Female contact modulates male aggression via a sexually dimorphic GABAergic circuit in *Drosophila*

Quan Yuan1–3, Yuanquan Song1,3, Chung-Hui Yang1,2, Lily Yeh Jan1 & Yuh Nung Jan1

Intraspecific male-male aggression, which is important for sexual selection, is regulated by environment, experience and internal states through largely undefined molecular and cellular mechanisms. To understand the basic neural pathway underlying the modulation of this innate behavior, we established a behavioral assay in *Drosophila melanogaster* and investigated the relationship between sexual experience and aggression. In the presence of mating partners, adult male flies exhibited elevated levels of aggression, which was largely suppressed by prior exposure to females via a sexually dimorphic neural mechanism. The suppression involved the ability of male flies to detect females by contact chemosensation through the pheromone-sensing ion channel ppk29 and was mediated by male-specific GABAergic neurons acting on the GABA\(\text{A}\) receptor RDL in target cells. Silencing or activating this circuit led to dis-inhibition or elimination of sex-related aggression, respectively. We propose that the GABAergic inhibition represents a critical cellular mechanism that enables prior experience to modulate aggression.

Aggression is an innate behavior important for both individual survival and group fitness. Although animals display a wide variety of aggressive behavior, with species- and gender-specific sensory input and motor output patterns1,2, the level of aggression is largely defined by the environment, prior experience and internal states of the animal3. These external and internal factors may influence common underlying mechanisms associated with the balance of survival and the reproductive needs of individuals2 and modulate the behavioral output accordingly. To understand the central mechanism underlying the regulation of aggression, we investigated the modulation of intraspecific aggression by prior experience in *Drosophila melanogaster*, a useful model for genetic studies of aggression4–7.

When encountering opponents in competition for territory, food and mating partners, male fruit flies display stereotypical aggressive behavior, quantifiable by both human inspection and computer surveillance8,9. Previous studies have demonstrated that specific sensory inputs elicit or temper aggression depending on the social context8,9. For example, acute exposure of the male-specific pheromone 11-cis-vaccenyl acetate (cVA) promotes aggression when one male encounters another male5, whereas chronic cVA exposure during long-term social grouping reduces aggression6. However, the central neural pathway that is responsible for the execution of the behavior, as well as the mechanism that allows experience to modify the intensity and duration of the behavior, remains elusive. As one of the primary motivations of male aggression is to win over mating partners10, the presence of females generally elicits elevated levels of male-male aggression. Indeed, we found that naive males without prior exposure to females were more aggressive toward each other in the presence of virgin females. Notably, our behavioral analyses revealed that prior contact with females strongly modified the subsequent behavioral choice of males and markedly suppressed this sex-related male-male aggression. Our behavioral assay, combined with recent advances in circuit mapping techniques in *Drosophila*, allowed us to study the basic cellular and molecular events involved in the experience-dependent modulation of sex-related aggression, and revealed a strong inhibitory mechanism mediated by GABA signaling pathways.

**RESULTS**

**Suppression of male aggression by prior sexual experience**

To assess male-male aggression, we recorded the actions of a pair of wild-type male flies for 2 h and quantified intensive aggressive interactions as previously described8,9 (Fig. 1a,b). We found very similar levels of aggression quantified on the basis of either the duration or frequency of the fighting episodes (Fig. 1c and Supplementary Fig. 1c), and the differences among groups could be readily detected in a 30-min window (60–90 min after introducing the flies into the test chamber; Fig. 1b,c and Supplementary Fig. 1c). We therefore used the duration of aggressive behavior displayed during this 30-min window as a measure of aggression in the following analyses.

To address the influence of experience on male-male aggression, we subjected male flies to social and/or sexual experiences and then tested their level of aggression in a sex-related context. For these experiments, we used the following conditions (Fig. 1a and Supplementary Fig. 2a). First, we carried out our experiments using either pair-housed or single-housed flies. Newly eclosed males were housed either in pairs or by themselves for 7 d before aggression assays. The pair housing allowed long-term male-male social interactions and reduced the baseline aggression, as previously reported11 (Fig. 1b,c). Second, we measured aggression with or without sexual...
context. During the aggression assay, we tested males with or without the presence of virgin females (Fig. 1a and Supplementary Fig. 2a). In the absence of females, males displayed limited aggression, which may reflect aggression over territory or food (Fig. 1b,c). In the presence of virgin females, males first exhibited courtship behavior and copulated with virgin females within 30–40 min, and then displayed elevated aggressive behavior toward the other male (Fig. 1b,c), which we regarded as sex-related aggression. Finally, we used both experienced and naive flies. To introduce prior sexual experience, we housed males either in pairs or singly for 6 d and then housed them with virgin females for 24 h before aggression assays (experienced males) or with no female contact at all (naive males). Notably, the presence of females during the aggression assay substantially elevated male-male aggression for naive males, but failed to do so for experienced males (Fig. 1b,c and Supplementary Video 1), suggesting that there was an inhibitory effect of prior sexual experience on aggression in males. Notably, our results indicate that the inhibition acts specifically on sex-related aggression. We observed that naive males showed enhanced aggression only in the presence of females. There was no notable difference in the aggression levels between naive and experienced flies when there was no female present during the aggression assay (Fig. 1b,c). In addition, if the females were removed after copulation during the aggression assay, the males no longer exhibited aggressive behavior (Supplementary Fig. 3). Furthermore, virgin females were more effective than mated females at inducing aggression (Supplementary Fig. 1a). This was not a result of a lack of interest of males in courting mated females, but rather because males spent most of the time courting the unreceptive mated females, as demonstrated by their high courtship index measured at 70–75 min (Supplementary Fig. 1b), leaving them with little time for aggressive interactions. In the case of virgin females, the males exhibited limited re-mating attempts after copulation (Supplementary Fig. 1b), coincident with the elevated engagement of aggression. These observations raise the possibility of a guarding behavior in these males after successful courtship encounter with their mating partners. This hypothesis warrants further investigation in future studies.

The inhibition was quantified via the inhibition index, defined as (AggressionN – AggressionN)/AggressionN, which was consistently close to 1 for most of the genotypes that we tested (Figs. 1d and 2). Thus, prior experience with females largely suppressed sex-related male-male aggression. The suppression on sex-related aggression appeared to be specific for the aggressive behavior; it was not an artifact resulting from fatigue exhibited by the experienced males after sexual interactions with females for 24 h, as our analyses of the courtship index and climbing index revealed no differences between the experienced and naive male flies (Supplementary Fig. 4a).

A previous report suggested that prior fighting experience influences the likelihood that a male fly will initiate subsequent fights, as well as the outcomes, reminiscent of the establishment of dominance in animals with social hierarchy12. However, the inhibition index was comparable for the single- or pair-housed flies (Fig. 1d), indicating that any hierarchy established between males housed in pairs has little to do with this inhibition of sex-related male aggression. Moreover, when we brought together two male flies that were housed in pairs, but not with each other (mixed group), thereby being socially experienced, but having no established hierarchy with each other, they exhibited as much inhibition of the sex-related aggression as did males that were raised together (Supplementary Fig. 4b). Taken together, these observations indicate that previous exposure to females suppressed the sex-related aggression of males regardless of the social interactions between the males.

Notably, the level of sex-related aggression remained suppressed for 2 d after the 24-h exposure to females and recovered at about 3–4 d (Fig. 2a and Supplementary Fig. 5a). However, this long-lasting inhibition did not seem to involve previously identified machineries for learning and memory, as classical memory mutants, such as rut and amn, and a courtship conditioning mutant, OrB2 knockout13, all had similar inhibition indices as that of the wild-type controls (Fig. 2b and Supplementary Fig. 5b).

ppk29 chemosensation is required for aggression inhibition

Clearly, the female exposure had strong influences on male flies’ subsequent behavior choice. To define the nature of the prior female exposure or the sexual experience that induced the inhibition, we carried out a series of behavioral tests. Substantial inhibition of male aggression was induced by housing the males for more than 10 h with females, but not with males (Fig. 2c and Supplementary Fig. 3). Notably, mating was not a necessary part of the experience, as aggression was suppressed by prior exposure to both virgin females and mated females, as well as virgin females expressing membrane-bound sex peptide, causing them to reject the male’s attempts to mate14,15. Nor was mating sufficient to suppress aggression; if females were removed shortly after mating, the mated males behaved similar to naive males and remained aggressive (Supplementary Fig. 3). Moreover, male aggression was suppressed by prior exposure with virgin females from D. pseudoobscura, but not D. virilis, which is more distantly related and possesses different body hydrocarbon profiles16, although these D. melanogaster males exhibited courtship interest toward females.
of both species, indicating a role of chemosensation in the female experience (Fig. 2c and Supplementary Fig. 3). Lastly, no inhibition of male aggression resulted from transferring males into vials that had previously been occupied by virgin females or housing males with females that were prevented from contacts by the males by a nylon mesh during the 24-h experience period (Fig. 2c and Supplementary Fig. 3). Thus, the prior experience with females that led to inhibition of aggression requires direct physical contact between the male and female during courtship, regardless of whether copulation occurs.

To identify the sensory pathway for this female contact–induced behavioral modulation, we used genetic approaches by screening a number of mutants, including Or67d knockout flies (which are unable to sense the male pheromone cVA17,18), GMR-hid flies (with compound eyes eliminated by cell death gene expression via an eye-specific promoter19), Orco knockout flies (which have defective olfactory function that disrupts their behavioral and electrophysiological responses to a wide range of odors20) and ppk29 knockout flies (which have defects in sensing female pheromones through direct physical contacts21). Notably, inhibition of male aggression was only reduced by mutation of the recently identified pheromone-sensing sodium channel ppk29, which is expressed in a specific group of sensory neurons innervating the sensory bristles on the legs of male flies (Fig. 2d and Supplementary Fig. 5c). Moreover, silencing the ppk29 neurons, via expression of the potassium channel Kir2.1 (ref. 22), also impaired the inhibition of aggression (Fig. 2d and Supplementary Fig. 5c). Furthermore, blocking synaptic transmission of the ppk29-expressing, male-specific sensory neurons specifically through the combination of the fruFLP allele and the active form of the Tetanus toxin (TNTact) transgene, which restricts expression to just the ppk29+ fru+ neurons21,22, resulted in a similar loss of inhibition, whereas the control group that expressed the inactive form of the toxin (TNTina) exhibited no impairment of inhibition (Fig. 2d and Supplementary Fig. 5d).

Previous studies have shown that cuticular hydrocarbons act as pheromones and serve critical functions in sex and species recognition for Drosophila23. These genetic manipulations of male sensory pathways, taken together with multiple tests that illustrate the importance of direct physical contact and the female body hydrocarbon profile in inhibitory effects, suggest that sex-related male-male aggression is inhibited by prior female experience through contact-dependent chemosensation.

fru+, d5-HT1B+ neurons mediate inhibition of aggression

Although the neural circuit controlling aggression remains unidentified in Drosophila, our observation of the experience-dependent inhibition of sex-related aggression provided an entry point to look into the central control of the aggressive behavior, as the identification of the inhibitory mechanism and its targets could eventually lead to the discovery of the main components of the aggression circuit. To search for neurons in the central brain that receive inputs from the periphery and mediate the female contact–induced inhibition, we screened a number of enhancer driver lines with Gal4 active in distinct groups of neurons in the fly nervous system, driving the expression of the potassium channel Kir2.1 and the temperature-sensitive Gal80ts, for spatially and temporally controlled neuronal silencing24. We kept the flies at 20 °C and then raised the temperature to 29 °C for 2 d before the assay to inactivate Gal80ts and silence the Gal4-expressing cells via Kir2.1 expression, and looked for those Gal4 lines that produced a disinhibition phenotype, the loss of inhibition by prior female encounter (Supplementary Fig. 2b). Among the enhancer driver lines that we tested, only one, d5-HT1B-Gal4, exhibited the disinhibition phenotype without affecting the courtship activity or baseline aggression (Fig. 3a and Supplementary Table 1). This enhancer line was generated using the upstream regulatory region of Drosophila serotonin receptor 1B (d5-HT1B), and targets the expression of transgenes in about 3,000 neurons in the fly brain25.

To identify the relevant cells in this large group of neurons expressing d5-HT1B–Gal4, we asked whether the male aggression circuit is sexually dimorphic and its cellular components are genetically defined by the sex-determinant gene fruitless (fru). Previous studies have identified fru+ neurons that vary in number and arborization patterns in the brain of adult male and female flies22,26. fruFLP, an allele generated by targeting a flippase transgene into the fru locus, is an effective genetic tool to label and manipulate fru+ neurons22. Using the fruFLP allele in combination with d5-HT1B–Gal4, we were able to identify a small number of fru+ d5-HT1B+ neurons in male flies.
including γ-neurons of mushroom bodies, a cluster of neurons located between antennal lobes and the subesophageal ganglion (SOG) region (~20), and a single neuron in the ventral nerve cord (VNC) (Fig. 3b and Supplementary Fig. 6a,c). A comparison of tissues collected from male and female flies 7 d after eclosion revealed that there were fewer fru d5-HT1B+ neurons in the female brain and VNC, and that these sexually dimorphic neurons in female brains had much less elaborate arbor, labeled with mCD8::GFP or Dscam17.1::GFP fusion proteins, as compared with those in male brains (Fig. 3b and Supplementary Fig. 6c). Notably, when we silenced fru d5-HT1B+ neurons in males by expressing TNT or Kir2.1, we observed a strong dis-inhibition phenotype, suggesting that these neurons are responsible for the experience-induced inhibition of aggression (Fig. 3c, Supplementary Fig. 7a, and Supplementary Videos 2 and 3). To further test this hypothesis, we asked whether activation of these fru d5-HT1B+ neurons could eliminate aggression. Indeed, expression of the heat-activated ion channel dTrpA1 led to reduced baseline aggression at 29 °C as compared with the 20 °C control. This reduction of baseline aggression persisted when the mushroom body neurons were excluded using MB-Gal80. The genotype for the manipulation is w; UAS>stop>dTrpA1; fru d5-HT1B-Gal4, without or with MB-Gal80 (n = 5 pairs of flies for each genotype), *** P < 0.0001, ** P = 0.0049, by Student’s t test. Error bars denote s.e.m.

sensory integration, learning and memory, and regulation of behaviors such as locomotion and sleep.13 We asked whether a Gal80 transgene expressed under a mushroom body–specific enhancer (MB-Gal80) could block the inhibitory effect caused by activation of fru d5-HT1B+ neurons. MB-Gal80 effectively inhibited mushroom body neurons from expressing transgenes such as mCD8::GFP (Supplementary Fig. 6b). However, at 29 °C, the flies’ baseline aggression was inhibited to the same extent with either dTrpA1 activation of all fru d5-HT1B+ neurons or dTrpA1 activation of just those fru d5-HT1B+ neurons that were not in mushroom bodies (Fig. 3d). Thus, it appears that the cluster of neurons above the SOG region in the brain, and possibly the lone neuron in the VNC, but not the mushroom body neurons, are critical for female contact–induced inhibition of aggression.

Given that the fru d5-HT1B+ neurons express a subtype of serotonergic mechanism is involved in the female contact–induced inhibition of aggression. We first administered the serotonin synthesis precursor 5-hydroxy-tryptophan (5-HTP), which elevates the serotonin level in flies and leads to increased baseline aggression.10 We found that 5-HTP treatment also enhanced baseline sex-related aggression in our behavioral assay, but did not alter the inhibition index (Fig. 4a,b). Moreover, the inhibition of male aggression was not altered by d5-HT1B receptor overexpression (UAS–d5-HT1B) or knockdown...
(UAS–d5-HT1B–RNAi) using the pan-neuronal driver elav-Gal4 (data not shown). Thus, it appears that the serotonergic system is not involved in female contact–induced inhibition of aggression.

**GABAergic neurotransmission mediates aggression inhibition**

We further tested other neurochemical systems, including the dopaminergic, octopaminergic, peptidergic and GABAergic systems, using both behavioral and anatomical approaches (Fig. 5 and Supplementary Table 1), and we identified the GABAergic system as a candidate for regulating aggression in flies. Aggression studies using mammalian models as well as observations in human patients have implicated the GABAergic system in the modulation of aggression. However, there is no consensus on the role of GABA in aggression.

In *Drosophila*, GABAergic interneurons are involved in the modulation of olfactory perception, learning and memory, and sleep. To test whether GABA is also involved in the modulation of aggression in flies, we used a Gal4 line driven by the Gad (Glutamic acid decarboxylase 1) enhancer to identify and manipulate the potential GABAergic neurons. We also used the frouFLP allele to specifically label the subset of fru* neurons that are GABAergic. Similar to fru* d5-HT1B* neurons, fru* GABA* neurons showed sexual dimorphisms in both the number and projection patterns, with a wide distribution in the adult brain and VNC, including clusters of neurons localized below antennal lobes and above the SOG region (Fig. 5a and Supplementary Fig. 8). Immunohistochemical studies using antibody to Gad revealed that some of the fru* d5-HT1B* neurons in the brain expressed Gad (Fig. 5b), but not the lone fru* d5-HT1B* neuron in the VNC (Supplementary Fig. 6c), raising the possibility that the clusters of fru* GABA* neurons above the SOG region may correspond to those fru* d5-HT1B* neurons implicated in mediating experience-induced inhibition. Indeed, behavioral analyses of the prior female contact–induced inhibition of aggression demonstrated that blocking chemical transmission in fru* GABA* neurons leads to dis-inhibition (Fig. 5c and Supplementary Fig. 7b), whereas activating these neurons suppressed baseline aggression (Fig. 5d). We also tested a Gal80 transgene driven by the Gad enhancer (Gad–Gal80) in combination with d5-HT1B–Gal4 and fruFLP. Gad–Gal80 effectively eliminated behavioral effects generated by either silencing (Fig. 5c and Supplementary Fig. 7b) or activating fru* d5-HT1B* neurons (Fig. 5d). Thus, the cluster of GABAergic fru* d5-HT1B* neurons located above the SOG...
Finally, we confirmed the involvement of GABA neurotransmission in the GABAergic fru+ d5-HT1B+ neurons in the inhibition of aggression by genetic manipulation of GABA levels in the d5-HT1B+ neurons. Knocking down the expression of either the GABA synthesis enzyme GAD or the vesicular GABA transporter for GABA reuptake VGAT specifically in d5-HT1B+ neurons produced the disinhibition phenotype, similar to the effects observed by silencing d5-HT1B+ neurons, suggesting that the release of GABA from d5-HT1B+ neurons is important for the inhibition of male aggression (Fig. 6a and Supplementary Fig. 9a).

To search for the downstream cellular and molecular targets of GABA-mediated inhibition of male aggression by prior encounters with females, we screened all of the GABA receptors using RNAi against GAD or VGAT led to disinhibition of aggression by genetic manipulation of GABA levels in the d5-HT1B+ neurons. Nonetheless, dTrpA1 expression in these neurons resulted in inhibition of aggression at 29 °C, suggesting that activation of fru+ Rdl+ neurons could overcome the GABAergic inhibition of aggression by the prior female encounter (Fig. 6b and Supplementary Fig. 9d). We also searched for a subgroup of fru+ Rdl+ neurons as the target of the GABAergic inhibition by studying the relationship between the dendrite branching pattern of fru+ Rdl+ neurons and the GABA distribution in the male fly brain and VNC (Supplementary Fig. 8b). However, our attempt was unsuccessful owing to the wide distribution of the GABA system in the fly nervous system. Future studies that employ more sophisticated genetic manipulations combined with the behavioral analyses may help to further delineate the circuitry.

**DISCUSSION**

Aggression is a complex behavior that is regulated by various internal and external stimuli. To date, however, studies have remained largely focused on the sensory pathways involved in regulating baseline aggression, with less examination of the central components of the underlying neural pathway. Moreover, the close relationship between sex and aggression has been a fascinating topic in both biology and literature, but their intertwined nature and the underlying neurobiological basis have remained elusive. In this study, using a behavioral genetics approach, we identified a previously unknown neural pathway that underlies the modulation of sex-related male-male aggression in *Drosophila* by prior contacts with females.

Our results suggest that prior female encounter through direct physical contacts activates the pheromone-sensing ppk29 neurons, resulting in inhibition of the central aggression circuit via GABAergic mechanisms involving the RDL GABA A receptor, thereby suppressing the behavioral output for male-male aggression (Supplementary Fig. 9e). The three levels of the neural pathway involved in this experience-dependent behavior modification all exhibited sexual dimorphism, consistent with the notion that morphological differences in male and female brains correlate with their distinct behavioral needs. We were able to modify the aggressive behavior output by manipulating the circuit at each of these three steps, which possibly represented the sequence of the information relay involved in the native behaviors, the sensory input, the information processing and the execution of the behavior. However, we recognize that the circuit

(a) Blocking GABA neurotransmission in d5-HT1B+ neurons using RNAi against GAD or VGAT led to dis-inhibition of aggression in (n = 5, 4, 5 and 4 pairs of flies for each genotype). *P = 0.0359, **P = 0.0139 by Student’s t test. Error bars denote s.e.m. (b) Inhibition of aggression was reduced in flies with Rdl knockdown pan-neuronally (Elav-Gal4>UAS-Rdl RNAi) or with the hypomorphic allele Rdl<sup>MB08800</sup>. In addition, activation of fru+ Rdl+ neurons by dTrpA1 at 29 °C resulted in dis-inhibition of male-male aggression induced by female contact (n = 4, 4, 5, 8, and 6 pairs of flies for groups from left to right). *P < 0.05, **P < 0.01, ***P < 0.001, by One-way ANOVA followed by Bonferroni’s multiple comparison test and Student’s t test (for Rdl-Gal4, fru<sup>FLP</sup>). Error bars denote s.e.m. (c) Neurons labeled by Rdl-Gal4 were widely distributed in the brain. Scale bar represents 40 μm. (d) Sexual dimorphisms of fru+ Rdl+ neurons in the fly brain. Representative images of the male and female brains are shown. nc82 co-staining (red) showed the neuropil. Scale bars represent 40 μm.

RNAi knockdown driven by elav-Gal4 or the hypomorphic allele arising from a P-element insertion, Rdl<sup>MB08800</sup>, showed the dis-inhibition phenotype (Fig. 6b and Supplementary Fig. 9b,c). We carried out anatomical and behavioral studies using a line in which Gal4 expression is driven by the upstream regulatory region of Rdl (Rdl-Gal4)<sup>14</sup> in combination with fru<sup>FLP</sup>. Because silencing the large number of fru+ Rdl+ neurons (Fig. 6c,d) via TNT or Kir2.1 results in lethality or severe courtship defects, it was difficult to assess female contact–induced inhibition of aggression of these flies. Nonetheless, dTrpA1 expression in these neurons led to dis-inhibition of aggression at 29 °C, suggesting that activation of fru+ Rdl+ neurons could overcome the GABAergic inhibition of aggression by the prior female encounter (Fig. 6b and Supplementary Fig. 9d). We also searched for a subgroup of fru+ Rdl+ neurons as the target of the GABAergic inhibition by studying the relationship between the dendrite branching pattern of fru+ Rdl+ neurons and the GABA distribution in the male fly brain and VNC (Supplementary Fig. 8b). However, our attempt was unsuccessful owing to the wide distribution of the GABA system in the fly nervous system. Future studies that employ more sophisticated genetic manipulations combined with the behavioral analyses may help to further delineate the circuitry.

**Figure 6** GABA neurotransmission, the GABA<sub>A</sub> receptor RDL and Rdl+ fru+ sexually dimorphic neurons mediate female experience dependent inhibition of aggression. (a) Blocking GABA neurotransmission in d5-HT1B+ neurons using RNAi against GAD or VGAT led to dis-inhibition of aggression. Scale bars represent 40 μm.
components elucidated by our experiments are clearly only parts of the machinery responsible for aggression modulation. In addition, by identifying RDL as a molecular target for aggression regulation, our study provides an entry point for characterizing the missing link of aggression studies, namely the central neurons that respond to experience-dependent modulation and mediate the execution of aggressive behaviors. Thus, our work provides new insights regarding the intricate interactions between sexual experience and aggression and delineates the underlying mechanisms to inform potential means to suppress excessive aggression.

The female contact–dependent suppression of male aggression may also be viewed as a form of learning-induced plasticity. The learning procedure in this case requires extended physical interactions between the male and the female (over 10 h), which could consist of repeated sessions of male courtship attempts and female rejection. As we did not observe obvious defects in aggression suppression in genetic mutants with deficits in courtship conditioning, such as homer, eag, Shaker and orb2 mutants (Fig. 2b and data not shown), this experience-induced suppression of aggression is likely different from conventional courtship conditioning. Another interesting feature of this suppression is that it is long term, yet reversible, lasting up to 2 d after the female encounter. However, it also differs from well-studied long-term memory formation, as no defect was observed in amm mutants, which is required for long-term memory formation. Our results implicate the fru+ d5-HT1B+ and GABA+ cluster of neurons in the central brain as the regulators of this suppression, but it remains to be determined whether these neurons are involved in the initiation, acquisition, execution or consolidation phase(s) of this behavior, what takes form as the underlying ‘memory trace’ and whether plasticity is manifested at the level of the number of neurons activated, neurite arborization, neuronal activity or some other aspect of neuronal signaling.

Notwithstanding the emergence of Drosophila as a successful genetic model for aggression studies and the extensive characterization of its stereotymical motor display of aggression, the strong influence of genetic background over baseline aggression and locomotor activity often complicates Drosophila aggression studies. Our assay avoids such difficulties by consistently eliciting aggression in naive male flies in the presence of females and by inducing a strong suppression of aggression in males with prior female encounter. The small variations among different genetic backgrounds in our behavioral assay make it possible to identify critical cellular and molecular components involved in the regulation of aggression by experience.

One purpose of studying aggression regulation in animal models is to eventually understand the basis of human violence and establish venues to reduce or prevent it. Psychophysiological studies suggest that the failure to maintain an appropriate level of aggression in humans is associated with impaired executive cognitive processes or emotion regulation. As an innate behavior built largely on pre-determined neural pathways, aggression in Drosophila males can be modulated by prior exposure to females through GABAergic inhibition. Our study raises the possibility that an ancient and basic machinery of the central neuronal circuitry, GABAergic inhibition, could be part of a conserved mechanism to modulate the level of aggression in males and ensure proper balance between reproductive competition and individual survival.

METHODS

Methods and any associated references are available in the online version of the paper.
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**ONLINE METHODS**

**Fly stocks and rearing conditions.** Fly stocks are maintained in the standard medium in a circadian and humidity controlled 25 °C incubator, unless otherwise noted. Wild-type, mutant and transgenic fly lines used are from the following sources: Canton-S was from B. Greenspan (University of California, San Diego), GMR hid was from J. Blau (New York University), MB-Gal80 was from S. Waddell (University of Oxford), Gad1-Gal4 and UAS-RdL-RNAi were from L. Griffith (Brandeis University), Orb2/- was from K. Kelemen (Research Institute of Molecular Pathology), Orco/- was from L. Voshall (Rockefeller University), ppk29/- and ppk29-Gal4 were from K. Scott (University of California, Berkeley), Or67d/-, Or67d/-, fru-Gal4, fruFLP, UAS-stop=mcD8:: GFP, UAS-stop=dscam17.1:: GFP, UAS-stop=TNTactive, UAS-stop=TNTinactive, UAS-stop=Kir2.1 and UAS-stop=dTrpA1 were from B. Dickson (IMP), Gad1-Gal80 was from T. Kitamoto (University Iowa), RdL-Gal4 was from J. Simpson (Howard Hughes Medical Institute, Janelia Farm), UAS-mSP was from T. Aigaki (Tokyo Metropolitan University), Trh-Gal4 was from E. Kravitz (Harvard (Howard Hughes Medical Institute, Janelia Farm), UAS-GAD-RNAi, UAS-VGAT-RNAi, UAS-GABAAR1-RNAi, UAS-GABAAR2-RNAi, UAS-GABAB1-RNAi and UAS-Lcch3-RNAi were from VDRC stock center, and Drosophila pseudoobscura and Drosophila viridis were from DGRG stock center.

**Experimental design.** Detailed experimental schemes are illustrated in Supplementary Figure 2. Newly eclosed males were collected and reared in pairs for 7 d before the behavioral assays. Virgin females were collected shortly after eclosion and reared at –20 females per vial for 7 d before assays. The day before the aggression assay, males were either housed together with females (1:1 ratio) as the experienced group, or not, as the naive group. During the aggression assay, both groups were divided and tested in two configurations, males only and two males with two wild-type virgin females. The flies were anesthetized with CO2 briefly and loaded in a chamber with grape juice agar medium similar to that described previously. Their activities were videotaped for 2 h. Aggression duration and frequency were quantified by visual inspection and scoring of aggressive male–male interactions, including shoving, lunging, boxing, tussling and head-buttting. To score the aggression phenotypes without considering locomotion or courtship defects, and to ensure the two males had the same courtship experiences, we included only those cases in which both males copulated with both virgin females. For most of the analyses, aggression quantification was performed in the 60–90 min time window, given that aggression was most consistent in this time period and copulation was usually finished in the first 40 min. Summed duration and frequency are presented in bar graphs. Inhibition index was defined as (AggressionN – AggressionE)/AggressionN: the aggression duration of naive males minus that of experienced males and normalized by the duration of naive males. If the value is 1, it means that the aggression is completely inhibited in the experienced group; if the value is 0, it means that the experienced group showed the same level of aggression, that is, disinhibition. Courtship index quantification was performed in the 70–75 min time window for the experiments shown in Supplementary Figure 1b, and the 1-min period right before copulation for the experiments shown in Supplementary Figure 4a.

In all experiments involving genetic manipulations, we compared genotypes in the same genetic background. Behavioral assays were performed in behavior chambers made from cuvettes filled with silicone with a thin layer of grape juice agar medium on top. Chambers were controlled by an adhesive film after flies were loaded, then placed in a light-controlled incubator at the desired temperature during the video recording. In the experiment shown in Figure 4, male flies were fed with 50 mM 5-HTP, which was mixed into food, after eclosion until assay. The aggression duration for every 5 min of scoring are plotted in Figure 1b and Supplementary Figure 1a.

For the experiment shown in Figure 2a, the males were housed with females at various time points (on days 6, 4, 3 or 1) for 24 h and then were separated from the females and reared for the number of days (0, 2, 3 or 5 d) until behavior testing. For the experiment shown in Supplementary Figure 1a, males were also tested with mated females. Under this configuration, males spent most of their time attempting to mate with the mated females and displayed low levels of aggression. For the experiments with temporally inhibiting neuronal activity with Kir2.1 shown in Figure 3, the males with the corresponding genotypes were raised at 20 °C for 5 d and were switched to the non-permissive temperature 29 °C for 2 d until assay. For the experiments with temporally activating neurons with dTrpA1 shown in Figures 3, 5 and 6, the males with the corresponding genotypes were raised at 20 °C until the behavioral testing at 29 °C.

Climbing assay was performed as described previously with the following modifications. Briefly, ten flies raised in pairs at 25 °C for 7 d after eclosion were placed in a 25-ml pipette that was sealed at the top with cotton (n = 30 flies per group). The flies were gently knocked to the bottom of the pipette, which was then turned and stood straight for a rack. The percentage of the flies to cross the 20-cm height after 10 s was recorded. Five trials were completed for each group and the results were averaged for statistical analysis using Student’s t test. Data are presented as mean ± s.e.m.

All behavioral experiments were carried out on the morning of the experimental day.

**Immunohistochemistry.** The procedures for dissection, fixation, immunohistochemistry on adult brains were as described previously. We used rat antibody to CD8 (Caltag Laboratories, MCD8000, 1:200), mouse antibody to GFP (Invitrogen, 1:100) and rabbit antibody to DAPI (Caltag Laboratories, MCD0800, 1:200), mouse antibody to nc82 (DSHB, 1:100) and rabbit antibody to GAD (F. Jackson (Tufts University), 1:200) for primary antibodies. For the representative images shown, each experiment has been successfully reproduced at least three times and was performed on multiple days.

**Confocal imaging.** Confocal images were taken on a Leica SP5 Confocal Microscope. Serial optic sections of 0.5 or 1 µm thickness (depending on the required resolution) were obtained from fixed whole-mount adult brain samples.

**Semi-quantitative reverse transcription–PCR.** Semi-quantitative RT-PCR was carried out for RdL and α-tubulin according to the manufacturer’s protocols. The primer sequences were as follows: two regions of the RdL transcripts were amplified with primers RdL 339-512 5'-tgagctacgtcctggta-3' and 5'-ctgctgaaatgtagtgcac-3'; RdL 914-1073 5'-caacgtttgactacct-3' and 5'-atgacgctctggtc-3'; RdL 669-760 5'-tactttcgttctcctggtg-3' and 5'-tattttctttcctcctcctcctc-3'.

**Statistical analysis.** No statistical methods were used to pre-determine sample sizes, but our sample sizes are similar to those reported in previous publications. Two-tailed unpaired Student’s t test was performed for comparison between two groups of samples, and one-way ANOVA followed by Bonferroni’s multiple comparison test was performed for comparisons among three or more groups of samples. The data meet the assumptions of the tests. The variance has been tested in each group of the data and the variance is similar among genotypes. Data distribution was assumed to be normal but this was not formally tested. Data collection and analysis were not performed blind to the conditions of the experiments. The data were collected and processed randomly.

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