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Control of Male Sexual Behavior in *Drosophila* by the Sex Determination Pathway

Review

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Understanding how genes influence behavior, including sexuality, is one of biology's greatest challenges. Much of the recent progress in understanding how single genes can influence behavior has come from the study of innate behaviors in the fruit fly *Drosophila melanogaster*. In particular, the elaborate courtship ritual performed by the male fly has provided remarkable insights into how the neural circuitry underlying sexual behavior — which is largely innate in flies — is built into the nervous system during development, and how this circuitry functions in the adult. In this review we will discuss how genes of the sex determination pathway in *Drosophila* orchestrate the developmental events necessary for sex-specific behaviors and physiology, and the broader lessons this can teach us about the mechanisms underlying the development of sex-specific neural circuitry.

Introduction

Innate behaviors refer to the actions of an animal that manifest themselves without prior experience, and thus by implication are genetically inherited. Yet how does gene expression control the development and function of the nervous system so that a gene's action influences some discernible aspect of behavior? Understanding this requires a model system that is both genetically and behaviorally tractable. Male courtship behavior in the fruit fly *Drosophila melanogaster* is an example of an innate behavior, and has long been used as a model to study the relationship between brain, behavior, and the genes that control their development and function.

The courtship ritual of the male fly consists of a sequence of behaviors (Figure 1). It begins with visual and olfactory cues attracting the male to a female and stimulating him to begin the early steps of orienting and following [1–3]. The ritual continues as the male taps the female with his forelegs, allowing him to detect non-volatile pheromones on the female's abdomen [4–7]. Next, the male extends and vibrates his wing to sing a species-specific courtship song composed of "sine" and "pulse" songs [8]. Sine song is a humming sound that is thought to increase female receptivity [9,10]. Pulse song consists of a train of pulses, where the time between consecutive pulses is known as the interpulse interval (IPI) [8]. The IPI is a species-specific parameter of the courtship song; females will mate most quickly when presented with homo-, as opposed to hetero-, specific pulse song [8,11,12]. If the female is receptive she will slow down, allowing the male to lick her genitals [2]. Finally, the male bends his abdomen to attempt copulation, and if the female is receptive they will copulate. During copulation the male transfers a complex mixture of sperm, which fertilizes the female, and seminal fluids, which induce an increase in ovulation and a decrease in her receptivity [13].

That these behaviors are innate is shown by the fact that wild-type males raised in isolation are capable of performing the entire behavioral sequence upon introduction to a female. This does not mean that courtship behavior is fixed, since the levels of courtship displayed by the male can be modified depending on prior experience with a female — this phenomenon is called courtship conditioning [14]. In this review, we will discuss how *Drosophila* male sexual behavior is an excellent paradigm with which to explore the genetic, developmental, and neural logic underlying complex behaviors, and how the brain develops as a sexual organ.

Many genes impact male sexual behavior [6,15–18]. Generally these genes are pleiotropic, affecting a variety of sexual and non-sexual phenotypes. However, one class of genes intrinsically connected with the determination of male sexual behavior is that of the sex determination pathway.

Drosophila Sex Determination and Courtship Behavior

Sexuality extends far beyond having different types of genitalia or body size. For a fly to be a fully reproductive male or female, it not only must look and smell like the sex dictated by its genes, but also must behave like that sex. The ability to perform sex-specific behaviors is dependent on a sexually dimorphic nervous system [17,19]. This dimorphism is determined by the same cascade of genes that directs male and female sexual morphology [20–22] (Figure 2). Briefly, the ratio of X chromosomes to autosomes specifies the sex of each cell by activating (in XX females), or repressing (in XY males) the *Sex lethal* (*Sxl*) gene. In females, Sxl protein regulates splicing of pre-mRNA transcribed from the *transformer* (*tra*) gene, so that an active Tra protein is expressed. Tra is not expressed in males. The presence or absence of Tra, in combination with Transformer-2 (Tra-2), controls the form or presence of the transcriptional regulators *doublesex (dsx)* and *fruitless (fru)*, which in turn determine most aspects of 'maleness' and 'femaleness'.

The prevailing view is that *fru* and *dsx* define a branch point downstream of *tra* in the sex determination pathway, such that male sexual behavior is entirely determined by *fru*, whereas somatic sexual differentiation in both sexes outside the central nervous system (CNS) is determined by *dsx* [17,19] (Figure 2).

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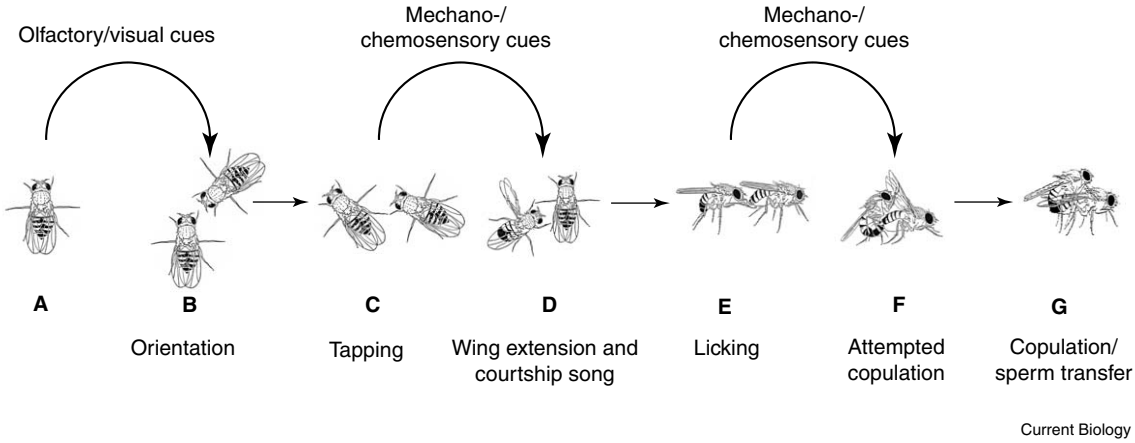

Figure 1. Courtship steps performed by male *Drosophila*.

(A) Target female prior to detection by the male fly. (B) The male fly orients to the female, (C) taps her abdomen with his foreleg, (D) extends his wing to sing a species-specific courtship song, (E) licks her genitals with his proboscis, (F) attempts copulation, and (G) copulation occurs.

However, we will argue that both genes are necessary for a complete male courtship repertoire [23], and recent evidence suggests that these genes act co-operatively in the development of neural circuitry underlying male sexual behavior [24]. Our current knowledge of how *fru* and *dsx* regulate courtship has come from behavioral analysis in males and females expressing mutations at the respective loci. Further insights into how these genes function in specifying male sexual behavior can be inferred by the temporal and spatial patterns of *fru* and *dsx* expression in both the CNS and the peripheral nervous system (PNS). Moreover, for *fru,* it has been possible to manipulate subsets of Fru-expressing cells to determine how they affect sexual behavior.

fruitless is Central to Male Courtship Behavior

It is well established that the performance of male courtship behavior is in large part regulated by *fru* [17,19,25,26]. *fru* is a pleiotropic gene with at least two major functions: one that controls male sexual behavior [24–32] and another that is essential for viability in both sexes [24,33–35]. *fru* encodes a set of transcriptional regulators, each containing a BTB protein–protein interaction domain and one of four alternatively spliced Zinc fingers [27,28,36]. Transcripts from the most distal *fru* promoter (P1) are sex-specifically spliced under the control of Tra [30]. These isoforms, collectively called Fru^M, are translated only in males and expressed in approximately 2% of the CNS [36,37]. The Fru^M isoforms are functionally different in that the presence of specific Zinc-finger DNA binding domains confers functional activity and specificity [24,36,38,39]. *fru* exploits these multiple isoforms through spatially and temporally controlled expression of either a single isoform, or a combination of isoforms, to control specific phenotypic outcomes [24]. This mechanism of alternative splice choice and differential isoform expression is central to the diversity of *fru* function, and goes a long way to explain how this single locus can control a complex behavior like courtship.

The demonstration that Fru^M is central to male courtship behavior has come from the observation that mutations at the *fru* locus that specifically affect male courtship behavior are always associated with a global reduction in the levels of Fru^M expression, an absence of expression in subsets of Fru^M-expressing neurons, or an absence of specific Fru^M isoforms [24–26,30,31,36]. These expression deficits correlate with a variety of courtship problems, including severe reduction or absence of courtship towards females, failure to produce the pulse-song component of courtship song, increased levels of inter-male courtship, and failure to attempt copulation [19,25–30,36,40,41]. Although certain *fru* mutant combinations allow copulation to occur, such mutant animals often fail to transfer sperm and seminal fluids [32]. Most *fru* mutants are sterile as a result of a combination of these defects. Further support that Fru^M proteins are critical to building the potential for male courtship has come from the observation that a female constitutively expressing Fru^M in her nervous system performs the early steps of the male courtship ritual, including initiation, orientation, following, and wing extension towards wild-type females [25,26]. However, in the later steps of courtship these "she-males" barely perform licking and never attempt copulation [25], and it is not yet known whether females expressing Fru^M are able to produce courtship song. The lack of attempted copulation cannot simply be a consequence of the larger female abdomen precluding bending of the abdomen for copulation, since earlier sexual mosaic studies showed that animals with a fully male CNS and an enlarged gravid female abdomen still attempted copulation [42]. In addition, although males completely lacking Fru^M do not show any sexual interest towards females, they form courtship chains when grouped with other mutant males, during which they exhibit much of the courtship ritual. This intriguing phenotype indicates that *fru* mutant males retain the ability to perform courtship, although only in this peculiar context. It appears that Fru^M can specify the earlier steps of courtship behavior, but it is clear that for a complete

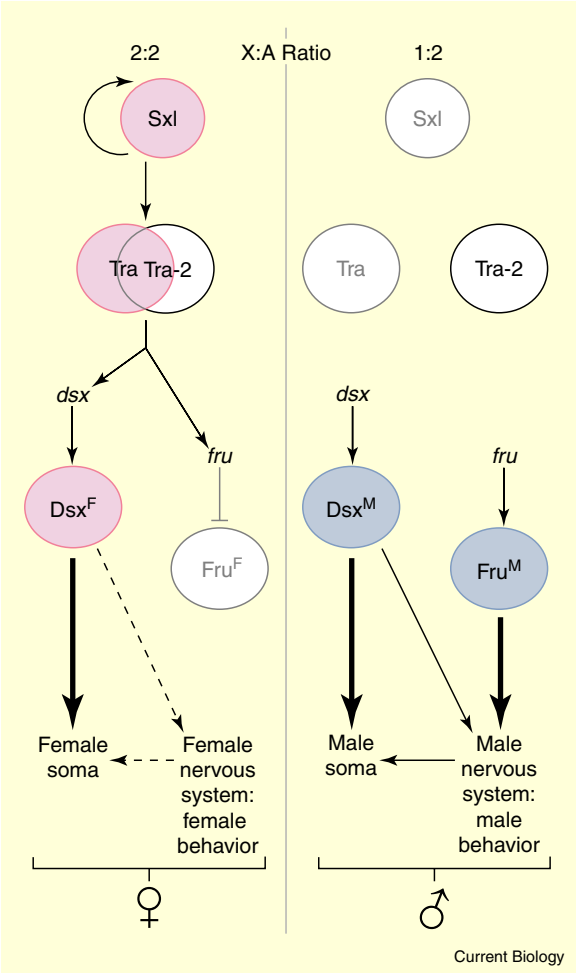

Figure 2. Schematic of the sex determination hierarchy highlighting the functional activities of *dsx* and *fru*.

Black lines or colors indicate active; grey indicates inactive or non-functional. Female-specific proteins are pink, male-specific proteins are blue, and non-sex-specific proteins are white. The *Sex lethal* gene is maintained "on" in females by positive auto-regulatory splicing of its own pre-mRNA. Sex Lethal (Sxl) protein also controls female-specific splicing of *transformer* pre-mRNA to generate the Transformer (Tra) protein [45,46]. Tra, in conjunction with Transformer-2 (Tra-2, active in both sexes), regulates the splicing of *doublesex* (*dsx*) and *fruitless* (*fru*) so that a functional female-specific form of Doublesex (Dsx^F) protein is produced and the female-specific *fru* pre-mRNAs are not translated into a functional Fruitless (Fru^F) protein [36,37]. Dsx^F largely determines female somatic structures and external morphology [19–22], though it has also been shown to impact female-specific behaviors arising from the nervous system (indicated by dashed arrow) [105]. Non-neuronal female somatic tissues may in turn be influenced by affects of Dsx^F on the nervous system (dashed arrow). In the absence of Tra, *dsx* and *fru* messages are spliced into functional male Dsx^M [43,44] and Fru^M [37,36] proteins. Dsx^M largely determines male somatic structures and external morphology [19–22], while Fru^M is required for expression of male behaviors arising from the male nervous system [24–32]. However, Dsx^M has also been shown to be required for the complete development of specific Fru^M-expressing neurons in the male nervous system [24]. Conversely, Fru^M's activity within male-specific structures of the peripheral nervous system may also indicate a function for *fru* within the developing soma of the fly, as in the induction of the MOL [24–26,56]. *dsx* appears above *fru* in the linear schematic because its expression precedes that of Fru^M.

behavioral repertoire there are further male-specific components required that must involve the function of additional genes.

doublesex: An Ignored Link?

One obvious candidate gene for acting in concert with *fru* in the determination of male sexual behavior is *dsx*. As is the case for *fru*, the sex-specific alternative splicing of *dsx* pre-mRNA is regulated by Tra and Tra-2 proteins [43,44] (Figure 2). Both the male- and female-specific *dsx* mRNAs encode Zinc-finger proteins — Dsx^M and Dsx^F, respectively — which share identical DNA-binding domains but differ in their carboxyl termini [45,46]. Wild-type *dsx* function is responsible for directing almost all aspects of somatic sexual differentiation outside the nervous system in both sexes [20,22]. However, *dsx* alone is not sufficient to control male sexual behavior, as chromosomally female flies (XX) expressing only the male form of *dsx*, Dsx^M, are male in appearance, but do not exhibit male behaviors [47]. This observation gave rise to the hypothesis that a *dsx*-independent pathway, the *fru* branch, was responsible for the development of male behaviors [15,47]. However, males lacking *dsx*, which are intersexual in appearance, perform male courtship at diminished levels and completely fail to generate a sine song [23]. Therefore *dsx* must also play a part in controlling male sexual behavior [15,23,47,48], a possibility consistent with the discovery that *dsx* is expressed in the nervous system [49]. These observations suggest that like *fru*, *dsx* influences the formation and/or function of the nervous system in mediating aspects of male sexual behavior. Yet how do *fru* and *dsx* generate a sexually dimorphic nervous system, and what can we learn from these genes to understand how complex behavior may be encoded?

The Brain as a Sexual Organ, Part I: Development

Remodelling of the CNS for Male Sexual Behavior During Metamorphosis

Flies undergo two waves of development, embryogenesis and metamorphosis, resulting in dramatic changes in morphology and behavior to suit the different needs of the larval and adult life stages. During metamorphosis, the CNS is reprogrammed to direct either female- or male-specific adult behaviors [50]. Fru^M is expressed from the onset of metamorphosis specifically in the nervous system [37] and is pivotal to the programming of the brain for male sexual behavior. Although *dsx* is expressed earlier in the larval CNS, its neural expression peaks during the pupal stage [49]. Thus the expression of both genes in the CNS coincides perfectly with the period critical to the determination of male sexual behavior [50]. It follows that by studying how these genes control the developmental decisions that generate a sexually dimorphic nervous system, we should be able to understand how they ultimately influence male-specific behaviors.

Sex and Death: Programmed Cell Death

The processing of information related to adult behaviors is controlled by dedicated interneurons formed during metamorphosis [51,52]. Some of these

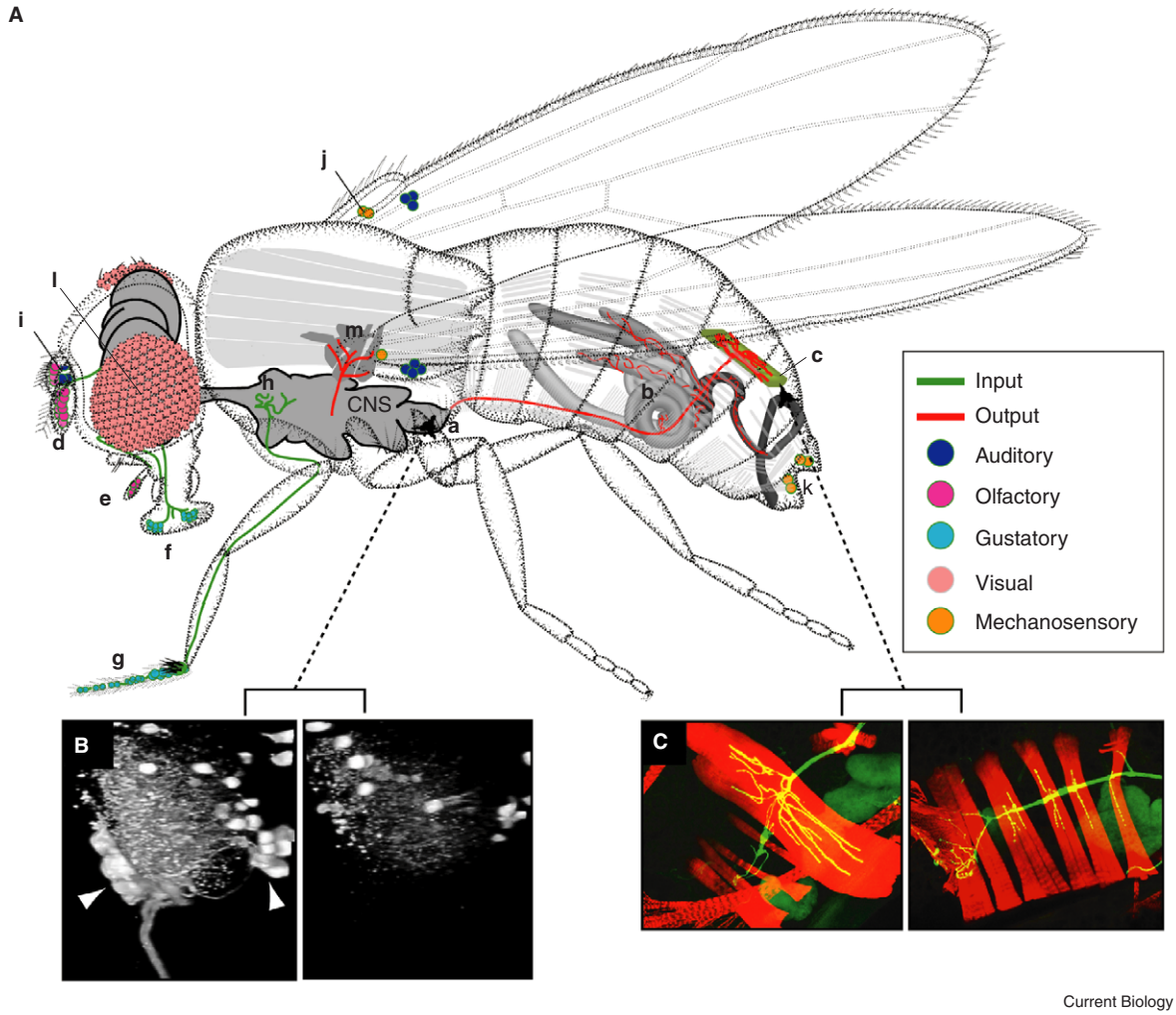

Figure 3. Sensory input and motor output of male sexual behavior.

(A) Sensory neurons expressing Fru^M [26,57] (colored circles) detect sex-specific signals and convey this information (green lines) to the central nervous system (solid dark grey) where it is processed to trigger motor output (red lines) for the different steps in male courtship behavior. Letters on the cartoon represent: a, male serotonergic neurons (also marked by arrow); b, male internal reproductive organs; c, muscle of Lawrence; d, trichoid sensillae in the antennae; e, maxillary palps; f, proboscis; g, leg sensory bristles; h, sexually dimorphic terminals of leg's gustatory neurons; i, Johnston's organ; j, tegula; k, external genitalia; l, compound eye; m, motor neuron of the indirect flight muscles. (B) Sexually dimorphic serotonin expression in the adult abdominal ganglion ("a" in the schematic). Samples are viewed laterally; posterior at bottom. Two male serotonergic clusters are indicated by arrowheads. (C) Dorsal abdominal musculature (red) of adults. Motor neurons are in green (and yellow) in forefront. Figure adapted from [24,106].

interneurons develop only in males, through the action of Fru^M [53]. The neuronal stem cell that produces these neurons proliferates in both sexes during the larval stage. In females, most of resulting daughter cells die during a wave of programmed cell death (PCD) occurring throughout the CNS at the onset of metamorphosis [54]. In males, Fru^M expression early in metamorphosis ensures survival of a cluster of 30 of these clonally related neurons (designated *fru*-mAL) by locally inhibiting PCD. Fru^M continues to act on these interneurons by remodelling their dendritic arborizations [53]. In females, the few surviving neurons from this lineage exhibit a lateralized dendritic arborization pattern, while most of the male-specific interneurons show bilateral arborization. These dendrites are located in a region innervated by gustatory neurons from the PNS and it is probable that the male dendritic pattern mediates behavioral changes by allowing sensory inputs to be processed in a male-specific manner; such remodelling has been shown in other systems to result in behavioral changes [51].

Fru^M-regulated PCD may also be used to control the development of two functionally related clusters of serotonergic motor neurons in the male abdominal ganglion (Figure 3A, part a; and 3B) that innervate the internal reproductive organs [24,31,32]. Fru^M expression is required for differentiation of the full complement of these neurons, for their organization into the two separate clusters, and to control expression of serotonin in these cells during metamorphosis [24,31]. Fru^M must be responsible for their neurogenesis or survival, given that some of these serotonergic neurons appear to be completely missing in males lacking Fru^M [24]. Given that Fru^M inhibits PCD in brain

interneurons, it might also exploit this cell-fate mechanism to establish these sex-specific motor neurons in the abdominal ganglion. Indeed, a similar remodelling takes place in the moth *Manduca sexta*, where a sex-specific pattern of cell death occurs in motor neurons that innervate the reproductive organs [55]. However, as the majority of neurons that express Fru^M in males also exist in females, PCD cannot explain all of the differences that exist between the sexes [26,37,56,57].

Neurogenesis

Adult neurons are born between the larval stage and the end of metamorphosis [54]. While Fru^M is not expressed until the late larval stage [37], *dsx* is expressed early on in the CNS and controls specific aspects of neurogenesis [49]. The male product of *dsx*, Dsx^M, acts on a group of neuronal stem cells in the abdominal ganglion to prolong neurogenesis into early metamorphosis; in females, these stem cells stop dividing at the late larval stage [58]. Thus, although Dsx^M acts on neuronal proliferation, exploiting a mechanism different from Fru^M-regulated PCD, the end result is the same: more neurons are generated in males than in females. Critically, animals that express Fru^M but not Dsx^M express only half the number of male serotonergic neurons in the abdominal ganglion, showing that expression of Dsx^M is also required for complete formation of these neurons [24]. The male-specific neurons induced by Dsx^M thus seem to provide a substrate on which Fru^M can act. Therefore the CNS is not a sexually neutral canvas on which Fru^M acts alone to build the substrate for male sexual behavior.

Inductive Signalling

Another developmental mechanism exploited by Fru^M is inductive signalling, which occurs when one group of cells influences the development of another. From a limited non-sex-specific pool of myoblasts, a group of muscle fibres in the abdomen develops either into a larger male-specific abdominal muscle called the muscle of Lawrence (MOL) (Figure 3A, c), or into 4–5 smaller muscles in females [59–61]. The male-specific patterning of myoblasts is induced by Fru^M expression in a motor neuron shared by males and females (Figure 3C) [24,36,56]. This inductive mechanism may occur elsewhere in the CNS, where certain Fru^M-neurons could trigger the sex-specific development of other neurons during metamorphosis. For example, inductive signalling may be exploited in generating the sexually dimorphic synaptic terminals of Fru^M-neurons that innervate the antennal lobes [57]. Expression of Fru^M increases the size of these glomeruli by increasing the number, and/or size, of synaptic connections of both the olfactory receptor neuron axonal terminals and the interneuron dendritic terminals [57]. Both of these synaptic partners express Fru^M [57], but genetic feminization (and thus cell-specific elimination of Fru^M expression) of only the olfactory receptor neurons is needed to reduce the size of the glomerulus to female dimensions [62]. Therefore control of male sexual behavior by Fru^M may be a result of *fru* acting directly in the neurons in which it is expressed, or indirectly through inductive signalling on non-*fru* expressing cells.

These examples show that Fru^M and Dsx^M use a variety of mechanisms to create a sexually dimorphic nervous system. If one thinks in terms of the behavioral function of the nervous system, then these genes must act to organize its component neurons to direct a unified process controlling the highly coordinated sexual behavior of adult males.

The Brain as a Sexual Organ, Part II: Function

By the end of development, flies are equipped with a CNS to suit their sex. The performance of sexual behavior is a highly dynamic process requiring the recognition and processing of specific inputs, coupled with the appropriate motor outputs. In neurobiological terms, cues coming from the opposite sex are detected by sensory neurons and communicated to interneurons that process this information and in turn instruct an appropriate motor output through dedicated motor neurons. The expression of Fru^M in each of these neuronal types suggests that they are organized into a circuit to receive, process, and transfer the information that controls male sexual behavior [24,26,56,57]. Although *dsx* is clearly expressed in the CNS, little is known about its expression in sensory structures of the PNS [49].

Sexually Dimorphic Sensory Inputs and Their Role in Modulating Sexual Behavior

Both sexual partners send and receive sensory information during courtship and need to respond to specific cues in different ways. This can be accomplished either by producing a different response to the same cue, or by exercising differing abilities to detect a given cue. Therefore, not only are sex-specific sensory cues exchanged, but the sensory structures responsible for receiving the cues are different as well.

Different olfactory and gustatory cues are emitted in a sex-specific manner [6,7]. Males detect volatile female pheromones critical for mate recognition with their olfactory system prior to physical contact, after which pheromone signalling appears to occur mostly through the exchange of non-volatile pheromones using gustatory sensillae [6,63]. Olfactory signals are perceived by two external olfactory sensory structures: the antennae and the maxillary palps (Figure 3A, d and e), which are connected to the CNS via the antennal lobes [64]. The antennae and the maxillary palps contain olfactory sensillae that are the putative sites for stimulatory or inhibitory female pheromone reception, respectively [2,64,65]. Gustatory receptors are located in the proboscis and on the leg sensory bristles (Figure 3A, f and g), and come into contact with the female abdomen during licking and tapping [7]. The distal part of the male foreleg appears to be used to discriminate receptive females from mated ones, and to distinguish between homo- and heterospecific females [4]. Compared to females, males possess on their antennae more trichoid sensillae, which have been implicated in pheromone reception, and possess on their forelegs twice the number of taste sensillae [66,67]. Therefore, males possess specialized sensory structures for the detection of female-specific cues.

Fru^M is expressed in odorant receptor neurons (ORNs) that sense female pheromones and participate in triggering male courtship [26,57]. These ORNs

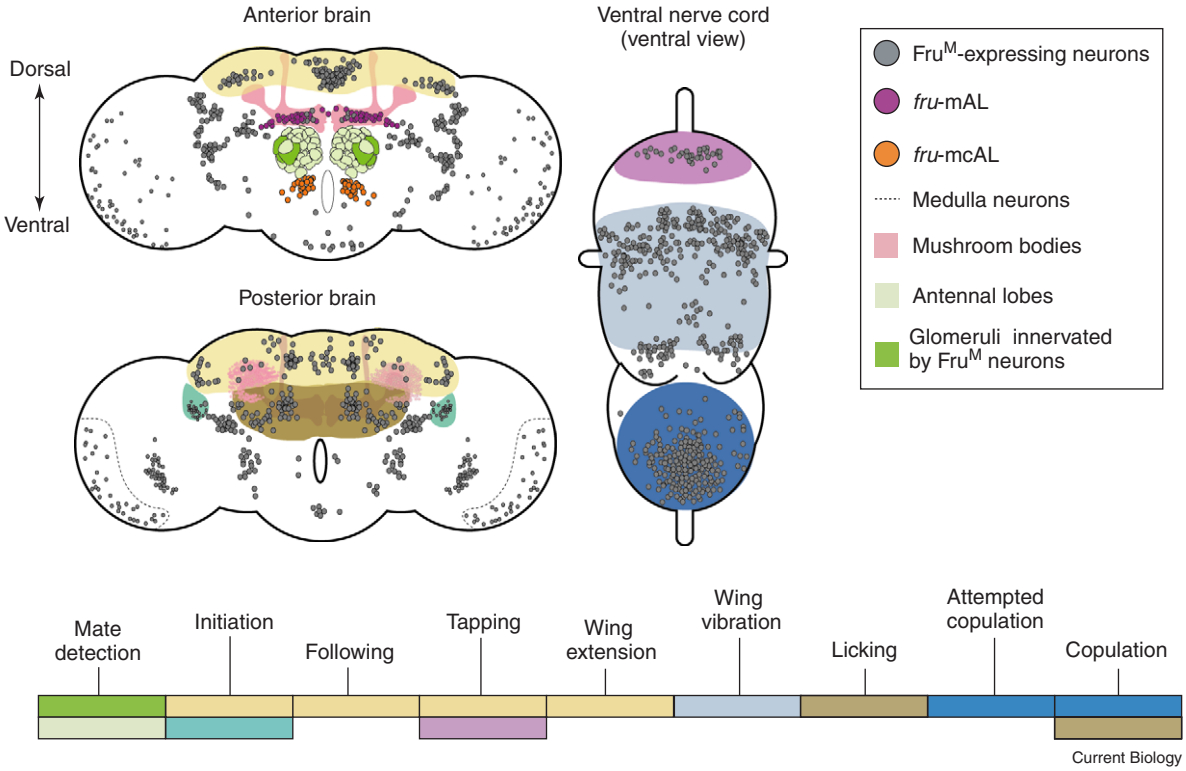

Figure 4. Overlap of Fru^M-expressing neurons with neural sites controlling male courtship behavior.

Areas of the adult male CNS associated with the control of specific courtship steps are color-coded and refer to the courtship steps indicated below and to the legend on the right. Fru^M immunoreactivity in neuronal nuclei of the adult male CNS is represented by grey and colored dots [37,56]. Colored dots refer to Fru^M neurons described in the text. The antennal lobes (light green) are involved in partner discrimination [85,86] and specific target glomeruli (darker green) of Fru^M neurons are involved in mate recognition [26,57]. A cluster of cells in the lateral dorsal brain (teal) regulates courtship initiation [87]. The dorsal posterior brain (yellow) is involved in the initiation of courtship, following, tapping, and wing extension [42,84,86]. The posterior midbrain (brown) affects licking and copulation [42,84,86]. In the ventral nerve cord, the prothoracic ganglion (light purple) is a site associated with the control of tapping [86]; the meso-thoracic ganglion (light blue) is hypothesized to be the control center for wing vibration for song [82]; and the abdominal ganglion (dark blue) controls attempted copulation and copulation [42,84,86].

innervate trichoid sensillae and send axonal projections to three glomeruli of the antennal lobes (Figure 4) [26,57]. These glomeruli are larger in males, and Fru^M directly controls their size [57,62]. The enlargement of these glomeruli may not simply be due to an increased number of ORNs in males [57], but may also reflect a more complex neuronal connectivity related to a male-specific function, and functionally may lead to a greater sensitivity for detection of female pheromonal cues. Three types of ORNs, expressing different odorant receptors, have been traced to these sexually dimorphic glomeruli and are thus candidate pheromone receptors [68,69]. However, Fru^M does not control expression of these receptors, as they are expressed in both males and females [68,69]. Therefore Fru^M affects the structure of the ORNs, rather than the type of receptor they express. Although the neurophysiology underlying this is not well understood, it is a clear example of a male-specific sensory structure set up by Fru^M. Another Fru^M function in olfaction is suggested by its expression in the maxillary palps (Figure 3A, e). Normally, males show less interest in mated females [2], but males missing their palps court mated females at high frequency [65]. Fru^M-expression in a group of ORNs on the maxillary palps (Figure 3A, e) may facilitate reception of mated-female compounds, and the relay of inhibitory signals to the male brain.

Dsx^M, in contrast, controls the expression of the male-specific gustatory receptor, Gr68a [70]. This receptor is expressed specifically in neurons of the PNS innervating the taste sensillae of the forelegs (Figure 3A, g), and is functionally involved in the transition from tapping to the later stages of courtship [70]. Therefore both *dsx* and *fru* enable the peripheral nervous system to sense female-specific stimuli. Moreover, since Fru^M is also expressed in gustatory neurons of the male foreleg [26,57], it may share a function with Dsx^M in enabling males to sense inhibitory or stimulatory female pheromones. Another area of interest includes a group of sensory neurons innervating the taste sensillae, which have been shown to have sexually dimorphic central projections (Figure 3A, h) under the control of Tra [71]. It remains to be seen whether this is mediated by Fru^M and/or Dsx^M. Together, these results persuasively show a role for both *fru* and *dsx* in determining sensory input reception, but much remains to be discovered about the individual roles of these two genes, as well as their interplay, in sensing sex-specific cues.

As with olfaction and gustation, males and females perform different behaviors in response to visual and auditory cues. These systems are less well understood, but expression of Fru^M in structures related to these senses (Figure 3A, i and j) implies a function in sex-specific processing of both types of signals. Upon hearing a courtship song, males will initiate indiscriminate courtship, whereas females will slow down and their receptivity will increase [9,10]. The tegula is a mechanosensory organ of the wing that has been shown to regulate wing-beat frequency in the locust [72]. Expression of Fru^M in the *Drosophila* tegula suggests a similar role in the regulation of wing-beat frequency, and therefore song.

Males exhibit complex processing of visual information. This is illustrated by two observations. Firstly, female movements and shape can elicit male courtship [73,74]. Secondly, a male continually tracks female movements during courtship [1]. Fru^M is not expressed in the eyes (Figure 3A, l), but in higher-order processing neurons directly connected to these sensory organs [26,53,57]. It follows that Fru^M may control male-specific development of neurons involved in sex-specific visual recognition and tracking [75]. Indeed, Fru^M is expressed in giant neurons innervating the lobula, an optic lobe neuropil that is slightly enlarged in *D. melanogaster* males [76,77] and is involved in tracking in other Diptera [78]. Moreover, Fru^M inhibits PCD in neurons of the medulla (Figure 4), a visual neuropil that is activated by motion stimuli [79].

Motor Output for Male Sexual Behavior

Motor neurons, through their action on the limbs, wings, proboscis and abdominal muscles, control the movements observed during courtship. That Fru^M is expressed in all these motor neurons suggests a direct function in muscular control [26,56,57]. This is also indicated by the fact that females expressing Fru^M can perform nearly all male courtship steps, such as extending their forelegs to tap (even though they do not possess the correct receptors to sense females), and extending their wing towards target females [25]. However, the behavior of these 'she-males' shows that Fru^M function by itself cannot induce all male behaviors, as these females do not attempt copulation [25]. As discussed, an enlarged abdomen does not preclude attempted copulation [42], so Fru^M alone is not sufficient to set-up the neuronal substrate for this behavior. It is clear that different neural control centers direct sex-specific motor outputs, such as attempted copulation and copulation in males, and ovulation and egg-laying in females. Since Fru^M and Dsx^M proteins are co-expressed in a large number of neurons in the abdominal ganglion [24], a region involved with copulation, and are both required for the differentiation of serotonergic neurons that control sperm transfer during copulation [24], we speculate that both proteins are required to build the complete neuronal circuitry controlling copulatory behavior.

Further compelling evidence of *fru*/*dsx* co-operation comes from several studies on the molecular and neuroanatomical basis of courtship song production. Early anatomical and physiological studies found that the activity of a set of thoracic muscles, the direct flight muscles (DFM), is directly related to song production [80]. However, it has yet to be determined how the motor neurons innervating the DFM (mnDFM) direct the production of courtship song (Figure 3A, m). Several of the mnDFM neurons have cell bodies that lie in the ventral region of the mesothoracic ganglion (MsG) of the ventral nerve cord (VNC) in the adult CNS [81] (Figure 4). Intriguingly, gynandromorph studies in *Drosophila* using male–female mosaics showed that this same region of the VNC needed to be male for individuals to perform a wild-type song (provided the head was also male) [82]. Therefore, the neural foci of courtship song may lie in the ventral region of the MsG. Provocatively, Dsx^M and Fru^M are both expressed within this region, and mutations in both genes cause song defects: *dsx* mutants lack the sine-song component of song, while *fru* mutants lack the pulse-song component [23,29,30,37,51].

Finally, the male-specific serotonergic motor neurons located in the abdominal ganglion (Figure 3A, part a; and 3B) offer insight into the ongoing function of Fru^M neurons during sexual behavior. These neurons, through their innervation of the male internal reproductive organs, control the synchronized transfer of sperm and seminal fluids during copulation [32,83]. These Fru^M-neurons are organized into two opposing clusters (Figure 3B), which both innervate the same male reproductive organs [24]. These collateral innervations could coordinate and synchronize ejaculation from functionally related targets like the vas deferens, which control sperm emission from the testes, and the accessory glands, which produce seminal fluids [83]. It seems possible that this is a male-specific serotonergic circuit set up by Fru^M to control male reproductive physiology.

Processing: Coordinating Inputs with Outputs

Perhaps the most challenging aspect of studying courtship behavior is to understand the processing that coordinates specific sensory inputs with the appropriate motor output responses, and ensures the performance and coordination of all courtship steps. Behavioral and neural analyses of sex mosaics have shown that male courtship is controlled by disparate brain centers (Figure 4) [26,42,57,82,84–87]. Fru^M expression is found in groups of neurons located in all of the neural sites described in Figure 4 [26,37,56,57] and it seems probable that the behavioral changes observed after changing the sexual identities of groups of these neurons are mediated by a change in Fru^M expression. Indeed, suppression of Fru^M expression or function in targeted areas of the CNS seems to support the notion that distinct groups of Fru^M-neurons are dedicated to specific steps of courtship behavior, which are correlated with the location of these neurons in specialized regions of the brain (Figure 4).

The Mushroom bodies (Mb) (Figure 4), a prominent brain structure receiving input from a variety of sensory structures, are central to courtship conditioning [88], a reduction in male courtship levels following rejection by a mated female [14]. Expression of Fru^M in a group of neurons intrinsic to the Mushroom bodies is required for courtship suppression, linking Fru^M expression and function in these specialized structures to an

experience-dependent modification of male behavior [26,29]. Another example we described earlier is the cluster of Fru^M interneurons, called *fru*-mAl, created by Fru^M inhibition of cell death [53] (Figure 4). Given that their dendrites send projections to the suboesophageal ganglion, a region innervated by gustatory neurons, these male-specific interneurons might process pheromones following tapping or licking [53]. This cluster of neurons is absent in animals bearing fru^1, a mutation that does not affect Fru^M-expression in all brain regions but leads to robust inter-male courtship [29,31,40]. High levels of inter-male courtship are also observed when these neurons are feminized or when their synaptic transmission is blocked [89,90]. It thus appears that these male-specific interneurons function to block sustained courtship between males. Finally, a cluster of Fru^M-expressing neurons (designated *fru*-mcAL) that form part of the median bundle (Figure 4) appears to control the sequential execution of the courtship steps [41]. The median bundle receives sensory input from different sensory systems activated during different steps of the courtship sequence, and Fru^M function likely enables these neurons to control the male-specific processing of this information. The question now is to determine the basic mechanisms, either physiological and/or neuroanatomical, underlying the specific behaviors controlled by these centers.

We began this section by stating that Fru^M neurons might form a circuit. Simply put, this would mean that Fru^M neurons are organized such that they convey information in a linear way, from the perception of a female to copulation. An example of this may be found in experiments that have shown the following: Fru^M-expressing ORNs in the male antennae (Figure 3A, d), when functionally ablated, result in a decrease in courtship initiation [57]; these ORNs connect to Fru^M interneurons, whose functional ablation also results in low levels of courtship initiation [91]; and Fru^M-interneurons connect to the lateral-dorsal brain, an identified focus for courtship initiation (Figure 4) [86,87]. Although these results appear to identify a linear circuit, these experiments center on olfactory cues to the exclusion of other sensory cues. Most types of sensory modalities can stimulate courtship initiation [6], even acoustic signals from the courtship song of another male [10]. This reflects the fact that the brain center for courtship initiation is the site of convergence for all the sensory systems: auditory, gustatory, olfactory, and visual [42,87,92]. Another argument against linear processing is that feminization of brain structures only reduces the intensity of male courtship, but never completely suppresses it. Male-specific courtship behavior is effectively suppressed only when almost all of the brain is female [42,93]. The brain is a highly interconnected and highly interactive system, and sexual behavior, as with any other complex behavior, is likely controlled through distributed processing by separate and mutually reinforcing units [93]. For example, by restoring *fru* function in a distinct subset of its normal expression pattern, it is possible to restore the mating and fertility of certain mutant males to near wild-type levels while leaving the earlier courtship steps at their mutant levels [24]. The neuronal substrates underlying male sexual behavior are better described as being organized into a network whose function may be deconstructed into a number of simpler, interconnected elements.

Evolution of Sexually Dimorphic Behaviors: A Perspective

The rich and varied functions of *fru* and *dsx* in the *Drosophila* nervous system spur us to ask to what degree these genes have been conserved in terms of their structural and functional evolution in other species. Parallel studies in other species are essential to elucidate the basic mechanisms of gene regulation, and reveal potential important differences among species. Significantly, uncovering potential adaptive changes in *fru* and *dsx* regulation might contribute to our understanding of the origin of species-specific sexual behaviors.

While it is well established that *dsx* is both structurally and functionally conserved throughout the animal kingdom [94,95], *fru* has currently only been found in insect lineages [39,96]. *fru*'s mechanism of sex-specific splicing and isoform choice is conserved between *Drosophilidae* and the mosquito (animals separated by 250M years of evolution) [39]. Moreover, it has been possible to rescue specific *Drosophila fru* mutant phenotypes, including behavioral phenotypes, by ectopic expression of a male mosquito ortholog [24,39]. Fru conservation has been found in insect species as diverse as *Tribolium castaneum* and *Apis mellifera*, though it is still to be determined whether a sex-specific role for the gene is employed throughout the insect species [39,97]. It is worth noting again that *fru*, unlike *dsx*, possesses a non-sex-specific developmental function essential for viability, which may be the ancestral role of this gene, and perhaps the sex-specific activity of *fru* has been co-opted into the sex determination pathway at a later stage. Given its conserved features, could *fru* be the prototypic gene of male sexual behavior among insects — including those with vastly different lifestyles? Since Fru proteins are highly conserved, the origin of distinct sexually dimorphic behaviors might come from evolutionary changes in the regulation of Fru^M [39,98]. In this regard, further functional evolution of *fru* is exemplified by Fru^M's action in the induction of the MOL. This muscle may be found in lineages that predate the expansive radiation of the *Drosophilidae,* and, given the homologous structure observed in the mosquito *Anopheles gambiae* [39], may represent a primitive male-specific anatomical feature of dipterans. Curiously, the muscle has been lost from a number of separate *Drosophila* lineages, including portions of the species subgroup to which *D. melanogaster* belongs; whether these evolutionary events included changes in structure or expression of Fru is unknown [99]. Therefore induction of the MOL is not just sex-specific but also species-specific, which might indicate that the presence or absence of this male-specific structure is a result of sexual selection pressures such as female mating preferences [100]. The exact physiological role of the MOL is unclear but it may regulate mating length by facilitating unbending of the abdomen at the end of copulation [24,32]. However, given that a specific Fru^M isoform drives MOL induction, and that this

isoform is conserved throughout the species expressing *fru*, it follows that regulatory elements controlling the expression of Fru^M in the motor neuron necessary for MOL induction may have been modified so that this structure's presence varies even between quite similar species [24,39,101].

Finally, a recent quantitative trait loci (QTL) analysis of the difference in mean interpulse interval (IPI), a critical species-specific component of the male courtship song, between *Drosophila simulans* and *D. sechellia* revealed that at least one QTL for phenotypic differences overlapped the genomic region containing *dsx* and *fru* [102]. In light of the roles discussed earlier of *dsx* and *fru* in song production, further studies may provide new insights into the evolution of reproductive isolation and speciation.

The co-operative activities of *fru* and *dsx* represent cross-talk in the sex determination pathway. Perhaps because interconnected networks are more accepting of 'tinkering,' small changes can become fixed within the system, and these changes may then modulate the outcome of the network [103,104]. The system's potential ability to accept changes, coupled with the functional diversity provided by alternative splicing, isoform heterogeneity, and spatio-temporal regulation of *fru* and *dsx*, gives insight into how these genes may function as speciation determinants through adaptive evolutionary modification of their activities within the male nervous system.

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