REVIEW

Molecular and neural mechanisms regulating sexual motivation of virgin female *Drosophila*

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Abstract

During courtship, multiple information sources are integrated in the brain to reach a final decision, i.e., whether or not to mate. The brain functions for this complex behavior can be investigated by genetically manipulating genes and neurons, and performing anatomical, physiological, and behavioral analyses. *Drosophila* is a powerful model experimental system for such studies, which need to be integrated from molecular and cellular levels to the behavioral level, and has enabled pioneering research to be conducted. In male flies, which exhibit a variety of characteristic sexual behaviors, we have accumulated knowledge of many genes and neural circuits that control sexual behaviors. On the other hand, despite the importance of the mechanisms of mating decision-making in females from an evolutionary perspective (such as sexual selection), research on the mechanisms that control sexual behavior in females has progressed somewhat slower. In this review, we focus on the pre-mating behavior of female *Drosophila melanogaster*, and introduce previous key findings on the neuronal and molecular mechanisms that integrate sensory information and selective expression of behaviors toward the courting male.

Keywords *Drosophila* · Sexual receptivity · Behavioral switching · Decision-making · Sensory integration

Introduction

Mating is an important biological event that has a major impact on the evolutionary fitness and survival of sexually reproducing species. In particular, females are responsible for their choice of mates. A male, therefore, displays multiple cues and signals to attract a female's attention, and courts her in a way that maximizes his value throughout the courtship. In *Drosophila melanogaster*, one of the best model animals for genetic analysis of sexual behaviors, males exhibit conspicuous courtship behavior to provide multimodal stimuli for arousing sexual motivation in females [1]. Upon encountering a courtship partner, females exhibit signatures of pre-mating rejection such as flapping, escaping, kicking, fending, and ovipositor extrusion to the courting male [2, 3]; during this time, females evaluate male response to judge the favorableness of copulation [4, 5]. As the female evaluates the sexual information of the male and becomes more sexually receptive, she decreases locomotion and eventually accepts copulation [6–12]. These pre-mating responses are instinctive, with both external stimuli and internal states determining the female's decision-making and behavioral choices, from pre-mating rejection to mating acceptance, via a multistep neuromodulatory system and neural circuitry. Although research into the underlying mechanisms which influence female mating behaviors has advanced substantially in recent years, much remains unclear regarding the exact nature of these mechanisms. Here we focus on the pre-mating behavior of *Drosophila melanogaster* females and review past and recent studies that have expanded our knowledge of the molecular and neural basis of sexual behavior in virgin females.

Males provide multiple cues to increase female receptivity

In the early stages of the courtship ritual, the female fly exhibits a rejection response consisting primarily of an escape response and an aggression response [6]. These encounter responses exhibited by the female are necessary to avoid adverse mating, and allow the female fly to recognize

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the nature of the encounter, whether the male is indeed a conspecific male, and whether it is worthy of becoming a mate. To this end, female flies evaluate males by detecting a variety of male derived signals. In *Drosophila melanogaster*, these signals are mainly comprised of pheromonal (gustatory and olfactory) and acoustic cues (Fig. 1a).

Gustatory and olfactory pheromonal cues

In *Drosophila melanogaster*, cuticular hydrocarbons and non-hydrocarbons play multiple roles as pheromonal cues, such as aggregation signals for food sources, species and sex recognition, and aphrodisiacs or sexual repulsion [13–15].
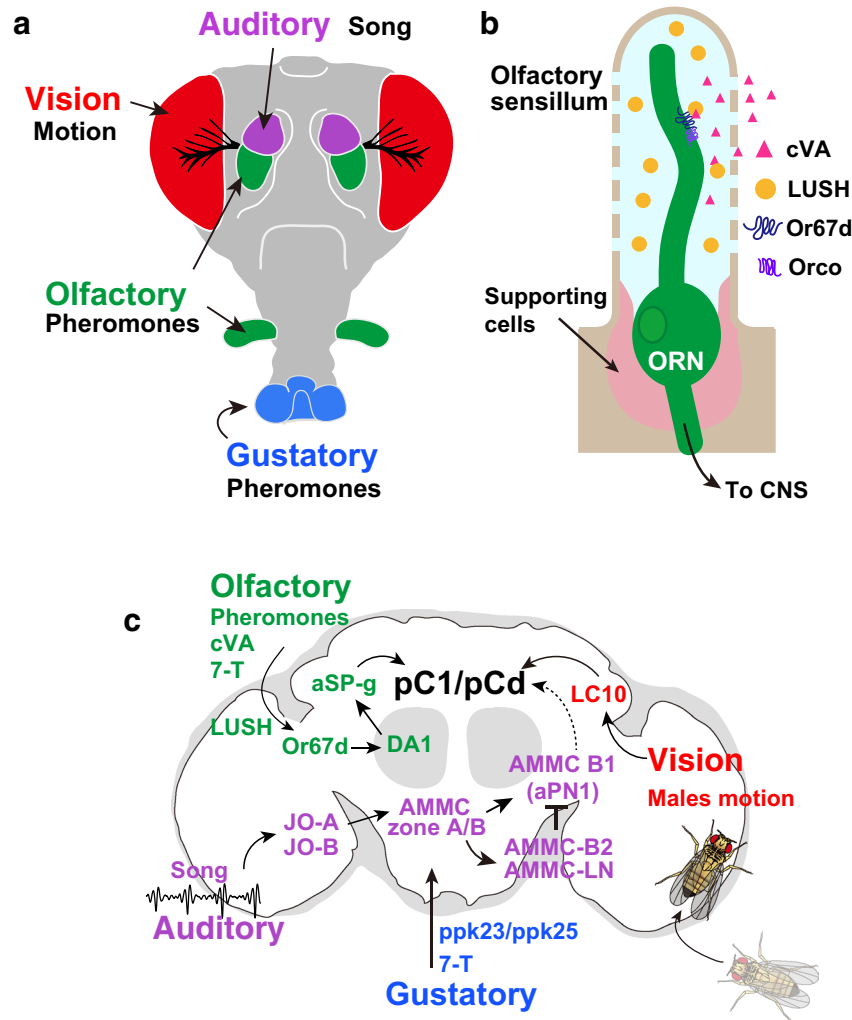

Fig.1 Sensory signals that influence female receptivity. **a** Sensory organs of the *Drosophila* head are depicted in different colors. Red, compound eyes perceive visual information such as male's courtship motion; green, third antennal segment and maxillary palps house olfactory receptor neurons (ORNs). Volatile pheromones are detected by specific olfactory receptors (ORs); purple, second antennal segment houses auditory mechanosensory neurons called Johnston's organ (JO) neurons [54, 55]; blue, gustatory receptor neurons are located in labellum. **b** An insect olfactory sensillum is illustrated. 2–4 ORNs are housed in a sensillum. The sensillum is filled with sensillum lymph secreted from supporting cells at the base of the sensillum. LUSH is an olfactory binding protein which binds with cVA and transfers it to Or67d. Orco is a co-receptor for ORs [24, 25]. **c** A schematic of the female brain depicting the flow of multiple sensory information during courtship. Olfactory information detected by Or67d is transmitted to DA1 olfactory glomerulus. A downstream of olfactory projection neurons (PNs) aSP-g transfers cVA information from DA1 to pC1/pCd, a potential integration center for female receptivity [20, 22, 29, 30]. Song information is received by JO-A/B primary auditory neurons. JO-A and JO-B project to the antennal mechanosensory and motor center (AMMC) zones A and B, respectively [40, 55]. Secondary auditory neuron AMMC-B1 (aPN1) transfers song information towards pC1/pCd via unidentified interneurons [57]. AMMC-B2 and AMMC-LN are local interneurons that suppress AMMC-B1 depending on the inter-pulse interval (IPI) of the song [49]. Lobular columnar 10 (LC10) cells transfer visual information of the male's courtship motion to pC1/pCd [76, 77]. Non-volatile pheromones are detected by gustatory receptor neurons expressing *ppk-23/ppk-25* [16]. 7-T, 7-tricosene; cVA, *cis*-11-octadecanyl acetate (*cis*-vaccenyl acetate)

Gustatory and olfactory sensory neurons redundantly participate in the detection of the pheromonal cues of males. Ion channels *ppk-23* and *ppk-25*, which belong to the degenerin/epithelial sodium channel (DEG/ENaC) family, and their expressing gustatory receptor neurons are required for detection of gustatory aphrodisiac pheromones that increase female receptivity for mating [16]. Multiple male pheromones are thought to be detected by *ppk-23* and *ppk-25* expressing gustatory receptor neurons; however, the identity of the pheromone itself remains unclear [16]. With regards to sex recognition and aphrodisiacs, males produce male-specific pheromones 7-tricosene (7-C23:1 or 7-T) and *cis*-11-octadecenyl acetate (*cis*-vaccenyl acetate, cVA). Female flies copulate with shorter latency to males with 7-T applied to their cuticle surface, suggesting that 7-T is an aphrodisiac pheromone for females [17]. A gustatory receptor, Gr32a, has been reported as a 7-T receptor in male flies [18, 19]. In males, 7-T signals via Gr32a are required for aggressive behavior and inhibit male–male courtship [18, 19]. In contrast to the clear role of 7-T as an aphrodisiac for females, its receptor Gr32a is not required for the control of receptivity [18, 19], suggesting that there is an unknown 7-T receptor in females. It has been reported that antennae, the main olfactory organ in flies, are required for females to sense concentration differences in 7-T [17]. Genetic exploration of olfactory receptor neurons may lead to the discovery of novel 7-T receptors.

The olfactory system is also important for the sensing of volatile pheromones. The male-specific volatile pheromone cVA is detected by olfactory receptor neurons expressing an olfactory receptor *Or67d* or *Or65a*, with Or67d and Or65a corresponding to acute and chronic responses, respectively [20–23]. The action of cVA on receptors is mediated by odorant binding protein LUSH, which facilitates binding to the Or67d receptor in the olfactory sensillar lymph [24, 25] (Fig. 1b). Another odorant binding protein, Obp69a, dynamically changes its expression level depending on male–female social interaction. Although it is unclear if Obp69a and cVA interact, Obp69a is known to regulate sexual behaviors induced by cVA, such as female receptivity [26].

Or67d olfactory receptor neurons transfer male information to the DA1 glomerulus in the antennal lobe, the primary olfactory center in the fly brain (Fig. 1c). It is ethologically interesting that the pheromonal signal of cVA at DA1 is enhanced by odor signals other than pheromones, such as the complex food odor vinegar, which additively increases neural response and enhances female sexual receptivity [27, 28]. This synergistic action of different odors has been proposed to be caused by interneurons that connect different projection neurons with electrical synapses [27]. DA1 projection neurons then transmit signals to aSP-g neurons, which activate pC1/pCd neurons, the main circuit for integration of sexual information and output regulation for the

female sexual behavior [20, 22, 29, 30]. In addition to providing an aphrodisiac function for females, 7-T and cVA also play roles as anti-aphrodisiacs to repel other males during future copulation attempts [31–34], via transference with the male sex fluid during copulation and storage in the female reproductive tract [35–37].

Auditory cues

During courtship, male flies generate a courtship song via wing vibrations. This courtship song has a significant impact on female mating propensity [38]. Indeed, ablation of male wings significantly reduces the success rate of mating, with concurrent playback of a conspecific courtship song restoring it [11]. Similarly, defects in female hearing reduce sexual receptivity [17, 39, 40]. The courtship song consists of both sine and pulse songs; the first is a humming sound around 160 Hz, with the second being short pulses generated as pulse trains with a species-specific inter-pulse interval (IPI) [41, 42]. The IPI contributes to species recognition, and thus also has a significant impact on sexual selection [43, 44]. The courtship song is genetically defined, but there is a certain degree of variation depending on the genetic background. Furthermore, males flexibly adjust their song bout patterning depending on social context provided by the target female [45, 46]. The existence of such song variation and adjustability also indicates that it is possible to investigate the mechanisms of genetic and neural control of the song production systems [47, 48]. Song playback experiments have demonstrated that female receptivity is increased by exposure to artificial songs that fit within the original range of conspecific IPI [49, 50] (Fig. 2a–c). Moreover, mating decisions are primarily revealed through either opening of the vaginal plates or ovipositor extrusion of females, both of which are indeed triggered in response to male courtship song and dependent upon the female's mating status [51, 52]. These findings suggest that courtship song contains important sexual information for informing mating decisions. The tuning of song preference of female is comparatively broad in *D. melanogaster*, but is far narrower in other species [42, 53]. Although it had previously been believed that song preference is genetically fixed, recent findings have demonstrated that the wide-ranging preference of IPI is not a lifelong trait but instead reliant on auditory experience to songs containing conspecific IPI pulses, which can reshape song preferences within a fairly confined range [50] (Fig. 2d).

In fruit flies, the courtship song is perceived by the mechanosensory neurons of the Johnston's organ (JO), located within the antennal ears [54, 55] (Fig. 1a). These mechanosensory neurons, so-called JO neurons, project to the antennal mechanosensory and motor center (AMMC), the primary auditory center in the fly brain. In the AMMC, the
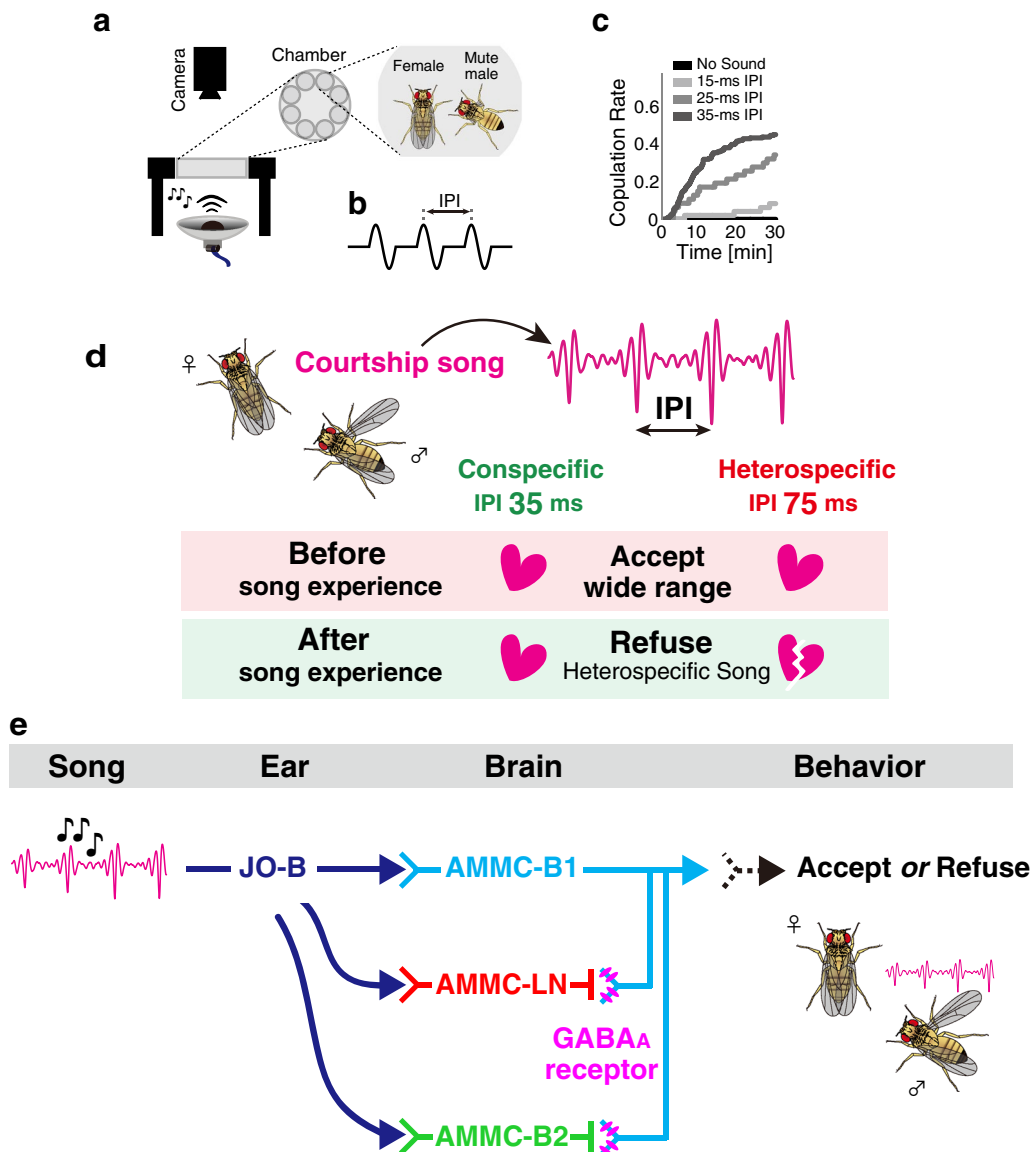
Fig. 2 Exposure to a courtship song alters female receptivity. **a** Setup for song playback experiments. A virgin female is paired with a wing-clipped (mute) male. Artificial song is broadcasted from a loudspeaker [49, 50]. **b** Artificial song. The mean inter-pulse interval (IPI) of *D. melanogaster* song is about 35 ms. **c** Copulation rate during the song playback. A song with 35 ms IPIs maximally accelerates copulations. Modified from Yamada et al. (2018) [49]. **d** Auditory experiences with conspecific songs fine-tune song preferences. Proper auditory experience refines courtship song preference, with females decreasing responses for heterospecific songs [50]. **e** A model of the auditory pathways involved in relay of song signals to modulate female receptivity. In this pathway, JO-B neurons transfer song information to the AMMC-B1 neurons. Two subsets of GABAergic interneurons, AMMC-LN and AMMC-B2, modulate the response pattern of the AMMC-B1 neurons via GABAA receptors. Modified from Yamada et al. (2018) [49]

JO projection region forms a tonotopic map in which the major auditory regions are denoted as zones A and B [40, 55] (Fig. 1c).

Recent studies have revealed the auditory neural pathway required for conspecific song detection [56, 57]. In this pathway, JO neurons projecting to the AMMC zone B (JO-B neurons) transfer song information to the AMMC-B1 neurons (also known as aPN1), a major type of secondary auditory neurons in the song-relay pathway [57]. When song signals activate AMMC-B1 neurons via JO-B neurons, females increase their receptivity for copulation. This increase, however, is counter-balanced partly by two subsets of GABAergic interneurons, namely AMMC-LN and AMMC-B2. AMMC-LN and AMMC-B2 neurons suppress AMMC-B1 neuron activation, via $GABA_A$ receptors, to song stimuli carrying a faster rhythm than a conspecific song. Through such

interactions, these GABAergic interneurons tune the song response of the AMMC-B1 neurons [49] (Figs. 1c and 2e).

No sexual dimorphisms have so far been observed in these primary and secondary auditory neurons. In male flies, AMMC-B1 neurons transmit song information to the third-order auditory interneuron vPN1, which then transmits signals to the pC1 neuron cluster, the integration center for multisensory information relating to courtship [58]. The female equivalent of vPN1 interneuron(s) remains to be identified.

Recently, a comprehensive map of neural activity of the *Drosophila* brain has been established, showing that neural activity in response to the courtship song is distributed throughout the brain and that the representation of auditory stimuli is diverse across brain regions [59]. This analysis also suggested that females have pulse-responsive neurons, equivalent to vPN1 neurons, in the same brain regions as males [59]. Recent advances of whole-brain connectomes, resources of molecular-genetic tools, and gene expression databases will facilitate anatomical and functional tracing of the un-revealed circuits [60–62]. Indeed, new subsets of auditory interneurons have been identified by the application of the *split-Gal4* intersectional technique [63–66].

Exposure to a conspecific song permits virgin females to allow copulation, in contrast to mated females that typically refuse to mate when exposed to the song. vpoENs are cholinergic interneuron that respond to conspecific song and promotes the opening of the vaginal plate, which is the sign for accepting mating [66]. vpoINs are GABAergic interneurons that also responds to conspecific song but suppress the vaginal opening [66]. Both vpoEN and vpoIN connect to the command-type neuron vpoDNs, which control the motor control of vaginal plate opening (see the following section) [66].

Potential involvement of visual information in pre-mating behaviors

Substantial evidence suggests that females evaluate males based primarily on olfactory, gustatory, and auditory information and make appropriate mating decisions. Visual information may also be important for this evaluation process. Males provide visual information via dynamic movement and appearance characteristics, such as pigmentation, colors, shapes, and size. Body size is one of the most straightforward characteristics which suggests individual strength, but has recently been shown to be of low determinative value during partner selection by females [67–70]. The visual appeal of wings, which are important in other *Drosophila* species for successful mating [71–73], has been found to be a visual factor that influences the selection of reproductive partners in *D. melanogaster* females [74].

Although there is still a lack of knowledge about the sexual attractiveness of male visual features as a criterion for the mate choice, whole-brain connectomics and functional identification of neural circuits are gradually revealing the intersection between visual information and female mating decisions. Connectome analysis of the adult brain has suggested indirect connections between lobular columnar cells (LCs), which convey visual information, and pC1d, which is a potential integration center for female sexual behavior [75]. One such type of LC, LC10 neurons, are required for the tracking of a fly-sized object in males [76, 77] (Fig. 1c). Interestingly, the response properties of LC10 neurons to such objects are qualitatively and quantitatively similar in males and females [77]. If the movement pattern of males during courtship is detected by LC10 neurons, and thereby influences pC1d activity in females and hence female mating receptivity, this could provide insight into the female sexual preferences for male visual characteristics. LC10 motion detection has been observed in males as well as females [76]. In males at least, LC10 activation stimulated by detecting female movement induces the initiation of courtship, and its activity is enhanced by sexual arousal [77]. The role of these LCs in the pre-mating behavioral choices of females, rejection or acceptance, remains to be clarified.

Internal states and mating experience affect receptivity

Age has a significant effect on virgin female receptivity. The first day after the adult *Drosophila* emerge from the pupa (day 0), females show extremely low receptivity to mating [78]. However, from day 2 onwards, female behavior changes to exhibit high receptivity [78]. The mechanisms which control this repressed receptivity are shrouded in mystery. A major issue to be resolved is whether this receptivity simply depends on immaturity or if other mechanisms, e.g. endocrine control, are involved [79, 80].

A rich nutritional environment, such as yeast containing food, increases sexual receptivity in virgin females [81]. Yeast odor acetic acid, sensed by the ionotropic receptor Ir75a, can affect female receptivity only when yeast amino acids are present in the food substrate (Fig. 3). Thus both the perception of the hedonic value of a food (in this case smell) and the perception of its nutrient content are needed simultaneously to elicit receptivity in females [81].

Mating significantly changes the behavior of females. For instance, egg-laying rate increases, while receptivity to mating decreases. These post-mating responses persist for approximately a week in *D. melanogaster* [78]. The post-mating reduction of female receptivity is induced by sex peptide (SP), which is delivered to the female via the male's seminal fluid during copulation [82]. SP is received by SP receptors expressed in sex peptide sensory neurons (SPSNs) that connect the uterus to the abdominal ganglion [83–86] (Fig. 3). Downstream of the SPSNs are SP abdominal

Fig. 3 Internal states and mating experience affect female receptivity. A yeast odor acetic acid and caloric nutrition sugar increase female receptivity via pC1 [81]. An ionotropic olfactory receptor Ir75a detects acetic acid. Internal sugar receptor Gr64a detects nutrition sugar. Sex peptide sensory neurons (SPSNs) connect uterus to the abdominal ganglion [83–86]. Peptide hormone myoinhibitory peptide (Mip) expressing interneurons, ventral abdominal lateral (vAL) and ventral abdominal medial (vAM), mediate sex peptide (SP) information transfer from SPSNs to SP abdominal ganglion (SAG) neurons [88]. Activation of SAG enhances female receptivity. SP suppresses SPSNs and SAG activity and induces post-mating behaviors of mated females. Male flies deposit mating plugs in female uteri, preventing female remating. Social interactions, such as crowded conditions, and food odor promote plug ejection by mated females, thus encouraging remating. A neuropeptide diuretic hormone 44 (Dh44) secreted from endocrine neurons in the brain inhibits mated female plug ejection [94–96]

ganglion (SAG) neurons located at the abdominal ganglion, whose axons project to the dorsal anterior cerebrum in the brain. SP induces female post-mating responses by inhibiting the activity of the SPSN and SAG neurons [87]. Additionally, a local circuit involved in post-mating behavioral switching has been identified in the abdominal ganglion. A key component is a group of neurons, the ventral abdominal lateral (vAL) and ventral abdominal medial (vAM) interneurons, which express a myoinhibitory peptide (Mip). These interneurons control female post-mating behavioral change by mediating SP information from SPSN to SAG neurons in the abdominal ganglion [88] (Fig. 3).

During and/or after mating, male flies deposit a mating plug in the female's uterus to prevent females from remating with other males and to allow time for sperm storage [89–91]. This mating plug, therefore, represents one of the key determinants of female remating propensity. The length of time between mating and females ejecting the plug depends on numerous external and/or internal states, such as social interactions and nutritional status [92, 93]. Neuropeptides also play a role, with diuretic hormone 44 (Dh44) delaying the timing of plug ejection by mated females and sustaining post-mating behaviors [94–96] (Fig. 3). Further studies are needed to clarify how these post-mating factors result in mating rejection and how their neural mechanisms crosstalk with the pre-mating rejection mechanisms shown by virgin females.

Cellular identities of neurons linked to receptivity

The development and implementation of sophisticated molecular-genetic tools which enable neuronal manipulations has led to the identification of the neural circuits and molecules regulating male sexual behavior, which is conspicuous and thus open for quantitative analysis [8, 97, 98]. Mature, receptive, and virgin females also exhibit a variety of distinct pre-mating behaviors in response to courtship stimuli, suggesting that a sexual dimorphic system should be involved in the neural and molecular bases of pre-mating behaviors [2, 3]. Whilst our understanding of the neural circuitry of female sexual behavior is still limited compared to equivalent male circuits, recent advances in female circuitry research have unveiled a number of molecular and neuronal components involved in integrating multiple sensory inputs and regulating motor outputs for female-specific behaviors.

One highly successful strategy employed over the past decade to identify neurons regulating sex-specific behaviors is morphological and functional dissection of neuronal populations which are molecularly defined as expressing master regulator genes for sexual dimorphism; fruitless (*fru*) and doublesex (*dsx*) [8, 99–102]. By utilizing the *Gal4/UAS* technique in combination with an intersectional approach, pC1 and pCd neurons have been identified as neuronal clusters regulating female receptivity from approximately 140 *dsx*-positive neurons in the female brain [103–106]. Intriguingly, calcium imaging analyses have revealed that pC1 neurons respond to multimodal sensory inputs, including both auditory song and olfactory cVA input. Furthermore, neuronal responses of pC1 neurons are enhanced by simultaneous inputs of courtship song and male pheromone cVA. Therefore, the function of pC1 neurons is not restricted to merely acting as a command-like controller for the copulation acceptance, but also appears to involve multimodal processing of male sensory information [103]. pC2 cluster neurons, another type of *dsx*-positive neurons, also play

an important role in determining female sexual receptivity [107]. The pC2 cluster consists of both lateral (pC2l) and medial (pC2m) type clusters [105]. pC2l/m clusters in both of male and female brains respond to pulse songs and induce sex-specific behaviors [106, 107] (Fig. 4).

A recent study utilized an intersectional approach by applying the *split-Gal4* technique to characterize vpoDNs, a pair of *dsx*-positive and *fru*-negative descending neurons in the female brain [66]. vpoDNs are command-type neurons involved in female vaginal plate opening behavior, with this occurring once females accept copulation [11, 47]. vpoDNs
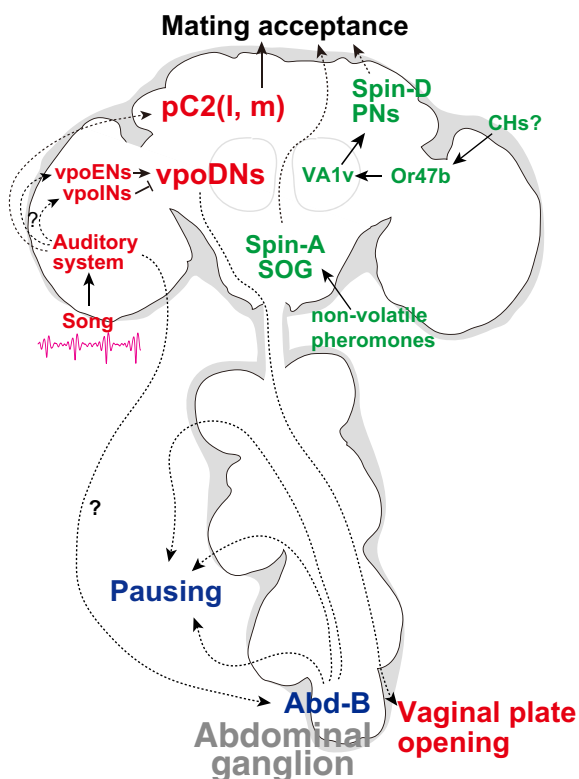
Fig. 4 Neurons promoting virgin female mating acceptance. Song promoting mating acceptance sections are labeled in red. Courtship song activates doublesex (*dsx*)-positive cluster of pC2 neurons (pC2l and pC2m), which increase female receptivity [106, 107]. Courtship song also activates neurons controlling vaginal plate opening. Cholinergic vpoENs and GABAergic vpoINs exert excitatory and inhibitory control over vpoDNs, respectively, and regulate the timing of vaginal plate opening [66]. Both vpoENs and vpoINs receive song information from unidentified auditory neurons. Green, specific subsets of spinster (*spin*)-expressing neurons promote female mating [110]. Olfactory receptor 47b (Or47b) perceives unidentified male cuticular hydrocarbons (CHs) and promotes female mating response. Spin-D neurons mediate Or47b information to increase virgin female mating acceptance. Spin-A neurons mediate information transfer of non-volatile pheromones received by gustatory systems to also promote virgin female mating acceptance. Blue, pausing behavior is increased before mating in virgin females. A homeobox transcription factor Abdominal-B (*Abd-B*) contributes to developing neural circuits regulating this pausing behavior [11]

integrate information about the male courtship song with the female's own mating status to control vaginal plate opening; however, it remains unknown how vpoDNs make decisions based on multiple information sources to determine the outcome of this final step of copulation acceptance [66] (Fig. 4).

Virgin females with mutations in the gene spinster (*spin*) show severe rejection responses to a courting male and rarely mate [108, 109]. Mosaic analysis with a repressible cell marker (MARCM) which generated *spin* mutant clones in female brains revealed two neuronal clusters, denoted as Spin-D and Spin-A clusters, that regulate female receptivity [110] (Fig. 4). The Spin-D cluster is a subset of the secondary olfactory neurons, i.e., projection neurons (PNs) [110]. Spin-D PNs receive odor information from the VA1v glomerulus as well as from other five glomeruli in the antennal lobe. The VA1v glomerulus receives inputs from *Or47b* expressing olfactory receptor neurons, which play a key role in determining the level of female sexual receptivity [110]. Although the ligand molecule for Or47b is not yet identified, *Or47b* neurons are known to at least respond to cuticular hydrocarbons extracted from conspecific flies [111]. This suggests that Spin-D neurons possibly mediate pheromonal cues to activate female mating acceptance.

The other cluster, Spin-A, is comprised of local neurons located in the suboesophageal ganglion (also known as gnathal ganglia), which houses the primary center for gustatory-sensory processing [110]. This finding suggests that the Spin-A cluster may contribute to the sensory perception of non-volatile pheromones. Notably, neither *fru* nor *dsx* are expressed in Spin-A neurons, suggesting that they are not sexually dimorphic [110].

When a female is ready to accept a male's courtship, her escape speed decreases and her frequency of pausing increases, similar to that seen in pre-mating preparatory behavior [47]. A homeobox transcription factor Abdominal-B (*Abd-B*) contributes to the development of neural circuits regulating virgin female pausing behavior during the courtship ritual [11] (Fig. 4). *Abd-B* neural cell bodies reside within the abdominal ganglion. Again, although a subset of these *Abd-B* neurons overlay with *fru* expression, this *fru* subset of *Abd-B* neurons do not contribute to virgin female pausing behavior [11]. How nonsexual dimorphic neurons contribute to sexual behavior in both sexes remains to be elucidated.

Cellular identities of neurons involved in virgin female pre-mating rejection

The neuronal underpinnings of pre-mating responses of females are still being investigated. Pre-mating responses, such as flicking of the wing, kicking, fending, decamping, and ovipositor extrusion, can be categorized and detected as behavioral elements [2, 3]. With the exception of ovipositor

extrusion, each component of the pre-mating response is reminiscent of aggressive behavior, and indeed aggressive behavior is associated with reproduction [112–115]. Like other animals, both male and female *D. melanogaster* show aggressive behaviors to gain multiple resources for survival and reproduction [116–119]. Relevant genetic and neuronal elements have been investigated, and the neural circuits and genes regulating these aggressive behaviors have been in part characterized [120, 121].

Most recently, it has been shown that central transmembrane channel-like (*Tmc-L*) expressing neurons (CTNs) in the mesothoracic ganglion of the ventral nerve chord elicit a female defensive behavior (a swift kick [122]), mediated via tactile sensation of wing margin mechanosensilla [123] (Fig. 5a). In virgin females, a GABAergic

subset of *dsx*-positive neurons inhibits CTNs, resulting in a reduced defense response [123]. In contrast, the defense response of mated females is enhanced. After mating, CTNs-dependent defense responses are enhanced via the peptide hormone Leucokinin (Lk), a human tachykinin homologue [123]. Future elucidation of the neural systems underlying the integration of male courtship cues/signals to control female state-dependent rejection responses is highly anticipated.

On the central brain side, the pC1 cluster has been found to be involved in female aggression as well as mating behavior [75, 124]. In females, a sex-determination gene, *dsx*, plays important roles in the development of the nervous system that controls female aggressive behaviors as well as mating behavior [5, 8, 101, 103, 125, 126].
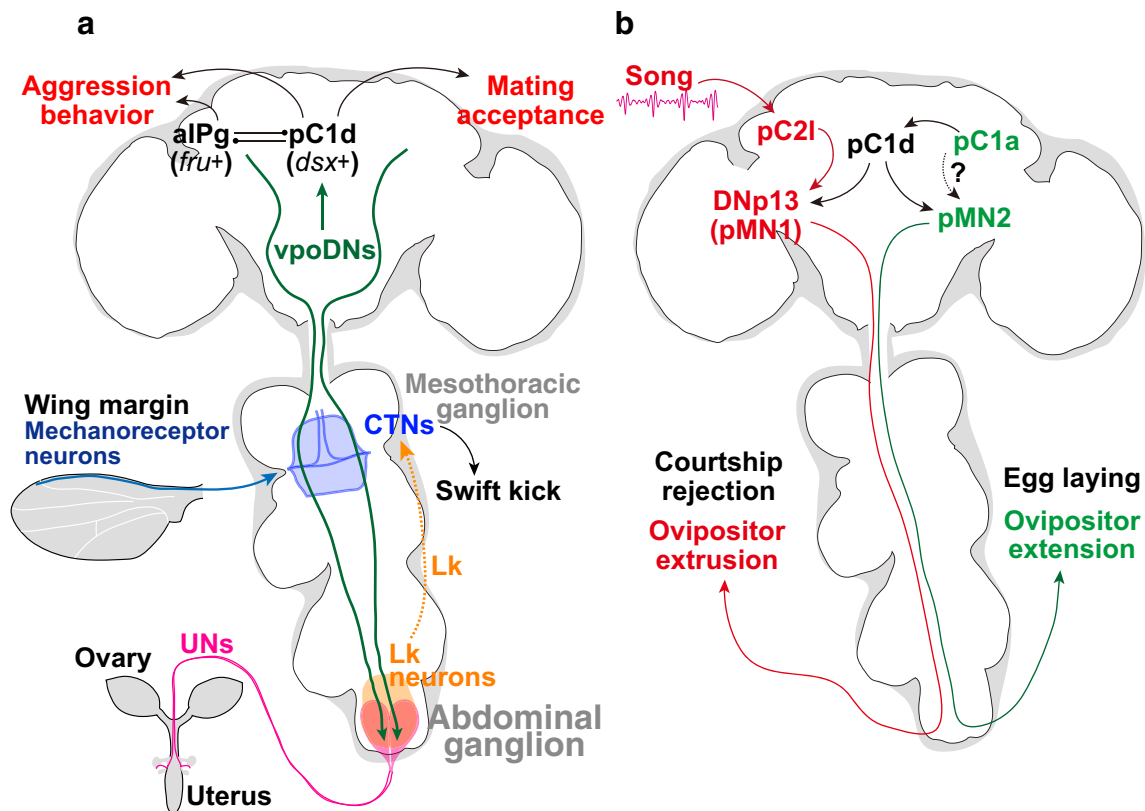
Fig. 5 Neurons promoting virgin female pre-mating rejection responses. **a** Role of neural control of aggression behavior and mating acceptance in virgin females. pC1d neurons promote mating acceptance in virgin females. One of the critical components of this mating acceptance is the opening of the vaginal plate. Vaginal plate opening is regulated by vpoDNs, which connect to pC1d. pC1d reciprocally connects to aIPg *fru*-positive neurons. Both pC1d and aIPg are important circuitry nodes for aggression behaviors [75]. It is clear that control of both aggression behavior and mating acceptance by pC1d is also important for pre-mating behavior in virgin females, but the underlying mechanisms remain unclear. vpoDNs project to the abdominal ganglion. In the mesothoracic ganglion, central transmembrane channel-like (*Tmc-L*) expressing neurons (CTNs) receive

mechanosensory signals derived from wing margin mechanoreceptor neurons and elicit swift kick responses. In virgin females, *dsx*-positive GABAergic neurons suppress CTNs activity [123]. On the other hand, a peptide hormone Leucokinin (Lk) activates CTNs dependent on the activity of uterus neurons (UNs) in mated females [123]. **b** Two modes of neural control derived from pC1d regulate the female ovipositor. Ovipositor extrusion, a female courtship rejection response, is regulated by command-like neurons DNp13 (pMN1) [52, 106, 129]. Upstream neuron of DNp13 is pC2l, a song activated neuron. Ovipositor extension is required for egg-laying behavior of mated females. pMN2 is a command-like neuron which regulates ovipositor extension [106]. Both pMN1 and pMN2 are downstream of pC1d [65]

Indeed, a group of neurons in the *dsx*-positive pC1 cluster promotes the persistence of hyper-aggression in females [127].

Recently, Schretter et al. identified two important circuitry nodes, aIPg and pC1d neurons, for regulation of female aggressive behaviors [75] (Fig. 5a). The aIPg cluster contains sexually dimorphic *fru*-positive neurons, whilst pC1d is a *dsx*-positive neuron that belongs to the pC1 cluster [75]. Both neurons are reciprocally connected and act as the command-type neurons of aggression behavior of females; activation of each induces persistent aggressive behavior, whilst its suppression inhibits aggressive behavior [75]. The extent to which the aggressive behavior regulated by aIPg and pC1d overlap with the control of pre-mating behavior has yet to be clarified, but inference based on the role of pC1d in promoting mating [103] could suggest considerable commonality between pre-mating rejection and aggressive behaviors in females. Indeed, neurons involved in oviposition and mating receptivity, such as pC1a and vpoDN, synapse to pC1d [66, 75]. As for mating acceptance, ovipositor control is also implicated in the behavioral response of females towards courting male [3, 52]. Ovipositor extension occurs at the time of egg laying in mated females, whereas ovipositor extrusion occurs irrespective of mating state during courtship [3, 128]. In both cases, the ovipositor extends, but is controlled by different clusters of descending neurons. MARCM analysis applied to narrow down the number of potential *dsx*-positive neurons identified pMN2 as a subset of descending neurons playing a critical role for ovipositor extension in egg-laying behavior [106]. DNp13 (also known as pMN1) is a subset of descending neurons having command-type functions for ovipositor extrusion during courtship [52, 106, 129] (Fig. 5b). In the ovipositor extrusion circuit, a *dsx*-positive cluster (pC2l neurons) regulates the action of pMN1 [52, 106]. Like the neurons involved in opening of the vaginal plate, both pC2l and pMN1 are activated by exposure to the courtship song [52]. It is notable that the reaction of males to ovipositor extrusion varies depending on the female mating status [52]. Ovipositor extrusion exhibited by mated females provides anti-aphrodisiacs, such as 7-T and cVA, and thus has an inhibitory effect on male courtship [52]. Although ovipositor extrusion of virgin females does not suppress male courtship, it may provide a cue for males to determine female mating status [52].

Involvement of visual and motor center in pre-mating behavioral switching

Although how visual information, such as the appearance and movement of a courting male, is computed to derive value judgments in females is not yet understood, the region of the brain that integrates visual information in flies is known. In the *Drosophila* brain, the ellipsoid body (EB), a substructure of the central complex (CX) located at the center of the fly brain, integrates multisensory information, primarily visual motion, and is responsible for the coordinated control of motor output [130]. In addition, EB is involved in the neural circuits for visual and olfactory learning, as well as in homeostatic regulation of behaviors related to hunger and sleep [131–137]. The EB has recently been found to contain a functional circuit that controls behavioral transition from pre-mating rejection to subsequent mating acceptance in virgin *Drosophila* females [138]. The major group of neurons that constitute the EB are called ring (R) neurons, whose axonal fibers form annular, layered neural structures located at the anterior-most region of CX in the central brain [139, 140] (Fig. 6a). R-neuron dendrites form a glomerular structure called a bulb and receive synaptic input from descending neurons in the anterior visual pathway [141]. Anatomical and developmental analyses have detected multiple types of R-neurons with different axonal morphology and comprising different EB ring layers [139–143]. These layers of R-neurons in the EB communicate with each other and form a neural circuit which regulates female behavioral switching from rejection to acceptance during the pre-mating period [138]. This circuit is driven by dopaminergic inputs and consists of R2/R4m and R4d.

PPM3, a cluster of dopaminergic neurons, extends their axons to the EB and their dendrites to the superior medial protocerebrum (SMP), in which neurons process multiple information sources derived from courting male, such as the male pheromone and the courtship song [29, 103]. PPM3 inputs a dopamine (DA) signal to both R2/R4m and R4d neurons (Fig. 6b). Thermogenetic activation of PPM3 prolongs the time of pre-mating rejection [138] (Fig. 6c). The R-neurons to which PPM3 is connected have different roles in influencing female pre-mating behavior. Cholinergic R4d neurons promote rejection responses from virgin females toward a courting male [138]. GABAergic and glutamatergic R2/R4m neurons are connected unidirectionally toward R4d neurons [143]. Inhibiting GABAergic signaling in R4d neurons by knocking down the GABA$_A$ receptor subunit, Resistance to dieldrin (Rdl), causes the female to engage in persistent rejection behaviors toward the courting male [138] (Fig. 6d). Activation of R2/R4m neurons promotes mating acceptance, whilst suppression induces a significant increase in mating latency [138] (Fig. 6e). R2/R4m neurons thus act in the opposite manner to R4d neurons.

The probability of behavioral transition from female rejection to acceptance increases as male courtship proceeds. Therefore, information accumulation is thought to be involved in the temporal control of this behavioral transition. This temporal control could be explained by a retrograde signal from R4d to its suppressor R2/R4m [138] (Fig. 6f). Activation of R4d by DA input leads to rejection responses from
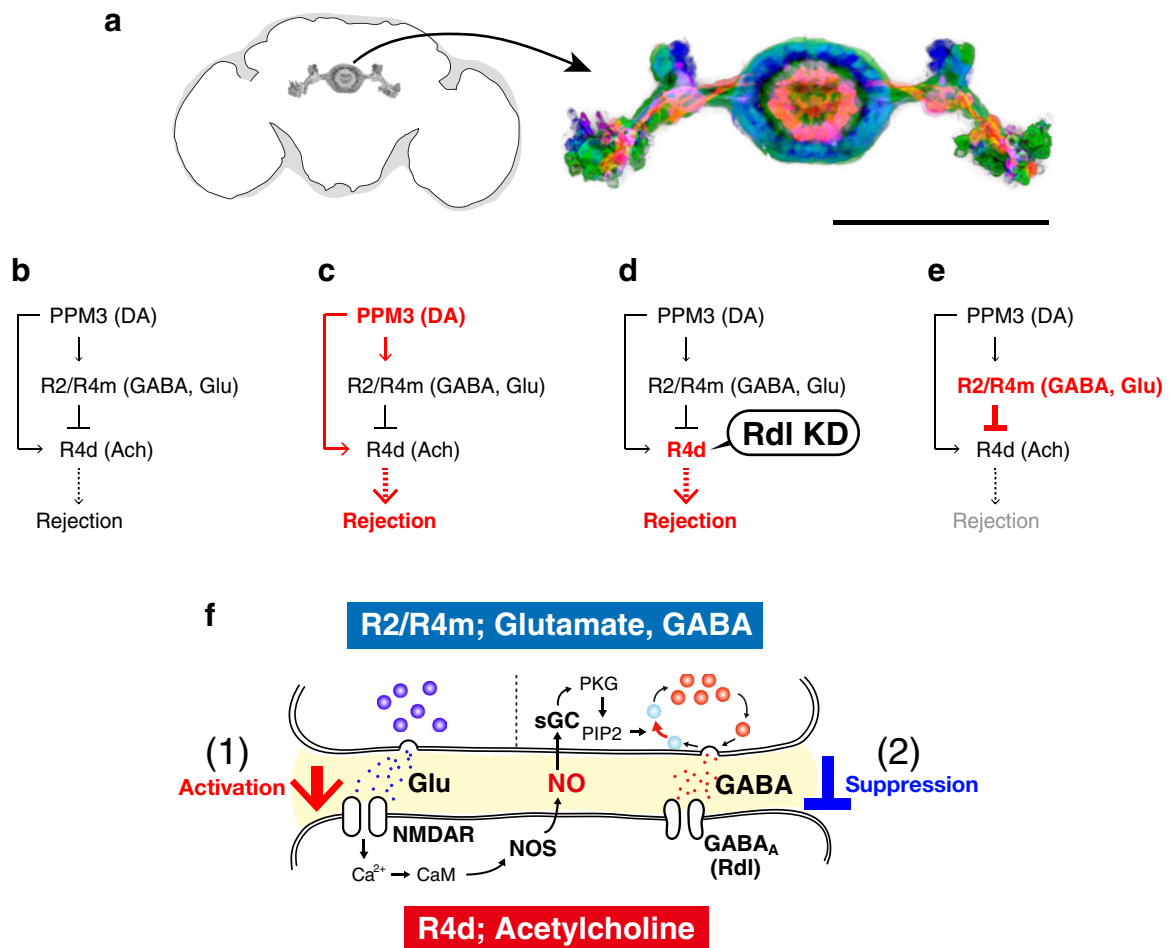
Fig. 6 An ellipsoid body circuit involved in pre-mating behavioral switching. **a** Ellipsoid body (EB) of female *Drosophila melanogaster*. Left, EB is located in the center of the brain; Right, the different ellipsoid ring neuron groups are visualized by different colors. Green, R3/R4d neurons; Blue, R2/R4m neurons; Red, R1 neurons. Scale bar; 50 μm. **b–e** A schematic diagram of the neural circuitry which controls virgin female pre-mating rejection [138]. **b** The feedforward circuit formed within the EB consists of the dopaminergic PPM3, the glutamatergic and GABAergic R2/R4m, and the cholinergic R4d. DA, dopamine; Glu, glutamate; Ach, acetylcholine. **c** Activation of PPM3 induces rejection behaviors. **d** Resistance to dieldrin (Rdl) knock-down (KD) induces hyper-rejection responses. **e** Activation of R2/R4m suppresses R4d, which attenuates rejection responses. **f** Potential retrograde signaling from R4d to R2/R4m. R2/R4m unidirectionally synapses to R4d. (1) Glutamate release from R2/R4m activates N-methyl-D-aspartate receptor (NMDAR), which triggers NOS activation via Ca^{2+}/CaM signaling pathway in R4d. (2) Activated NOS generates NO, which retrogradely affects sGC activity in the pre-synaptic R2/R4m. sGC signals potentially facilitates synaptic transmission of GABA. Adequate GABA release from R2/R4m weakens the R4d-mediated rejection response. Modified from Ishimoto and Kamikouchi (2020) [138]

a virgin female, as observed during the pre-mating response toward a first-time courting male. Glutamate signaling from R2/R4m to R4d activates N-methyl-D-aspartate (NMDA) receptors in R4d that facilitate nitric oxide synthase (NOS) reactions that generate nitric oxide (NO) (Fig. 6f). NO diffuses via the synaptic cleft back to R2/R4m and activates soluble guanylyl cyclase (sGC), which likely facilitates synaptic release of R2/R4m as a result of retrograde NO signaling [144] (Fig. 6f). When R2/R4m GABA release is sufficiently enhanced, R4d activity is suppressed via Rdl and rejection transitions to acceptance [138].

These properties of the PPM3/R-neuron circuit support the previously suggested idea that lateral inhibition from one type of R-neuron to another type of R-neuron helps fine-tune circuit output and behavioral shifts between distinct states, such as from pre-mating rejection to acceptance [145], though the exact visual features relevant for circuit operation remains to be investigated. The command-type descending neuron vpoDN integrates excitatory and inhibitory inputs to control proper timing of vaginal plate opening for the acceptance of mating [66] (Fig. 4). It is tempting to assume that R4d neurons provide the vpoDN with inhibitory information to sustain pre-mating rejection responses.

How male pheromonal information acts on PPM3 neurons and R-neurons from the SMP region needs to be physiologically investigated, but at the very least, bidirectional (excitatory and inhibitory) responsiveness of EB to the courtship pulse song have been shown [59]. It will also be important to examine the song-response properties of individual R-neurons and their respective roles in sexual behaviors. Neither *fru* nor *dsx* are expressed in PPM3 and R-neurons, as in the spin-A cluster, which regulates female mating receptivity [98, 105]. Also, no morphological sexual dimorphism has been detected in these neurons [143]. Therefore, anatomical and functional identifications of how the PPM3/R-neuron circuitry is linked to command-type circuits that perform female-specific behaviors are required.

Evolutionarily conserved functions in sexual communication between the central complex and the basal ganglia

Regarding the anatomical similarity of laminated structures and the functional similarity of sensory integration, motor coordination, and action selection for adaptive behaviors, Strausfeld and others have proposed anatomical and functional analogy between the insect CX containing EB/R-neuron circuit and the vertebrate basal ganglia [146–149]. In the CX, various neurotransmitters such as DA, acetylcholine, glutamate, and GABA are functionally involved in the EB/R-neuron circuitry to achieve neural control of the decision of virgin females to continue rejection or to switch to mating acceptance. In the basal ganglia, DA regulates the activity of the nucleus accumbens (NAc) and the ventral tegmental area via the differential modulation of glutamate and GABA release [150–153]. In monogamous rodents, sexual interactions activate these neurochemical systems in the NAc of females, resulting in the formation and the maintenance of social bonding with males [154–157]. These pre-mating female brain mechanisms which determine mating partners show striking similarities between the fly CX/EB and the mammalian basal ganglia/NAc; further research could pave the way for the unraveling of evolutionary similarities and diversity of brain functions across the animal kingdom.

Conclusions and perspectives

In the pre-mating phase, mature, receptive, virgin female flies integrate the sensory information provided by the courting male flies and from there assess the value of their mates, including their species and sex, before deciding whether to accept or reject his courtship. Because of the current paucity of knowledge about the brain systems that control sexual decision-making in pre-mating females, it is difficult to identify and extract general principles of causality between the genes, circuits, and behaviors that underlie sexual communication with the courting male. Newly established technologies such as the whole-brain connectome, single-cell gene expression profiling, genome editing, and visualization of brain molecular activity, in addition to conventional research methods of genetics, molecular neural manipulation and behavioral analysis, are, however, enabling the elucidation of each and every complex brain function. Although sexual preferences and sexual behaviors are innate behaviors, suggesting they are to some extent genetically programmed and hardwired, mating behaviors are also flexible and sometimes modified by learning in response to situations such as internal states and environments. Understanding how these modulations are enacted and how sexual desire affects decision-making remains an open question to be addressed by future research.

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