Neurosci. Bull. November, 2022, 38(11):1277–1291
https://doi.org/10.1007/s12264-022-00908-8

www.neurosci.cn
www.springer.com/12264

ORIGINAL ARTICLE

Serotonin Signaling Modulates Sexual Receptivity of Virgin Female *Drosophila*

Baoxu Ma[1,2] · Rencong Wang[1,2] · Yaohua Liu[3] · Bowen Deng[4] · Tao Wang[1] · Fengming Wu[1,2] · Chuan Zhou[1,2,5]

Received: 7 December 2021 / Accepted: 13 April 2022 / Published online: 5 July 2022

Abstract The choice of females to accept or reject male courtship is a critical decision for animal reproduction. Serotonin (5-hydroxytryptamine; 5-HT) has been found to regulate sexual behavior in many species, but it is unclear how 5-HT and its receptors function to regulate different aspects of sexual behavior. Here we used *Drosophila melanogaster* as the model animal to investigate how 5-HT and its receptors modulate female sexual receptivity. We found that knockout of tryptophan hydroxylase (*Trh*), which is involved in the biosynthesis of 5-HT, severely reduced virgin female receptivity without affecting post-mating behaviors. We identified a subset of sexually dimorphic *Trh* neurons that co-expressed fruitless (*fru*), in which the activity was correlated with sexual receptivity in females. We also found that 5-HT_{1A} and 5-HT_7 receptors regulate virgin female receptivity. Our findings demonstrate how 5-HT functions in sexually dimorphic neurons to promote virgin female receptivity through two of its receptors.

Keywords Female sexual receptivity · Serotonin · 5-HT · *Fruitless* · Neurochemical · 5-HT receptors · *Drosophila*

Introduction

Sexual behavior in *Drosophila melanogaster* is an excellent model in which to investigate the neuronal basis underlying social behavior because they are innate and robust [1–3]. Wild-type male and female flies can achieve copulation without social learning experiences during adulthood [4, 5]. *Drosophila* sexual behaviors include stereotypic male courtship rituals such as orienting to a female, extending an ipsilateral wing to produce courtship songs, tapping and licking the female, attempting copulation, and finally copulation [6, 7]. The neural circuit involved in male courtship behavior has been dissected in recent years owing to advances in genetic technology [8–11]. However, studies on female sexual behavior are far fewer than those on males.

Much progress has been made in recent years on how female flies perceive the presence of males and their courtship, and integrate auditory, olfactory, and mechanosensory cues, to decide whether to be receptive or not [12–15]. Such a decision is also dependent on the maturity and mating status of the female. Sexually immature females exhibit rejection behaviors by running away, flicking wings, or kicking the courting male [16, 17]. After sexual maturity, virgin females make the decision to copulate with courting males and exhibit sexual receptivity,

Baoxu Ma, Rencong Wang, and Yaohua Liu contributed equally to this work.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s12264-022-00908-8.

✉ Fengming Wu
wufengming@ioz.ac.cn

✉ Chuan Zhou
zhouchuan@ioz.ac.cn

1 State Key Laboratory of Integrated Management of Pest Insects and Rodents, Institute of Zoology, Chinese Academy of Sciences, Beijing 100101, China

2 University of Chinese Academy of Sciences, Beijing 100101, China

3 Department of Plant Protection, Shanxi Agricultural University, Jinzhong 30801, China

4 Chinese Institute for Brain Research, Zhongguancun Life Sciences Park, Beijing 102206, China

5 Institute of Molecular Physiology, Shenzhen Bay Laboratory, Shenzhen 518132, China

which is a proxy metric to evaluate the willingness of females to mate [18–20]. Recently-mated females display post-mating behaviors by reducing receptivity and increasing egg-laying [8, 21, 22]. Female post-mating behaviors are triggered by the male seminal fluid peptide (sex-peptide, SP) and regulated by SP-responsive neurons which express fruitless (*fru*), doublesex (*dsx*), and pickpocket (*ppk*) [23–26]. Despite this progress in the sensory and integrative circuits for female sexual behavior [26–29], very little is known about how internal factors modulate virgin female receptivity.

Internal factors that modulate the function of neuronal circuits often use neurochemical systems including neuropeptides and neurotransmitters [30]. Serotonin (5-hydroxytryptamine; 5-HT), which is one of the highly-conserved neurotransmitters across species, is involved in a range of behaviors including cognition, reward, learning, and memory, as well as male and female sexual behavior [31–35]. Although 5-HT is known to be involved in mammal sexual behavior, its exact function in regulating sexual behavior is unclear. In *Drosophila*, 5-HT has also been shown to regulate a variety of complex behaviors including aggression, sleep, and feeding [36–38], but whether and how it regulates female sexual receptivity is unknown. Thus, it is of particular importance to investigate the function of 5-HT in female sexual receptivity using the *Drosophila* model.

Sexual behaviors in *Drosophila* are largely controlled by two pivotal regulatory genes, *fru* and *dsx*, that control most aspects of sexual development and behavior [10, 39, 40]. Sex-specific *dsx* transcripts are translated in both sexes to produce the sex-specific proteins Dsx^M or Dsx^F, which control male and female differentiation, respectively [15, 41–43]. In contrast, *fru* proteins (Fru^M) control male courtship and are male-specific [1, 5, 44, 45]. Although Fru^M proteins are not produced in females, neurons expressing the *fru* transcript (fru^+) are crucial for female sexual receptivity, as silencing these fru^+ neurons impairs female receptivity [44, 46]. Recent studies have also revealed the importance of dsx^+ neurons in controlling virgin female receptivity and post-mating behaviors in mated females [24, 27, 47, 48].

In this study, we showed that 5-HT signaling modulates female sexual receptivity at both the molecular and the neural circuit levels. Knockout and knockdown of tryptophan hydroxylase (*Trh*), which is involved in the biosynthesis of 5-HT, decreased virgin female receptivity. Activation of the entire population of Trh^+ neurons enhanced sexual receptivity in virgin females but had no effect on sexual receptivity in mated females. We identified a group of sexually dimorphic Trh^+fru^+ neurons in the posterior lateral protocerebrum (PLP) to be a crucial 5-HT-releasing site in the regulation of female sexual receptivity. Analysis of Ca^{2+} activity in 5-HT-PLP neurons revealed stronger activity in virgin flies than in mated flies. Furthermore, we found two 5-HT receptors, 5-HT_{1A} and 5-HT_7, that might be crucial for female sexual receptivity.

Materials and Methods

Fly Culture and Strains

All *D. melanogaster* strains were reared on standard medium at 25°C and 60% humidity in a 12-h light/dark photoperiod unless otherwise described. All the knockout lines in this study for screening have been published [49]. The following strains were obtained from Dr. Yi Rao's lab (Peking University, Beijing, China): *isoCS* (wild-type), *Trh-GAL4*, *elav-GAL4*;*UAS-dicer2*, *elav-GAL4*, and *UAS-5-HT1A*. The *UAS-stingerGFP* and *UAS-Redstinger* lines were gifts from Dr. Yufeng Pan's lab (Southeast University, Nanjing, China). *UAS-PACα* was a gift from Dr. Yan Zhu's lab (Institute of Biophysics, Chinese Academy of Sciences). The following strains were from the Bloomington *Drosophila* Stock Center: *UAS-Kir2.1* (BL#6596), TRIC (BL#61679), *UAS-mCD8-GFP* (BL#5137), *UAS-shi^{ts}* (BL#44222), and *UAS-Trh-RNAi* (BL#33612).

Behavioral Assays

Female receptivity assays were conducted as previously described [15]. In brief, individual virgin females (8–10 days old) were paired with a naïve wild-type male courter (*isoCS*) (8–10 days old). Before they were paired, females and males were separately introduced into a two-layer courtship chamber (10 mm diameter × 3 mm height per layer), which was divided by a removable transparent strip. The assay was recorded with a resolution of 1280 pixels × 720 pixels (1.78:1) at 30 frames/s for 30 min using cameras (VIXIA HF R500, Canon, Tokyo, Japan). The number of receptive females and the time of receptivity for individual females were analyzed manually.

In the egg-laying assay, 3–4 virgin or mated females (~8 days old) were transferred to a vial with fresh medium left for 48 h at 25°C and 60% humidity under a 12-h light/dark cycle, and the number of eggs laid per female during 48 h was counted manually. To collect mated females, individual females were aspirated into the courtship chamber to allow copulation with a wild-type male before the egg-laying tests.

In the re-mating assay, we obtained mated females by pairing virgin females with wild-type males (both ~8 days old). The mated females were collected as above, transferred to food vials, and left for 48 h before re-mating tests with a new wild-type male of the same age for 1 h. The percentage of re-mating females was analyzed manually.

The locomotion assay was applied at 25°C and 60% humidity. Individual virgin females were transferred to the

courtship chambers without males and recorded for 10 min. The locomotor speed was analyzed using MatLab software (MathWorks Inc., MA, USA) as described previously [50].

All behavioral assays were run from 11:00 to 15:00. The food medium was replaced every 2–3 days to ensure freshness.

Light-induced Experiments

In *PACα* (photoactivated adenylyl cyclase α) experiments, flies were crossed on standard medium, and the vials were wrapped in aluminum foil to avoid light. Female progeny (<8 h) were isolated in darkness for 8–10 days. Prior to behavioral tests, *PACα*-expression was activated by blue light (420 nm, 1200 mW/cm^2, 5 s; Denjoy, DY-400-4, Changsha, China).

In *CsChrimson* experiments, flies were crossed on 0.2 mmol/L retinal-containing medium (Sigma-Aldrich, St. Louis, USA) in darkness. Virgin females were immediately transferred to 0.4 mmol/L retinal-containing medium and isolated in darkness for 8–10 days. Female receptivity tests were performed in darkness (control) or with red light activation (620 nm, 0.03 mW/mm^2; Kemai Vision Technology, Dongguan, China) during a 30-min observation period. The assay was recorded by an industrial camera (Stingray F080B ASG, Allied Vision Technologies, Stadtroda, Germany) equipped with an infrared light source (860-nm IR LED, Kemai Vision Technology) for illumination.

Temperature-induction Experiments

In *TrpA1* and *UAS-shi^{ts}* experiments, virgin females were maintained at 22°C for 8–10 days. Before the behavioral assay, the flies were concurrently introduced into chambers at 30°C or 21°C for 20 min. 30°C was the activation temperature in *TrpA1* activation experiments but a restrictive temperature in *UAS-shi^{ts}* inactivation experiment.

Generation of *UAS-5-HT7*

pJFRC28-5XUAS-IVS-GFP-p10 (# 12073; Fungene Biotechnology, Shanghai, China) was used for the generation of the pJFRC28-*UAS-5-HT7* (*UAS-5-HT7*) construct. pJFRC28-5XUAS-IVS-GFP-p10 plasmid digested within NotI and XbaI was used to excise the coding sequence of GFP. Using the Gibson Assembly, the complementary DNA (cDNA) of 5-HT7 was cloned in the described plasmid. The right upstream of ATG codon added the Kozak sequence, and the *UAS-5-HT7* construct was injected into the attP40 site using phiC31 integrase-mediated transgenesis. The construct was confirmed using DNA sequencing and PCR. The primers used for cloning *5-HT7* cDNA were as follows:

- UAS-5-HT7-forward:

TCTTATCCTTTACTTCAGGCGGCCGCCACCATGGCT TTATCTGGACAGGACTG

- UAS-5-HT7-reverse:

GTTATTTTAAAAACGATTCATTCTAGATTAAGAGAA AGCTCTCCCTCGC

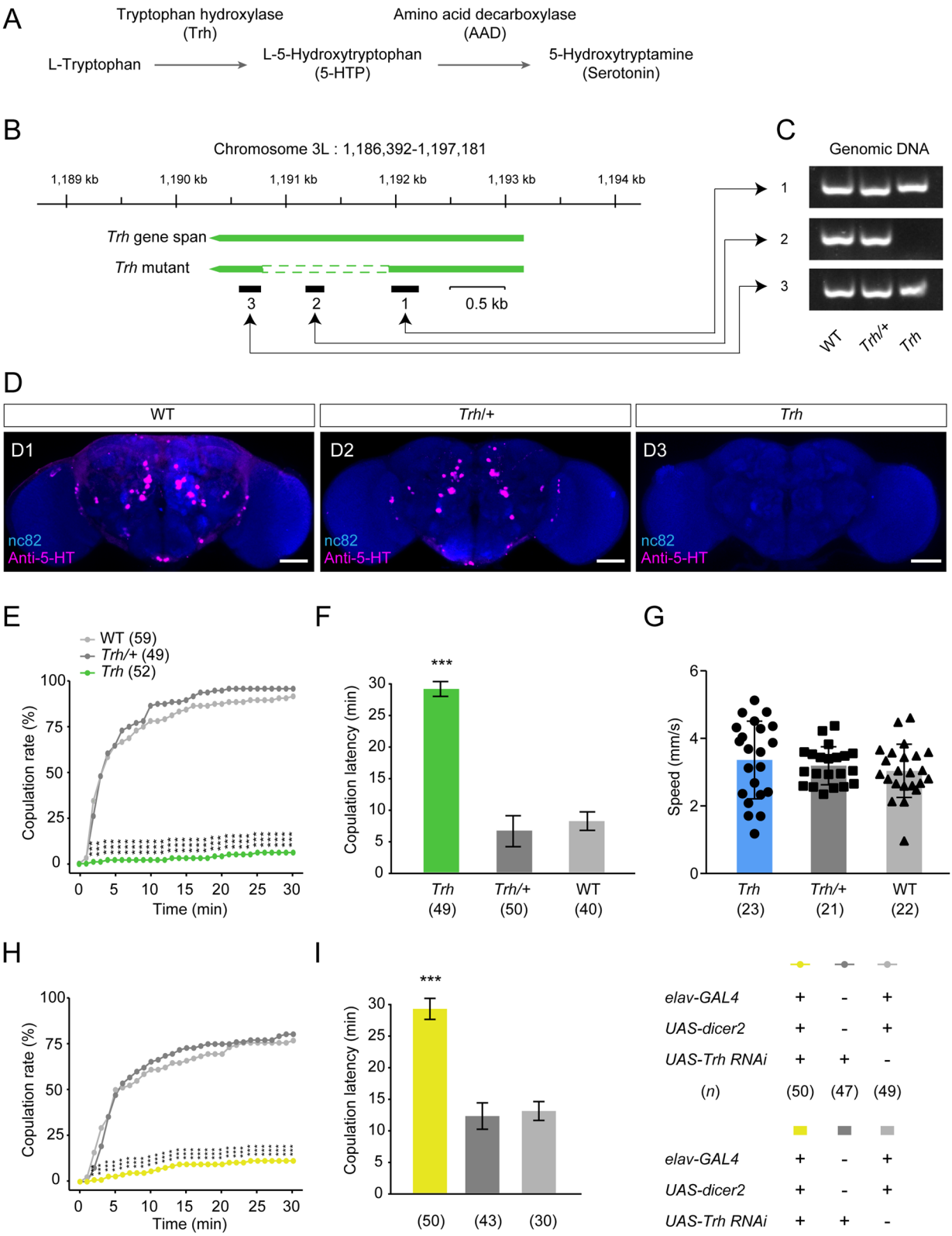
Confirmation of Transgenic Flies

Genomic DNA was extracted from the whole body of adult flies. Individual flies were crushed with a pestle in 50 μL DNA extraction reagent. After incubation at 95°C for 10 min, the samples were centrifuged at 12,000 r/min for 10 min at room temperature. The supernatant of DNA was collected and added to the mixing system to conduct PCR, according to the manufacturer's instructions. The mix contained 25 μL 2× MightyAmp buffer, 1 μL MightyAmp DNA polymerase, and 5 μL 10× Additive for high specificity, and was adjusted to 50 μL. Primers used in regions 1–3 (Fig. 1B) were as follows:

- Region 1 forward: GGCTACGGTGGATATTCCAAG
- Region 1 reverse: CATTCAGGCTGTTGTGGAGC
- Region 2 forward: GAGAGGTGGCCTCTGTGAAC
- Region 2 reverse: CGGTGCCCCTTTGAACG
- Region 3 forward: AGGGAACAGATTCTCGGGAC
- Region 3 reverse: ACTTCTTGGTGCAGTGCCTC

Immunostaining

Flies were dissected in phosphate-buffered saline (PBS), and then the brains were fixed in 2% (weight/volume) paraformaldehyde (PFA) (Electron Microscopy Sciences, Hangzhou, China) for 55 min at room temperature. Then, the samples were washed five times in PBS with 0.3% Triton (PBST) for 15 min and incubated in blocking solution [5% (volume/volume) goat serum (Sigma-Aldrich) diluted in 0.3% PBST] for 1 h at room temperature. The brains were then incubated with the primary antibody (diluted in blocking solution) for >24 h at 4°C, and washed five times in 0.3% PBST for 15 min before incubation with the secondary antibody (1:500, diluted in blocking solution) overnight at 4°C. The samples were washed five times in 0.3% PBST for 15 min and fixed in 4% PFA for >4 h at room temperature. The brains were washed five times with 0.3% PBST for 15 min at room temperature and were placed on a poly-L-lysine-coated coverslip in 0.3% PBST. The brains were then immersed in 30%, 50%, 75%, 95%, and 100% ethanol. The brains were immersed three times in xylene for 5 min and mounted on glass slides using dibutylphthalate polystyrene xylene (DPX)



(Sigma-Aldrich) for imaging. Images were generated on a Zeiss 710 confocal microscope (Carl Zeiss, Oberkochen, Germany) and were processed using Fiji software (https://imagej.net/Fiji).

◂**Fig. 1** The *Drosophila Trh* gene is essential for virgin female receptivity. **A** 5-HT biosynthesis process. **B** Schemata of the *Trh* gene locus and mutants generated by *Trh* knockout. Black bars indicate target regions 1–3 in the PCR analysis in **C**. **C** PCR verification of regions 1–3 in **B** from *Trh* mutant genomic DNA samples. **D** Neuropil expression of the *Trh* gene in female adult brains of wild-type (WT) (**D1**), heterozygous (**D2**), and homozygous *Trh* mutant flies (**D3**), immunostained with anti-5-HT antibody (magenta) and nc82 antibody (blue) (scale bars, 50 μm). **E**, **F** The copulation rate is decreased (**E**) and the copulation latency is prolonged (**F**) in *Trh*-knockout mutants within a 30 min observation period. **G** *Trh* mutants do not differ in locomotor speed from WT and heterozygous control females. **H**, **I** Knockdown of *Trh* expression reduces virgin female receptivity. The copulation rate is decreased (**H**) and the copulation latency is prolonged (**I**) by knocking down *Trh* expression pan-neuronally. ***P <0.001, otherwise no significant difference (χ^2 test for copulation rate; Kruskal-Wallis with *post hoc* Mann-Whitney U test for copulation latency). n values are shown in parentheses. Error bars, ±SEM.

The antibodies used were mouse anti-nc82 (1:50; Developmental Studies Hybridoma Bank, Iowa City, USA), chicken anti-GFP (1:1000; Life technologies, Carlsbad, USA), rabbit anti-RFP (1:500; Invitrogen, Waltham, USA), and rabbit anti-5-HT (1:500; Life technologies). The secondary antibodies were Alexa Fluor goat anti-chicken 488 (1:500; Life technologies), Alexa Fluor goat anti-rabbit 488 (1:500; Life technologies), Alexa Fluor goat anti-mouse 546 (1:500; Life technologies), and Alexa Fluor goat anti-rabbit 633 (1:500; Invitrogen).

Drug Treatment in Rescue Experiments

The procedure in the 5-hydroxytryptophan (5-HTP; Sigma-Aldrich) feeding experiment is shown in Fig. 2A. Virgin females were reared on normal food for 8 days after eclosion. Two days before behavioral tests and immunostaining analysis, the flies were divided into two treatment groups. In the control group (5-HTP$^-$), females were put on control mock food containing 2% agar and 10% sucrose; in the 5-HTP feeding group (5-HTP$^+$), the flies were reared on drug-containing food in which 2 mg/mL 5-HTP was dissolved in the mock food.

Transcriptional Reporter of Intracellular Ca^{2+} (TRIC) Assay

Virgin females with the genotype *10XUAS-mCD8::RFP/13XLexAop2-mCD8::GFP;nSyb-MKII::nlsLexADBDo;UAS-p65AD::CaM/Trh-GAL4* were collected within 8 h after eclosion until TRIC assay. For mated females, 8-days-old virgin females of given genotypes were transferred to courtship chambers and paired with wild-type male flies. The females that copulated successfully within 30 min were collected for further TRIC analysis. Adult female brains from these two groups (virgin and mated) were dissected, and the whole brain was perfused with a saline solution containing (in mmol/L) 103 NaCl, 3 KCl, 4 $MgCl_2$, 1.5 $CaCl_2$, 26 $NaHCO_3$, 1 NaH_2PO_4, 5 N-tri-(hydroxymethyl)-methyl-2-aminoethane-sulfonic acid, 20 glucose, 17 sucrose, and 5 trehalose, adjusted to pH 7.3.

Images were acquired using a confocal microscope (Nikon A1R+, Nikon, Toyko, Japan) with a 40× water immersion objective. The Ca^{2+} signal was indicated by the fluorescence intensity. 488-nm and 546-nm light was used to excite GFP and RFP, respectively. The regions of interest (ROIs) were manually defined in the PLP cluster area and were analyzed using NIS-Elements D (Nikon; https://www.microscope.healthcare.nikon.com/en_EU/products/software). The relative TRIC signal of selected ROIs (GFP signal/RFP signal) was used to compare neural activity in virgin and mated females.

Statistical Analysis

Statistical analysis and graphics were applied with the R system 4.0.2 (https://www.r-project.org/), MatLab software (MathWorks Inc., MA, USA), and GraphPad Prism 7 software (GraphPad Software, San Diego, USA). The χ^2 test was used to compare the copulation rate of different groups at various time points. The Mann-Whitney U test was applied for two-group comparisons. Kruskal-Wallis with the *post hoc* Mann-Whitney U test was used to compare the differences between multiple groups. All data are shown as the mean ± SEM. Sample sizes are indicated in the figures. Statistical significance was set at P <0.01.

Results

5-HT Modulates Virgin Female Receptivity

In *Drosophila*, it has been found that virgin female receptivity is associated with the release of dopamine (DA), drosulfakinin (DSK), and SIFamide (SIFa) [51–54]. To identify the role of other neurochemicals involved in regulating virgin female receptivity, we screened 108 chemoconnectome (CCT) knockout lines generated by the CRISPR-Cas9 system [49].

Preliminary screening (unpublished data) showed that virgin female receptivity might be regulated by the *Trh* gene that encodes an enzyme catalyzing the first and rate-limiting step of 5-HT biosynthesis (Fig. 1A) [55, 56]. We confirmed the *Trh* knockout line by using PCR analysis at the *Trh* locus in genomic DNA samples (Fig. 1B, C) and by detecting the immunoreactivity of 5-HT in the central brain (Fig. 1D). 5-HT immunoreactivity was found in the brain of wild-type and heterozygous flies (Figs. 1D), but was absent in homozygous *Trh*-knockout flies (Fig. 1D). We found that knockout

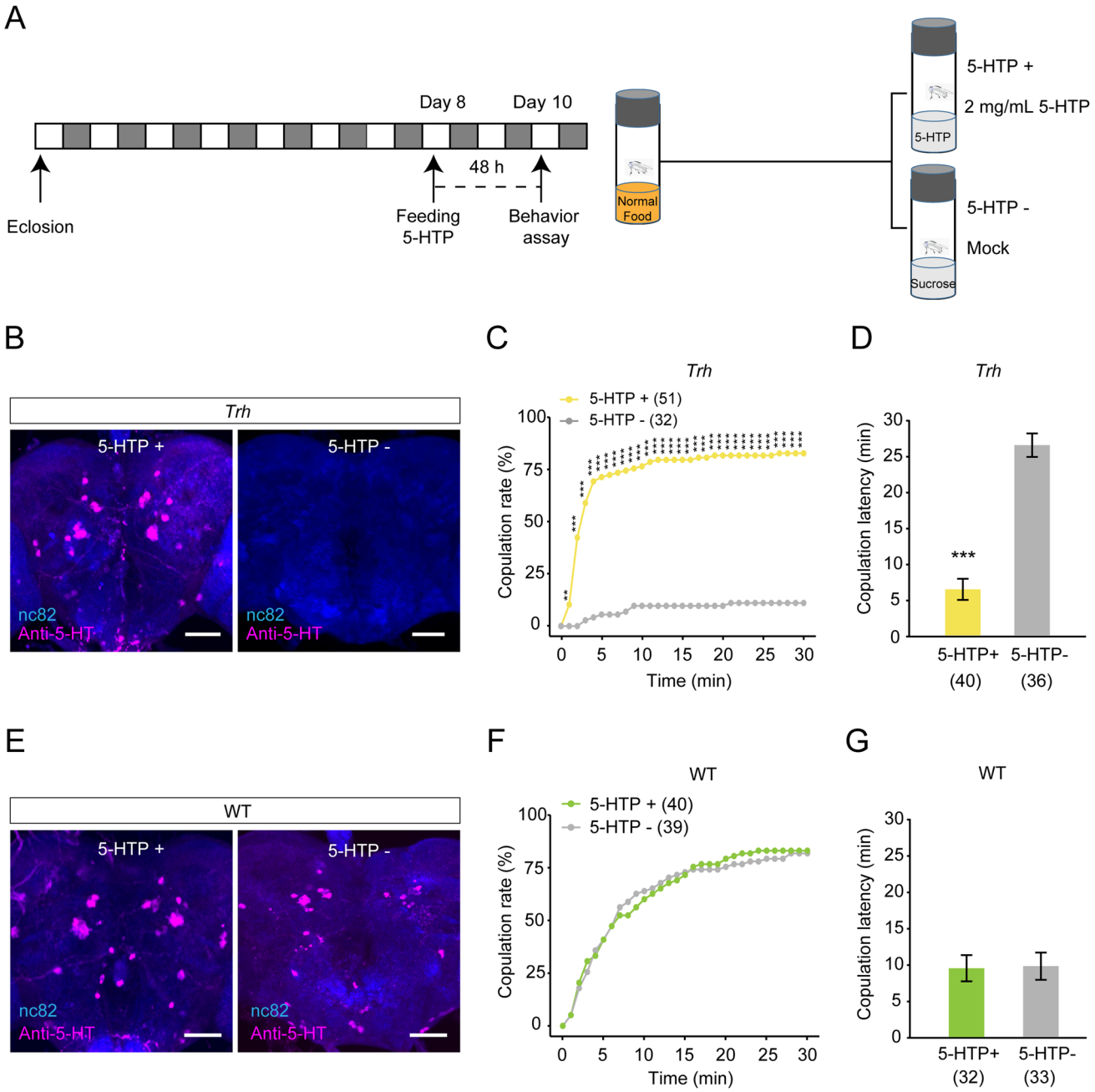


Fig. 2 Acutely feeding 5-HTP restores 5-HT expression and virgin female receptivity in *Trh*-knockout mutants. **A** The protocol of the feeding assay in the rescue experiment. **B** Immunoreactivity of 5-HT is restored in the brain of *Trh* mutant females after feeding 5-HTP for 48 h (scale bars, 50 μm). **C**, **D** Virgin female receptivity is restored by feeding *Trh* mutants with 5-HTP. In *Trh*-knockout mutants, the copulation rate is elevated to the normal level (**C**) and the copulation latency is shortened (**D**). **E** Immunoreactivity of 5-HT in wild-type females is not significantly affected by feeding 5-HTP (scale bars, 50 μm). **F**, **G** Sexual receptivity of wild-type virgin females is not affected by 5-HTP feeding. Wild-type females show a comparable copulation rate (**F**) and copulation latency (**G**) with or without 5-HTP feeding. ***P <0.001, **P <0.01, otherwise no significant difference (χ^2 test for copulation rate; Mann-Whitney U test for copulation latency). n values are shown in parentheses; error bars, ±SEM.

of *Trh* reduced the virgin female copulation rate and prolonged the copulation latency compared to heterozygous and wild-type control females (Fig. 1E, F). In contrast, we found that *Trh*-knockout females showed locomotor activity comparable to control females (Fig. 1G). Furthermore, knocking down *Trh* expression pan-neuronally using the *elav-GAL4* driver reduced the copulation rate and increased

the copulation latency in virgin females (Fig. 1H, I). Given that *Trh* heterozygous and wild-type females displayed similar phenotypes, we mainly used wild-type females as control flies in later experiments.

To determine whether restoration of the 5-HT expression level could rescue the sexual receptivity of *Trh*-knockout females, we performed pharmacological rescue experiments by feeding them with 5-HTP (Fig. 2A). After feeding with 2 mg/mL 5-HTP, 5-HT immunofluorescence was restored in the brain of *Trh* mutant females (Fig. 2B). Furthermore, both copulation rate and copulation latency were rescued to normal levels in *Trh* mutant females (Fig. 2C, D). In contrast, 5-HT immunofluorescence and female sexual receptivity were not significantly affected in wild-type females by feeding with 5-HTP (Fig. 2E–G). These results indicate that 5-HT is crucial for virgin female receptivity in *Drosophila*.

To determine whether 5-HT specifically regulates virgin female receptivity, or affects sexual behavior in both virgin and mated females, we next tested egg-laying and re-mating behaviors in *Trh*-knockout or -knockdown females. We found that *Trh*-knockout virgin females did not show increased egg-laying (Fig. S1A), or any re-mating behavior after mating, like wild-type controls (Fig. S1B). We also observed similar phenotypes in *Trh* RNAi-knockdown females (Fig. S1C, D). Thus, 5-HT specifically regulates virgin female receptivity but not post-mating behaviors.

Trh$^+$ Neurons Regulate Virgin Female Receptivity

We used a *UAS-mCD8GFP* reporter to visualize the expression pattern of the newly-generated *Trh-GAL4* [36] (Fig. 3A). The *Trh-GAL4* labeled 5-HT clusters in the brain similar to those reported by previous studies [57, 58]. We mapped the distribution of *Trh*$^+$ neurons in the central brain including the anterior dorsomedial protocerebrum (ADMP), anterior lateral protocerebrum (ALP), anterior medial protocerebrum (AMP), lateral subesophageal ganglion (SEL), lateral protocerebrum (LP), medial subesophageal ganglion (SEM), posterior medial protocerebrum, dorsal (PMPD), medial (PMPM), and ventral (PMPV) posterior medial protocerebrum, and posterior lateral protocerebrum (PLP) (Fig. 3B).

We then analyzed whether *Trh*$^+$ neurons are involved in the modulation of female sexual receptivity. We activated all *Trh*-expressing neurons by using PACα [59], which specifically enhances intracellular cAMP levels after blue light stimulation, and also has a more moderate activation effect than *CsChrimson* [60] or *dTrpA1* [61], since the *Trh*-GAL4 labels a large number of neurons and strong activation of these neurons may have side-effects. We found that activation of *Trh-GAL4* neurons increased copulation rate and decreased copulation latency in *UAS-PACα/Trh-GAL4* virgin females (Fig. 3C, D). In contrast, there was no significant change in copulation rate and copulation latency after blue light stimulation in *UAS-PACα/+* or *Trh-GAL4/+* control females (Fig. 3E–H).

To further confirm whether *Trh*$^+$ neurons are necessary for virgin female receptivity, we silenced these neurons by expressing the inwardly-rectifying K$^+$ channel (Kir2.1) [62]. *UAS-Kir2.1/Trh-GAL4* virgin females exhibited a dramatic reduction in copulation rate and a prolonged copulation latency compared to control females (Fig. 3I, J). We also found that blocking neurotransmission from *Trh*$^+$ neurons expressing the temperature-sensitive *shibire*^{ts} (*shi*^{ts}) [63] significantly reduced virgin female receptivity (Fig. S2). Furthermore, neither activation nor inactivation of *Trh*$^+$ neurons affected sexual receptivity in mated females (Tables S1, S2). Taken together, our findings indicate that the activity of *Trh*$^+$ neurons positively regulates sexual receptivity in virgin, but not mated females.

Trh$^+$*fru*$^+$neurons Mediate Virgin Female Receptivity

We next set out to narrow down the serotonergic neurons that promote virgin female receptivity. As previous studies revealed crucial roles of *fru* or *dsx* neurons in regulating female receptivity [15, 41, 46], we tried to specifically label and manipulate *Trh*$^+$∩*fru*$^+$ or *Trh*$^+$∩*dsx*$^+$ neurons. We first applied the FLP/FRT intersectional strategy [64] to restrict expression in overlapping *Trh*$^+$ and *fru*$^+$ neurons (Fig. 4A). *UAS>stop>CsChrimson*; *fru*^{LexA} *LexAop2-FlpL/Trh-GAL4* virgin females, in which the overlapping *Trh*$^+$ and *fru*$^+$ neurons (referred to as *Trh*$^+$*fru*$^+$ neurons hereafter) express the optogenetic effector *CsChrimson* [60], displayed a much higher copulation rate and decreased copulation latency with red light stimulation (Fig. 4B, C). In contrast, control *UAS>stop>CsChrimson*; *fru*^{LexA} *LexAop2-FlpL/+*, or *Trh-GAL4/+* virgin females did not exhibit red light-induced changes in receptivity (Fig. 4D–G). We also used the thermogenetic effector *TrpA1* [61] to activate the *Trh*$^+$*fru*$^+$ neurons, and found that heat-induced activation of *Trh*$^+$*fru*$^+$ neurons slightly but significantly enhanced virgin female receptivity (Fig. S3). These results indicate that activation of *Trh*$^+$*fru*$^+$ neurons is able to promote virgin female receptivity.

We next tried to silence the *Trh*$^+$*fru*$^+$ neurons using the same intersectional strategy to express the inwardly-rectifying K$^+$ channel Kir2.1 [62]. Silencing the *Trh*$^+$*fru*$^+$ neurons dramatically reduced copulation rate and increased copulation latency in virgin females compared with control females (Fig. 4H, I). The reduction of receptivity in these females was not due to locomotor activity as

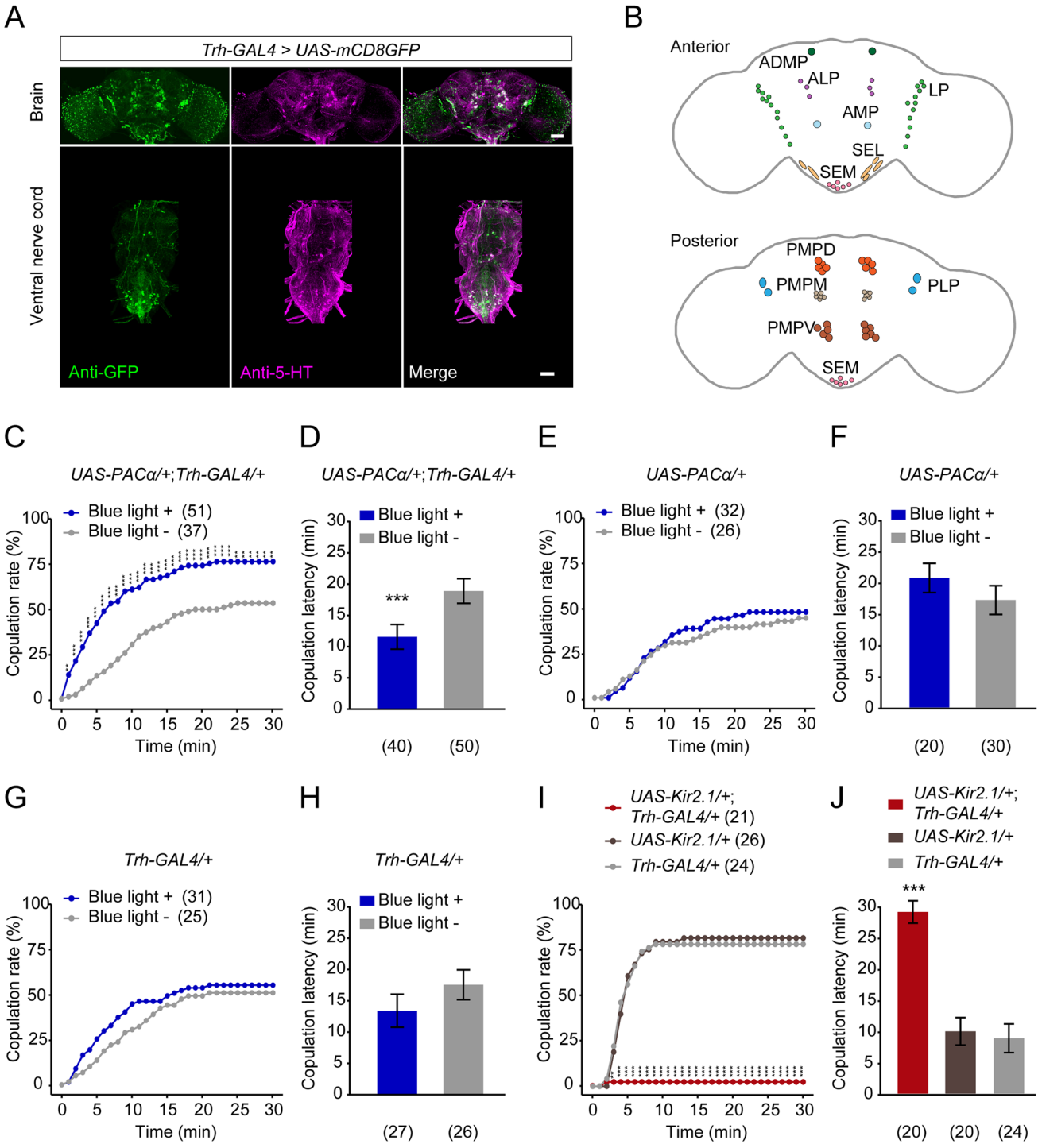


Fig. 3 *Trh*$^{+}$ neurons regulate virgin female receptivity. **A** Expression pattern of the *Trh* gene and *Trh-GAL4* visualized with anti-5-HT antibody (magenta) and anti-GFP (green) in a *UAS-mCD8-GFP/Trh-GAL4* female brain and VNC (scale bars, 50 μm). **B** Anterior (upper) and posterior views (lower) of *Trh*$^{+}$ neurons labeled by *Trh-GAL4* in the brain. **C**, **D** The copulation rate is increased (**C**) and the copulation latency is shortened (**D**) after blue light stimulation in *UAS-PACα/Trh-GAL4* virgin females. **E**, **F** *UAS-PACα/+* control females do not display a significantly changed copulation rate (**E**) and copulation latency (**F**) after blue light stimulation. **G**, **H** *Trh-GAL4/+* control females show a comparable copulation rate (**G**) and copulation latency (**H**) with or without blue light stimulation. **I**, **J** *UAS-Kir2.1/Trh-GAL4* virgin females exhibit a decreased copulation rate (**I**) and prolonged copulation latency (**J**) relative to control females. ***P <0.001, **P <0.01, otherwise no significant difference (χ^2 test for copulation rate analysis; Mann-Whitney U test for **D**, **F**, and **H**; Kruskal-Wallis with *post hoc* Mann-Whitney U test for **J**). n values are shown in parentheses; error bars, ±SEM. ADMP, anterior dorsomedial protocerebrum; ALP, anterior lateral protocerebrum; AMP, anterior medial protocerebrum; LP, lateral protocerebrum; PLP, posterior lateral protocerebrum; PMPD, dorsal, PMPM, medial, and PMPV, ventral posterior medial protocerebrum; SEL, lateral and SEM, medial subesophageal ganglion.

they displayed a walking speed comparable with control females (Fig. 4J). We also used the same intersectional strategy to silence the overlapping *Trh*$^+$ and *dsx*$^+$ neurons, but did not find any significant change in virgin female receptivity (Fig. S4A, B). Thus, these findings indicate that *Trh*$^+$*fru*$^+$ neurons are crucial for virgin female receptivity.

Sexually Dimorphic *Trh*$^+$*fru*$^+$ PLP Neurons Promote Virgin Female Receptivity

The above results demonstrated a crucial role of *Trh*$^+$*fru*$^+$ neurons in regulating virgin female receptivity. To visualize the *Trh*$^+$*fru*$^+$ neurons, we first applied double-labeling in *Trh-GAL4/UAS-stingerGFP*; *fru*^{LexA}*/LexAop-Redstinger* flies, and found that a subset of PLP neurons was co-labeled by *Trh*$^+$ and *fru*$^+$ in the brain of females (Fig. 5A, B). In addition, we applied the FLP/FRT intersectional strategy to express GFP in both sexes of *UAS>stop>mCD8-GFP*; *fru*^{LexA} *LexAop2-FlpL/Trh-GAL4* flies. We observed GFP expression in ~3 pairs of PLP neurons (Fig. 5C) and a few neurons in the ventral nerve cord (VNC) in females (Fig. 5C); in contrast, we observed 1–2 pairs of PLP neurons as well as a few other neurons in the brain (Fig. 5C) and VNC in males (Fig. 5C). Thus, there might be female-specific *Trh*$^+$*fru*$^+$ PLP neurons that regulate virgin female receptivity. We also used the same strategy to visualize *Trh*$^+$*dsx*$^+$ neurons, and observed sparse expression in the brain and VNC (Fig. S4C). Nevertheless, these *Trh*$^+$*dsx*$^+$ neurons were not involved in virgin female receptivity (Fig. S4A, B). In addition, we used the above strategy to express nsyb-GFP and Dscam-GFP and localized presynaptic and postsynaptic sites of the *Trh*$^+$*fru*$^+$ PLP neurons in females (Fig. S5).

To further test the role of *Trh*$^+$*fru*$^+$ PLP neurons in female receptivity, we monitored the neural activity of PLP neurons by expressing TRIC [65] in virgin and mated females. The TRIC signal in PLP neurons was significantly stronger in virgins than that in mated females (Fig. 5D). Thus, the spontaneous activity of *Trh*$^+$*fru*$^+$ PLP neurons is higher in virgin females, which might reflect an internal state of female receptivity. Together, these results demonstrate that a subset of sexually dimorphic *Trh*$^+$*fru*$^+$ PLP neurons promotes sexual receptivity in virgin females.

5-HT$_{1A}$ and 5-HT$_7$ Receptors Regulate Virgin Female Receptivity

There are five types of 5-HT G-protein-coupled receptors (GPCRs): 5-HT$_{1A}$, 5-HT$_{1B}$, 5-HT$_{2A}$, 5-HT$_{2B}$, and 5-HT$_7$ [36, 66–68]. These receptors, which are mammalian orthologs of the 5-HT receptor family, are expressed widely in the central nervous system [69] (Fig. S6), and regulate complex behaviors [37, 70–72]. To identify which 5-HT receptors are involved in virgin female receptivity, we used knockout lines of each 5-HT receptor and found that the copulation latency of *5-HT1A*- and *5-HT7*-knockout females was significantly longer than in the wild-type control females (Fig. 6A). In addition, knockout of *5-HT1A* and *5-HT7*, but not *5-HT1B, 5-HT2A*, or *5-HT2B*, significantly reduced the copulation rate in virgin females (Fig. 6B–F).

Given that female sexual receptivity was reduced in the *5-HT1A* and *5-HT7* mutants, we analyzed whether overexpressing *5-HT1A* or *5-HT7* in *5-HT1A* or *5-HT7* mutants could restore female receptivity. We used *elav-GAL4* to drive the expression of *UAS-5-HT1A* or *UAS-5-HT7* in *5-HT1A* or *5-HT7* mutant flies, respectively. We found that the copulation rate was increased, and copulation latency was reduced in *5-HT1A* mutant females with *elav-GAL4*-driven expression of *5-HT1A* (Fig. 6G, H). Meanwhile, the copulation rate and the copulation latency of *5-HT7* knockout females were also restored to normal levels with *elav-GAL4*-driven expression of *5-HT7* (Fig. 6I, J). These results suggest that 5-HT$_{1A}$ and 5-HT$_7$ receptors are involved in virgin female receptivity.

Discussion

In animals, males often initiate courtship, and females decide whether to accept or reject copulation. Acceptance by females is a prerequisite for reproductive success, which is determined not only by external factors but also by internal sexual motivation. Monoamine neurotransmitters and neuropeptides have been found to regulate female receptivity, such as dopamine [51, 53], octopamine [27], DSK [54], and SIFa [52]. Here, we showed that 5-HT signaling plays a critical role in virgin female receptivity. Both the knockout and *RNAi* knockdown of *Trh* reduced the receptivity. 5-HT may regulate virgin female receptivity through two of the 5-HT receptors, 5-HT$_{1A}$ and 5-HT$_7$. Furthermore, we identified ~3 pairs of sexually dimorphic *Trh*$^+$*fru*$^+$ PLP neurons in the female brain that promote sexual receptivity in virgin females.

5-HT is a well-known conserved molecule, which participates in regulating sexual behavior in a wide range of species [73]. In mammals, a fraction of 5-HT is produced in the central nervous system to regulate male sexual behavior, such as ejaculation and orgasm [74–77]. Moreover, 5-HT is required for male sexual preference: male mice lacking 5-HT prefer to court males rather than females [78]. Although the role of 5-HT has been

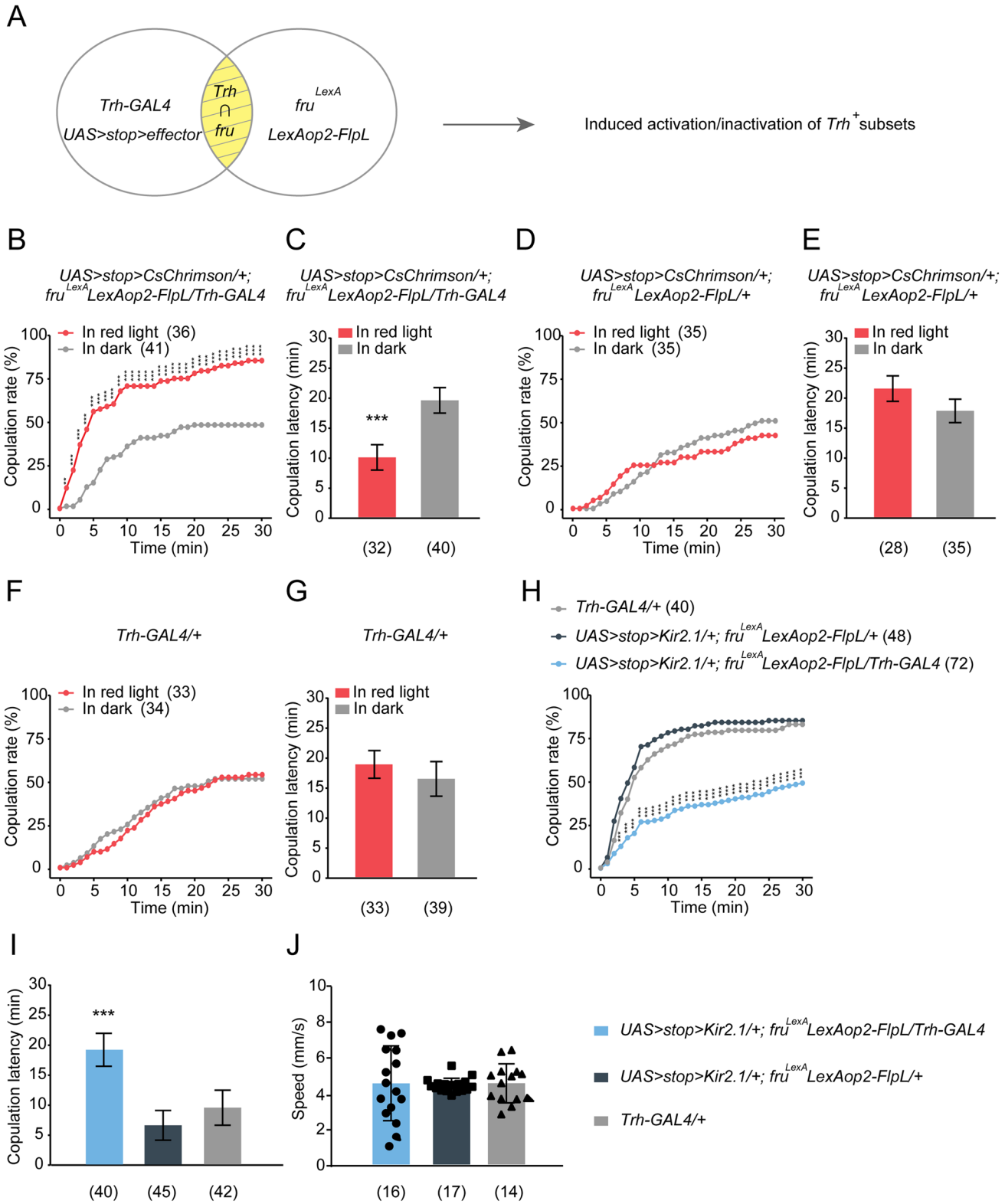


Fig. 4 *Trh*$^{+}$*fru*$^{+}$ neurons mediate virgin female receptivity. **A** The intersectional strategy used to narrow down the serotonergic neurons. FLP is driven by *fru*^{LexA}*LexAop* to excise the transcriptional stop cassette, allowing the expression of specific effectors in intersecting neurons (*Trh*∩*fru*). **B**, **C** Activation of *Trh*$^{+}$*fru*$^{+}$ neurons with red light increases the copulation rate (**B**) and decreases the copulation latency (**C**) in *UAS>stop>CsChrimson*; *fru*^{LexA} *LexAop2-FlpL/Trh-GAL4* virgin females. **D–G** *UAS>stop>CsChrimson*; *fru*^{LexA} *LexAop2-FlpL/+* or *Trh-GAL4/+* control virgin females do not show a red light-induced change in copulation rate or copulation latency. **H**, **I** Silencing the *Trh*$^{+}$*fru*$^{+}$ neurons decreases copulation rate (**H**) and prolongs copulation latency (**I**) in virgin females compared with control females. **J** Inactivation of *Trh*$^{+}$*fru*$^{+}$ neurons in these females does not affect locomotor speed. ***P <0.001, **P <0.01, otherwise no significant difference (χ^2 test for copulation rate; Mann-Whitney U test for **C**, **E**, and **G**; Kruskal-Wallis with *post hoc* Mann-Whitney U test for **I** and **J**). n values shown in parentheses; error bars, ±SEM.

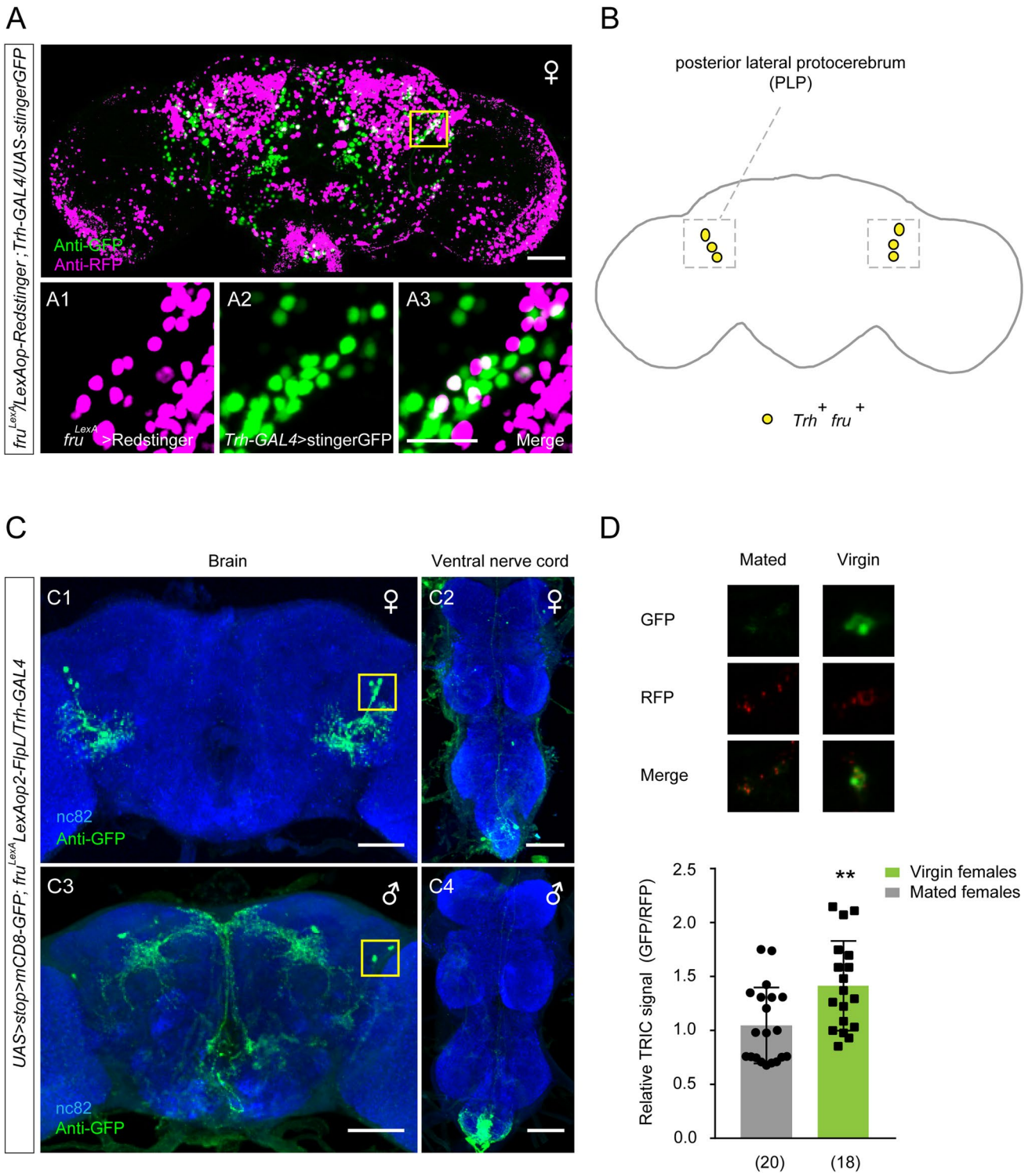


Fig. 5 Identification of sexually dimorphic $Trh^{+}fru^{+}$ PLP neurons. **A** The cell cluster of PLP neurons (yellow box) is labeled by *Trh-GAL4/UAS-stingerGFP* (green) and fru^{LexA}/*LexAop-Redstinger* (magenta) [scale bars, 50 μm (upper) and 20 μm (lower)]. **B** Schematic of overlapped $Trh^{+}fru^{+}$ PLP neurons (yellow dots) in the brain of females. **C** Neurons co-expressing Trh^{+} and fru^{+} visualized with anti-GFP (green) in females and males. Anti-nc82 (blue) indicates the neuropil of the central nervous system. Yellow boxes indicate cell bodies of the $Trh^{+}fru^{+}$ PLP neurons (scale bars, 50 μm). **D** The TRIC signal in PLP neurons is significantly stronger in virgin females than that in mated females. $^{**}P < 0.01$ (Mann-Whitney *U* test). *n* values shown in parentheses; error bars, ±SEM.

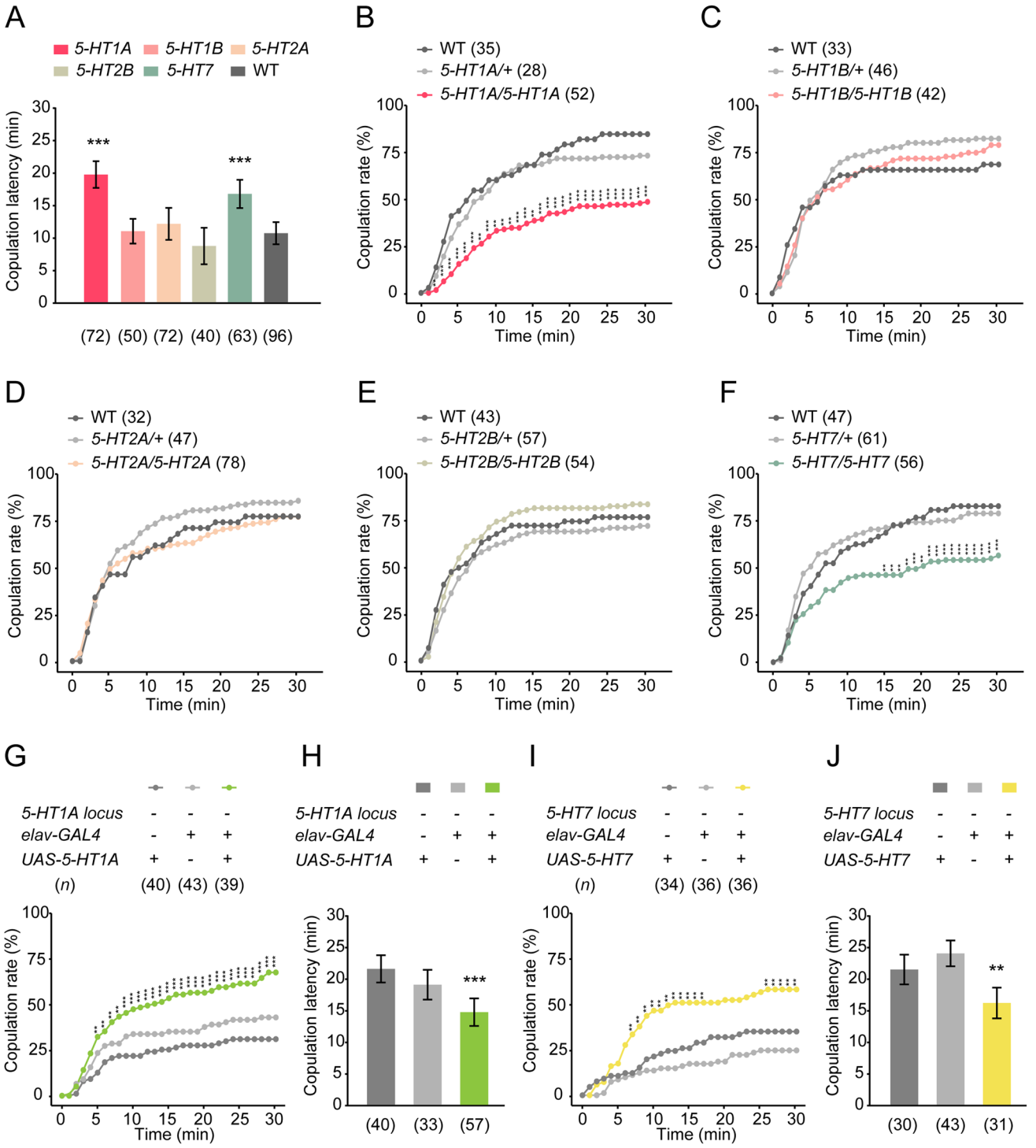


Fig. 6 5-HT_{1A} and 5-HT_7 receptors are involved in virgin female receptivity. **A** The copulation latency is extended in *5-HT1A*- and *5-HT7*-knockout females compared to wild-type control females. **B–F** Copulation rate of females with 5-HT receptor knockout during a 30-min observation period. Knockout of *5-HT1A* (**B**) and *5-HT7* (**F**), but not *5-HT1B* (**C**), *5-HT2A* (**D**), or *5-HT2B* (**E**), significantly reduces the copulation rate in virgin females. **G**, **H** The copulation rate (**G**) is increased, and the copulation latency (**H**) is reduced in *5-HT1A* mutant females with the *elav-GAL4*-driven expression of *5-HT1A*. **I**, **J** The *elav-GAL4*-driven expression of *UAS-5-HT7* in *5-HT7*-knockout females also restores the copulation rate (**I**) and copulation latency (**J**) to normal levels. ***P <0.001, **P <0.01, otherwise no significant difference (Kruskal-Wallis with *post hoc* Mann-Whitney U test for copulation latency; χ^2 test for copulation rate). n values are shown in parentheses; error bars, ±SEM.

unraveled in the modulation of male sexual behavior, little is known about its role in female sexual behavior. We found that *Trh* knockout females showed a dramatic reduction in receptivity, which was rescued by acutely feeding 5-HTP before the receptivity assay. Loss of 5-HT specifically impaired virgin female receptivity but not post-mating behaviors. Furthermore, the spontaneous activity of a subset of 5-HT-releasing neurons was stronger in receptive virgin females. We speculate that 5-HT is required to maintain proper activity in sex-promoting neurons, and thus serves as a positive regulator for sexual motivation in virgin females.

All 5-HT receptors, 5-HT_{1A}, 5-HT_{1B}, 5-HT_{2A}, 5-HT_{2B}, and 5-HT_7, play coordinated roles in serotonin signaling to modulate diverse complex behaviors including aggression, locomotion, and sleep [36, 79, 80]. Notably, knockout of either *Trh* or individual 5-HT receptors did not result in any evident developmental deficit in flies, which suggests that the role of 5-HT signaling in a variety of behaviors is not due to a developmental deficit. We found that the 5-HT_{1A} and 5-HT_7 receptors, but not the 5-HT_{1B}, 5-HT_{2A}, or 5-HT_{2B} receptors, are involved in virgin female receptivity. Knockout of 5-HT_{1A} or 5-HT_7 reduced the receptivity, although not as severely as knockout of *Trh*, suggesting that 5-HT receptors might have parallel and redundant roles in virgin female receptivity. We also noted that knockout of 5-HT_{2B} induced a slight increase in the female copulation rate. It has been reported that 5-HT_{2B} regulates the amount of sleep and sleep homeostasis [36, 81], while sleep significantly influences female mating behaviors. Whether 5-HT_{2B} functions to coordinate female sleep and sexual behavior awaits further investigation.

Approximately 90 serotonergic neurons are present in the central brain and are divided into several clusters into distinct brain regions [57, 58]. Distinct clusters of serotonergic neurons modulate various behaviors, such as walking, long-term memory, and feeding [38, 69]. Previous reports have indicated that female sexual receptivity is regulated by fru^+ neurons [23, 46, 82], which encouraged us to subdivide the serotonergic neurons involved in sexual receptivity by intersecting with fru^+ neurons. We identified Trh^+fru^+ neurons in the PLP cluster to be crucial for virgin female receptivity. Interestingly, there were more Trh^+fru^+ PLP neurons in females than in males, suggesting the involvement of female-specific Trh^+fru^+ PLP neurons in female receptivity. Such dimorphism of Trh^+fru^+ PLP neurons might be regulated by the presence/absence of Fru^M protein in males and females, respectively, as found in other sexually dimorphic fru^+ neurons [83–85]. We also found that 5-HT functions in sexually dimorphic neurons to mediate male courtship behavior (unpublished data). Thus, 5-HT signaling regulates both male and female sexual behavior through sexually dimorphic neural circuits. Future studies may reveal how 5-HT functions in each sex to mediate different aspects of sexual behavior, possibly through distinct 5-HT receptors.

Acknowledgments We thank Dr. Yi Rao (Peking University), Dr. Yufeng Pan (Southeast University), Dr. Jue Xie (Chinese Institute for Brain Research), Dr. Pengxiang Wu (Institute of Zoology, Chinese Academy of Sciences), Dr. Yan Zhu (Institute of Biophysics, Chinese Academy of Sciences), and Dr. Yu Mu (Institute of Neuroscience, Chinese Academy of Sciences) for valuable comments on this manuscript. We also thank Shaowei Hu and Yantong Yang for technical guidance and for providing fly stocks, and Bingcai Wang and Na Xiao for editing software. We also thank the members of Chuan Zhou's Laboratory for their comments and discussions. We thank the Bloomington Stock Center for fly stocks. This work was supported by the National Natural Science Foundation of China (Y711181133 and Y711241133) and the State Key Laboratory of Integrated Management of Pest Insects and Rodents at the Institute of Zoology of the Chinese Academy of Sciences (Y652751E03).

Conflict of interests The authors claim that there is no conflict of interest.

References

1. Dickson BJ. Wired for sex: the neurobiology of *Drosophila* mating decisions. Science 2008, 322: 904–909.
2. Billeter JC, Levine JD. Who is he and what is he to you? Recognition in *Drosophila melanogaster*. Curr Opin Neurobiol 2013, 23: 17–23.
3. Pavlou HJ, Goodwin SF. Courtship behavior in *Drosophila melanogaster*: Towards a 'courtship connectome.' Curr Opin Neurobiol 2013, 23: 76–83.
4. Ito H, Fujitani K, Usui K, Shimizu-Nishikawa K, Tanaka S, Yamamoto D Sexual orientation in *Drosophila* is altered by the satori mutation in the sex-determination gene *fruitless* that encodes a zinc finger protein with a BTB domain Proc Natl Acad Sci U S A 1996, 93: 9687–9692.
5. Peng Q, Chen J, Pan Y. From *fruitless* to sex: On the generation and diversification of an innate behavior. Genes Brain Behav 2021, 20: e12772.
6. Greenspan RJ. Understanding the genetic construction of behavior. Sci Am 1995, 272: 72–78.
7. Sturtevant AH. Experiments on sex recognition and the problem of sexual selection in *Drosoophilia*. J Animal Behav 1915, 5: 351–366.

8. Greenspan RJ, Ferveur JF. Courtship in *Drosophila*. Ann Rev Genet 2000, 34: 205–232.
9. Pan Y, Baker BS. Genetic identification and separation of innate and experience-dependent courtship behaviors in *Drosophila*. Cell 2014, 156: 236–248.
10. Villella A, Hall JC. Neurogenetics of courtship and mating in *Drosophila*. Adv Genet 2008, 62: 67–184.
11. Gao C, Guo C, Peng Q, Cao J, Shohat-Ophir G, Liu D. Sex and death: Identification of feedback neuromodulation balancing reproduction and survival. Neurosci Bull 2020, 36: 1429–1440.
12. Datta SR, Vasconcelos ML, Ruta V, Luo S, Wong A, Demir E, *et al*. The *Drosophila* pheromone cVA activates a sexually dimorphic neural circuit. Nature 2008, 452: 473–477.
13. Deutsch D, Clemens J, Thiberge SY, Guan G, Murthy M. Shared song detector neurons in *Drosophila* male and female brains drive sex-specific behaviors. Curr Biol 2019, 29: 3200-3215.e5.
14. Liu C, Zhang B, Zhang L, Yang T, Zhang Z, Gao Z, *et al*. A neural circuit encoding mating states tunes defensive behavior in *Drosophila*. Nat Commun 2020, 11: 3962.
15. Zhou C, Pan Y, Robinett CC, Meissner GW, Baker BS. Central brain neurons expressing doublesex regulate female receptivity in *Drosophila*. Neuron 2014, 83: 149–163.
16. Aranha MM, Vasconcelos ML. Deciphering *Drosophila* female innate behaviors. Curr Opin Neurobiol 2018, 52: 139–148.
17. Cook R, Connolly K. Rejection responses by female *Drosophila melanogaster*: Their ontogeny, causality and effects upon the behaviour of the courting male. Behaviour 1973, 44: 142–165.
18. Ferveur JF. *Drosophila* female courtship and mating behaviors: Sensory signals, genes, neural structures and evolution. Curr Opin Neurobiol 2010, 20: 764–769.
19. Spieth HT. Courtship behavior in *Drosophila*. Ann Rev Entomol 1974, 19: 385–405.
20. Manning A. The control of sexual receptivity in female *Drosophila*. Animal Behav 1967, 15: 239–250.
21. Kubli E. Sex-peptides: Seminal peptides of the *Drosophila* male. Cell Mol Life Sci 2003, 60: 1689–1704.
22. Wolfner MF. Tokens of love: Functions and regulation of *Drosophila* male accessory gland products. Insect Biochem Mol Biol 1997, 27: 179–192.
23. Häsemeyer M, Yapici N, Heberlein U, Dickson BJ. Sensory neurons in the *Drosophila* genital tract regulate female reproductive behavior. Neuron 2009, 61: 511–518.
24. Rezával C, Pavlou HJ, Dornan AJ, Chan YB, Kravitz EA, Goodwin SF. Neural circuitry underlying *Drosophila* female postmating behavioral responses. Curr Biol 2012, 22: 1155–1165.
25. Yang CH, Rumpf S, Xiang Y, Gordon MD, Song W, Jan LY, *et al*. Control of the postmating behavioral switch in *Drosophila* females by internal sensory neurons. Neuron 2009, 61: 519–526.
26. Haussmann IU, Hemani Y, Wijesekera T, Dauwalder B, Soller M. Multiple pathways mediate the sex-peptide-regulated switch in female *Drosophila* reproductive behaviours. Proc Biol Sci 2013, 280: 20131938.
27. Rezával C, Nojima T, Neville MC, Lin AC, Goodwin SF. Sexually dimorphic octopaminergic neurons modulate female postmating behaviors in *Drosophila*. Curr Biol 2014, 24: 725–730.
28. Ding Z, Haussmann I, Ottiger M, Kubli E. Sex-peptides bind to two molecularly different targets in *Drosophila melanogaster* females. J Neurobiol 2003, 55: 372–384.
29. Yapici N, Kim YJ, Ribeiro C, Dickson BJ. A receptor that mediates the post-mating switch in *Drosophila* reproductive behaviour. Nature 2008, 451: 33–37.
30. Luo L. Principles of neurobiology, 2nd edn. Garland Science, New York, 2020, pp 80–87.
31. Ferguson J, Henriksen S, Cohen H, Mitchell G, Barchas J, Dement W. "Hypersexuality" and behavioral changes in cats caused by administration of p-chlorophenylalanine. Science 1970, 168: 499–501.
32. Obara Y, Fukano Y, Watanabe K, Ozawa G, Sasaki K. Serotonin-induced mate rejection in the female cabbage butterfly Pieris rapae crucivora. Naturwissenschaften 2011, 98: 989–993.
33. Soares MC, Paula JR, Bshary R. Serotonin blockade delays learning performance in a cooperative fish. Animal Cogn 2016, 19: 1027–1030.
34. Ma Q. Beneficial effects of moderate voluntary physical exercise and its biological mechanisms on brain health. Neurosci Bull 2008, 24: 265–270.
35. Zhu Y, Wu X, Zhou R, Sie O, Niu Z, Wang F, *et al*. Hypothalamic-pituitary-end-organ axes: Hormone function in female patients with major depressive disorder. Neurosc. Bull 2021, 37: 1176–1187.
36. Qian Y, Cao Y, Deng B, Yang G, Li J, Xu R, *et al*. Sleep homeostasis regulated by 5HT2b receptor in a small subset of neurons in the dorsal fan-shaped body of *Drosophila*. Elife 2017, 6: e26519.
37. Alekseyenko OV, Chan YB, Fernandez MP, Bülow T, Pankratz MJ, Kravitz EA. Single serotonergic neurons that modulate aggression in *Drosophila*. Curr Biol 2014, 24: 2700–2707.
38. Albin SD, Kaun KR, Knapp JM, Chung P, Heberlein U, Simpson JH. A subset of serotonergic neurons evokes hunger in adult *Drosophila*. Curr Biol 2015, 25: 2435–2440.
39. Yamamoto D. The neural and genetic substrates of sexual behavior in *Drosophila*. Adv Genet 2007, 59: 39–66.
40. Lee G, Hall JC, Park JH. *Doublesex* gene expression in the central nervous system of *Drosophila melanogaster*. J Neurogenet 2002, 16: 229–248.
41. Rideout EJ, Dornan AJ, Neville MC, Eadie S, Goodwin SF. Control of sexual differentiation and behavior by the doublesex gene in *Drosophila melanogaster*. Nat Neurosci 2010, 13: 458–466.
42. Waterbury JA, Jackson LL, Schedl P. Analysis of the doublesex female protein in *Drosophila melanogaster*: Role on sexual differentiation and behavior and dependence on intersex. Genetics 1999, 152: 1653–1667.
43. Williams TM, Selegue JE, Werner T, Gompel N, Kopp A, Carroll SB. The regulation and evolution of a genetic switch controlling sexually dimorphic traits in *Drosophila*. Cell 2008, 134: 610–623.
44. Stockinger P, Kvitsiani D, Rotkopf S, Tirián L, Dickson BJ. Neural circuitry that governs *Drosophila* male courtship behavior. Cell 2005, 121: 795–807.
45. Yamamoto D, Koganezawa M. Genes and circuits of courtship behaviour in *Drosophila* males. Nat Rev Neurosci 2013, 14: 681–692.
46. Kvitsiani D, Dickson BJ. Shared neural circuitry for female and male sexual behaviours in *Drosophila*. Curr Biol 2006, 16: R355–R356.
47. Wang K, Wang F, Forknall N, Yang T, Patrick C, Parekh R, *et al*. Neural circuit mechanisms of sexual receptivity in *Drosophila* females. Nature 2021, 589: 577–581.
48. Wang F, Wang K, Forknall N, Parekh R, Dickson BJ. Circuit and behavioral mechanisms of sexual rejection by *Drosophila* females. Curr Biol 2020, 30: 3749-3760.e3.
49. Deng B, Li Q, Liu X, Cao Y, Li B, Qian Y, *et al*. Chemoconnectomics: mapping chemical transmission in *Drosophila*. Neuron 2019, 101: 876-893.e4.
50. Wu F, Deng B, Xiao N, Wang T, Li Y, Wang R, *et al*. A neuropeptide regulates fighting behavior in *Drosophila melanogaster*. Elife 2020, 9: e54229.
51. Ishimoto H, Kamikouchi A. A feedforward circuit regulates action selection of pre-mating courtship behavior in female *Drosophila*. Curr Biol 2020, 30: 396-407.e4.
52. Terhzaz S, Rosay P, Goodwin SF, Veenstra JA. The neuropeptide SIFamide modulates sexual behavior in *Drosophila*. Biochem. Biophys. Res Commun 2007, 352: 305–310.

53. Neckameyer WS. Dopamine modulates female sexual receptivity in *Drosophila melanogaster*. J Neurogenet 1998, 12: 101–114.
54. Wu S, Guo C, Zhao H, Sun M, Chen J, Han C, *et al*. Drosulfakinin signaling in *fruitless* circuitry antagonizes P1 neurons to regulate sexual arousal in *Drosophila*. Nat Commun 2019, 10: 4770.
55. Kuhn DM, Rosenberg RC, Lovenberg W. Determination of some molecular parameters of tryptophan hydroxylase from rat midbrain and murine mast cell. J Neurochem 1979, 33: 15–21.
56. Neckameyer WS, White K. A single locus encodes both phenylalanine hydroxylase and tryptophan hydroxylase activities in *Drosophila*. J Biol Chem 1992, 267: 4199–4206.
57. Alekseyenko OV, Lee C, Kravitz EA. Targeted manipulation of serotonergic neurotransmission affects the escalation of aggression in adult male *Drosophila melanogaster*. PLoS One 2010, 5: e10806.
58. Pooryasin A, Fiala A. Identified serotonin-releasing neurons induce behavioral quiescence and suppress mating in *Drosophila*. J Neurosci 2015, 35: 12792–12812.
59. Schröder-Lang S, Schwärzel M, Seifert R, Strünker T, Kateriya S, Looser J, *et al*. Fast manipulation of cellular cAMP level by light *in vivo*. Nat Methods 2007, 4: 39–42.
60. Klapoetke NC, Murata Y, Kim SS, Pulver SR, Birdsey-Benson A, Cho YK, *et al*. Independent optical excitation of distinct neural populations. Nat Methods 2014, 11: 338–346.
61. Hamada FN, Rosenzweig M, Kang K, Pulver SR, Ghezzi A, Jegla TJ, *et al*. An internal thermal sensor controlling temperature preference in *Drosophila*. Nature 2008, 454: 217–220.
62. Baines RA, Uhler JP, Thompson A, Sweeney ST, Bate M. Altered electrical properties in *Drosophila* neurons developing without synaptic transmission. J Neurosci 2001, 21: 1523–1531.
63. Kitamoto T. Conditional disruption of synaptic transmission induces male-male courtship behavior in *Drosophila*. Proc Natl Acad Sci U S A 2002, 99: 13232–13237.
64. Bohm RA, Welch WP, Goodnight LK, Cox LW, Henry LG, Gunter TC, *et al*. A genetic mosaic approach for neural circuit mapping in *Drosophila*. Proc Natl Acad Sci U S A 2010, 107: 16378–16383.
65. Gao XJ, Riabinina O, Li J, Potter CJ, Clandinin TR, Luo L. A transcriptional reporter of intracellular Ca^{2+} in *Drosophila*. Nat Neurosci 2015, 18: 917–925.
66. Colas JF, Launay JM, Kellermann O, Rosay P, Maroteaux L. *Drosophila* 5-HT2 serotonin receptor: Coexpression with fushitarazu during segmentation. Proc Natl Acad Sci U S A 1995, 92: 5441–5445.
67. Witz P, Amlaiky N, Plassat JL, Maroteaux L, Borrelli E, Hen R. Cloning and characterization of a *Drosophila* serotonin receptor that activates adenylate cyclase. Proc Natl Acad Sci U S A 1990, 87: 8940–8944.
68. Saudou F, Hen R. 5-hydroxytryptamine receptor subtypes in vertebrates and invertebrates. Neurochem Int 1994, 25: 503–532.
69. Howard CE, Chen CL, Tabachnik T, Hormigo R, Ramdya P, Mann RS. Serotonergic modulation of walking in *Drosophila*. Curr Biol 2019, 29: 4218–4230.e8.
70. Ganguly A, Qi C, Bajaj J, Lee D. Serotonin receptor 5-HT7 in *Drosophila* mushroom body neurons mediates larval appetitive olfactory learning. Sci Rep 2020, 10: 21267.
71. Gasque G, Conway S, Huang J, Rao Y, Vosshall LB. Small molecule drug screening in *Drosophila* identifies the 5HT2A receptor as a feeding modulation target. Sci Rep 2013, 3: 2120.
72. Siddiqui A, Niazi A, Shaharyar S, Wilson CA. The $5HT_7$ receptor subtype is involved in the regulation of female sexual behaviour in the rat. Pharmacol. Biochem Behav 2007, 87: 386–392.
73. Berger M, Gray JA, Roth BL. The expanded biology of serotonin. Annu Rev Med 2009, 60: 355–366.
74. Dominguez JM, Hull EM. Serotonin impairs copulation and attenuates ejaculation-induced glutamate activity in the medial preoptic area. Behav Neurosci 2010, 124: 554–557.
75. Giuliano F. 5-hydroxytryptamine in premature ejaculation: Opportunities for therapeutic intervention. Trends Neurosci 2007, 30: 79–84.
76. Gradwell PB, Everitt BJ, Herbert J. 5-hydroxytryptamine in the central nervous system and sexual receptivity of female rhesus monkeys. Brain Res 1975, 88: 281–293.
77. Uphouse L. Pharmacology of serotonin and female sexual behavior. Pharmacol Biochem Behav 2014, 121: 31–42.
78. Liu Y, Jiang Y, Si Y, Kim JY, Chen ZF, Rao Y. Molecular regulation of sexual preference revealed by genetic studies of 5-HT in the brains of male mice. Nature 2011, 472: 95–99.
79. Yuan Q, Joiner WJ, Sehgal A. A sleep-promoting role for the *Drosophila* serotonin receptor 1A. Curr Biol 2006, 16: 1051–1062.
80. Alekseyenko OV, Chan YB, Okaty BW, Chang Y, Dymecki SM, Kravitz EA. Serotonergic modulation of aggression in *Drosophila* involves GABAergic and cholinergic opposing pathways. Curr Biol 2019, 29: 2145-2156.e5.
81. Xia M, Li Z, Li S, Liang S, Li X, Chen B, *et al*. Sleep deprivation selectively down-regulates astrocytic $5\text{-}HT_{2B}$ receptors and triggers depressive-like behaviors via stimulating $P2X_7$ receptors in mice. Neurosci Bull 2020, 36: 1259–1270.
82. Sakurai A, Koganezawa M, Yasunaga K, Emoto K, Yamamoto D. Select interneuron clusters determine female sexual receptivity in *Drosophila*. Nat Commun 1825, 2013: 4.
83. Kimura KI, Ote M, Tazawa T, Yamamoto D. Fruitless specifies sexually dimorphic neural circuitry in the *Drosophila* brain. Nature 2005, 438: 229–233.
84. Yamamoto D, Sato K, Koganezawa M. Neuroethology of male courtship in *Drosophila*: From the gene to behavior. J. Comp. Physiol. A Neuroethol. Sens Neural Behav Physiol 2014, 200: 251–264.
85. Kimura KI, Hachiya T, Koganezawa M, Tazawa T, Yamamoto D. Fruitless and doublesex coordinate to generate male-specific neurons that can initiate courtship. Neuron 2008, 59: 759–769.