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 (Billette 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; Billette 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, 2005; Manoli et al., 2005). FruM is expressed only in postsynaptic neurons, including ~1-2% of the neurons in the 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 behavior (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 mechanosensory 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 DsxL are required in fores leg GRNs in order for their axons to cross the midline, whereas DsxM and DsxF have less prominent roles in midline crossing by foreleg GRNs. We show that midline crossing by foreleg GRNs of both sexes sends axonal projections in a foreleg-specific fashion (Billeter et al., 2006b; Lee and Hall, 2001).

Furthermore, sexual dimorphisms in at least two distinct aspects of foreleg GRN development are known. First, the number of gustatory receptor neurons (GRNs) in the foreleg is sex dependent (Manoli et al., 2005; Stockinger et al., 2005). Finally, ddx acts in concert with FruM to masculinize the circuitry underlying male behaviors (Billeter et al., 2008). A rigorous analysis of sexually dimorphic neural circuitry underlying male behaviors (Billeter et al., 2006b; Lee and Hall, 2001).

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/; poxn-Gal4-14-1-TM6B were crossed to w; 3XP3-DsRed; ddx+/+; TM6B/TM6B males. To generate ddx-masculinized females, w; 3XP3-DsRed; ddx+/+; TM6B/TM6B males were crossed to w; UAS-mCD8::GFP; FRT82B ddx/; poxn-Gal4-14-1-TM6B females. poxn-Gal4-14-1-TM6B females (fru was provided by B. Dickson). To examine ddx-masculinized females in a ddx-null background, w; 3XP3-dsRed;+/CyO; FRT82B ddx/; poxn-Gal4-14-1-TM6B were crossed to w; UAS-mCD8::GFP; FRT82B ddx/; poxn-Gal4-14-1-TM6B females. To examine the effect of ddx dose on midline crossing, w; UAS-mCD8::GFP; poxn-Gal4-14-1-TM6B females were crossed to either fru4-80/TM6B or fru4-80/TM6B males. To knockdown the fruM expression in GRNs, w; UAS-fruMIR, UAS-mCD8::GFP/CyO; UAS-fruMIR, fru4-80 were crossed to poxn-Gal4-14-1/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-TM6B 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-roboTM3 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 fruP1.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 Hybridaoma Bank)] was performed as described (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 fruP1.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′ KpnI and 3′ SacI to the fruM start codon and an ~2.5 kb fragment with 5′ Nhel and 3′ Sfl 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 J. Sekelsky, University of North Carolina, 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′ Nhel sites. These latter fragments were then cloned into the backbone vector above. This donor construct was then transformed into embryos using standard protocols.

FruM-specific 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 fruP1.LexA

To verify that fruP1.LexA is a genetic null, males carrying fruP1.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 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-fru**

A synthesized fragment containing a coding sequence for the AU1 epitope tag (5’-CCCAAGCTTGGACTGATACTTACCGGATATCTATAAGGTACACG-3’) was subcloned into pBskII (+) using KpnI and HindIII (pBskII-AU1). PCR using primers 5’-CCCGAAATTCTATGATGGCGACGTCACAGGATTAT-3’ and 5’-CCCAAGCTTGGGTAGTGGAGTCTCGTCAAATTGTCAGTGGAGCAGGCG-3’ was performed, using as a template a plasmid containing the fru 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 fru, and the AU1 epitope was added in frame following a short linker (fru::AU1). fru::AU1 was then cut from pBskII (+) using EcoRI and KpnI and subcloned into pUAST.

**Generation of UAS-fru**

PCR amplification using primers 5’-TCTAGAAGACCGAGTACCATCCTCTC-3’ and 5’-TCTAGAAGACCGAGTACCATCCTCTC-3’ was performed to amplify a 308 bp fragment specific to the C exon of fruitless while simultaneously adding Xhol sites, using as a template a plasmid containing the fru 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 UAS-fru**

The tdTomato gene was amplified from the plasmid pRSEb-tdTomato (a gift from R. Tsien; Shaner et al., 2004) using the primers 5’-ACCCGATATGGTGAGAATCTTGCGTACAGTGTGATGAGGCGAGAAG-3’ and 5’-TAGAGCCGTCGAGCTTCTCAGTGGGAAGTGAGACGTTTCCCAATCCAGCTCGTCCAT3’. A nuclear-localization sequence was amplified from the plasmid pStinger (Barolo et al., 2000) using the primers 5’-AAAGAGCCGACACGAAAGCCGATC-3’ and 5’-GTCTCATCGCCTTCTGATGAGGCGATC-3’. A -208-bp fragment containing the nuclear localization sequence was subcloned into a tdTomato expression vector (gift of Liqun Luo lab, Stanford University, USA), which had been modified to carry an FRT-tdTomato::nls (gift of Liqun Luo lab, Stanford University, USA). The resulting transgene, in which FRT-tdTomato::nls is flanked by FRT sequences (Song et al., 1991) was subcloned into pUAST-tdTomato using XbaI and HindIII.

**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).

**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 to 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, pinn, 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 are 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 sexually 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+/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 (dsx1/dsx1-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...
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+/+, poxn-Gal4/TM6B); dsx− (UAS-mCD8::GFP, FRT82B dsx−, poxn-Gal4/dsxM+/;), dsxM (UAS-mCD8::GFP, FRT82B dsx+, poxn-Gal4/dsxM+); dsx− and dsx+ are siblings. dsxM 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 dsxM 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**

Control of sex-specific axon morphology

**Material and Methods**

**fru**

It is unknown which neuron subtypes express fru.

**fru**

fru expression is restricted to a subset of the five neurons found in each gustatory sensillum. We also examined overlap between poxn-Gal4 and fru expression in the adult, and consistently saw fru expression in 2 (occasionally 3) cells per gustatory sensillum (see Fig. S1 in the supplementary material).

**fru** is necessary for midline crossing by GRN axons

We next asked whether fru is responsible for the sex-specific regulation of GRN axon morphology by examining GRN axons in the VNCs of males deficient for fru. Using three different null genotypes: fru, fru, and fru, we examined fru expression in neurons in the VNC, so it is possible that the midline-crossing phenotype observed is due to cell non-autonomous fru 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 function is necessary in the GRNs, we used poxn-Gal4-driven expression of UAS-fruIR (Manoli and Baker, 2004) to reduce fru transcript levels specifically in GRNs [poxn-Gal4 is expressed in only a few cells in the VNC and these do not express fru (data not shown)]. Such males had few or no contralateral GRN projections (Fig. 4), indicating that Fru is required in the GRNs to promote midline crossing by their axons in the VNC.

**fru** is necessary and sufficient for midline crossing by GRN axons

As noted above, fru 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, FruC, is necessary for midline crossing by examining males lacking just the Fru isoform. For this we used the hetero-allelic combination fru/ fru (Billeter et al., 2006b). Nearly all such males had a complete loss of midline crossing (Fig. 4), demonstrating the necessity of Fru function in VNC neurons, rather than the GRNs. However, we failed to observe a statistically significant reduction in midline crossing in fru/+ males.

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necessity of FruMC. A similar result was seen when RNAi directed against exon-C (UAS-fruIR) was driven by poxn-Gal4. Thus fruMC is necessary for midline crossing by the GRN axons (Fig. 4).

To address whether FruMC 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 fru isoforms regardless of chromosomal sex (Demir and Dickson, 2005). Surprisingly, no midline crossing was observed in these fruMC::AU1 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. We therefore 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::AU1 alleles, whereas females produce fruM mRNA from only the fruMC::AU1 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 fruM-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 (ie. FruMC::AU1 is supplied by poxn-Gal4 at a level high enough to overcome the repressive effect of Dsx). Alternatively, Dsx might function to repress 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 for 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 FruM 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-roboRNAi, UAS-robo2RNAi or UAS-robo3RNAi (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
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 of midline-crossing males. Knocking down robo in fruM-null 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 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.

**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 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, no GRNs cross the midline because of both repression by $Dsx^\alpha$ and because $Fru^{MC}$ is not present to repress Robo signaling.

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 $Dsx^\alpha$ 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 $Fru^{MC}$ 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 $fru^{aro}$, 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 $fru^{aro}$ we showed that the absence of contralateral GRN projections was due to $Dsx^\alpha$ 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 $fru^{aro}$ 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 $Dsx^\alpha$ 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, $Dsx^\alpha$ 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 $Dsx^\alpha$.

**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 $fru^{aro}$-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 $robo$ 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 $comm$-specific genes, 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 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 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 during male courtship and/or aggression. 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 (Roberson, 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

Supplementary material for this article is available at http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.045047/-/DC1

References


### Table S1. Effect of fruP1.LexA on male courtship behavior

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Ci (m → f)</th>
<th>% fertile males</th>
<th>ChI</th>
<th></th>
<th></th>
<th></th>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Day 1</td>
<td>Day 2</td>
<td>Day 3</td>
<td>Day 4</td>
<td>Day 5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>w; lexAop-rCD2::GFP; fruP1.LexA/+</td>
<td>44±6 (24)</td>
<td>100 (20)</td>
<td>0±0 (3)</td>
<td>0±0</td>
<td>0±0</td>
<td>0±0</td>
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<tr>
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<td>0 (20)</td>
<td>0±0 (4)</td>
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<tr>
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<td>not tested</td>
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<td>0±0</td>
<td>0±0</td>
<td>0±0</td>
<td></td>
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</table>

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 \(fru^P1\text{LexA}\)

<table>
<thead>
<tr>
<th>Genotype</th>
<th>% with detectable Fru(^+) in CNS</th>
<th>% with detectable SHT in neurons innervating internal genitalia</th>
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</thead>
<tbody>
<tr>
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<td>100 (6)</td>
</tr>
<tr>
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<td>0 (10)</td>
</tr>
<tr>
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<td>0 (12)</td>
</tr>
<tr>
<td>XY; (fru^P1\text{LexA}fru^\text{4-40})</td>
<td>0 (10)</td>
<td>0 (10)</td>
</tr>
<tr>
<td>XY; (fru^P1\text{LexA}fru^\text{mat15})</td>
<td>0 (10)</td>
<td>0 (10)</td>
</tr>
</tbody>
</table>