

Social regulation of aggression by pheromonal activation of Or65a olfactory neurons in *Drosophila*

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When two socially naive *Drosophila* males meet, they will fight. However, prior social grouping of males reduces their aggression. We found olfactory communication to be important for modulating *Drosophila* aggression. Although acute exposure to the male-specific pheromone 11-*cis*-vaccenyl acetate (cVA) elicited aggression through Or67d olfactory receptor neurons (ORNs), chronic cVA exposure reduced aggression through Or65a ORNs. Or65a ORNs were not acutely involved in aggression, but blockade of synaptic transmission of Or65a ORNs during social grouping or prior chronic cVA exposure eliminated social modulation of aggression. Artificial activation of Or65a ORNs by ectopic expression of the *Drosophila* gene *TrpA1* was sufficient to reduce aggression. Social suppression of aggression requires subsets of local interneurons in the antennal lobe. Our results indicate that activation of Or65a ORNs is important for social modulation of male aggression, demonstrate that the acute and chronic effects of a single pheromone are mediated by two distinct types of ORNs, reveal a behaviorally important role for interneurons and suggest a chemical method to reduce aggression in animals.

Both genes and environment are important for behaviors. Among the environmental factors, social influences are particularly interesting. Social experience can modify social behaviors of animals from insects to mammals^{1,2}. Lack of social interactions, or social isolation, induces changes not only in animals' behavior but also in their physiology, which can be detrimental to their health. In humans, social isolation influences emotion, cognition and interpersonal relationships³. Rodents reared in isolation show physiological and behavioral abnormalities, collectively referred to as isolation syndrome⁴⁻⁶. In nematode, isolation causes reduced body size, dampened mechanosensory responses and altered neural connectivity^{7,8}.

Previous studies in *Drosophila* have shown that social isolation or grouping can affect a variety of behaviors, including circadian activity, sleep, mating, chemical communication, memory formation and oviposition site selection (for a review, see ref. 1). Pheromonal communication is an important aspect of conspecific interactions of many species. In flies, pheromones are involved in regulating gender-selective behaviors. Four olfactory receptors, Or47b, Or65a, Or67d and Or88a, are known to respond to fly odors⁹. Or67d is important in courtship and aggression¹⁰⁻¹², whereas the functional roles of the other three receptors remain largely unknown.

Male-male aggression of *Drosophila* is controlled by genetically determined neural circuitry, but is also modulated by social interactions in adulthood. In all of the animal models tested, social isolation increases aggression¹³. The existence of social modulation of aggression in *Drosophila*¹⁴⁻¹⁷ allowed us to use genetic and molecular approaches to study its mechanisms. Here, we examined the roles of a pheromone and ORNs in social regulation of *Drosophila* aggression.

The male-specific pheromone cVA acts on Or67d¹⁸ to increase female receptivity¹¹ and inhibit male courtship^{10,11}. A recent study has shown that cVA enhances male aggression¹². We found that cVA functions through two different ORNs in a temporally differential manner to regulate aggression: acutely through Or67d ORNs and chronically through Or65a ORNs. We also found that activation of Or65a ORNs underlies social suppression of aggression.

RESULTS

Social suppression of aggression by chronic cVA exposure

To test for potential contribution of olfaction in social regulation of male aggression, we examined whether prior exposure of male flies to the odors of other flies could modulate aggression. Newly eclosed male flies were isolated for 6 d. On the seventh day, we placed a test male into a nylon mesh tube that separated it from ~60 males or females in the same vial. After group-housing for a day, the test flies were assayed for aggression (Fig. 1).

Males with prior exposure to males (group III) fought substantially less than males that had no prior exposure (group I). The latency to initiate fighting was significantly lengthened ($P < 0.001$) and the frequency was reduced ($P < 0.001$) in group III flies. In contrast, exposure to females (group II) did not change male aggression (Fig. 1). To directly investigate the involvement of olfaction, we studied cVA, the only known male-specific volatile pheromone^{19,20}, and found substantially more cVA in the cuticles of mature males than in those of immature males (Supplementary Fig. 1a,c,e). Socially naive mature males rarely fought immature males, and addition of cVA on immature males elicited more aggression from mature

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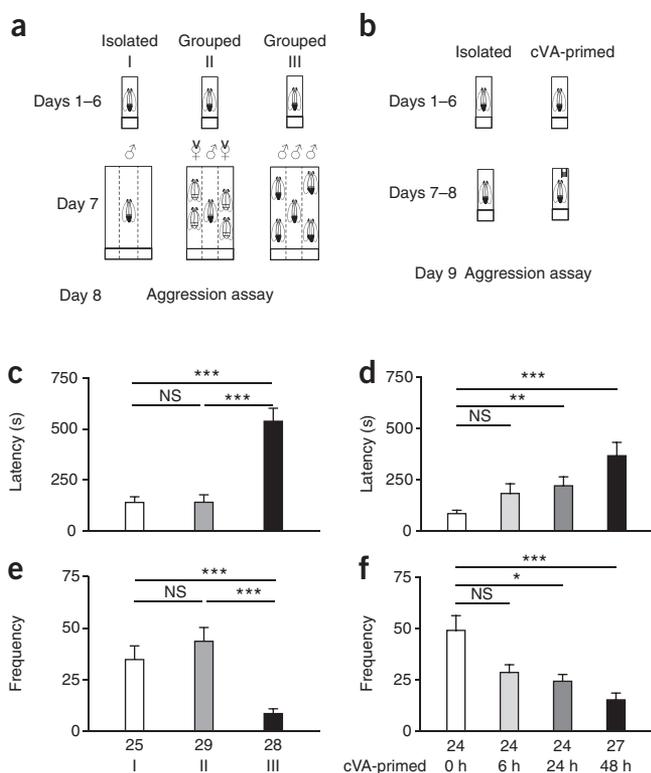


Figure 1 Social regulation of aggression mimicked by male odor or cVA. **(a)** Schematic illustration of social exposure. Group I males were isolated in nylon mesh tubes without exposure to other flies, group II males were isolated in nylon mesh tubes and exposed to mature virgin females, and group III males were isolated in nylon mesh tubes and exposed to other mature virgin males. **(b)** Schematic illustration of chronic cVA exposure. In the experimental group, a capillary containing 2–3 μ l of cVA was inserted into each tube. In the control group, male flies were not exposed to cVA. **(c–f)** Chronic male odor or cVA exposure reduced aggression. Chronic male exposure for 1 d lengthened fighting latency **(c)** and reduced lunging frequency **(e)** (Kruskal-Wallis test, followed by Dunns post test, *** $P < 0.001$; NS, not significant, $P > 0.05$). cVA exposure for 6 h slightly reduced aggression, whereas exposure for 24 h or more significantly suppressed aggression **(d,f)** (Kruskal-Wallis test, followed by Dunns post test, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$). All values are mean + s.e.m. Numbers below each bar represent the number of pairs of flies tested.

To reduce variations in EAGs, we recorded at the same position in the left antenna of each male fly (**Fig. 2a**). Different dilutions of cVA were delivered with a custom-made seven-channel olfactometer²⁴. Isolated, grouped and cVA-primed flies showed negative potentials in response to cVA (**Fig. 2b**). Response amplitude was defined as the peak cVA-stimulated voltage change. No difference in response amplitude was found between isolated, grouped or cVA-primed flies when cVA was diluted at 10^{-12} , 10^{-9} and 10^{-6} (**Fig. 2c**). We further analyzed the response area, which was quantified by summing the changed voltages during cVA stimulation. We detected no reduction in response area in grouped and cVA-primed flies compared with isolated ones at the concentrations tested (**Fig. 2d**). Failure to detect habituation in EAGs prompted us to investigate other mechanisms that could mediate social suppression of aggression.

Or65a ORNs required for chronic cVA suppression of aggression

Or67d ORNs are strongly activated by cVA^{9,11,18}. We confirmed the recent report¹² that Or67d receptors are involved in mediating the acute effect of cVA in promoting male aggression (**Supplementary Figs. 2 and 3**). cVA can also activate Or65a ORNs⁹. However, the functional importance of cVA activation of Or65a ORNs is unknown. Only one functional role has been proposed for Or65a ORNs in courtship²⁵, but it remains controversial¹⁰. We examined Or67d and Or65a ORNs to determine their roles in mediating the inhibitory effect of chronic cVA on intermale aggression.

To examine the roles of Or67d or Or65a ORNs during the period of cVA chronic exposure, we used *Or67d*- and *Or65a*-driven *GAL4* to

males (**Supplementary Fig. 2**). This result supports the idea that cVA promotes aggression in acute male encounters¹².

Is social suppression of aggression attributable to a reduction of cVA on male cuticle? We measured the cuticular hydrocarbon profiles of male flies. Although we confirmed an increase of cVA in adult males compared with immature males (**Supplementary Fig. 1**), we did not detect any difference in cVA levels between isolated and group-housed male flies (**Supplementary Fig. 1**).

We then examined the effects of prior chronic exposure to cVA on aggression. cVA priming for 24 h or longer substantially lengthened the fighting latency and reduced lunging frequency (**Fig. 1**). In contrast, we failed to find changes in aggression when flies were exposed to several other fly cuticular components (data not shown). These results indicate that chronic cVA exposure before aggression suppresses aggression.

No detectable EAG changes caused by social grouping or cVA

Because cVA activation of ORNs expressing Or67d is required for acute triggering of male aggression¹², and because ORNs can adapt on long-term odor stimulation^{21,22}, the simplest explanation for the effect of prior chronic cVA exposure is that social grouping or chronic cVA causes ORNs to habituate and thus reduce their responses. To investigate this possibility, we measured the electroantennograms (EAGs), which reflect receptor potentials in the fly antenna²³.

Figure 2 Electrophysiological responses to cVA in isolated, grouped and cVA-primed flies. **(a)** Illustration of the EAG recording site on the left antenna (anterior view). **(b)** Averaged response waveforms of all recordings ($n = 18$, 15 and 13 for isolated, grouped and cVA-primed flies, respectively) when different concentration of cVA was applied. Scale bar represents 0.5 mV and 1 s. **(c,d)** Modulus of response amplitude **(c)** and area of response waveforms **(d)** on cVA stimulation at different cVA concentrations. No significant differences were detected between isolated, grouped and cVA-primed flies at the concentration tested (Kruskal-Wallis test, $P > 0.05$). All values are mean + s.e.m.

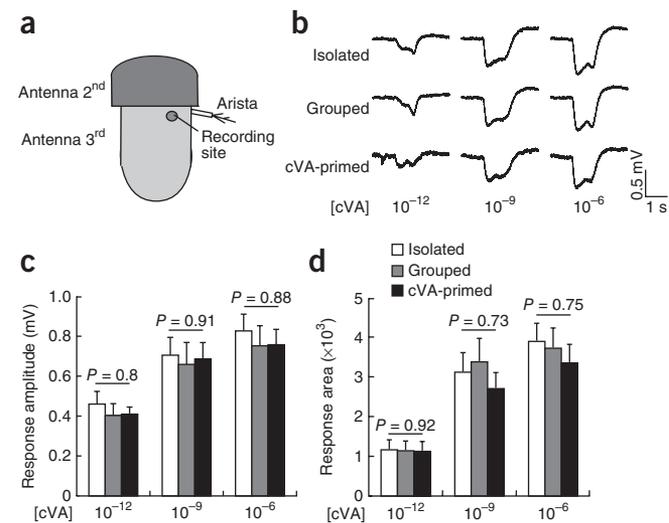
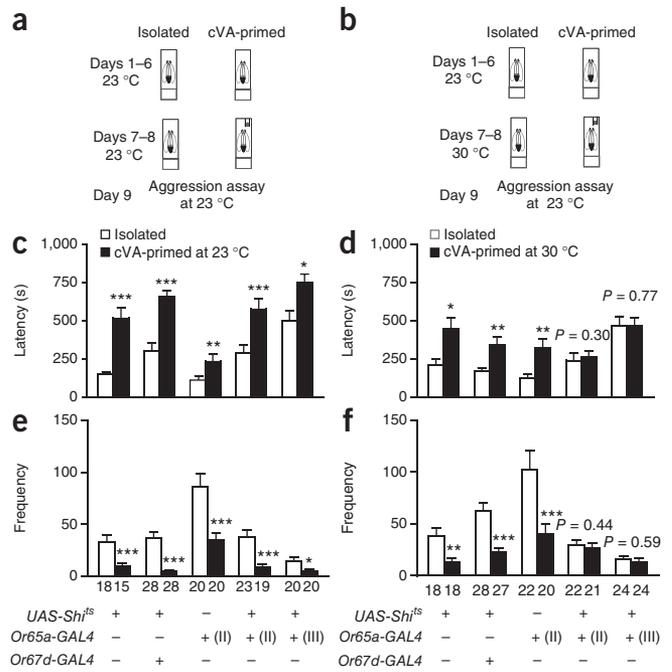


Figure 3 Aggression-suppressing effect of chronic cVA exposure mediated by Or65a ORNs. (a,b) Schematic illustration of experimental design. (c–f) When *Or67d-GAL4/+; UAS-Shi^{ts}/+* and control flies were kept at 23 °C, chronic cVA exposure reduced their aggression. The aggression-suppressing effect of cVA could also be observed when the flies were shifted to 30 °C during the cVA exposure time window (Mann-Whitney test, **P* < 0.05, ***P* < 0.01, ****P* < 0.001). At 23 °C, cVA exposure could suppress aggression of *Or65a-GAL4/+; UAS-Shi^{ts}/+* and *Or65a-GAL4/UAS-Shi^{ts}* flies (Mann-Whitney test). When *Or65a-GAL4/+; UAS-Shi^{ts}/+* and *Or65a-GAL4/UAS-Shi^{ts}* flies were shifted to 30 °C, cVA exposure during this time window could no longer suppress aggression. (II) and (III) denote chromosomes of *Or65a-GAL4* transgene insertions. All values are mean + s.e.m. Numbers below each bar represent the number of pairs tested.

express *Shibire^{ts}* (*Shi^{ts}*)²⁶. Spatial control via *GAL4* (Supplementary Fig. 4) and temporal control via temperature shifting make the *GAL4/Shi^{ts}* system an ideal tool for studying the roles of ORNs in social regulation of aggression because chronic cVA treatment could be separated from the (later and acute) period in which cVA triggers aggression. Males carrying *Or67d* or *Or65a-GAL4* and *UAS-Shi^{ts}* were isolated for 6 d before they were divided into two groups. One group was kept at 23 °C while the other was shifted to 30 °C for 2 d. Half of the flies in each group were exposed to cVA during these 2 d. Aggression was then assayed at 23 °C (Fig. 3). In the group of flies that were shifted to 30 °C, neural transmission in *Or67d* or *Or65a* ORNs was specifically blocked during the cVA exposure period, but not during the aggression assay.

When *Or67d-GAL4/+; UAS-Shi^{ts}/+* or *Or65a-GAL4/+; UAS-Shi^{ts}/+* flies were kept at the permissive temperature, chronic cVA reduced aggression as expected (Fig. 3). When *Or67d-GAL4/+; UAS-Shi^{ts}/+* flies were shifted to the restrictive temperature, prior cVA exposure was still able to reduce aggression (Fig. 3), indicating that *Or67d* ORNs are not involved in chronic cVA suppression of aggression. However, when *Or65a-GAL4/+; UAS-Shi^{ts}/+* flies were shifted to restrictive temperature, cVA exposure did not reduce aggression (the



results were reproduced with two *Or65a-GAL4* lines; Fig. 3). We did not observe any change in the effects of chronic cVA exposure in any of the control groups (lacking either *Or65a-GAL4* or *UAS-Shi^{ts}*, at either 23 °C or 30 °C; Fig. 3).

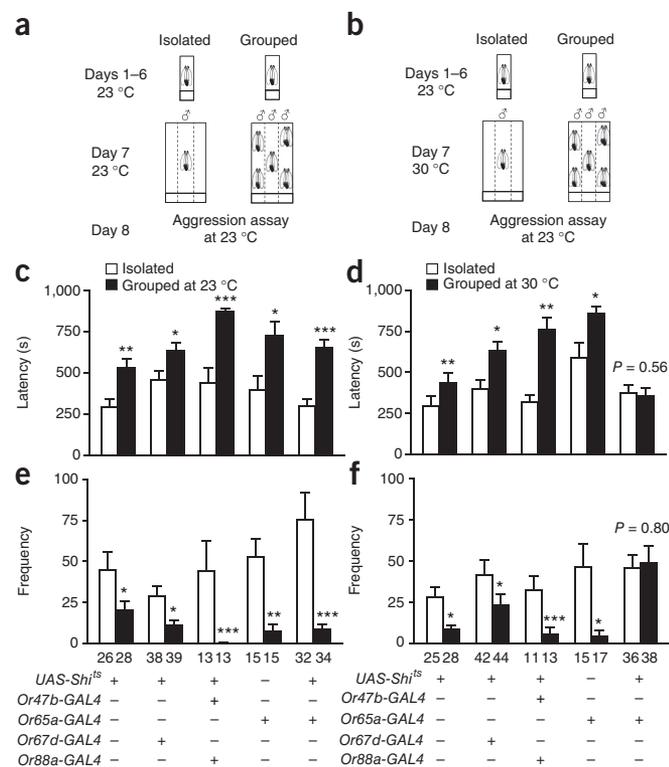
When *Or65a* ORNs were ablated by diphtheria toxin (*DTI*)²⁷ expressed in *Or65a-GAL4/+; UAS-DTI/+* flies, chronic cVA exposure could not suppress aggression, whereas cVA still suppressed aggression in the control groups (Supplementary Fig. 5). These results indicate that *Or65a* ORNs are involved in mediating the suppressive effect of chronic cVA exposure on aggression.

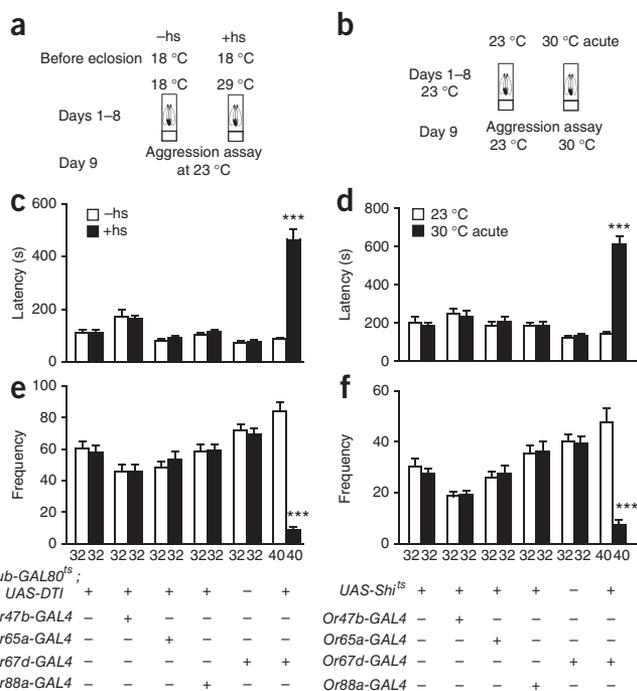
Role of Or65a ORNs in social suppression of aggression

Although we found that *Or65a* ORNs are required for the effect of chronic cVA exposure, it remains possible that social regulation of aggression by prior exposure to other males may involve other neurons, as cVA is only one of the male pheromones. It is known that ORNs expressing any one of the four receptors, *Or47b*, *Or65a*, *Or67d* or *Or88a*, are responsive to male odors⁹. We therefore sought to test which, if any, of the four types of ORNs participate in the social regulation of aggression.

We expressed *Shi^{ts}* in *Or67d*, *Or65a*, *Or47b* or *Or88a* ORNs by combining *UAS-Shi^{ts}* with specific *GAL4* lines (Supplementary Fig. 4). We isolated newly eclosed male flies for 6 d. On day 7, individual flies were transferred into nylon mesh tubes. The rest of the experiment was carried out as described above (Fig. 3), except that the flies were exposed to other males instead of cVA (Fig. 4).

Figure 4 Aggression-suppressing effect of chronic male exposure mediated by Or65a ORNs. (a,b) Schematic illustration of experimental design. (c–f) At 23 °C, 1 d of male exposure reduced aggression of flies of all genotypes. When the first four genotypes of flies were shifted to 30 °C during the male exposure time window, their aggression was also significantly reduced (Mann-Whitney test, **P* < 0.05, ***P* < 0.01, ****P* < 0.001). At 23 °C, 1 d of male exposure suppressed aggression of *Or65a-GAL4/+; UAS-Shi^{ts}/+* flies (Mann-Whitney test). When *Or65a-GAL4/+; UAS-Shi^{ts}/+* flies were shifted to 30 °C, male exposure during this time window no longer suppressed aggression. All values are mean + s.e.m. Numbers below each bar represent the number of pairs tested.





At the permissive temperature, chronic male exposure reduced aggression in all of the genotypes that we tested (Fig. 4). Disrupting synaptic transmission of Or47b, Or88a or Or67d ORNs by shifting the flies to the restrictive temperature during the period of male exposure did not alter the social suppression of male aggression (Fig. 4). In contrast, blockade of neurotransmission of Or65a ORNs during the social exposure period eliminated the effect of social suppression of aggression (Fig. 4). We observed no changes in social suppression of aggression in any of the control genotypes at either temperature (Fig. 4), suggesting a specific role for Or65a ORNs.

Or65a ORNs not required for triggering aggression

We used two approaches to investigate whether Or65a ORNs are involved in aggression *per se*. One was to ablate Or65a ORNs permanently and the other was to transiently inhibit their neurotransmission.

The TARGET (temporal and regional gene expression targeting) system²⁸ was used to express *DTI* in Or65a ORNs. As a control, *DTI* was introduced into Or67d ORNs. When *Or65a-GAL/tub-GAL80^{ts}; UAS-DTI/+* flies were kept at the permissive temperature, the *DTI* gene was not expressed. When flies were shifted to the restrictive temperature for *GAL80^{ts}*, *DTI* was expressed and Or65a ORNs were ablated in adult flies. Similarly, we were able to specifically ablate Or67d ORNs in adult flies before aggression assays. Ablation of Or65a ORNs did not affect aggression of flies that were kept in isolation. In contrast, flies lacking Or67d ORNs had longer latency in fighting and lower frequency in lunging (Fig. 5). These results confirm previous findings that Or67d ORNs are required for eliciting aggression¹² and suggest that Or65a ORNs are not required for triggering aggression in the acute phase. We could not detect any role for Or47b or Or88a ORNs in acute triggering of aggression (Fig. 5).

We used *Shi^{ts}* to block neurotransmission in Or65a, Or67d, Or47b and Or88a ORNs (Fig. 5). In the acute phase, Or67d ORNs, but not Or65a, Or47b or Or88a ORNs, were involved in aggression, which supports our *DTI* findings.

Figure 5 Or65a ORNs not required for aggression *per se*. (a) Schematic illustration of experimental design. Before eclosion, flies were reared at 18°C. Newly eclosed male flies were isolated for 8 d. Half of the flies were kept at 18°C, and the other half were heat-shocked (hs) at 29°C. On day 9, aggression was assayed at 23°C. (b) Schematic illustration of experimental design. All flies were reared and isolated at 23°C for 8 d. On day 9, aggression of flies was assayed at either the permissive or restrictive temperature. (c-f) Killing or acute silencing Or65a ORNs did not affect aggression. When *Or67d-GAL4/tub-GAL80^{ts}; UAS-DTI/+* male flies were heat-shocked, their aggression was significantly reduced. Ablation Or65a ORNs did not affect aggression (c,e) (Mann-Whitney test, *** $P < 0.001$). When *Or67d-GAL4/+; UAS-Shi^{ts}/+* flies were assayed at 30°C, their aggression was reduced (Mann-Whitney test). Acute disruption of synaptic transmission of Or65a ORNs did not affect aggression (d,f). All values are mean + s.e.m. Numbers below each bar represent the number of pairs tested.

Aggression suppressed by prior activation of Or65a ORNs

Elimination of the aggression-suppressing effect of chronic cVA and social exposure by silencing Or65a ORNs (Figs. 3 and 4) suggests that chronic cVA and social exposure activates Or65a ORNs to suppress male aggression. If this hypothesis is correct, then activation of Or65a ORNs in flies without prior social exposure or cVA treatment should be sufficient to recapitulate the effect of social or cVA exposure. In other words, aggression in single-housed flies would be inhibited if their Or65a ORNs were activated artificially.

We introduced *TrpA1* into Or65a or Or67d ORNs. *TrpA1* encodes a transient receptor potential cation channel which is closed at 23°C and opened at 27°C²⁹. *Or67d-GAL4/UAS-TrpA1* and *Or65a-GAL4/UAS-TrpA1* flies were isolated and kept at 23°C for 6 d. On day 7, flies were divided into two groups. One group was kept at 23°C while the other group was transferred to 27°C. We assayed for aggression at 23°C 2 d later (Fig. 6). We found that when *Or67d-GAL4/UAS-TrpA1* males were kept at 27°C for 2 d before assay at 23°C, aggression was not affected (Fig. 6). However, 2-d heat treatment of *Or65a-GAL4/UAS-TrpA1* flies significantly reduced their aggression: latency was significantly lengthened ($P < 0.05$) and lunging frequency was significantly reduced ($P < 0.01$). These results indicate that chronic activation of Or65a ORNs before the aggression assay is sufficient to mimic the effect of chronic social or cVA exposure in suppressing aggression.

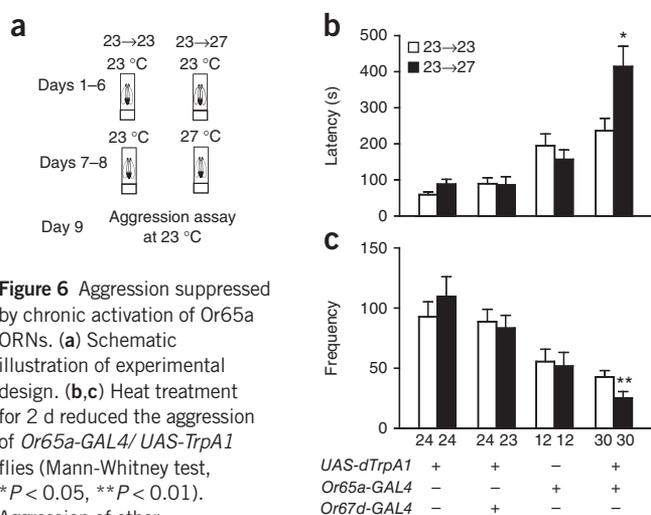


Figure 6 Aggression suppressed by chronic activation of Or65a ORNs. (a) Schematic illustration of experimental design. (b,c) Heat treatment for 2 d reduced the aggression of *Or65a-GAL4/UAS-TrpA1* flies (Mann-Whitney test, * $P < 0.05$, ** $P < 0.01$). Aggression of other genotypes was not affected by the heat treatment. All values are mean + s.e.m. Numbers below each bar represent the number of pairs tested.

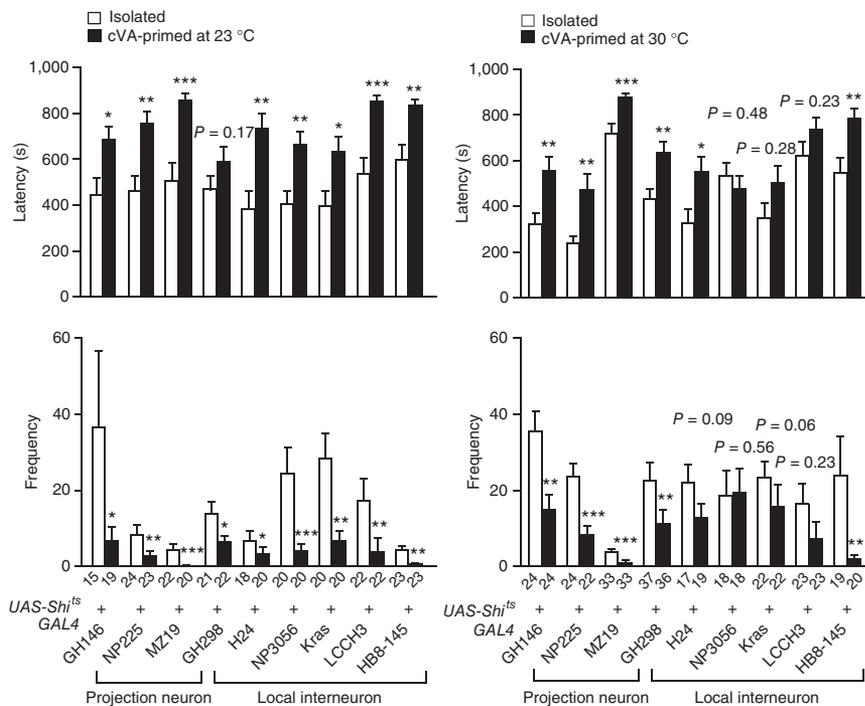


Figure 7 Local circuit plasticity in antennal lobe required for social modulation of aggression. When different combinations of *GAL4* and *UAS-Shi^{ts}* flies were kept at 23 °C, chronic cVA exposure significantly reduced aggression. Disruption synaptic transmission in *NP3056-GAL4* neurons by shifting *NP3056-GAL4/UAS-Shi^{ts}* flies to 30 °C during the cVA exposure time window totally eliminated the aggression-suppressing effect of chronic cVA exposure. A moderate attenuation of aggression-suppressing effect of chronic cVA exposure was detected in *H24-GAL4*, *Kras-GAL4* and *LCCH3-GAL4* lines, but not *GH298-GAL4* and *HB8-145-GAL4* lines. When synaptic transmissions in *GH146-GAL4*, *NP225-GAL4* and *MZ19-GAL4* neurons were blocked during cVA exposure time window, social modulation was not affected (Mann-Whitney test, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$). All values are mean + s.e.m. Numbers below each bar represent the number of pairs tested.

DISCUSSION

Our results reveal a molecular mechanism for social modulation of behavior. We conclude that the Or65a ORNs, although not involved in the acute phase of aggression, mediate the effect of social grouping

in suppressing male aggression, through a mechanism requiring local interneurons in the antennal lobe. We found that cVA has two distinct roles in male aggression that are not only temporally different, but also functionally opposite: acute cVA enhances aggression, whereas chronic cVA reduces aggression. Furthermore, our results indicate that these two roles are mediated by different ORNs.

Our findings have revealed the importance of olfactory neurons that sense pheromones. It was recently shown that cVA is required for triggering aggression in males through Or67d¹². We confirmed the roles of cVA (**Supplementary Fig. 2**), Or67d ORNs (**Fig. 5** and **Supplementary Fig. 6**) and the Or67d receptor (**Supplementary Fig. 3**) in the acute phase of aggression. Or67d mutant flies rarely fought and acute cVA exposure could not enhance their aggression (**Supplementary Fig. 3**). Reintroduction of Or67d expression in the ORNs rescued male responsiveness to acute cVA exposure (**Supplementary Fig. 3**). Silencing Or67d ORNs or ablating Or67d ORNs attenuated aggression of male flies (**Fig. 5**). Acute activation of Or67d ORNs by *TrpA1* increased aggression (**Supplementary Fig. 6**). Thus, cVA acts on Or67d ORNs to signal the presence of competitors and trigger aggressive behavior of male flies.

Our studies of all four known pheromone-responsive neurons (Or47b, Or65a, Or67d and Or88a ORNs) indicate that Or65a ORNs are important for mediating the effect of prior social exposure on male aggression. These findings have three particularly important aspects in regards to Or65a ORNs. First, two different types of neurons mediated different effects of cVA on the same behavior. Second, prior chronic exposure of social odor activated Or65a ORNs. Finally, Or65a ORNs were exclusively involved in the chronic phase of social regulation of aggression (**Figs. 3** and **4**), but were not involved in the acute phase of aggression (**Fig. 5**).

Our findings raise questions that should be addressed in the future. How does the social information delivered by the Or65a pathway modulate the neural circuit underlying aggression? Or65a ORNs project to the DL3 glomerulus in the antennal lobe^{34,35} forming synaptic connections with projection neurons, which send axons to higher

Local interneurons in social suppression of aggression

Peripheral ORNs in the antenna project to the antennal lobe, where they are connected by two types of neurons: projection neurons, which relay olfactory information to higher brain regions, and local interneurons, which are involved in local processing of olfactory information. To investigate the cellular site of behavioral modulation, we examined the role of projection neurons and local interneurons in social suppression of aggression. We used nine *GAL4* lines: *GH146*, *NP225*, *MZ19*, *GH298*, *H24*, *NP3056*, *Krasavietz* (*Kras*), *LCCH3* and *HB8-145*. *GH146* labels approximately 90 projection neurons that contribute to 32 unique olfactory channels, including the four known pheromone-responsive channels³⁰. *NP225* labels approximately 70 projection neurons that contribute to 35 glomeruli³¹. *MZ19* labels projection neurons innervating DA1 and VA1d glomeruli³². *GH298*, *H24* and *NP3056* each label a cluster of overlapping local interneurons and a combination of these three labels almost all of the ipsilateral-projecting local interneurons³³. *Kras*, *LCCH3* and *HB8-145-GAL4* label subsets of *NP3056-GAL4*-positive local interneurons³³.

We combined each *GAL4* line with the *UAS-Shi^{ts}* line before testing social modulation of aggression by selectively disrupting synaptic transmission during the cVA exposure period (similar to the experiment described in **Fig. 3a,b**). We found that disruption of synaptic transmission in *GH146-GAL4*, *NP225-GAL4* and *MZ19-GAL4* neurons, which included projection neurons contacting with Or65a and Or67d ORNs, did not alter social suppression of aggression (**Fig. 7**). Disruption of *NP3056-GAL4* neurons totally eliminated social suppression of aggression. A moderate effect was detected for *H24-GAL4*, *Kras-GAL4* and *LCCH3-GAL4* neurons. No effect was detected when *GH298-GAL4* and *HB8-145-GAL4* neurons were disrupted (**Fig. 7**). Although we found no evidence for the involvement of projection neurons in behavioral modulation, our results are consistent with the possibility that subsets of local interneurons may underlie social suppression of aggression.

brain regions. In the lateral horn, the region processing pheromones is segregated from the region processing fruit odors³⁰. Modulation could happen at any of the steps after the ORNs. However, it is attractive to consider modulation in the antennal lobe. Odor exposure has been found to cause behavioral adaptation in an odor-specific manner³⁶. Peripheral sensitivity to the odors was not affected, whereas synaptic loss and volume reduction of certain glomeruli in the central brain were detected³⁶. The DL3 glomerulus is immediately adjacent to the DA1 glomerulus³¹, which is formed by connection between axons from the Or67d ORNs and dendrites from projection neurons. One possibility is that the Or67d ORNs send a signal to trigger aggression, whereas the Or65a ORNs send a modulatory signal relayed in the antennal lobe to inhibit the signal from the Or67d ORNs. Interaction between the two channels could potentially be mediated by local interneurons in the antennal lobe³³. Both global inhibition and glomerulus-specific interactions have been detected in odor information processing in the local circuit³⁷. Antagonizing GABA_B receptors in antennal lobe results in a pronounced elevation of DA1 glomerulus activity in response to cVA stimulus³⁸. Branch patterns of projection neurons are stereotypical, whereas local interneurons are much more diversified and have great wiring variability^{30,33,39,40}. Our findings of subsets of local interneurons involved in social suppression of aggression support the hypothesis that lateral interaction between Or67d and Or65a olfactory channels is responsible for fine-tuning aggressive behavior. When Or67d and Or65a ORNs were simultaneously activated, we indeed found antagonism between them (**Supplementary Fig. 6**). Acute activation of Or67d ORNs alone enhanced aggression, whereas acute activation of Or65a alone did not reduce aggression. Notably, when Or67d and Or65a ORNs were activated simultaneously, the effect of Or65a ORNs overpowered that of Or67d ORNs (**Supplementary Fig. 6**). Considering that the ORN–projection neuron synapses are strong and postsynaptic projection neuron activity is nonlinearly amplified⁴¹, it is possible that, when acutely activating Or65a ORNs alone, the presynaptic lateral inhibition to Or67d ORNs is not sufficient to affect the activity of postsynaptic projection neurons innervating DA1 glomerulus. However, when the synaptic strength of Or67d channel is artificially elevated, a presynaptic lateral inhibition may scale down the postsynaptic responses. This explanation is consistent with the observation that simultaneous activation of Or65a and Or67d ORNs keeps aggression at, but not below, the basal level.

What are the other molecules involved in social modulation of aggression and are they functionally related? Previous work has identified *Cyp6a20*, which encodes cytochrome P450 (refs. 14,42). One possibility is that *Cyp6a20* may modulate the presentation of cVA or its derivatives to ORNs. The cAMP pathway was reported to be involved in mediating the structural and functional adaptive changes in response to prolonged odor exposure³⁶. Delta-dependent Notch activation in ORN termini has been observed in response to chronic odor stimulation⁴³. Notch activation is reversible and odor specific; olfactory receptor and ORN activity are indispensable for Notch activation and the effect is modulated by local interneuron activity⁴³. Serotonin was found to regulate neural responses in the antennal lobe⁴⁴. Serotonin promotes inhibitory local interneurons and reduces neurotransmitter release from ORNs⁴⁴. Another study found that, when octopamine neurons were potentiated, group-housed flies exhibited more aggressive behaviors, whereas single-housed flies were not affected⁴⁵. It remains to be tested whether Or65a, *Cyp6a20*, cAMP, Notch, serotonin and octopamine are functionally related in social regulation of aggression.

In addition to aggression, pheromonal-olfactory modality also contributes to social modulation of other behaviors. Group-housed flies exhibit extended sleep time only when they can smell well⁴⁶. Social cues

communicated by olfactory modality could reset the circadian activity of flies^{47,48}. It will be interesting to identify the social cues and corresponding chemosensory channels responsible for these social modulations.

METHODS

Methods and any associated references are available in the online version of the paper at <http://www.nature.com/natureneuroscience/>.

Note: Supplementary information is available on the Nature Neuroscience website.

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AUTHOR CONTRIBUTIONS

Y.R. conceived the project. Y.R., W.L. and X.L. designed the experiments. W.L. and X.L. performed the experiments. J.G. and Z.Y. synthesized cVA. Y.-H.Z. and J.-X.Z. performed the experiments shown in **Supplementary Figure 1**. W.L., X.L. and Y.R. wrote the paper.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Fly stocks and rearing conditions. All fly stocks were reared on standard corn meal at 23 °C and 60% humidity on a 12-h light:12-h dark cycle. Unless otherwise indicated, newly eclosed flies were isolated in Eppendorf tubes containing 0.5 ml of food before further manipulations.

OrX-GAL4; UAS-DTI; tub-GAL80^{ts} flies were reared at 18 °C, heat-shocked at 29 °C and assayed at 23 °C. The permissive temperature for *OrX-GAL4; UAS-Shi^{ts}* flies was 23 °C and the restrictive temperature was 30 °C. *OrX-GAL4; UAS-TrpA1* flies were kept at 23 °C and heat treatment was carried out at 27 °C.

Or67d-GAL4, Or65a-GAL4, Or47b-GAL4 and *Or88a-GAL4* were generously provided by L. Vosshall (Rockefeller University). *UAS-Shi^{ts}* was from P. Shen (University of Georgia). *UAS-DTI, GH146-GAL4, GH298-GAL4, H24-GAL4, NP3056-GAL4, Kras-GAL4, LCCH3-GAL4* and *HB8-145-GAL4* were from L. Luo (Stanford University). *NP225, MZ19* were from T. Lee (Janelia Farm). *UAS-TrpA1* was from P. Garrity (Brandeis University). *Or67d^{GAL4[2]}* and *UAS-Or67d* were from B. Dickson (Research Institute of Molecular Pathology). *tubP-GAL80^{ts}* and *UAS-mCD8::GFP* lines were ordered from the Bloomington Stock Center.

Aggression assay. The size of the fighting chamber was 13 mm in diameter and 7 mm in height. A patch of food (diameter, 8 mm; height, 3.5 mm) was placed in the center of the chamber. Agarose (0.8%) was filled into the circular space surrounding the food patch. The height of the agarose layer was 3.5 mm. The food patch was prepared as described previously⁴⁵.

In the aggression assay, pairs of male flies were introduced into the fighting chamber by spontaneous phototaxis and anti-geotaxis tendencies of flies. After flies entered the chamber, the entrance was covered up with 20-mm × 20-mm glass slides. Behaviors were recorded by a digital video camera for the next 15 min.

We measured the aggression of male flies with two indices: latency and frequency. Latency is the time from introduction of the males into the chambers until the initiation of the first agonistic encounter (in most cases, a lunge). Frequency describes the number of lunges over 15 min. Lunging is the most frequently observed aggressive pattern of male flies. It consists of a stereotyped behavioral pattern in which one fly rears up on its hind legs and snaps down onto its opponent⁴⁹.

Chronic cVA exposure. Newly eclosed flies were individually isolated in Eppendorf tubes for 6 d at 23 °C. On day 7, flies were transferred into new tubes. A capillary (inner diameter, 0.5 mm) containing 2–3 μl of cVA was inserted into each tube. cVA volatilized from the openings of the capillaries and male flies in the tubes were exposed to cVA for the next 2 d. On day 9, aggression was assayed.

Fly odor exposure. Newly eclosed flies were individually isolated into Eppendorf tubes for 6 d. On day 7, individual flies were transferred into nylon mesh tubes (nylon mesh was ordered from Beijing HongYahong Wire Mesh). Seven nylon mesh tubes were inserted into the food layer of a glass vial. For the experimental group of flies, 60–80 wild-type 7–10-d-old CS mature males or virgin females were introduced into the space surrounding the nylon mesh tubes. For the control group of flies, no other flies were introduced into the glass vial. After 1 d of fly odor exposure, aggression was assayed.

Neuronal ablation with DTI in the TARGET system. *OrX-GAL4; UAS-DTI; tub-GAL80^{ts}* males were reared at 18 °C. The experimental groups of flies were isolated and shifted to 29 °C after eclosion. Flies were heat-shocked for 12–15 h every day, for 8 d. Control groups of flies were isolated from eclosion onwards and were kept at 18 °C. The Eppendorf tubes were changed every 3–4 d. After heat-shock treatment, the corresponding ORNs were completely eliminated (data not shown). Heat shock by itself could not change aggression of *tub-GAL80^{ts/+}; UAS-DTI/+* flies.

Histochemistry and confocal imaging. *OrX-GAL4; UAS-mCD8::GFP* flies were anesthetized and dissected in cold phosphate-buffered saline (PBS). Whole-mount brains were fixed in 4% paraformaldehyde (wt/vol) for 1 h at 24–26 °C, washed three times in PBS for 10 min, washed in PBST (PBS containing 0.5% Triton X-100, vol/vol) for 20 min five times, blocked in PBST/S (PBST containing 5% heat-inactivated normal goat serum, vol/vol) for 1 h at 24–26 °C, incubated with the primary antibody in PBST/S for 36 h at 4 °C, and washed in PBST for 20 min five times. Brains were then incubated with the secondary antibody in PBST/S for 3 h in darkness at 24–26 °C, and thoroughly washed five times in

PBST for 20 min. Finally, the brains were mounted in 50% glycerol (vol/vol) and imaged on Zeiss LSM5 Pascal confocal microscope. Images were processed by Zeiss software and Adobe Illustrator software (Adobe). Monoclonal antibody nc82 (Developmental Studies Hybridoma Bank) was diluted to 5% (vol/vol) in PBST/S. The secondary antibody was fluorescence-labeled goat antibody to mouse IgG (Molecular Probes). The concentration was 0.02% (vol/vol) diluted in PBST/S.

Electrophysiological recording from the antenna. We used similar strategies to record EAG in flies to those described previously²³. Male 3–5-d-old flies were held in a truncated 200-μl pipette tip. The anterior part of the fly's head and antennae were exposed and fixed on a glass slide with dental wax. A recording electrode with a tip diameter of 1–2 μm filled with *Drosophila* Ringer's was inserted into the proximal region of the third segment of the antenna. A broken glass micropipette with a sharp open end was inserted into the eye to serve as the reference electrode. Electrophysiological signals were amplified (Axoclamp 2B, Axon Instruments), band-pass filtered at 0.1–100 Hz (Brownlee Amplifier model 440), and then digitized at 6 kHz for offline analysis.

Odor delivery. Room air was filtered with active charcoal and directed at the fly at a constant flow rate of 400 ml min⁻¹. cVA was stored in headspace vials with different dilutions in mineral oil and delivered with a custom-made seven-channel olfactometer²⁴ at 120 ml min⁻¹. Flow rates were controlled by flow meters (Botion LZB-2) and monitored digitally (FMA 1700/1800 series, Omega). The diameter of the odor delivery tube was 1.5 mm. The end of the delivery tube was 5 mm from the fly. cVA with different concentrations were presented at 50-s intervals (three trials per concentration). Teflon tubing was used to reduce cross-contamination.

Electrophysiological data analysis. Data analysis was carried out using custom-written programs in Matlab (Mathworks). Voltage data were corrected for baseline drift by subtracting the data acquired 3 s before stimulation for each trial. Because control stimuli (air from the mineral oil) elicited varied response from different antennae, we further subtracted the value of response strength to controls for each repetition. Thus, the corrected data were used to calculate the maximal response amplitude and area.

Cuticular hydrocarbon extraction. Flies were cold anesthetized (15 min at –20 °C) before solvent extraction. Each fly carcass was gently rinsed with distilled water and dried with filter paper. Flies were individually transferred into a glass vial containing 60 μl of dichloromethane (purity >99.5%, Dima Technology) and kept at 0 °C for 12 h. Extracts from each fly were transferred to another vial and stored at –20 °C until chemical analysis.

Gas chromatography–mass spectrometry (GC-MS) analysis of cuticular hydrocarbon profiles. Qualitative identification of fly hydrocarbons was performed by analytical GC-MS (Agilent Technologies) using an Agilent Technologies Network 6890N gas chromatography system coupled with a 5973 mass selective detector and the MS Library (National Institute of Standards and Technology 2002, Agilent Technologies 2002, Windows 2000). The gas chromatography had an HP5-MS column (a nonpolar column, 30 m long × 0.25-mm internal diameter × 0.25-μm film thickness, Agilent Technologies), carrier gas helium at 1.0 ml min⁻¹, the injector port was set at 280 °C. We programmed the gas chromatography oven at 5 °C min⁻¹ from 50 to 280 °C. The mass spectrometry was in the electron impact mode (70 eV), and the transfer line was set at 280 °C. In total, 1 μl of extracts was injected in the splitless mode. We made tentative identifications using the MS Library and diagnostic ions. We further confirmed *n*-tetradecanoic acid, (*Z*)-9-hexadecenoic acid, hexadecanoic acid, (*Z,Z*)-9,12-octadecadienoic acid, oleic acid, (*Z*)-11-vaccenyl acetate, (*Z*)-9-tricosene, (*Z*)-7-tricosene, (*Z*)-9-pentacosene and (*Z*)-7-pentacosene by matching their retention times and mass spectra with authentic analogs (all purity >95%, purchased from ACROS Organics or synthesized by us). Identities were further confirmed with a polar column (DBWAX, 30 m long × 0.25-mm internal diameter × 0.25-μm film thickness, Agilent Technologies), for which we programmed the gas chromatography oven at 5 °C min⁻¹ from 50 to 250 °C.

Quantitative analysis was further performed by analytical GC-FID (Agilent Technologies) using an Agilent Technologies Network 7890A gas chromatography system equipped with 7683B automatic liquid sampler. The gas chromatography

had an HP5-MS column (a nonpolar column, 30 m long \times 0.25-mm internal diameter \times 0.3- μ m film thickness, Agilent Technologies). The injector and detector temperatures were 280 °C and 300 °C, respectively. The temperature program was the same as above mentioned. Samples (~3.0 μ l) were injected in the splitless mode with purge delay time of 0.7 min. To measure the relative abundances of the compounds, we converted the peak area (obtained by the GC-FID) of a particular compound into a percentage of the summed peak area using all the detected volatile gas chromatography peaks from fly bodies.

Statistics. All statistical analysis was done using Prism 5 (GraphPad Software). Nonparametric Mann-Whitney test was used to compare two columns of data. Kruskal-Wallis test followed by Dunns post test was used to compare multiple columns of data.

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