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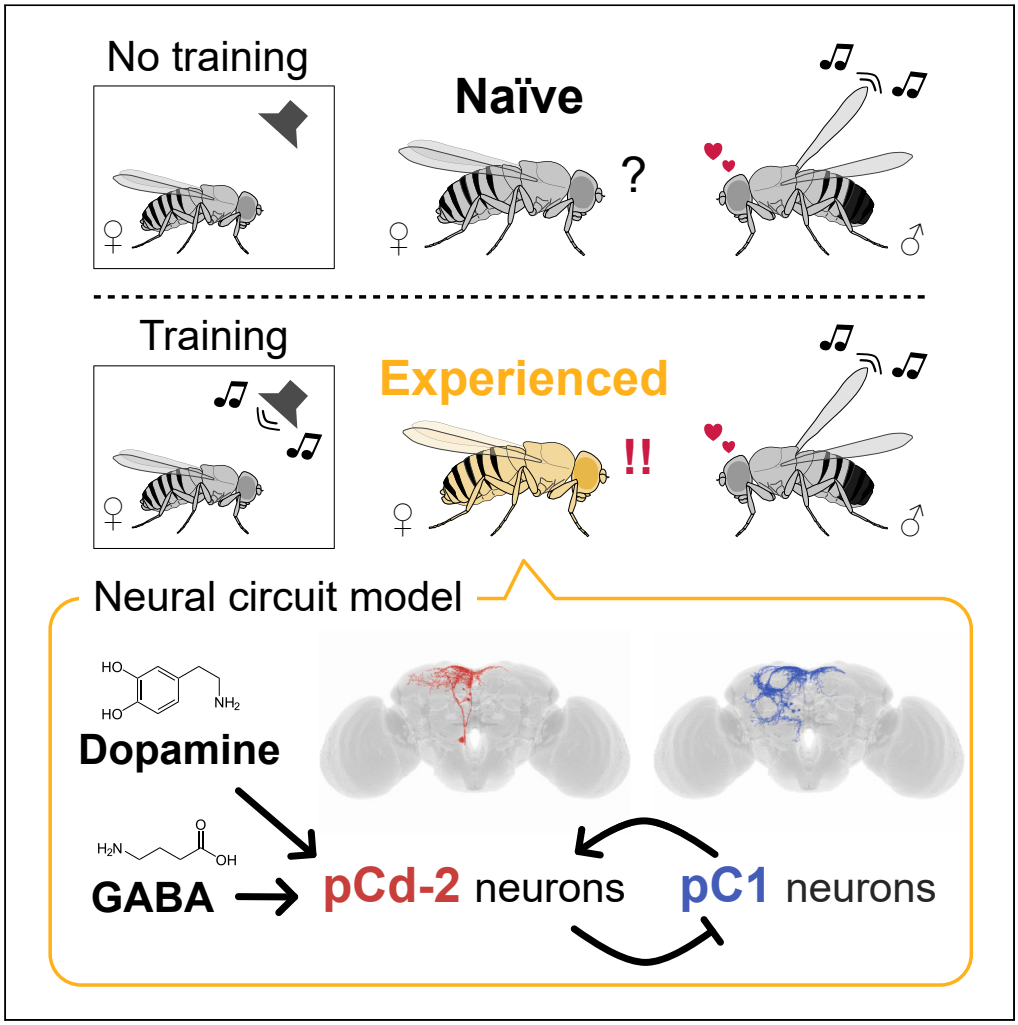

Keisuke Imoto, Yuki Ishikawa, Yoshinori Aso, Jan Funke, Ryoya Tanaka, Azusa Kamikouchi

kamikouchi@bio.nagoya-u.ac.jp

Highlights

Fruit flies acquire song preference following prior song exposure

GABAergic signals from *doublesex*$^{+}$ neurons are necessary for song preference learning

pCd-2 neurons express *doublesex* and form reciprocal circuits with pC1 neurons

GABA and dopamine contribute differentially to song preference learning

Imoto et al., iScience 27, 110266
July 19, 2024
https://doi.org/10.1016/j.isci.2024.110266

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Neural-circuit basis of song preference learning in fruit flies

Keisuke Imoto,[1] Yuki Ishikawa,[1] Yoshinori Aso,[2] Jan Funke,[2] Ryoya Tanaka,[1] and Azusa Kamikouchi[1,3,4,*]

SUMMARY

As observed in human language learning and song learning in birds, the fruit fly *Drosophila melanogaster* changes its auditory behaviors according to prior sound experiences. This phenomenon, known as song preference learning in flies, requires GABAergic input to pC1 neurons in the brain, with these neurons playing a key role in mating behavior. The neural circuit basis of this GABAergic input, however, is not known. Here, we find that GABAergic neurons expressing the sex-determination gene *doublesex* are necessary for song preference learning. In the brain, only four *doublesex*-expressing GABAergic neurons exist per hemibrain, identified as pCd-2 neurons. pCd-2 neurons directly, and in many cases mutually, connect with pC1 neurons, suggesting the existence of reciprocal circuits between them. Moreover, GABAergic and dopaminergic inputs to *doublesex*-expressing GABAergic neurons are necessary for song preference learning. Together, this study provides a neural circuit model that underlies experience-dependent auditory plasticity at a single-cell resolution.

INTRODUCTION

Many animals, ranging from humans to birds to insects, produce sounds to communicate with others of the same species. Each species typically has its own sounds, such as voices, calls, or songs. Accordingly, the auditory capability to discriminate key sound communication features is indispensable.[1] Studies of vocal learning in infants or juvenile birds have revealed that such discrimination abilities are shaped by the interaction between nature and nurture, relying on innate abilities and experience-dependent auditory plasticity, respectively.[2,3] Human infants hone their capability to make phonetic distinctions by repeated exposure to their native language.[4] The human brain starts becoming attuned to the native language a few days after birth, due to prenatal and/or short-term postnatal exposure to the native language.[5,6] Similarly, juvenile songbirds develop their auditory discrimination ability during song learning by hearing tutor songs in the two months after birth.[3] Other animals, such as mice and frogs, also utilize courtship sounds for mating.[7,8] The neural circuit mechanism of how the animal brain is tuned to the unique conspecific communication sound, however, has just started to be identified,[9,10] and the cellular basis of sound-experience-dependent learning remains to be elucidated.

As observed in the mating communication of many animals, male fruit flies (*Drosophila melanogaster*) emit various signals to attract females, including the near-field sound known as the courtship song, which is produced by wing vibrations.[11,12] The major component of the courtship song of *D. melanogaster* is the pulse song, i.e., a repetition of sound pulses.[13] The inter-pulse interval (IPI) of the pulse song differs among sibling *Drosophila* species and significantly affects female mate choice, with increased mating receptivity to conspecific courtship songs rather than heterospecific songs.[12,14] The auditory pathways from sensory neurons to higher-order brain neurons are organized to tune selectively to the conspecific song and thus facilitate mating acceptance in female flies.[15–17] Several neurons in this circuit express the sex-specific transcription factor *doublesex* (*dsx*), which plays a role in their sexual differentiation.[17–19] In the fly brain, *dsx*-expressing neurons form several clusters (six clusters in both males and females, with four male-specific and one female-specific),[18] and previous studies have reported the importance of *dsx*-expressing neurons in mating behaviors.[20] Among them, female pC1 neurons, which innervate the lateral protocerebral complex and superior-medial protocerebrum (SMP) in the brain, serve as a key regulator of female mating behavior by enhancing copulation receptivity when activated.[19]

Our previous study showed that both male and female fruit flies acquire a preference for songs with conspecific IPIs over songs with heterospecific IPIs following prior exposure to the conspecific song.[21] Further studies in females revealed the importance of the neurotransmitter γ-aminobutyric acid (GABA), as GABAergic inputs to pC1 neurons are necessary for song preference learning.[21] Studies on the equivalent neuronal mechanism in songbirds have also suggested GABA to be a key regulator in forming auditory memories during the song-learning process: some higher-order auditory cortical neurons become tuned to the tutor song through the recruitment of GABAergic inhibition when

[1]Graduate School of Science, Nagoya University, Nagoya, Aichi 464-8602, Japan
[2]Janelia Research Campus, Howard Hughes Medical Institute, Ashburn, VA 20147, USA
[3]Institute of Transformative Bio-Molecules (WPI-ITbM), Nagoya University, Nagoya, Aichi 464-8602, Japan
[4]Lead contact
*Correspondence: kamikouchi@bio.nagoya-u.ac.jp
https://doi.org/10.1016/j.isci.2024.110266

exposed in early life.[10] However, the neural circuit basis of how sound exposure tunes the higher-order integration center that controls the auditory responses remains unclear in either case.

To address this issue, we used a fruit fly model to identify GABAergic neurons within neural circuits that mediate song preference learning. First, using an intersectional strategy we showed that GABAergic neurons that express *dsx* are responsible for song preference learning. These neurons are distributed both in the brain and in the ventral nerve cord (VNC), with those in the brain identified as pCd-2 neurons. Second, mining of the *Drosophila* hemibrain connectome database revealed synaptic connections between pCd-2 neurons and pC1 neurons, including reciprocal connections. Finally, we showed that *dsx*-expressing GABAergic neurons receive GABAergic and dopaminergic inputs via $GABA_A$ receptors (Rdl) and Dop1R2 receptors, respectively, that contribute differently to song preference learning. Taken together, this study suggests that reciprocal circuits between pC1 and pCd-2 neurons serve as a hub integrating sensory and internal states, allowing flexible control over female copulation. Consequently, this study proposes at a single-cell resolution the fundamental neural circuit that underlies song preference learning in fruit flies.

RESULTS

doublesex-expressing GABAergic neurons control song preference learning

Approximately 6,000 GABAergic neurons are present in the adult fly brain.[22] To identify GABAergic neurons involved in song preference learning, we focused on the *dsx*-expressing neurons (approximately 66 and 315 neurons in the brain and VNC of females, respectively),[23] which are involved in female mating receptivity. To genetically label *dsx*-expressing GABAergic neurons, we utilized an intersectional strategy to generate a *dsx*∩*Gad1* driver-1, in which *Gad1* encodes the rate-limiting enzyme in the GABA synthesis pathway (Figures 1A and 1B; see STAR methods for fly strains). We found *dsx*-expressing GABAergic neurons in the female brain and the VNC (Figure 1A): in the brain, labeled neurons (three or four cells per hemibrain) have cell bodies at the posterior-dorsal side of the brain, ventral to the protocerebral bridge, and extend neurites to the superior medial protocerebrum (SMP) and ipsilateral gnathal ganglia (GNG). In the VNC, labeled neurons (approximately >100 neurons per side) have cell bodies in the abdominal ganglion.

Next, we examined whether *dsx*-expressing GABAergic neurons contribute to song preference learning. The song preference learning paradigm comprised a training session and a subsequent test session (Figure 1C).[21] During the training session, female flies in the experienced condition were exposed to an artificial conspecific song, whereas those in the naive condition were kept without sound exposure. During the test session, female flies in both conditions were paired with mute males and exposed to one of two artificial songs (conspecific or heterospecific song). In this test, the cumulative copulation rate serves as a readout of female receptivity for the courting male (Figure 1D; see also STAR methods). To assess the effect of song exposure, we defined the song preference learning index (LI) based on the accelerated failure time (AFT) model.[24,25] In brief, the LI value represents the magnitude of learning: when the LI and its confidence intervals (CIs) deviate from 1, the flies are detected as showing song preference learning (see STAR methods; LI of wild-type flies shown in Figure S1).

Using the *dsx*∩*Gad1* driver-1, we suppressed *Gad1* expression specifically in *dsx*-expressing GABAergic neurons (see STAR methods for fly strains). Control flies without any knockdown showed song preference learning: naive flies rapidly initiated copulation behaviors after being exposed to either the conspecific or heterospecific song (NC and NH, respectively), whereas experienced flies showed a lower copulation rate during exposure to the heterospecific song but not the conspecific song (EH and EC, respectively) (Figure 1D, left). The AFT model detected a significant interaction between song experience and test song type, indicating that control flies showed experience-dependent reduction of female receptivity to the heterospecific song (Figure 1E; LI = 2.18; 95% CI, 1.20 to 3.97; $p = 0.011$; log-logistic AFT model; see STAR methods). In contrast, *Gad1* knockdown females lost the experience-dependent reduction of copulation (Figure 1D, right): they showed a high copulation rate under all conditions, and no significant interaction was detected (Figure 1D, right and 1E; LI = 1.26; 95% CI, 0.67 to 2.37; $p = 0.48$; log-logistic AFT model). We next combined the *Otd-FLP*, *tubP FRT-Gal80-FRT* strain with *dsx*∩*Gad1* driver-1 (referred to as *dsx*∩*Gad1* driver-2 hereafter). Although the *dsx*∩*Gad1* driver-2 failed to silence marker expression in the VNC, it labeled the same set of neurons in the brain (Figures S2 and S3). Moreover, this *dsx*∩*Gad1* driver-2 phenocopied the original *dsx*∩*Gad1* driver-1 strain in terms of song preference learning when *Gad1* expression was knocked down (Figure S4; see Figure S5 for another RNAi strain). These results show that suppression of GABA synthesis in *dsx*-expressing GABAergic neurons abolished song preference learning, indicating that neuronal signals from *dsx*-expressing GABAergic neurons are involved in song preference learning. Interestingly, the neurites of these *dsx*-expressing GABAergic neurons in the brain spatially overlapped with pC1 neurites in the SMP.[18] Previous studies have reported the importance of *dsx*-expressing neurons in the brain, including pC1 neurons, in regulating female receptivity.[19] Because GABAergic input to pC1 neurons contributes to song preference learning,[21] we hypothesized that *dsx*-expressing GABAergic neurons in the brain are responsible for this GABAergic input to pC1 neurons.

pCd-2 neurons connect to pC1 neurons

Previous studies identified seven to eight clusters of *dsx*-expressing neurons in the female brain, i.e., pC1, pC2l, pC2m, pCd-1, pCd-2, pMN1, pMN2, and aDN clusters, with distinct cell-body locations and neurite morphologies.[17,18,26] The number, cell-body locations, and neurite morphologies of neurons in the brain labeled by both *dsx*∩*Gad1* drivers resemble those of pCd-2 neurons and are distinct from other *dsx*-expressing neuronal clusters (Figures 1A and S2),[18,26] indicating that the labeled neurons in the brain belong to the pCd-2 cluster. To verify the synaptic connections between pCd-2 neurons and pC1 neurons suggested by the spatial overlap between their neurites, we performed GFP reconstitution across synaptic partners (GRASP) analysis[27] by expressing $spGFP_{1-10}$ and $spGFP_{11}$ in pCd-2 neurons and pC1 neurons, respectively. In all cases tested ($N = 4$), reconstructed GFP signals (GRASP signals) were detected at the posterior side of the SMP (Figure 2A). It is thus highly likely that pCd-2 neurons have direct synaptic connections with pC1 neurons.

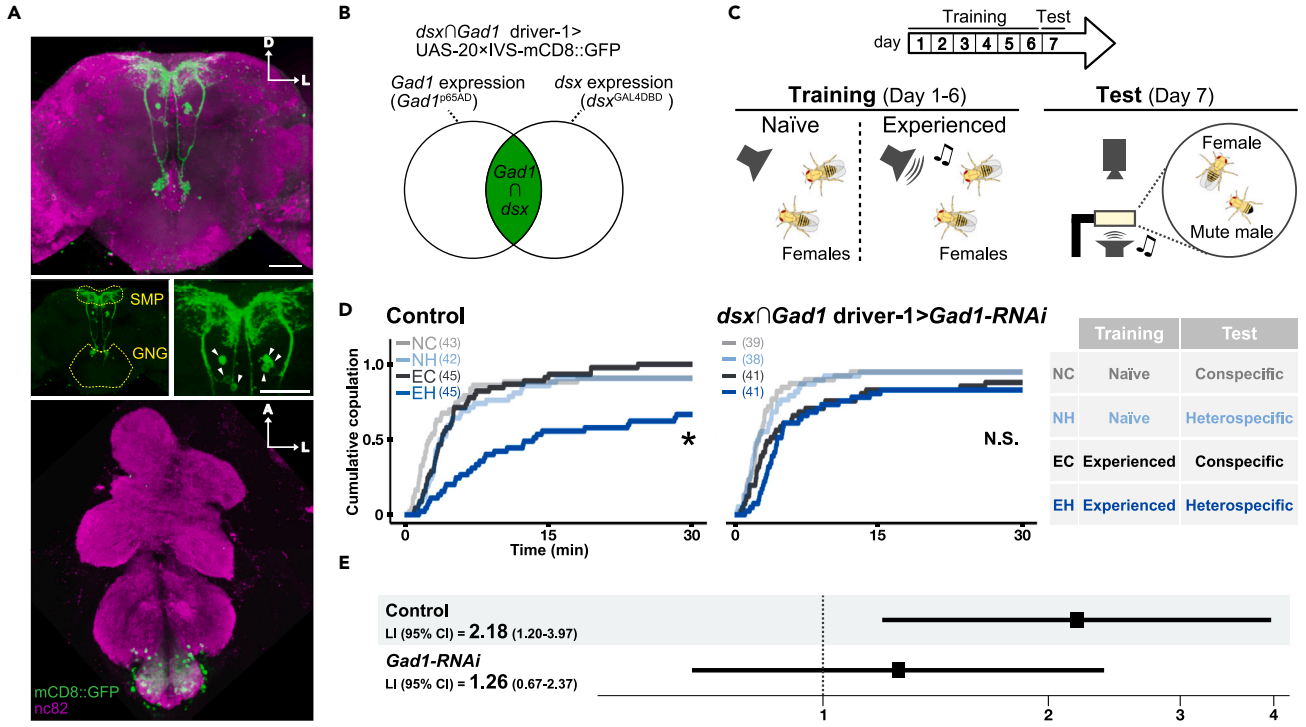

Figure 1. *doublesex*-expressing GABAergic neurons are necessary for song preference learning

(A) Expression pattern of *dsx*∩*Gad1* driver-1 in the brain (top and middle-left; anterior view) and ventral nerve cord (bottom; ventral view) in females. A magnified view of the cell bodies (arrowheads) is shown in the middle-right. SMP, superior medial protocerebrum; GNG, gnathal ganglia. Scale bars, 50 μm. A, anterior; D, dorsal; L, lateral (the same in the following Figures).

See also Figures S2 and S3.

(B) Venn diagram of the genetic intersection. The population labeled by the *dsx*∩*Gad1* driver-1 is shown in green.

(C) Experimental scheme for song preference learning. In the training session, females in the experienced condition are exposed to the conspecific song for the first 6 days after eclosion, whereas naive females are kept in silence. During the test session on the 7th day, females of both conditions are paired with mute males and exposed to either conspecific or heterospecific song.

(D) Song preference learning in *Gad1* knockdown females. The number of trials for each group is shown in parentheses. NC, naive flies tested with the conspecific song; NH, naive flies tested with heterospecific song.; EC, experienced flies tested with the conspecific song; EH, experienced flies tested with heterospecific song. Not significant (N.S.), $p > 0.05$; *, $p < 0.05$; log-logistic AFT model.

See also Figure S1.

(E) The learning index (LI) estimated based on the cumulative copulation rate using log-logistic AFT model. The horizontal axis uses a natural logarithm scale (see STAR methods for details). The squares indicate estimated LI, and horizontal lines indicate 95% confidence intervals (CIs) (same in the following Figures).

To reveal the synaptic connections between pC1 and pCd-2 neurons at the single-cell level, we analyzed the FlyEM dataset, an EM-based connectome dataset of an adult female fly brain (hemibrain: v1.2.1).[28] pC1 neurons are composed of five cells, namely, pC1a, pC1b, pC1c, pC1d, and pC1e[29] (see Table S2 for the FlyEM neuron IDs). These subtypes have been suggested to control different types of female behaviors, i.e., aggression and copulation receptivity.[17] To identify pCd-2 neurons labeled by the *dsx*∩*Gad1* drivers in the FlyEM dataset, we searched for neurons whose morphologies resemble those of pCd2 neurons and have GABA synapses to pC1 neurons (threshold = 10 synapses; Figure 1A; Table S2) using a neurotransmitter classification algorithm.[30] We identified four neurons in a hemibrain, named SMP286, SMP287, SMP294, and SMP297, which share comparable cell body locations and projection patterns with pCd-2 neurons (Figures 2B–2F; FlyEM neuron IDs are listed in Table S2). The distributions of postsynaptic sites in these four neurons, mapped in the FlyEM, were consistent with the pCd-2 dendritic regions labeled by a postsynaptic marker DenMark[31] driven by the *dsx*∩*Gad1* driver-1 (Figure S6). Thus, we classified these four neurons as pCd-2 neurons, GABAergic neurons projecting to pC1 neurons.

By investigating synaptic connections between these four pCd-2 neurons and pC1 neurons with the FlyEM dataset (Figure 2B), we classified pCd-2 neurons into two types. One is the intensive connection type (SMP286 and SMP287), which has many synapses with several types of pC1 neurons (Figures 2G and 2H). The other is the sparse connection type (SMP294 and SMP297), with fewer synapses with a subset of pC1 neurons (Figures 2I and 2J). Among the intensive connection-type pCd-2 neurons, SMP286 has many bilateral output synapses to pC1 neurons, while the number and variety of ipsilateral outputs exceed that of contralateral outputs. Importantly, SMP286 receives intensive synaptic inputs from pC1a neurons of both brain sides, indicating reciprocal synaptic connections between SMP286 and bilateral pC1a neurons. The other intensive connection-type pCd-2 neuron, SMP287, has output synapses mainly to the ipsilateral pC1d neuron but receives many

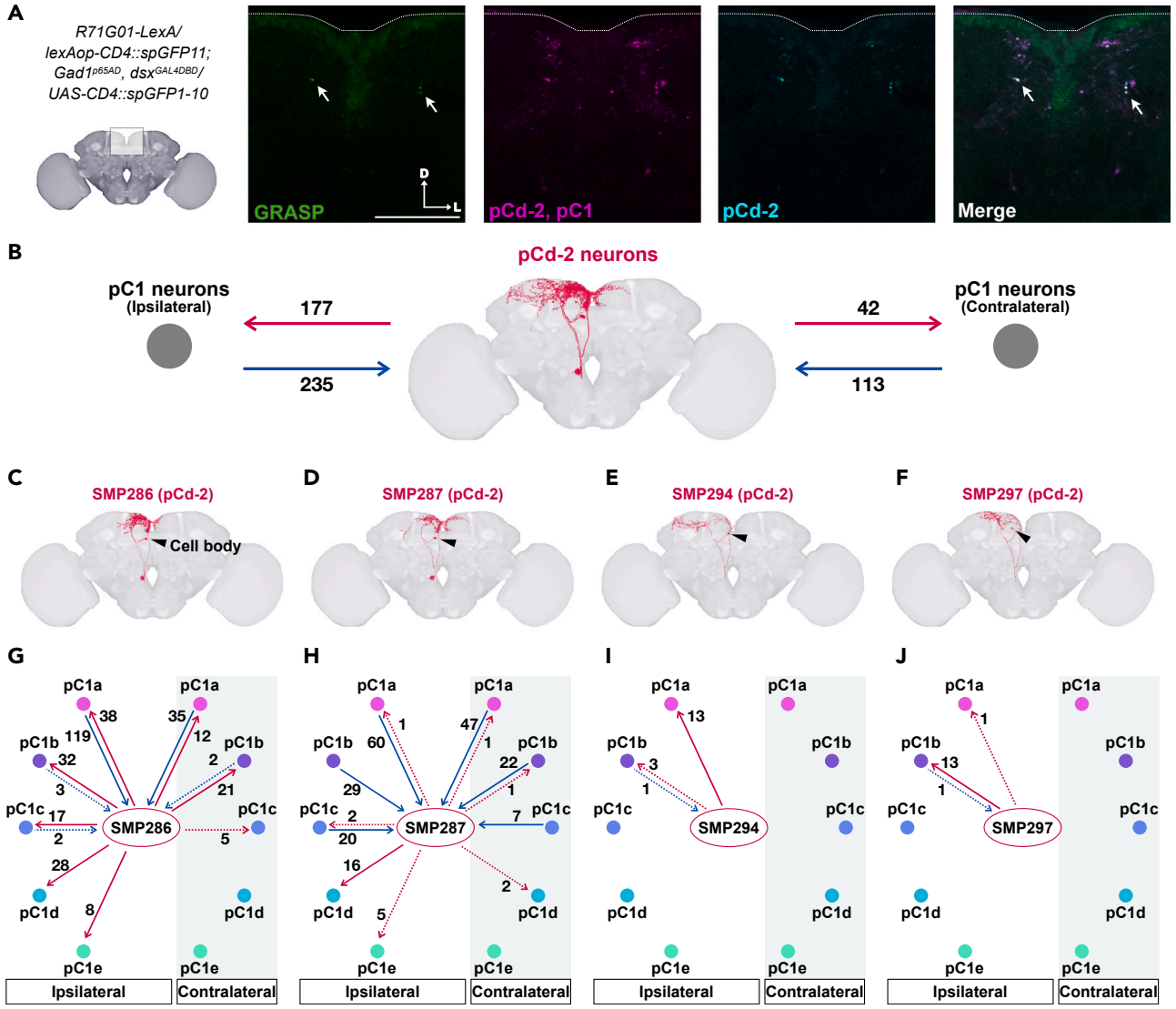

Figure 2. Synaptic connections between pCd-2 and pC1

(A) GRASP between pCd-2 neurons and pC1 neurons. The brain region shown in the middle to right panels is outlined in the left panel. GRASP signals (green), $spGFP_{1-10}$ and $spGFP_{11}$ expression in pCd-2 neurons and pC1 neurons, respectively (magenta), $spGFP_{1-10}$ expression in pCd-2 neurons (light blue), and merged image are shown (see Table S1 for the genotype). Scale bar, 50 μm.

(B) Synaptic connections between pC1 neurons and pCd-2 neurons. Red and blue arrows depict the output and input synapses of pCd-2 neurons to/from pC1 neurons, respectively. Numbers on arrows indicate the number of synapses.

See also Figure S6.

(C–F) Single pCd-2 neurons in the right hemibrain of the FlyEM dataset.

See also Figure S8.

(G–J) Synaptic connections between pCd-2 neurons and pC1 neurons based on the FlyEM dataset. Numbers on arrows indicate the number of synapses. Weak connections (fewer than six synapses)[17] are shown in dotted arrows.

See also Figures S7 and S12.

synaptic inputs bilaterally from pC1a/b/c neurons. In contrast, the sparse connection-type pCd-2 neurons have output synapses almost exclusively to pC1 (Figures 2I and 2J). pC1a and pC1b receive inputs from ipsilateral SMP294 and SMP297, respectively, suggesting a specific regulation from these pCd-2 neurons to a corresponding pC1 neuron. Taken together, GRASP and the FlyEM connectome analyses revealed the synaptic connectivity between pC1 and pCd-2 neurons. Interestingly, there are direct synaptic connections from SMP294 to SMP286 and SMP286 to SMP287 but not vice versa, suggesting a unidirectional information flow among these three pCd-2 neurons (Figure S7A; Table S3). It is possible that all types of pCd-2 neurons are involved in the GABAergic control of pC1 neurons, while the specific combination of target pC1 neurons and synaptic weights differ between each type of pCd-2 neuron (Figures 2G–2J and S8). Furthermore, two of the pCd-2

neurons (SMP286 and SMP287) receive many direct synaptic inputs from pC1 neurons, suggesting the existence of reciprocal connectivity between these pCd-2 neurons and pC1 neurons that together exhibit feedback and lateral inhibition motifs.

FlyEM database mining also revealed that two intensive-type pCd-2 neurons have further direct synaptic connections with other neurons involved in female copulation receptivity (Figure S7B). SMP286 and SMP287 receive synaptic inputs from SAG neurons, activation of which is known to depolarize pC1 neurons and increases female receptivity.[29,32,33] SMP286 sends synaptic output to vpoDN, which controls vaginal plate opening to accept copulation (Table S3).[33] vpoDN also receives direct synaptic input from pC1 neurons,[33] suggesting a feedforward circuit motif from SAG-neurons to vpoDN neurons through pC1-pCd-2 reciprocal circuits (Figure S7B). These findings suggest that two intensive-type pCd-2 neurons are embedded in the neural circuit composed of SAG, pC1, and vpoDN, possibly to modulate female receptivity and copulation acceptance.

GABA and dopamine signaling to pCd-2 neurons

Our findings suggest that pCd-2 neurons send GABAergic signals to pC1 neurons to suppress female receptivity in response to heterospecific song exposure. To further explore how pCd-2 activity is modulated by upstream neurons, we screened neurotransmitter receptors expressed in pCd-2 neurons using a single-cell transcriptome of adult fly brains.[22,34] To focus on the gene expression profiles of pCd-2 neurons, we selected cells that express *Gad1*, *dsx,* and *elav* genes, among which *elav* serves as a pan-neuronal marker.[35] Many neurons in this population expressed several types of receptors, including $GABA_A$-type receptor (Rdl), dopamine 1-like receptor 1 (Dop1R1), dopamine 1-like receptor 2 (Dop1R2), and/or dopamine 2-like receptor (Dop2R) (Figure S9).

To investigate whether and how these receptors in pCd-2 neurons contribute to song preference learning, we examined the effect of knockdown of each receptor using the *dsx*∩*Gad1* driver-2. Knockdown of either *Dop1R1* or *Dop2R* did not abolish song preference learning, suggesting that they are not or only minimally involved in this process (Figure S10). In contrast, *Rdl* knockdown females lost the learning phenotype (Figures 3A and 3B; *Rdl* knockdown group: LI = 0.80; 95% CI, 0.42 to 1.51; p = 0.49; control group: LI = 2.39; 95% CI, 1.11 to 5.13; p = 0.026; log-logistic AFT model). The phenotypic changes observed following *Rdl* knockdown by *dsx*∩*Gad1* driver-2 resembled that of *Gad1* knockdown, with experienced females showing as high a copulation rate to the heterospecific song as to the conspecific song (Figure 3A vs. Figures 1D and S4A). Interestingly, flies with *Dop1R2* knockdown by *dsx*∩*Gad1* driver-2 exhibited a different type of learning disruption (Figure 3C). In the experienced condition, *Dop1R2* knockdown females exhibited lower copulation rates to both the conspecific and heterospecific songs than naive flies, which led to the loss of the learning phenotype (Figures 3C and 3D; *Dop1R2* knockdown group: LI = 0.70; 95% CI, 0.33 to 1.49; p = 0.35; control group: LI = 2.30; 95% CI, 1.06 to 4.99; p = 0.039; log-logistic AFT model). We used restricted mean time lost (RMTL) as an indicator of the cumulative copulation rate to evaluate this change, where a larger RMTL reflects higher copulation acceptance (Figure 3E; see STAR methods for details).[14] We found that the response of experienced females to the conspecific song, but not to the heterospecific song, was significantly reduced in the *Dop1R2* knockdown group when compared to that of the control group (Figure 3E; conspecific song: $p = 1.62e^{-12}$; heterospecific song: p = 0.32; restricted mean survival time adjusted by Bonferroni). This phenotype contrasted with that in the *Rdl*-knockdown females, which lost the experience-dependent reduction in response to the heterospecific song (Figure 3E; conspecific song: p = 0.057; heterospecific song: p = 0.0022; restricted mean survival time adjusted by Bonferroni correction). These findings support the model that GABAergic and dopaminergic inputs to pCd-2 neurons, through Rdl and Dop1R2 receptors, respectively, play different modulatory roles to control song preference learning through pC1 neurons: GABAergic input to pCd-2 via Rdl receptors is necessary to suppress copulation behavior in response to heterospecific song exposure, and dopaminergic input through Dop1R2 receptors is necessary to facilitate behavioral responses to the conspecific song in experienced flies. While investigating synaptic connections using the FlyEM dataset, we discovered that some pCd-2 neurons (SMP286 and SMP287) receive synaptic input from other pCd-2 neurons, as well as a dopaminergic neuron cluster known as PAL (Figures S7A and S7C; Table S3). GABAergic and dopaminergic modulations of these pCd-2 neurons may thus be mediated via signaling from other pCd-2 neurons and PAL neurons, respectively.

DISCUSSION

Here, we demonstrate that *dsx*-expressing GABAergic neurons are responsible for song preference learning in female fruit flies. In the brain, pCd-2 neurons are identified as *dsx*-expressing GABAergic neurons. pCd-2 neurons have direct connections with pC1 neurons, which regulate female sexual receptivity. A functional analysis of neurotransmitter receptors suggested that GABAergic and dopaminergic inputs to *dsx*-expressing GABAergic neurons are involved in this learning. These findings propose a neural circuit model that contributes to song response behaviors at the single-cell resolution level (Figure 4; but see limitations of the study for a possible involvement of *dsx*-expressing GABAergic neurons in the VNC). In this model, song responses of females are regulated by an interaction between innate and experience-dependent pathways (Figure 4A).[21] The innate pathway starts with auditory sensory neurons, which transmit song information finally to pC1 neurons to regulate female receptivity. The experience-dependent pathway modifies the innate pathway through GABAergic pCd-2 neurons, which make reciprocal connections with pC-1 neurons (Figure 4B). GABAergic pCd-2 neurons receive GABAergic input through $GABA_A$ receptors and dopaminergic input mediated by Dop1R2 receptors. GABAergic signaling to pCd-2 plays a key role in suppressing mating receptivity to the heterospecific song in the experienced flies, whereas dopamine signals facilitate, and thus maintain, the receptivity for the conspecific song after the experience (Figure 4B). It implies that pCd-2 neurons gate the song response of female flies based on prior sound experiences. One notable feature of the behavioral phenotype of song preference learning in wild-type flies is that the experience affects only the heterospecific song response. This specificity might be achieved via separate GABAergic and dopaminergic modulation pathways, and its underpinning mechanism should be explored in the future.

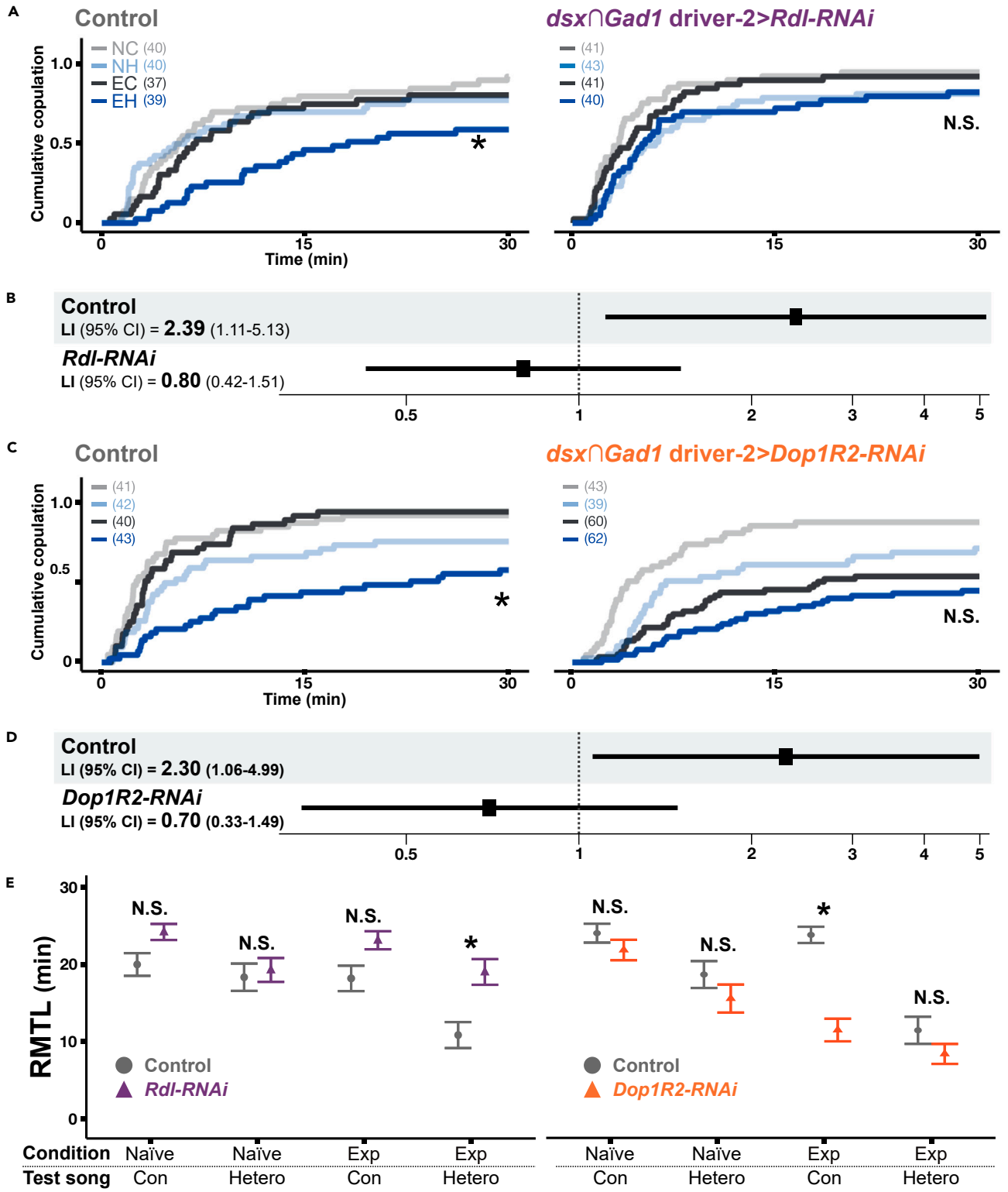

Figure 3. Involvement of GABA and dopamine receptors in song preference learning

(A) Cumulative copulation rates in control and *Rdl* knockdown groups. NC, naive flies tested with the conspecific song; NH, naive flies tested with heterospecific song.; EC, experienced flies tested with the conspecific song; EH, experienced flies tested with heterospecific song. The number of trials in each group is shown in parentheses.

Figure 3. *Continued*

(B) Learning index (LI) in control and *Rdl* knockdown groups.

(C) Cumulative copulation rates in control and *Dop1R2* knockdown groups.

(D) LI in control and *Dop1R2* knockdown groups.

(E) Restricted mean time lost (RMTL) of cumulative copulation rate for each group. Plots display the average (circle or triangular dot in each box) and standard errors (horizontal bars). Exp, experienced; Con, conspecific song; Hetero, heterospecific song. Log-logistic AFT model (A–D) and restricted mean survival time with Bonferroni correction (E) were used. N.S., not significant; $*p < 0.05$.

See also Figures S9, S10, and S12.

The neural circuit between pCd-2/pC1 neurons

In this study, we found that a subset of pCd-2 neurons forms reciprocal circuits with pC1 neurons (Figures 2B and 2G–2J). The pCd-2 neurons are GABAergic and therefore likely form inhibitory connections to cholinergic pC1 neurons, which, in turn, establish excitatory connections back to pCd-2 neurons (Figure 4B).[29] The reciprocal circuits between these two neuronal types thus likely provide both feedback and lateral inhibitions to pC1 neurons that act as decision-makers for copulation. These inhibitory motifs are commonly observed in the brains of a wide range of animals. In mammals, for example, feedback inhibition in cortical circuits has been suggested to play a significant role in decision computations.[36,37] Lateral inhibition, on the other hand, can mediate competitive interactions between neurons, often leading to contrast enhancement.[38] Accordingly, a possible scenario in flies is that the pCd-2/pC1 reciprocal circuits adjust the copulation decision-making signal based on prior sound experiences through contrasting activity patterns among pC1 subtypes. Further investigation is necessary to explore how pCd-2 neurons modify the activity profile of these reciprocal circuits, as well as to examine how each of the pCd-2/pC1 cluster neurons are functionally integrated for copulation decision-making.

pCd-2 neurons observed in this study were found in both males and females (Figure S11), suggesting that these neurons are shared between the sexes. This observation is consistent with a previous study, which reported that pCd-2 neurons also exist in males with a slight sexual dimorphism in neurite structures.[18] Because male flies also exhibit song preference learning,[21] pCd-2 neurons may play a key role in song preference learning in both sexes. However, a female-specific population of pCd-2 neurons, labeled by a distinct set of hemi-drivers, has been reported: these female-specific pCd-2 neurons control post-mating changes in food preference under the regulation of pC1 neurons[39] and are located downstream of the SAG-pC1 pathway, which alters its mode after copulation.[33] It will be interesting to further investigate if and how the pCd-2/pC1 reciprocal circuits presumably underlying song preference learning in female brains interact with the SAG-pC1-pCd-2 pathway involved in post-mating food preference.

Two different pathways shape song preference after a song experience

Our findings suggest that GABA and dopamine inputs to pCd-2 neurons are mediated by $GABA_A$ (Rdl) and Dop1R2 receptors (Figure 4B). Rdl encodes a subunit of ligand-gated chloride channel $GABA_A$, whereas Dop1R2 is a G-protein-coupled receptor that presumably belongs to the D1-like dopamine receptor group.[40] It is known that dopamine signaling via Dop1R2 increases intracellular calcium levels and cAMP,[41] whereas GABAergic signaling through $GABA_A$ receptors containing Rdl subunits induces fast inhibition due to chloride influx.[42] Such distinct signaling cascades in pCd-2 neurons may contribute to the different responses to two song types in experienced females (Figure 4B), though these signaling properties remain to be characterized and validated in pCd-2 neurons.

A candidate source of GABAergic signals to pCd-2 neurons is the pCd-2 neurons themselves, as our connectome analysis detected synaptic connections within pCd-2 neurons (Figure S7A; Table S3). In the mammalian brain, interactions between GABAergic neurons contribute to synchronized oscillation of cortical neuron activity that is proposed to facilitate neural plasticity.[43,44] Similarly, interactions between GABAergic neurons in flies can potentially contribute to song-experience-dependent neural plasticity. On the other hand, dopaminergic signals to pCd-2 neurons are possibly derived from PAL neurons (Figure S7C; Table S3). The PAL cluster, located in the superior lateral protocerebrum in the fly brain,[45] is one of the dopaminergic neuron clusters involved in olfactory learning in both males and females.[46] During mating behaviors, a dopamine neuron denoted aSP4 in the PAL cluster in males transmits signals to P1 neurons, a male-specific subset of pC1 neurons, via Dop1R2. While this pathway contributes to promoting male mating drive,[47] the involvement of the PAL cluster in female mating motivation is not well understood.

A previous report has shown that an interaction between GABA and dopamine signaling is important for controlling *Drosophila* female pre-mating behaviors.[48] This interaction involves dopaminergic PPM3 neurons, GABAergic R2/R4m neurons, and cholinergic R4d neurons, constituting a circuit motif known as a feedforward motif with a repressor, specifically the incoherent type 1 feedforward loop (I1-FFL). In the I1-FFL circuit, an activator *X* activates a target *Z* and simultaneously activates another target *Y*, which inhibits target *Z* (Figure S7D). The neural circuit involving dopaminergic PAL (activator *X*), GABAergic pCd-2 (target *Y* that inhibits the target *Z*), and cholinergic pC1 neurons (target *Z*) identified in this study aligns well with the I1-FFL circuit motif, as the PAL cluster has a synaptic output to both pCd-2 and pC1 neurons (Figure S7D). The I1-FFL motif is widely observed in various biological systems, including gene and protein regulation networks, metabolic pathways, and neural networks, spanning from bacteria to humans.[49,50] The findings of this study corroborate the importance of the I1-FFL motif in regulating biological functions.

In zebra finches, an interaction between dopamine and GABA was reported in a brain region known as the caudomedial nidopallium (NCM), the higher-level auditory cortex in the avian brain that serves as a possible storage site of tutor song memories.[51] Most neurons in the NCM co-express D1 receptor (D1R) and GABA. *Ex vivo* slice recordings and *in vivo* electrophysiological recordings from NCMs suggested that dopamine signals via D1R modulate the amplitude of GABAergic currents in NCM neurons and stimulus-specific neural plasticity. A

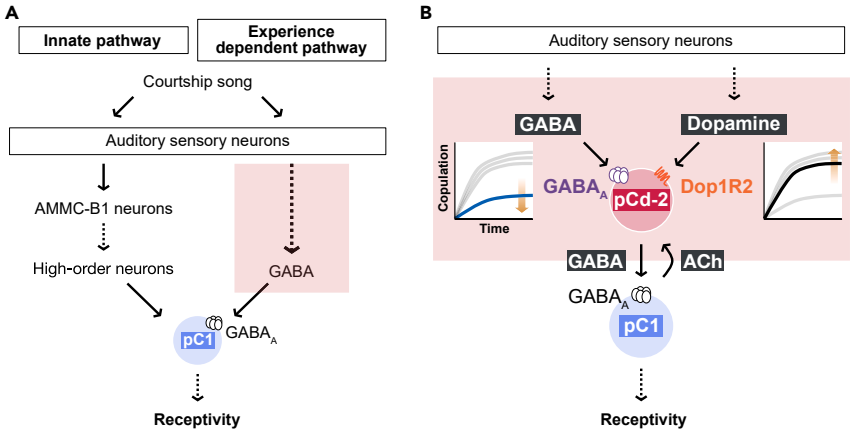

Figure 4. Neural circuit model for song preference learning in flies

(A) A model for experience-dependent tuning of song responses in fruit flies. Flies fine-tune their innate IPI preference through auditory experiences. Modified from[21] with permission. Red shaded area is shown in greater detail in (B).

(B) A model for the neural circuit mechanism of experience-dependent modulation. Song preference learning in flies involves at least two distinct mechanisms, the experience-dependent suppression of the response to the heterospecific song (blue line in the copulation plot) and the maintenance of the response to the conspecific song (black line in the copulation plot) after experience. These two mechanisms are mediated by GABA and dopamine, respectively, transmitted to pCd-2 neurons. pC1 neurons are excitatory cholinergic neurons transmitting acetylcholine (ACh).[29]

similar modulation might occur in fly song preference learning, in which dopamine signals to pCd-2 via Dop1R2 possibly modulate GABAergic signals to pC1 neurons. It should be noted, however, that the present study does not clarify the extent to which pCd-2 neurons express, and are thus modulated through, Rdl ($GABA_A$) and Dop1R2 receptors, highlighting the importance of analyzing gene expression in individual pCd-2 neurons.

Possible role of pCd-2 neurons in modulating the balance of mating-related decisions via pC1 neurons

When exposed to courtship songs, female flies typically escape from males before finally accepting copulation attempts.[48] During this pre-copulation period, virgin females sometimes engage in aggressive behaviors.[17] These aggressive behaviors toward the courting male are driven by pC1d/e neurons, whereas mating receptivity is promoted by other pC1 neurons (i.e., pC1a/b/c) to facilitate copulation acceptance.[17] Our study shows that pC1d/e neurons receive synaptic inputs from intensive-type pCd-2 neurons (i.e., SMP286 and SMP287), whereas other pC1 neurons, pC1a/b/c, receive inputs from all pCd-2 neurons except SMP287 (Figures 2G–2J; see Figure S12 for details). Because pCd-2 neurons are GABAergic, these pCd-2 neurons are likely to inhibit the target pC1 neurons and thus adjust the balance between aggressive behaviors and copulation acceptance in female flies. Notably, two intensive-type pCd-2 neurons receive many synaptic inputs from pC1a/b/c neurons, suggesting that pC1a/b/c neurons suppress pC1d/e neurons via these pCd-2 neurons. Experiences of hearing conspecific songs might affect the landscape of pC1 activations, which would be mediated by mutual interactions between pCd-2 and pC1 neurons. In addition to reciprocal connections with pC1 neurons, pCd-2 neurons directly connect to the vpoDN (Figure S7B). pCd-2 neurons thus modulate the activity landscape of the SAG-pC1-vpoDN pathway, which switches the female state from rejection to copulation acceptance. In this scenario, pCd-2 neurons are likely to play a role in inhibiting or gain-controlling this pathway if flies have prior auditory experiences. The EM database analysis also showed that one of the pCd-2 neurons, SMP286, receives direct synaptic inputs from circadian neurons involved in sleep regulation (i.e., LPN; see Table S3).[52] These anatomical connections together imply that pCd-2 neurons serve as an integration hub for external sensory stimuli and internal states to achieve flexible control of mating receptivity. Unraveling the overall function of pCd-2 neurons in female copulation decision-making requires further investigation.

GABAergic inhibitions in the mating circuits of fruit flies

The female brain contains eight clusters of *dsx*+ neurons: pC1, pC2l, pC2m, pCd-1, pCd-2, pMN1, pMN2, and aDN clusters. Mating acceptance was suppressed upon the inactivation of all these clusters, as well as individually in pC1 or pCd-1 cluster.[19,20] These previous reports have pointed to an overall receptivity-promoting role of *dsx*+ neurons in the female brain. However, this study suggests a specific cluster, pCd-2, functions to inhibit female receptivity. This finding, in conjunction with earlier studies, suggests that *dsx*+ neuron populations in the female brain contribute to the modulation of mating motivation, exerting either positive or negative influences to achieve flexible control over mating receptivity.

In male flies, neurons expressing the *fruitless* (*fru*) gene play a crucial role in regulating mating behaviors. The activation of all *fru*+ neurons robustly induced courtship behavior,[53] suggesting an overall mating-promoting role similar to that of female *dsx*+ neurons. Notably, GABAergic inhibition motifs have been identified among the *fru*+ neuron populations in the male brain. These motifs are presumed to contribute to gain-controlling the response to excitatory courtship stimuli and courtship learning in males.[54] Considering that *fru*- and

dsx-expressing neurons regulate mating behavior respectively in males and females, the function of GABAergic inhibitions in modulating male mating behavior aligns with the finding in this study that GABAergic signals in *dsx*+ neural circuit modulate female mating behavior based on prior sound experiences (Figure 4B). Our finding, coupled with previous findings on the *fru*+ neural circuit in males,[54] underscores the significance of inhibitory signals in the flexible control of mating behaviors in both sexes.

dsx-expressing GABAergic neurons in the VNC

Although our neural circuit analysis strongly supports the model that pCd-2 neurons contribute to song preference learning, it does not exclude the possibility that *dsx*-expressing GABAergic neurons in the VNC also contribute to this learning phenotype. In the female VNC, *dsx*-expressing neurons exclusively exist in the abdominal ganglion.[23] Previous reports have shown that neurons in the abdominal ganglion, namely Abd-B neurons, are necessary for mating receptivity in females.[55] In this study, silencing *dsx*-expressing GABAergic neurons by *tetanus-toxin* expression dramatically decreased mating receptivity (Figure S13). Although it is not clear if female Abd-B neurons express *dsx* and *Gad1*, these studies together with our findings suggest that *dsx*-expressing GABAergic neurons in the VNC might modulate overall mating receptivity.

Perceptual learning in mammals, birds, and flies

"Perceptual learning" describes how perceptual discrimination ability improves with training.[56,57] A broad range of sensory skills improve with practice during perceptual learning, such as language acquisition, musical abilities, and visual discrimination.[4,58–61] Studies using animal models have shown that perceptual learning is associated with changes in sensory cortex activity.[62–64] Notably, the involvement of GABA in perceptual learning is observed in the auditory cortex of Mongolian gerbils, in which the infusion of muscimol, a selective agonist for $GABA_A$ receptors, prevents perceptual learning in the sound discrimination task.[57] More peripherally, GABAergic granule cells in the olfactory bulb are responsible for olfactory perceptual learning of mice.[65] These granule cells are the main source of lateral inhibition in the olfactory bulb and modulate pattern separation of mitral cells, which then project their axons to higher structures in the brain. Studies in this circuit further suggested the importance of inhibitory (i.e., granule cells) and excitatory (i.e., mitral cells) reciprocal connections in olfactory perceptual learning.[65] The neural circuit model for song preference learning in fruit flies also involves the inhibitory-excitatory reciprocal circuit that includes GABAergic signals through $GABA_A$ receptors as a key motif, supporting the idea that the circuit mechanisms underlying this learning are shared between vertebrates and flies.

A "sensitive period," which typically occurs during development including pre- and postnatal stages, has been identified during which the effect of experience on the brain to improve perceptual discrimination ability is particularly strong.[66,67] Studies using human and other vertebrate models have suggested that during the sensitive period, a balance between excitation and inhibition (E/I balance) is established, which typically requires appropriate sensory inputs.[66–68] Particularly, the maturation of the inhibitory system plays a dominant role in shaping sensory perception during the sensitive period.[69] In the primary auditory cortex of rodents, maturation of the inhibitory system during postnatal development enables excitation and inhibition to become highly correlated and balanced, which is accelerated by sound experiences.[70] In the higher-level auditory cortex of songbird, GABAergic inhibitions matured by tutor song experiences form selective responses to the tutor song.[10] Although the present study has not assessed the developmental aspect of song preference learning of flies, exploring the maturation process of the GABAergic signals to/from pCd-2 neurons in the fly model would provide key insights into a general neural-circuit mechanism on how sensory experiences establish the E/I balance that shapes information processing during development. An interesting future direction is to test if the neural circuit motif found in this study also underlies the experience-dependent auditory plasticity observed in vertebrates.

Limitations of the study

Our research supports the hypothesis that pCd-2 neurons are responsible for song preference learning and have reciprocal synapses with pC1 neurons. Although it provides behavioral/anatomical evidence in this regard, this study does not show whether the neural responses of pCd-2/pC1 to courtship songs are altered depending on prior auditory experiences. Furthermore, although our analyses suggest the functional role played by pCd-2 neurons (via continuous RNAi-mediated knockdown assays), the timescale over which these neurons contribute to song preference learning remains unclear. Finally, our data do not exclude the possibility that the *dsx*-expressing GABAergic neurons in the VNC are involved in the song preference learning phenotype.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2024.110266.

ACKNOWLEDGMENTS

We thank Yusuke Miwa, Ken-ichi Kimura, and Daisuke Yamamoto for *Otd-FLP*; *tubulin^{P} FRT-GAL80-FRT* fly strain; Mako Murai, Takuro S Ohashi, Xiaodong Li, Yukina Chiba, Yoichi Oda, Matthew P Su, Naotoshi Nakamura, and Shingo Iwami for discussions; Bloomington Drosophila Stock Center for fly stocks; and Developmental Studies Hybridoma Bank for antibodies. This study was supported by MEXT KAKENHI Grants-in-Aid for Scientific Research (B) (Grant JP20H03355 to A.K.), Scientific Research on Innovative Areas "Evolinguistics" (Grant JP20H04997 to A.K.), Grant-in-Aid for Transformative Research Areas (A) "iPlasticity" (Grant JP21H05689 and JP23H04228 to A.K.), "Evolutionary theory for constrained and directional diversities" (Grant JP20H04865 to Y.I.), Grant-in-Aid for Early-Career Scientists (Grants JP21K15137 to R.T.), Grant-in-Aid for Transformative Research Areas (A) Hierarchical Bio-Navigation (Grant JP22H05650 to R.T.), JST SPRING (Grant JPMJSP2125 to K.I.), JST PRESTO (Grant JPMJPR21S2 to Y.I.) and JST FOREST (Grant JPMJFR2147 to A.K.), Japan.

AUTHOR CONTRIBUTIONS

K.I.: conceptualization, methodology, software, validation, formal analysis, investigation, data curation, visualization, writing—original draft; R.T.: conceptualization, methodology, validation, supervision, funding acquisition, writing—review and editing; Y.A.: methodology, formal analysis, writing—review; J.F.: methodology, formal analysis; Y.I.: methodology, software, validation, formal analysis, supervision, visualization, funding acquisition, writing—review and editing; A.K.: conceptualization, methodology, validation, investigation, supervision, project administration, funding acquisition, writing—original draft.

DECLARATION OF INTERESTS

The authors declare no competing interests.

DECLARATION OF GENERATIVE AI AND AI-ASSISTED TECHNOLOGIES IN THE WRITING PROCESS

During the preparation of this work, the authors used ChatGPT in order to improve language. After using this tool, the authors reviewed and edited the content as needed and take full responsibility for the content of the publication.

Received: November 28, 2023
Revised: March 27, 2024
Accepted: June 11, 2024
Published: June 13, 2024

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
anti-GFP (Rat Monoclonal)	NACARAI TESQUE, INC	RRID: AB_221569 (1:1000)
anti-GFP (Mouse Monoclonal)	Sigma-Aldrich	RRID: AB_259941 (1:300)
nc82-s (mouse monoclonal; supernatant)	DSHB	RRID: AB_2314866 (1:20)
anti-CD4 (rabbit polyclonal)	Sigma-Aldrich	RRID: AB_1078466 (1:300)
anti-GFP (chicken polyclonal)	Abcam	RRID: AB_300798 (1:200)
anti-DsRed (rabbit polyclonal)	Clontech	RRID: AB_10013483(1:1000)
anti-rat-Alexa 488 (goat polyclonal)	Jackson ImmunoResearch	RRID: AB_2338362 (1:300)
anti-mouse-Alexa 555 (goat polyclonal)	Thermo Fisher Scientific	RRID: AB_141780 (1:300)
anti-chicken-Alexa 488 (goat polyclonal)	Thermo Fisher Scientific	RRID: AB_2534096 (1:300)
anti-rabbit-Alexa 555 (goat polyclonal)	Thermo Fisher Scientific	RRID: AB_2535850 (1:300)
anti-mouse-Alexa 647 (goat polyclonal)	Thermo Fisher Scientific	RRID: AB_2535805 (1:300)
Deposited data		
Raw and analyzed data	This paper; Mendeley Data	Mendeley Data: https://doi.org/10.17632/ywrmpn7b4z.1
Experimental models: Organisms/strains		
Drosophila: Canton-S	Gift from Dr. K. Ito	Hotta-lab strain
Drosophila: Gad1-p65.AD, dsx-Gal4DBD	made in this paper	RRID: BDSC_60322[23]
Drosophila: UAS-Gad1-TRiP-RNAi attP40	Bloomington Drosophila Stock Center	RRID:BDSC_51794[71]
Drosophila: UAS-Gad1-TRiP-RNAi attP2	Bloomington Drosophila Stock Center	RRID:BDSC_28079[72]
Drosophila: TRiP-BG attP40	Bloomington Drosophila Stock Center	RRID:BDSC_36304
Drosophila: TRiP-BG attP2	Bloomington Drosophila Stock Center	RRID:BDSC_36303
Drosophila: Otd-FLP, tubP FRT-Gal80-FRT	Gift from Dr.Miwa	RRID: BDSC_38880[73]
Drosophila: 20XUAS-IVS-mCD8::GFP	Bloomington Drosophila Stock Center	RRID:BDSC_32194
Drosophila: UAS-DenMark	Bloomington Drosophila Stock Center	RRID:BDSC_33064
Drosophila: R71G01-LexA	Bloomington Drosophila Stock Center	RRID:BDSC_54733
Drosophila: lexAop-CD4::spGFP11; UAS-CD4::spGFP1-10	made in this paper	Gift from Kristin Scott
Drosophila: UAS-Rdl Trip RNAi attp40	Bloomington Drosophila Stock Center	RRID:BDSC_52903[74]
Drosophila: UAS-Dop1R1 Trip RNAi attp2	Bloomington Drosophila Stock Center	RRID:BDSC_31765[75]
Drosophila: UAS-Dop1R2 Trip RNAi attp2	Bloomington Drosophila Stock Center	RRID:BDSC_26018[76]
Drosophila: UAS-Dop2R Trip RNAi attp2	Bloomington Drosophila Stock Center	RRID:BDSC_26001[77]
Software and algorithms		
Fiji (version: 2.9.0/1.53t)	Image J[78]	RRID: SCR_002285
VVDViewer (version 1.5.10)	Dr. Takashi Kawase	RRID:SCR_021708: https://github.com/takashi310/VVDViewer
The Audacity Team (version 2.0)	Audacity Team	RRID: SCR_00719: https://www.audacityteam.org/
Others		
R (version 4.1.0)	R Core Team	RRID: SCR_001905: https://www.r-project.org/
Neuprint	Janelia Research Campus	https://neuprint.janelia.org/
Virtual Fly Brain	Virtual Fly Brain Project	RRID:SCR_004229: https://www.virtualflybrain.org/

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and regents can be directed to the lead contact, Azusa Kamikouchi (kamikouchi@bio.nagoya-u.ac.jp).

Materials availability

Newly generated *Drosophila* strain (*dsx*∩*Gad1* driver-1 and driver-2 strains) can be made available to other researchers.

Data and code availability

- Original data have been deposited on **Mendeley** at (https://doi.org/10.17632/ywrmpn7b4z.1).
- Original codes are also available at **Mendeley** (https://doi.org/10.17632/ywrmpn7b4z.1).
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Fly stocks

Drosophila melanogaster were reared on standard yeast-based media at 25°C in 40%–60% relative humidity and a 12-h light/dark cycle. For fly strain details see key resources table and Table S1. The *dsx*∩*Gad1* driver-1 was generated by chromosomal recombination of two hemi-drivers, *Gad1*^{p65AD} and *dsx*^{GAL4DBD}. *Gad1*^{p65AD} replicates the expression pattern of *Glutamic acid decarboxylase 1* (*Gad1*), a gene encoding the rate-limiting enzyme in the GABA synthesis pathway, whereas *dsx*^{GAL4DBD} replicates the *dsx* expression pattern.[23,79] The *dsx*∩*Gad1* driver-2 was generated by combining the *dsx*∩*Gad1* driver-1 with a flippase-mediated *GAL80* strain[80] which has two transgenes *Otd-FLP*[73] and *tubulin*^{P} *FRT-GAL80-FRT*. We used *Canton-S* as the wild-type strain. For knockdown experiments, *UAS-RNAi* strains, selected based on previous studies, were used with corresponding background strains as controls (from the Transgenic RNAi Project, see key resources table for details). All males used in the behavioral experiments were of the wild-type strain. They were collected within 8 h after eclosion, had their wings clipped under ice anesthesia, and kept singly until the copulation test was conducted as described previously.[81]

METHOD DETAILS

Sound stimulus

We used artificial pulse songs, which are comprised of repetitions of a 1-s pulse burst followed by a 2-s pause.[81] Inter-pulse intervals are 35 ms for the conspecific song and 75 ms for the heterospecific song. Intrapulse frequency was 167 Hz in both songs. We delivered the sound stimuli using loudspeakers (FF225WK, FOSTEX, Foster Electric Company, Tokyo, Japan during training; Daito Voice AR-10N, Tokyo Cone Paper MFG. Co. Ltd in copulation tests). The mean baseline-to-peak amplitudes of the particle velocity were 6.6–8.6 mm/s during training and around 9.2 mm/s during the copulation test.

Training

We define "training" as the process that exposes flies to external artificial sounds.[21] Females were collected within 8 h after eclosion under ice anesthesia and kept in groups of 8–10 in training capsules at 24°C–26°C and 40–60% humidity. Training capsules are made from plastic straws about 70 mm long with a diameter of 14 mm, with edges covered with mesh and food provided at the bottom. In the experienced training condition, females were exposed to the conspecific song for 6 days. Naive females were kept in training capsules for 6 days without song exposure. We replaced the capsule every 2–3 days (within 60 h) during the training. After training, females were kept without song exposure for 14–18 h and then subjected to the copulation test.

Copulation test

We conducted the copulation test within 4 h after light onset (ZT 0–4) at 24°C–26°C and 40–60% humidity. 7-day-old wing-clipped males were used as a mating partner. A male and a female were gently aspirated into one of the eight chambers of the experimental plate[81] without anesthesia. The experimental plate was placed above a loudspeaker at a distance of 39 mm, and playback of sound stimuli started immediately. Fly pairs were recorded for 35 min at 15 fps with a web camera (Logicool HD Webcam C270, Tokyo, Japan). Copulation was defined as the following criteria: (1) the male mounts the female for more than 5 min, (2) the mounted female decreases locomotor activity, and (3) opens her wings during the mounting.[14]

Immunohistochemical analysis

The brains and VNCs of 5- to 7-day-old females were dissected in phosphate-buffered saline (PBS; pH 7.4), fixed for 1 h in 4% paraformaldehyde/PBS (TAKARA BIO INC. Cat#T900), and subjected to antibody labeling.[82] Primary and secondary antibodies are listed in key resources table. In the GRASP experiment, reconstituted GFP (GRASP signals comprised of GFP1-10 and GFP11) and GFP1-10 were labeled with monoclonal and polyclonal GFP antibodies, respectively. We additionally used CD4 antibody to label both CD4GFP1-10 and CD4GFP11 fragments.

Confocal microscopy and image processing

Samples were imaged using an FV1200 laser-scanning confocal microscope (Olympus) equipped with either a silicone-oil immersion 30× or 60× objective lens (UPLSAPO 30×s, NA = 1.05; UPLSAPO 60×s, NA = 1.30; Olympus). Serial optical sections were obtained every 0.84 μm with a resolution of 512 × 512 pixels (0.83 μm/pixel). For three-dimensional (3D) image reconstruction, confocal image datasets were processed with VVDViewer (version 1.5.10). Image size, contrast, and brightness were adjusted using Fiji (version 2.9.0).[78]

Detecting synaptic connections using the FlyEM database

GABAergic neurons that synapse onto pC1 neurons (Table S2) were extracted by combining the Hemibrain v1.2.1 datasets (obtained in the FlyEM project; Scheffer et al., 2020) with a neurotransmitter classification tool.[30] In order to identify pCd-2 neurons from the extracted GABAergic neuron population, we assessed whether cell bodies were located at the posterior-dorsal side of the brain and projected to the superior medial protocerebrum (SMP) and ipsilateral GNG, with a trajectory similar to that of pCd-2 neurons described in a previous study.[18] Since the Hemibrain v1.2.1 dataset was derived from electron microscopy (EM) sections of a significant portion of the right hemibrain and a small portion of the left hemibrain, two pCd-2 neurons in the left hemibrain (i.e., SMP294 and SMP297) were not identified. The projection patterns of pCd-2 and pC1 neurons were visualized using Virtual Fly Brain (Figures 2B–2F).[83] The neurons were registered to the JRC2018 template.[84]

The number of synapses between two neurons was obtained from the NeuPrint web interface (https://neuprint.janelia.org/). According to a previous study,[17] paired neurons with 6 or more synapses connecting them were considered to be connected (Table S3).

Gene expression profile of *dsx+/gad1+/elav+* cells

To verify the gene expression profile of pCd-2 neurons, we analyzed a fly brain single-cell transcriptome dataset published previously (GEO accession: GSE107451; GSE107451_DGRP-551_w1118_WholeBrain_157k_0d_1d_3d_6d_9d_15d_30d_50days_10X_DGEM_MEX.mtx.tsv.tar.gz)[22] using R software (ver. 4.3.0). Expression levels were calculated using the NormalizeData function of Seurat package (ver. 4.3.0.1) with the LogNormalize method and default parameter settings (scale.factor = 10000). To characterize gene expression profiles of pCd-2 neurons, the 32 cells that express *elav* (a pan-neuronal marker), *gad1*, and *dsx* were selected using the WhichCells function (parameter: expression >0) of Seurat. The expression level of neurotransmitter receptor genes, including *rdl* for $GABA_A$-type receptor-expressing cells and *dop1r1*, *dop1r2,* and *dopr2* were plotted by pheatmap package (ver. 1.0.12).

QUANTIFICATION AND STATISTICAL ANALYSIS

Copulation test data were analyzed using R (version 4.1.0). We evaluated the cumulative copulation rates and copulation latencies (i.e., time to start copulation) of all groups simultaneously, using an accelerated failure time (AFT) model[24,25] included in the flexsurv package (version 2.2.1) of R. The learning index (LI) and 95% confidence interval (CI) were estimated using log-logistic AFT model. Briefly, a lack of song preference learning results in an LI of 1, whereas LI > 1 indicates that flies demonstrate the learning phenotype (See the following "data analysis using the AFT model" for details). To evaluate the cumulative copulation rate and copulation latency of each group, we also utilized restricted mean time lost (RMLT)[85] calculations using the survRM2 package (version 2.2.1) of R.[14] Calculated *p*-values were adjusted by Bonferroni corrections. Statistical significance was defined as $*p < 0.05$ and N.S., not significant.

Data analysis using the AFT model

We evaluated the cumulative copulation rates and copulation latencies of all of the four groups (i.e., NC, NH, EC, and EH in Figures 1, 3, S1, S4, S5, and S10) simultaneously, using an accelerated failure time (AFT) model.[24,25] Prior to analysis, we tested several distributions to formulate the AFT model framework with our datasets. We tested Weibull, exponential, log-normal, normal, logistic, and log-logistic distributions and found that the log-logistic distribution gave the smallest values for both the Akaike's Information Criterion (AIC) and Bayesian Information Criterion (BIC) in all datasets. Therefore, the log-logistic distribution was used throughout the analyses.

The AFT model assesses the effect of covariates or interactions on the time to copulate (i.e., copulation latency), T. In this study, we defined two types of covariates: χ_1, the existence of conspecific song exposure in the training session (naive vs. experienced), and χ_2, the test song type (conspecific song vs. heterospecific song). Since each covariate has two factors, we set one factor as *0* and the other as *1* for each covariate. The effect of the interaction between covariates χ_1 and χ_2 is considered to be the effect of song preference learning. Using the log-logistic AFT model, copulation latency T is described as follows:

$$T = e^{\left(\beta_0+\beta_1\chi_1+\beta_2\chi_2+\gamma\chi_1\chi_2+\sigma\varepsilon\right)}$$

where $\boldsymbol{\varepsilon}$ is the residual that corresponds to log-logistic distribution and $\boldsymbol{\sigma}$ is the scale parameter. To assess song preference learning, we focused on the interaction of the two covariates, χ_1 and χ_2. In particular, e^{γ}, an acceleration factor of the interaction, was used as the learning Index, LI. The results of the analysis are described in Table S4.