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A Frazzled/DCC-Dependent Transcriptional Switch Regulates Midline Axon Guidance

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Abstract

Precise wiring of the nervous system depends on coordinating the action of conserved families of proteins that direct axons to their appropriate targets. Slit-Robo repulsion and Netrin-DCC (Frazzled) attraction must be tightly regulated to control midline axon guidance in vertebrates and invertebrates, but the mechanism mediating this regulation is poorly defined. Here we show that the Fra receptor has two genetically separable functions in regulating midline guidance in *Drosophila*. First, Fra mediates canonical chemoattraction in response to Netrin, and second, it functions independently of Netrin to activate *commissureless* transcription, allowing attraction to be coupled to the down-regulation of repulsion in pre-crossing commissural axons.

Establishing precise midline circuitry is essential to control rhythmic and locomotor behaviors (1, 2). Conserved signals that regulate axon guidance at the midline include attractive cues such as Netrins, and repulsive cues such as Slits, Semaphorins and Ephrins (3, 4). In *Drosophila*, Netrin attracts many commissural axons to the midline through activation of the Frazzled (Fra)/DCC (Deleted in Colorectal Cancer) receptor (5–8), while the repellant Slit and its receptor Roundabout (Robo) prevent commissural axons from re-crossing (9, 10). Commissureless (Comm) controls midline crossing by negatively regulating surface levels of Robo on pre-crossing commissural axons (11–13). Comm is expressed transiently in commissural neurons as their axons traverse the midline, where it sorts Robo to endosomes (12). Once across the midline, *comm* expression is extinguished, resulting in increased levels of Robo on the growth cone. How *comm* expression is spatially and temporally regulated to gate midline crossing is unknown.

While characterizing the structural requirements for Fra-mediated axon attraction, we observed that neuronal expression of a dominant negative form of Fra (FraΔC) leads to a dose-dependent “commissureless” phenotype (14). Searching for candidate genes that modify this phenotype, we found that removing one copy of *comm* enhances the midline crossing defects caused by expressing *UASFraΔC* (fig. S1), suggesting a role for Fra in regulating Comm during midline guidance. Consistent with this idea, removing one copy of

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Table S1

comm in hypomorphic *fra* mutants increases the commissural defects as shown by thin or missing commissures in many segments, as well as an increased frequency of non-crossing defects in a subset of commissural neurons: the eagle neurons (Fig. 1 and Table S1). Similar genetic interactions are also observed using additional alleles of both *fra* and *comm* (fig. S2 and Table S1). These dose-dependent genetic interactions suggest that *fra* and *comm* may function in the same pathway to control commissural axon guidance.

How could Fra regulate the function of Comm? Because *comm* mRNA is up-regulated in commissural neurons as their axons cross the midline and DCC has been shown to mediate Netrin-induced axon outgrowth and turning through activation of the MAP Kinase and Calcineurin/NFAT signaling cascades (15, 16), we tested whether Fra regulates *comm* mRNA expression. Examination of *comm* mRNA in *fra* mutant nerve cords by real time PCR reveals a 12-fold reduction of total *comm* mRNA relative to wild type (fig. S3). To analyze *comm* mRNA expression with single cell resolution, we focused on the eagle neurons. At stage 14 in wild-type or *fra*/+ embryos, when the Eagle axons are crossing the midline, they have high *comm* RNA expression in their cell bodies (Fig. 2, A–C). However, in *fra* mutants *comm* mRNA is reduced in both the EW and EG neurons (Fig. 2, fig. S4, and S5). *comm* mRNA reduction in *fra* mutants is unlikely to be secondary to the failure of these axons to cross the midline, since a similar reduction is observed in EWs that have normal trajectories (Fig. 2). This implies that crossing the midline is not sufficient to induce *comm* transcription. Furthermore, the down-regulation of *comm* mRNA is likely a reflection of reduced transcription, rather than reduced mRNA stability, since we detect a similar reduction of *comm* pre-mRNA expression using a *comm* intron probe for hybridization (fig. S6). Finally, *comm* mRNA reduction in *fra* mutants is specific to commissural neurons since *comm* mRNA expression in the midline glia is not affected (Fig. 2).

Fra has non cell-autonomous functions (17, 18), so we tested whether Fra is required exclusively in commissural neurons to control *comm* transcription. Expressing a *UASFra-Myc* transgene in the eagle neurons of *fra* mutants not only rescues the guidance defects of EWs as previously reported(14), but also rescues *comm* mRNA expression (Fig. 2, G to I and fig. S4). *comm* mRNA expression is also recovered in the few EWs (1.8%) that are not rescued and *comm* mRNA levels are normally regulated when the EW axons are prevented from crossing the midline by mis-expressing the Robo receptor, indicating that crossing the midline is not necessary to induce *comm* expression (fig. S4 and S7). During axon migration, growth cones of ipisilateral neurons extend long filopodia that reach all the way across the midline (19), suggesting that even when commissural axons extend ipsilaterally they could still have access to midline signals.

In contrast to *wild type* Fra, expression of FraΔC in *fra* mutants does not rescue *comm* mRNA expression (fig. S8). In fact, expressing *UASFra*Δ*C* in the eagle neurons of wild type animals results in a decrease in *comm* expression in EWs; an observation consistent with FraΔC's function as a dominant negative (fig. S9). Altogether these results support a cell autonomous requirement for Fra to activate *comm* transcription in commissural neurons as they cross the midline, and furthermore this effect is dependent on an intact cytoplasmic domain.

To test whether Fra is sufficient to induce *comm* mRNA expression, we over-expressed Fra in a subset of ipsilateral neurons, the apterous (Ap) neurons. In wild type embryos, the Ap neurons do not express *comm*. Only stochastic expression of *comm* can be detected at late stages in these neurons (stage 16 and 17) (Fig. 3, A and C arrows) (12). Over-expressing a *UASFra-myc* transgene in the Ap neurons frequently induces ectopic *comm* mRNA expression (16% of hemi-segments contain Ap neurons that express *comm*, n = 160 hemi-segments) (Fig. 3, D and F arrows). In addition Fra expression causes the Ap axons to cross the midline in many segments (35%, n=18) (Fig. 3E asterisks). Therefore, Fra is both necessary and sufficient for *comm* mRNA expression in subsets of neurons *in vivo*.

Since Netrins are the ligands for DCC to activate downstream gene transcription during vertebrate axon outgrowth and turning, we tested whether Netrins are required for *comm* transcription. Unexpectedly, there is no reduction of *comm* mRNA in the eagle neurons of *netAB* mutants compared to *netAB*/+ siblings (Fig. 4 and fig. S8). Even in the EWs that fail to cross the midline, *comm* mRNA is expressed normally, again arguing that midline crossing is not required to induce *comm* transcription (Fig. 4, D and F arrows). In addition, expressing either a *UASMyr-Fra-Myc* transgene that removes the entire extracellular domain of Fra (and therefore its ability to bind Netrin) or a *UASFraΔP1ΔP2ΔP3-Myc* transgene can also rescue *comm* mRNA expression (fig. S8). Accordingly, the midline crossing defects of the EW axons in these embryos are partially rescued, resulting in a milder phenotype (Table S1). The conserved cytoplasmic P3 motif of Fra is required for Netrin-mediated attraction (14). Therefore, FraΔP1ΔP2ΔP3 loses its chemoattractive function, but still retains the ability to activate *comm* transcription. These results support the idea that Netrins are not the ligands for Fra to induce *comm* transcription, and indicate that chemoattraction and the regulation of *comm* expression are controlled by distinct regions of the Fra cytoplasmic domain. Moreover, the transcriptional activation of *comm* appears to be independent of any of the conserved P motifs.

Together these results suggest that to ensure midline crossing, Fra signaling has dual functions in commissural neurons: first it mediates Netrin-dependent axon attraction and second it leads to Netrin-independent activation of *comm* transcription. Comm, in turn, down-regulates Robo levels on commissural axons, allowing midline crossing (fig. S10). If this model is correct, the guidance defects observed in *fra* mutants should be due to a combination of the loss of attraction and a failure to activate *comm* transcription, and at least four genetic predictions can be made. First, *fra* mutants should have more severe EW commissural guidance defects than *netAB* mutants. Second, expressing *UASComm* transiently in commissural neurons should partially rescue the guidance defects in *fra* mutants and these partially rescued *fra; UASComm* mutant animals should have similar guidance defects to *netAB* mutants. Third, *fra, robo* double mutants should display the same severity of defects as *fra; UASComm* animals. Finally, expressing *UASComm* in commissural neurons of *netAB* mutants should have no effect on the midline crossing defects.

To test these predictions, we compared the EW axon guidance defects in the genotypes described above and a phenotypic analysis was performed blind to genotype (Fig. 5). As predicted, the EW guidance defects in *fra* mutants are significantly stronger than those in

netAB mutants (Fig. 5, B, F and H; Table S1). Expressing *UASComm* in the eagle neurons of *fra* mutants partially rescues the EW guidance defects leading to a phenotype similar to that observed in *netAB* mutants (Fig. 5, C and I; Table S1). Similarly, the EW guidance defects in *fra, robo* double mutants are also less severe than *fra* single mutants (Fig, D and I; Table S1) (20). Finally, over-expression of *UASComm* in *netAB* mutants does not affect the guidance defects (Fig. 5, F and G; Table S1). These observations strongly support a Netrin-independent role for Fra in triggering *comm* transcription. Fra-dependent transcriptional regulation is unlikely to be the only mechanism to activate *comm* expression, since *fra* mutants have less severe commissural guidance defects than *comm* mutants.

Preventing conflicting signals at the midline from confusing navigating axons is fundamental to neuronal development. One mechanism that may allow axons to coordinate their responses to conflicting attractive and repulsive signals has been described in cultured *Xenopus* spinal neurons, where Slit induces a physical interaction between Robo and DCC/Fra (21). This direct receptor-receptor interaction silences Netrin attraction and this mechanism is proposed to prevent post-crossing commissural axons from re-crossing the midline (21). Here, we provide *in vivo* evidence supporting a distinct mechanism to regulate axon responses: two conserved guidance receptor signaling pathways (Fra and Robo) are coupled through a transcriptional event in pre-crossing commissural neurons to prevent premature repulsive responses, and therefore ensure midline crossing. Although transcriptional regulation by Netrin-DCC signaling is required for embryonic axon outgrowth and turning *in vitro*, it is less clear whether it is relevant *in vivo*. Furthermore, to our knowledge no transcriptional target gene(s) important for axon pathfinding has been identified. Here we show that Fra signaling triggers a transcriptional event *in vivo*, and identify a specific target gene- *comm*- a key regulator of repulsion at the *Drosophila* midline.

Surprisingly, Fra-mediated transcriptional activation is Netrin-independent, raising the question of whether there is an extrinsic midline signal required to activate Fra-dependent *comm* transcription. The spatial/temporal *comm* expression pattern is tightly associated with midline crossing, strongly suggesting the existence of such a midline signal. At first glance, our finding that Fra-induced *comm* transcription can be restored by expression of a myristolated Fra cytoplasmic domain seems inconsistent with this idea. While it may be tempting to conclude from this observation that the regulation of comm is strictly ligand independent, it is also possible (and in our view likely given the tight temporal window of comm expression) that the myrFracyto construct is either constitutively active or that it can associate with a co-receptor. Indeed, a similar construct when expressed in *C. elegans* leads to constitutive activity (22), and myristolated guidance receptor cytoplasmic domains have been shown to be competent to interact with co-receptors in a ligand-dependent manner (23, 24). Identifying the signals that trigger *fra* to activate *comm* transcription and determining how these events are restricted to commissural neurons are of high future priority.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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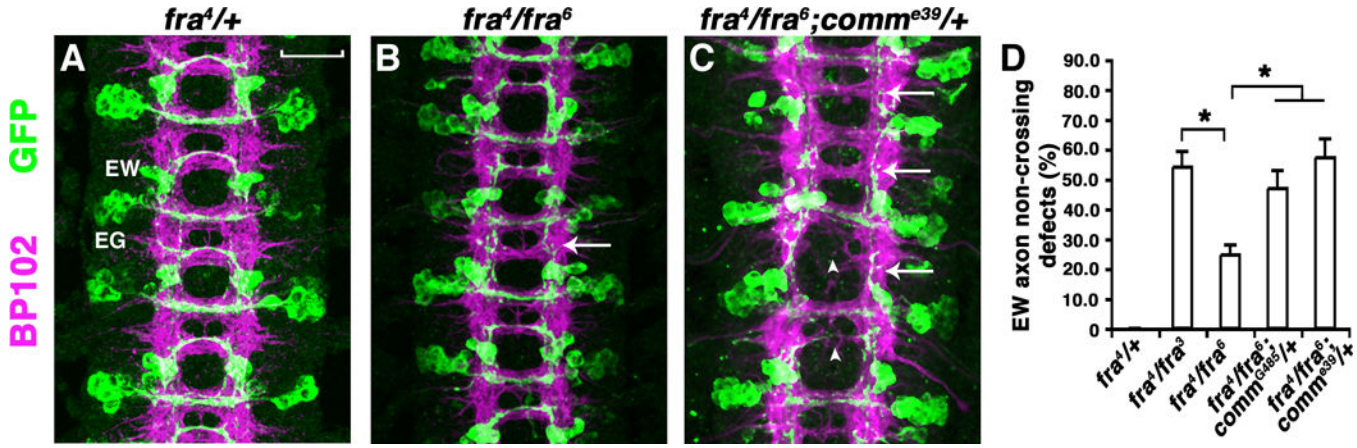

Fig. 1. Genetic interaction between *fra* and *comm*

(A to C) Stage 16 *eglGal4::UASTau-MycGFP* embryos stained with MAb-BP102 (magenta) to display all CNS axons and anti-GFP (green) to visualize the eagle neurons. Anterior is up. (A) In fra^4/+ or fra^4/+; $comm^{e39}$/+ embryos, EW and EG neurons (white labels) project their axons across the midline in almost every segment. (B) fra^4/fra^6 mutants have normal commissure formation and a mild EW axon non-crossing defect (arrow). (C) Compared to fra^4/fra^6, fra^4/fra^6; $comm^{e39}$/+ embryos have missing and thin commissures in many segments (arrowheads) and many EW axons also fail to cross the midline (arrows). (D) Quantification of EW axon non-crossing defects. Error bars represent standard error of the mean. Asterisks denote $p < 0.02$ in a Student's t test. Scale bar in (A), 20 microns.

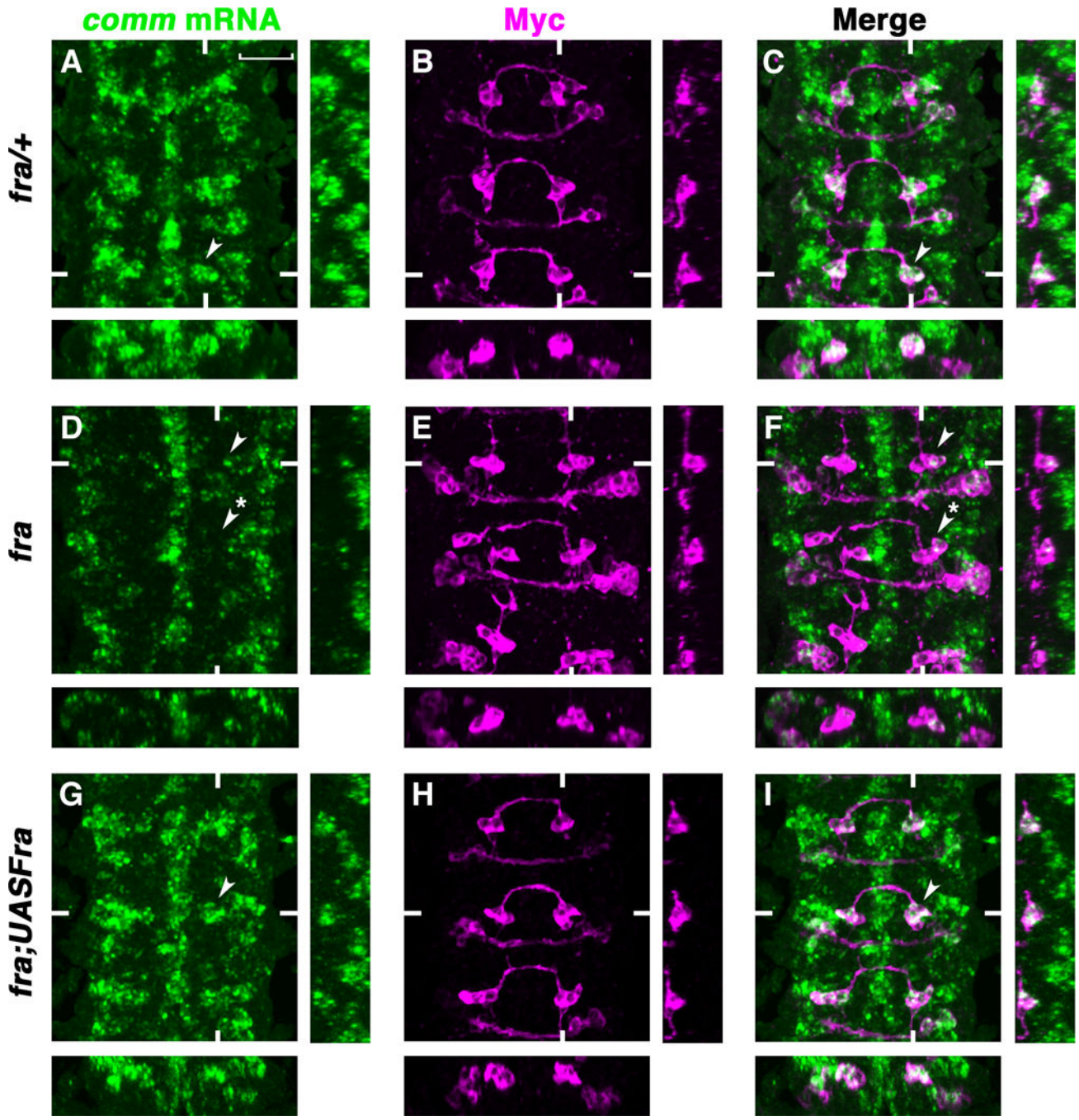

Fig. 2. Fra is required cell-autonomously for *comm* mRNA expression

(A to I) Stage 14 *eglGal4::UASTau-MycGFP* embryos double-labeled with RNA in situ probes for *comm* (green) and anti-Myc (magenta) to visualize the eagle neurons. Anterior is up. Confocal sections of the EWs are shown. White hash marks indicate the positions of the XZ and YZ sections. (A to C) *comm* mRNA expression in the EWs of *fra*/+ embryos (arrowheads). (D to F) *comm* mRNA is reduced in the EWs of fra^4/fra^3 mutants (arrowhead, EW with crossing defect; starred arrowhead, EW that projects normally). (G to I) Expressing

UASFra-Myc in the eagle neurons of *fra* mutants rescues *comm* mRNA expression in the EWs (G and I arrowheads). Scale bar in (A), 20 microns.

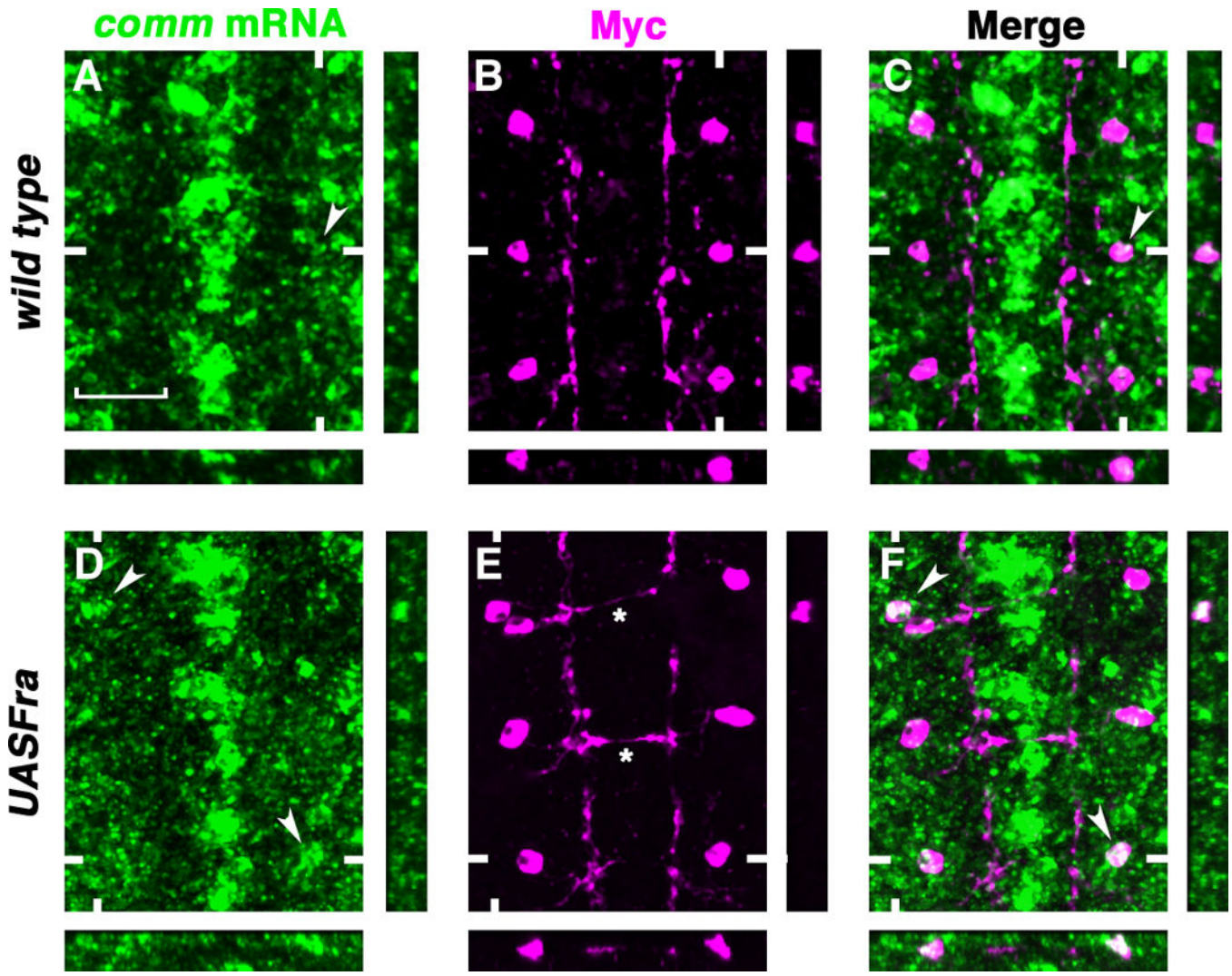

Fig. 3. Fra is sufficient to induce *comm* mRNA expression

(A to F) Stage 16 *aptGal4::UASTau-MycGFP* embryos double-labeled with RNA in situ probes for *comm* (green) and anti-Myc (magenta) to visualize the Ap neurons. Anterior is up. Confocal sections of the Aps are shown. White hash marks indicate the positions of the XZ and YZ sections. (A to C) Stochastic *comm* mRNA expression in the Ap neurons (arrowheads). (D to F) Expressing *UASFra-Myc* in the Ap neurons induces *comm* mRNA expression frequently [arrowheads in (D) and (F)] and leads to ectopic midline crossing in many segments [asterisks in (E)]. Scale bar in (A), 20 microns.

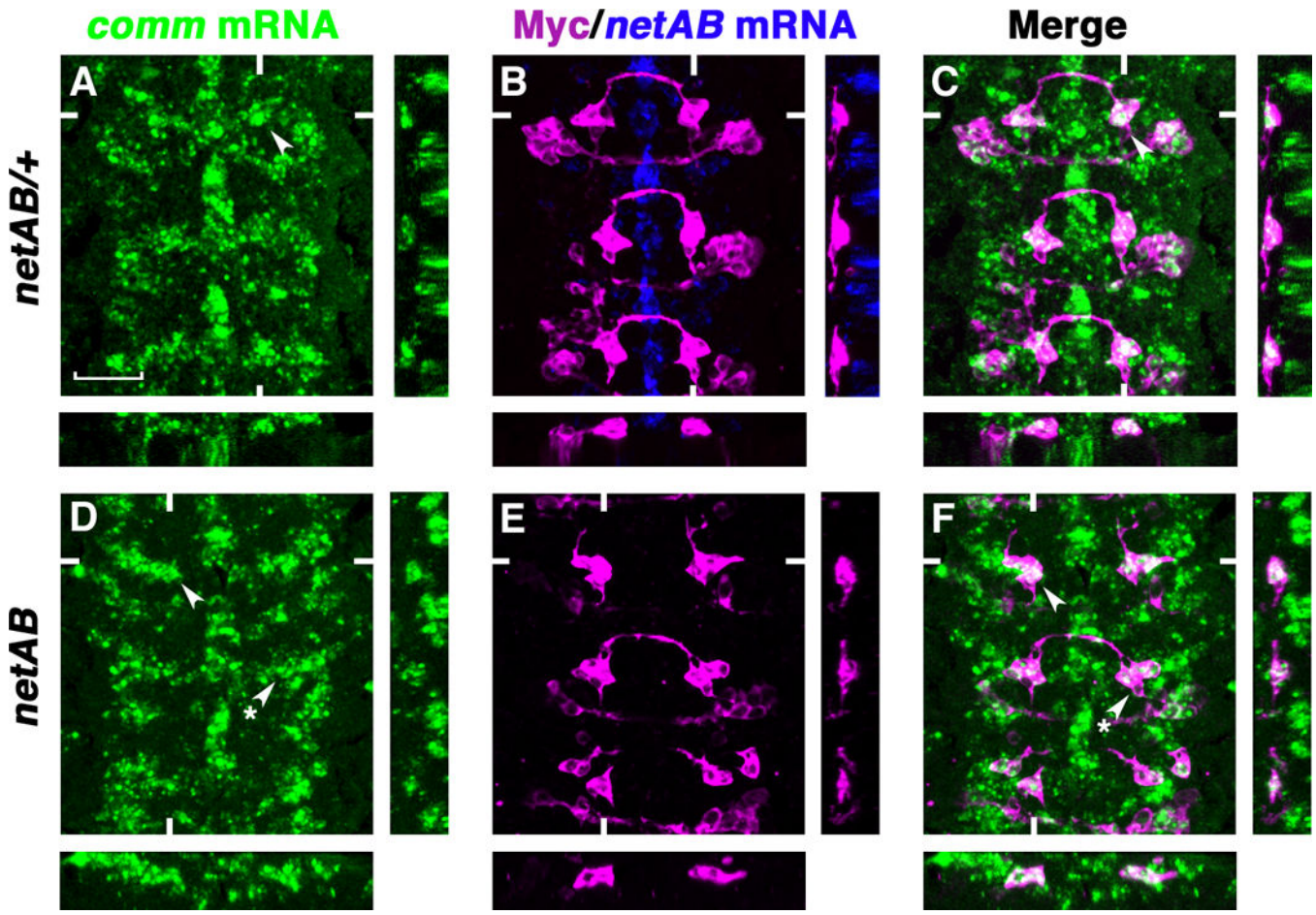

Fig. 4. Netrins are not required for *comm* mRNA expression

(A to F) Stage 14 *eglGal4::UASTau-MycGFP* embryos triple-labeled with RNA in situ probes for *comm* (green) and *netrinAB* (blue), and anti-Myc (magenta) to visualize the eagle neurons. Anterior is up. Confocal sections of the EWs are shown. White hash marks indicate the positions of the XZ and YZ sections. (A to C) *comm* mRNA expression in the EWs of *netAB/+* embryos (arrowheads). (D to F) *netAB* mutants have normal levels of *comm* mRNA expression (arrowheads indicate an EW that has crossing defects and starred arrowheads indicate an EW that projects normally). Scale bar in (A), 20 microns.

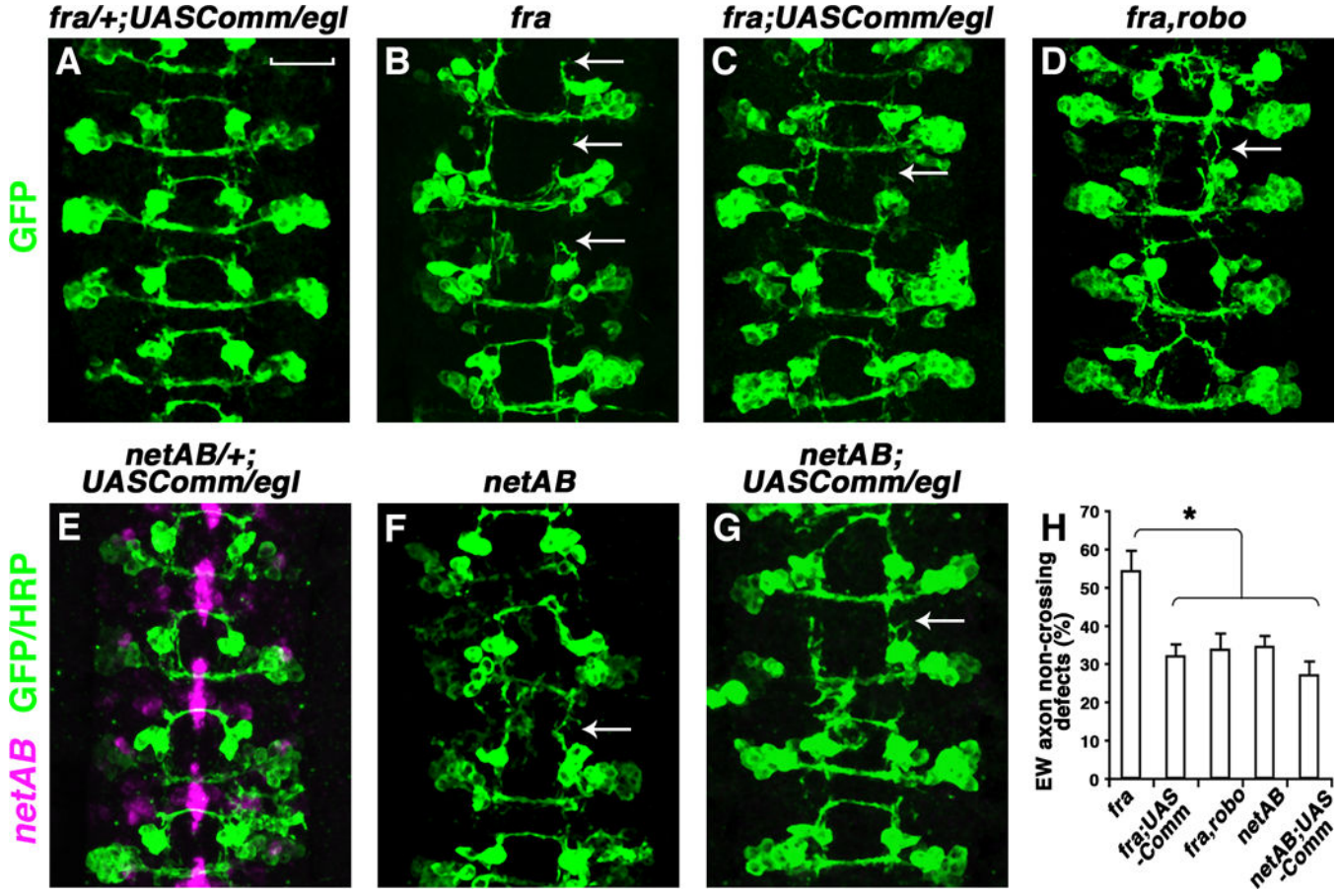

Figure 5. Expression of Comm partially rescues guidance defects in *fra* mutants

(A to G) Stage 16 *eglGal4::UASTau-MycGFP* embryos stained anti-GFP (green). Embryos in (E to G) were also labeled with RNA in situ probes for *netrinAB* (Magenta). Anterior is up. Over-expressing *UASComm* in the eagle neurons partially rescues the EW guidance defects in *fra* mutants [compare arrows in (B) and (C)], but not in *netAB* mutants [compare arrows in (F) and (G)]. The EW guidance defects in *fra,robo* mutants are also partially rescued [compare arrows in (B) and (D)]. Over-expressing *UASComm* in *fra/+* or *netAB/+* does not affect the trajectories of eagle neurons (A) and (E). (H) Quantification of EW axon non-crossing defect. Error bars represent standard error of the mean. Asterisk denotes $p < 0.001$ in a Student's t test. Scale bar in (A), 20 microns.