Social Experience Shapes Fighting Strategies for Reproductive Success

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Abstract

Social isolation generally increases aggression but decreases mating competition, resulting in an intricate and ambiguous relationship between social experience, aggression, and reproductive success. In male *Drosophila*, aggression is often characterized by lunging, a frequent and comparatively gentle combat behavior. Here, we establish a behavioral paradigm for studying a less frequent but more vigorous fighting form known as tussling. We discover that while social enrichment decreases lunging, aligning with past observations, it heightens the more forceful tussling behavior. These two forms of aggression rely on different olfactory receptor neurons, specifically Or67d for lunging and Or47b for tussling. We further identify three pairs of central pC1 neurons that specifically promote tussling. Moreover, shifting from lunging to tussling in socially enriched males facilitates better territory control and mating success, mitigating the disadvantages associated with aging. These findings reveal how social experience shapes fighting strategies to optimize reproductive success.

Keywords: Social experience; reproductive competition; aggression; *Drosophila melanogaster*; neural circuit; behavioral strategy

Introduction

Competition for resources such as territory, food, and mates is crucial for animal survival and reproduction [1-4]. Among diverse competitive strategies, physical combat is a prevalent form of fighting to repel rivals and regulated by both genetic factors and social environment [1, 5]. Understanding how social experience influences combat behaviors in animals to maximize their survival and reproduction remains a fundamentally important yet inadequately understood question.

In the fruit fly, *Drosophila melanogaster*, inter-male aggression includes low-intensity fights such as lunging, in which one fly rears up on its hind legs and snaps down onto its opponent, as well as high-intensity fights like tussling, where two males tumble over each other [6-8]. It is noteworthy that the majority of studies focus on the low-intensity lunging behavior, owing to its higher frequency and ease of observation. Substantial studies have identified regulatory genes and the neural circuit underlying the lunging-based aggressive behaviors [7, 9]. In particular, the male-specific volatile pheromone 11-*cis*-vaccenyl acetate (cVA) acutely promotes lunging behavior via its olfactory receptor Or67d [10]. Interestingly, chronic cVA exposure in socially-enriched males reduces the level of lunging via another olfactory receptor Or65a [11]. Consistently, it has been found that social isolation increases lunging frequency by altering the expression of genes such as *Cyp6a20* [12], *Drosulfakinin* (*Dsk*)[13], and *Tachykinin* (*Tk*) [14]. Social isolation also increases male aggression in mice [15, 16] and even in humans [17]. In mice, the TK homologue Tachykinin 2 (Tac2) responds to social isolation and promotes inter-male aggressive behaviors [16]. These findings contribute to the consensus that social isolation escalates aggression, whereas social enrichment mitigates it in animal models.

Meanwhile, social enrichment increases male mating success by enhancing the sensitivity of *Or47b*-expressing olfactory receptor neurons (ORNs) to fly pheromones common to both sexes [18, 19]. Artificially activating *Or47b* ORNs also improves mating advantage in a competitive assay [20]. Group housing and age-dependent juvenile hormone (JH) jointly increase the expression of the male-specific proteins from *fruitless* (fru^M), which is a master gene for male courtship behaviors [21-23], in the *Or47b*-expressing ORNs [19, 24, 25]. Notably, courtship levels in single-housed and group-housed wild-type males are not significantly different [26]. In fact, group-housing decreases excitability of the courtship-promoting P1 neurons [27], which express both terminal genes in the sex determination hierarchy, *fru* and *doublesex* (*dsx*). These findings raise a question on how social enrichment, which suppresses lunging-based aggression and does not increase courtship intensity, would facilitate mating competition.

Here, we establish a behavioral paradigm for studying inter-male tussling in *Drosophila* and discover that this infrequent yet intense form of combat is enhanced by social enrichment and is crucial for territory dominance and mating competition. This process requires olfactory receptor Or47b, but not Or67d or Or65a. We further identify

three pairs of central *dsx*-expressing pC1 neurons that specifically promote tussling. Moreover, we demonstrate that socially enriched males can overcome mating disadvantages associated with aging.

Results

A Paradigm for High-intensity Aggression in *Drosophila*

While studying the lunging-based aggression in *Drosophila*, we occasionally observed the high-intensity tussling behavior in male flies. We reasoned that the intensity, instead of the frequency, could be decisive for a combat. Thus, although tussling is a less frequent behavior, it could serve a vital function.

To induce a higher level of tussling for further manipulation, we first introduced a fixed live virgin female in the same behavioral chamber for assaying lunging. The female fly was immobilized in the middle of the food, with its head embedded in the food and its body exposed to enhance male competition (Figure 1A). We found that males that were group-housed for 7 days showed a significantly higher frequency of tussling behavior and reduced latency to tussle in the presence of an embedded virgin female (Figure 1B). We next compared tussling in male flies of different ages. We observed a significantly higher proportion of 14-day-old males raised in groups that showed tussling behavior during the 2-hour observation period, compared to the 7-day-old males (Figure 1B). Furthermore, 2-day-old males never displayed tussling behavior. In contrast, 14-day-old males showed lowed level of lunging behavior than 7-day-old males did (Figure 1—figure supplement 1A and 1B). Previous studies have shown that, in addition to mating resources, food quality is another crucial factor affecting aggressive behaviors [28]. Therefore, we next assayed tussling behavior in 14-day-old males in the presence of an embedded virgin female with different concentrations of sucrose or yeast in the food during the behavioral test. We found that these males displayed similar levels of tussling behavior with different concentrations of sucrose in the food (Figure 1C). On the contrary, male tussling intensity increased significantly with higher yeast concentrations such that more than 70% of males displayed this high-intensity fighting behavior (Figure 1D, see Video 1). Taken together, these results establish an efficient behavioral paradigm for the high-intensity tussling behavior and indicate that older males display more tussling but less lunging than younger ones.

Social Enrichment Inhibits Low-intensity Lunging but Enhances High-intensity Tussling

Previous studies have shown that social enrichment inhibits aggressive behaviors, specifically the low-intensity lunging, in male flies. Therefore, we wondered whether social enrichment might also inhibit the high-intensity tussling behavior in males. To answer this question, we compared both fighting behaviors in single-housed (SH) and group-housed (GH) 14-day-old males (Figure 2A). Lunging and tussling events from each group of representative samples were exhibited in raster plots for comparison

(Figure 2B). We scored lunging behavior in the first 10-min of test, due to its relatively higher occurrence, and tussling behavior during the whole 2-hour observational period since males take much longer time to initiate tussling (Figure 1D). Interestingly, we found that while SH males performed more frequent lunging behavior in 10 min than GH males did, which is consistent with previous findings, they rarely showed tussling behavior. In contrast, GH males displayed a large number of tussling events within 2 hours. Note that a tussling event may last up to several minutes, indicative of its high intensity, in contrast to lunging events that were often transient (Figure 2B). Statistical analysis of tussling and lunging behaviors showed that GH males exhibited reduced tussling latency and enhanced tussling frequency, in addition to increased lunging latency and decreased lunging frequency, compared to SH males (Figure 2C-F). These results indicate that social enrichment inhibits low-intensity lunging but promotes high-intensity tussling in male flies.

***Or47b* ORNs Mediate Social Experience-induced Tussling Behavior**

To further investigate the sensory basis underlying the opposite regulation of lunging and tussling behaviors by social experience, we tried to examine the role of cVA-sensitive neurons, as well as other *fru*^{M}-expressing ORNs, in mediating intermale aggression. Two cVA sensory neurons, *Or67d* and *Or65a* ORNs, play crucial roles in enhancing the lunging-based aggression in SH males, in which *Or67d* ORNs mediate acute cVA stimulation and promote male aggression[10], while *Or65a* ORNs mediate chronic cVA stimulation and inhibit male aggression in GH males [11]. In addition, the *fru*^{M}-expressing *Or47b* ORNs are sensitive to a class of pheromones common to both sexes, such as palmitoleic acid (PA), and their sensitivity is enhanced under group rearing conditions[19]. *Ir84a* ORNs, which are also *fru*^{M}-positive, are involved in the perception of mixed food odors and promote male courtship behavior[29]. We silenced *Or67d*, *Or65a*, *Or47b* or *Ir84a* ORNs by expressing the inwardly rectifying potassium channel Kir2.1 and assayed lunging and tussling behaviors in 14-day-old males. We found that silencing *Or47b* ORNs, but not *Or67d*, *Or65a* or *Ir84a* ORNs, significantly decreased male tussling, compared to the control (Figure 3A, B). In contrast, silencing *Or67d* ORNs, but not other ORNs, significantly reduced male lunging (Figure 3C, D). These loss-of-function results suggest that *Or47b* ORNs mediate male tussling behavior, whereas *Or67d* ORNs regulate male lunging behavior.

To elucidate the potential role of the Or47b receptor in mediating tussling behavior, we knocked down its expression in *Or47b* ORNs. Our results showed a significant decrease in male tussling behavior when the Or47b receptor expression was reduced (Figure 3E, F), indicating that the functionality of *Or47b* ORNs in tussling is dependent on the Or47b receptor. As previous studies suggested that the male-specific *fru*^{M} sustains *Or47b* expression in an activity-dependent manner [19], we next knocked down *fru*^{M} expression in *Or47b* ORNs and assayed male tussling. The efficiency of *Or47b* and *fru*^{M} RNAi knockdown was both validated by quantitative PCR (Figure 3—figure supplement 1A and 1B). We observed a slight increase of tussling latency in *fru*^{M}

161 knocked-down males, although not significantly different from the control (Figure 3E,
162 F), suggesting that Or47b, rather than Fru^M, plays a more crucial role in mediating male
163 tussling.
164 To further confirm that the effect of social enrichment on tussling depends on the
165 activity of *Or47b* ORNs, we artificially activated *Or47b* ORNs by expressing the
166 bacterially derived sodium channel (NaChBac)[30] in 14-day-old SH males. We found
167 that these SH males with enhanced activity of *Or47b* ORNs showed a significant
168 increase in tussling behavior compared to control SH males. Interestingly, the level of
169 tussling in these SH males with activated *Or47b* ORNs was indistinguishable from that
170 in GH males (Figure 3G, H). These gain-of-function results indicate that social
171 enrichment acts through *Or47b* ORNs to promote male tussling. Consistent with this,
172 we observed substantially more downstream neurons of *Or47b* ORNs in GH males than
173 those in SH males (Figure 3—figure supplement 2A and 2B), as visualized by the
174 trans-Tango technique [31].
175
176 **Distinct Central Brain Neurons for Lunging and Tussling**
177 We next set out to identify the central neurons involved in regulating the high-intensity
178 tussling. We found that silencing the mushroom body neurons via Kir2.1 did not affect
179 male tussling, suggesting that the experience-dependent tussling behavior is not
180 dependent on the classical learning and memory center. Surprisingly, inactivation of
181 the previously-identified aggression-promoting $P1^a$ and TK neurons did not affect
182 tussling behavior (Figure 4A, B). These results imply that the central circuit for tussling
183 behavior may be distinct from the previously reported aggression circuits. As *dsx*-
184 expressing pC1 neurons, also termed P1 if co-expressing fru^M, are crucial for sexual
185 and aggressive behaviors in both males and females, we also silenced two subsets of
186 pC1 neurons ($pC1^{SS1}$ and $pC1^{SS2}$), whose functions were previously characterized in
187 females but not in males. We found that inactivation of $pC1^{SS2}$ but not $pC1^{SS1}$ neurons
188 significantly reduced the tussling behavior in male flies (Figure 4A, B). The cell bodies
189 of $pC1^{SS2}$ and $P1^a$ neurons are both located in the center of posterior brain and project
190 to overlapping but distinct parts of the lateral protocerebral complex (Figure 4C-E).
191 These results suggest that $pC1^{SS2}$ neurons may be key central neurons regulating
192 tussling behavior.
193 To further compare the function of $P1^a$ and $pC1^{SS2}$ neurons in regulating male
194 behaviors, we performed optogenetic activation experiments on these neurons
195 expressing *CsChrimson*. Compared to control male pairs that did not show any
196 significant change upon red light stimulation (Figure 4F-H), optogenetic activation of
197 $P1^a$ neurons induced acute courtship behavior as indicated by unilateral wing extension
198 (UWE), and upon red light removal, there was a significant increase in lunging but not
199 tussling (Figure 4I-K), consistent with previous findings. In contrast, optogenetic
200 activation of $pC1^{SS2}$ neurons induced strong and acute tussling behavior, with more
201 than 50% of the time during red light in tussling, and the tussling behavior ceased
202 immediately upon removal of red light (Figure 4L, see Video 2). Activation of $pC1^{SS2}$

203 neurons did not significantly affect either male lunging or courtship (Figure 4M, N).
204 These results indicate that $pC1^{SS2}$ neurons acutely promote the high-intensity tussling
205 behavior.
206 Previous studies have revealed a crucial role of $P1^{a}$ neurons in promoting a
207 persistent internal state that enhances male aggression. To test whether $P1^{a}$ neurons can
208 promote both lunging and tussling behaviors, we further performed thermal activation
209 experiments. We found that activation of $P1^{a}$ neurons via dTrpA1 at 30˚C induced male
210 tussling, just like activation of $pC1^{SS2}$ neurons did (Figure 4—figure supplement 1A
211 and 1B). As thermal activation but not optogenetic activation of $P1^{a}$ neurons induced
212 tussling, we reasoned that vision might be a key factor for this discrepancy, since all
213 optogenetic experiments were performed in dark or red light. Thus, we re-performed
214 these thermal activation experiments in dark and found that activation of $pC1^{SS2}$ but not
215 $P1^{a}$ neurons induced tussling, although the level of tussling by $pC1^{SS2}$-activated males
216 was lower compared to that in light, suggesting a crucial role of vision in male tussling.
217 These optogenetic and themogenetic activation experiments suggest that $P1^{a}$ neurons
218 can promote both lunging and tussling behaviors, but their function on tussling requires
219 visual stimulation, while $pC1^{SS2}$ neurons play a more direct role in acutely promoting
220 male tussling.
221
222 **Territory and Mating Advantage in Tussling-favored Experienced Males**
223 As our results showed that GH males and SH males were more likely to perform the
224 low-frequency, high-intensity tussling and the high-frequency, low intensity lunging,
225 respectively, we wondered which fighting strategy was more practical in the
226 competition for territory and mating resources.
227 To address this question, we first designed an experimental paradigm for territory
228 competition (Figure 5A). A circular territory, which is provided with food and a fixed
229 virgin female and separated by a wall with a small door open to the outer area, was
230 competed by two tester males. We manually analyzed the first 10 winning events; each
231 was defined as one male successfully displacing the other from the inner resource area.
232 The winning index was calculated as the proportional difference in the number of wins
233 between each pair of males. In control groups such as two GH males of the same age,
234 we observed some 'winner takes all' phenomenon (one male won nearly all of the 10
235 encounters), but overall, the winning index was close to zero. We further found that
236 while GH 7-day-old males (G7) won slightly, but not significantly, more than SH 7-
237 day-old males (S7) (Figure 5B), older G14 males had a significant advantage over the
238 S14 males (Figure 5C and Figure 5—figure supplement 1, see Video 3).
239 Next, we conducted further investigations into the role of social experience and the
240 two types of fighting behaviors in mating competition (Figure 5D). Two males and one
241 virgin female were loaded into the behavioral chamber at the same time and recorded
242 for courtship behaviors. We found that G7 males had no significant advantage over S7
243 males (Figure 5E); however, G14 males displayed a significantly increased mating
244 advantage over S14 males (Figure 5F, see Video 4). Together, these results suggest that

the tussling-favored GH males are more competitive in territory control and mating compared to the lunging-favored SH males.

The above results showed that GH males were more competitive compare to SH males, especially in older males, suggesting that social experience may play a potential role in altering age-related mating competition. To test this hypothesis, we first tested mating competition between males of different ages under the same rearing conditions. The results showed that younger males, whether reared in group or isolation, had a greater mating advantage compared to older males (G7 > G14, S7 > S14, G14 > G21, S14 > S21) (Figure 5G). These results indicate that there is generally a disadvantage in mating competition associated with aging. We next investigated whether the mating advantage induced by social experience could compensate for the mating disadvantage caused by aging. We tested pairs of males with different ages and rearing conditions (G7 vs. S14, S7 vs. G14, G14 vs. S21, S14 vs. G21) for mating competition. We found that while 14-day-old males were less competitive than 7-day-old males regardless of the rearing condition, indicative of the aging-related disadvantage, 21-day-old GH males were significantly more competitive than 14-day-old SH males (G21 > S14) (Figure 5H). These results indicate that long-term social enrichment in older males increases their territorial and mating competition, thus overcoming their mating disadvantage associated with aging (Figure 6).

Discussion

Regulation of physiology and behavior by social experience is an important feature across animal species. Here, we found that social experience shapes the fighting strategies of male flies, in which GH males tend to reduce the low-intensity lunging and increase the high-intensity tussling, which enhances their territorial and mating competition. We further showed that the two forms of fighting are mediated by distinct sensory and central neurons. The complex implications of these findings are discussed below.

Previous studies have found that social isolation generally enhances aggression but decreases mating competition in animal models, presenting a paradox between social experience, aggression and reproductive success. Our results resolved this paradox by dissecting aggressive behaviors into the well-studied, frequently observed lunging behavior and the less frequent but high-intensity tussling behavior. We established a new behavioral paradigm for studying tussling behavior and revealed that social experience oppositely regulates lunging and tussling. It is important to note that the frequency of lunging decreases while the frequency of tussling increases from 7-day-old to 14-day-old males, suggesting a shift in fighting strategy with age. Our results further suggest that social experience may enhance male mating competition by shifting their fighting strategy to the low-frequency, high-intensity tussling, which may be more effective in combat, although direct evidence is still lacking. Interestingly, we found that the mating advantage gained through social enrichment can even offset the mating

disadvantage associated with aging, further supporting the vital role of shifting fighting strategies in experienced, aged males. This dissection of the two fighting forms rectifies our understanding of how social experiences regulates aggression and reproductive success.

Our results revealed distinct neural circuits underlying the two forms of fighting, spanning from primary sensory neurons to central regulatory neurons. Specifically, silencing the cVA-sensing Or67d neurons reduced male lunging but not tussling, whereas inactivating Or47b neurons impaired male tussling but not lunging. Notably, Or47b neurons respond to pheromones common to both sexes, and the sensitivity of these sensory neurons is enhanced by social enrichment [18, 19, 21]. Unlike the Or67d-dependent lunging, which is primarily male-induced and socially inhibited, the Or47b-dependent tussling is promoted by social enrichment with either male or female flies. Recently, it has been found that female flies increase aggression towards mating pairs or mated females, depending on Or47b and Or67d, respectively [32, 33]. These results suggest a common function of Or47b and Or67d neurons in both sexes for regulating aggression. However, whether these neurons mediate different fighting patterns in females and how they respond to social experience in females remain unknown. Nevertheless, our results on these two sensory pathways in males provide crucial evidence for dissecting their functions.

Regarding central regulatory neurons, we identified a novel class of pC1 neurons, $pC1^{SS2}$, that specifically promote male tussling. In male flies, there are ~60 pairs of *dsx*-expressing pC1 neurons, including the well-characterized $P1^{a}$ neurons that also express fru^{M} [34, 35]; however, there are only three pairs of $pC1^{SS2}$ neurons whose function is both necessary and sufficient for inducing tussling in males. The three pairs of $pC1^{SS2}$ neurons may have distinct function from $P1^{a}$ neurons in several ways. (1) $P1^{a}$ neurons promotes both male courtship and aggression [36, 37], whereas $pC1^{SS2}$ neurons specifically promote aggression but not courtship. A recent study also identified $pC1^{SS2}$ neurons as aggression-promoting, but their roles in different fighting modes are not depicted [38]. (2) $P1^{a}$ neurons promotes both lunging and tussling, although their function on tussling requires additional visual stimulation. In contrast, $pC1^{SS2}$ neurons specifically promote tussling, and the presence of light enhances but is not absolutely necessary for tussling. These results align with previous findings that visual stimuli amplify P1 activation and related behaviors [39-41], and visual stimulation is important for male aggression [42, 43]. (3) Male lunging is induced after $P1^{a}$ activation; whereas male tussling is induced during $pC1^{SS2}$ activation. These results suggest that $P1^{a}$ neurons may not directly regulate aggression but instead induce a persistent state that promote aggression in general, while $pC1^{SS2}$ neurons may play a more direct role in mediating tussling. Future studies are needed to further dissect the seemingly parallel sensory and central neuronal pathways involved in lunging and tussling, and to explore how these pathways interact during social experience and aging to optimize reproductive success.

Materials and Methods

Fly Stocks

Flies were maintained at 22 or 25 °C in a 12 h:12 h light: dark cycle. *Canton-S* flies were used as the wild-type strain. *Or67d-GAL4* (BDSC_9998), *Or65a-GAL4* (BDSC_9993), *Or47b-GAL4* (BDSC_9984), *IR84a-GAL4* (BDSC_41734), *TK-GAL4* (BDSC_51795), *R19B03-GAL4* (BDSC_49830), *UAS-dTrpA1* (BDSC_26263), *UAS-Kir2.1* (BDSC_6595), *UAS-CsChrimson* (BDSC_82181), *UAS-myrGFP* (BDSC_32198) and *trans-Tango* (BDSC_77124) flies were obtained from Bloomington *Drosophila* Stock Center (BDSC). *UAS-NaChBac* [30], fru^{GAL4} [44], $P1^{a}$-*spGAL4* [45], $pC1^{SS1}$-*spGAL4* [46], $pC1^{SS2}$-*spGAL4* [46] and *UAS-fruMi* [47] were used as previously described. *UAS-Or47b RNAi* (THU_2599) was obtained from TsingHua Fly Center.

Male aggression assay

For the aggression assays presented in Figure 1, the prepared food was evenly spread into the chambers of two paradigms. There was no female in the traditional paradigm, while a 3-5 day old virgin female was gently fixed in the center of the food with its abdomen outside in the new paradigm. Then two male testers were placed into the upper chambers separated from the food (with or without a fixed female) by a film barrier. All testers were loaded by cold anesthesia. After a 30-minute adaptation, the film was removed, and the aggressive behavior was recorded for 2 hours. The aggression assays in Figure 2 and 3 were performed by using the new paradigm. The aggression assays in Figure 1—figure supplement 1 was performed by using the traditional paradigm.

The food recipes for the male aggression assay were listed below. A mixture of 2.5% yeast, 2.5% sucrose and 1% agar dissolved in apple juice was used for Figure 1B; a mixture of 2.5% yeast, different concentrations of sucrose (0, 2.5%, 5% or 10%) and 1% agar dissolved in apple juice was used for Figure 1C; a mixture of different concentrations of yeast (0, 2.5%, 5% or 10%), 2.5% sucrose and 1% agar dissolved in apple juice was used for Figure 1D; A mixture of 10% yeast and 1% agar dissolved in apple juice was used for other experiments.

dTrpA1-mediated activation experiments

For thermogenetic activation assay showed in Figure 4—figure supplement 1, two 5-7 days old males were loaded into the circular chamber (d=15mm) under cold anesthesia and separated with a transparent film. Before the recording start, the chamber was transferred to the recording platform at a specific temperature (22°C or 30°C) for 30 minutes adaptation. After that, the film was removed, and the behavior was recorded for 1 hour. The 10-minute video after film extraction was used for behavioral analysis.

CsChrimson-mediated optogenetic activation experiments

370 For optogenetic activation assay showed in Figure 4, males were raised at 25°C on
371 standard fly food, collected after eclosion and housed in the dark for 5-7 days on food
372 containing 0.4 mM all *trans*-Retinal (MFCD00001550, Sigma-Aldrich, St. Louis, MO).
373 The specific stimulation protocols are showed in the schematic (Figure 4F). In brief,
374 Two 5-7 days old males were loaded into the circular chamber (d=10mm) under freeze
375 anesthesia and separated with a transparent film in the dark. Then the chamber was
376 moved on the plane light source (IR channel: 865nm and red-light channel: 630nm) for
377 15 mins adaptation. Before video recording, the IR light channel was switched on and
378 adjusted to the appropriate brightness for recording. The film was removed before the
379 recording start, with the red-light channel switched on (constant red light, 0.02
380 mW/mm^2) during the 2nd and 5th minutes of the experiment. The statistics for the
381 tussling, lunging, and unilateral wing extension (UWE) are as follows. For tussling time
382 and UWE time, we used LifesongX software to calculate the percentage of the total
383 time these behaviors occurred per minute. For lunges, we counted the number of
384 lunging occurrences per minute.
385
386 **Territorial competition experiment**
387 For the territorial competition experiment in Figures 5A-C, the behavioral setup was
388 produced using 3D printing technology, as illustrated in the schematic, with a
389 transparent cover plate placed on the top. The preparation of food and female were the
390 same as the new aggression paradigm. The female was fixed in the center of the food
391 and two male testers were placed separately in the two small chambers on both sides.
392 Male wings were marked 24 hours before the experiment. Before initiating the
393 experiment, the two barriers were closed. After a 30-minute adaptation, the barriers
394 were opened to allow the males access to the central large chamber, and were closed
395 once the males went inside, and the territorial behavior was recorded for 2 hours. The
396 winning index stand for territorial control ability was calculated by the following
397 example formula: (num. of wins by GH males – num. of wins by SH males)/10
398 encounters * 100%. The first 10 encounters occurring after 1 hour of recording were
399 used for statistical analysis. The winning event was defined as a male driving the other
400 male out of the food area.
401
402 **Mating competition experiment**
403 For the mating competition experiment shown in Figure 5D-H, the wings of males were
404 marked 24 hours prior to the experiment using the same method described above.
405 During the experiment, a 3-5 days old virgin female was placed into the chamber under
406 freeze anesthesia and covered with a transparent film. Then the unmarked males were
407 loaded into the chamber in the same manner and also covered with a transparent film.
408 Finally, the marked males were loaded. After 30 minutes adaptation, two films were
409 removed, and the behavior was recorded for 1 hour. The chambers without successful
410 copulation during the 1-hour period were excluded from further analysis. Each assay
411 included 120 efficient chambers at least, and 10 random chambers were measured as

one sample. The copulation advance index was calculated by the following example formula: (num. of GH winners – num. of SH winners)/10 chambers * 100%). The winner was defined as the male that completed the first copulation with female.

Quantitative Real-time PCR

Adult fly samples were frozen in liquid nitrogen and decapitated by vortex. The heads were then separated from the bodies using metal sieves. Each sample, consisting of 40 frozen heads, was used for total RNA preparation 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 $2^{\wedge-\Delta CT}$ method using *actin* as control. Each sample was run in triplicate. Each experiment was repeated three times using independent sets of genetic crosses. Primers used for RT-qPCR quantification were:

actin forward: 5′-GTCGCGATTTAACCGACTACCTGA-3′
actin reverse: 5′-TCTTGCTT CGAGATCCACATCTGC-3′
fru-P1 forward: 5′-GTGTGCGTACGTTTGAGTGT-3′
fru-P1 reverse: 5′-TAATCCTGTGACGTCGCCAT-3′
Or47b forward: 5′-CAAATCTCAGCCTTCTGCGG-3′
Or47b reverse: 5′-GATACTGGCACAGCAAACTCA-3′

Tissue Dissection, Staining, and Imaging

Brains 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 day at 4°C, then washed in PAT, and incubated in secondary antibodies diluted in 3% NGS for 4 h at room temperature and 1 day at 4°C. The tissues were then washed thoroughly in PAT and mounted for imaging. The antibodies used were Rabbit anti-HA (1:1000), Mouse anti-GFP (1:1000), nc82(1:50) and Donkey anti-Mouse, Alexa Fluor 488 (1:500), Donkey anti-Rabbit, Alexa Fluor 555 (1:500). Samples were imaged on Zeiss 900 confocal microscopes using ZEN and processed with Fiji software.

Brain image registration

The protocol for standard brain registration is described previously [48]. Confocal images for brains containing $P1^{a}$ neurons and $pC1^{SS2}$ neurons were registered onto a standard reference brain, which was generated by the images of nc82 stained brains.

Statistics

Experimental flies and genetic controls were tested under the same conditions, and data were collected from at least two independent experiments. Statistical analysis was performed using GraphPad Prism 9 (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 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 group matched data that were normally-distributed, the Paired *t* test signed rank test was used for pairwise comparisons, and RM one-way ANOVA was used for comparison among multiple groups, followed by RM one-way ANOVA multiple comparisons. For group matched data that were not normally-distributed, the Wilcoxon matched-pairs signed rank test was used for pairwise comparisons, and the Friedman test was used for comparison among multiple groups, followed by Dunn's multiple comparisons. For qPCR experiments, the average relative expression of three independent experiments was analyzed using Mann-Whitney test. For one group compared with zero, we used one sample *t* test. The Chi-square test was used to compare male tussling percentage between two groups.

Data availability

All data generated or analyzed during this study are included in the article and its supporting information files. Fly stocks and reagents used in this study are available from the corresponding author upon reasonable request.

Author contributions

C. Gao, Q. Peng and Y. Pan conceived the study; C. Gao and M. Ma performed all experiments and analyzed the data with help from J. Chen and X. Ji; Q. Peng and Y. Pan supervised the study; C. Gao and Y. Pan wrote the manuscript with input from all authors.

Competing interests

The authors declare no competing interests.

Acknowledgments

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Figures and figure legends

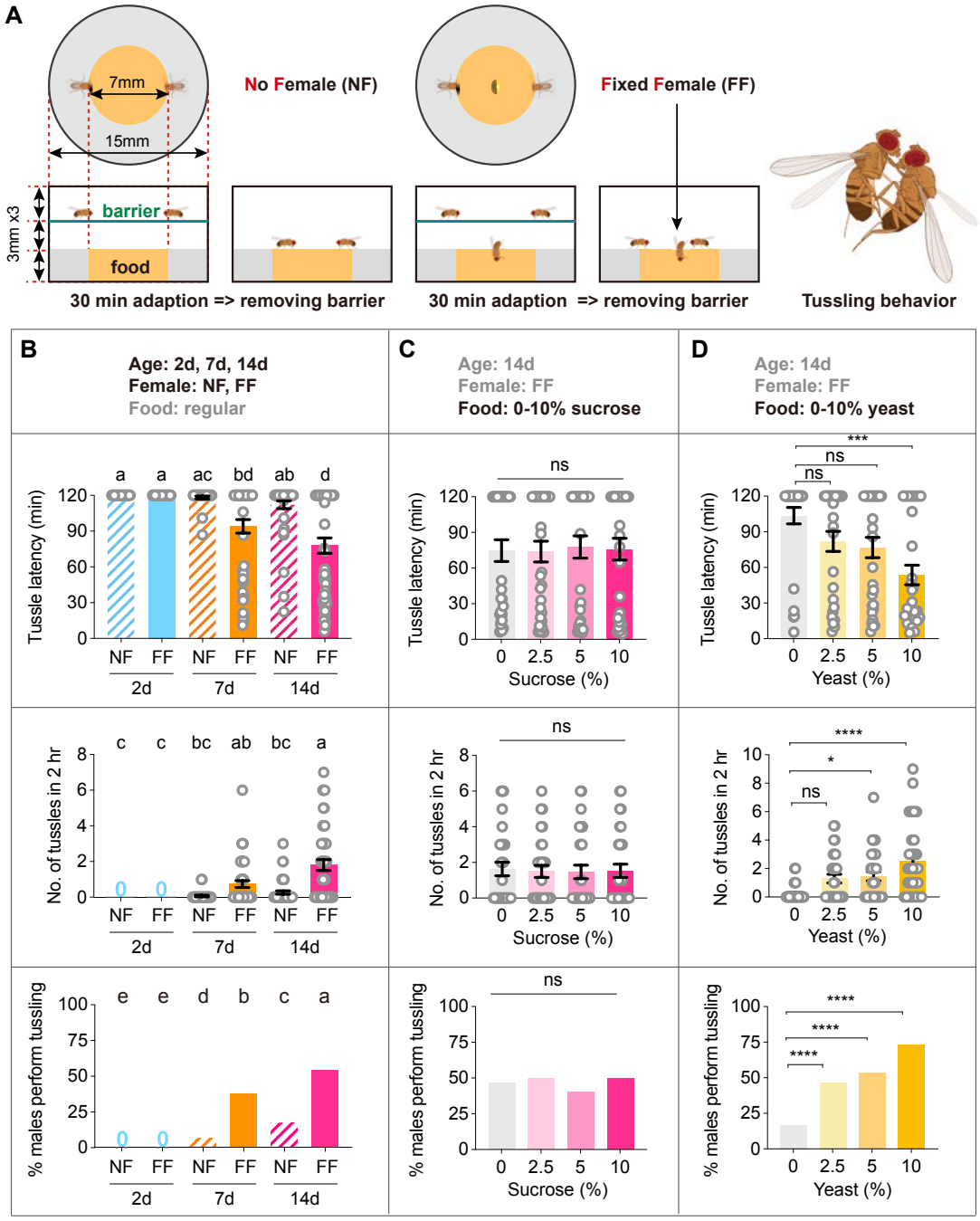

Figure 1. Establishing a paradigm for tussling behavior in male *Drosophila*. (A) Diagrams of two fighting paradigms for *Drosophila* tussling behavior. The left diagram shows a traditional fighting paradigm with no female (NF) in the food area and a new fighting paradigm with a fixed female (FF) in the center of the food area. Before the experiment began, two male flies were placed on the upper layer, separated from the food by a film. After a 30-minute adaptation, the film was removed to start recording. The right diagram shows a pair of tussling male *Drosophila*.

(B) The influence of age and female factors on male tussling behavior. From top to bottom, the tussle latency, the number of tussles, and the proportion of tussles occurring are shown respectively. n=30, 30, 30, 46, 46, 46 from left to right. Bars sharing the same letter are not significantly different according to Kruskal-Wallis test (tussle latency, number of tussles) and Chi-square test (proportion of tussles occurring).

(C) Sucrose concentration of food have no effect on male tussling behavior. n=30 for each group, n.s., not significant, Kruskal-Wallis test (tussle latency, number of tussles), and Chi-square test (proportion of tussles occurring).

(D) The influence of yeast concentration on male tussling behavior. n=30 for each group, n.s., not significant, *$P < 0.05$, ***$P < 0.001$, ****$P < 0.0001$, Kruskal-Wallis test (tussle latency, number of tussles), and Chi-square test (proportion of tussles occurring). Error bars correspond to SEM.

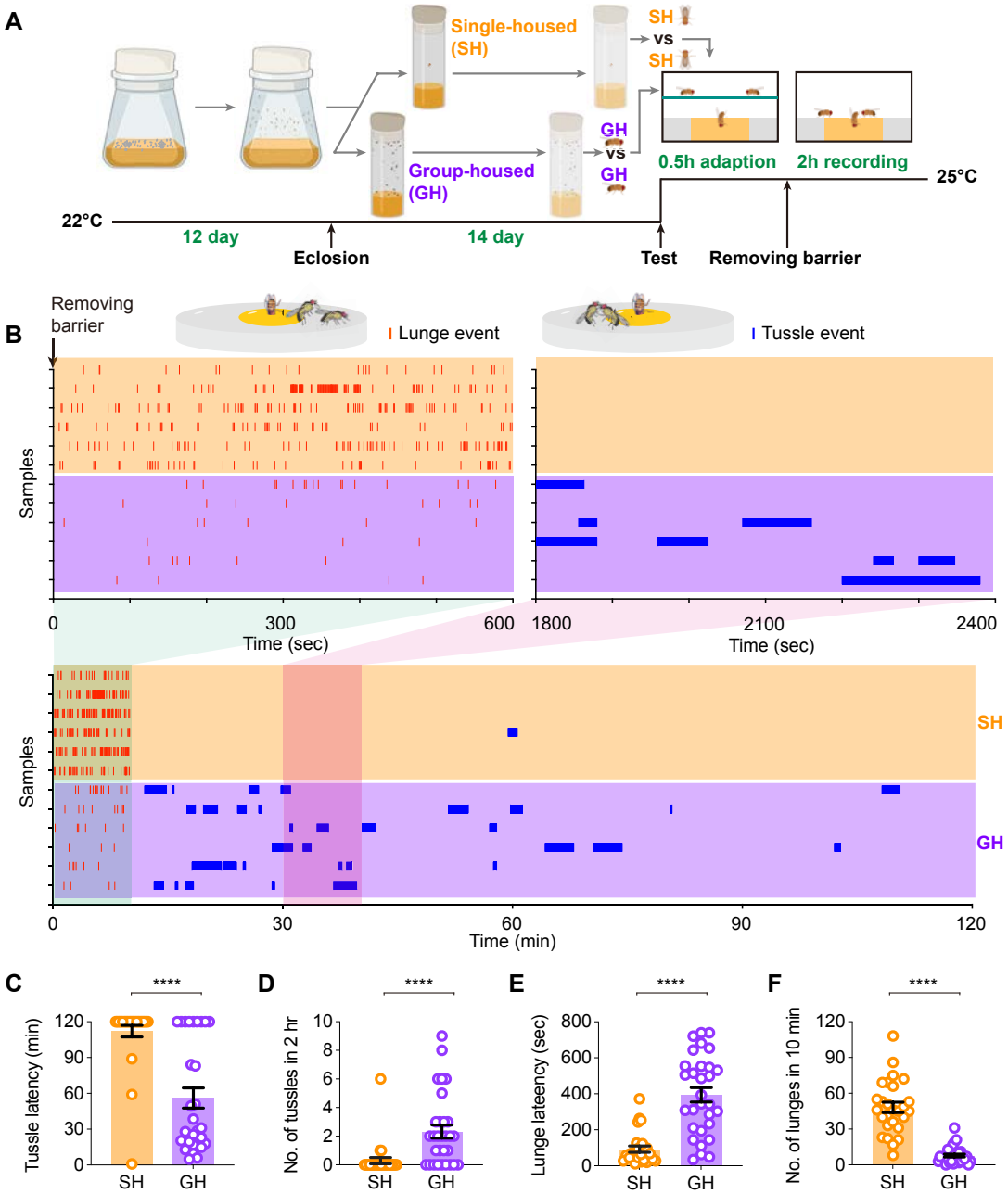

Figure 2. Social enrichment inhibits lunging behavior but promotes tussling behavior in males.

(A) Schematic diagram of *Drosophila* rearing conditions and experimental procedure.

(B) Representative raster plots illustrating lunge events in the first 10 minutes and tussle events over 2 hours in single-housed (SH) and group-housed (GH) males.

(C, D) Latency (C) and number (D) of tussling behavior in SH and GH males, n=27, 30 for SH and GH males, respectively, ****P < 0.0001, Mann-Whitney test for both.

(E, F) Latency (E) and number (F) of lunging behavior in SH and GH males, n=27, 30 for SH and GH males, respectively, ****P < 0.0001, Mann-Whitney test for both. Error bars correspond to SEM.

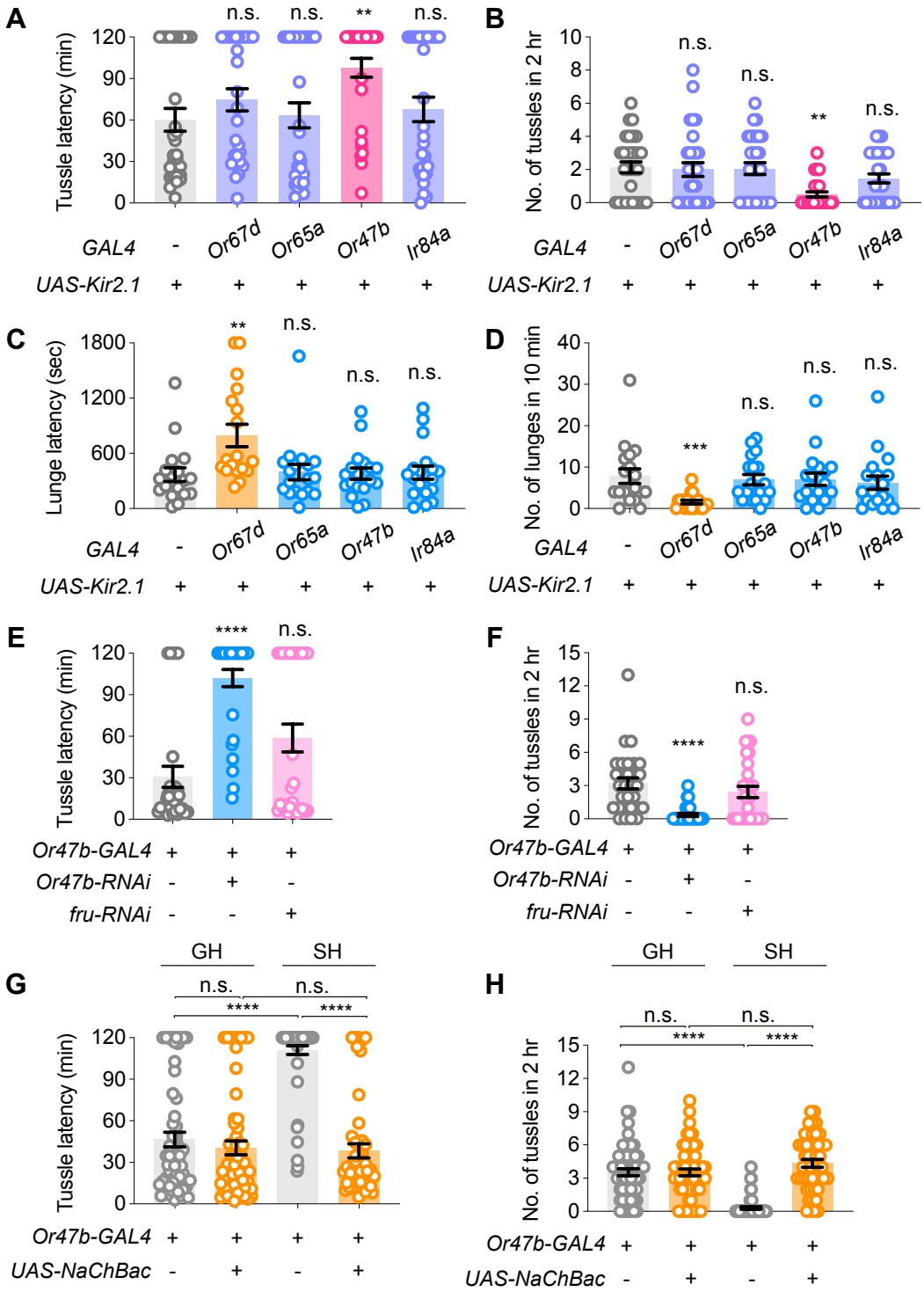

Figure 3. Or47b neurons are required for male tussling but not lunging.

(A, B) The effect of inactivating different sensory neurons on latency (A) and number (B) of male tussling behavior, n=30 for each group, n.s., not significant, **P < 0.01, Kruskal-Wallis test.

(C, D) The effect of inactivating different sensory neurons on the latency (C) and number (D) of male lunging behavior, n=30 for each group, n.s., not significant, **P < 0.01, ***P < 0.001, Kruskal-Wallis test.

(E, F) The effect of knocking down *Or47b* or *fru*^{M} expression in Or47b ORNs on the latency (E) and number (F) of male tussling behavior, respectively. n=30 for each group, n.s., not significant, ****P < 0.0001, Kruskal-Wallis test.

(G, H) The effect of activating Or47b ORNs on latency (G) and number (H) of tussling behavior under conditions of GH or SH for 14 days, n=60, 60, 60, 49 respectively, n.s., not significant, ****P < 0.0001, Kruskal-Wallis test. Error bars correspond to SEM.

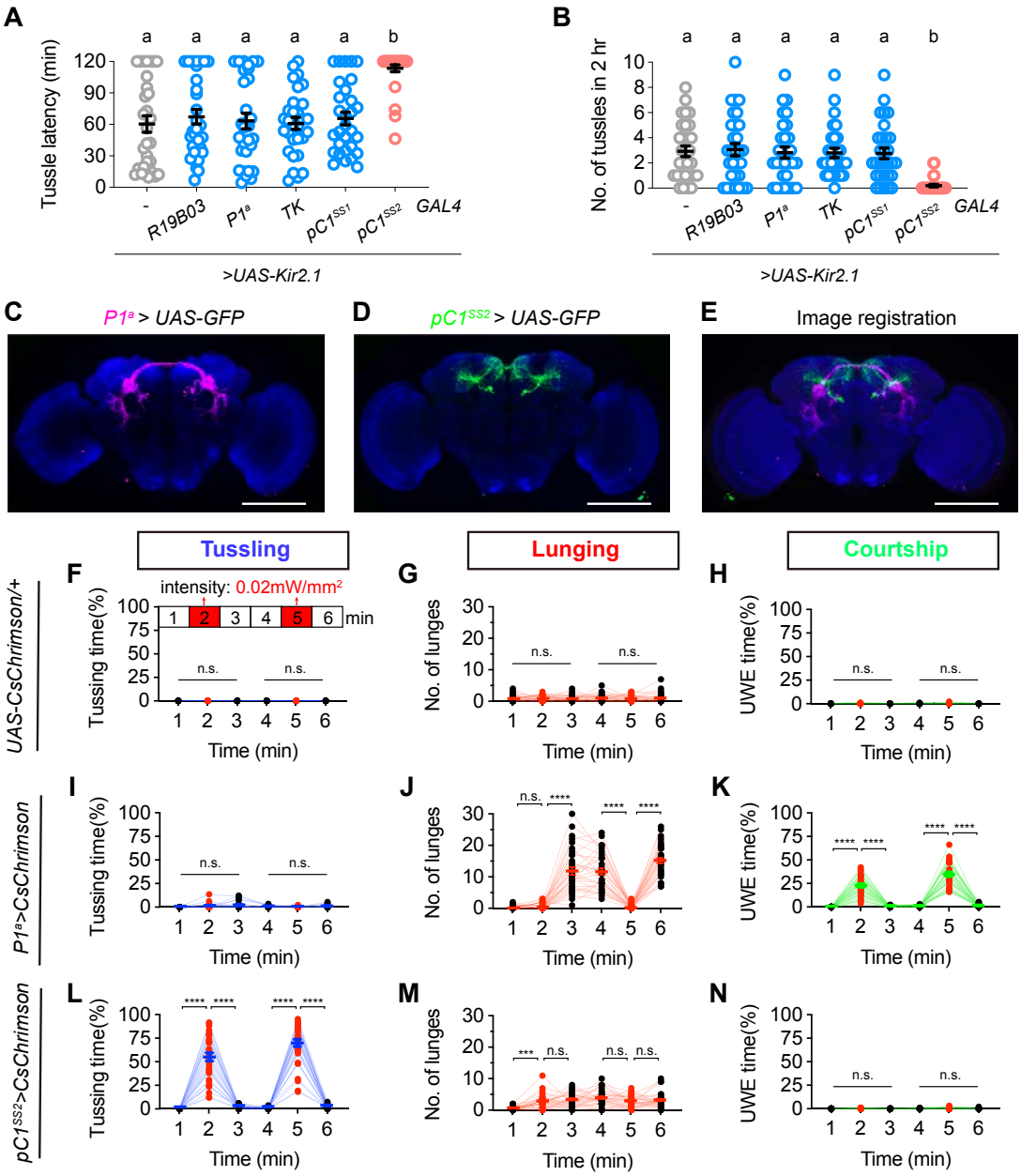

Figure 4. A subset of pC1 neurons specifically promote male tussling behavior.

(A, B) The effect of inactivating candidate interneurons on latency (A) and number (B) of male tussling behavior, n=30 for each group, bars sharing the same letter are not significantly different according to Kruskal-Wallis test.

677 (C-E) The expression pattern of *P1ᵃ-spGAL4* (C) and *pC1^{SS2}-spGAL4* (D) in male brain,
678 and (E) the registration of P1ᵃ (magenta) and pC1^{SS2} (green) neurons in a standard brain.
679 The GFP expression indicates P1ᵃ (magenta) and pC1^{SS2} (green) neurons in the male
680 brain counterstained with nc82 (blue). Scale bars, 100μm.
681 (F-H) Statistics on tussling (F), lunging (G) and unilateral wing extension (UWE, H)
682 by *UAS-CsChrimson/+* males. Red light activation with a light intensity of 0.02
683 mW/mm^2 for 1 minute was applied at the 2nd and 5th minutes of this and subsequent
684 experiments. n=32 for each group, n.s., not significant, Friedman test.
685 (I-K) Statistics on tussling (I), lunging (J) and UWE (K) in *P1ᵃ>UAS-CsChrimson*
686 males. n=32 for each group, n.s., not significant, ****P < 0.0001, Friedman test.
687 (L-N) Statistics on tussling (L), lunging (M) and UWE (N) in *pC1^{SS2}>UAS-*
688 *CsChrimson* males. n=32 for each group, n.s., not significant, ***P < 0.001, ****P <
689 0.0001, Friedman test.
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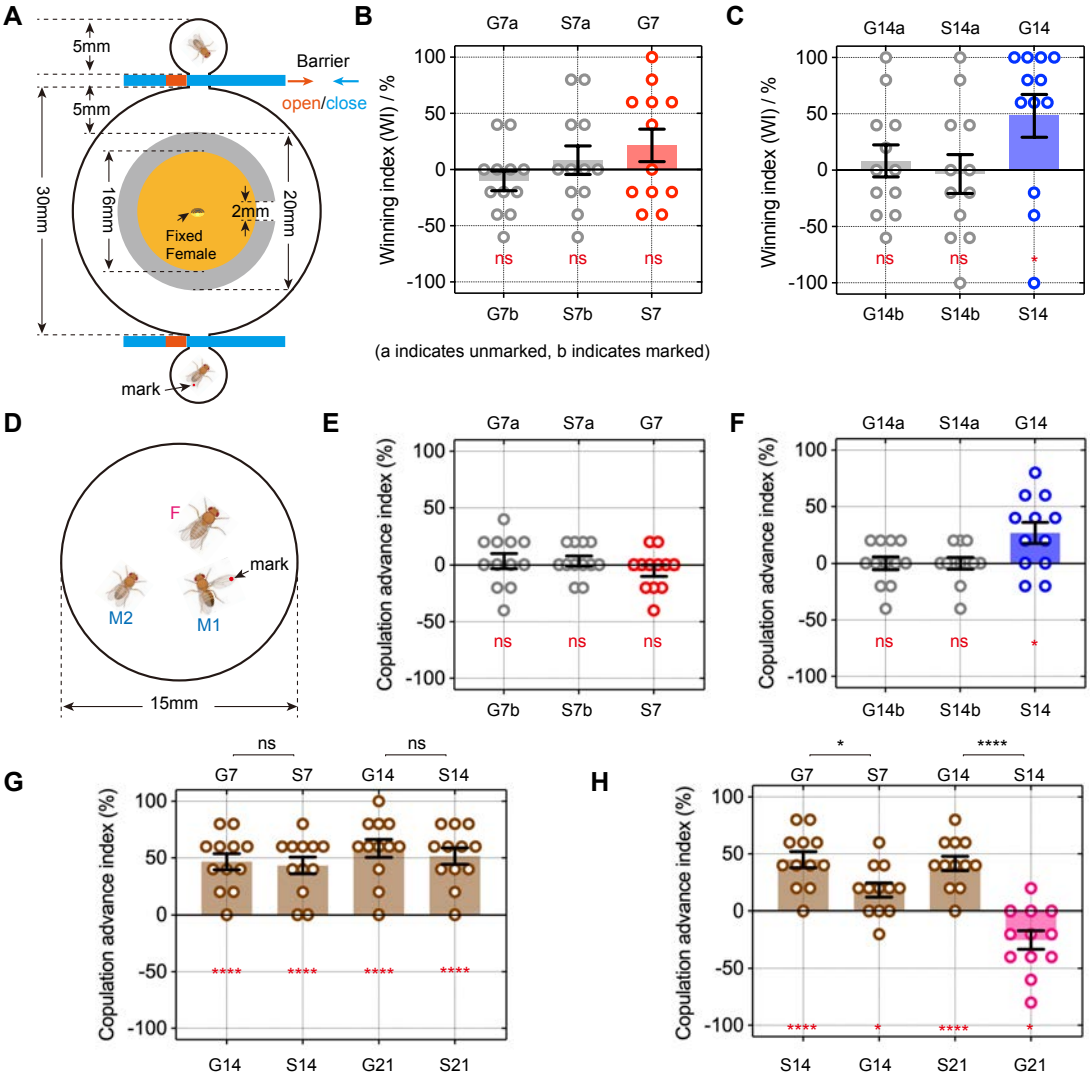

691
692 **Figure 5. Social enrichment enhances male territorial control and mating**
693 **competition.**

(A) Schematic diagram of the paradigm for testing territorial control in males.
(B) The territorial control test of 7-day-old GH (G7) and SH (S7) males. G7a and G7b stand for marked G7 male and unmarked G7 male, respectively. n=12 for each group, n.s., not significant, one sample *t* test.
(C) The territorial control test of 14-day-old GH (G14) and SH (S14) males. n=12 for each group, n.s., not significant, *P < 0.05, one sample *t* test.
(D) Schematic diagram of the paradigm for testing male mating advantage.
(E) The mating competition test between G7 and S7 males. n=12 for each group, n.s., not significant, one sample *t* test.
(F) The mating competition test between G14 and S14 males. n=12 for each group, n.s., not significant, *P < 0.05, one sample *t* test.
(G and H) The mating competition test between young males and old males, n=12 for each group, n.s., not significant, *P < 0.05, ****P < 0.0001, one sample *t* test for comparison within group (red labeling), Mann-Whitney test for comparison between groups (black labeling). Error bars correspond to SEM.

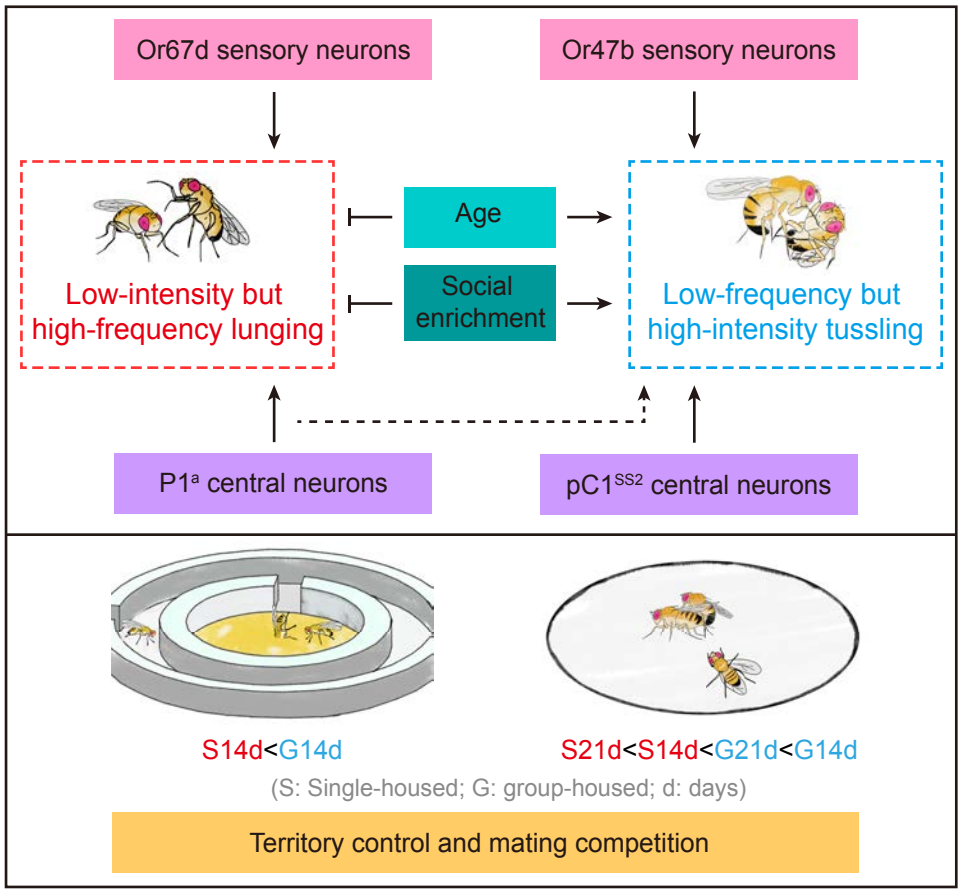

Figure 6. Summary model of fighting strategies and reproductive success in male *Drosophila*.

Age and social enrichment inhibit the low-intensity, high-frequency lunging while promoting the low-frequency, high-intensity tussling. This shift in fighting strategies among experienced, aged males enhances their territorial control and mating

716 competition, even offsetting aging-related mating disadvantages. Lunging and tussling
717 behaviors are regulated by distinct sensory and central neurons.
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Supplementary Information

Supplementary figures and legends

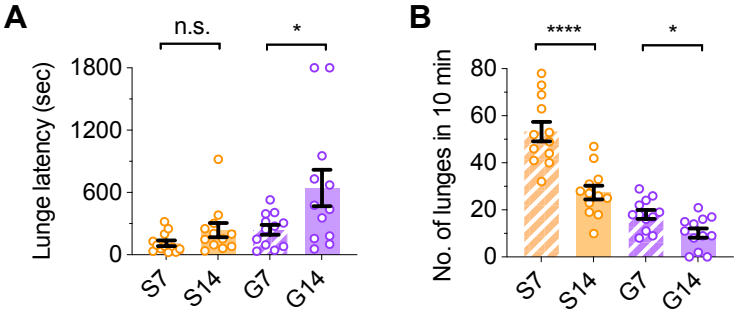

Figure 1—figure supplement 1. Aging suppresses lunging behavior in male *Drosophila*.
(A) The lunge latency in S7, S14, G7 and G14 males, n=12 for each group, n.s., not significant, *P < 0.05, Mann-Whitney test.
(B) The number of lunges in S7, S14, G7 and G14 males within 10 minutes, n=12 for each group, *P < 0.05, ****P < 0.0001, Mann-Whitney test. Error bars correspond to SEM.

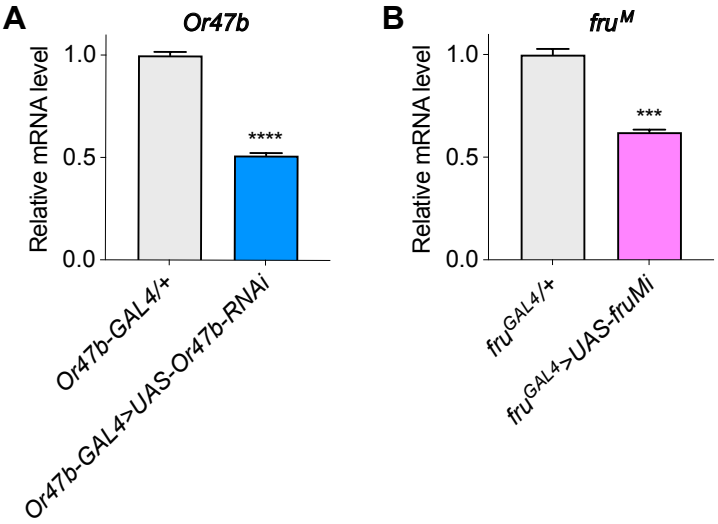

Figure 3—figure supplement 1. Validation of the *Or47b* and *fru*^{M} RNAi efficiency.
Validation of *UAS-Or47b-RNAi* (A) and *UAS-fruMi* (B) efficiency by quantitative qPCR. n=9 for each of three replicates, ****P < 0.0001, ***P < 0.001, Mann-Whitney test. Error bars correspond to SEM.

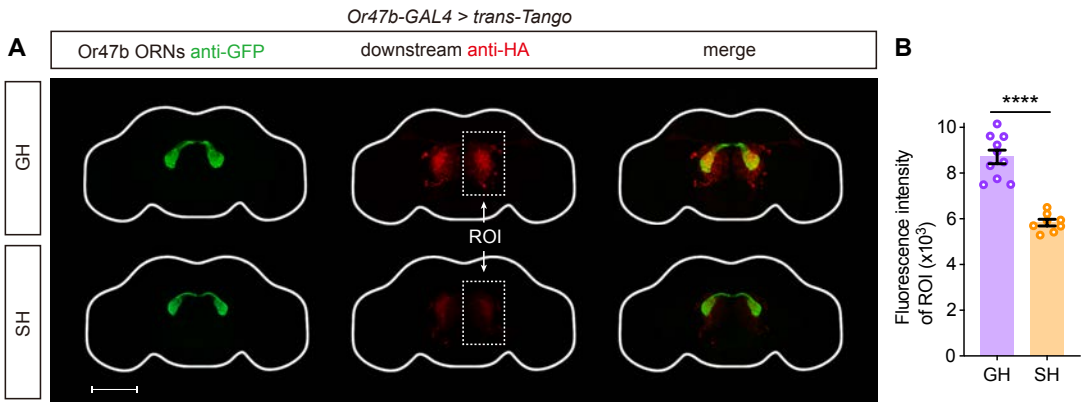

Figure 3—figure supplement 2. Group housing increases downstream signals of Or47b ORNs.

(A) Projection of Or47b ORNs (green) and their downstream neurons (red) in GH and SH conditions. Scale bar, 100μm.

(B) The fluorescence intensity of neurons downstream of Or47b ORNs is stronger under GH conditions compared to SH conditions. Both groups of male testers were 14 days old, n=10 and 8, respectively, ****P < 0.0001, Mann-Whitney test. Error bars correspond to SEM.

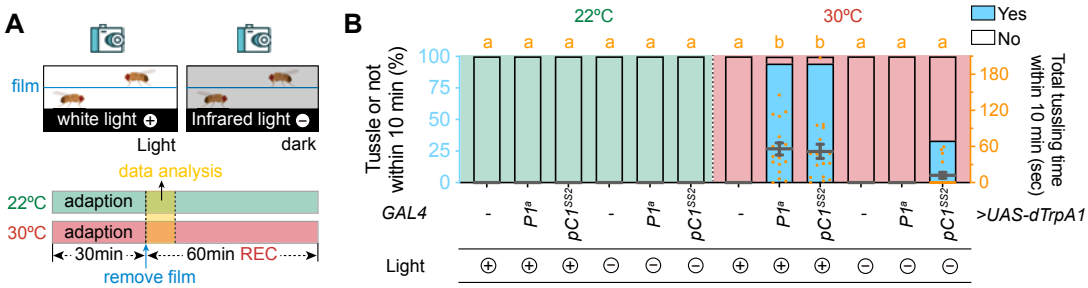

Figure 4—figure supplement 1. light-dependent initiation of male tussling behavior.

(A) Schematic diagram of the thermal activation experiment.

(B) The percentage of tussling occurrence (left) and total tussling time (right) within 10 mins. n=18 for each group, bars sharing the same letter are not significantly different according to Kruskal-Wallis test based on the data of total tussling time.

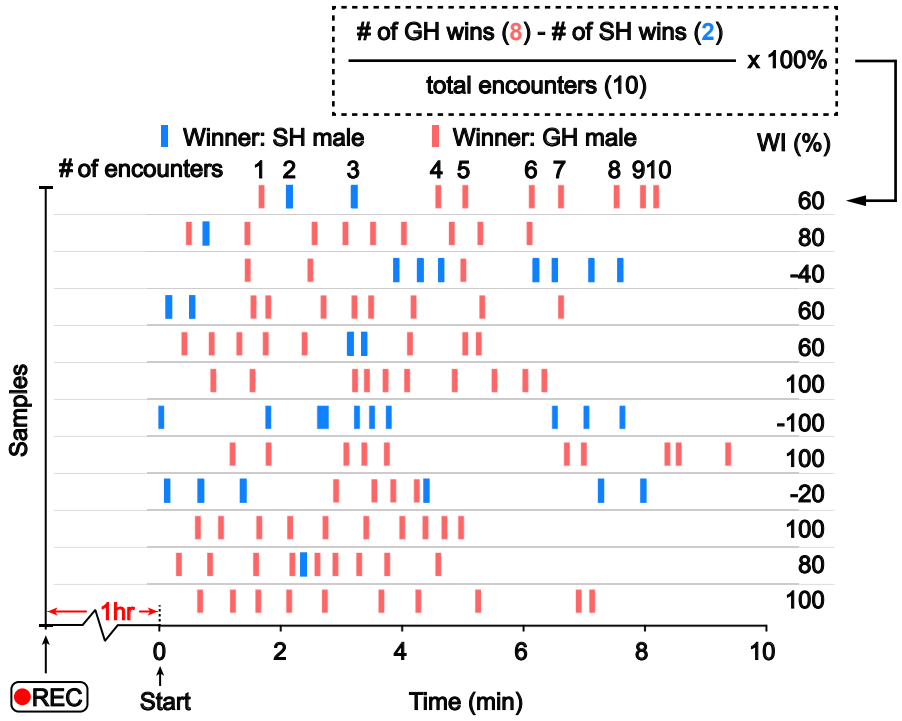

Figure 5—figure supplement 1. Winning events of representative samples for territorial competition.

The blue and red bars represent the wins for S14 males and G14 males, respectively. An example formula is provided for calculating the winning index in the dashed box.

Supplementary video legends

Video 1. Tussling behavior between two 14-day-old group-housed (G14) wild-type males.

Video 2. Optogenetic activation of pC1^{SS2} neurons acutely induces tussling in males.

Video 3. In a territory competition paradigm, a 14-day-old group-housed (G14, marked) wild-type male consistently outcompetes a 14-day-old single-housed (S14, unmarked) wild-type male.

Video 4. In a mating competition paradigm, a 14-day-old group-housed (G14, unmarked) wild-type male outperformed a 14-day-old single-housed (S14, marked) wild-type male and successfully copulated with the female.