship between attraction and repulsion during midline axon guidance, we generated fly strains carrying various combinations of loss-of-function mutations in *slit*, *robo*, *fra*, and *Netrin*. In *Drosophila*, *Netrin* is encoded by two genes, *NetrinA* and *NetrinB*; hereafter, we refer to the two genes as *Netrin* for simplicity. Initially, we analyzed double-mutant embryos using BP102 as a pan-axonal marker or anti-FasII to recognize ipsilateral axons and compared their phenotypes to wild-type animals and single mutants (Fig. 1). In wild-type *Drosophila* embryos, immunostaining with the BP102 MAb reveals a ladder-like axon scaffold with longitudinal axons forming the rails of the ladder and commissural axons forming the rungs, whereas anti-FasII staining reveals three parallel bundles of longitudinal axons on either side of the midline (Fig. 1*A*,*E*). The attractive Netrin receptor Fra is expressed broadly and at high levels on many axons in the CNS, including both commissural and longitudinal portions of axons, whereas the Robo receptor is expressed broadly and at high levels along the longitudinal axon connectives but is kept at low levels on the commissural portions of axons (Fig. 1*I*,*M*) (Kolodziej et al., 1996; Kidd et al., 1999).

Mutations in *fra* that eliminate Fra protein expression result in reduced or absent axon commissures as well as characteristic breaks in the longitudinal connectives; however, many axons still cross the midline normally in *fra* mutants, and the majority of the crossing defects are observed in the posterior commissure (Fig. 1*B*,*F*,*J*) (see below) (Kolodziej et al., 1996; Forsthoefel et al., 2005). The longitudinal restricted expression pattern and levels of Robo are unaltered in *fra* mutants, indicating that loss of *fra* does not influence the repulsive pathway at the level of receptor expression or localization (Fig. 1*N*). Protein null mutations in *robo* lead to the opposite kind of phenotype, in which too many axons cross the midline. Axon commissures appear thicker than wild-type and are often fused together, whereas FasII-positive neurons are observed to wander back and forth across the midline (Fig. 1*C*,*G*) (Kidd et al., 1998a). In contrast to *fra*, in which mutant phenotypes show a high degree of segment-to-segment variability, essentially all segments in all embryos show defects in *robo* mutants (Fig. 1*C*,*G*) (Kidd et al., 1998a). We also tested whether Fra protein expression was in any way dependent on *robo* function and found no apparent differences in either levels or localization (Fig. 1*K*).

Simultaneous reduction of attraction and repulsion results in guidance defects that combine aspects of both single-mutant phenotypes

In light of the previously published data indicating that *fra*, *robo* double mutants are indistinguishable from *fra* single mutants (Bhat, 2005), we were surprised to find that embryos with mutations in *fra* and *robo* clearly exhibit defects that combine aspects of both mutant phenotypes; with some segments exhibiting the thickened and fused commissures characteristic of *robo* mutants and some segments showing thin or missing posterior commissures characteristic of *fra* mutants (Fig. 1*D*,*L*,*P*). This is true of multiple different allelic combinations of *fra* and *robo* (see Materials and Methods). In addition, clear defects in midline crossing of FasII-positive axons are observed in the double mutants, although these crossing defects do not appear to be as severe as *robo* single mutants (Fig. 1*H*). Importantly, immunostaining confirms the absence of both Fra and Robo proteins in these double mutants (Fig. 1*L*,*P*). Furthermore, out-crossing our *fra*, *robo* double mutants to either *fra* or *robo* single mutants resulted in phenotypes that were identical to the single-mutant *fra* and *robo* phenotypes described above (Fig. 1).

We next extended our analysis to an examination of *Netrin; slit* and *fra*, *slit* double mutants and found that both of these double mutants much more closely resemble the *slit* single-mutant phenotype in which all axons collapse onto and grow

along the CNS midline (Fig. 2) (data not shown). In these experiments, two different strategies were used to eliminate Netrin function. First, we used an X-chromosomal deletion that is known to completely remove both *Netrin* genes, as well as several additional genes (Mitchell et al., 1996). Second, we used specific *NetAB* double mutants generated by homologous recombination (Brankatschk and Dickson, 2006). In both types of *Netrin; slit* double mutants, we confirmed the absence of Netrin and Slit by fluorescence mRNA *in situ* and immunostaining, respectively (Fig. 2*H*,*J*) (data not shown). In contrast to the *fra*, *robo* double mutants, in *Netrin*, *slit* or *fra*, *slit* double mutants it was more difficult to discern aspects of the *fra* and *Netrin* phenotypes (Fig. 2*D*). This is likely attributable to the fact that when only *robo1* is absent, *robo2* can provide some repulsive activity, whereas in *slit* mutants, the repulsion is completely lost (Kidd et al., 1999; Rajagopalan et al., 2000; Simpson et al., 2000). Consistent with this idea, *fra*, *robo*, *robo2* triple mutants showed phenotypes that were very similar to those seen in *fra*, *slit* double mutants (data not shown). Nevertheless, there does seem to be a slight reduction in the extent of axon collapse in double mutants compared with *slit* alone (Fig. 2*D*). Together, our phenotypic analysis of compound mutants that simultaneously reduce attraction and repulsion support the idea that these two signaling systems function largely independently in regulating midline crossing.

Neuronal expression of either Robo or Fra in the double-mutant background restores the phenotype to that observed in single mutants

Because our double-mutant data directly contradicts the previously published work of Bhat (2005), we sought additional confirmation of the veracity of our double-mutant strains. Previous studies have established that the axon guidance phenotypes of both *robo* and *fra* can be significantly rescued by expressing these genes in postmitotic neurons using the Gal4-UAS system (Kolodziej et al., 1996; Kidd et al., 1998a; Garbe et al., 2006). Therefore, we tested whether transgenic expression of either Fra or Robo in the double mutants would "rescue" the phenotype and restore it to one that more closely resembled the single mutants (Fig. 3). This is clearly the case: reintroducing *fra* reverts the double mutant to a more *robo*-like phenotype, whereas reintroducing *robo* shifts the phenotype back toward *fra* (Fig. 3*B*,*C*,*H*,*I*). In fact, pan-neural

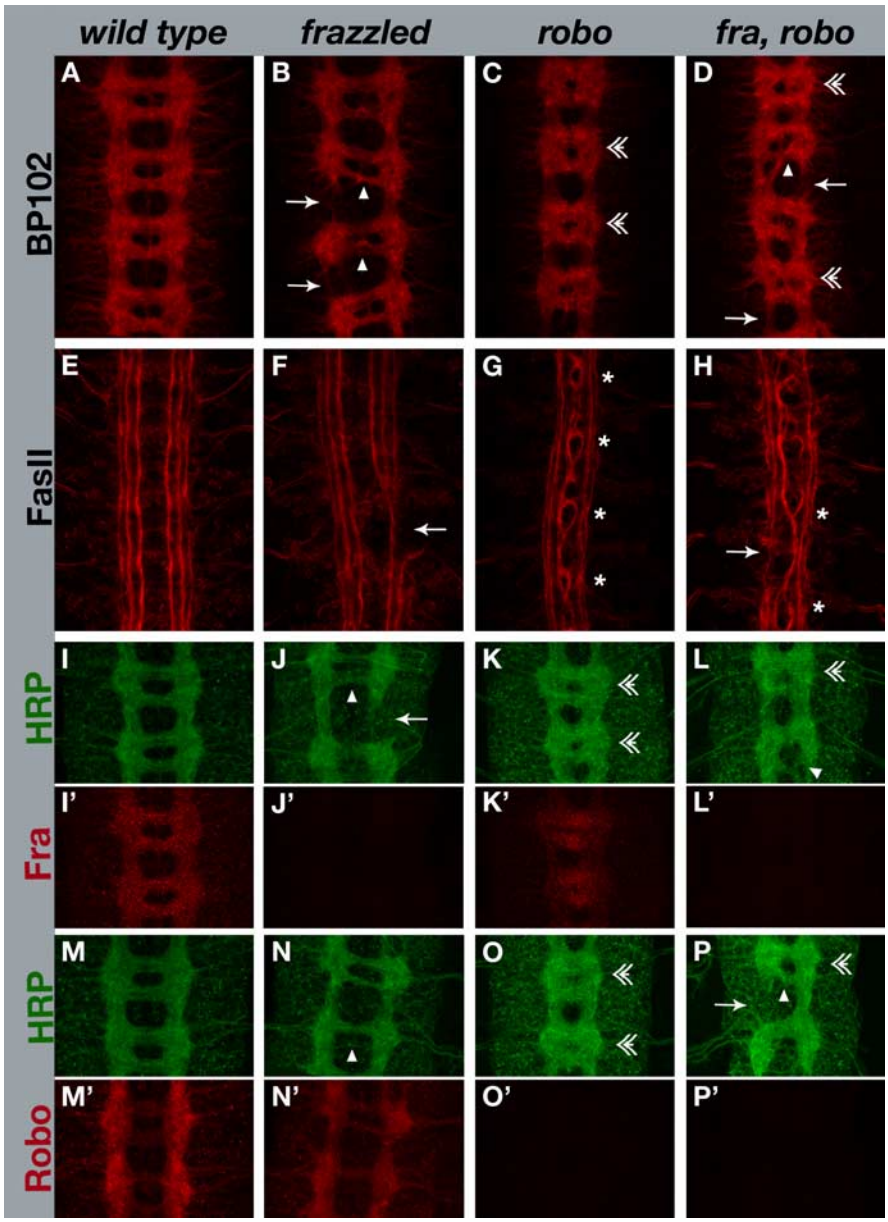

Figure 1. *fra*, *robo* double-mutant embryos display a combination of single-mutant phenotypes. For all panels, anterior is up. Arrows represent longitudinal breaks, arrowheads represent thinning of commissures, feathered arrowheads represent condensed/fused commissures, and asterisks represent segments with recrossing longitudinal bundles. Genotypes are listed on top, and antibodies are listed on the left. ***A–D***, Stage 16 embryos stained with MAb BP102. ***A***, Wild-type embryos stained with BP102 exhibit a ladder-like CNS scaffold with distinct thick anterior and posterior commissures and continuous longitudinal tracks on each side of the midline. ***B***, *fra* mutant embryos have many segments with thin commissures suggesting reduced midline attraction. *fra* mutant embryos also display longitudinal breaks. ***C***, *robo* mutant embryos display fused anterior and posterior commissures. ***D***, *fra*, *robo* double mutants ($n > 50$ embryos) exhibit a combination of single-mutant phenotypes; for example, thin and/or condensed commissures and longitudinal breaks. ***E–H***, Late stage 16–17 embryos stained with anti-FasII. ***E***, In wild-type embryos, three distinct fascicles on each side of the midline never cross and remain ipsilateral. ***F***, *fra* mutants show longitudinal breaks of these FasII-positive fascicles. ***G***, Medial FasII-positive bundles frequently cross and recross the midline in *robo* mutants. ***H***, *fra*, *robo* double mutants ($n > 50$ embryos) have a combination of single-mutant phenotypes such as longitudinal breaks and medial FasII-positive bundles crossing the midline. ***I–L***, All embryos stained with anti-HRP (green) to show the axon scaffold and anti-Fra (red). ***I***, ***I′***, Wild-type embryos have a normal axonal scaffold and are positive for anti-Fra. ***J***, ***J′***, *fra* mutant embryos have a disrupted scaffold and are negative for anti-Fra. ***K***, ***K′***, *robo* mutants are positive for anti-Fra. ***L***, ***L′***, *fra*, *robo* double mutants are indeed negative for anti-Fra and display a combination of single-mutant phenotypes. ***M–P***, All embryos stained with anti-HRP (green) to show the axon scaffold and anti-Robo (red). ***M***, ***M′***, Wild-type embryos are positive for anti-Robo. ***N***, ***N′***, *fra* mutants are positive for anti-Robo. ***O***, ***O′***, *robo* mutants are negative for anti-Robo. ***P***, ***P′***, *fra*, *robo* double-mutant embryos are negative for anti-Robo and show a combination of single-mutant phenotypes. To further confirm the presence of each single mutation in the fra^3 or fra^4, *robo/CyWg* stocks, we generated the single-mutant phenotypes in this figure by individually crossing flies from each single mutant to the double-mutant stock.

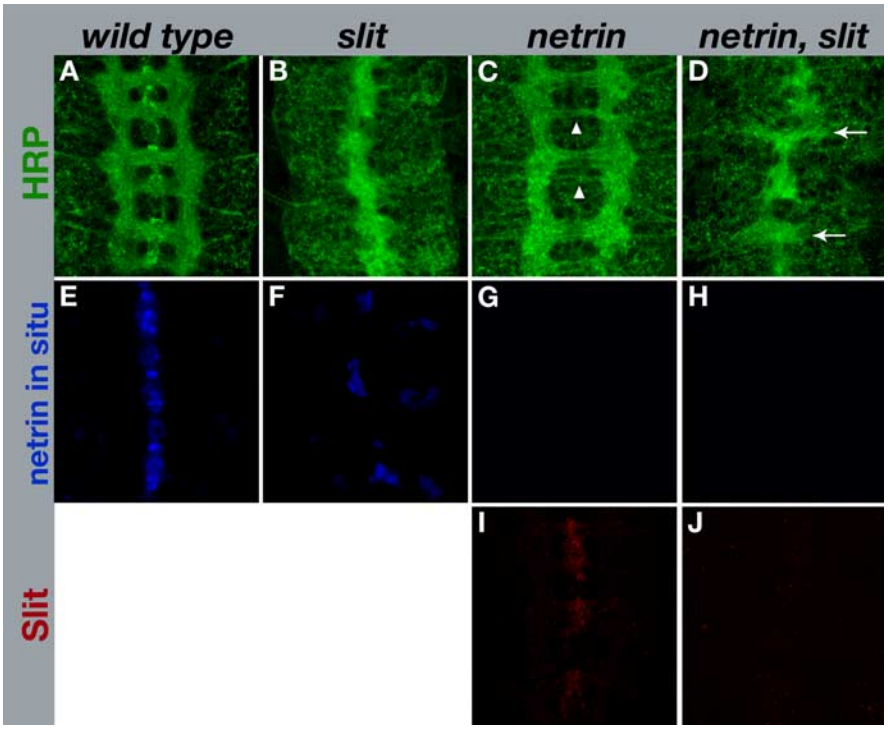

Figure 2. Netrin mutants are not epistatic to *slit*. ***A–D***, Stage 15 animals stained with anti-HRP (green) to reveal all axons. Anterior is up. ***A***, Wild-type embryos have a normal axonal scaffold. ***B***, Without the repulsive ligand Slit, all axons collapse at the midline. ***C***, *Netrin* mutant embryos have thinning or absent commissures (arrowheads) consistent with a loss of midline attraction. ***D***, Similar to *slit* single mutants, in *Netrin*, *slit* double mutants ($n > 15$ embryos), all axons collapse at the midline. Some segments appear less severe than *slit* single mutants, and a broadening of the axon pile at the midline is observed (arrows). ***E–H***, Same embryos as in ***A–D*** but instead detecting *Netrin* mRNA (blue). Wild-type (***E***) and *slit* single mutants (***F***) are positive for *Netrin* mRNA. In contrast, *Netrin* single mutants (***G***) and *Netrin*, *slit* double mutants (***H***) are negative for *Netrin* mRNA. Notice that although *Netrin*, *slit* double mutants are negative for *Netrin* mRNA, they still exhibit a *slit* mutant phenotype. Note also the disorganization of the midline glia in *slit* mutants in ***F***. ***I***, ***J***, *Netrin* single mutants are positive for Slit, whereas *Netrin*, *slit* double mutants are negative, indicating that the double mutants are indeed mutant for *slit*. Unfortunately, for technical reasons, we could not image the Slit protein in either wild-type or *slit* single mutants in these triple-labeled embryos because the Cy5 signal detecting *Netrin* mRNA interfered with Cy3 (Slit) detection. Notice that the Cy5 "bleed-through" signal is also present in ***A***, demonstrating that it was excited by all lasers.

expression of Robo in the *fra*, *robo* double mutant not only rescues the ectopic midline crossing but also appears to significantly exacerbate the loss of attraction associated with *fra* mutants (Fig. 3*I*).

To further evaluate whether misexpression of Robo can enhance the defects in *fra* mutants, we misexpressed Robo in all neurons with *elavGal4* in either wild-type (*fra*/+ heterozygotes) or *fra* mutants. Consistent with previous data (Kidd et al., 1998b; Bashaw et al., 2000), overexpressing one copy of a *UASRoboMyc* transgene with *elavGal4* in wild-type animals did not lead to significant guidance defects, nor did we observe ectopic Robo protein in the axon commissures (supplemental Fig. 1, available at www.jneurosci.org as supplemental material). In contrast, similar misexpression of *UASRoboMyc* in *fra* mutants led to a dramatic disruption in commissure formation, affecting both anterior and posterior commissures (supplemental Fig. 1, available at www.jneurosci.org as supplemental material). The fact that we can enhance the complete loss of *fra* attraction suggests that panneural expression of Robo repels axons independently of any influence on *Netrin–fra* signaling, a finding that is consistent with an additive influence of *fra* attraction and *robo* repulsion. Importantly, ectopic Robo expression is unable to repel axons in *fra*, *slit* double mutants, arguing that the Robo repulsive gain-of-function phenotype is strictly dependent on *slit* (supplemental Fig. 1, available at www.jneurosci.org as supplemental material).

In addition to analysis of double mutants, Bhat performed additional genetic experiments to bolster the argument that Slit–Robo signaling functions primarily to inhibit Netrin attraction (Bhat, 2005). In particular, evidence was presented that the gain-of-function ectopic axon attraction phenotype associated with expression of a chimeric receptor consisting of the extracellular domain of Robo and the cytoplasmic domain of Fra (Bashaw and Goodman, 1999) was completely dependent on midline-expressed *Netrin*. We repeated these experiments with the identical transgenic inserts used in the Bhat study and found no such Netrin dependence: misexpression of the Robo–Fra chimera resulted in ectopic attraction in the presence or absence of *Netrin* (supplemental Fig. 2, available at www.jneurosci.org as supplemental material).

Together, these observations are inconsistent with the idea that loss of Netrin-mediated midline attraction is epistatic to loss of midline repulsion. Our genetic analysis, mRNA and protein expression analysis, and transgenic rescue data leave little doubt that our double mutants remove the relevant gene functions. Importantly, although our data preclude the possibility that *slit–robo* function is mediated solely through inhibition of *Netrin–fra*, examination of ipsilateral pioneer posterior corner cell (pCC) neurons in *fra*, *robo* or *fra*, *slit* double mutants is consistent with the proposal of Hiramoto and Hiromi (2006) that one function of *slit–robo* signaling is to prevent axons from responding to relocalized Netrin during the pioneering of the longitudinal connectives. In contrast to *robo* mutants in which the pCC pioneer crosses the midline almost all of the time (~96%), in *fra*, *robo* double mutants, this ectopic crossing is significantly reduced to ~60% (Table 1). To extend this observation, we further reduced repulsion in *fra* mutants by removing *slit* and found that in *fra*, *slit* double mutants there was no significant difference in the extent of pCC ectopic midline crossing compared with *slit* alone (Table 1). This is likely attributable to the fact that axon commissures do not form at all in *slit* mutants, and thus Netrin is presumably not relocalized in a way that it can influence the behavior of the longitudinal pioneers (Hiramoto and Hiromi, 2006). Our data are consistent with the idea that Robo signaling likely acts simultaneously to repel pioneers from midline expressed Slit, as well as to prevent longitudinal pioneer axons from responding to Netrin as they navigate the segment boundary.

Robo repels specific subsets of ipsilateral axons independently of Fra

Although our analysis of simultaneously removing attraction and repulsion clearly reveals aspects of both kinds of phenotypes (es-

pecially in the case of *fra*, *robo* double mutants), the defects observed in late-stage embryos with BP102 and FasII are so severe and affect so many axons that they are very difficult to analyze quantitatively. We were able to circumvent this by quantifying the guidance defects in pCC pioneers in early stages, in which single axon resolution is possible (Table 1, see above). To attain similar resolution in later navigating ipsilateral neurons, we analyzed the trajectory of small subsets of CNS interneurons in *fra* and *robo* single- and double-mutant combinations using *apterousGal4* (*apGal4*) to label three ipsilateral neurons per hemisegment (O'Keefe et al., 1998).

In wild-type or *fra* mutant embryos, the apterous (ap) neurons in the eight abdominal segments of the embryo project axons toward the midline before turning anteriorly along the outer edge of the medialmost FasII axon bundle; they do not cross the midline (Fig. 4*A*,*B*). In contrast, in *robo* mutants, the ap neurons extend across the midline and then wander back and forth or stay at the midline, often tracking FasII-positive axon bundles that are ectopically crossing (Fig. 4*C*). This defect can be rescued by providing *robo* function specifically in the ap neurons in an otherwise mutant background, indicating that Robo guides later projecting neurons independently of its earlier function in the longitudinal pioneers (Garbe et al., 2006). Interestingly, a time course analysis has shown that midline crossing defects in the ap neurons can arise at two different times. In the complete absence of Robo (or Slit), almost all of the ap axons extend medially, directly across the midline at stage 15, never making their correct anterior turn (Table 1), whereas in embryos mutant for *β-spectrin* or carrying genetic combinations that partially limit Slit–Robo signaling, the ap axons initially make the correct ipsilateral anterior turn, but several hours later, at stage 17, ectopic midline crossing is observed (Garbe et al., 2006). We have interpreted these findings to suggest that Slit–Robo repulsion is required continuously to establish and maintain appropriate pathway selection (Garbe et al., 2006). The early-stage ap axon guidance defect provides an easily quantifiable assay for *robo* repulsive function. In *fra*, *robo* double mutants the majority of ap axons project directly across the midline, again arguing for *fra*-independent Robo-mediated midline repulsion (Fig. 4*D*, Table 1). The observation that the midline crossing defect of the ap neurons in *fra*, *robo* double mutants can be rescued by resupplying *UASRobo* to the ap neurons further supports a *fra*-independent repulsive function (data not shown).

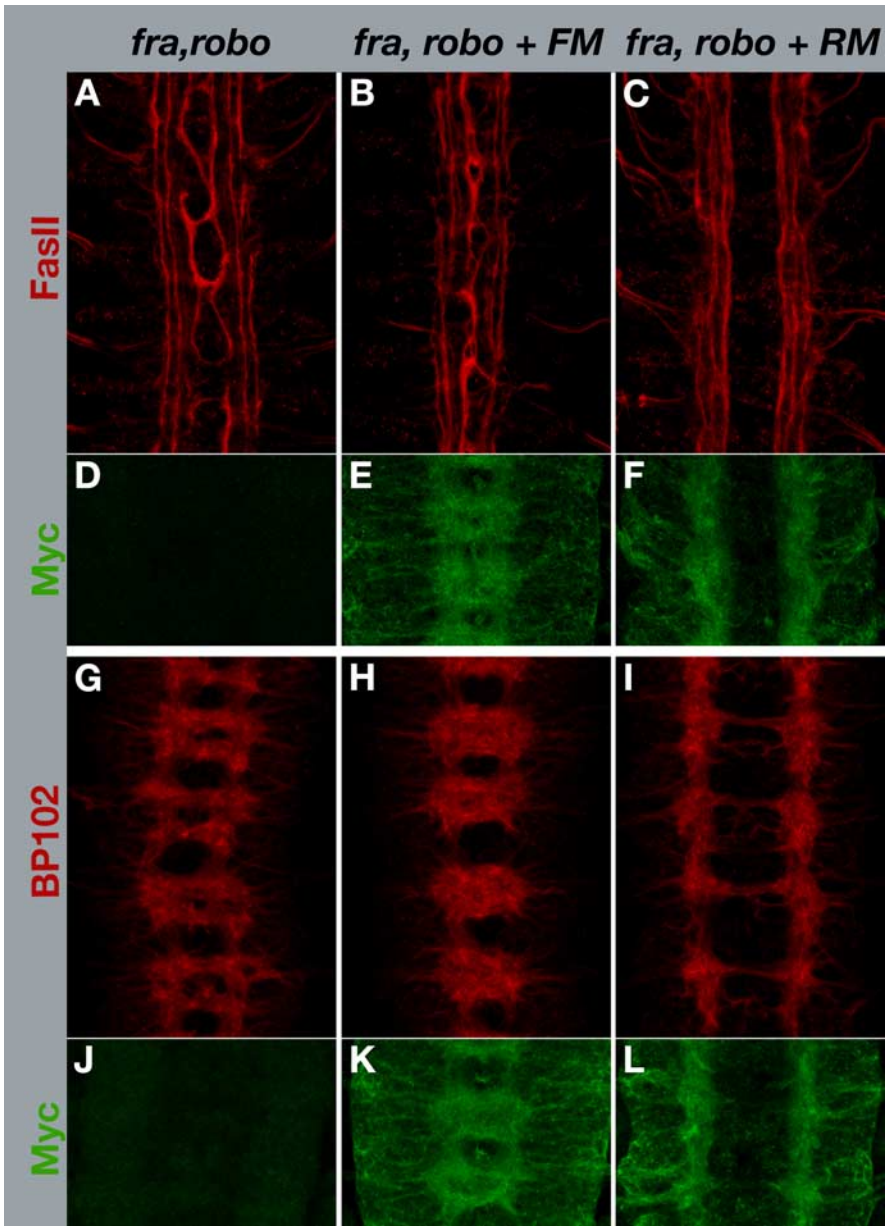

Figure 3. The *fra*, *robo* double-mutant phenotypes can be "rescued" by individually expressing each receptor. ***A–C***, Late stage 16 embryos stained with anti-FasII. All embryos are homozygous for *fra* and *robo*. Anterior is up. ***A***, *fra*, *robo* double-mutant embryos exhibit many FasII-positive axons inappropriately crossing the midline. ***B***, More axons seem to ectopically cross and stay at the midline when Fra is misexpressed in all axons ($n = 16$ embryos). ***C***, When Robo is overexpressed in all axons, FasII-positive neurons are rescued and no longer cross the midline ($n = 12$ embryos). ***D–F***, Anti-Myc staining demonstrating *UASFra-Myc* (*FM*) (***E***) or *UASRobo-Myc* (*RM*) (***F***) in the above embryos. ***G–I***, Mid-stage 16 embryos stained with MAb BP102. All embryos are homozygous for *fra* and *robo*. Anterior is up. ***G***, *fra*, *robo* double mutants show a combination of single-mutant phenotypes; for example, condensed and/or thinning commissures and longitudinal breaks. ***H***, The double-mutant phenotype can be rescued back to *robo* single mutants by expressing Fra in all neurons ($n = 17$ embryos). Often, this phenotype seems qualitatively stronger than *robo* single mutants suggesting Fra can attract additional axons independently of Robo. ***I***, When Robo is expressed in the *fra*, *robo* double-mutant background, the phenotype is rescued back to the *fra* single-mutant phenotype ($n = 18$ embryos). Often, this phenotype is considerably more severe than *fra* single mutants, suggesting that Robo is able to repel commissural axons independently of *fra*. ***J–L***, Anti-Myc staining demonstrating *UASFra-Myc* (*FM*) (***K***) or *UASRobo-Myc* (*RM*) (***L***) in the above embryos.

Commissural axons recross the midline in *robo* mutants in the absence of *fra*

To more clearly define the role of *Netrin–fra* attraction and *slit–robo* repulsion in the guidance of precrossing and postcrossing commissural axons, we next examined the trajectories of the Egl commissural interneurons in different single- and double-

Table 1. Quantification of midline crossing defects

	Ipsilateral axon projections		Contralateral axon projections	
Genotype	Ectopic pCC crossing[a]	Ectopic ap crossing[b]	EW crossing defects[c]	EG recrossing[d]
Wild type	0/88 (0%) $n = 8$	0/99 (0%) $n = 9$	0/96 (0%) $n = 12$	0/96 (0%) $n = 12$
fra	0/110 (0%) $n = 10$	0/88 (0%) $n = 8$	257/372 (69%) $n = 49$	0/80 (0%) $n = 10$
robo	143/147 (97%) $n = 15$	206/217 (95%) $n = 23$	0/96 (0%) $n = 12$	134/208 (64%) $n = 26$
fra, *robo*	115/186 (61%)* $n = 19$ $p = 6.15e-07$	162/193 (84%)* $n = 19$ $p = 0.0001$	163/307 (53%)** $n = 41$ $p = 8.60e-05$	107/232 (46%)* $n = 18$ $p = 9.02e-06$
slit	166/176 (94%) $n = 18$	ND	ND	ND
fra, *slit*	313/351 (89%) $n = 36$	ND	ND	ND

In all cases, defects are indicated as number of defective segments divided by the total segments scored to give a percentage. *n* represents the number of embryos of the specified genotype that were analyzed. *Statistically different from robo alone or **fra alone in a two-sample Student's *t* test with $p < 0.01$. *p* values are indicated. ND, Not determined.

[a]Late stage 12 to early stage 13 embryos stained with anti-Fas2 were scored.

[b]Stage 15 embryos in which the ap neurons were labeled with GFP were scored for ectopic crossing defects.

[c]Stage 16 embryos in which the EW neurons were labeled with GFP were scored for defects in normal midline crossing. Defects include a complete loss of or a noticeable thinning of the commissural EW bundle. Only the eight abdominal segments were scored.

[d]Stage 17 embryos in which the EG neurons were labeled with GFP were scored for abnormal recrossing at the midline.

mutant combinations. In wild-type embryos, the Egl neurons comprise two clusters of neurons: one cluster (the EG neurons) contains 10–12 cells that extend their axons across the midline in the anterior commissure, and the second cluster (the EW neurons) consists of four cells, three of which are serotonergic interneurons that project axons across the midline in the posterior commissure (the other cell is a motor neuron) (Fig. 4*E*) (Higashijima et al., 1996; Dittrich et al., 1997). Lineage tracing has established that the EW neurons are derived from neuroblast 7–3 and that they make local synaptic connections immediately after crossing the midline, whereas the EG commissural interneurons are derived from neuroblast 3–3 and that they cross the midline and then project anteriorly for several segments traveling near the medial edge of the longitudinal connective (Fig. 4*E*,*I*) (Bossing et al., 1996; Schmidt et al., 1997; Schmid et al., 1999). In *fra* mutants, the EW neurons frequently fail to cross the midline, whereas the EG axonal projections in the anterior commissure are unaffected both precrossing and postcrossing (Fig. 4*F*,*J*, Table 1). In contrast, *robo* mutants exhibit defects that are most prominent in postcrossing EG neurons, in which inappropriate recrossing and circling around the midline are observed in late-stage embryos (Fig. 4*G*,*K*). Interestingly, in *fra*, *robo* double mutants, there is a small but significant decrease in the number of EW axons that fail to cross the midline compared with *fra* single mutants, suggesting that in a wild-type situation this repulsive function of Robo in precrossing commissural EW axons may be counterbalanced by *fra* attraction (Fig. 4*H*, Table 1). Similarly, postcrossing EG axons exhibit a slight reduction in midline recrossing and circling behavior compared with *robo* single mutants (Fig. 4*L*, Table 1). We believe that these subtle shifts in the percentage of defects in the double mutants reflect alterations in the balance of attraction and repulsion.

Discussion

Here, we have investigated how attraction and repulsion are coordinated during midline axon guidance in *Drosophila* to determine whether Slit and Robo function to mediate repulsion or alternatively whether they function to inhibit Netrin–Fra attraction. We generated double mutants that simultaneously remove the attractive and repulsive ligands and receptors and verified the absence of each of the proteins in our double mutants using molecular and genetic criteria. Analysis of their phenotypes using markers for large groups of axons, as well as markers for specific subsets of ipsilateral and contralateral neurons, reveals a combination of guidance defects that include aspects of both single-mutant phenotypes. Furthermore, gain-of-function experiments indicate that Robo can repel CNS axons in the absence of *fra* function, arguing for an independent repulsive function of Slit and Robo. In addition, we found that postcrossing commissural neurons recross the midline in *robo* mutants, and this behavior is also largely independent of *Netrin–fra* attraction. Together, our data support at least two distinct functions for Slit–Robo signaling in the regulation of midline crossing; one role to repress *Netrin–fra* function during the guidance of longitudinal pioneer neurons at the segment boundary and a second major *Netrin–fra*-independent repulsive role in preventing ectopic midline crossing.

Independent functions of Netrin–Fra and Slit–Robo regulate midline crossing

Our study was inspired in part by the provocative findings of Bhat (2005) indicating that mutations in *Netrin* and *fra* were epistatic to mutations in *slit* and *robo*; that is, compound mutants between components of these two signaling pathways resulted in phenotypes indistinguishable from single mutants in *fra* or *Netrin*. These observations were surprising because loss of *slit* function leads to profound guidance defects in essentially all CNS axons, whereas loss of *Netrin* or *fra* causes much milder defects (Harris et al., 1996; Kolodziej et al., 1996; Mitchell et al., 1996; Bashaw and Goodman, 1999; Hummel et al., 1999; Forsthoefel et al., 2005; Brankatschk and Dickson, 2006). Indeed, many commissural neurons cross the midline normally in the absence of *Netrin–fra* signaling. So how could a mutation that affects only some neurons be epistatic to a mutation that affects all neurons? The data presented here directly contradict the published double-mutant analysis (Bhat, 2005) and support the more parsimonious explanation that Robo repels axons independently of Netrin–Fra attraction.

Here, it should be noted that several clear examples of independent repulsive functions for Slit and Robo have been described in both *C. elegans* and Vertebrates (Brose and Tessier-Lavigne, 2000; Zou et al., 2000; Stein and Tessier-Lavigne, 2001; Yu et al., 2002). Furthermore, studies in *C. elegans* have suggested that Slit repulsion and Netrin attraction act cooperatively during ventral guidance and that DCC/UNC-40 can actually potentiate SAX-3/Robo repulsion independently of Unc-6/Netrin (Hao et al., 2001; Yu et al., 2002). The most likely explanation for the discrepancy between our data and that reported by Bhat is the misidentification of the double-mutant embryos in the earlier study.

The role of Robo in longitudinal pioneer axons

Hiramoto and Hiromi have observed that the abnormal midline crossing of specific pioneer ipsilateral neurons seen in *robo* mu-

tants can be strongly suppressed by simultaneous removal of *Netrin* or *fra*, suggesting that the *robo* defect results from a failure to inhibit a response to redistributed Netrin, rather than a loss of midline repulsion (Hiramoto et al., 2000; Hiramoto and Hiromi, 2006). In this context, Robo is proposed to function to prevent longitudinally extending pioneer axons from responding to Netrin that has been relocalized to commissural axons through interactions with Fra (Hiramoto et al., 2000). We also observed a significant reduction in pioneer crossing in *fra*, *robo* double mutants compared with *robo* single mutants, suggesting that defects in longitudinal pioneers are not strictly caused by loss of repulsion. To investigate this possibility further, we also determined the incidence of longitudinal pioneer defects in *fra*, *slit* double mutants, a background in which all Slit-dependent repulsion is eliminated. In contrast to *fra*, *robo* double mutants, *fra*, *slit* double mutants exhibited the same high levels of ectopic crossing as observed in *slit* single mutants, indicating that completely removing midline repulsion masks the function of Robo in preventing abnormal responses to redistributed Netrin. Together, our genetic results suggest that Slit–Robo signaling likely acts simultaneously to repel pioneer neurons from midline-expressed Slit, as well as to prevent longitudinal pioneer axons from responding to Netrin as they navigate the segment boundary.

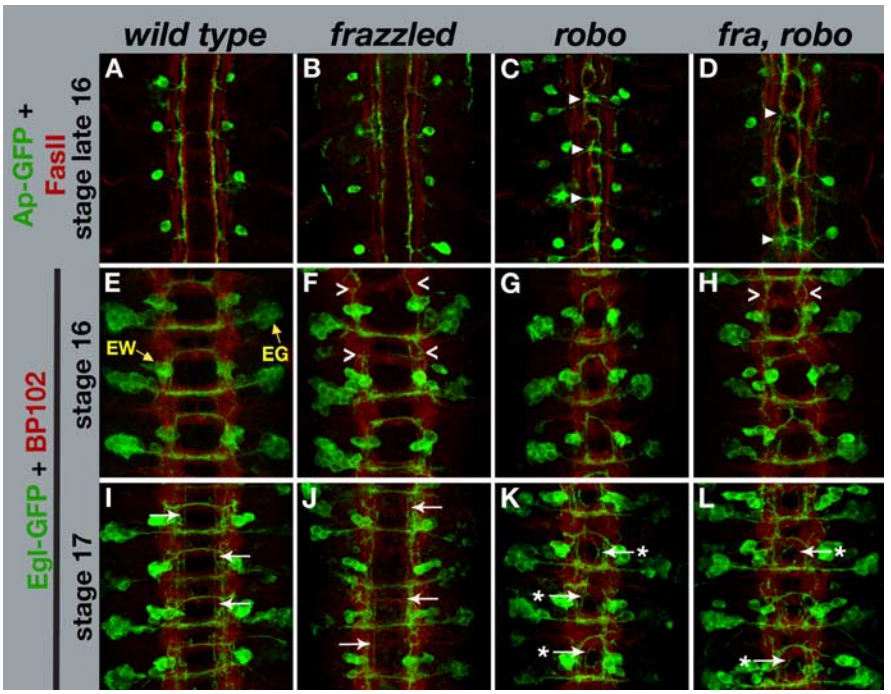

Figure 4. Commissural axons recross the midline in *robo* mutants independent of *fra*. ***A–D***, Late stage 16–17 embryos stained with anti-FasII and anti-GFP. Anterior is up. ***A***, Wild-type embryos have a bundle of ap axons on each side of the midline that remain ipsilateral. ***B***, Similar to wild type, ap axons in *fra* mutants do not cross the midline. ***C***, In *robo* mutant embryos, we see many ap axons ectopically crossing the midline (arrowheads). ***D***, Similar to *robo* mutant embryos, *fra*, *robo* double mutants exhibit bundles of ap axons inappropriately crossing the midline (arrowheads). This is consistent with Robo having direct repulsive activity in late-extending neurons even in the absence of the midline attractive receptor Fra. ***E–H***, Stage 16 embryos stained with MAb BP102 and anti-GFP. Anterior is up. ***E***, Wild-type embryos contain two commissural bundles of eagle neurons that always cross the midline; EW crosses in the posterior commissure and EG in the anterior (yellow label). ***F***, In *fra* mutants, the EW bundle of axons often fails to cross the midline (carrots). ***G***, In *robo* mutants, although the cell bodies are closer to the midline, EW neurons cross the midline like wild type. ***H***, Similar to *fra* single mutants, *fra*, *robo* double-mutant embryos exhibit EW neurons that fail to cross the midline (carrots) consistent with Fra playing a direct role in attracting these axons across the midline. ***I–L***, Stage 17 embryos stained with MAb BP102 and anti-GFP. Anterior is up. ***I***, Later in development, after the EG neurons have crossed the midline, they send axons anteriorly in a longitudinal fascicle (arrows). ***J***, Similar to wild-type, *fra* mutants also extend the EG neurons anteriorly once across the midline (arrows). ***K***, In *robo* mutants, instead of extending anteriorly, the EG axons "recross" the midline (starred arrows), suggesting that Robo is required to repel the EG neurons away from the midline after crossing once. ***L***, In *fra*, *robo* double mutants, the EG neurons also recross the midline (starred arrows), suggesting that Fra is not essential for this "reattraction" and is consistent with Robo playing a direct role in axonal repulsion independent of the attractive function of Fra. Genotypes of individual panels are as follows: ***A***, *apterousGal4* (*apGal4*), *UAS-TauMycGFP (UTMG)/+*; ***B***, fra^4, *apGal4/*fra^3, *UTMG*; ***C***, $robo^{Ga285}$, *apGal4/*$robo^{Ga285}$, *UTMG*; ***D***, fra^4, $robo^{Ga285}$, *apGal4/*fra^3, $robo^{Ga285}$, *UTMG*; ***E***, ***I***, *UTMG/+; eagleGal4 (eglGal4)/+*; ***F***, ***J***, fra^3/fra^4, *UTMG; eglGal4/+*; ***G***, ***K***, $robo^{Ga285}/robo^{Ga285}$, *UTMG; eglGal4/+*; ***H***, ***L***, fra^3, $robo^{Ga285}/fra^4$, $robo^{Ga285}$, *UTMG; eglGal4/+*. See Table 1 for quantification.

Guidance at the *Drosophila* midline: balancing attraction and repulsion or hierarchical receptor interactions?

It has been proposed that navigating growth cones measure the relative levels of attractive and repulsive cues to arrive at the correct decisions (Tessier-Lavigne and Goodman, 1996). In this scenario, the relative influence of attractive and repulsive cues can be biased in one direction or another by regulating the complement of guidance receptors that are expressed on the surface of the growth cone. Experimental manipulations in which the levels of guidance cues and receptors are either increased or decreased, singly or in combinations, have provided substantial evidence in support of this idea; in particular in the context of selective axon fasciculation, midline axon guidance, and target selection (Lin et al., 1994; Tessier-Lavigne and Goodman, 1996; Winberg et al., 1998; Bashaw and Goodman, 1999). Recent work has suggested an alternative (although certainly not mutually exclusive) idea that at certain times during the trajectory of an axon, attractive and repulsive signals may be more intimately connected, with one response overriding another through direct receptor–receptor interactions (Stein and Tessier-Lavigne, 2001).

A number of findings presented here support the model that the relative levels of attractive and repulsive influences play an important role in instructing the decision of whether or not to cross the midline. First, our observation that simultaneous removal of attraction and repulsion leads to defects that combine aspects of removing either one supports the idea that these two signaling systems act independently. Furthermore, the fact that pan-neural misexpression of *robo* in the absence of *fra* (or in *fra*, *robo* double mutants) results in much stronger disruptions in midline crossing than when *fra* is present argues that (1) Robo can repel additional axons independently of Fra attraction, and (2) that the ability of Robo to ectopically repel axons can be counteracted by the independent attractive influence of Fra. Similarly, misexpression of *fra* in the *fra*, *robo* double mutant results in midline crossing defects that appear to be qualitatively more severe than *robo* single mutants, whereas misexpression of *fra* in wild-type embryos has no effect (data not shown), again suggesting that ectopic attraction mediated by *fra* can be counteracted by the independent repulsive function of Robo.

Implications for silencing

The "silencing" model provides an elegant explanation for how a postcrossing commissural neuron might couple the upregulation of Robo repulsion to the downregulation of attraction to ensure high-fidelity guidance (Stein and Tessier-Lavigne, 2001) and is consistent with the finding that postcrossing commissural hindbrain neurons lose responsiveness to Netrin (Shirasaki et al., 1998). Our observation that postcrossing commissural axons in *robo* mutants can recross the midline in the absence of *fra*-mediated axon attraction has important implications for the mechanism by which commissural axons normally avoid recrossing. Under its strictest interpretation, the silencing model proposed by Stein and Tessier-Lavigne posits that postcrossing commissural neurons require Slit–Robo function to downregulate the attractive response to midline-expressed Netrin and that this function is essential to prevent recrossing (Stein and Tessier-Lavigne, 2001). The fact that the majority of EG commissural axons recross in the absence of *fra* function would suggest that at the *Drosophila* midline, silencing of *fra* is not absolutely required to prevent recrossing and therefore would not be consistent with the extreme interpretation of silencing as stated above. Here it is important to point out that despite considerable conservation in the molecules and mechanisms mediating midline guidance in vertebrates and invertebrates, there are significant differences in the two systems. In particular, the mechanism of Robo regulation in precrossing axons appears to be distinct in the two systems, with the Commissureless protein inhibiting Robo function in commissural axons in the fly, whereas the variant Robo family member Rig-1 appears to fulfill this role in vertebrates (Kidd et al., 1998b; Keleman et al., 2002; Sabatier et al., 2004).

Therefore, we propose two mutually exclusive possibilities: (1) either Robo silencing of Fra does not occur at the *Drosophila* midline (or at least not in these particular commissural neurons), or (2) silencing does occur, but either Robo repulsion on its own is sufficient to prevent recrossing, or there are additional attractive functions that must also be silenced in postcrossing axons. Currently, we are unable to distinguish between these possibilities, although our previous observation that Robo repulsion is required continuously throughout embryonic development to maintain appropriate pathway selection, together with the double-mutant analyses we describe here, support an independent repulsive function of Robo in regulating axon crossing at the midline. Future studies in *Drosophila* and Vertebrates, for example, with the analysis of mice bearing mutations in both Robo and DCC receptors, should shed additional light on how much of a role silencing plays during midline guidance *in vivo*.

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