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Published in:
Current Biology

DOI:
10.1016/j.cub.2007.07.047

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Document Version
Publisher's PDF, also known as Version of record

Publication date:
2007

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):

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The Sex-Determination Genes *fruitless* and *doublesex* Specify a Neural Substrate Required for Courtship Song

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Summary

Courtship song is a critical component of male courtship behavior in *Drosophila*, making the female more receptive to copulation and communicating species-specific information [1–6]. Sex mosaic studies have shown that the sex of certain regions of the central nervous system (CNS) is critical to song production [7]. Our examination of one of these regions, the mesothoracic ganglion (Msg), revealed the coexpression of two sex-determination genes, *fruitless* (fru) and *doublesex* (dsx). Because both genes are involved in creating a sexually dimorphic CNS [8, 9] and are necessary for song production [10–13], we investigated the individual contributions of *fru* and *dsx* to the specification of a male CNS and song production. We show a novel requirement for *dsx* in specifying a sexually dimorphic population of *fru*-expressing neurons in the Msg. Moreover, by using females constitutively expressing the male-specific isoforms of *fru* (*FruM*), we show a critical requirement for the male isoform of *dsx* (*DsxM*), alongside *FruM*, in the specification of courtship song. Therefore, although *FruM* expression is sufficient for the performance of many male-specific behaviors [14], we have shown that without *DsxM*, the determination of a male-specific CNS and thus a full complement of male behaviors are not realized.

Results and Discussion

Courtship behavior in *Drosophila melanogaster* consists of a sequence of behaviors performed by males to interest females in copulation. The male orients to the female, follows her, taps her abdomen with his foreleg, sings a species-specific courtship song, licks her genitals, attempts copulation, and finally copulates [15, 16]. Sex mosaic studies have shown that the sex of the central nervous system (CNS) is critical to the performance of these behaviors, suggesting that sex determination in the CNS is required for male sexual behavior in flies [7, 15, 17–21]. In particular, one sex-determination gene, *fruitless* (fru), is a key regulator of many steps in the courtship ritual [10, 12–14, 22]. Transcripts derived from the *fru* P1 promoter are spliced in females by the sex-specific splice factor Transformer (Tra) in conjunction with the non-sex-specific Transformer-2 (Tra-2), introducing a premature stop codon into female P1 transcripts. In males, a default splice occurs, giving rise to a class of male-specific *fru* isoforms (*FruM* proteins) [10, 13, 22–25] that are expressed in the CNS and peripheral nervous system (PNS) [23, 25–28] in regions associated with male-specific behaviors.

The constitutive expression of *FruM* isoforms in females triggers many male-specific courtship behaviors [14]. However, these females perform subnormal amounts of courtship and do not attempt copulation [14], suggesting that *fru* alone cannot specify all male courtship behaviors.

We examined the role of *doublesex* (*dsx*), another sex-determination gene, in the specification of male sexual behavior. *dsx* transcripts also undergo sex-specific splicing by Tra, producing male- and female-specific isoforms: *DsxM* and *DsxF*, respectively [29, 30]. *dsx* is responsible for somatic sexual differentiation [15, 31–33] and aspects of sex-specific development in the CNS [8]. *dsx* is also expressed in the CNS and is necessary for wild-type courtship song in males [11, 34]. Recently, *dsx* was shown to act alongside *fru* in the differentiation of male-specific neurons in the abdominal ganglion [25]; however, few other studies have examined the relative contributions of both *fru* and *dsx* in specifying a male-specific CNS and regulating male sexual behavior. Therefore, this study examined the individual contributions of both genes in specifying courtship song.

**FruM** Is Not Sufficient for Courtship Song

Courtship song in *Drosophila melanogaster* is male-specific and is critical to stimulating the female [4, 6]. It consists of a humming sound called sine song, and a rhythmically patterned pulse song, which together stimulate the female to mate, reducing the time to copulation [4, 6]. Pulse song also communicates species-specific information, allowing females to recognize conspecific males [1–3].

*FruM* mutant males lack pulse song [10, 12, 13], and constitutive *FruM* expression in the CNS of *fruM* and *frustra* females induces the performance of many steps of the male courtship ritual [14], suggesting an important role for *FruM* in specifying courtship song. To determine the contribution of *FruM* in the specification of courtship song, we analyzed song production in females of genotypes *fruM* and *frustra*.

Song analysis was based on 29 *fruM* and *frustra* females because most *fruM* and *frustra* females did not perform sufficient courtship behavior or song for analysis (total n = 61; Table S1 in the Supplemental Data available online). In accordance with [14], the wing-extension indices (WEIs) of these 29 *fruM* and *frustra* females were
females capable of wild-type courtship song [36, 37], 

DsxM Is Required to Specify Courtship Song Production.

To dissect the individual contributions of both FruM and fru

Figure 1. Representative Song Traces of fruM-/- Males and Females

Representative song traces from a 5–7-day-old fruM-/-/Df(3R)fru4-40 female (A) and a fruM-/-/Df(3R)fru4-40 female (B) produced in the presence of a single wild-type Canton S (CS) virgin female. Each song trace represents a fraction of a 5 min recording for a given individual. Wing extension during the recording is indicated below the song trace. Bouquets of pulse song ("P") and sine song ("S") are recorded above the song trace. "F" indicates a noise caused by the CS target falling.

not significantly different from wild-type and control fruM and fruM-/- males; however, we found a significant decrease in the song index (SI) (the percentage of time spent singing during wing extension) (p < 0.05) (Table S1). Also, the fruM and fruM-/- females' pulse song was highly aberrant (Figure 1). The number of pulse trains per minute (PTPM), mean pulses per train (MPPT) and interpulse interval (IPI) were all significantly lower compared to wild-type and control males (Table S1). Most striking, however, was the complete absence of sine song in these females (n = 29). Although the fruM and fruM-/- females were capable of wild-type wing extension, they spent significantly less time singing during courtship and produced song of poor quality. Thus, FruM expression alone cannot specify wild-type song production.

DsxM Is Required to Specify Courtship Song

To dissect the individual contributions of both FruM and Dsx to the specification of courtship song, we analyzed males lacking FruM and Dsx (genotype fruM,In(3R)dsex23/fruM, Df(3R)dsex19). These double mutants had a courtship index (CI) of 0 toward females (n = 11) and no song. We have shown that FruM expression in females is not sufficient for courtship song. Likewise, the expression of DsxM in females is also not sufficient for song [35]. Thus neither fru nor dsex alone can specify courtship song. In fact, only the presence of both FruM and DsxM, as in transformer (tra) mutant females, renders females capable of wild-type courtship song [36, 37], a finding we confirm. Together, these results demonstrate a previously unrecognized requirement for DsxM, in conjunction with FruM, in specifying courtship song.

Dsx and FruM Colocalize in the CNS

Studies with male-female mosaics have shown that in gynandromorphs with a male head, the ventral thoracic ganglia of the adult CNS (including the mesothoracic ganglion [Msg]) must also be male for courtship song [7]. This suggests that the neural foci of courtship song are located in the ventral thoracic ganglia and that the sex of this region is critical to song. fru and dsex are both expressed in neurons located in this region, and mutations in both genes cause song defects [10–13, 23, 34]. In the abdominal ganglion (Abg) of the CNS, FruM and Dsx were shown to colocalize in a proportion of neurons and play critical roles in the development of male-specific clusters of serotoninergic neurons [25]. Therefore, we asked whether FruM and Dsx were also coexpressed in the thoracic ganglia, and whether they act in parallel (if expressed in different neurons) or in concert (if expressed in the same neurons) to determine the neuronal substrate for courtship song in the CNS.

We determined that Dsx and FruM colocalize in the Msg of the CNS (Figure 2B). Colocalization occurred in a subset of Dsx-expressing neurons (TN1 cluster; nomenclature as per [34]) from the pupal stage onward. The number of neurons coexpressing Dsx and FruM in 2-day-old male pupae was 17.4 ± 1.7 per hemisegment (mean ± standard deviation [SD]; n = 10), and was not significantly different in 5-day-old adults (p < 0.05). Colocalization occurred in a further two subsets of Dsx-expressing neurons in the posterior brain, pC1 and pC2 (nomenclature as per [34]), in addition to previously reported colocalization in the Abg [25] (Figure 1). Given the critical importance of the sex of the ventral ganglia (including the Msg) to song production, the colocalization of FruM and Dsx in this region suggests that sexually dimorphic developmental mechanisms might be operating in the Msg, contributing to the sex-specific nature of courtship song production.

The Neuromuscular Junctions of the Direct Flight Muscles Are Not Sexually Dimorphic

Electrophysiological studies show that the activity of seven of the direct flight muscles (DFMs) is directly related to the beating of the wing during song [38]. These seven DFMs are the basalar muscles B1–B4, the anterior muscles of the first and third axillaries AX1a and AX3a, and the sternobasal muscle SB (nomenclature as per [38]). The axonal morphology and cell-body location of the motor neurons innervating six of these DFMs (mnDFMs) has also been reported [39]. The cell bodies of these six mnDFMs lie in the ventral thoracic ganglia, five having cell bodies in the Msg [39]. We therefore investigated whether male-specific song production could be attributed to fru- and/or dsex-regulated sexually dimorphic characteristics of these motor neurons.

First, we asked whether any of the mnDFMs were fru or dsex expressing. By using fruGAL4, a GAL4 driver expressing in all fru neurons [28], we determined that only mnB3/B4 (a single motor neuron innervating both B3 and B4 [39]) was fruGAL4 positive, and thus is a fru neuron (Figures 3A and 3B). This neuron was fruGAL4 positive in both males and females, and the innervation was not obviously sexually dimorphic. However, because some dsex-expressing neurons in the Msg are not fru expressing, we examined the axonal morphology of all mnDFMs to eliminate the possibility of sex-specific DFM innervation.

The axonal morphology and expression of common neurotransmitters at the neuromuscular junction (NMJ)
Kimura et al. [9] showed that FruM expression prevented the sexually dimorphic production of song. Where might the critical difference(s) then lie?

The Mesothoracic Ganglion Is Sexually Dimorphic

Kimura et al. [9] showed that FruM expression prevented reaper-mediated programmed cell death [41, 42] in a cluster of cells, resulting in more neurons in this cluster in males. DsxM, on the other hand, prolongs neuroblast divisions in the Abg of males [8], again resulting in more neurons in males. Thus, sexual dimorphisms might be present in regions in which Dsx and FruM colocalize, as suggested by the ability of FruM and Dsx to generate sexually dimorphic neuronal populations [8, 9, 25]. Given that our investigation found no obvious sex-specific dimorphisms in the mnDFMs, the dimorphism might lie in a population of interneurons. Therefore, the Msg was examined so that it could be determined whether a sexually dimorphic population of neurons was present.

By using fruGAL4, which expresses in both males and females [28], to drive a GAL4-responsive UAS-LacZ.NZ reporter, we quantified the number of β-Gal-positive neurons in males and females (Figure 4A). The number of β-Gal-positive neurons was significantly higher in males, with 136.4 ± 3.3 cells per hemisegment (n = 10) versus 111.6 ± 3.1 cells per hemisegement in females. Previously, Lee et al. [23] reported a sexual dimorphism in the number of neurons expressing fruP1 transcripts in the Msg. Together, these results suggest that a sexually dimorphic population of neurons is present in the Msg; therefore, we examined the individual contributions of FruM and Dsx in the creation of this difference in fruGAL4-positive neuron number in the Msg.

FruM Expression Alone Cannot Create a Sexually Dimorphic Msg

We found a sexually dimorphic number of fruGAL4-expressing neurons in the Msg, a region of the CNS central to song production [7] and in which FruM and Dsx colocalize. To determine the individual contributions of dsc and fru in the creation of this sexually dimorphic number of neurons, we examined fruM and fruAla females to see if FruM expression alone abolishes the observed difference in neuronal number in the Msg between the sexes. We found that the number of FruM-expressing neurons in the Msg of these females was significantly reduced in comparison to wild-type and control males (Figure 4B; Table S2). Furthermore, this decrease in FruM-expressing neurons was comparable to the difference in neuron number observed in the Msg of fruGAL4 males and females driving the UAS-LacZ.NZ reporter.

Our results demonstrate that the difference in neuronal populations of males and females in the Msg lies in a subpopulation of FruM-expressing neurons, and that FruM expression alone cannot eliminate this difference. Thus FruM expression cannot, by itself, dictate the creation of the sexually dimorphic population of neurons in the Msg. We therefore asked whether Dsx, which colocalizes with FruM in the Msg, plays a role in the specification of this sexually dimorphic population of neurons, helping to determine the full complement of FruM neurons.

DsxM Is Required for Wild-Type FruM Expression

dsx affects the sex-specific development of other regions of the CNS [8, 25]. To determine whether dsx contributes to creating the sex-specific population of neurons in the Msg, we tabulated the number of FruM-expressing neurons in the Msg of dsx null and dsx heterozygote control males. We found that dsx mutant males had significantly fewer FruM-expressing neurons in the Msg than did wild-type and control males, demonstrating that Dsx is indeed required to obtain a full complement of FruM-expressing neurons (Figure 4B;...
Because fruM and fruTRA females (who express the female-specific isoform of dsx, DsxX) do not have a full complement of FruM-expressing neurons in the Msg, we have demonstrated a critical role for DsxM in the creation of a sexually dimorphic Msg. In fact, only when both FruM and DsxM are present, as in tra mutant females, can a full complement of FruM-expressing neurons in the Msg be obtained (Figure 4B). Thus, we have demonstrated a previously unrecognized requirement for Dsx M in the specification of a population of FruM-expressing neurons in the Msg.

DsxM prolongs the division of neuroblasts in the Abg of males, resulting in more neurons in the male Abg [8]. Also in the Abg, DsxM plays a critical role alongside FruM in the differentiation of a male-specific serotonergic population of neurons [25]. Our findings suggest that DsxM operates in a similar manner in the Msg and the posterior brain (E.J.R. and S.F.G., unpublished data) to create sexually dimorphic neuronal numbers.

These differences in neuronal populations suggest a common developmental theme in colocalization regions, where DsxM generates a sexually dimorphic population of neurons, which is exploited by FruM to fashion a male-specific behavioral neural network [25] (cf. [9, 15]).

It is not clear why the absence of a sexually dimorphic population of FruM-expressing neurons in the Msg is associated with striking defects in courtship song because our results suggest that this population of FruM-expressing neurons does not directly innervate the DFMs. We propose that the FruM-expressing neurons form at least part of a male-specific neural network responsible for controlling the production of courtship song.

Thus, although FruM expression can specify many male-specific behaviors, we show that without DsxM, the determination of a complete male-specific CNS, and therefore a full complement of male behaviors, is not realized. This additional gene function is critical to
understanding complex sex-specific phenotypes compared to previous interpretations of function, where *fru* has been described as the only gene needed for a "genetic switch" to male sexual behavior in *Drosophila*. Significantly, it adds to the growing evidence that *fru* and *dsx* are both necessary for a complete male courtship repertoire, in both neural and nonneural tissues [11, 25, 34, 43–47].

Supplemental Data
Experimental Procedures, one figure, and two tables are available at http://www.current-biology.com/cgi/content/full/17/17/1473/DC1/.

Acknowledgments
We are grateful to Adriana Villella, John Rieffel, and Jeff Hall (Brandeis University) for generously providing, and assisting in the use of, LifeSongX software. We thank Gyunghee Lee for the generous gift of Dsx antibody, Barry Dickson for *fru*^Dtra* and *fru*^M* flies, and Paul Schedl for *dsx*^1* flies. We thank Megan Neville, Tony Dornan, Jeff Hall, and Michelle Arbeitman for comments on the manuscript.

E.J.R., J.-C.B., and S.F.G. were supported by grants from the Wellcome Trust.

Received: June 21, 2007
Revised: July 19, 2007
Accepted: July 20, 2007
Published online: August 23, 2007

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Figure 4. Sexually Dimorphic Neuron Numbers in the Mesothoracic Ganglion
(A) Mean number of nuclei expressing β-Gal per hemisegment (±SD) in the Msg of the CNS in 5–7-day-old adult males and females. The mean number of nuclei is calculated from 10 hemisegments. *fru*^GAL4* drives the expression of β-Gal in both males and females. ***indicates a significant difference (p < 0.05).
(B) Mean number of nuclei expressing FruM per hemisegment (±SD) in the PrMs cluster of FruM-expressing neurons (nomenclature as per [23]) in 5–7-day-old adult flies. The mean number of nuclei per hemisegment is calculated from 10 hemisegments per genotype. ***indicates a significant decrease from wild-type and control males (p < 0.05). The presence or absence of FruM, DsxM, and DsxF is noted below each histogram bar.


