




Sex and Death: Identification of Feedback Neuromodulation Balancing Reproduction and Survival

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Abstract Some semelparous organisms in nature mate as many times as they can in a single reproductive episode before death, while most iteroparous species including humans avoid such suicidal reproductive behavior. Animals naturally pursue more sex and the possible fatal consequence of excessive sex must be orchestrated by negative feedback signals in iteroparous species, yet very little is known about the regulatory mechanisms. Here we used *Drosophila* male sexual behavior as a model system to study how excessive sex may kill males and how the nervous system reacts to prevent death by sex. We found that continuous sexual activity by activating the *fruitless*-expressing neurons induced a fixed multi-step behavioral pattern ending with male death. We further found negative feedback in the fly brain to prevent suicidal sexual behavior by expression changes of the neurotransmitters acetylcholine and gamma-aminobutyric acid, and neuropeptide

F. These findings are crucial to understand the molecular underpinnings of how different organisms choose reproductive strategies and balance reproduction and survival.

Keywords *Drosophila* · Reproduction · Survival · NPF · GABA · Acetylcholine

Introduction

Most species, including humans, balance sexual behaviors and survival, avoiding excessive sexual activity based on their past experience and internal physiological state [1–3]; however, there are some semelparous organisms, such as the insectivorous marsupials, that mate as many times as they can in a single reproductive episode that eventually kills them [4]. While the evolutionary mechanism of suicidal reproduction in semelparous animals is of much interest, it is hard to study the molecular and neuronal mechanisms underlying such behavior due to the very limited genetic and neurological tools in these animals.

Drosophila melanogaster is iteroparous with survival and reproduction well balanced [2]. Male courtship in *Drosophila* is a well-studied innate behavior, which is largely controlled by the sex-specific transcription factors (FRUM and DSXM in males, and DSXF in females) encoded by the *fruitless* (*fru*) and *doublesex* (*dsx*) genes [5–7]. FRUM is responsible for most aspects of male courtship [8–10], and is expressed in a dispersed subset of ~2000 neurons including sensory neurons, interneurons, and motor neurons that are interconnected to form sex circuitry [9–12]. Recently, much progress has been made on how sensory cues are integrated by the male-specific P1 neurons that initiate male courtship, and how experiences may alter P1 excitability to modulate courtship [13–20]. In

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particular, mating experience decreases further courtship through dopamine modulation of P1 neurons [2, 21, 22], providing a potential mechanism to avoid excessive sexual behavior in male flies. However, there is no evidence that sexual activity *per se* in male flies perturbs their survival, although exposure to female pheromones does [23, 24], making it hard to study the molecular and neuronal mechanisms underlying suicidal reproduction despite the advanced genetic tools in this species [25].

We previously showed that mild activation of all *fruM* neurons induces courtship behaviors in solitary males, which perform courtship behaviors until death [26]. In this study, we used two genetic models, one in the above males with *fruM* neurons activated that are semelparous, and the other in wild-type males that are iteroparous. Combining these two genetic models, we investigated how excessive sexual activity accelerates death in male flies, and how the central nervous system reacts to prevent such behaviors. We found that continuous performance of sexual behaviors induces a fixed behavioral pattern ending with male death. We further found a negative feedback in the fly brain involving acetylcholine, gamma-aminobutyric acid (GABA) and neuropeptide F (NPF) to prevent suicidal sexual behavior.

Materials and Methods

Fly Stocks

Flies were maintained at 22°C or 25°C in a 12h:12h light:dark cycle. Canton-S flies were used as the wild-type strain. *fruGAL4* [10], *fruLexA* [27], *UAS-dTrpA1* (II) [28], *UAS-CsChrimson* (attp2) [29], *UAS-fruMi* (attp2) [30], *UAS-fruMiScr* (attp2) [30], *R19B03-Gal4* (attp2) [31], *NPF-GAL4* (BDSC#25682), and *Crz-GAL4* (BDSC#51976) have been described previously. RNAi lines (*UAS-GABA-B-R3-RNAi*, *UAS-Dsk-RNAi*, *UAS-CCKLR-17D3-RNAi*, *UAS-Oct-beta-2R-RNAi*, *UAS-NPF-RNAi*, *UAS-NPFRI-RNAi*, *UAS-Trh-RNAi*, and *UAS-Dop1RI-RNAi* all at attp2, and *UAS-Gad1-RNAi* and *UAS-ChAT-RNAi* at attp40) have been described previously [32] and were from Tsinghua Fly Center at Tsinghua University.

Male Courtship Assay

In courtship assays for Fig. 4, 4–8-day-old wild-type virgin females were loaded individually into cylindrical chambers (diameter: 1 cm; height: 3 mm per layer) as courtship targets, and 4–6-day-old test males were then gently loaded into the chambers after cold anesthesia and separated from target females by a transparent film. The chambers were warmed at 27°C for 4 h allowing dTRPA1-mediated

neuronal activation, and transferred to 22°C for 30 min, 1 h, or 2 h before the 10-min courtship test. The courtship index (CI), which is the percentage of observation time a male fly performs courtship, was used to measure courtship towards female targets, and measured manually using LifeSongX software.

In the courtship assays for Fig. 5, 4–8-day-old wild-type virgin females were loaded into large cylindrical chambers (diameter: 4 cm; height: 3 mm per layer) as courtship targets (10 females per chamber), and 4–6 day-old-test males were then individually loaded into chambers after cold anesthesia, and separated from target females by a transparent film until courtship testing for 5 h continuously at 25°C.

Feeding Assay

Feeding was assayed using food with a blue dye. In brief, in a regular feeding assay test (Fig. 2C), flies were starved for 24 h on 1% aqueous agar food at 22°C, then transferred to 1% FD & C Blue 1 food (2.5% sucrose, 2.5% yeast extract, and 0.5% agar; Sigma-Aldrich, St. Louis, MO) at 27°C for 30 min (the food was pre-warmed at 27°C for 3 h). The feeding test shown in Fig. 2A was the same but used non-starved males; that in Fig. 2B used non-starved males and allowed feeding at 27°C for 12 h. To quantify food intake, the absorbance of the ingested blue dye was measured at 630 nm using a 96-well microplate spectrophotometer.

Survival Assay

Adult male flies were collected within 12 h after eclosion and group housed (10–15 males) for 3–4 days before the survival test. In the survival assay for Figs 1A, B, and 5I, individual test males were loaded with a certain number of virgin females (1M+1F, 1M+4F, 1M+7F, or 1M+10F) into empty vials without food, and controls with the same group size of males (2M, 5M, 8M, or 11M). The survival of males was scored every 6 h at 25°C. Only the 1M+10F condition was used for the survival assays shown the Figs 1B and 5I. The sample size for all experiments was 10, and each sample contained 8–11 males. For all M+F groups (1M+1F, 1M+4F, 1M+7F or 1M+10F), 10 vials were used as one sample, thus there were 100 males in each group. For the 2M group, 5 vials were used as one sample (100 males; for the 5M group, 2 vials were used as one sample (100 males; and for the 8M and 11M groups, each vial was a sample (80 and 110 males).

The survival assay shown in the Fig. S1 was almost the same as above, but 1% agar + 1% sugar medium in vials at 22°C was provided. The number of dead males was scored

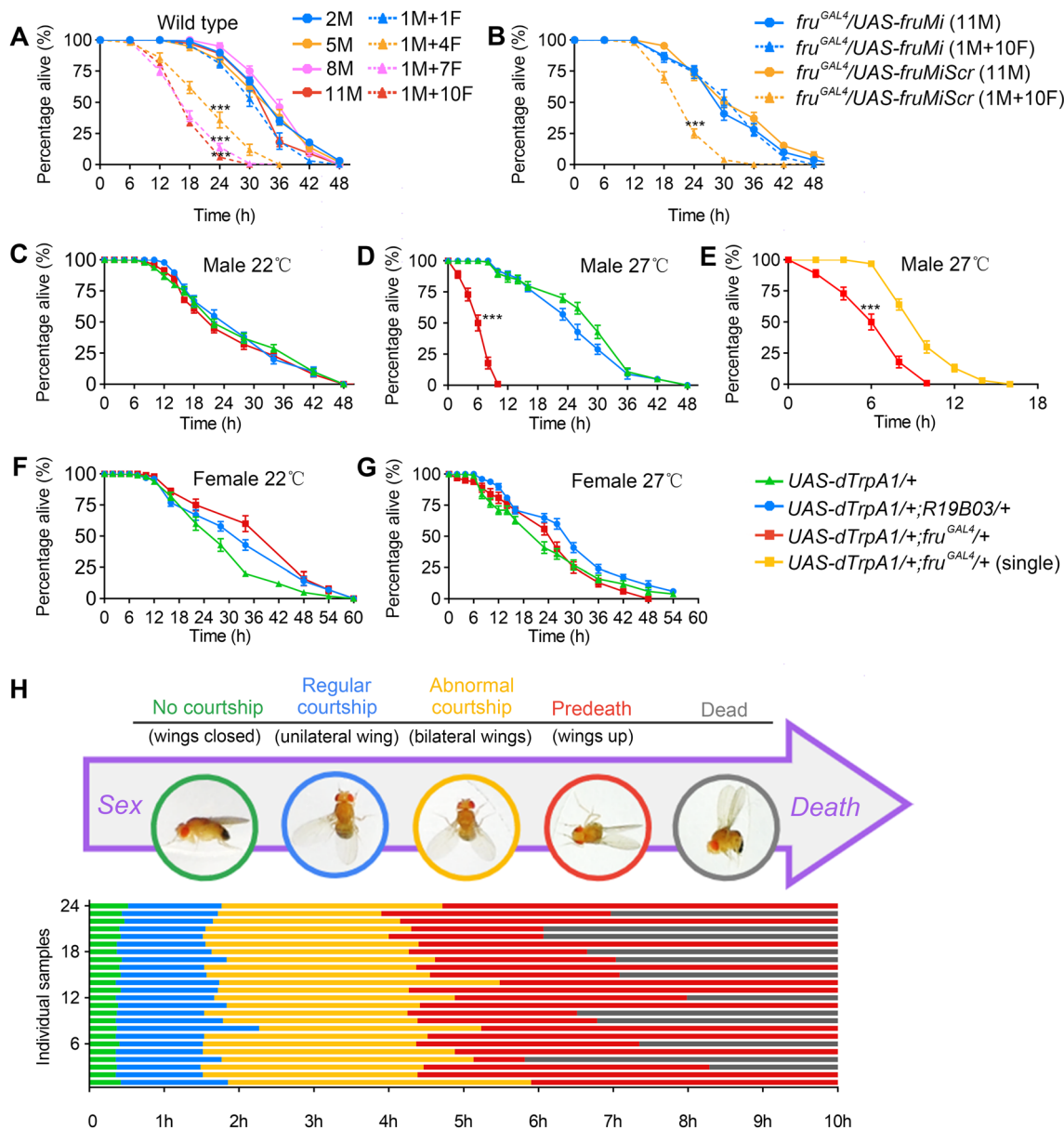


Fig. 1 Coupling sex and death in *Drosophila* males. **A** Survival rates of wild-type males under conditions allowing different levels of sexual activity. Males die much sooner when group-housed with increasing numbers of virgin females (M, males; F, virgin females). Flies were housed in vials without food and assayed at 25°C. *N* = 10 for each. Each *n* consists of 10 vials of 1M+1F, 1M+4F, 1M+7F, 1M+10F, 5 vials of 2M, 2 vials of 5M, or one vial of 8M or 11M. ****P* < 0.001 for 1M+4F vs 5M, 1M+7F vs 8M, or 1M+10F vs 11M at the 24-h time point, Mann Whitney U test. **B** Males with *fru^M* knocked down survive similarly when grouped with 10 virgin females or in groups of 11 males. *n* = 10 for each. Each *n* consists of 10 vials of 1M+10F or one vial of 11M. ****P* < 0.001, Mann Whitney U test. **C, D** Mildly activating all *fru^m* neurons at 27°C induces continuous

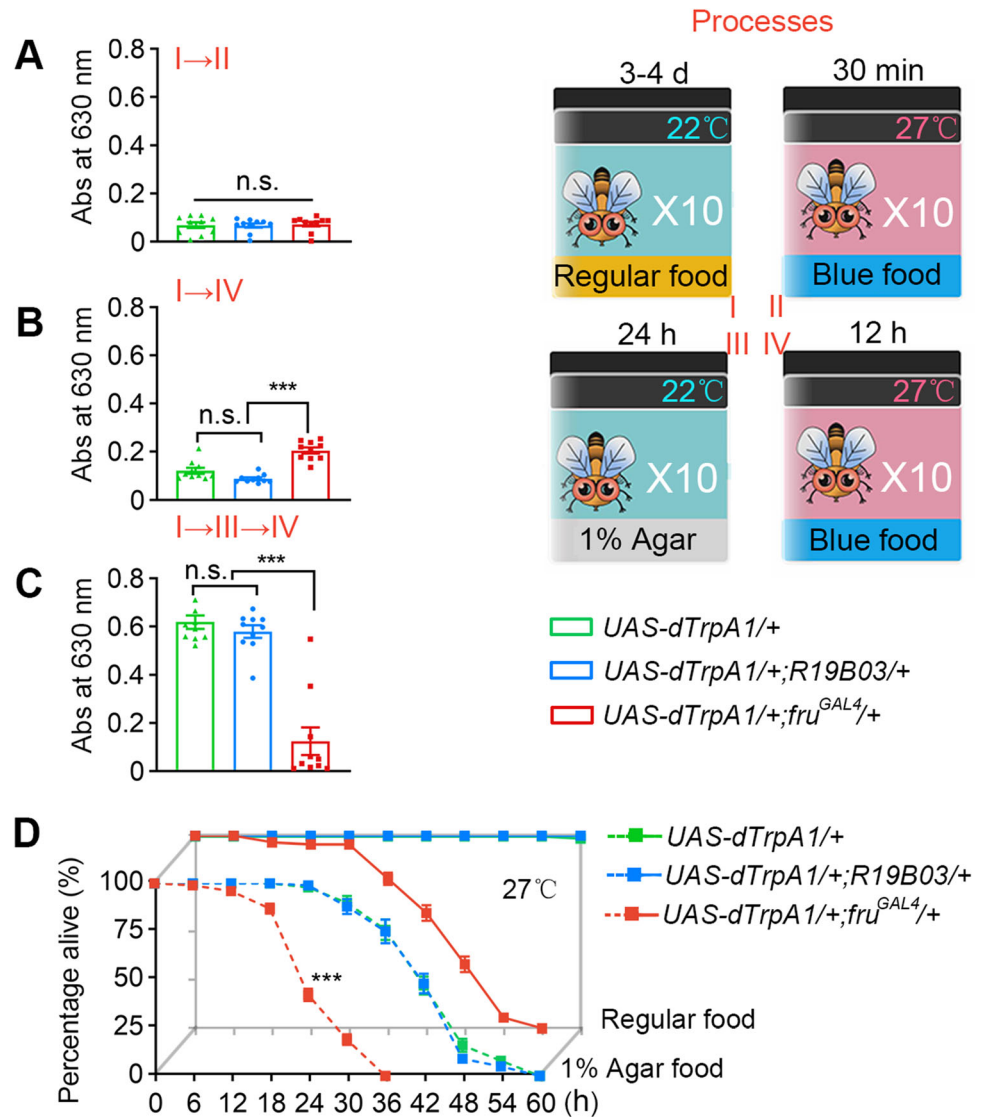
courtship and accelerates male death. All males were housed in groups of 10 without food. *n* = 10 for each. ****P* < 0.001 at 6 h, Mann Whitney U test. **E** Males with *fru^M* neurons activated die much more quickly in groups of 10 males than in isolation. *n* = 10 for each. Each *n* consists of one vial of 10 males or 10 vials of single-housed males. ****P* < 0.001 at 6 h, Mann Whitney U test. **F, G** Activating *fru^M* counterpart neurons in females does not accelerate female death. *n* = 10 (10 females/group) for each. **H** Activating *fru^M* induces a series of stereotypical behavior patterns from regular courtship display to male death. Flies were individually housed in round chambers without food and recorded at 27°C. *n* = 24. Error bars indicate SEM.

twice a day. Flies were transferred to new vials with medium every 2 days.

In the survival assays for Figs 1C–G, 2D, and 3E, I: groups of 10 males (or 10 females in Fig. 1F, G) were

housed in vials without food (Fig. 1C–G), or in a nutritional environment (1% agar for the 3 agar groups in Fig. 2D; regular food for the other 3 groups in Figs 2D and 3E, I) at specified temperatures. Flies housed in vials with

Fig. 2 Abnormal feeding in continuously courting males. **A–C** Males with activated *fru^M* neurons eat more in the short term, but much less in the long term. Feeding assays are illustrated on the right. $n = 10$ (10 flies/group) for each. n.s., not significant, $***P < 0.001$, Kruskal–Wallis test, post hoc Dunn's multiple comparisons test. **D** Feeding alleviates but does not prevent death in males with activated *fru^M* neurons. $n = 10$ (10 flies/group) for each. $***P < 0.001$ at 24 h, Mann Whitney U test. Error bars indicate SEM.



food were transferred to new food vials every 2 days, and dead flies were removed and counted. Ten replicate vials were established for each group, a total of 100 flies.

In the survival assays for Fig. 3H, flies were raised on regular food or food with 0.2 mmol/L ATR (all-trans-retinal, MFCD00001550, Sigma-Aldrich, St. Louis, MO) throughout development and adulthood in the dark. Test males were group-housed (10 flies per vial) and exposed to 620-nm red light (40 Hz, 8 ms duration, 0.071 mW/mm²). Flies were transferred to new vials containing fresh food every 2 days, and dead flies were removed and counted. Ten replicate vials were established for each group (total, 100 males).

Ejaculation Assay

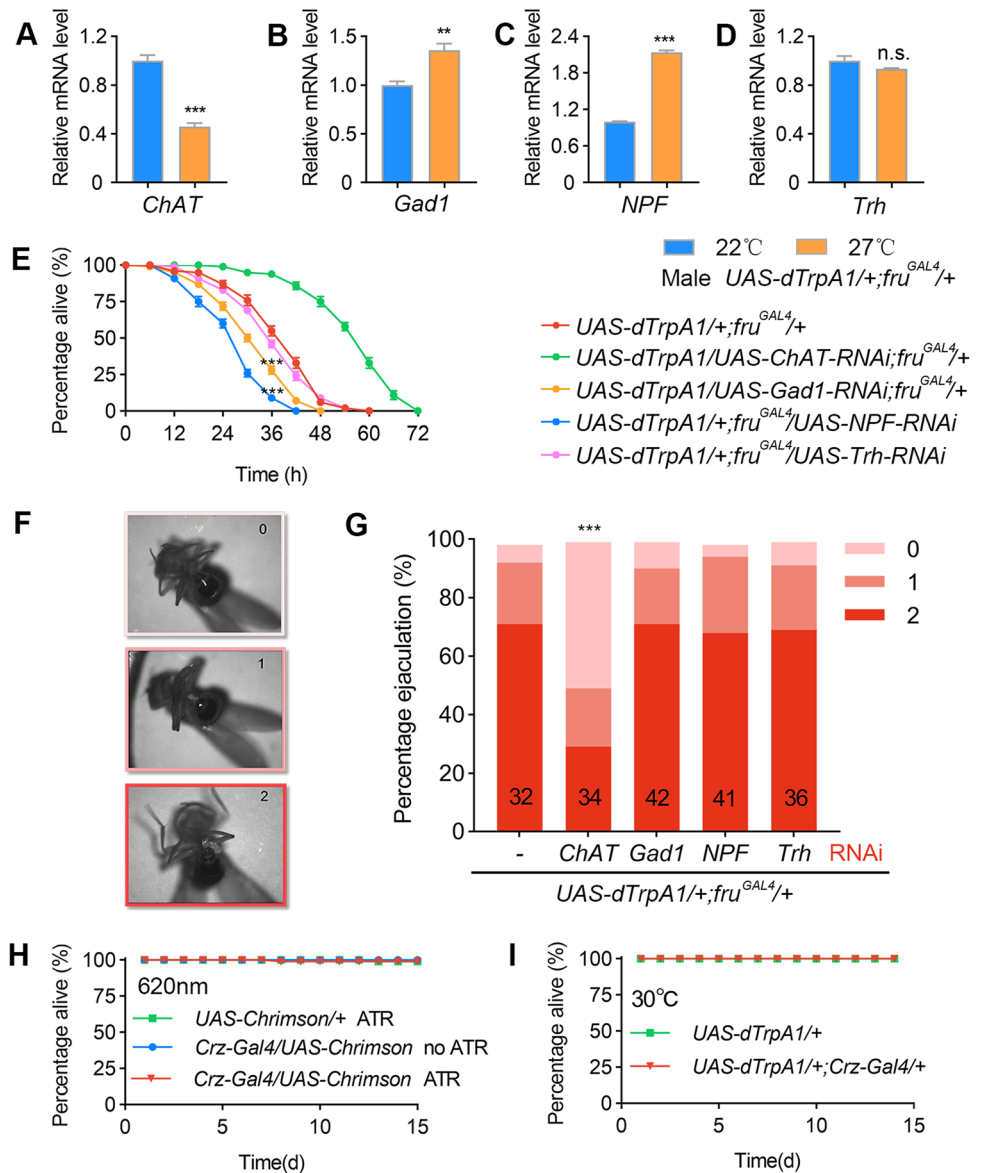
To evaluate ejaculation, males were loaded individually into cylindrical chambers (diameter, 2 cm; height, 3 mm)

after ice anesthesia, and allowed to recover at 22°C for 30 min, then transferred to 27°C for 30 min. Ejaculation levels (0, 1, or 2; see Fig. 3F) were manually scored under a microscope.

Body Weight

A few 1.5-mL centrifugal tubes were numbered and each weighed as M_0 . Then 20 males were loaded into each tube after CO₂ anesthesia, and weighed as M_1 . The same 20 males were transferred from the centrifugal tube to an empty vial, transferred to 27°C for 6 h, and then returned to the original centrifugal tube and weighed as M_2 . The percentage weight loss was $(M_2 - M_1)/(M_1 - M_0) \times 100\%$. Five replicates were measured for each group.

Fig. 3 Counterbalancing molecular changes induced by excessive sexual output. **A–D** Continuous activation of *fru^M* neurons decreases *chat* expression and increases *Gad1* and *NPF* expression in male brains. Groups of 10 males were housed in food vials for 12 h at 27°C. *n* = 3 per group. n.s., not significant, ***P* < 0.01, ****P* < 0.001, unpaired *t*-test. **E** Knocking down *chat* alleviates, while knocking down *Gad1* or *NPF* further accelerates, the fatal effect of *fru^M* neuronal activation in males. All test males were housed in food vials in groups of 10 at 27°C. *n* = 10 (10 flies/group) for each. ****P* < 0.001 at 36 h, Mann Whitney U test. **F** Evaluation of ejaculation in individual males. 0 refers to no ejaculation, 1 and 2 refer to ejaculation with different volumes. **G** Knocking down *chat* significantly reduces the level of ejaculation in males with activated *fru^M* neurons. *n* as indicated inside bars. ****P* < 0.001, χ^2 test. **H, I** Activation of *Crz* neurons *via* light-sensitive CsChrimson (**H**) or temperature-sensitive dTrpA1 (**I**) does not affect mortality over a 15-day period on regular food or food with ATR. *n* = 10 each, each *n* consists of 10 flies. Error bars indicate SEM.



Quantitative Real-time PCR

Fly samples from neuronal activation and mating experiments were frozen in liquid nitrogen and decapitated by vigorous vortexing. The heads were then separated from the bodies using metal sieves. Each sample, consisting of 30 frozen heads, was used for total RNA using TRIzol reagent (15596026, Thermo Fisher Scientific, Waltham, MA). We purified total RNA using a DNA-freeTM Kit (AM1906, Thermo Fisher Scientific, Waltham, MA) and performed reverse transcription using SuperScriptTM IV (18091050, Thermo Fisher Scientific, Waltham, MA) to obtain cDNA used for templates. Quantitative PCR was performed on the Roche LightCycler® 96 Real-Time PCR machine using AceQ qPCR SYBR Green Master Mix (Q121-02, Vazyme, Nanjing). Transcript levels were

analyzed by the $2^{-\Delta\Delta CT}$ method using *Actin* as an internal control. Each sample was run in triplicate. Each experiment was repeated three times using independent sets of genetic crosses. Primers used for RT-PCR quantification were:

<i>Actin</i> Forward	5'-GTCGCGATTTAACCGAC-TACCTGA-3'
<i>Actin</i> Reverse	5'-TCTTGCTT CGAGATCCACATCTGC-3'
<i>Chat</i> Forward	5'-TGAATATGCCTTGAGCTGTGC-3'
<i>Chat</i> Reverse	5'-TCGTCGAGAATTCCGCAAAC-3'
<i>Gad1</i> Forward	5'-GTGCCACCACATTGAAGTACC-3'
<i>Gad1</i> Reverse	5'-AGACCGTTGGACAGCTGATTG-3'

<i>Trh</i> Forward	5'-TCCATTCTACACACCAGAACCG-3'
<i>Trh</i> Reverse	5'-ACTGGGCAAAACTGGAGTTG-3'
<i>NPF</i> Forward	5'-CCTCATTAACGCGAGCAAAT-3'
<i>NPF</i> Reverse	5'-ATCGCTGATGGATATCCTGAGG-3'

Tissue Dissection, Staining, and Imaging

Brains of 4–6 day-old males and females were dissected in Schneider's insect medium (S2) and fixed in 4% paraformaldehyde in 0.5% Triton X-100 and 0.5% bovine serum albumin in phosphate-buffered saline (PAT) for 30 min at room temperature. After 4 × 10-min washes, tissues were blocked in 3% normal goat serum (NGS) for 60 min, then incubated in primary antibodies diluted in 3% NGS for 4 h at room temperature and 1–2 days at 4°C, then washed in PAT, and incubated in secondary antibodies diluted in 3% NGS for 4 h at room temperature and 1–2 days at 4°C. The tissues were then washed thoroughly in PAT and mounted for imaging. The antibodies used were rabbit anti-NPF (1:500; RB-19-0001, RayBiotech, Norcross, GA), rabbit anti-GFP (1:1000; A11122, Invitrogen, Waltham, MA), and secondary Alexa Fluor 488 antibodies (1:500, A21206, Invitrogen, Waltham, MA). Samples were imaged at ×20 magnification on Zeiss 710 confocal microscopes using ZEN (<https://www.zeiss.com/microscopy/int/products/microscope-software/zen.html>) and processed with Fiji software (<https://imagej.net/Fiji/Downloads>).

Statistics

Experimental flies and genetic controls were tested under the same conditions, and data are collected from at least two independent experiments. Statistical analysis was performed using GraphPad Prism 8 (<https://www.graphpad.com/scientific-software/prism/>) as indicated in each figure legend. Data were first verified for normal distribution by the D'Agostino–Pearson normality test. For normally-distributed data, Student's *t* test was used for pairwise comparisons, and one-way ANOVA was used for comparisons among multiple groups, followed by Tukey's multiple comparisons. For data not normally-distributed, the Mann-Whitney U test was used for pairwise comparisons, and the Kruskal–Wallis test was used for comparison among multiple groups, followed by Dunn's multiple comparisons. For RT-PCR experiments, the average relative expression of three independent experiments was analyzed using the unpaired *t*-test. The χ^2 test was used to compare two groups in the male ejaculation assay.

Results

Coupling Sex and Death: A *Drosophila* Model

Previous studies revealed that male courtship behavior *per se* does not affect life span in male *Drosophila* [23, 24]. We wondered if the excessive expression of courtship behaviors (successive mating in a short period of time) would be costly, and tested the survival rate of individual wild-type *Canton-S* (*w^{ts}*) males housed with increasing numbers of virgin females (1M+1F, 1M+4F, 1M+7F, or 1M+10F). We also tested the survival rates of groups of males with the same group size as above (2M, 5M, 8M, or 11M). We found that male flies grouped with 4, 7, or 10 virgin females died more quickly than those grouped with males or with only one female, if no food was provided (Fig. 1A). To confirm that this effect was induced by sexual activity, we used male flies with *fru^M* knockdown by expressing RNAi targeting *fru^M* [30] using *fru^{GALA}*, which rarely showed any courtship behavior when grouped with females during the 2-day test period. We found that such males had the same survival rates when grouped with 10 virgin females or in groups of 11 males (Fig. 1B). We also tested groups of *w^{ts}* flies (1M+10F, or 11M) under low nutrition conditions (1% agar + 1% sugar), and found that individual males housed with 10 females died more quickly than groups of 11 males (Fig. S1). However, we did not find a survival difference in males housed with females (1M+10F) or males (11M) within a 30-day period if regular food was provided. These results indicate that excessive sexual activity with multiple females is costly to male survival under low nutrition conditions but has no effect on survival if fed on regular food.

Although the above results revealed an effect of excessive sexual activity on survival, wild-type male flies would not court until death. We previously showed that activation of ~2,000 *fru^M*-expressing neurons at 29°C using the warmth-activated cation channel dTRPA1 [28] induces continuous courtship behaviors and eventually kills males [26]. Thus we used such a model to couple sex and death in male flies, and study how excessive sexual activity would be fatal and how flies avoid such behavior. We tested the survival rate of groups of 11 males without food, and found that all showed similar survival rates at the permissive temperature (22°C, Fig. 1C); however, half of the *UAS-dTrpA1/+; fru^{GALA}/+* males at 27°C performing continuous courtship behaviors died in ~6 h, much more quickly than control *UAS-dTrpA1/+* males (Fig. 1D). We used 27°C, rather than the 29°C previously used [26], as it allowed much longer survival of *UAS-dTrpA1/+; fru^{GALA}/+* males, suitable for further genetic manipulations (see below). We also tested the survival rate of male flies in

which ~2,000 mushroom body (MB) neurons (labeled by *RI9B03*) were activated at 27°C, and did not find any survival deficit (Fig. 1D). Furthermore, individual *UAS-dTrpA1/+; fru^{GAL4}/+* males survived longer than groups of such males, probably due to the lack of energy-consuming chasing behaviors that were only found in groups of such males (Fig. 1E). We found no survival deficit in *UAS-dTrpA1/+; fru^{GAL4}/+* females at 27°C with all *fru^{GAL4}* neurons activated (Fig. 1F, G). These results indicated that it is the continuous output of sexual behaviors, but not the continuous activation of a large number of neurons (MB neurons in males and females; *fru^{GAL4}* neurons in females), that kills flies in hours.

To reveal how continuous sexual activity results in quick death in males, we recorded individual *UAS-dTrpA1/+; fru^{GAL4}/+* males at 27°C for 10 h, and found a stereotypic behavioral expression from sex to death in all males: initially they closed their wings and did not show any courtship in the first few minutes after transfer to 27°C, and performed intensive courtship behaviors including unilateral wing extension and abdominal bending for 1–2 h. Later, all males began to extend and vibrate the wings bilaterally, indicating abnormal courtship, for 2–3 h, followed by a pre-death phase when males stopped walking, raised both wings, and bent their abdomen to attempt copulation, until motionless and dead (Figs 1H and S2).

As sexual activity is energy-consuming, we tested how 6-h continuous expression of courtship behaviors might change body weight. We found that after 6-h activation of *fru^M* neurons at 27°C, males lost ~20% of their body weight, while 6-h activation of *fru^M* neurons in females, or 6-h activation of MB neurons in males or females, like control *UAS-dTrpA1/+* males or females, lost <10% of their body weight (Fig. S3A–D), which may be due to dehydration during this period. That 6-h activation of *fru^M* neurons induces ~10% more weight loss in males than control flies indicates that continuous sexual activity is associated with energy expenditure that burns ~10% of their body weight in 6 h.

Feeding Alleviates but Does not Prevents Death From Sex

The above results show how continuous activation of *fru^M* neurons accelerates male death if food is not provided, and we next tested whether these males could eat, and if so, if feeding could prevent death. We first transferred fed males to 27°C, allowing feeding on blue food for 30 min. Males with activated *fru^M* neurons or MB neurons, or control *UAS-dTrpA1/+* males, all showed similarly low levels of feeding (Fig. 2A). However, when we transferred fed males to 27°C and allowed 12-h feeding on blue food, we

found that males with activated *fru^M* neurons ate more than males with activated MB neurons or control *UAS-dTrpA1/+* males (Fig. 2B), which may be due to the higher energy consumption and need for food in males with activated *fru^M* neurons. In contrast, when starved males were allowed 12-h feeding on blue food at 27°C, those with activated *fru^M* neurons ate significantly less than males with activated MB neurons or control *UAS-dTrpA1/+* males (Fig. 2C). These results demonstrate that while performing intensive courtship behaviors, males with activated *fru^M* neurons can eat, so feeding and courtship behaviors are not exclusive at least in this context, although the level of feeding is much lower than control males over 12 h. Indeed, males with activated *fru^M* neurons at 27°C survived much longer if provided food [Fig. 2D, 50% of males survived for >36 h with regular food, and ~18 h with 1% agar, compared to ~6 h without any food (Fig. 1D)]. Thus males with activated *fru^M* neurons have a lower level of feeding, and such feeding alleviates but does not prevent death.

Altered Neural Transmission Upon Excessive Sexual Activity

As flies, like most animals, do not court and mate until death, we reasoned that there must be feedback signals upon excessive sexual activity to inhibit further such behaviors. We first used real-time PCR to assess changes in the mRNA expression of major neurotransmitters, as well as neuropeptide F (NPF) that is a reward signal for mating [33, 34], in the brains of *UAS-dTrpA1/+; fru^{GAL4}/+* males after 12-h at 27°C. We found reduced expression of *choline acetyltransferase (ChAT)*, increased expression of the GABA synthesis enzyme *glutamic acid decarboxylase 1 (Gad1)*, and *NPF*, and unaltered expression of *tryptophan hydroxylase (Trh)* (Fig. 3A–D). These results indicated that continuous sexual activity resulted in reduced excitatory acetylcholine (ACh) signaling, and enhanced inhibitory GABA signaling, as well as elevated NPF signaling. To test if these expression changes play a role in survival during the activation of *fru^M* neurons, we knocked down *ChAT*, *Gad1*, *NPF*, or *Trh* in *UAS-dTrpA1/+; fru^{GAL4}/+* males using RNAi [32], and assayed their survival rates at 27°C on food. We found that the lethal effect of activating *fru^M* neurons was alleviated by knockdown of *ChAT*, but accelerated by knockdown of *Gad1* or *NPF* (Fig. 3E). Together, these results indicate that feedback neuromodulation (reduced ACh and increased GABA and NPF) by continuous sexual activity is protective and enhances male survival.

We noted that all the above males at 27°C performed intensive courtship behaviors in groups of 10 males, and found no difference, so we further tested the ejaculation

rates of individual males at 27°C. We found that knock-down of *ChAT*, but not *Gad1*, *NPF*, or *Trh*, significantly decreased the ejaculation rates of *UAS-dTrpA1/+; fru^{GAL4}/+* males (Fig. 3F, G), suggesting a role of ACh-dependent ejaculation in the mortality of these males. To test if excessive ejaculation alone kills males, we activated *Corazonin* (*Crz*) neurons, which induce ejaculation but no other courtship behaviors in isolated males [34, 35], and found no survival deficit during the 15-day test period using either optogenetic (Fig. 3H) or thermogenetic activators (Fig. 3I). Together, these results suggest that continuous display of courtship behaviors other than ejaculation (wing extension and abdominal bending) is more fatal to males, although ejaculation might enhance this fatal effect.

Negative Feedback Prevents Continuous Sexual Activity

To test if prior experience of excessive sexual activity inhibits further courtship behaviors, we first kept individually-housed *UAS-dTrpA1/+; fru^{GAL4}/+* males at 27°C for 4 h, allowing continuous courtship expression, then

transferred them to 22°C for 30 min, 1 h, or 2 h, during which all males stopped courting. We then introduced virgin females to test male courtship behavior after the above experience (Fig. 4A). We found that males with 4-h experience of *fru^M* neuronal activation, even after 0.5–2 h recovery, showed much reduced courtship of virgin females (Fig. 4B–D; the percentage of time males displayed any courtship behavior was ~13% after 1 h of recovery, compared to ~85% in control males). We reasoned that the altered neural transmission described above might be involved in courtship inhibition after continuous courtship expression, so we separately knocked down *ChAT*, *Gad1*, *NPF*, *Trh*, and some other transmission related genes in *UAS-dTrpA1/+; fru^{GAL4}/+* males. All males tested showed reduced courtship levels (compared to the control CI of ~85%) after 4-h continuous courtship expression and 1-h recovery; however, we found that knocking down *NPF* in *fru^M*-expressing neurons significantly alleviated the courtship inhibition effect (Fig. 4E; the CI was ~40% with *NPF* knockdown, and ~10% with intact *NPF*). Together, these results indicate that excessive sexual activity induces expression changes of certain

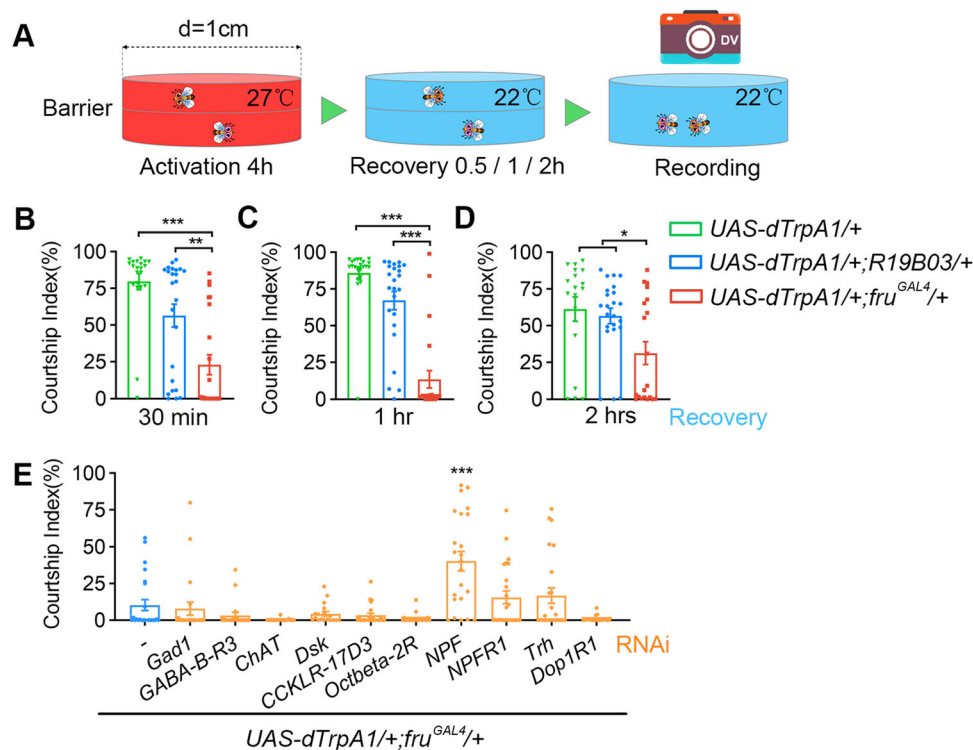


Fig. 4 NPF represses courtship after excessive sexual output. **A** Schematic for experiments assaying courtship inhibition after 4 h of continuous sexual output. **B–D** Reduced male courtship toward virgin females after 4 h of continuous activation of *fru^M* neurons. For 0.5 h recovery, *n* = 21, 24, and 23, *P* < 0.001, Kruskal-Wallis test. ***P* < 0.01, ****P* < 0.001, *post hoc* Dunn's multiple comparisons test. For 1 h recovery, *n* = 22, 24, and 23, *P* < 0.001, Kruskal-Wallis test.

****P* < 0.001, *post hoc* Dunn's multiple comparisons test. For 2 h recovery, *n* = 18, 23, and 20, *P* = 0.032, Kruskal-Wallis test. **P* < 0.05, *post hoc* Dunn's multiple comparisons test. **E** Knocking down *NPF* partially restores male courtship that was suppressed by 4-h activation of *fru^M* neurons. *n* = 24, 22, 20, 24, 18, 23, 22, 23, 24, 24, and 22. ****P* < 0.001, Mann-Whitney U test compared with control without RNAi (blue). Error bars indicate SEM.

transmission genes including *NPF*, which in turn inhibit further courtship behaviors.

The above results used males with *fru^M* neurons forcefully activated for 4 h, but whether this applied to regular males without artificial neuronal activation was uncertain. Thus we placed individual males with 10 virgin females, and recorded their courtship behaviors for 5 h (Fig. 5A). We found that control *fru^{GAL4/+}* males, as well as males with *Gad1*, *NPF*, or *Trh* knocked down, mated multiple times; however, males with *ChAT* knocked down in *fru^M* neurons rarely courted and never mated (Fig. 5B). Further analysis of their courtship behaviors showed that

control *fru^{GAL4/+}* males, as well as those with *Trh* knocked down, reduced their further courtship after each mating, such that their CI was $\sim 15\%$ after 4 successive matings (Fig. 5C, CI-5). Although males with *Gad1* knocked down reduced their courtship after mating, this reduction was not as severe as in control males (Fig. 5C, CI-5; $\sim 48\%$ after 4 successive matings). Surprisingly, males with *NPF* knocked down displayed indistinguishable levels of courtship even after 4 successive matings (Fig. 5C; initial CI-1 $\sim 83\%$, and CI-5 after 4 matings $\sim 73\%$, not significantly different). Furthermore, while control *fru^{GAL4/+}* males and those with *Trh* knocked down had an average of ~ 4

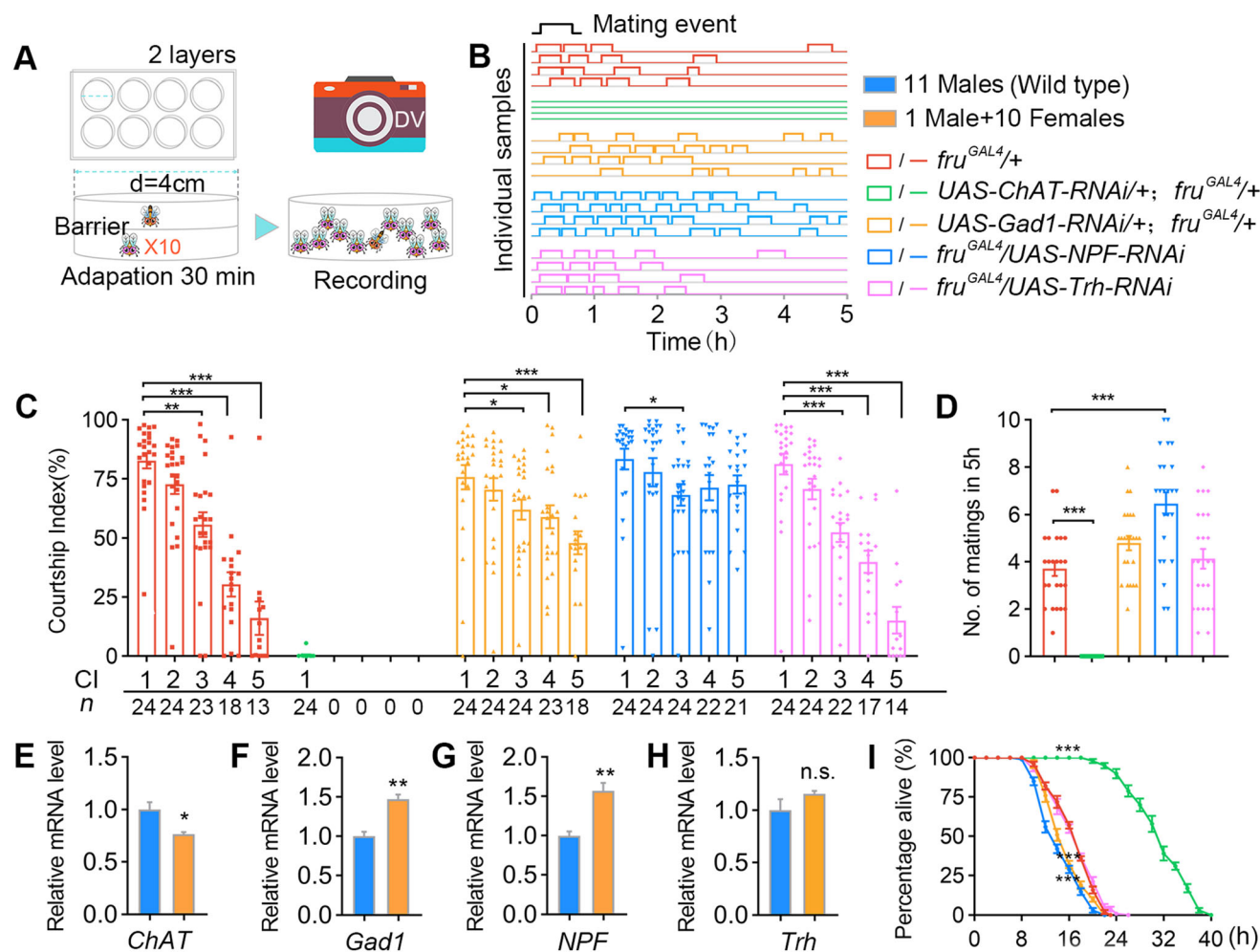


Fig. 5 GABA and NPF inhibit excessive sexual activity in wild-type males. **A** Behavioral setup allowing up to 10 matings (1M+10F) in a 5-h test. **B** Examples of male mating activity in 5 h. Genotypes as indicated by colors below. **C** Courtship indices of males in the 1M+10F environment. CI-1 indicates courtship in the first 10 min after the male and females are introduced; CI-2, CI-3, CI-4, and CI-5 indicate 10-min courtship after the 1st, 2nd, 3rd, and 4th matings, respectively. Kruskal-Wallis test, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, *post hoc* Dunn's multiple comparisons test. *N* as indicated below. **D** Numbers of matings by males during the 5-h test. Knockdown of *NPF* significantly increases the number of matings.

n = 24 per group, $P < 0.001$, one-way ANOVA, $***P < 0.001$, *post hoc* Tukey's multiple comparisons test. **E-H** Wild-type males housed with 10 virgin females have reduced *chat* expression and elevated *Gad1* and *NPF* expression in their brains compared with groups of 11 males. Flies were housed in vials without food for 12 h at 25°C. *N* = 3 per group. n.s., not significant, $*P < 0.05$, $**P < 0.01$, unpaired *t*-test. **I** Knocking down *Gad1* or *NPF* accelerates, while knocking down *ChAT* slows down death in males housed with 10 virgin females without food. *n* = 10 for each. Each *n* consists of 10 vials of 1M+10F. $***P < 0.001$ at 16 h, Mann Whitney U test. Error bars indicate SEM.

successive matings with 10 virgin females within 5 h (3.7 ± 0.3 and 4.1 ± 0.4 , respectively), males with *NPF* knocked down showed more successive matings (6.5 ± 0.5 , Fig. 5D). Males with *Gad1* knocked down showed slightly more successive matings (4.8 ± 0.3), but not significantly different from controls (Fig. 5D). Together, these results reveal crucial roles of GABA and NPF in inhibiting further sex after excessive sexual experiences.

As we showed that knockdown of *NPF* in *fru^M* neurons eliminated the courtship inhibition effect after excessive sexual activity, we tried to identify the specific *NPF* and *fru^M* co-expressing neurons involved in courtship inhibition. Anti-NPF staining in male and female brains revealed three pairs of male-specific *NPF* neurons (Fig. S4A, B). These neurons were also faithfully labeled through genetic intersectional labelling between *fru^{LexA}* and *NPF-GAL4* (Fig. S4C, D). Consistent with our findings, a recent study uncovered the role of these male-specific *NPF* neurons in inhibiting courtship [36].

We further found that, after 5 h of experience with 10 virgin females, the mRNA level of *ChAT* decreased, while those of *Gad1* and *NPF* increased in the brains of wild-type males (Fig. 5E–H), which is consistent with our previous findings in males with activated *fru^M* neurons. We then measured the survival rate of males housed in the 1M+10F environment, and found that males with *ChAT* knocked down that rarely courted survived much longer than control *fru^{GAL4}/+* males, while males with *Gad1* or *NPF* knocked down died slightly quicker (Fig. 5I), probably due to their continuously high level of courtship and mating. Thus, using two independent genetic models, we showed that ACh, GABA, and NPF are key modulators that respond to excessive sexual activity, and prevent continuous sex that may result in male death.

Discussion

In this study, we established a model in which *Drosophila* males perform continuous sexual behaviors until death, mimicking the behavior of semelparous animals during the mating season. We also identified ACh, GABA, and NPF as key feedback modulators in the brain that respond to excessive sexual activity and prevent semelparous mating.

Previous studies imply that mating does not affect lifespan in *Drosophila* [23, 24]. We showed that while single mating (1M+1F) does not affect survival, multiple matings (1M+4F, 1M+7F, and 1M+10F) do affect male survival, at least under lower nutrition conditions, and this is probably due to the energy expenditure incurred during courtship display and mating. In an extreme example, males with activated *fru^M* neurons performed intense courtship behaviors for 6 h and lost $\sim 10\%$ of their body

weight, compared to males that did not perform courtship behaviors. However, whether this effect is specific to sexual behaviors or could also be caused by other kinds of strenuous physical activity still needs further investigation.

A striking phenomenon was that while males with activated *fru^M* neurons at 27°C performed intensive courtship behaviors, they were able to eat, although significantly less, consistent with a previous finding that sexually-aroused males with ~ 23 pairs of male-specific P1 neurons activated have reduced feeding [37]. This phenomenon indicates that sexual and feeding behaviors are not mutually exclusive. Indeed, males with activated *fru^M* neurons at 27°C survived ~ 30 h longer if provided food (median survival time ~ 36 h with food, and 6 h without food).

Males of most species decrease further sexual activity after mating to prevent excessive sex; such a process must evolve feedback signals that respond to mating, or changes in metabolism or immunity, and decrease sexual drive through the nervous system. Indeed, we found a spontaneous negative feedback mechanism used by male flies to protect them from excessive sex. On one hand, the enzyme for the generally excitatory neurotransmitter ACh, *ChAT*, was lower and the enzyme for the inhibitory neurotransmitter GABA, *Gad1*, was higher in males with activated *fru^M* neurons or experienced with 10 virgin females. On the other hand, knockdown of *ChAT* in *fru^M* neurons alleviated, and knockdown of *Gad1* in *fru^M* neurons accelerated death in the above males. These results reveal how the nervous system responds to excessive sexual activity by balancing the excitatory and inhibitory signals.

We also found that the neuropeptide NPF as an important modulator that prevents excessive sexual activity. First, *NPF* expression was increased in males with activated *fru^M* neurons or males housed with 10 virgin females, consistent with previous notions that NPF is a reward signal for mating [33, 34]. Second, knockdown of *NPF* partially restored courtship behaviors in males with *fru^M* neurons activated for 4 h. Furthermore, wild-type males with *NPF* knocked down failed to decrease courtship even after 4 successive matings, such that they could mate up to 10 times (the maximum, as there were 10 females in total) in 5 h. Third, knockdown of *NPF* accelerated the deaths caused by *fru^M* neuronal activation or grouping with 10 females. Thus, while *NPF* functions as a reward signal for mating, its cumulative increase after mating experiences inhibits further sex. Recently, Liu and colleagues found that disrupting *NPF* signaling increases sexual activity, and identified subsets of *NPF* and *fru^M* co-expressing male-specific neurons that inhibit courtship [36]. Meanwhile, another recent study by Zhang and colleagues found *NPF* signaling to be courtship-promoting, although they were involved in recurrent circuitry for

sexual satiety [22]. Our results are generally consistent with the former study that NPF functions as a repressor of courtship, at least when it becomes excessive, although these studies used different behavioral protocols. Future study of how NPF possesses dual roles, first as a reward signal, then an inhibitor for further sex, probably through distinct NPF and/or NPF_R neurons, would help to interpret these discrepancies.

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Conflict of interest All authors claim that there are no conflicts of interest.

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