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 (FruM) acts in foreleg GRNs to promote midline crossing by their axons, whereas midline crossing is repressed in females by female-specific Dsx (DsxF). In addition, midline crossing by these neurons might be promoted in males by male-specific Dsx (DsxM). Finally, we (1) demonstrate that the *roundabout* (*robo*) paralogs also regulate midline crossing by these neurons, and (2) provide evidence that FruM 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 *fruitless* (*fru*) and *doublesex* (*dsx*) (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 DsxF protein. In males, *fru* and *dsx* mRNAs are translated into FruM and DsxM proteins. DsxF and DsxM are zinc-finger transcription factors that have the same DNA binding domain but different carboxy termini (Burtis and Dickson, 1989; An et al., 1996; Erdman et al., 1996). In the case of *fru*, at least three FruM 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 FruM 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). FruM is expressed only in motoneuronal neurons, including ~1-2% of the neurons in the central nervous system (CNS). *frum* expressing neurons are found largely in clusters throughout the brain and ventral nerve cord (VNC) (Lee et al., 2000). In addition, *frum* 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 *frum* expressing neurons are dedicated to courtship (Manoli et al., 2005; Stockinger et al., 2005). Taken together, these findings support the hypothesis that the *frum* expressing neurons comprise the circuitry subserving male courtship behavior (Ryner et al., 1996; Baker et al., 2001).

*frum* expression peaks during pupal development (Lee et al., 2000), suggesting that it regulates neuronal differentiation during metamorphosis. For most groups of *frum* 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 *frum* expressing CNS neurons revealed few differences between the sexes in the gross neuroanatomical features of the *frum* circuitry, suggesting that the FruM proteins largely function to regulate fine neuronal connectivity or neural physiology (Manoli et al., 2005; Stockinger et al., 2005). Independently, it was...
shown that fruM 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 (Billette et al., 2006b; Lee and Hall, 2001).

Although fruM is the master regulator of male courtship behavior, ddx function is also important for courtship behavior. One component of courtship song, sine song, requires DsxM function (Villella and Hall, 1996; Rideout et al., 2007). In addition, XY ddx 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, ddx is expressed in the CNS in 350-450 cells, the majority of which are neurons (Lee et al., 2002).

Indeed, ddx 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 FruM in many neurons in the abdominal ganglion (Billette et al., 2006b). In the periphery, ddx regulates the development of certain gustatory and mechanoreceptor sense organs in the foreleg and genitalia (Hildreth, 1965), and the FruM proteins are expressed in the neurons of these sense organs (Manoli et al., 2005; Stockinger et al., 2005). Finally, ddx acts in concert with FruM to masculinize parts of the CNS, suggesting that DsxM (and the absence of DsxF) is required to fully masculinize the circuitry underlying male behaviors (Billette 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 fruM and ddx.

The gustatory receptor neurons (GRNs) in the foreleg are an attractive starting point for unraveling the roles of ddx and fruM in sensory neuron development. The exchange of gustatory information during tapping appears to be important for species and sex discrimination (Spietti, 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 ddx (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), suggesting that DsxM (and the absence of DsxF) is required to fully masculinize the circuitry underlying male behaviors (Billette 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 fruM and ddx.

The gestatory receptor neurons (GRNs) in the foreleg are an attractive starting point for unraveling the roles of ddx and fruM in sensory neuron development. The exchange of gustatory information during tapping appears to be important for species and sex discrimination (Spietti, 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 ddx (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 ddx and fruM, but to different degrees. FruM is required in foreleg GRNs in order for their axons to cross the midline, whereas DsxM and DsxF 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 FruM 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 ddx mutants, w; UAS-mCD8::GFP; FRT82B ddx^1/2 poxn-Gal4-14-1-7/TM6B females were crossed to w; 3XP3-DsRed; ddx^6/TM6B males. To generate ddx-masculinized females, yw; 3XP3-DsRed; ddx^6/TM6B males were crossed to w; UAS-mCD8::GFP; FRT82B ddx^1/2 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 ddx^1/2dx^6-;B1 and ddx^1/2dx^6D flies.

To generate flies null for fruM, w; UAS-mCD8::GFP; fruP1LexA, poxn-Gal4-14-1-7/TM6B males were crossed to either fruM^101/2 fruM^100/2 fruM^1TM6B females. To examine fruM-masculinized females, w; UAS-mCD8::GFP; fruM^100/2; fruM^107/2;MKRS males were crossed to poxn-Gal4-14-1-7/7TM6B females (fruM^100/2 was provided by B. Dickson). To examine fruM-masculinized females in a ddx-null background, w; 3XP3-dsRed; +/CyO; FRT82B ddx^6/2 fruM^107/2 TM6B males were crossed to w; UAS-mCD8::GFP; FRT82B ddx^1/2 poxn-Gal4/6TM6B females. To examine the effect of fruM dose on midline crossing, w; UAS-mCD8::GFP; poxn-Gal4-14-1-7/7TM6B females were crossed to either fruM^100/2TM6B or fruM^101/2TM6B males. To knockdown the fruM transcript in GRNs, w; UAS-fruIR, UAS-mCD8::GFP/CyO; UAS-fruIR fruM^100 fruM^101 males were crossed to poxn-Gal4/6TM6B 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 fruP1LexA 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 Hybirdoma 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 fruP1LexA 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’ Kan and 3’ SacII to the fruM start codon and an ~2.5 kb fragment with 5’ NheI and 3’ StuI that begins with codon 3 of fruM were independently subcloned, sequence-verified 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’ SacII and 3’ Xbal, 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’ Xbal and 3’ NheI sites. These latter fragments were then cloned into the backbone vector above. This donor construct was then transformed into embryos using standard protocols.

For flies, 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 fruP1LexA
To verify that fruP1LexA is a genetic null, males carrying fruP1LexA 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 CO2 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 FruM protein and 5HT were performed as described by Manoli (Manoli and Baker, 2004).

Generation of UAS-fruMC::AU1
A synthesized fragment containing a coding sequence for the AU1 epitope tag (5’-CCAAGCGTTGAGGATGAGTTCAGCTTGAGCAGGCG-3’) (Lim et al., 1990) was subcloned into pBSKII(+) using KpnI and HindIII (pBSKII-AU1). PCR using primers 5’-CGGGAATTCGATAGCACTTACGCTCTTAC-3’ and 5’-AGCTGGCAGGTGTTCAAGTTGAGCAGGCG-3’ was performed, using as a template a plasmid containing the fruMC sequence (Song et al., 2002). This PCR product was subcloned into pBSKII-AU1 using EcoRI and HindIII, producing a construct in which the stop codon was removed from fruMC, and the AU1 epitope was added in frame following a short linker (fruMC::AU1). fruMC::AU1 was then cut from pBSKII(+) using EcoRI and subcloned into pUAST.

Generation of UAS-fruIR
PCR amplification using primers 5’-TCTAGAAGACGAGATACCATCTCCTCT-3’ and 5’-TCTAGAGGTGTGGGGAGTAGAAGTGGG-3’ was performed to amplify a 308 bp fragment specific to the C exon of fruIR (Murphey, 1989), suggesting that GRN axon guidance is regulated by fruIR and/or dsx and/or Spt20 (Lee and Carthew, 2003) using the standard procedure available through FlyBase. The resulting inverted repeat construct was in the 5’-3’ orientation.

Generation of lexAop-FRT-tdTomato::nls
The tdTomato gene was amplified from the plasmid pBSEub-tdTomato (a gift from R. Tsien; Shaner et al., 2004) using the primers 5’-ACCCTATGGGTGAGCAACGCTCCAGAAGCTTTAT-3’ and 5’-TAGAGCGCTGACGTCTTCTCCTGTCTCTCTGCATCT-3’. A nuclear-localization sequence was amplified from the plasmid pStinger (Barolo et al., 2000) using the primers 5’-AGGAGCAGCCACAGAAGCTCTTCTTCTCCTGCTCTTCT-3’ and 5’-AGTACCCATGTTACGCTCTTCTCCTGCTCTTCTGCTCT-3’ and fused to the C-terminus of tdTomato by overlapping PCR. The resulting tdTomato::nls fragment was cloned into pCR2.1 (Invitrogen) and fused to the C-terminus of FruM, which is expressed in GRNs, neurons in the central brain, and the ventral nerve cord. The resulting transgene, Stp-FRTtdTomato::nls, was inserted into the genome using standard transformation techniques. A second chromosome P[lexAop-FRT-Stp-FRTtdTomato::nls] insertion was selected for germ-line transmission. The resulting transgene, P[lexAop-FRT-Stp-FRTtdTomato::nls], was then recombined with P[UAStinger] (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 FruM and DsxM 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 fruM (Fig. 1A).

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.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, 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+/TM6B) males had many midline-crossing GRN axons, whereas only a single thin contralateral projection was observed in 12 control (dsx+/TM6B) females (Fig. 2). In dsx mutant individuals (dsx+/dsxmo+R1), 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 material).
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 dsx1, poxn-Gal4/+/D); dsx– (UAS-mCD8::GFP, FRT82B dsx1, poxn-Gal4/dsx+/+); dsx+ (UAS-mCD8::GFP, FRT82B dsx1, poxn-Gal4/dsx+/+); dsx+ and dsx– are siblings. dsx+ 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– females.

Fig. 3. fruP1.LexA expression in the foreleg. (A,B) Composite confocal images of a w; lexAop-rCD2::GFP, fruP1.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) fruP1.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 fruP1.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.

fru\textsuperscript{M} is expressed in both male-specific and sex-nonspecific GRNs

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

In the foreleg, fru\textsuperscript{P1.LexA} drove lexAop-rCD2::GFP in a pattern indistinguishable from other reporters of fru\textsuperscript{M} expression (fru\textsuperscript{Gal4} and fru\textsuperscript{P1.Gal4}, data not shown). In both sexes, fru\textsuperscript{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\textsuperscript{P1.LexA} was expressed in neurons in every tarsal segment (Fig. 3A,B). Of those neurons that expressed fru\textsuperscript{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\textsuperscript{P1.LexA}-expressing neurons whose dendritic projections converged were considered to belong to a single gustatory sensillum.

To determine which gustatory sensilla contain Fru\textsuperscript{M}-expressing neurons, we compared counts of fru\textsuperscript{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 fruP1.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 fruP1.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 fruP1.LexA+ GRNs from fruP1.LexA+ neurons of the sex comb in the distal part of T1. These counts suggest that fruM is expressed in a subset of neurons innervating every gustatory sensillum in the T1-T4 segments of the male foreleg. We also saw fruP1.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 fruP1.LexA expression indicates that fruM is expressed in both male-specific GRNs as well as those GRNs homologous between males and females. Accordingly, we also saw fruP1.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, fruP1.LexA was most often expressed in two neurons, although expression was occasionally seen in three neurons (Fig. 3C). Thus, fruM expression is restricted to a subset of the five neurons found in each gustatory sensillum. We also examined overlap between poxn-Gal4 and fruP1.LexA in the adult, and consistently saw fruP1.LexA expressed in 2 (occasionally 3) cells per gustatory sensillum (see Fig. S1 in the supplementary material). It is unknown which neuron subtypes express fruM.

fruM is necessary for midline crossing by GRN axons

We next asked whether fruM is responsible for the sex-specific regulation of GRN axon morphology by examining GRN axons in the VNCs of males deficient for fruM, using three different null genotypes: fruP1.LexA/fru4-15, fruP1.LexA/fru4-40 and fruP1.LexA homozygotes. Contralateral projections were nearly absent in these males, indicating that fruM regulates GRN axon morphology (Fig. 4). Midline crossing was not completely abolished in one fruP1.LexA/fru4-15 male, so it might be that occasional GRN axons can cross the midline in the absence of fruM function. This is consistent with our observations that some dse-masculinized females (which also lack FruM but express DsxM) had a few contralateral GRN projections (Fig. 2A,B). Finally, fruM function in these neurons might be dose-dependent, as fruP1.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 fruP1.LexA/+ or fruP1.LexA/TM6B males.

fruM is expressed in neurons in the VNC, so it is possible that the midline-crossing phenotype observed is due to cell non-autonomous fruM 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 fruM function is necessary in the GRNs, we used poxn-Gal4-driven expression of UAS-fruIR (Manoli and Baker, 2004) to reduce fruM transcript levels specifically in GRNs poxn-Gal4 is expressed in only a few cells in the VNC and these do not express fruM (data not shown). Such males had few or no contralateral GRN projections (Fig. 4), indicating that FruM is required in the GRNs to promote midline crossing by their axons in the VNC.

Fig. 4. FruMC 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: fruP1LexA/+ (XY, UAS-mCD8::GFP+; fruP1LexA, poxn-Gal4/TM6B); fruP1LexA (XY, UAS-mCD8::GFP+; fruP1LexA, poxn-Gal4/FRU15 (XY, UAS-mCD8::GFP+; fruP1LexA, poxn-Gal4/FRU15); fruP1LexA/fru4-40 (XY, UAS-mCD8::GFP+; fruP1LexA, poxn-Gal4/FRU4-40); UAS-fruIR (XY, UAS-fruIR, UAS-mCD8::GFP+; UAS-fruIR, fru4-40; poxn-Gal4); fruP1LexA/fru4-40 (XY, UAS-mCD8::GFP+; fruP1LexA, poxn-Gal4/FRU15); UAS-fruIR (XY, UAS-mCD8::GFP+; fruP1LexA, poxn-Gal4/FRU4-40); UAS-fruIR (XY, UAS-mCD8::GFP+; fruP1LexA, poxn-Gal4/FRU15); fruP1LexA/fru4-40 (XY, UAS-mCD8::GFP+; fruP1LexA, poxn-Gal4/FRU15); siblings of XY fruP1LexA/fru4-40. (B) Confocal images demonstrating that fruP1LexA homoyzygotes (upper right) phenocopy individuals in which fruP1LexA is paired with two different alleles that (1) cannot produce any functional fru transcript (fru4-15) or (2) do not produce functional proteins from transcripts containing exon C (fruC). Thus, males lacking either (1) all fruM proteins or (2) FruMC proteins only do not form contralateral gustatory receptor neuron (GRN) projections. Midline images are at the same scale as in previous figures.

FruMC is necessary and sufficient for midline crossing by GRN axons

As noted above, fruM 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, FruMC, is necessary for midline crossing by examining males lacking just the FruMC isoform. For this we used the hetero-allelic combination fruP1.LexA/fru4-40 (Billetter et al., 2006b). Nearly all such males had a complete loss of midline crossing (Fig. 4), demonstrating the
necessity of FruMC. A similar result was seen when RNAi directed against exon-C (UAS-fruMCIR) was driven by poxn-Gal4. Thus fruMC is necessary for midline crossing by the GRN axons (Fig. 4).

To address whether FruM is sufficient for midline crossing, we first examined the VNCs of females carrying the fruMC::AU1 allele, whose transcripts are spliced into mRNAs encoding the fruMC isoforms regardless of chromosomal sex (Demir and Dickson, 2005). Surprisingly, no midline crossing was seen in these fruMC females (Fig. 5), suggesting that the FruM proteins alone are not sufficient. We reasoned that FruM-dependent midline crossing might be repressed by Dsx in chromosomal females. Therefore we examined dsx+/fruMC::AU1 females, which lack dsx function and contain FruM activity. Contralateral GRN projections were observed in 10 out of 11 of these females, demonstrating that Dsx represses midline crossing by GRN axons (Fig. 5). The dsx+/fruMC::AU1 females had, on average, less midline crossing than their male siblings, which might reflect a difference in the levels of FruM proteins between males and females of this genotype: males produce fruM mRNA from both the wild-type and fruMC alleles, whereas females produce fruM mRNA from only the fruMC allele.

To specifically ask whether the FruMC isoform is sufficient to promote GRN midline crossing, we used poxn-Gal4 to express an epitope-tagged version of FruMC (UAS-fruMC::AU1) in GRNs of fruMC-null males and females. Male-typical midline crossing was restored in these males, indicating that FruMC 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. This result could be due to overexpression (i.e., FruMC::AU1 is supplied by poxn-Gal4 at a level high enough to overcome the repressive effect of Dsx). Alternatively, Dsx might function in repressing midline crossing via a mechanism that is bypassed by the poxn-Gal4-driven expression of FruMC::AU1 (see Discussion).

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). Axons only cross the midline when Robo signaling is low or absent in the growth cone, indicating that Robo normally represses midline crossing in females. Interestingly, 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
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); fru4-40, UAS-roboRNAi (UAS-mCD8::GFP+; poxn-Gal4, fru4-40); UAS-robo2RNAi (UAS-mCD8::GFP+; poxn-Gal4, fru4-40); UAS-robo3RNAi (UAS-mCD8::GFP+; poxn-Gal4, fru4-40). 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-robo2RNAi 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 fruM-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 FruM and Robo in GRN midline crossing. First, FruM might act through the Robo-signaling pathway. Alternatively, FruM could functionally contribute to midline crossing through a Robo-independent mechanism. If FruM promotes midline crossing by suppressing Robo activity, then knocking down robo in a FruM-null animal should restore some midline crossing. Conversely, if FruM acts independently of Robo-signaling to regulate midline crossing, then modifying Robo activity should not alter the FruM-null phenotype. When we drove UAS-RoboRNAi with poxn-Gal4 in FruM-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 FruM 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 FruM function. Specifically, FruM 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 DsxM have somewhat fewer contralateral GRN projections, and (2) DsxM prevents the appearance of contralateral GRN axons in females.

The finding that FruM regulates GRN axon midline crossing is consistent with previous findings that, in some neurons, FruM 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 FruM is to support the formation of male-specific connections, and
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 FruM 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 FruMC in female GRNs using poxn-Gal4 is sufficient to induce midline crossing, suggesting that the sex-nonspecific GRNs are not intrinsically nonresponsive to FruM.

With respect to the latter result, it is worth considering the contrast between females that are masculinized with fruΔava, where we observed no contralateral GRN projections, and females in which poxn-Gal4 is used to drive the expression of UAS-fruMC::AU1 in females, where we observed GRN midline crossing. In the case of females masculinized by fruΔava we showed that the absence of contralateral GRN projections was due to Dsx expression. This could prevent midline crossing in a manner that was epistatic to fruM.

One attractive explanation for the difference between these two situations is based on the fact that masculinization by fruΔava occurs via FruM produced from the endogenous fruitless locus, whereas masculinization by UAS-fruMC::AU1, occurs via fruMC expressed from a UAS construct that contains none of the untranslated sequences present in endogenous fruM transcripts. Thus, it might be that the difference in midline crossing seen in these two situations is due to Dsx directly regulating fruM expression through noncoding fru sequences that are present in the endogenous fru gene, but absent in the fru cDNA expressed from UAS-fruMC::AU1. It is not likely that DsxM represses fruM transcription, as we see fruM expressed in GRNs in both males and females. Thus, if fruM is downstream of dsx in these cells, Dsx probably affects the processing or translation of fruM transcripts through sequences not present in the UAS-fruMC::AU1 construct. Alternatively, differences between these two situations in expression levels or patterns of expression might result in differences in the ability of FruM versus FruMC to overcome a parallel repressive effect of Dsx.

**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 fruM-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 fruM regulate midline crossing? Our data indicate that robo lies genetically downstream of fruM. The most straightforward mechanistic explanation is that FruM suppresses the activity of the Robo signaling pathway. We can envision several ways that this might occur. First, fruM 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 fruM regulates comm, it does so subtly. It is more probable that fruM regulates the expression of either other regulators of robo signaling, robo itself, or robo effectors. We are currently pursuing strategies to identify candidate FruM targets that might be involved in regulating midline crossing.

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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 FruMC directly or indirectly represses Robo signaling, although DsxM also has a (possibly indirect) positive effect. In females, no GRNs cross the midline because of both repression by Dsx and because FruMC is not present to repress Robo signaling.
How does midline crossing by GRN axons affect gustatory perception?

Given that male-typical GRN morphology requires fruM, and that fruM 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 that lead to courtship behavior. Previous reports have shown that fruM-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 fruM, dxx, 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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