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Midline crossing by gustatory receptor neuron axons is regulated by *fruitless*, *doublesex* and the Roundabout receptors

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SUMMARY

Although nervous system sexual dimorphisms are known in many species, relatively little is understood about the molecular mechanisms generating these dimorphisms. Recent findings in *Drosophila* provide the tools for dissecting how neurogenesis and neuronal differentiation are modulated by the *Drosophila* sex-determination regulatory genes to produce nervous system sexual dimorphisms. Here we report studies aimed at illuminating the basis of the sexual dimorphic axonal projection patterns of foreleg gustatory receptor neurons (GRNs): only in males do GRN axons project across the midline of the ventral nerve cord. We show that the sex determination genes *fruitless* (*fru*) and *doublesex* (*dsx*) both contribute to establishing this sexual dimorphism. Male-specific Fru (Fru^M) acts in foreleg GRNs to promote midline crossing by their axons, whereas midline crossing is repressed in females by female-specific Dsx (Dsx^F). In addition, midline crossing by these neurons might be promoted in males by male-specific Dsx (Dsx^M). Finally, we (1) demonstrate that the *roundabout* (*robo*) paralogs also regulate midline crossing by these neurons, and (2) provide evidence that Fru^M exerts its effect on midline crossing by directly or indirectly regulating Robo signaling.

KEY WORDS: Axon guidance, *fruitless*, Midline crossing, *roundabout*, Sex determination, *Drosophila*

INTRODUCTION

Nervous system sexual dimorphisms are likely to underlie many sex-specific behaviors found in diverse animal species. Although environmental factors might play a role, many sexually dimorphic behaviors are innate, and thus the development of the circuitry subserving these behaviors is likely to be genetically specified (Baker et al., 2001; Greenspan, 1995). Innate sexual behaviors in genetically tractable organisms offer unique opportunities to identify the neuronal circuitry underlying sexual behaviors, unravel how this circuitry is genetically specified and elucidate the contributions of neuronal sexual dimorphisms to these behaviors.

In *Drosophila melanogaster*, male courtship behaviors are largely innate, as males raised in isolation will, when presented with a virgin female, readily perform the stereotyped behaviors that comprise courtship. Given that male courtship behavior is both sex-specific and innate, it is perhaps not surprising that it is controlled by the genetic program that regulates all aspects of sexual differentiation (Billeter et al., 2006a; Manoli et al., 2006).

Drosophila somatic sexual development is governed by a hierarchy of sex determination regulatory genes that terminates with *doublesex* (*dsx*) and *fruitless* (*fru*; Fig. 1A) (Christiansen et al., 2002; Manoli et al., 2006; Billeter et al., 2006a; Yamamoto, 2007; Dickson, 2008). *dsx* and *fru* are sex-specifically regulated at the level of pre-mRNA splicing, resulting in male- and female-specific mRNAs. In females, the female-specific *fru* mRNA is not translated (Usui-Aoki et al., 2000; Lee et al., 2000), whereas the female-specific *dsx* mRNA is translated into the Dsx^F protein. In males, *fru* and *dsx* mRNAs are translated into Fru^M and Dsx^M proteins. Dsx^F and Dsx^M are zinc-finger transcription factors that have the same DNA binding domain but different carboxy termini (Burtis and Baker, 1989; An et al., 1996; Erdman et al., 1996). In the case of *fru*, at least three Fru^M proteins are produced through the usage of three alternative 3′ exons, which encode alternative pairs of zinc-fingers (Ryner et al., 1996; Usui-Aoki et al., 2000).

The Fru^M proteins are both necessary and sufficient for nearly all aspects of male courtship behavior (Ryner et al., 1996; Anand et al., 2001; Demir and Dickson, 2005; Manoli et al., 2005). Fru^M is expressed only in postmitotic neurons, including ~1-2% of the neurons in the central nervous system (CNS). *fru^M*-expressing neurons are found largely in clusters throughout the brain and ventral nerve cord (VNC) (Lee et al., 2000). In addition, *fru^M* is expressed in subsets of the primary sensory neurons in the olfactory, gustatory, auditory and mechanosensory systems (Manoli et al., 2005; Stockinger et al., 2005). Finally, the *fru^M*-expressing neurons are dedicated to courtship (Manoli et al., 2005; Stockinger et al., 2005). Taken together, these findings support the hypothesis that the *fru^M*-expressing neurons comprise the circuitry subserving male courtship behavior (Ryner et al., 1996; Baker et al., 2001).

fru^M expression peaks during pupal development (Lee et al., 2000), suggesting that it regulates neuronal differentiation during metamorphosis. For most groups of *fru^M*-expressing neurons found in males there are homologous neurons in females (Ryner et al., 1996; Manoli et al., 2005; Stockinger et al., 2005). The initial characterizations of *fru^M*-expressing CNS neurons revealed few differences between the sexes in the gross neuroanatomical features of the *fru^M* circuitry, suggesting that the Fru^M proteins largely function to regulate fine neuronal connectivity or neural physiology (Manoli et al., 2005; Stockinger et al., 2005). Independently, it was

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shown that *fru*^{M} regulates the morphology and survival of certain CNS neurons (Kimura et al., 2005; Kimura et al., 2008) and is necessary for the proper differentiation of a group of serotonergic neurons (Billeter et al., 2006b; Lee and Hall, 2001).

Although *fru*^{M} is the master regulator of male courtship behavior, *dsx* function is also important for courtship behavior. One component of courtship song, sine song, requires Dsx^{M} function (Villella and Hall, 1996; Rideout et al., 2007). In addition, XY *dsx* mutant individuals show decrements in the performance of many steps of courtship, although they are able to proceed through courtship up to and including attempted copulation (Taylor et al., 1994; Villella and Hall, 1996). Consistent with a neural etiology of these courtship behavior defects, *dsx* is expressed in the CNS in 350-450 cells, the majority of which are neurons (Lee et al., 2002). Indeed, *dsx* governs the sex-specific pattern of proliferation of a small group of abdominal neuroblasts (Taylor and Truman, 1992), and the Dsx proteins are co-expressed with Fru^{M} in many neurons in the abdominal ganglion (Billeter et al., 2006b). In the periphery, *dsx* regulates the development of certain gustatory and mechanoreceptor sense organs in the foreleg and genitalia (Hildreth, 1965), and the Fru^{M} proteins are expressed in the neurons of these sense organs (Manoli et al., 2005; Stockinger et al., 2005). Finally, *dsx* acts in concert with *fru*^{M} to masculinize parts of the CNS, suggesting that Dsx^{M} (and the absence of Dsx^{F}) is required to fully masculinize the circuitry underlying male behaviors (Billeter et al., 2006b; Rideout et al., 2007; Sanders and Arbeitman, 2008; Kimura et al., 2008). A rigorous analysis of sexually dimorphic neural development should therefore account for both *fru*^{M} and *dsx*.

The gustatory receptor neurons (GRNs) in the foreleg are an attractive starting point for unraveling the roles of *dsx* and *fru*^{M} in sensory neuron development. The exchange of gustatory information during tapping appears to be important for species and sex discrimination (Spieth, 1974; Bray and Amrein, 2003; Miyamoto and Amrein, 2008), providing a clear behavioral context. Moreover, sexual dimorphisms in at least two distinct aspects of foreleg GRN development are known. First, the number of gustatory sense organs, called gustatory sensilla, in the first four tarsal segments (T1-T4) of the foreleg is higher in males than in females (Nayak and Singh, 1983). We have found that this sexual dimorphism is controlled by *dsx* (D.J.M., unpublished results). Second, whereas foreleg GRNs of both sexes send axonal projections to the VNC, only in males do these projections cross the midline (Possidente and Murphey, 1989).

We focus here on the sexually dimorphic midline crossing by foreleg GRN axons. We show that midline crossing by foreleg GRN axons is regulated by both *dsx* and *fru*, but to different degrees. Fru^{M} is required in foreleg GRNs in order for their axons to cross the midline, whereas Dsx^{M} and Dsx^{F} have less prominent roles in promoting and repressing midline crossing, respectively. We also (1) demonstrate that the *roundabout* (*robo*) genes act in the foreleg GRNs to regulate midline crossing, and (2) provide genetic evidence that Fru^{M} promotes midline crossing through the direct or indirect repression of Robo signaling.

MATERIALS AND METHODS

Fly stocks used

Unless otherwise indicated, all crosses were at 25°C under standard conditions. To examine GRN projections in *dsx* mutants, *w*; *UAS-mCD8::GFP*; *FRT82B dsx*^{1}, *poxn-Gal4-14-1-7/TM6B* females were crossed to *w, 3XP3-DsRed; dsx*$^{m+R13}$*/TM6B* males. To generate *dsx*-masculinized females, *yw, 3XP3-DsRed; dsx*^{D}*/TM6B* males were crossed to *w; UAS-mCD8::GFP; FRT82B dsx*^{1}, *poxn-Gal4-14-1-7/TM6B* females. *poxn-Gal4-14-1-7* was provided by M. Noll (Boll and Noll, 2002). The X-chromosomal transgene *3XP3-DsRed*, a gift from O. Schuldiner (Schuldiner et al., 2008), was used to distinguish the chromosomal sex of *dsx*^{1}*/dsx*$^{m+R13}$ and *dsx*^{1}*/dsx*^{D} flies.

To generate flies null for *fru*^{M}, *w; UAS-mCD8::GFP; fru*$^{P1.LexA}$, *poxn-GAL4-14-1-7/TM6B* males were crossed to either *fru*$^{4-40}$*/TM6B* or *fru*^{sat15}*/TM6B* females. To examine *fru*^{M}-masculinized females, *w; UAS-mCD8::GFP; fru*$^{\Delta tra}$*/MKRS* males were crossed to *poxn-Gal4-14-1-7/TM6B* females (*fru*$^{\Delta tra}$ was provided by B. Dickson). To examine *fru*^{M}-masculinized females in a *dsx*-null background, *w, 3XP3-dsRed; +/CyO; FRT82B dsx*^{1} *fru*$^{\Delta tra}$*/TM6B* males were crossed to *w; UAS-mCD8::GFP; FRT82B dsx*^{1}, *poxn-Gal4/TM6B* females. To examine the effect of *fru*^{M} dose on midline crossing, *w; UAS-mCD8::GFP; poxn-Gal4-14-1-7/TM6B* females were crossed to either *fru*$^{4-40}$*/TM6B* or *fru*^{sat15}*/TM6B* males. To knockdown the *fru*^{M} transcript in GRNs, *w; UAS-fru*^{M}*IR, UAS-mCD8::GFP/CyO; UAS-fru*^{M}*IR, fru*$^{4-40}$ males were crossed to *poxn-Gal4/TM6B* females, and progeny were raised at 29°C.

For *robo* RNAi experiments, males were crossed to *w; UAS-mCD8::GFP; poxn-Gal4-14-1-7* females and progeny were raised at 29°C. To knockdown transcript levels for all three *robo* family genes, we used *w; UAS-RoboRNAi, UAS-Robo2RNAi, UAS-Robo3RNAi/ T(2;3)SM6-TM6B* (provided by P. Garrity; Tayler et al., 2004). Stocks to knockdown each *robo* paralog individually were obtained from the Bloomington stock center. To overexpress *robo* and *robo2*, we used *w; UAS-robo/TM3* and *w; UAS-Robo2* (P. Garrity), respectively.

Preparation and examination of tissues

Tissues were dissected in PBS and fixed in 4% paraformaldehyde in PBS for 30-45 minutes, then rinsed in PBS before being mounted in Vectashield. To examine *poxn-Gal4* and *fru*$^{P1.LexA}$ expression in the foreleg, pupae were removed from their pupal case and fixed at the times indicated. Following fixation, pupae were wholemounted, ventral side up. For examination of *poxn-GAL4*-expressing GRN axon morphology, ventral nerve cords were dissected from 2- to 5-day-old adults. Antibody staining [1:1000 dilution of rabbit α-GFP (Invitrogen) and 1:40 dilution of mouse α-Robo (13C9, *Drosophila* Studies Hybridoma Bank)] was performed as described by Truman (Truman et al., 2004). Imaging was performed on a Zeiss LSM 510 confocal microscope and Z-stacks were analyzed and collapsed using ImageJ. Images were cropped and rotated with Adobe Photoshop.

Generation of *fru*$^{P1.LexA}$ through homologous recombination

Our procedure for ends-out homologous recombination (Gong and Golic, 2003) has been previously described (Manoli et al., 2005). Briefly, an ~3 kb fragment with 5′ *Kpn*I and 3′ *Sac*II 5′ to the *fru*^{M} start codon and an ~2.5 kb fragment with 5′ *Nhe*I and 3′ *Stu*I that begins with codon 3 of *fru*^{M} were independently subcloned, sequence-verifed and then cloned into the *pWhiteOut2* vector (a gift from J. Sekelsky, University of North Carolina, USA) to create a backbone vector for homologous recombination. The LexA:VP16 coding sequence, a gift from the laboratory of L. Luo (Stanford University, USA), was subcloned with 5′ *Sac*II and 3′ *Xba*I, and a transcription stop cassette containing the SV40-polyA and *D. melanogaster* α*-tubulin* transcription termination sequence (Stockinger et al., 2005) was subcloned with 5′ *Xba*I and 3′ *Nhe*I sites. These latter fragments were then cloned into the backbone vector above. This donor construct was then transformed into embryos using standard protocols.

Ectopic LexA:VP16 expression was examined by crossing donor lines to a LexA-responsive GFP reporter line, LexA-hrGFP, a gift from Gunter Merdes (Loewer et al., 2004). Donors without ectopic LexA:VP16 expression were used for mobilization as previously described (Manoli et al., 2005), with LexA-driven expression of GFP used as a primary screen for mobilization and proper integration. Integration of the LexA construct into the *fruitless* locus was also verified by PCR, followed by sequencing.

Experiments for testing *fru*$^{P1.LexA}$

To verify that *fru*$^{P1.LexA}$ is a genetic null, males carrying *fru*$^{P1.LexA}$ and either a wild-type allele of *fru* or previously characterized deletions of the *fru* locus were tested for courtship behavior. Briefly, males were collected at eclosion and aged individually for 4-6 days at 25°C and 12:12 hours light:dark. For testing, males and females were lightly anesthetized with CO_2 and loaded separately into circular mating arenas (10 mm diameter, 8 mm depth). All

flies were allowed three hours to recover prior to observation. All behavioral tests were conducted at 25°C and 50% humidity, between circadian stages ZT7 and ZT10. To test fertility, a single male of the indicated genotype was raised in isolation for 3-5 days post-eclosion, then placed in a new vial with three virgin Canton-S females. Vials were checked for progeny after 5 days. Only those vials in which the male and at least one female were still alive after 5 days were counted. Chaining assays were performed as described by Villella (Villella et al., 1997). Immunohistochemistry and analysis of Fru^M protein and 5HT were performed as described by Manoli (Manoli and Baker, 2004).

Generation of *UAS-fru^{MC}::AU1*

A synthesized fragment containing a coding sequence for the *AU1* epitope tag (5′-CCCAAGCTTGCAGCAGATACTTACCGATACATCTAATA-AGGTACCGG-3′) (Lim et al., 1990) was subcloned into *pBSKSII(+)* using *Kpn*I and *Hin*dIII (*pBSKII-AU1*). PCR using primers 5′-CCGGAAT-TCATGATGGCGACGTCACAGGATTAT-3′ and 5′-CCCAAGCTTTG-CTGCAATGGATGAGTTCAGCTTGAGCAGGCG-3′ was performed, using as a template a plasmid containing the *fru^{MC}* sequence (Song et al., 2002). This PCR product was subcloned into *pBSKII-AU1* using *Eco*RI and *Hin*dIII, producing a construct in which the stop codon was removed from *fru^{MC}*, and the *AU1* epitope was added in frame following a short linker (*fru^{MC}::AU1*). *fru^{MC}::AU1* was then cut from *pBSKII(+)* using *Eco*RI and *Kpn*I and subcloned into *pUAST*.

Generation of *UAS-fru^{C}IR*

PCR amplification using primers 5′-TCTAGAAACGCGAGTAC-CATCCTCT-3′ and 5′-TCTAGAGGTGTGGGAGTGAAAGTGG-3′ was performed to amplify a 308 bp fragment specific to the C exon of *fruitless* while simultaneously adding *Xba*I sites, using as a template a plasmid containing the *fru^{MC}* sequence (Song et al., 2002). This fragment was inserted into *pWiz* (Lee and Carthew, 2003) using the standard procedure available through FlyBase. The resulting inverted repeat construct was in the 5′-3′/3′-5′ orientation.

Generation of *lexAop-FRT-tdTomato::nls*

The *tdTomato* gene was amplified from the plasmid *pRSEtb-tdTomato* (a gift from R. Tsien; Shaner et al., 2004) using the primers 5′-ACCGGTATGG-TGAGCAAGGGCGAGGAGGTCATCAAA-3′ and 5′-TAGAGCGCTG-GCGATGCCTTCTGTGCCTGCTCTTTGCCTTGTACAGCTCGTCCAT-3′. A nuclear-localization sequence was amplified from the plasmid *pStinger* (Barolo et al., 2000) using the primers 5′-AAGAGCAGGCACAGAA-GGCAT-3′ and 5′-GTACCGGTCATTAGCGTCTTCGTTCACTGCGA-CTT-3′ and fused to the C-terminus of *tdTomato* by overlapping PCR. The resulting *tdTomato::nls* fragment was cloned into *pCR2.1* (Invitrogen) and then sequence-verified. Next, we used the flanking *Age*I sites to clone *tdTomato::nls* into the transformation vector *pLexOT* (gift of Liqun Luo lab, Stanford University, USA), which had been modified to carry an FRT-(transcriptional stop)-FRT cassette (Stockinger et al., 2005). The resulting plasmid was introduced into the *Drosophila* genome using standard transformation techniques. A single second chromosome *P{lexAop-FRT-Stp-FRTtdTomato::nls}* insertion was selected for germ-line flip-out of the transcriptional stop cassette. The resulting transgene, *P{lexAop-FRT-tdTomato::nls}*, was then recombined with *P{UAS-stinger}* (Barolo et al., 2000), allowing visualization of overlap between *Gal4* and *LexA*.

RESULTS

Foreleg GRN axons have *dsx*-independent, sexually dimorphic morphology

The foreleg GRN axons project into the prothoracic leg neuromere in the VNC. In males, many of these projections cross the midline, but no midline crossing is seen in females (Possidente and Murphey, 1989; Boll and Noll, 2002). In females (XX) lacking *tra* or *tra2* function (which express Fru^M and Dsx^M and develop somatically as males), some foreleg GRN axons cross the midline (Possidente and Murphey, 1989), suggesting that GRN axon guidance is regulated by *dsx* and/or *fru^M* (Fig. 1A).

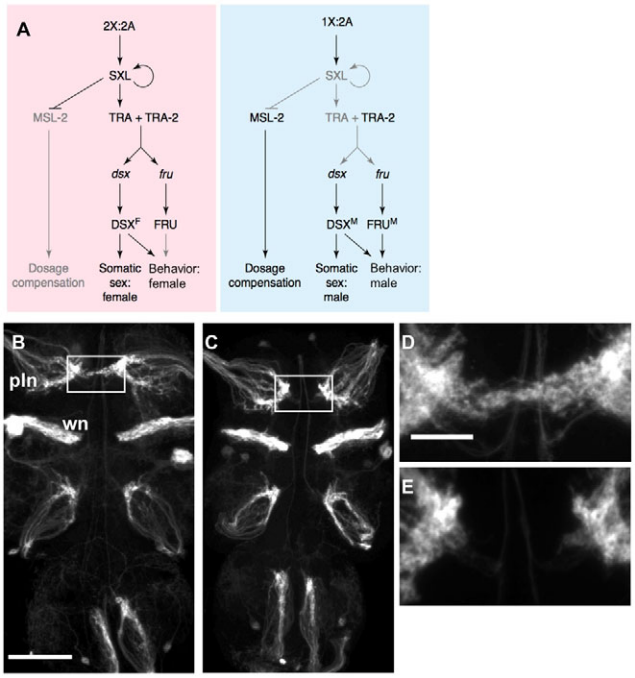


Fig. 1. Sex and taste in *Drosophila*. (**A**) Sex determination hierarchy. The X chromosome-to-autosome ratio activates (black) or leaves inactive (gray) a splicing cascade regulating the development of somatic sex and behavior. (**B**) Confocal image of gustatory receptor neuron (GRN) axons in the adult male ventral nerve cord (VNC), labeled with *poxn-Gal4*-driven *UAS-mCD8::GFP*. Image is oriented anterior toward the top. (**C**) *poxn-Gal4* in an adult female VNC. (**D**,**E**) Commissural regions of B and C, respectively. Note the commissural GRN projections in the male. pln, prothoracic leg neuromere; wn, wing neuromere. Scale bars: 100 μm in B; 25 μm in D,E.

Two simple models can account for the male-specific presence of GRN axons that cross the midline. First, it could be that GRNs able to send axonal projections across the midline are present only in males. Under this model, as *dsx* alone regulates the number of gustatory sensilla (and hence GRNs) (D.J.M., unpublished results), *dsx* should indirectly control midline-crossing by GRN axons. Alternatively, it could be that GRN axon morphology is sex-specifically regulated independently of the establishment of gustatory sensilla.

To examine the axon morphology of the entire population of foreleg GRNs, we used *poxn-Gal4-14-1-7* (hereafter referred to as *poxn-Gal4*), which is expressed in GRNs, neurons in the central brain, and a small number of cells in the VNC (Boll and Noll, 2002). GRN projections from the wings and legs were clearly labeled when *poxn-Gal4* is used to drive *UAS-mCD8::GFP* expression, and the presence of contralateral foreleg GRN projections in males (Fig. 1B,D), and the absence of these projections in females (Fig. 1C,E), are evident.

We then investigated the effect of *dsx* on GRN axon morphology. As expected, control sibling (*dsx^1*/TM6B) males had many midline-crossing GRN axons, whereas only a single thin contralateral projection was observed in 12 control (*dsx^1*/TM6B) females (Fig. 2). In *dsx* mutant individuals (*dsx^1*/*dsx^{m+R13}*), males had a much higher level of midline-crossing than females, similar to what is seen in *dsx$^+$* control males and females. This difference was visually evident (Fig. 2B) and was quantified by measuring the average fluorescence of the commissural region (Fig. 2A; see Fig. S3 in the supplementary

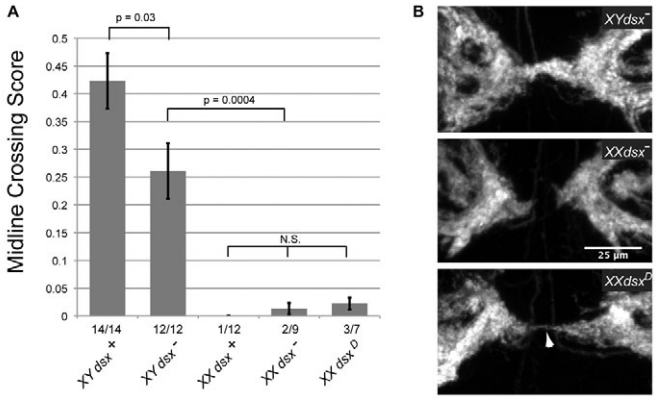
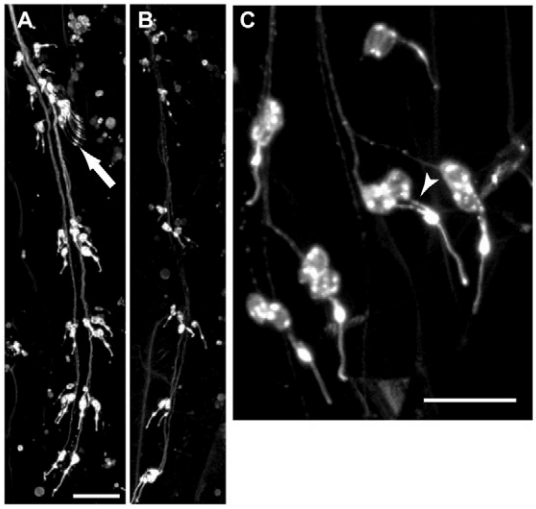


Fig. 2. Foreleg GRN axon morphology is sex-specifically regulated independent of *dsx*. (**A**) Average level of GRN midline crossing by genotype. The midline crossing score is the average fluorescence of the commissural region divided by the average fluorescence just lateral to the midline (see Fig. S3 in the supplementary material). Also shown for each genotype is the fraction of total scored individuals that show any midline crossing, regardless of level, with the denominator indicating the *n* for the experiment. Genotype abbreviations: dsx$^+$ (*UAS-mCD8::GFP*; *FRT82B dsx^1*, *poxn-Gal4/TM6B*); dsx$^-$ (*UAS-mCD8::GFP*; *FRT82B dsx^1*, *poxn-Gal4/dsx^{m+R13}*); *dsx^D* (*UAS-mCD8::GFP*; *FRT82B dsx^1*, *poxn-Gal4/dsx^D*). *dsx$^+$* and *dsx$^-$* are siblings. *dsx^D* are from a separate cross. Error bars indicate s.e.m. *t*-tests were used to obtain *P*-values. N.S.=not significant. (**B**) Images of foreleg gustatory receptor neuron (GRN) projections as in Fig. 1. The GRN projections of *dsx*-null animals are sexually dimorphic. The bottom panel shows an example of the midline crossing (arrowhead) seen in three of the seven *dsx^D* females.



D

Number of poly-innervated sense organs

	Male		Female	
	poxn-Gal4 +	*fru$^{P1.LexA}$* +	*poxn-Gal4* +	*fru$^{P1.LexA}$*+
T1	10.5 ±0.3	9.5 ±0.3	6.9 ±0.1	7.0 ±0.2
T2	7.3 ±0.3	7.2 ±0.1	4.4 ±0.2	4.0 ±0.0
T3	6.0 ±0.0	6.0 ±0.1	4.0 ±0.0	4.0 ±0.1
T4	6.3 ±0.6	6.0 ±0.2	4.4 ±0.2	2.1 ±0.1
T5	not counted	2.0 ±0.0	not counted	2.1 ±0.1

Fig. 3. *fru$^{P1.LexA}$* expression in the foreleg. (**A**,**B**) Composite confocal images of a *w*; *lexAop-rCD2::GFP*; *fru$^{P1.LexA}$*/+ male (A) and female (B) foreleg, 28 hours after puparium formation (APF). Arrow in A is pointing to the neurons of the sex comb. (**C**) *fru$^{P1.LexA}$* expression in male tarsal segment 3 (T3) at 28 hours APF. Expression is seen in pairs of neurons. Arrowhead points to two dendrites, arising from two different neurons, which come together to project into a single bristle. These features are indicative of a gustatory sensillum. (**D**) Average number (±s.e.m.) of sensilla-containing multiple *fru^{LexA}*-expressing neurons in each segment of the foreleg, compared with the number of *poxn-Gal4*+ (gustatory) sensilla. Scale bars: 50 μm in A,B; 25 μm in C.

material). Furthermore, although *dsx*-null females did not differ significantly from their *dsx$^+$* sibling females with respect to the midline crossing phenotype, *dsx*-null males were observed to have less midline crossing than their *dsx$^+$* male siblings. We conclude that *dsx* function is not necessary for establishing sex-specificity in GRN axon morphology, although Dsx^M might promote midline crossing. It is worth recalling that *dsx* specifies the sexual dimorphism in the number of gustatory sensilla seen in wild-type animals (D.J.M., unpublished results) and that XX and XY animals lacking *dsx* function have the same number of gustatory sensilla. Thus, *dsx* might have distinct roles in (1) specifying the number of gustatory sensilla, and (2) regulating the axonal morphology of their neurons.

We also examined *XX; dsx^D*/*dsx^1* individuals, which develop a full set of male-specific foreleg GRNs. Three of seven *XX; dsx^D*/*dsx^1* individuals had some midline-crossing GRN projections, but many fewer such axons than observed in control males (Fig. 2). Thus, although Dsx^M function might contribute somewhat to midline crossing, it is not sufficient to produce the wild-type pattern of male GRN axon morphology.

fru^M is expressed in both male-specific and sex-nonspecific GRNs

We next examined the role of *fru^M* in foreleg GRN midline crossing. We first asked whether *fru^M* is expressed the right place and time to regulate GRN axon guidance. For this purpose, we used a new *fru* allele (*fru$^{P1.LexA}$*) into which sequence coding for the bacterial transcription factor *LexA* fused with the VP16 activation domain (Lai and Lee, 2006) was inserted by homologous recombination at the first codon of the *fru^M* open reading frame. This insertion allows for the expression of LexA::VP16 in the same pattern as transcripts from the *fru^M* promoter (*P1*), which is transcribed in both males and females. *fru$^{P1.LexA}$* can be used to label *fru^M*-expressing neurons in males and the homologous neurons in females when combined with *lexAop-rCD2::GFP*, a reporter containing LexA binding sites (Lai and Lee, 2006). *fru$^{P1.LexA}$* has the expected characteristics of a *fru^M*-null allele (see Tables S1 and S2 in the supplementary material).

In the foreleg, *fru$^{P1.LexA}$* drove *lexAop-rCD2::GFP* in a pattern indistinguishable from other reporters of *fru^M* expression (*fru^{Gal4}* and *fru$^{P1.Gal4}$*; data not shown). In both sexes, *fru$^{P1.LexA}$* expression was first seen in neurons at around 18-20 hours after puparium formation (APF) in tarsal segments T3-T5, with little or no expression seen in T1-T2. By 24-28 hours APF, *fru$^{P1.LexA}$* was expressed in neurons in every tarsal segment (Fig. 3A,B). Of those neurons that expressed *fru$^{P1.LexA}$*, most were found in groups of two or more (Fig. 3C). Because mechanosensory organs are only singly innervated, whereas gustatory sensilla are multiply innervated, groups of *fru$^{P1.LexA}$*-expressing (*fru$^{P1.LexA}$*+) neurons whose dendritic projections converged were considered to belong to a single gustatory sensillum.

To determine which gustatory sensilla contain Fru^M-expressing neurons, we compared counts of *fru$^{P1.LexA}$*+ sensilla with counts of *poxn-Gal4*+ sensilla, given that (1) *poxn-Gal4* is expressed in all GRNs (Boll and Noll, 2002), and (2) our own counts of *poxn-*

Gal4+ sensilla (Fig. 3D) are comparable to counts of total gustatory sensilla based on bristle morphology (Nayak and Singh, 1983; Meunier et al., 2000). The number of foreleg gustatory sensilla containing $fru^{P1.LexA}$+ neurons in males did not significantly differ from counts of *poxn-Gal4*+ sensilla in males in segments T2-T4 (Fig. 3D; P>0.5, Welch two-sample t-test). In T1, the difference between putative $fru^{P1\text{-}LexA}$+ gustatory sensilla and *poxn-Gal4*+ sensilla was small (~1), but statistically significant (P=0.04, Welch two-sample t-test), and probably reflects difficulty in distinguishing $fru^{P1.LexA}$+ GRNs from $fru^{P1.LexA}$+ neurons of the sex comb in the distal part of T1. These counts suggest that fru^{M} is expressed in a subset of neurons innervating every gustatory sensillum in the T1-T4 segments of the male foreleg. We also saw $fru^{P1.LexA}$ expression in neurons innervating two gustatory sensilla in T5, where sexual dimorphisms in GRN number/morphology have not been previously reported. The male pattern of $fru^{P1.LexA}$ expression indicates that fru^{M} is expressed in both male-specific GRNs as well as those GRNs homologous between males and females. Accordingly, we also saw $fru^{P1.LexA}$ expressed in GRNs in females, but in fewer gustatory sensilla than seen in males, reflecting the sexual dimorphism in gustatory sensilla number.

Within each gustatory sensillum, $fru^{P1.LexA}$ was most often expressed in two neurons, although expression was occasionally seen in three neurons (Fig. 3C). Thus, fru^{M} expression is restricted to a subset of the five neurons found in each gustatory sensillum. We also examined overlap between *poxn-Gal4* and $fru^{P1.LexA}$ in the adult, and consistently saw $fru^{P1.LexA}$ expressed in 2 (occasionally 3) cells per gustatory sensillum (see Fig. S1 in the supplementary material). It is unknown which neuron subtypes express fru^{M}.

fru^{M} is necessary for midline crossing by GRN axons

We next asked whether fru^{M} is responsible for the sex-specific regulation of GRN axon morphology by examining GRN axons in the VNCs of males deficient for fru^{M}, using three different null genotypes: $fru^{P1.LexA}/fru^{sat15}$, $fru^{P1.LexA}/fru^{4\text{-}40}$ and $fru^{P1.LexA}$ homozygotes. Contralateral projections were nearly absent in these males, indicating that fru^{M} regulates GRN axon morphology (Fig. 4). Midline crossing was not completely abolished in one $fru^{P1.LexA}/fru^{sat15}$ male, so it might be that occasional GRN axons can cross the midline in the absence of fru^{M} function. This is consistent with our observations that some *dsx*-masculinized females (which also lack Fru^{M} but express Dsx^{M}) had a few contralateral GRN projections (Fig. 2A,B). Finally, Fru^{M} function in these neurons might be dose-dependent, as $fru^{P1.LexA}$/TM6B males tended to have reduced midline crossing when compared with +/TM6B males, although we failed to observe a statistically significant reduction in midline crossing in $fru^{4\text{-}40}$/+ or fru^{sat15}/+ males.

fru^{M} is expressed in neurons in the VNC, so it is possible that the midline-crossing phenotype observed is due to cell non-autonomous fru^{M} function in VNC neurons, rather than the GRNs. However, gynandromorph experiments suggest that it is the sex of the GRN that determines whether its axon is able to cross the midline (Possidente and Murphey, 1989). To verify this and to ask whether fru^{M} function is necessary in the GRNs, we used *poxn-Gal4*-driven expression of *UAS-*fru^{M}*IR* (Manoli and Baker, 2004) to reduce fru^{M} transcript levels specifically in GRNs [*poxn-Gal4* is expressed in only a few cells in the VNC and these do not express fru^{M} (data not shown)]. Such males had few or no contralateral GRN projections (Fig. 4), indicating that Fru^{M} is required in the GRNs to promote midline crossing by their axons in the VNC.

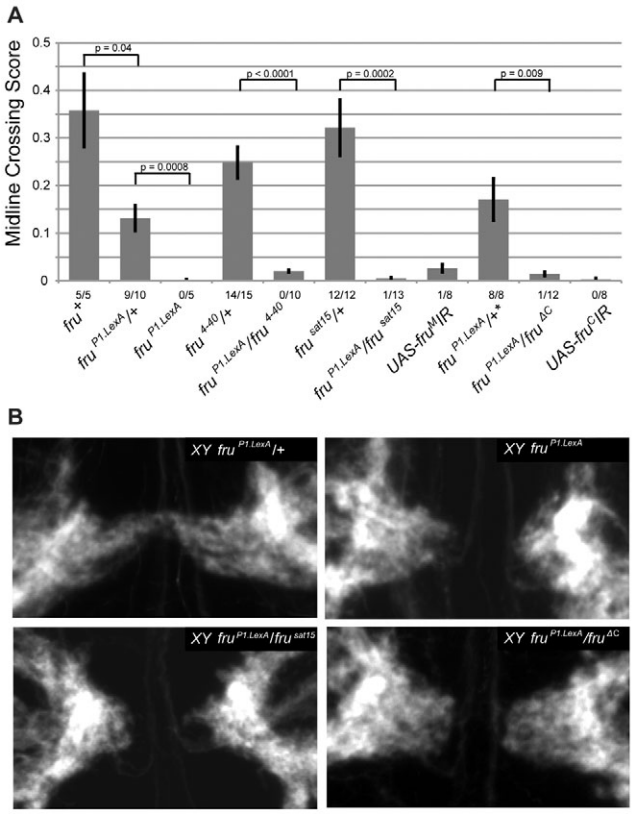


Fig. 4. Fru^{MC} regulates midline crossing by GRN axons. (**A**) Average level of midline crossing by genotype. Results shown as in Fig. 2A. All data shown are for males (XY). Note that low, but non-zero, midline crossing scores (<0.05) might be due to noise and not necessarily indicative of midline crossing (see Fig. S3 in the supplementary material). Genotype abbreviations: $fru^{P1.LexA}$/+ (+/Y; *UAS-mCD8::GFP*/+; $fru^{P1.LexA}$, *poxn-Gal4/TM6B*); $fru^{P1.LexA}$ (+/Y; *UAS-mCD8::GFP*/+; $fru^{P1.LexA}$, *poxn-Gal4*/$fru^{P1.LexA}$); $fru^{P1.LexA}/fru^{sat15}$ (+/Y; *UAS-mCD8::GFP*/+; $fru^{P1.LexA}$, *poxn-Gal4*/fru^{sat15}); $fru^{P1.LexA}/fru^{4\text{-}40}$ (+/Y; *UAS-mCD8::GFP*/+; $fru^{P1.LexA}$, *poxn-Gal4*/$fru^{4\text{-}40}$); *UAS-*fru^{M}*IR* (+/Y; *UAS-*fru^{M}*IR,UAS-mCD8::GFP*/+; *UAS-*fru^{M}*IR*, $fru^{4\text{-}40}$/*poxn-Gal4*); $fru^{P1.LexA}/fru^{\Delta C}$ (+/Y; *UAS-mCD8::GFP*/+; $fru^{P1.LexA}$, *poxn-Gal4*/$fru^{\Delta C}$); *UAS-*fru^{C}*IR* (+/Y; *UAS-mCD8::GFP*/+; $fru^{P1.LexA}$, *poxn-Gal4*/*UAS-*fru^{C}*IR*); $fru^{P1.LexA}$/+* (+/Y; *UAS-mCD8::GFP*/+; $fru^{P1.LexA}$, *poxn-Gal4/TM6B*, siblings of XY $fru^{P1.LexA}/fru^{\Delta C}$). (**B**) Confocal images demonstrating that $fru^{P1.LexA}$ homozygotes (upper right) phenocopy individuals in which $fru^{P1.LexA}$ is paired with two different alleles that (1) cannot produce any functional *fru* transcript (fru^{sat15}) or (2) do not produce functional proteins from transcripts containing exon C ($fru^{\Delta C}$). Thus, males lacking either (1) all Fru^{M} proteins or (2) Fru^{MC} proteins only do not form contralateral gustatory receptor neuron (GRN) projections. Midline images are at the same scale as in previous figures.

Fru^{MC} is necessary and sufficient for midline crossing by GRN axons

As noted above, fru^{M} mRNAs encode three transcription factors that possess alternative zinc-finger domains and thus probably differ in their downstream targets. We asked whether the male-specific Fru protein containing the exon-C zinc-finger domain, Fru^{MC}, is necessary for midline crossing by examining males lacking just the Fru^{MC} isoform. For this we used the hetero-allelic combination $fru^{P1.LexA}/fru^{\Delta C}$ (Billeter et al., 2006b). Nearly all such males had a complete loss of midline crossing (Fig. 4), demonstrating the

necessity of Fru^{MC}. A similar result was seen when RNAi directed against exon-C (*UAS-fru^{C}IR*) was driven by *poxn-Gal4*. Thus *fru^{MC}* is necessary for midline crossing by the GRN axons (Fig. 4).

To address whether Fru^{M} is sufficient for midline crossing, we first examined the VNCs of females carrying the *fru$^{\Delta tra}$* allele, whose transcripts are spliced into mRNAs encoding the *fru^{M}* isoforms regardless of chromosomal sex (Demir and Dickson, 2005). Surprisingly, no midline crossing was seen in these *fru$^{\Delta tra}$* females (Fig. 5), suggesting that the Fru^{M} proteins alone are not sufficient. We reasoned that Fru^{M}-dependent midline crossing might be repressed by Dsx^{F} in chromosomal females. We therefore examined *dsx^{1} fru$^{\Delta tra}$/dsx^{1}* females, which lack *dsx* function and contain Fru^{M} activity. Contralateral GRN projections were observed in 10 out of 11 of these females, demonstrating that Dsx^{F} represses midline crossing by GRN axons (Fig. 5). The *dsx^{1} fru$^{\Delta tra}$/dsx^{1}* females had, on average, less midline crossing than their male siblings, which might reflect a difference in the levels of Fru^{M} proteins between males and females of this genotype: males produce *fru^{M}* mRNA from both the wild-type and *fru$^{\Delta tra}$* alleles, whereas females produce *fru^{M}* mRNA from only the *fru$^{\Delta tra}$* allele.

To specifically ask whether the Fru^{MC} isoform is sufficient to promote GRN midline crossing, we used *poxn-Gal4* to express an epitope-tagged version of Fru^{MC} (*UAS-fru^{MC}::AU1*) in GRNs of *fru^{M}*-null males and females. Male-typical midline crossing was restored in these males, indicating that Fru^{MC} expression in the GRNs is sufficient to promote midline crossing (Fig. 5). Surprisingly, the female siblings of these males also exhibited robust midline crossing, even though these animals express Dsx^{F}. This result could be due to overexpression (ie. Fru^{MC}::AU1 is supplied by *poxn-Gal4* at a level high enough to overcome the repressive effect of Dsx^{F}). Alternatively, Dsx^{F} might function to repress midline crossing via a mechanism that is bypassed by the *poxn-Gal4*-driven expression of Fru^{MC}::AU1 (see Discussion).

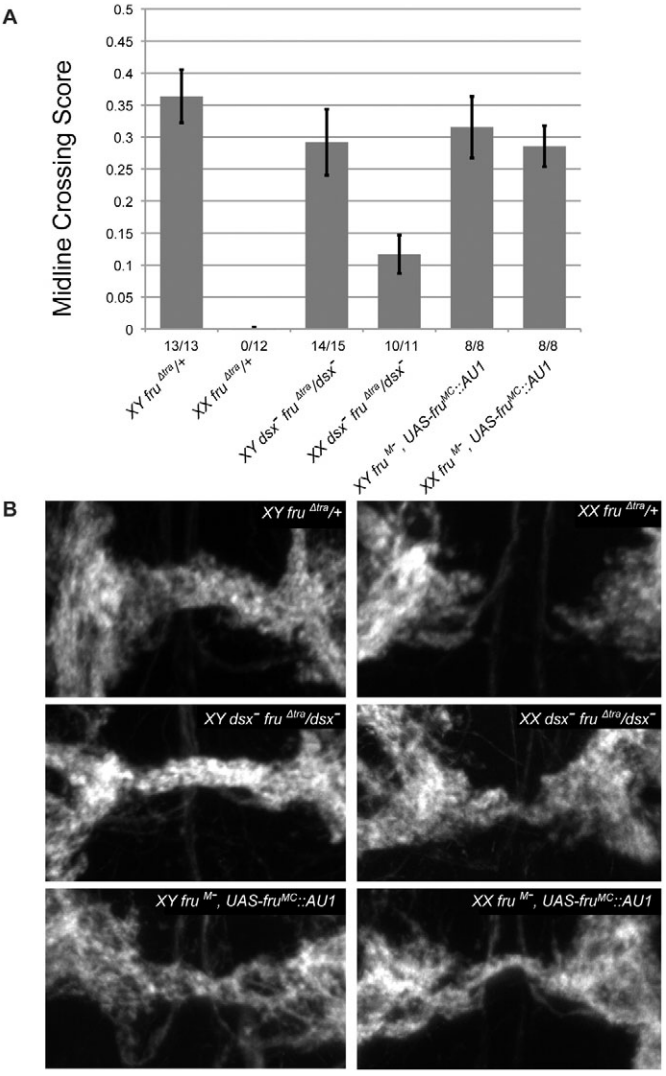


Fig. 5. Dsx^{F} represses midline crossing in females expressing *fru^{M}* from the endogenous locus but not in females in which *fru^{MC}* is expressed using *poxn-Gal4*. (**A**) Average level of midline crossing by genotype. Results summarized as in previous figures. Genotype abbreviations: XY; *fru$^{\Delta tra}$/+* (+/Y; *UAS-mCD8::GFP/+; fru$^{\Delta tra}$/poxn-Gal4*); XX; *fru$^{\Delta tra}$/+* (*w/+*; *UAS-mCD8::GFP/+; fru$^{\Delta tra}$/poxn-Gal4*); XY; *dsx^{-} fru$^{\Delta tra}$/dsx^{-}* (*w/Y*; *UAS-mCD8::GFP/+*; *FRT82B dsx^{1} fru$^{\Delta tra}$/FRT82B dsx^{1}, poxn-Gal4*); XX; *dsx^{-} fru$^{\Delta tra}$/dsx^{-}* (*w/w, 3XP3-DsRed; UAS-mCD8::GFP/+*; *FRT82B dsx^{1} fru$^{\Delta tra}$/FRT82B dsx^{1}, poxn-Gal4*); *fru^{M-}, UAS-fru^{MC}::AU1* (*UAS-mCD8::GFP/+*; *fru$^{P1.LexA}$, poxn-Gal4/UAS-fru^{MC}::AU1, fru^{sat15}*). Males (XY) and females (XX) of each genotype are siblings. (**B**) Representative images of the genotypes listed above. Females carrying the *fru$^{\Delta tra}$* allele show gustatory receptor neuron midline crossing only when they lack *dsx* function. Expressing Fru^{MC} by using *poxn-Gal4* to drive *UAS-fru^{MC}::AU1* bypasses or overcomes this repressive effect of Dsx^{F}.

Robo signaling regulates GRN axon morphology

In the embryonic nervous system, both the midline crossing and lateral positioning of axons are regulated by Robo signaling (reviewed by Dickson and Gilestro, 2006). Slit protein is present at the midline and acts as a repellent, activating the Robo, Robo2 and Robo3 receptors. Axons only cross the midline when Robo signaling is low or absent in the growth cone. The Robo receptors are also expressed in the CNS during late larval life (Tayler et al., 2002) and metamorphosis (Brierley et al., 2009) and Slit/Robo signaling has also been shown to regulate the patterning of the leg neuropil (Brierley et al., 2009). Moreover, Robo is expressed in GRNs while they are entering the leg neuropil (see Fig. S2 in the supplementary material). Thus, we asked whether Robo signaling regulates GRN axon guidance and, if so, whether Fru^{M} might modulate Robo signaling.

To examine the possible roles of individual *robo* paralogs in the GRNs, we reduced their expression level by the *poxn-Gal4*-driven expression of *UAS-robo.RNAi, UAS-robo2.RNAi* or *UAS-robo3.RNAi* (Tayler et al., 2004). When just *robo* expression is reduced, males displayed an increase in GRN axonal projections in the commissural region, suggesting that the level of *robo* expression in foreleg GRNs is important in establishing the appropriate male axon morphology (Fig. 6A,B). Moreover, 6 out of 10 of the sibling females of the same genotype had visible midline crossing, indicating that Robo normally represses midline crossing in females. Strikingly, RNAi-mediated knock-down of *robo2* and *robo3* had no clear effect in females and caused reduced midline crossing by the foreleg GRN axons in males (Fig. 6A,B). These results suggest that Robo2 and Robo3 promote midline crossing in this context, contrary to our expectations based on their described roles as receptors of repulsive cues (Rajagopalan et al., 2000b; Simpson et al., 2000a), but consistent with the observations that Robo2 can oppose Robo function (Simpson et al., 2000b) and help axons to cross the midline (Rajagopalan et al., 2000a).

When we simultaneously reduced the function of all three *robo* genes by RNAi, GRN axon morphology was severely disrupted, with axons collapsing onto the midline (Fig. 6B). This effect was

DEVELOPMENT

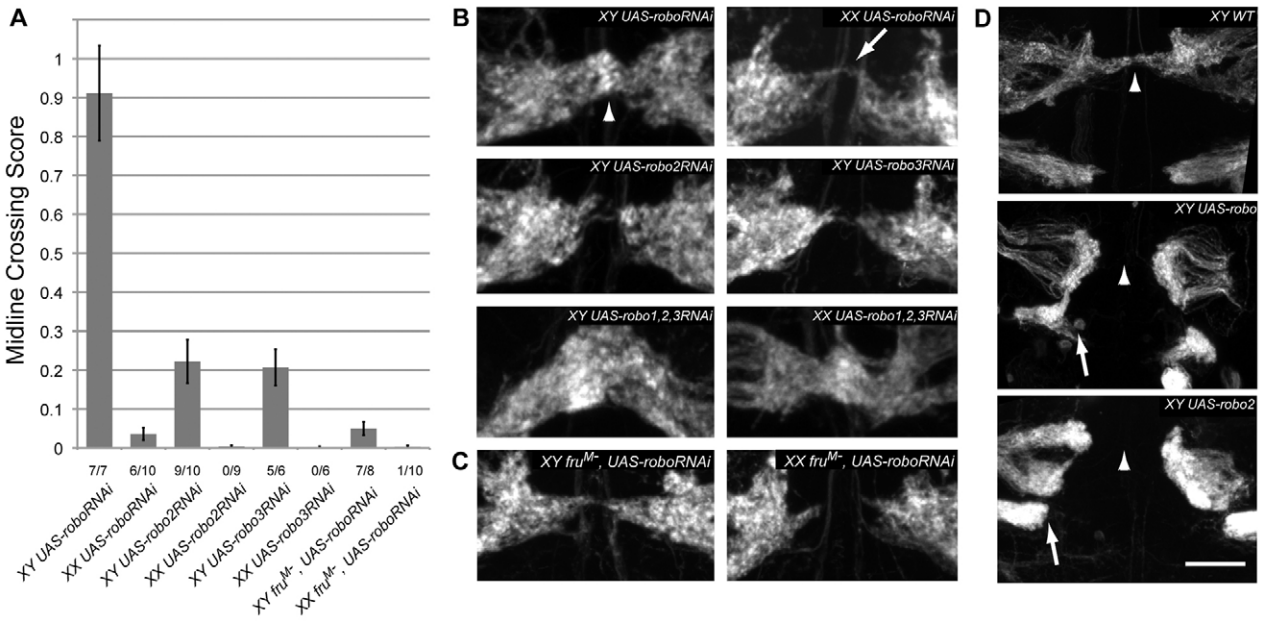


Fig. 6. Foreleg GRN axon morphology is regulated by Robo signaling. (**A**) Average level of midline crossing by genotype. Results summarized as in previous figures. Males (+/*Y*) and females (+/*w*) of the same genotype were siblings. Genotype abbreviations: *UAS-roboRNAi* (*UAS-mCD8::GFP/+*; *poxn-Gal4/UAS-robo.RNAi*); *UAS-robo2RNAi* (*UAS-mCD8::GFP/+*; *poxn-Gal4/UAS-robo2.RNAi*); *UAS-robo3RNAi* (*UAS-mCD8::GFP/+*; *poxn-Gal4/UAS-robo3.RNAi*); fru^{M-}, *UAS-roboRNAi* (*UAS-mCD8::GFP/+*; *poxn-Gal4,* $fru^{P1.LexA}$*/UAS-robo.RNAi,* fru^{4-40}). Commissure fluorescence is increased in *UAS-roboRNAi* males, but decreased in *UAS-robo2RNAi* and *UAS-robo3RNAi* males. Knocking down *robo* in $fru^{P1.LexA}/fru^{4-40}$ males restored some midline crossing in nearly all males but levels remained low. (**B**) Confocal images of gustatory receptor neuron (GRN) projections in which *robo*, *robo2*, *robo3*, or all three were knocked down by RNAi. *UAS-roboRNAi* males (top left panel) often had a bright spot of fluorescence directly over the midline (arrowhead), suggesting accumulation of GRN axon branches at the midline, and 6 out of 10 *UAS-roboRNAi* females exhibited some midline crossing (top right panel, arrow). Some *UAS-robo2RNAi* and *UAS-robo3RNAi* males appeared to have drastically reduced midline crossing (middle two panels). GRN axon morphology was severely disrupted in both males and females when all three *robo* genes were knocked down (bottom two panels), with a substantial accumulation at the midline. (**C**) Knocking down *robo* in fru^{M}-null males restores a low level of midline crossing (left panel), but their sibling females (right) did not see such an increase. (**D**) Confocal images of GRN projections in the ventral nerve cord, but with an expanded field of view to show projections from both the foreleg and wing. All three images are at the same scale. Overexpression of either Robo or Robo2 results in a complete loss of midline crossing (arrowheads), and also causes the GRNs of the wing to terminate in a more lateral region than in wild-type animals (arrows). Scale bar: 50 μm.

much more severe than when we targeted any one gene, suggesting that *robo*, *robo2*, and *robo3* must act in concert, and at appropriate levels, in order for GRNs to achieve their appropriate morphology.

Finally, we asked whether overexpression of *robo* or *robo2* could lead to disruption of GRN axon morphology. In males in which *robo* was overexpressed in GRNs, no GRN axons crossed the midline (Fig. 6D), consistent with a negative role for Robo in midline crossing by the GRN axons. Overexpression of Robo often appeared to cause the GRNs to project more laterally than in wild-type animals, a feature that was especially noticeable in the GRNs originating from the wing (Fig. 6D). This supports a role for Robo in the positioning of GRN axons within the neuropile, in addition to simply regulating midline crossing. Interestingly, overexpression of *robo2* gave a similar phenotype (Fig. 6D), even though *robo* and *robo2* differ in their loss-of-function phenotypes.

We can imagine two scenarios with respect to the functional relationship between Fru^{M} and Robo in GRN midline crossing. First, Fru^{M} might act through the Robo-signaling pathway. Alternatively, Fru^{M} could functionally contribute to midline crossing through a Robo-independent mechanism. If Fru^{M} promotes midline crossing by suppressing Robo activity, then knocking down *robo* in a Fru^{M}-null animal should restore some midline crossing. Conversely, if Fru^{M} acts independently of Robo-signaling to regulate midline crossing, then modifying Robo activity should not alter the Fru^{M}-null phenotype. When we drove *UAS-RoboRNAi* with *poxn-Gal4* in Fru^{M}-null males, a low level of midline crossing could be seen in 7 out of 8 males (Fig. 6C). The simplest interpretation of this result is that Fru^{M} exerts its effect on midline crossing by either directly or indirectly regulating Robo signaling.

DISCUSSION

fru and *dsx* cooperate to specify sexually dimorphic GRN morphology

We have shown that the male-specific presence of contralateral GRN projections is primarily due to Fru^{M} function. Specifically, Fru^{MC} acts in foreleg GRNs to promote the crossing of the VNC midline by their axons. We also identify a role for *dsx* in this dimorphism as (1) males that lack Dsx^{M} have somewhat fewer contralateral GRN projections, and (2) Dsx^{F} prevents the appearance of contralateral GRN axons in females.

The finding that Fru^{M} regulates GRN axon midline crossing is consistent with previous findings that, in some neurons, Fru^{M} regulates axonal morphology (Datta et al., 2008; Kimura et al., 2005; Kimura et al., 2008). Regulation of axonal morphology is likely to alter synaptic connectivity, suggesting that one of the roles of Fru^{M} is to support the formation of male-specific connections, and

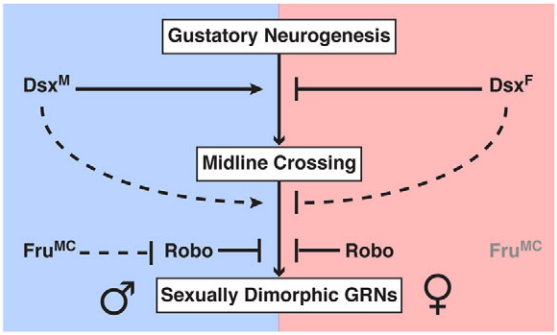


Fig. 7. Model for the sexual differentiation of the foreleg GRNs. First, the Dsx proteins establish a sexual dimorphism in the number of foreleg gustatory receptor neurons (GRNs) (D.J.M., unpublished results). In males, many GRN axons cross the midline primarily because Fru^{MC} directly or indirectly represses Robo signaling, although Dsx^{M} also has a (possibly indirect) positive effect. In females, no GRNs cross the midline because of both repression by Dsx^{F} and because Fru^{MC} is not present to repress Robo signaling.

possibly prevent the formation of female-specific connections, between neurons that are present in both sexes. Determining how such changes alter information processing will contribute to understanding how the potential for male courtship behavior is established.

It is also notable that *dsx* plays a role in regulating sexually dimorphic midline crossing, given that it also specifies the sexual dimorphism in gustatory sensilla number in the foreleg. It might be that *dsx* regulates gustatory sensilla development independently of its regulation of GRN axon morphology. That *dsx* can independently specify multiple sexual dimorphisms within particular cell lineages has been previously shown for the foreleg bristles that comprise the sex comb teeth of the male foreleg and their homologous bristles in the female (Belote and Baker, 1982). There, *dsx* was shown to function at one time to determine the sex-specific number of bristles that are formed and at another time to determine their sex-specific morphology. In support of a similar sequential role in the developing GRNs, *dsx* is expressed in the gustatory sense organ precursor cells and continues to be expressed in the terminally differentiated GRNs (C. Robinett, personal communication).

It is also possible that the effect of *dsx* on the presence of contralateral GRN projections is indirect. The two pools of gustatory sensilla, those that are male-specific and those that are homologous between males and females, might differ in their competence for midline crossing (i.e. only the male-specific GRNs will cross the midline when Fru^{M} is expressed). We think that this is not the case for two reasons. First, *dsx* is expressed in the GRNs throughout their development (C. Robinett, personal communication), consistent with a role in regulating axon guidance. Second, the expression of Fru^{MC} in female GRNs using *poxn-Gal4* is sufficient to induce midline crossing, suggesting that the sex-nonspecific GRNs are not intrinsically nonresponsive to Fru^{M}.

With respect to the latter result, it is worth considering the contrast between females that are masculinized with $fru^{\Delta tra}$, where we observed no contralateral GRN projections, and females in which *poxn-Gal4* is used to drive the expression of *UAS-fru^{MC}::AU1* in females, where we observed GRN midline crossing. In the case of females masculinized by $fru^{\Delta tra}$ we showed that the absence of contralateral GRN projections was due to Dsx^{F} functioning to prevent midline crossing in a manner that was epistatic to fru^{M} function. One attractive explanation for the difference between these two situations is based on the fact that masculinization by $fru^{\Delta tra}$ occurs via Fru^{M} produced from the endogenous *fruitless* locus, whereas masculinization by *UAS-fru^{MC}::AU1*, occurs via fru^{MC} expressed from a *UAS* construct that contains none of the untranslated sequences present in endogenous fru^{M} transcripts. Thus, it might be that the difference in midline crossing seen in these two situations is due to Dsx^{F} directly regulating fru^{M} expression through noncoding *fru* sequences that are present in the endogenous *fru* gene, but absent in the *fru* cDNA expressed from *UAS-fru^{MC}::AU1*. It is not likely that Dsx^{M} represses fru^{M} transcription, as we see $fru^{P1.LexA}$ expressed in GRNs in both males and females. Thus, if fru^{M} is downstream of *dsx* in these cells, Dsx^{F} probably affects the processing or translation of fru^{M} transcripts through sequences not present in the *UAS-fru^{MC}::AU1* construct. Alternatively, differences between these two situations in expression levels or patterns of expression might result in differences in the ability of Fru^{M} versus Fru^{MC} to overcome a parallel repressive effect of Dsx^{F}.

Regulation of *robo* signaling as a possible mechanism for sexually dimorphic GRN development

We have also found that *robo*, *robo2* and *robo3* are involved in GRN axon guidance. Of these three genes, *robo* appears to be most important in regulating GRN midline crossing because only reductions in levels of *robo* transcript result in midline crossing in females or fru^{M}-null males. Reducing levels of *robo2* and *robo3* transcripts in addition to *robo* enhances the *robo* phenotype but individual reductions of *robo2* or *robo3* function have the opposite effect, a reduction in midline crossing, suggesting that these receptors function to promote crossing in the presence of wild-type levels of *robo* expression.

It is not surprising that *robo* differs in function from *robo2* and *robo3* with respect to foreleg GRN development. *robo2* and *robo3* are more similar in sequence to each other than to *robo*, and *robo* contains two cytoplasmic motifs not found in its paralogs (Simpson et al., 2000b; Rajagopalan et al., 2000b). Furthermore, functional differences have been recognized since the original reports of *robo2* and *robo3* (Simpson et al., 2000b; Rajagopalan et al., 2000a). Finally, *robo2* might promote midline crossing if pan-neuronally overexpressed at low levels and yet repress midline crossing when overexpressed at high levels (Simpson et al., 2000b). This ‘switch’ in function might explain why we see reduced midline crossing under conditions of both *robo2* overexpression and reduction.

Given that the Robo receptors play such an important role in GRN development, how might fru^{M} regulate midline crossing? Our data indicate that *robo* lies genetically downstream of fru^{M}. The most straightforward mechanistic explanation is that Fru^{M} suppresses the activity of the Robo signaling pathway. We can envision several ways that this might occur. First, fru^{M} might regulate *commissureless*, which itself participates in the midline crossing decision by regulating the subcellular localization of Robo (Keleman et al., 2005). We could not detect a sexual dimorphism in the subcellular localization of a Robo::GFP fusion protein in GRNs in either the axons or cell body [*UAS-robo::GFP* provided by B. Dickson (Keleman et al., 2005); data not shown], so if fru^{M} regulates *comm*, it does so subtly. It is more probable that fru^{M} regulates the expression of either other regulators of *robo* signaling, *robo* itself, or *robo* effectors. We are currently pursuing strategies to identify candidate Fru^{M} targets that might be involved in regulating midline crossing.

How does midline crossing by GRN axons affect gustatory perception?

Given that male-typical GRN morphology requires fru^M, and that fru^M has a major regulatory role for social behavior, one hypothesis is that the contralateral GRN projections in males play a role in mediating the processing of contact cues during male courtship and/or aggression. Previous reports have shown that fru^M-masculinized females, which do not have contralateral GRN projections, readily perform tapping and proceed to subsequent steps in the male courtship ritual (Demir and Dickson, 2005), and behave like males with respect to aggressive behaviors (Vrontou et al., 2006). Thus, contralateral GRN projections are not necessary for the initiation and execution of these male-specific behaviors. Nevertheless, midline crossing might still be important for mediating socially relevant gustatory information. For instance, amputation experiments suggest that the detection of contact stimuli is important for courtship initiation under conditions when the male cannot otherwise see or smell the female (Robertson, 1983) (D. H. Tran, personal communication).

It is possible that midline crossing by GRN axons facilitates the comparison of chemical contact cues between the two forelegs. Such a comparison might help the male to determine the orientation of another fly, which would be a useful adaptation for performing social behaviors in conditions of sensory deprivation, such as in the dark. Alternatively, midline crossing might simply be a mechanism to form additional neuronal connections that integrate gustatory information into circuits underlying male-specific behaviors. Armed with the results of the present study, we can now use fru^M, *dsx*, and the *robo* genes as handles for developing tools and strategies to specifically manipulate midline crossing in the foreleg GRNs, with the goal of understanding its importance with regard to male behavior.

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Competing interests statement

The authors declare no competing financial interests.

Supplementary material

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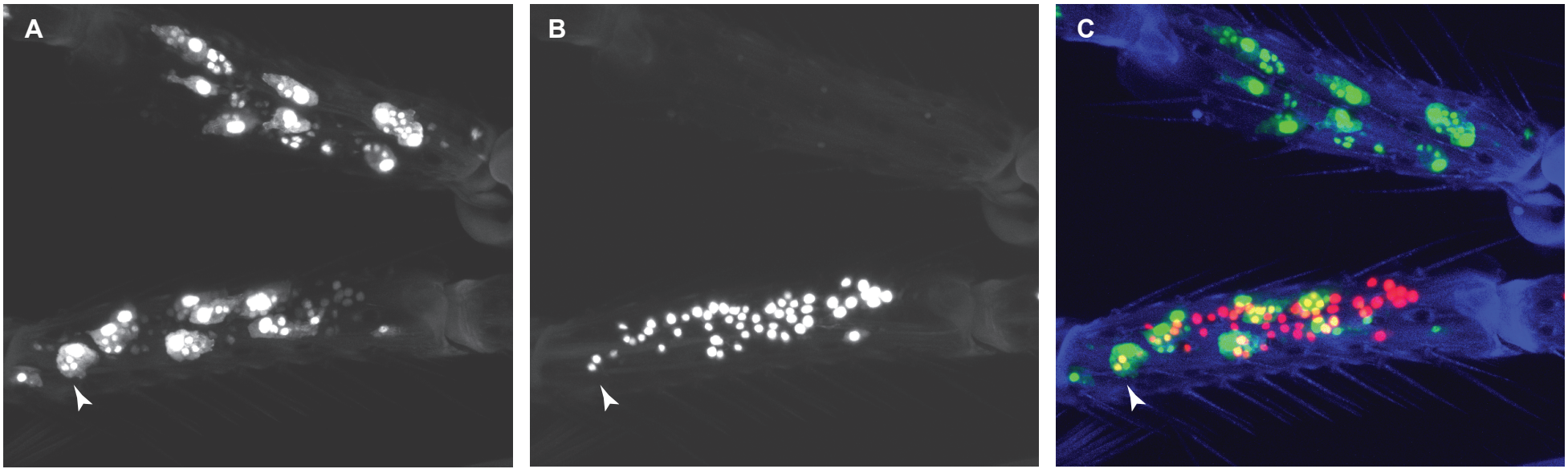
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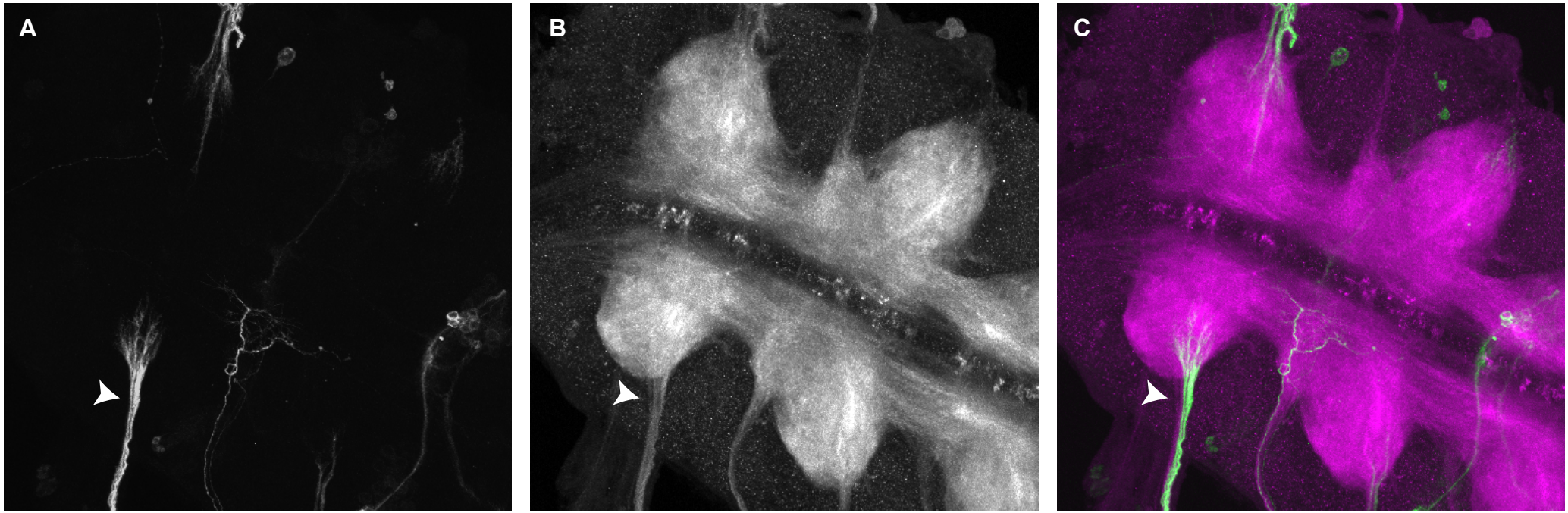
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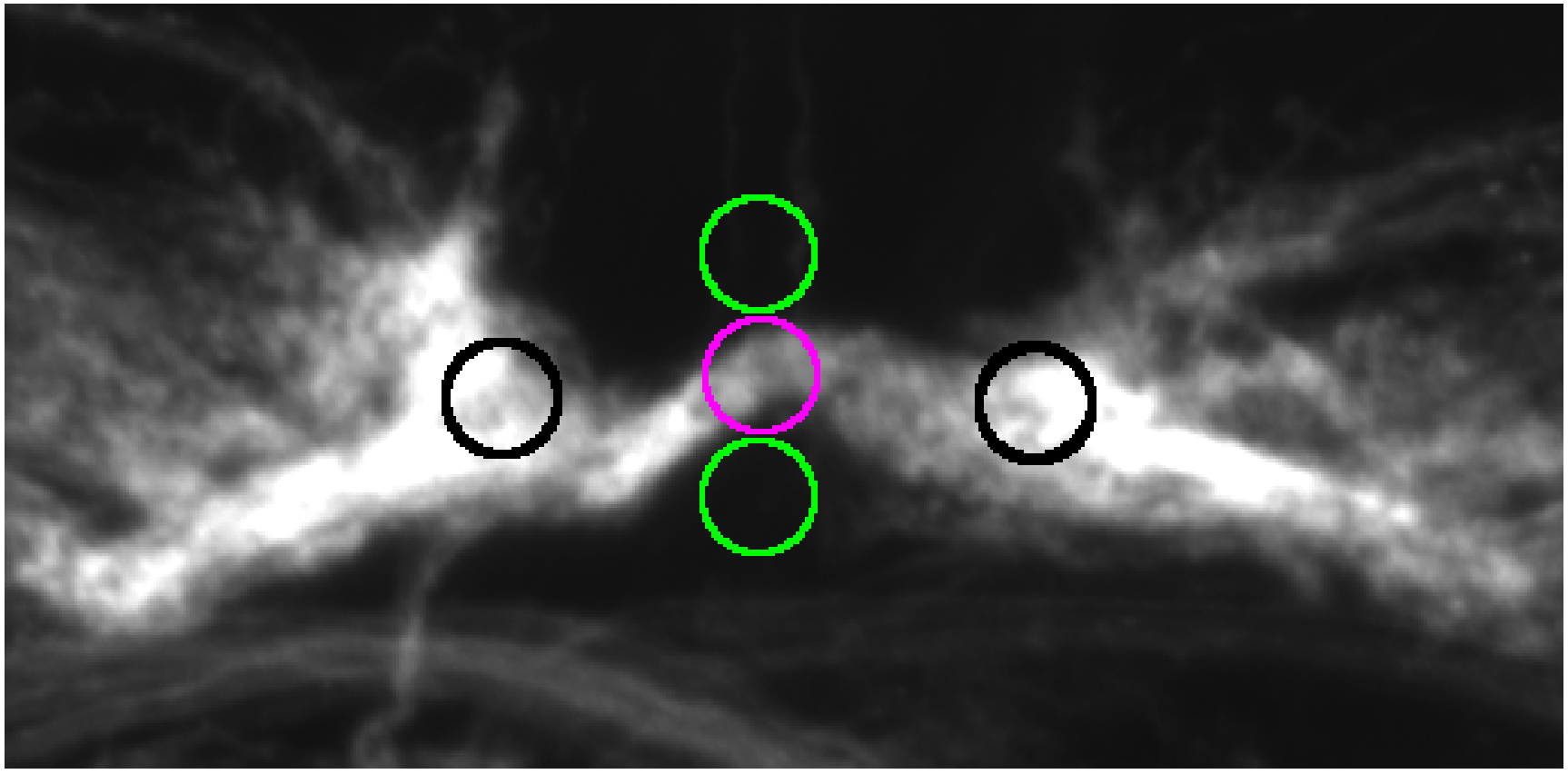

Table S1. Effect of *fru*$^{P1.LexA}$ on male courtship behavior

Genotype	CI (m → f)	% fertile males	ChI				
			Day 1	Day 2	Day 3	Day 4	Day 5
w; lexAop-rCD2::GFP; fru$^{P1.LexA}$/+	44±6 (24)	100 (20)	0±0 (3)	0±0	0±0	0±0	0±0
w; lexAop-rCD2::GFP; fru$^{P1.LexA}$/*fru*$^{4\text{-}40}$	0±0 (20)	0 (20)	0±0 (4)	4±2	50±11	46±12	63±13
w; lexAop-rCD2::GFP; fru$^{P1.LexA}$/*fru*^{sat15}	0±0 (20)	0 (20)	2±1 (4)	5±2	14±5	53±10	26±10
w; lexAop-rCD2::GFP/+; fru$^{P1.LexA}$/*fru*^{w24}	0±0 (20)	not tested	0±0 (3)	0±0	0±0	0±0	0±0

Means are shown ± s.e.m. Sample size is given in parentheses. ChI (*n*) refers to the number of groups of 8 males. CI, courtship index; ChI, chaining index.

Table S2. Immunohistochemistry in genotypes containing *fru*$^{P1.LexA}$

Genotype	% with detectable Fru^{M} in CNS	% with detectable 5HT in neurons innervating internal genitalia
XY; fru$^{P1.LexA}$/+	100 (6)	100 (6)
XX; fru$^{P1.LexA}$/+	0 (6)	0 (10)
XY; fru$^{P1.LexA}$/*fru*$^{P1.LexA}$	0 (10)	0 (12)
XY; fru$^{P1.LexA}$/*fru*$^{4-40}$	0 (10)	0 (10)
XY; fru$^{P1.LexA}$/*fru*^{sat15}	0 (10)	0 (10)