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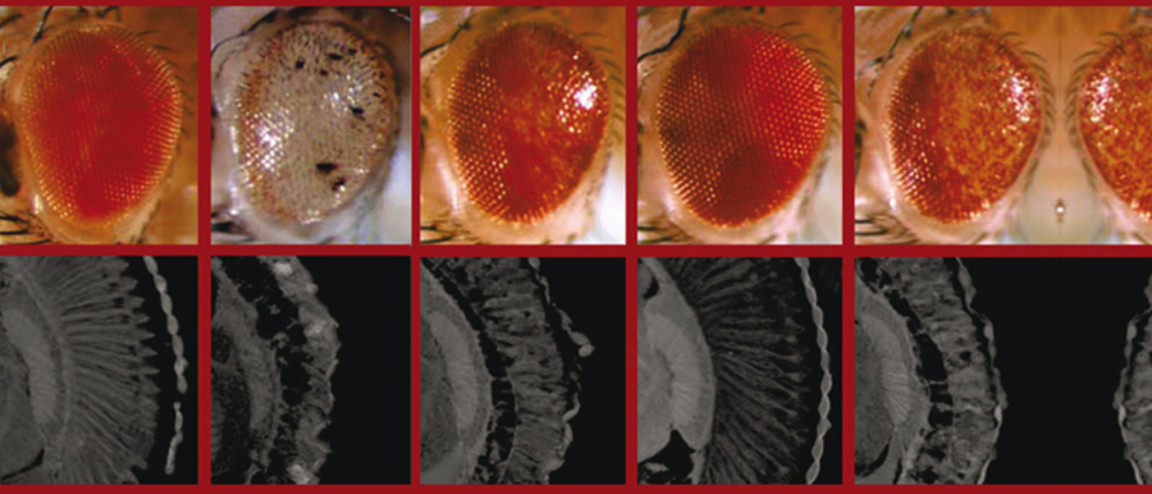

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NIGEL ATKINSON

THE ROLES OF *FRUITLESS* AND *DOUBLESEX* IN THE CONTROL OF MALE COURTSHIP

Brigitte Dauwalder

University of Houston, Department of Biology and Biochemistry, 369 SR2, Houston, TX 77204-5001, USA

Male courtship in *Drosophila melanogaster* is a robust innate behavior that is shaped by sensory input and experience. It is regulated by the general sex-determination pathway through the sex-specific forms of *fruitless* and *doublesex*. Recent findings have shown that both *fruitless* and *doublesex* are required for courtship. This chapter reviews the role of these proteins and the neurons that express them in the regulation of courtship behavior. In particular it discusses how *doublesex* and *fruitless* contribute to the generation of sexually dimorphic neurons, the role of cell death, and the emerging information about circuits that underlie the behavior.

I. Introduction

Courtship in *Drosophila melanogaster* consists of an innate set of behavioral steps that are shaped by sensory input and prior experience. The behavior is characterized by a series of steps that are performed sequentially and can relatively easily be observed and quantified in the laboratory. It is therefore well suited for genetic analysis and to address fundamental questions about the molecular control of

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complex behaviors. The mating behavior of *D. melanogaster* males has been described by Bastock and Manning (1955) and Spieth (1974) and reviewed in detail (Greenspan, 1995; Hall, 1994; Villella and Hall, 2008). This chapter will summarize the behavior and its control by the general sex-determination pathway, specifically the roles of *fruitless* and *doublesex*. It will then review the latest findings on the roles of some of the *fruitless* and *doublesex* expressing neurons and how sexual dimorphism is generated by cell death. Finally, the search for downstream targets is discussed, as well as the role of non-neuronal input through diffusible factors from the fat body.

II. The Behavior

A. Male–Female Courtship

A protocol on how to set up the behavioral assay has recently been published (Ejima and Griffith, 2007). In short, the following steps can be observed (Fig. 1):

1. *Orientation toward the female*. The time to first orientation toward the female is measured as latency. The time it takes the male to orient toward the female is highly dependent on a variety of sensory stimuli. Vision and pheromone detection, but also auditory cues play an important role (Ejima and Griffith, 2008; Griffith and Ejima, 2009b). Performing courtship assays in red light will increase latency because visual stimuli are absent. Assays performed in red light are therefore a sensitive way to examine the role and perception of pheromones. In bright light, where visual input plays a significant role, *white* mutant males, which have impaired vision, will show a long latency compared to w^+ males. This can be an issue with transgenic strains whose marker w^+ expression is very weak. Latency also depends on the size of the courtship chamber.
2. *Tapping* of the female's abdomen with the forelegs. Male forelegs have been shown to harbor gustatory cells. Three gustatory receptors have been identified with a role in courtship (Gr32a, Gr33a and Gr68). The behavior of mutants suggests that these receptors are involved in inhibiting male–male courtship, in the perception of inhibitory pheromones and the control of correct courtship song (Koganezawa *et al.*, 2010; Miyamoto and Amrein, 2008; Moon *et al.*, 2009).

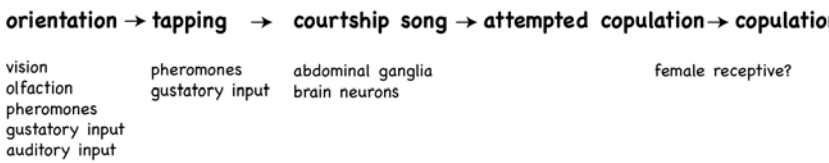

Fig. 1. Courtship steps displayed by *Drosophila melanogaster* males.

3. "*Singing*" of the courtship song by extension and vibration of the wing that is closest to the female. This song is male- and species-specific. This unilateral wing vibration produces a species-specific courtship song that consists of sine song (a humming sound) and a pulse song with rhythmic elements, which stimulate the female to mate (Von Schilcher, 1976). The pulse song contains species-specific information (Kyriacou and Hall, 1986; Kyriacou *et al.*, 1990). The song can be recorded and, when played to a virgin receptive female, stimulates her willingness to mate.
4. *Licking* of the female's genitalia.
5. *Attempted copulation:* Curling of the male's abdomen in attempts to copulate.
6. *Copulation*. Whether or not copulation occurs is mostly dictated by the female. If she is too young or has previously mated, and is therefore unreceptive, she will run away, kick the male with her legs and protrude her ovipositor, and copulation will generally not occur. If she is mature and ready to copulate, she will slow down, open her anal plates and copulate. Copulation takes about 20 min.

In general, although the different steps of courtship have been shown to be regulated by different regions of the central nervous system (CNS) by mosaic analysis, they are stereotypically displayed in this order. Spieth (1974) has suggested that each step may be involved in elevating arousal levels, with thresholds that trigger later steps.

Courtship assays are generally performed in plexigas chambers or small tubes. Overall courtship is measured as courtship index (CI), the fraction of time a male performs any of the courtship steps during the observation period. The "wing-expansion index" WEI measures the fraction of time a wing is extended toward the female. The CI measured for wild-type flies is dependent of the size of the chamber and, as mentioned before, whether or not the assay is performed in white or red light.

B. Male–Male Courtship or How to Discriminate Between Males and Females

Mature wild-type males generally do not court other males. This is mainly due to the inhibitory effect of male-specific pheromones on the cuticle of adult males, and the rejection behavior of courted males. Young males elicit courtship from mature males because the full repertoire of sex-specific pheromones is established during the first 2 days after eclosion. Young males also court males and females as the full behavioral competence may only be established after eclosion (McRobert and Tompkins, 1983). A number of mutations cause male–male courtship, often without affecting male–female courtship, indicating a loss of the ability to discriminate males and females. This may be an indication that multiple mechanisms are

in place in wild-type males to suppress male–male courtship. In this context it is interesting that the silencing of mushroom-body neurons, a structure not basically required for courtship (Kido and Ito, 2002), causes male–male courtship, suggesting an inhibitory function of these neurons (Kitamoto, 2002).

The discrimination between males and females is in large part mediated by sex-specific pheromones (Ferveur, 2005). As most of them have low volatility, recognition is probably mostly gustatory. Gustatory sensillae are present on the maxillary palp, the proboscis, and the forelegs, which all make contact with the female during courtship. Gustatory neurons of the legs project to thoracic ganglia and the subesophageal ganglion (SOG) (Possidente and Murphey, 1989), olfactory neurons to the glomeruli of the antennal lobes. Interestingly, sex-specific branching of neurons has been observed in several of these structures that is dependent on the sex-specific regulators *fruitless* and *doublesex* (see Section III.C). Moreover, although specific receptors and pheromones are playing an important role, intriguingly, the specificity of some of the responses may lie in the wiring and projections of specific neurons (see Section III.C). Besides pheromones, many sensory inputs contribute to efficient mate recognition and courtship. Vision, smell, taste, and auditory stimuli all contribute to the behavior (Griffith and Ejima, 2009b). In the absence of any one of these stimuli, courtship still proceeds, even though at lowered efficiency. Pheromones also play an important role in species recognition (Ferveur, 1997). In this context, an important question still largely unclear is how sender and receiver of the signal co-evolve. A recent study has suggested that the *desat1* gene involved in the biosynthesis of pheromones plays a role: a mutation in *desat1* simultaneously altered both sex pheromone emission and perception in *D. melanogaster* (Bousquet *et al.*, 2009).

C. The Role of the Female

Although the female seems to have a mainly passive role in courtship to the observer, she is the one that ultimately decides acceptance by slowing down and allowing copulation. Besides sensory input from the courting male (his courtship song, for example), major factors that influence female receptivity are her age (very young females are not sexually mature), and whether or not she has previously mated. Male accessory gland proteins that are transferred during copulation cause a profound change in the female's behavior and lead her to reject other males. These postmating behaviors of *D. melanogaster* females are well-studied and described (Ferveur, 2010; Kubli, 1992; Wolfner, 2009).

D. Males Remember: Courtship Conditioning

Although male courtship behavior is a fairly stereotyped innate behavior, it is shaped by sensory input as described above. It is also influenced by the previous

mating experience of the male. If a male encounters a female that rejects him because she is too young or already mated, the rejected male will form a lasting memory of the rejection. It has been shown that mated females carry cisVA on their cuticle that was transferred from the male during mating. cisVA is repulsive to males and its perception is associated with the rejection (Ejima *et al.*, 2007). If previously rejected males subsequently encounter a virgin female that is ready to mate, they show an increased latency to court. The memory decays over time. This associative learning ("courtship conditioning") has been shown to be dependent on the same genes and brain structures that are generally required for associative aversive learning in *Drosophila*. The courtship conditioning assay is therefore often used as a paradigm to study learning and memory (reviewed in Ejima and Griffith (2011); Griffith and Ejima (2009a)).

III. The Genes, the Neurons

A. Fruitless and Doublesex

The master regulators of the somatic sex-determination pathway also regulate sexual behavior (Belote and Baker, 1987; McRobert and Tompkins, 1985; Taylor *et al.*, 1994). Sex in *Drosophila* is determined cell autonomously by a cascade of sex-specific alternative splicing (Baker, 1989) (Fig. 2). The primary signal lies in the ratio of X chromosomes to autosomes. The XX:AA = 1 ratio in females leads to

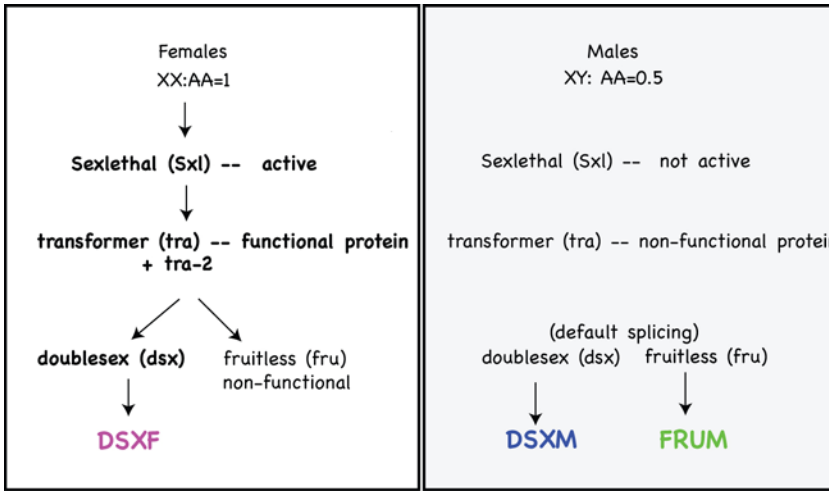

Fig. 2. Regulators of the *Drosophila melanogaster* sex-determination pathway.

the activation of *Sexlethal (Sxl)*, a splicing factor. *Sxl* autoregulates itself to produce an active protein that regulates splicing of the *transformer* (*tra*) pre-mRNA. Only *tra* RNA that is spliced in the female mode can make a functional protein (TRAF) because the default male splice form contains an early stop codon. TRAF is also a splicing factor that, together with TRA-2, regulates the sex-specific alternative splicing of the two downstream target pre-mRNAs *doublesex (dsx)* and *fruitless (fru)*. This leads to the production of the female-specific DSXF protein. The female-specific splicing of *fru* results in a nonfunctional FRU protein. In males, default splicing in the absence of TRA leads to the production of the male-specific DSXM protein. The default splicing of *fru* pre-mRNA in males leads to the generation of a functional male-specific FRUM protein. In summary, males end up with DSXM and FRUM, and females with DSXF (and no FRUM). In genotypically normal XX females that carry a mutation in *tra* or *tra-2*, *dsx* and *fru* are spliced in the male mode. These animals look like males and court like males, confirming that male courtship behavior is regulated by the sex-determination pathway. When *dsx* and *fru* mutants were examined for their courtship behavior, *dsx* mutants showed a reduction in courtship, but still courted (Villella and Hall, 1996). Furthermore, induction of DSXM in females did not initiate courtship. In contrast, strong *fruitless* mutant males no longer courted. Weaker *fru* mutants showed strongly reduced courtship toward females and they courted males, and these mutants became "famous" for the male courtship chains they formed (a male courting a male, followed by a courting male, followed by another courting male, and so on) (Gailey and Hall, 1989; Ito *et al.*, 1996; Ryner *et al.*, 1996). Based on these mutant findings, *fruitless* was long thought to be the only regulator of male courtship behavior. Fruitless is expressed in about 2000 neurons in the male nervous system as determined by antibodies (Lee *et al.*, 2000). This expression pattern was reproduced in transgenic flies in which Gal4 sequences had been introduced into the *fru* locus by homologous recombination such that expression of Gal4 was controlled by the *fru* P1 promoter (Dornan *et al.*, 2005; Manoli *et al.*, 2005; Stockinger *et al.*, 2005). The fru P1 promoter directs expression of the *fru* transcripts that are subject to sex-specific alternative splicing. In the *fru-Gal4* construct P1-controlled expression of Gal4 leads to the presence of Gal4 in the cells that normally express *fru* in both sexes. This is because the male-specific presence of FRUM is due to alternative splicing of the *fru* pre-mRNA and translational control, and not regulated at the level of transcription. The availability of Gal4 lines that express Gal4 specifically in *fru* neurons in both sexes has opened the door to extensive manipulation of these neurons by expression of a variety of UAS-transgenes. In addition, these constructs have made it possible to address an important question: Are the neurons that express FRUM in males male-specific neurons that are absent in females (in which case one would not expect to see Gal4 expression in females), or are the neurons present in females, but just not expressing FRUM? When the *fru-Gal4* flies were crossed to *UAS-reporters* and expression visualized, the answer was that the neurons

are generally present in both sexes (Dornan *et al.*, 2005; Manoli *et al.*, 2005; Stockinger *et al.*, 2005). This indicates that the neurons are present in both sexes, but probably work differently because they express FRUM in males, but not in females. Indeed at first glance it appeared that there were no sex-specific differences. However, as the analyses became more detailed since these initial experiments, a growing number of instances have been found in which the cell number in *fru* clusters differs between males and females, or where small clusters are only present in one sex. Most importantly, in a number of cases it has been found that the projection pattern of these neurons differs between males and females. This is likely to be of significant biological importance, influencing the way these neurons work and connect. Interestingly, in many of these instances it has also been shown that *doublesex* plays an important role in the generation of these differences (see below).

B. *dsx* and *fru* Are Both Required for Male Courtship Behavior

Another important transgene was used to address the question whether FRUM is sufficient to induce male courtship behavior. In this transgenic line the *fru* gene had been manipulated to always express the male FRUM protein, also in females (Demir and Dickson, 2005; Manoli *et al.*, 2005). Indeed, females that express FRUM from this transgene are capable of performing male courtship, even though at lower rate and with some steps missing. These experiments clearly demonstrated the very important role FRUM has in setting up the competence of the nervous system to display courtship. But what could be the factor(s) that are missing in the FRUM females in order to obtain complete male courtship behavior? In the past few years it has become increasingly clear that while *fruitless* has a very important role in conferring the ability to court in the nervous system, *doublesex* is also required for full and normal male courtship. Notably, the courtship "song" of FRUM females was not a real courtship song: It lacked "sine" song and showed aberrant features in the "pulse" song (Rideout *et al.*, 2007). As it turns out, both FRUM and DSXM are required and females that express both FRUM and DSXM (as is the case in *tra* mutant females) have normal male courtship song (Rideout *et al.*, 2007; von Schilcher and Hall, 1979). It has previously been shown that the male differentiation of the ventral thoracic ganglia is important for courtship song (von Schilcher and Hall, 1979). It is intriguing that a male-specific cluster of neurons is present in the thoracic ganglia that expresses both FRUM and DSXM (Lee *et al.*, 2002; Rideout *et al.*, 2007; Sanders and Arbeitman, 2008). The sexual dimorphism in cell number is dependent on both *fru* and *dsx* (Lee *et al.*, 2002; Rideout *et al.*, 2007; Sanders and Arbeitman, 2008) with a major role for *dsx*. In fact, it has been shown that the female-specific DSXF activates cell death genes that lead to the absence of these cells in females (Sanders and Arbeitman, 2008).

These sexually dimorphic neurons are good candidates for mediating courtship song. However, as they do not make direct contact with the flight muscles, their exact function and involvement in courtship song is not clear at present. There are other neurons in the thoracic ganglia that are sexually dimorphic: A group of serotonergic neurons that differ in their cell number in males and females in a DSXM- and FRUM-dependent way (Billeter *et al.*, 2006; Taylor and Truman, 1992), as well as a group of abdominal neuroblasts for which DSXM prolongs division in males (Billeter *et al.*, 2006; Taylor and Truman, 1992).

There is another important sexual dimorphism in the abdominal ganglia: Sexually dimorphic axons have been identified in the prothoracic ganglion that cross the midline in males, but not in females. These are the projections of foreleg gustatory receptor neurons (Boll and Noll, 2002; Possidente and Murphey, 1989). The differential midline crossing of their axons has been found to be dependent on both *fru* and *dsx*, with a major role for *fru* (Mellert *et al.*, 2009). This is one of the rare cases where a downstream effector has been identified that is involved in mediating the differential effects of *fru* and *dsx*: It has been found that the midline crossing of the gustatory receptor axons in males requires the presence of FRUM to repress *roundabout* (*robo*) signaling. *robo* receptors have a wide role in regulating axonal pathfinding. For the gustatory neurons, reduced *robo* signaling is required to allow midline crossing, and FruM appears to be involved in the reduction of an inhibitory signal that keeps the axons from crossing in females (Mellert *et al.*, 2009).

These findings are in keeping with previous mosaic mapping experiments that examined regions of the nervous system that need to be male in order for male courtship to occur. Mosaic mapping using different techniques has revealed anatomical foci in the ventral ganglia as well as the brain (Ferveur *et al.*, 1995; Hall, 1979; Hotta and Benzer, 1972). The above examples illustrate roles of the ventral ganglia in sensory perception, but also in motor output function, with courtship song as a prominent example.

C. Sexual Dimorphism in *fru* and *dsx* Expressing Neurons in the Brain

The crucial function of male brain regions in the regulation of the behavior that was found in the mosaic studies has since been confirmed many times. The constant refinement of these maps and the identification of neuronal cell groups with potential specific roles are a vibrant and exciting part of current research into the control of courtship behaviors. New tools and techniques are continuing to allow more detailed insight. Not surprisingly, *fru*, but also *dsx* expressing cells are found in the brain regions that are required for courtship. A number of brain clusters have been identified by now that are sexually dimorphic. The first one to be discovered was a FRUM expressing cluster of interneurons in a region just above the antennal lobe called mAL. Males and females differ in the number of

neurons and their projection patterns. It was found that the higher number of cells in males is caused by FRUM, which inhibits cell death of the neurons at the pupal stage and determines their projection pattern (Kimura *et al.*, 2005). Projections of these neurons are seen in the lateral protocerebrum and the region of the SOG and the use of a presynaptic marker suggests that the SOG is the input region, and the lateral protocerebrum the output site. The SOG receives input from gustatory neurons and it is an intriguing hypothesis that sex-specific gustatory neurons may make contact with mAL neurons. In fact, a foreleg gustatory neuron that expresses receptor Gr32a projects toward mLA extensions in the SOG (Koganezawa *et al.*, 2010). Males with inactivated Gr32a neurons or males in which the projections from this neuron have been interrupted fail to show the unilateral wing extension that is typical for *D. melanogaster* courtship and often extend both wings instead. Their altered courtship song is likely the cause for their lowered mating success. These findings suggest a pathway of information flow: from gustatory input to higher order integration centers in the brain to output neurons and abdominal neurons that mediate the wing extensions.

In addition to gustatory input, olfactory input plays a role in courtship as well. Olfactory receptor neurons project to the antennal glomeruli and receptor neurons and a subset of antennal glomeruli have been identified that express fru or are contacted by fru neurons (Datta *et al.*, 2008; Kimura *et al.*, 2005; Stockinger *et al.*, 2005). Changing the sex of antennal glomeruli that are contacted by *fru*-positive neurons induces male–male courtship, and silencing of these neurons leads to a drastic reduction in courtship also toward females (Stockinger *et al.*, 2005).

Another sexually dimorphic neuronal brain cluster that expresses *fru* is P1. P1 neurons are only present in males. They express both FRUM and DSXM and several studies have now shown their importance in the control of the first steps of courtship. P1 neurons are located in the posterior part of the brain, close to the mushroom bodies, an area that has been implicated in the initiation of courtship in the mosaic studies mentioned earlier. It has been found that FRUM is required to define the normal branching pattern of these neurons. In females, the P1 cluster is absent due to the presence of DSXF that triggers cell death of these neurons. When FRUM and DSXM are expressed in these cells in otherwise normal females by the induction of *tra* mutant clones (tra mutant cells assume a male fate and express both FRUM and DSXM), P1 neurons are present and the females will initiate courtship behavior, suggesting a role for these neurons in the initiation of courtship (Kimura *et al.*, 2008). Two recent studies have further corroborated the importance of this cluster in the initial steps of courtship. In one of the studies, activation of P1 neurons triggered tapping and wing extension, two early steps of courtship. A transgenic strain that expressed the heat-activatable channel dTRPA1 in *fru* neurons was used to create mosaic males in which dTRPA1 was present only in subsets of *fru* neurons. When the temperature was raised, some of the mosaic males initiated tapping and wing extension in the absence of a female, with

characteristics very similar to males encountering a female. These behaviors were highly correlated with expression of dTRPA1 in P1 neurons as well as a second cluster nearby, P2b, consisting of descending interneurons (Kohatsu *et al.*, 2011). Furthermore, touching of the foreleg of a tethered male with a female abdomen triggered neuronal activity in P1 neurons, suggesting a pathway from sensory input to integration in P1 neurons to potential output through P2b descending neurons.

TRPA1 induction was also used in the screening of driver lines that were expressed in subsets of *fru* neurons (von Philipsborn *et al.*, 2011). The males initiated wing extensions when P1 neurons and pIP10 neurons in the brain were activated by raised temperatures. The pIP10 neuron is a descending neuron with axons terminating in the mesothoracic ganglion with likely input from P1. It would therefore be well positioned to mediate the command for courtship song. Although activation of P1 and pIP10 neurons led to wing extension, it did not lead to structured courtship song. Three other types of neurons in the mesothorax were identified that appear to provide the typical song features of the courtship song. These results were similar to earlier experiments in which headless flies displayed wing extensions when abdominal *fru* neurons (in a pretty broad pattern) were activated through the activation of a light-activatable channel (Clyne and Miesenbock, 2008).

In contrast to *fru*, alternative splicing of *dsx* produces two different functional proteins in males and females (DSXM and DSXF). DSXM and DSXF are expressed in a sexually dimorphic pattern in the CNS as well (in addition to their expression in non-neuronal tissues) (Lee *et al.*, 2002; Sanders and Arbeitman, 2008). A central role for *dsx* expressing neurons in behavior was demonstrated recently by using *dsx-Gal4* transgenic flies in which Gal4 was placed into the *dsx* locus. The silencing of *dsx-Gal4* expressing neurons reduced male courtship drastically and abolished courtship song (Rideout *et al.*, 2010). Sanders and Arbeitman (2008) found that the sex-specific number of neurons that express *dsx* is determined by cell death that is regulated by the amount of DSX and by the presence of the specific male or female DSX isoform. For example, as a consequence of DSXF expression in females, cell death of some of the neurons occurs during metamorphosis (it is blocked in a mutant in which several cell death genes are deleted). Sex-specific cell death, or more specifically, cell death that occurs in females due the presence of DSXF, thus emerges as a recurrent and important mechanism that creates sexually dimorphic numbers of neurons (reviewed in Kimura (2011). Although the difference in cell numbers is fairly modest, the observed differences in projections from these neurons are pronounced and could significantly influence the potential interactions of these neurons with other neurons. Not surprisingly, the function of DSXF in regulating cell death does not depend on *fru* (Sanders and Arbeitman, 2008), as no Fru protein is expressed in females normally.

In summary, there are a number of possible mechanisms that can underlie the sexually dimporphic function of *fru* and *dsx*-expressing neurons (diagrammed in Fig. 3). In the male neurons that express FRUM and, in many instances, DSXM, it

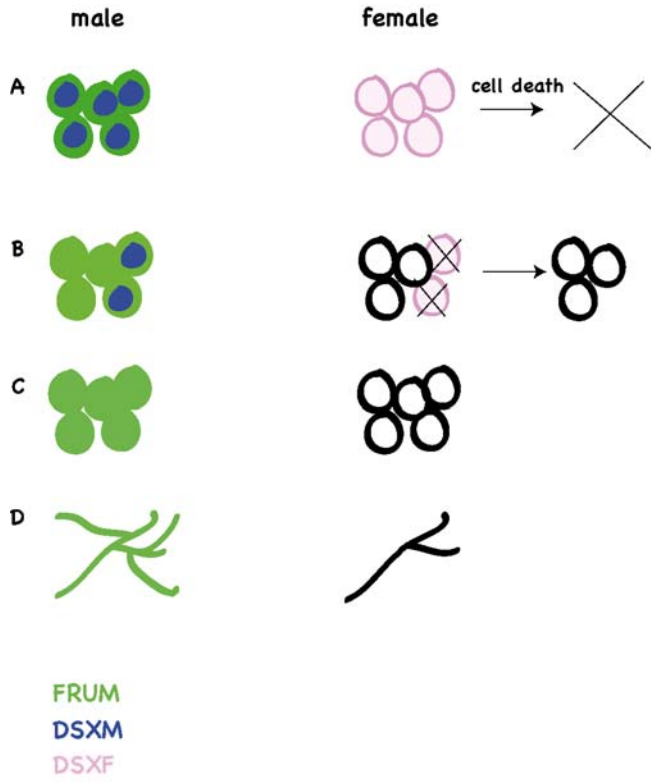

FIG. 3. Mechanisms that create sexual dimorphism in fru and dsx expressing neurons. (A) Absence of a particular cluster in females due to DSXF-mediated cell death in females. A cell cluster is shown in which all cells in the cluster express FRUM (green) and DSXM (blue) in males, and DSXF (pink) in females. DSXF-expressing cells undergo cell death. As a result, the cell cluster is absent in females, whereas it is present in males (no cell death occurs in the male cells that express FRUM and DSXM). (B) Reduced cell number in a particular cluster due to DSXF-mediated cell death of some of the neurons. Only some of the cells in the cluster express DSX (DSXM in males and DSXF in females). In females, the DSXF expressing cells undergo cell death. This leads to a different number of neurons in males and females. The remaining cells in the female are nevertheless qualitatively different from their male counterparts because they do not express FRUM. (C) Equal number of cells in a particular cluster. Although the cell number in the cluster is the same in males and females, neurons in the male express FRUM, the corresponding neurons in the female do not. (D) FRUM expressing neurons have different neuronal extensions than the corresponding female axons. Due to the presence of FRUM, projection patterns are different in males. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this book.)

is likely that these two regulators determine at least some of their function and projections. The mechanisms of this functional determination are unknown, as are the genes that mediate it. Also, what makes *fru* expressing clusters different from each other? The number of neurons that express both *fru* and *dsx* is limited

(Rideout *et al.*, 2007; Sanders and Arbeitman, 2008). Therefore, sexual dimorphism by the observed mechanism of DSX-mediated cell death is likely not going to account for all sexually dimorphic cell groups. In addition, it is worth noting that there are a number of *fru* neurons that do not show differences in cell number, or even projections, between males and females. *fru* neurons that are present in both sexes are nevertheless molecularly dimorphic: They express FRUM or DSXM in males, whereas their female counterparts do not express FRUM or DSXM (some will express DSXF). It will be of particular interest to define the function of these neurons in females.

An attractive hypothesis is that *fru* neurons and *dsx* neurons form behavioral circuits. What constitutes such circuits and how these circuits interact in the behaving adult animal is an important next question to be addressed. Experiments have recently identified a likely *fru* circuit that mediates the recognition and response to cisVA. cisVA, a volatile pheromone, is attractive to females and inhibitory to males (Ejima *et al.*, 2007; Kurtovic *et al.*, 2007; Vosshall, 2008). It is perceived equally in both male and females by Or67d olfactory receptor neurons, evoking the same electrical response, indicating that perception by the receptor is not sex-specific. Or67d receptor neurons project to the antennal glomerulus DA1, one of several glomeruli that are contacted by *fru*-positive neurons. (The antennal lobe glomeruli are the first-order processing center for incoming olfactory receptor neurons.) DA1 is contacted by projection neurons that project to the lateral horn of the protocerebrum. These neurons differ in their projection patterns and branching in the two sexes, a difference that is dependent on *fru* (Datta *et al.*, 2008; Kimura *et al.*, 2008; Kimura *et al.*, 2005). Using photoactivated GFP to visualize the proximity of neurons, and calcium-imaging and electrophysiology to monitor activity, Ruta *et al.* have shown that the projection neurons that contact DA1 are part of a circuit that responds to cisVA. In the lateral horn they contact 4 sexually dimorphic clusters of presumptive third-order neurons (Ruta *et al.*, 2010). These neurons in turn make contact with neurons that extend processes into the ventral ganglia that may then be involved in the behavioral response to cisVA. Similarly, as described above, significant insight has been gained into the possible circuits that involve the P1 cluster and its control of initiation of courtship and its first steps such as tapping and wing extensions (Kohatsu *et al.*, 2011; von Philipsborn *et al.*, 2011).

Extensive mosaic mapping of *fru* neurons in male and female brains has recently been carried out and a detailed map of these neurons and their potential connections established by (Cachero *et al.*, 2010; Yu *et al.*, 2010). Both groups have mapped the identified projections onto a standard brain. These maps will facilitate the mapping of functional circuits in the future. The studies have confirmed a fair number of sexual dimorphism between males and females. In addition to changes in cell numbers as outlined above, in many instances second and third-order neurons have different projection patterns in males and females.

D. What Are the Downstream Effectors of *fru* and *dsx*?

Experiments with *tra* and *tra-2* temperature-sensitive mutants have indicated that the critical period to establish the basic competence for male courtship behavior is during late pupal stages (Arthur *et al.*, 1998; Belote and Baker, 1987). The temperature-shift experiments examined whether courtship was being displayed but did not quantify it or look at individual components. It is not known to what degree *fru* and/or *dsx* are also required for the optimal adult function of these neurons. The experiments mentioned in the section above did not use conditional mutants that would allow an assessment whether *fru* and *dsx* are required mainly to set up neuronal cell numbers and connections, or whether they are still required for the optimal sex-specific functioning of the neurons in the adult male.

An important next level of analysis will be to address the molecular consequences of *fru* or/and *dsx* expression in these neurons. What are the downstream targets? Do subsets express different cell adhesion proteins or cell migration guidance proteins, signaling pathways, and neurotransmitters? What differentiates the circuits: the incoming stimuli, the way this incoming information is processed, the connections the neurons make with others, and integration across a network of neurons? What are the output pathways, and which molecular and electrophysiological determinants define the specific behavioral response?

A number of potential downstream genes regulated by both *fru* and *dsx* have been identified in several screens, and their roles verified in a few cases (Arbeitman *et al.*, 2004; Dauwalder *et al.*, 2002; Fujii and Amrein, 2002; Goldman and Arbeitman, 2007). Interestingly, although both *dsx* and *fru* are transcription factors, except for the female yolk protein (yp) genes that are directly regulated by binding of DSX (An and Wensink, 1995; Coschigano and Wensink, 1993), no other genes are known to date that are directly bound and regulated by these factors, leaving open the question of direct or indirect regulation. Therefore, we do not know how these target genes are regulated. Based on the aforementioned *yp* studies, binding sites for DSXF and DSXM in the *yp* genes are well described. *yp* protein genes contain DSX binding sites in their promoter regions that can be bound by both DSXF and DSXM in a competing manner. This led to the model of DSXF acting as an activator, and DSXM as a repressor. We do not know whether the sequences that mediate DSX control are the same in all cell types (in neurons, for example). They may vary depending on tissue-specific co-regulators. This is a particularly interesting question in light of the crucial role DSXF plays in controlling sex-specific cell death. Are cell death genes directly controlled by DSXF? Do they have DSX-binding sites in their promoters? In some of the instances where DSXF-mediated cell death has been examined, it has been observed that the presence of DSXM affected the outcome of cell death (Sanders and Arbeitman, 2008). This suggests the presence of regulatory elements with characteristics similar to those in the *yp* genes. We know even less about how FRU regulates gene

expression. No binding consensus sequences for FRU have been described. In the neurons that express both *fru* and *dsx*, some target genes may be activated by both transcription factors, as has been suggested for some targets. For example, in the case of the male-preferentially expressed *takeout* gene, it has been shown both *dsx* and *fru* are required for wild-type levels of the protein: transcript levels are reduced in *dsx* mutants, but they are also reduced in *fru* mutants (Dauwalder *et al.*, 2002). In addition, it was shown that expression of both DSXM and FRUM is required to express the gene at male levels in female fat body (Dauwalder, 2008). Interestingly, a number of identified *fru* targets that were identified in expression screens are expressed in the fat body, a secretory tissue that plays a role in courtship regulation (see below), but *fru* expression has so far not been observed in that tissue. It is therefore possible that *fru* regulation may occur indirectly, perhaps via diffusible factor(s) or/and as a consequence of neuronal activity in *fru* expressing neurons. As described earlier, a number of neurons express and require both *fru* and *dsx* and it is possible that both pathways may converge on some of the target genes directly or indirectly.

E. One Source of Likely Input Signals

1. *The Role of the Fat Body*

Although a major input into *fru* and *dsx* neurons that regulate courtship comes from sensory neurons, there is another source of input that is non-neuronal. The fat body, a major secretory tissue, has been shown to significantly contribute to courtship (Lazareva *et al.*, 2007). As mentioned earlier, females that express FRUM court other females, even though with reduced CI. However, when the fat body of these FRUM females is masculinized in addition, they have a near-normal CI. Similarly, when the fat body of normal males was feminized, the courtship of these males dropped significantly (Lazareva *et al.*, 2007). These results suggest that male-specific proteins from the fat body "talk to" the brain and contribute to the regulation of courtship behavior. Screens that examined downstream targets of *fru* and *dsx* have identified a number of genes that are expressed in the fat body. Intriguingly, fat body transcripts were also identified in experiments that examined genes whose expression changes in response to mating (Ellis and Carney, 2010a, 2010b). The biological function of most of them is not known yet. The best characterized ones to date are *fit*, *sx1*, *sx2*, and *takeout* (Dauwalder *et al.*, 2002; Fujii and Amrein, 2002). *takeout* is preferentially expressed in male head fat body and *takeout* mutant males have courtship defects. The Takeout protein is secreted into the hemolymph and has been shown to act as a secreted protein in courtship (Lazareva *et al.*, 2007). This is a likely mode of action also for other sex-specific genes from the fat body that interact with the nervous system to control courtship. Feminization of the fat body affects courtship significantly, beyond the effects seen

in *takeout* mutants. This implies a significant role of secreted circulating molecules in the regulation of neuronal activity. The fat body secretes proteins into the hemolymph and an important (and unanswered) question is how hemolymph proteins interact with the CNS. Takeout has also been shown to be involved in feeding behavior and the regulation of longevity (Bauer *et al.*, 2010; Galikova and Flatt, 2010; Ringo *et al.*, 1992; Sarov-Blat *et al.*, 2000; Wilson *et al.*, 2003). As these are other known important functions of the fat body, courtship genes expressed in that tissue may also serve as a link between reproduction and metabolic processes. The takeout protein has characteristics of a soluble carrier protein of lipophilic molecules, such as odorant-binding proteins. It is most similar in sequence to juvenile hormone-binding proteins from other insects. It is not known whether Takeout binds JH or whether JH has a role in the control of male courtship behavior. Interestingly, *apterous* and *Met* mutants, in which JH levels are altered, show courtship defects (Ringo *et al.*, 1992; Wilson *et al.*, 2003). It will be interesting to see whether there is a role for this hormone in adult behavior. Recently a function for ecdysone, another major insect developmental hormone, has been identified in the control of sex-specific, *fru* expressing neurons. Mutants in the EcR-A receptor displayed male-male courtship and were found to have a size reduction in two antennal lobe glomeruli that express *fru* (Dalton *et al.*, 2009).

In summary, courtship behavior offers an excellent model to study how complex behavior is regulated. The emerging picture is one of sex-specific neurons that are required to establish the competence for courtship behavior, and which respond to sensory and environmental input as well as diffusible, hormone-like factors. The molecular basis of how the relevant neuronal circuits are established and how they function continues to be an exiting area for future studies.

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