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CMLS Cellular and Molecular Life Sciences

# Review

# Sexual behavior mutants revisited: molecular and cellular basis of *Drosophila* mating

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**Abstract.** The study of *Drosophila melanogaster* by a combination of forward genetics with specific mutants, and reverse genetics, in which a given gene is expressed in an appropriate brain area to test its effect on behavior, provides a unique opportunity to explore the causal relationship between a particular gene, its function in the cell and the behavioral outcome at the organismic level. Enhanced male-to-male courtship has been shown to occur as a result of mutations in several different genes. For example, the *Voila* mutant exhibits intense GAL4 reporter expression in the tarsal gustatory sensilla, suggesting the importance of tapping by a male on the female abdomen with his forelegs. Feminization of parts of the antennal lobe and mushroom body by targeted expression of a female-determining gene *transformer*$^+$ (*tra*$^+$) drives the male to court other males. Mutations in the *tra* target gene *fruitless* (*fru*), which is expressed in the antennal lobe as well as the suboesophageal ganglion (the gustatory inputs are processed here), also induce homosexual courtship in males. These results suggest that sensory inputs mediated and/or processed by the tarsal receptors, suboesophageal ganglion, antennal lobe and mushroom body contribute to the regulation of male–female courtship. Mosaic analysis localized the neural center for male courtship behavior to the posterior dorsal brain, in which the sensory information processed by the aforementioned neural structures may be integrated. Another mosaic study mapped the neural center for female sexual behavior, as measured by her receptiveness to copulation, to the anterior dorsal brain. The issue as to how the mutations that reduce female sexual receptiveness, e.g. *dissatisfaction* (*dsf*), *spinster* (*spin*) and *chaste* (*cht*), affect the structure and/or function of this neural center deserves to be addressed urgently.

**Key words.** Courtship; sexual receptiveness; *Drosophila melanogaster*; sexual orientation; pheromones; sexual mosaicism.

## Introduction

The introduction of behavioral genetics has ushered in a new era. Even some of the personality traits and cognitive abilities of humans are now examined by rigorous linkage mapping, utilizing DNA polymorphism markers in conjunction with powerful statistical analyses [1, 2]. Typically, such higher-order brain functions, reflected as behavior, are polygenically controlled [3]. In certain cases, however, they are under the strong influence of

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single major genes [4, 5]. Even when such one-to-one correlation between a certain behavior and a gene is demonstrated, it is not always obvious how the products of such genes, which may have been identified at the molecular level, mediate the final phenotypic outcome. The biochemical processes involving the proteins in question, in the cells involved in the regulation of the respective behavior, need to be elucidated for a thorough understanding of the gene-behavior relationship [6].

The use of model organisms facilitates the establishment of a correlation between the gene, its biochemical function in the cell and behavior. *Drosophila melanogaster* is one of the best-suited organisms for this purpose, as its provides an excellent opportunity to probe complex, unexplored systems, such as the mechanisms generating sexual behavior, by means of forward genetic techniques [7].

Forward genetics refers to analyses that are initiated using mutants that are defective in specific aspects of behavior, and does not require any prior knowledge regarding the mechanism to be elucidated. Thus this approach is unbiased in that it identifies the molecular components that are necessary for the behavior, based simply on the disrupted gene inducing malfunctioning of the system required for a given behavior [8]. Furthermore, forward genetics is useful for exhaustive identification of the elements that compose a system, as exemplified by the success of saturation mutagenesis in the study of early embryogenesis in *Drosophila* [9].

On the other hand, the development of the P-element-mediated germline transformation technique [10] has opened up avenues for highly efficient reverse genetics in *Drosophila*, providing the means for rescue of mutant phenotypes by introducing and expressing the cloned gene (so as to prove the causal relationship between the gene and the phenotype), for insertional mutagenesis [11], for targeted overexpression of introduced [12] or intrinsic genes [13, 14], and for producing enhancer-trap or gene-trap reporters that label specific cells [15, 16].

In this review, we focus our attention on the mechanisms underlying the generation of sexual behavior in *D. melanogaster*, as revealed by a combination of forward and reverse genetic approaches. Special efforts are made to substantiate the classic views held on the neural centers for mating behavior as inferred from mosaic analysis and ablation experiments, with recent results obtained using newly isolated mutants and transgenic flies that are subjected to sexual transformation or targeted expression of cloned genes.

## Sensory basis

Mating behavior in *D. melanogaster* starts with the male orienting itself toward and following a female, a behavior often referred to as tracking [17, 18]. The tracking behavior is apparently guided visually, because mutant males with defects in vision tend to misorient in relation to the female, resulting in the termination of tracking, and consequently the whole sequence of courtship [19]. The importance of vision in this process was assessed by comparison of mating behavior in the light and in the dark. The pairs reach the stage of copulation with a significantly longer delay in the dark than in the light [20, 21]. A careful analysis by Sakai et al. [22] documented that the total time spent by males for following a female in the dark was only 100th of that in the light. Thus, it was inferred that the transition from the orientation to the following of the female by the male critically depends on vision.

After tracking, the male approaches the female to tap her abdomen with his forelegs, followed by unilateral wing vibration. The role of tapping in courtship has not been evaluated critically. It is, however, speculated that the male can 'taste' the body surface of the female by tapping it with his forelegs, which bear gustatory as well as mechanosensory bristles [23]. The forelegs of males have more gustatory bristles than those of females [23]. *Voila*, recovered as a mutant with an enhanced tendency to male-to-male courtship, exhibits preferential expression of the GAL4 reporter in central neurons linked to gustatory processing, and sensory neurons in the gustatory bristles of the prothoracic legs [24]. Feminization of these sensory elements of the *Voila* males with UAS-$tra^{+}$ induces dramatic reduction in courtship activity toward both females and males [24]. This observation suggests that gustatory sensation of pheromones obtained during tapping helps the male to continue courting [25].

Unilateral wing vibration (fig. 1) is regarded as a hallmark of male courtship in *D. melanogaster* and has been intensively studied behaviorally [26], physiologically [27] and genetically [28]. In this section, we concentrate on its sensory aspect. The impact of wing vibration in males upon females is primarily auditory [29, 30], although fluttering of the wings also accelerates dispersal of hydrocarbons from the body surface, thereby potentially facilitating olfactory communication between the male and female [20].

Unilateral wing vibrations produce two distinct types of sound, the 'pulse song' and the 'sine song', collectively called the courtship song [31]. The pulse song is composed of a series of single-peaked (with exceptions) pulses with a mean interpulse interval (IPI) of about 35 ms in *D. melanogaster* and of about 55 ms in *D. simulans* [32]. The IPI fluctuates regularly with a mean cycle of 55 s in *D. melanogaster*, in contrast to the 35-s cycle in *D. simulans* [32–34]. The existence of the IPI rhythm called into question by Crossley [35] and Ewing [36]. The other, independent group recently confirmed

its presence, using a new sensitive method of analysis [37]. Exposure of *melanogaster* virgin females to a simulated conspecific pulse song immediately before the introduction of males into the mating chamber improved their receptiveness [32]. For the promotion of copulation to occur as a result of prior exposure to the pulse song, an IPI drift with a 55-s cycle is required [38]. Neither songs with a fixed IPI nor those with a 35-s IPI cycle had any effect [32]. The promotion of copulation appears to result from reduced locomotion by the females after exposure to the song [31]. The sine song consists of a modified sine wave of 160 Hz and is equally effective for slowing down the female's movement [31]. Interestingly, the simulated male pulse song, even without a drift of the IPI cycle, enhances locomotor activity in males. The males exposed to the song are also stimulated to sing, with a consequently enhanced tendency toward courting other males in the absence of females [31]. Only the pulse song, and not the sine song, stimulates males to sing [31]. The ecological significance of the stimulatory effect of the male's song upon singing by other males is unclear. The existence of a singing male could be construed as being a good indication of the existence of females, as potential targets for courtship by other males.

Figure 1. A male and female pair engaged in mating behavior. The male is vibrating a wing while licking the female's genitalia. Also note the bent abdomen of the male (Photo courtesy of ICAM Co., Ltd).

Whatever the importance of the male song in inducing courtship, it provides a useful means to screen for mutants with defects in auditory mechanisms [39]. Eberl et al. [39] quantified the intensity of male-to-male courtship as a function of the sound intensity of the simulated pulse song (they termed it the 'audiogram') for 15 new mutants the males of which are less stimulated to court other males by the song. The *5L3* line, one of these mutants, shows an audiogram similar to that of wild-type flies after amputation of the aristae. The arista is a brushlike appendage on the antenna considered to be the transformer structure that resonates in the presence of sound, transferring its vibrations to the articulating stalk of the third segment to stimulate the Johnston's organ in the second segment, the presumed auditory sensilla. In wild-type males, the courtship-stimulatory effect of the male's song appears at the intensity of 70 dB and attains the peak at 75 dB, declining slowly thereafter to a plateau. In contrast to this, the aristaectomized wild-type males or *5L3* males are practically unresponsive to the simulated song even at the intensity of approximately 85 dB. Above this sound intensity, the song did stimulate courtship, and the effect increased gradually with increasing sound intensity up to 100 dB. The resemblance of the *5L3* audiogram profile with that of wild-type flies after amputation of the aristae implies that the function of aristae in transmitting vibrations to the Johnston's organ is somehow interfered with by the *5L3* mutation. The fact that both the surgical removal and mutational manipulation of the aristae alleviate the stimulatory effect of the simulated song upon the male's copulatory behavior emphasizes the importance of auditory inputs through the Johnston's organ in this behavior. It has not been reported whether the *5L3* mutant females show a lesser tendency to reduce locomotor activity upon exposure to the male's song, although it could reasonably be expected to be the case. On the other hand, there is ample evidence to indicate that removal of the aristae renders the females more locomotive, compared with wild-type females, in response to courtship songs, thus reducing female sexual receptiveness [40].

Another type of auditory signal is known to be exchanged between flies exhibiting mating behavior: the rejection signal [41]. This sound is generated during bilateral wing flicking by males and unreceptive young females. Because frequent flicking by the courtee considerably reduces the level of male-male interaction [42], the results from the audiogram paradigm described above may be affected by this factor.

When the courting male is not hampered by the rejection signal, he turns to the back of the female and licks her genitalia with his proboscis (fig. 1). Although licking likely mediates exchange of tactile and/or chemical signals between the male and the female, no systematic study has been done on the sensory physiology underlying it. Cobb and Ferveur [20] suggest, based on observations in *D. sechellia*, the possibility that the licking by the male is triggered by extrusion by the female of the ovipositor, which the male contacts with his proboscis. In *D. melanogaster*, extrusion was considered to be an expression of the female's unwillingness to copulate, most commonly exhibited by recently fertilized females [43, 44]. However, extrusion may be simply a posture of females to emit chemical substances. Such chemicals may be inhibitory as has been demonstrated in fertilized *melanogaster* females [45], or excitatory as suggested in *sechellia* females [20]. Fuyama and Ueyama [46] observed that virgin *melanogaster* females expressing the introduced sex peptide gene (thus unreceptive, see below) extrude repeatedly while the male is licking or attempting copulation, yet the males may or may not be discouraged by the extrusion. They showed that the removal of eggs from such females by the *ovo*$^{D}$ mutation eliminates the male-discouraging (inhibitory) effect of the extrusion, and the males continue to court vigorously these unreceptive females which exhibit repeated extrusion. On the contrary, a male who encounters a female who extrudes eggs tends to terminate his attempt at courtship quickly followed by vigorous preening of his face [46]. These observations collectively imply that extrusion per se does not encourage or discourage males from courting females, but chemical substances emitted during the extrusion do alter the subsequent behavior of the male. It remains to be determined how and why an egg is effective in discouraging males to court.

Following licking, the male attempts to copulate, mounting the female's back while gripping the female's raised wings with his forelegs. When the female is sufficiently receptive, she opens the vaginal plate, allowing the male to copulate. After stable copulation for 15–20 min, the male dismounts the female after genital uncoupling. Mechanosensory control of the termination of copulation has been well documented in certain insects. For example, tonic discharges of a stretch receptor neuron in response to a rapid increase in the volume of the bursa copulatorix due to sperm accumulation appear to signal termination of copulation in females [47]. In *Drosophila*, however, little is known regarding the sensory basis for disengagement.

To promote or repress sexual activity of flies, chemosensory stimulation by pheromones is crucial [48]. 5-Tricosene (5-T) is a volatile male-specific compound that inhibits courtship [49]. 5-T tends to delay male initiation of courtship with another male, serving as an olfactory cue for distinguishing males from females [50]. The main component of the female sex pheromones is 7,11-heptacosadiene (7,11-HD), while that of the male pheromones is 7-tricosene (7-T) in *D. melanogaster*: 7,11-HD induces wing vibration in males and 7-T inhibits it [48, 49]. 7,11-Nonacosadiene (7,11-ND) is a minor female pheromone which potentiates the action of 7,11-HD. It is suggested here that 7-T also has effects on females as a stimulant, because *nerd* mutant males, which produce significantly less 7-T than wild-type males, experience difficulties in inducing a female to be receptive [51]. Two components that are common to the pheromones of both sexes merit mention: 7-pentacosene (7-P) acts as a stimulant for males. The stimulatory effect of male-derived 7-P upon males is masked by 7-T coexisting in the males. 7-P and 7-T are the two main hydrocarbons in male flies [52]. Since the 7-P content is variable among different strains, the males from 7-P-rich populations attract courtship by males from other populations [53, 54]. 9-Pentacosene (9-P) exerts an excitatory effect on males in synergy with 7-P, promoting copulation [49].

Oenocytes have been suggested to be the site of pheromone production [55, 48]. This idea was supported by the results of an experiment conducted to feminize male oenocytes by targeted expression of a female-determining gene *transformer*$^{+}$ (*tra*$^{+}$) using GAL-4 enhance-trap lines. Selective feminization of oenocytes in males changed their pheromone profile to that characteristic for females, eliciting vigorous courtship from other (nonmanipulated) males [56]. The males with feminized oenocytes were not excited sexually by their own pheromones. Temporally controlled sexual transformation with *heat shock 70 promoter (hsp 70)*-driven *tra*$^{+}$ expression defined the critical period for development of the sex-specific pheromonal profile, that is, 12–48 h posteclosion (fig. 2).

The receptors for sex pheromones have not been identified at the molecular level in *D. melanogaster*, although the receptors for the contact chemicals are expected to be localized on the membrane of the sensory neurons of the gustatory sensilla on the tarsus or proboscis. However, it must be noted that olfaction, and not gustation by contact, also plays a role, because virgin females, and even males, attract males when placed in a trap container [57]. Recently, 12 [58]–16 [59] putative odorant receptor genes were identified in *Drosophila*. They encode novel members of seven transmembrane domain proteins that are selectively expressed in the antenna and/or maxillary palp. Over 100 odorant receptor proteins of this family are expected to exist in *Drosophila* [58]. None of the identified odorant receptor proteins in *Drosophila* has been assigned to function in pheromone reception.

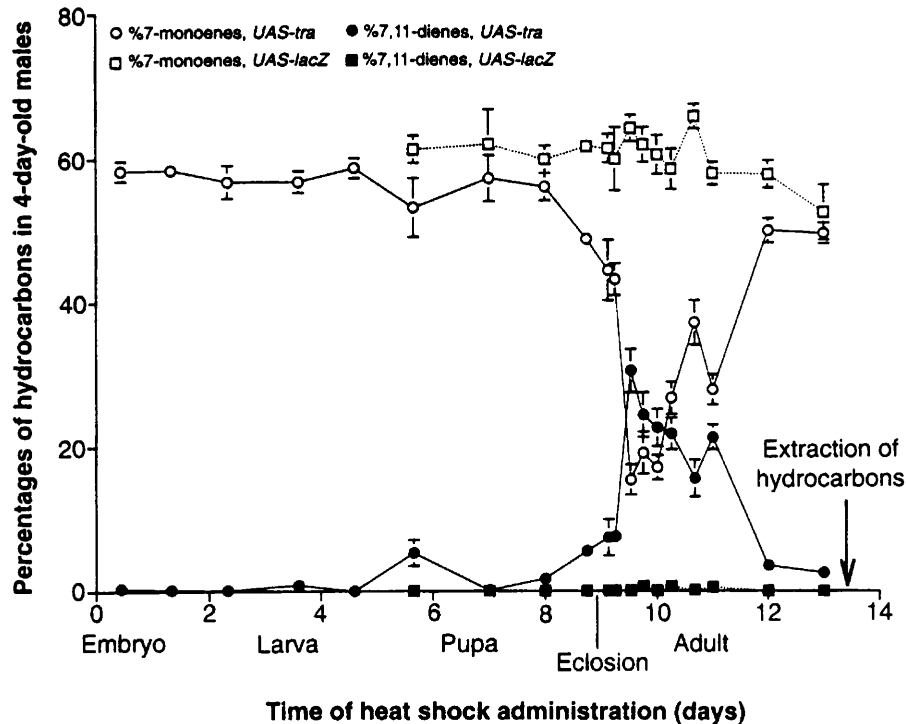


Figure 2. Production of sex pheromones in 4-day-old male flies as a function of temporal activation of *UAS-tra* or of *UAS-lacZ*. A single pulse of heat shock (37 °C) was applied for 2 h, at various times (or 6 h before pupariation). Each data point represents the mean percentage ( ± SE) of 7-monoenes (%7-T + %7-P) and of 7, 11-dienes (%7, 11-HD + %7, 11-ND) for 20 *hsp-GAL4 UAS-tra* individuals and for 10 *hsp-GAL4 UAS-lacZ* individuals. Control, non-heat-shocked *hsp-GAL4 UAS-tra* and *has-GAL4 UAS-lacZ* males yielded 52.8 ± 1.5 and 57.5 ± 2.3% of 7-monoenes [56].

Several putative odorant-binding proteins (OBPs) have been characterized at the molecular level [60–62]. OBPs presumably transport the volatile odorants across fluid-filled lumens in the sensilla to the receptor site on the neuronal membrane, although some other possible mechanisms are also suggested. Immunocytochemical analysis with specific antibodies against two such OBPs, OS-E and OS-F, was performed to determine their localization at the electron-microscopic level [61]. OS-E and OS-F were coexpressed in most of the sensilla trichodea and some sensilla basiconica in the ventrolateral region of the antenna [61]. The similarity of the *Drosophila* OBPs to the moth pheromone-binding protein was not strong [62]; OS-F is only 29% identical to the pheromone-binding protein, APR-1 from *Antheraea pernyi* [63]. No sex difference has been detected in the expression of cloned OBPs in *Drosophila* [64]. Identification of mutations that disrupt each of the OBP genes will allow us to determine whether either of these is involved in chemosensory signalling in sexual behavior.

## Motor control

The mating behavior of *D. melanogaster* is composed of several discrete acts, each of which requires complex and coordinated motor control. Among such motor acts, the generation of courtship songs by male flies has been studied genetically and physiologically in some detail. Some mutations are known to alter the song pattern, although they display additional anomalies in other neural functions such as vision or olfaction. This fact reflects pleiotropic functions of the respective genes. The first song mutant of *D. melanogaster* was *cacophony* (now known as $cac^s$), discovered by von Schilcher [29]. Although the sine song in *cac* is basically normal [65], the pulse song markedly deviates from the wild-type pattern: the interpulse interval (IPI) is significantly longer (e.g. the mean IPI for $cac^s/cac^{L-6}$ is 64 ms, whereas that for the wild-type is 41–45 ms; [65]), and the pulse tends to be multipeaked in contrast to the wild-type pulse, which is typically single-peaked [28, 66]. The *cac* gene was recently cloned and shown to encode

a voltage-gated calcium channel $\alpha$1 subunit (Dmca1A; [67]). The *cac* mutations were found to be allelic to *nightblind-A* (*nbA*), a series of mutations that are defective in visually mediated behaviors [68, 69], accompanied by aberrant electroretinographic (ERG) responses to light stimuli [70]. Although $cac^s$ homozygotes do not exhibit any detectable phenotype in terms of visually-guided behavior or ERG pattern, the $cac^s$ mutants exhibit visual abnormalities when tested in heteroallelic combinations: $cac^s/cac^{L-6}$ flies performed poorly in Y-tube phototaxis, and the ERG of $cac^s/cac^{L10}$ flies showed a reduced on-transient followed by an aberrant undershoot [65]. In the more severe *cac* mutants, both on- and off-transients were practically absent, and the slow sustained negative potential was almost eliminated [65]. The transients in ERG reflect the postsynaptic potentials generated in the large monopolar interneurons L1 and L2 [71], whereas the slow negative potential is the extracellularly recorded summed potentials derived from the retinula cells [72]. Thus the Dmca1A channel plays a role in electrogenesis in both pre- and postsynaptic sites. Conversely, $cac^{P73}$, originally isolated on the basis of visual defects, was found to generate pulse songs with a significantly reduced amplitude [65]. On the other hand, $cac^{H18}$ is considered to be a strong mutation in terms of vision, yet without detectable phenotypes in terms of courtship songs. Sequence comparisons of the *cac* gene between mutants and the wild-type revealed that $cac^s$ carried a single amino acid substitution in the conserved region of the gene, whereas $cac^{H18}$ had an amber mutation in exon I/IIa, which is subjected to alternative splicing so as to generate an isoform preferentially expressed in the eye [65]. Thus the phenotypic variations among different alleles appear to result from differential levels of residual wild-type gene products present in the systems for vision and courtship song.

Encouraged by the finding that malfunctioning of an ion channel can produce a rather specific alteration in the courtship song, other channel mutants were examined for possible anomalies in song generation. Among those tested, *slowpoke* (*slo*), a mutation in the gene coding a calcium-dependent potassium channel [73], was found to produce marked abnormalities in the courtship song: the pulses were multipeaked with extremely variable amplitudes [74]. The males carrying the *paralytic* (*para*) or *no action potential* (*nap*) mutation [75] showed milder alterations in the number of peaks composing a song pulse [74]. Taken together, these results indicate that hypomorphic mutations that alter ion channel functions tend to alter the number of peaks in the pulse song, with a relatively minor effect on the overall composition of the courtship song. It is somewhat surprising that mutations of all of the calcium, sodium and calcium-dependent potassium channels led to the same outcome, an increase in the number of peaks composing a song pulse. The finding that other song parameters such as the interpulse interval were less sensitive (though affected) than the number of peaks might indicate that the song pattern generator relies upon a mechanism in which voltage-gated ion channels with rapid kinetics play only a secondary role. This could be explained if the neurons composing the song pattern generator are principally nonspiking and thus interact via graded synaptic transmission just as is the case for the central pattern generator for locomotion activity in the cockroach [76] and the locust [77]. Alternatively, electrotonic propagation of signals between coupled neurons might be responsible for song pattern generation.

Besides *cac*, two other mutants have been isolated as courtship song variants, i.e. *dissonance* (*diss*) [78] and *croaker* (*cro*) [79]. Later analyses demonstrated that *diss* is an allele of *no-on-transient A* (*nonA*), a visual mutant and thus called $nonA^{diss}$ [80]. The ERGs of *nonA* flies are devoid of on- and off-transients, as the name implies. The *nonA* gene encodes a set of putative RNA-binding proteins that have two RNA-recognition motifs, RRM1 and RRM2 [80, 81]. Point mutations were introduced in these motifs of transgenes which in turn were used for germline transformations of flies for testing their ability to rescue the visual and courtship phenotypes of the *nonA* mutants [82, 83]. The result indicated that both the motifs must be intact to restore normal vision, whereas RRM2 is dispensable for singing [83]. This finding suggests that the *nonA* gene product has two separate functions in the visual and song generation mechanisms. Thus, *nonA* proteins may play specific roles in different neuronal populations even though they are expressed ubiquitously throughout the nervous system [80].

*cro* was isolated in our laboratory as a mutant, the males of which generate multipeaked pulse songs with prolonged IPIs [79]. In contrast to *cac* and *nonA* mutants, *cro* did not exhibit any abnormality in the ERG. Furthermore, axonal conduction, neuromuscular transmission and muscle action potentials operated by voltage-gated calcium and potassium channels were all normal in *cro* homozygous flies [79]. The *cro* mutants were also different from *cac* and *nonA* mutants in that they were impaired in flying [79]. Therefore the *cro* mutants have normal neuromuscular functions, yet display obvious defects in two types of motor outputs, namely singing and flight. This implies that *cro* motor defects have a central origin [84].

The observations in *cro* mutants raise the interesting question as to what extent the mechanisms underlying singing and flying overlap. Barnes et al. [85] examined courtship songs of the so-called flightless mutants that are unable to fly because of a reduced or zero wing beat

frequency. The *ISO-Q* flies cannot move their wings when using a fixed-wire tether, which, however, allows the wild-type flies to make hovering flight. The *KA16-II* flies can vibrate their wings, but the wing beat frequency was reduced to about half of that in the wild-type flies. In spite of the reduction in wing beat frequency, both *ISO-Q* and *KA16-II* males generated practically normal pulse and sine songs [85]. Based on these observations, Barnes et al. [85] argue that the neural circuits for flight and singing are separate.

The motor pattern generator for flight is likely to be located in the pro- and mesothoracic ganglia: intracellular current injection into an identified motoneuron innnervating the dorsal longitudinal flight muscles (DLMs) does affect the firing phase of other identified motoneurons also controlling the DLMs, presumably due to electrical coupling among these neurons [86, 87]. There is no comparable analysis of the putative pattern generator for singing. However, mosaic analysis with gynandromorphs [88] revealed that either the left or the right thoracic ganglion needs to be male for the generation of a normal pulse song, which is generated by both the left and the right wings.

Electromyographic recordings from the thoracic muscles during singing have provided important insights into the motor pattern generator for this behavior [27, 89]: the axillary muscles fire during pulse song IPIs and the basalar muscles fire synchronously with wing movements on the upstrokes of flight, the sine song and the pulse song. The sternobasalar muscle also fires on the upstroke during the pulse song. Importantly, however, the latter muscle does not fire during the sine song [89]. Moreover, lesions of this muscle lead to the generation of multipeaked song pulses just as seen in the songs of *cac* or *nonA$^{diss}$* males. Therefore, distinct though overlapped motor units are activated in the generation of pulse and sine songs. This is consistent with the observation that only the pulse song, but not the sine song, is affected by the *cac* and *nonA$^{diss}$* mutations [65]. It is interesting to recall that a discrete focus for the pulse song has been mapped in the thoracic ganglion by the analysis of sexual mosaics, which, in contrast, yielded diffuse foci for the sine song in both the brain and thoracic ganglion [88]. Thus, identification of the motoneurons innervating the sternobasalar muscle and of the central inputs to them could be expected to provide clues for identification of the pattern generator for the pulse song.

## Central mechanisms

The 'central mechanisms' here refer to the neuronal systems that provide instructions for precisely ordered behavioral repertories or that function as the decision maker for a particular behavioral repertory. A classic example of the neuronal system that acts as the instructor is the 'command neurons' in the cricket brain (with their descending fibers in the cervical connective), repetitive electrical stimulations at different frequencies which produce different song repertoires such as the rivaling song and the calling song [90]. The M (multimodal) neuron described by Heitler and Burrows [91] in the locust metathoracic ganglion represents a decision maker, since spiking of the high-threshold M neuron by summed inputs from a variety of sensory organs triggers jump decidedly. A question here is whether or not such 'executive neurons' exist in the control of *Drosophila* mating behavior. Due to the lack of electrophysiological data on the central neuronal activities (except for certain motoneurons and giant fibers) correlated to behavior, this still remains an open question. However, sexual mosaic analysis with internal tissue markers has been used to localize the brain areas responsible for the execution of mating behavior. With this method, the foci for male courtship behavior were extensively mapped by Hall [92]: the foci for tapping, following and wing extension were found at the posterior cortex of the dorsal brain, whereas the foci for courtship song and attempted copulation were located in the thoracic ganglion. Hall [92] did not specify the anatomical identity of the 'posterior dorsal brain' assigned by him as the focus of male mating behavior, although he discussed the possible importance of the mushroom body as the center for sensory (olfactory in particular) information processing.

Recent introduction of the GAL4-enhancer trap method [12] allowed us to adopt a new strategy for generating sex mosaicism: by just introducing the second transgene, *UAS-tra$^{+}$*, into the enhancer-trap male flies, only the cells expressing GAL4 can be sexually transformed into female by the action of the female-determinant *tra$^{+}$* (see the previous section). With such males having a partially feminized brain, Ferveur et al. [93] demonstrated that when feminized locally, most of the areas in the brain do not produce detectable changes in the males' behavior, with one exception; i.e. vigorous courtship toward males was observed in the males in which the three antennal lobe glomeruli, DM2, DA3 and DA4 had been feminized. Since the antennal lobe is the primary relay center for olfactory information from the antenna [94], this result emphasizes the importance of olfactory processing in discriminating females from males as the target for courtship by a male. Although there are nearly 50 glomeruli in the antennal lobe [95; Y. Kondoh, unpublished observation], only the three aforementioned were positively shown to be relevant to sexual orientation.

The observation by Ferveur et al. [93] was soon confirmed and extended by O'Dell et al. [96], who reported

bisexual courtship by males in whom part of the mushroom body had been feminized. The mushroom body is one of the major projection sites for the antennal lobe neurons [97], along with the lateral protocerebrum, or lateral horn [98]. Thus the possible role of the mushroom body in the determination of courtship targets by males is further emphasized [96].

In the experiments to feminize brain substructures, GAL4 expression in the adult was used to interpret the results [93]. Recent analysis [99] of the sensitive period of *tra*$^+$ action on behavioral feminization (but not target discrimination) indicated that sex types of the brain are determined between the late third larval instar to early pupal stage: when the male to female transformation was achieved in this stage by heat shock induction of *hs-tra*$^+$, after adult emergence, the males failed to show any courtship behavior (fig. 3). A similar induction of *hs-tra*$^+$ after emergence was unable to obliterate male sexual performance [99]. This result is different from the previous report by Belote and Baker [100], who claimed that *tra-2* inactivation after eclosion with a

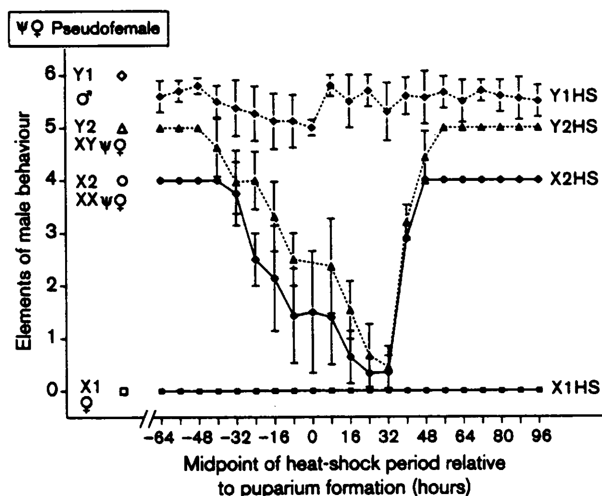


Figure 3. Aspects of male courtship behavior exhibited by an experimental animal when confronted with a single virgin female. Embryos (0–4 h) were collected at 25 °C and either kept at this temperature throughout development (open symbols, left), or subjected to a series of seven heat pulses (30 min each at 34 °C) separated by 7.5 h at 25 °C (filled symbols, HS, right). The position of the filled symbols along the abscissa indicates the time of the fourth heat shock, which is the midpoint of the series of pulses, relative to puparium formation (time 0). The ordinate gives the number and sequence of the elements of male behavior: the value 3, for example, means that orienting, following and wing vibration were observed. The experimental animals were *XX;tra*$^1$/*tra*$^1$*hs[tra-fem]* (X2) and *XY;tra*$^1$/*tra*$^1$*hs[tra-fem]* (Y2), and their siblings *XY;tra*$^1$/*TM6 tra*$^+$ (Y1, control) and *XX;tra*$^+$/*tra*$^1$*hs[tra-fem]* (X1, control). Each data point represents the mean ($\pm$SEM) of at least 30 flies [99].

temperature-sensitive allele can induce male courtship behavior in genetically female flies. It is important to demonstrate that the *tra-2*$^{ts}$ flies, when reared at a permissive temperature (16 °C), express the Tra-2 protein at a level sufficient for driving female development.

Courtship of mature males by males has been reported to occur in some mutants [24, 84, 101–103]. Some of them have sensory deficit, making it likely that inadequate orientation is ascribable to peripheral causes. In other cases, however, the homosexual orientation has a central origin. For example, the *fruitless* (*fru*) gene, mutations of which result in a homosexual tendency of different strengths in males depending on the molecular lesions [104, 105], has sex-specific forms of transcripts specifically expressed in a subset of central neurons [105]. The sequence analysis of the female-specific *fru* transcript identified a putative *tra* binding site [104], which is recognized by Tra and Tra-2 protein in vitro, leading to female-specific splicing [106]. The *fru*-expressing neurons were found in a variety of brain areas including the antennal lobe and the suboesophageal ganglion, receiving antennal olfactory afferents [107, 108] and gustatory projections, respectively. These brain regions are thus implicated in the discrimination of females from males by male flies during courtship. It is intriguing that some of the *fru* alleles lead to extremely low sexual drive [104, 109]. The presumptive trigger neurons for sexual behavior might differentiate by a mechanism dependent on the sex determination gene *fru* [110].

There are other mutations that reduce courtship activity in males [102, 111, 112]. These mutant males initiate courtship with females, but only a small fraction succeed in copulating [102, 111, 113]. Cloning of these genes and determination of their sites of action in the brain could be expected to provide clues to explore the origins of instructions for sexual behavior in the brain.

Mosaic analysis to examine female sexual behavior was carried out by Tompkins and Hall [114], who mapped the focus for receptiveness to copulation in the dorsal anterior brain, a focus different from that for male sexual behavior. There has been no corresponding study using the GAL4-UAS system.

Mutations leading to reduced sexual receptiveness in females have been isolated. For example, *dissatisfaction* (*dsf*), recovered by Finley et al. [115], is accompanied by aberrant motor innervation of uterine muscles, implying impairment of synaptogenesis in the central connections regulating female sexual receptiveness. Molecular analysis identified the *dsf* gene, which encodes a putative transcription factor homologous to the vertebrate Tailless protein [116]. Interestingly, an intron of the *dsf* gene contained a sequence similar to the Tra binding site, implying that *dsf* is the third target of Tra.

*dsf* messenger RNA (mRNA) was detected in only a limited number ( < 100) of cells located near the antennal lobe, lateral protocerebral neuropil and subesophageal neuropil [116]. We isolated *spinster* (*spin*) [117], mutant females of which exhibit unusually strong rejection behavior against courting males. Preliminary analysis of the expression patterns of these genes suggested their having roles in the central nervous system.

Involvement of dopaminergic transmission in the regulation of female sexual receptiveness was documented recently [118]. Virgin females treated with 3-iodo-tyrosine (3IY), a selective inhibitor of tyrosine hydroxylase that catalyzes dopamine synthesis [119, 120], exhibited enhanced mate refusal in response to courting males [118]. Dopamine was effectively depleted by 3IY in these flies as judged from the result of HPLC analysis [118]. Feeding the 3IY-treated females with L-dopa, the catalytic product of tyrosine hydroxylase, alleviated the effect of 3IY, restoring female receptiveness [118]. Since the tyrosine hydroxylase gene is strongly expressed in the mushroom body, Neckameyer [118] assumed that this brain structure is important for female receptiveness. In determining the anatomical site at which tyrosine hydroxylase is required for female receptiveness, classic mosaic analysis could be valid: patches of brain tissue that are null for tyrosine hydroxylase (i.e. *pale* homozygous patches) are expected to allow the fly to survive in adulthood (the *pale* mutation is lethal), which can be tested for female sexual behavior.

The role of the other major monoamine neurotransmitter octopamine in the regulation of female sexual receptiveness is less clear. Tyramine ß-hydroxylase (TBH) catalyzes the last step in octopamine biosynthesis. The females null for TBH mated normally, although they failed to lay eggs [121]. These flies are devoid of octopamine, as determined by HPLC [121]. The level of octopamine decreases to one-sixth of the wild-type in the *inactive* mutant [122]. The *inactive* females were found to be less receptive [123]. It is important to determine whether the effect of the *inactive* mutation on female receptiveness is a direct consequence of lowered octopamine levels.

Another chemical message known to increase female sexual receptiveness is the juvenile hormone (JH) synthesized in the corpus allatum [47]. Implantation of the ring gland, in which the *Drosophila* corpora allata are contained, into pupae resulted in precocious development of sexual receptiveness in eclosed female flies [124]. Earlier sexual receptiveness was also achieved by topical application of JH, or a synthetic JH analog, methoprene [125]. The topical application of JH or methoprene restored sexual receptiveness when the latter was reduced by mutations such as *apterous* [126] and *icebox* (*ibx*) [127]. These results are compatible with the hypothesis that JH plays a role in the development of female sexual receptiveness. Since JH is known to stimulate proliferation of mushroom body neurons in the adult orthopteroids [128], the effect of JH on female receptivity might result from its neurogenic action. The corpora allata are innervated by brain neurons [129, 130]. Some projections are received from the pars intercerebralis. It has been reported that destruction of the pars intercerebralis blocked female receptiveness [131].

Mature wild-type females exhibit marked rejection behavior when they are courted after being fertilized [43]. The switching of female behavior from receptive to nonreceptive by copulation is not a direct consequence of the presence of sperm in the female, but due to the presence of the sex peptide, a product of the male accessory gland, that is transferred into the female during copulation [44, 132, 133]. In addition to the sex peptide, several protein products of the male accessory gland have also been shown to translocate to the female during copulation [134–136]. An experiment to kill a certain class of cells with diphtheria toxin revealed that only the main cells in the accessory gland are associated with inhibition of female remating [137]. Ubiquitous overexpression of the sex peptide transgene in virgin females induces strong rejection behavior against males and stimulates ovulation [133].

Although efforts have been made to localize the target of the sex peptide by examining binding of the labeled peptide on tissue sections [138] or by targeted expression of the sex peptide gene in different brain areas of virgin females and subsequent behavioral assay [139], the site of its action has not been unequivocally identified. Assuming that the brain site controlling receptiveness is common to virgin and fertilized females, an experiment can be done to examine whether the sex peptide gene expressed under the *spin* gene promoter intensifies the rejection behavior of such virgin females.

## Conclusion

The combination of classical genetic and modern molecular tools and techniques have made *D. melanogaster* the unparalleled material for the study of complex higher-order processes, such as sexual behavior. Targeted gene expression utilizing the GAL4-UAS system provides a particularly powerful approach for the identification of anatomical sites linked to certain aspects of sexual behavior, because a given gene can be made active, inactive or dominant negative in defined cell populations. Only when the gene expression is driven in the right cells can the gene exert its effect. Conversely, most genes have multiple functions in different biological contexts, a property known as pleiotropy. Even if the gene has pleiotropic actions, local expression of the selected form of the complemen-

tary DNA (cDNA) would allow visualization of its role specific to sexual behavior. Thus this technique can be used to extract the casual relationship between the genes, cells and behaviors. Indeed, targeted feminization of a restricted set of neurons has led to the identification of neural substrates for bisexual courtship. Since the list of mating behavior mutants is continuously growing and more genes responsible for these mutations are expected to be cloned, the targeted expression system can be used to identify the cells in which the gene actually functions to control the relevant behavioral element.

The targeted expression system was successfully used to feminize anatomically defined brain areas, leading to identification of the glomeruli critically involved in sensory processing for correct sexual orientation in male flies. In contrast, the 'neural centers' for other aspects of mating behavior remain largely hypothetical. This is, in part, due to the paucity of information on the anatomy and physiology of neuronal connections in the *Drosophila* brain. A large collection of GAL4 enhancer trap lines are now being analyzed for defining the neuroanatomy: the reporter constructs *UAS-tau* and *UAS-tau:lacZ* have made it possible to label reproducibly fine neurites as well as axons and somata with extremely high resolution. 'Pulse'-labeling of single neuroblasts with conditional induction of GAL4 enables us to trace neuronal lineages from each neuroblast [94], and thus to identify unique interneurons in the brain.

In situ electrophysiological study of central neurons in the *Drosophila* brain is still in its primitive stage due to their small size, but other powerful methods for measuring neuronal activities, such as calcium imaging, may provide an alternative approach to functional analysis of the neuronal circuitry involved in sexual behavior [cf. 140, 141]. Rapid growth in the knowledge of the anatomy and physiology of the fly brain raises the exciting possibility of identifiable single neurons manipulated molecularly being studied during mating behavior. Such an approach could be expected to open a new decade of neuroethology.

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