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Suppression of male courtship by a *Drosophila* pheromone receptor

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Abstract

In male *Drosophila*, chemosensory cues control many aspects of social behavior. We found that males with a mutated *Gustatory receptor 32a* gene (*Gr32a*) show high courtship toward males and mated females, indicating that GR32a functions as a pheromone receptor for a male inhibitory pheromone. Notably, we discovered that tarsal *Gr32a*-expressing neurons were essential for courtship suppression and projected to the ventrolateral protocerebrum, implying direct communication of chemosensory neurons with a higher-order brain structure.

In the fruit fly *Drosophila melanogaster*, social behaviors are mediated through multiple sensory channels, including chemosensation, vision and audition¹. For example, chemicals released by both sexes are thought to function as stimulatory and inhibitory pheromones that are necessary to direct courtship toward appropriate individuals. The only *Drosophila* pheromone receptors associated with specific roles during courtship are GR68a and OR67d/OR65a^{2–4}. OR67d and OR65a are expressed in olfactory sensory neurons and recognize *cis*-vaccenyl acetate (cVa)^{4,5}, a volatile male pheromone, and OR67d is required in males to suppress male-male courtship⁴. *Gr68a* is specifically expressed in male taste neurons in the forelegs and is necessary for efficient male courtship toward females².

We generated a mutant fly strain by knocking out *Gr32a*, a close relative of *Gr68a*^{1,2}, using ends-out homologous recombination⁶ (Supplementary Methods online). Precise replacement of the *Gr32a* coding sequence by the mini-*white* gene was confirmed by Southern analysis and PCR (Fig. 1a). Both *Gr32a* mutant males and females were healthy, fertile and without an obvious taste phenotype (Supplementary Fig. 1 online). *Gr32a*^{−/−} and wild-type control males showed similar levels of courtship when paired individually with virgin female partners, suggesting that female pheromones were properly received in the absence of *Gr32a* (Fig. 1b). However, when two males competed for a single virgin female, the wild-type male outperformed the *Gr32a*^{−/−} males by a ratio of 4 to 1, a phenotype that was rescued by two genomic *Gr32a* transgenes (Fig. 1c). Movie recordings from these courtship competition experiments showed that *Gr32a*^{−/−} males frequently courted their competitor, in addition to

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AUTHOR CONTRIBUTIONS

T.M. and H.A. conceived the experiments. T.M. conducted all of the experiments. T.M. and H.A. wrote the paper.

the female (Fig. 1d and Supplementary Video 1 online). To further investigate this behavior, we carried out courtship experiments using passive, decapitated flies of either sex. Such flies are aggressively courted, but fail to display behavioral feedback that could confound precise measurement of sex appeal. Consistent with courtship experiments using intact females, decapitated, virgin females elicited the same courtship activity from *Gr32a*^{-/-} and wild-type males (Fig. 2a). In contrast, courtship toward decapitated males increased only in *Gr32a*^{-/-} males and included wing vibration and copulation attempts, behaviors that were not observed in wild-type males (Fig. 2b and Supplementary Table 1 online). Notably, the phenotype was rescued by two *Gr32a* transgenes (Fig. 2b and Supplementary Fig. 2 online).

As *Gr32a* is expressed in both males and females, one might expect a role for this receptor in female mating behavior. We compared female receptivity of wild-type and *Gr32a* mutant females, but did not find any obvious differences (Supplementary Fig. 2). Further behavioral analyses need to be conducted to address a possible role for *Gr32a* in females.

Mated females are less attractive than mature virgin females, as they receive inhibitory male pheromones during copulation^{7,8}. Although wild-type males showed a significant decrease in courtship toward mated females, *Gr32a*^{-/-} males showed the same high courtship index toward both types of females (Fig. 2a, c), indicating that *Gr32a* is required for discrimination between virgin and mated females. To determine the source of the pheromones, we compared courtship levels that were elicited by mated females (contain both cuticular- and seminal fluid-derived pheromones) with those elicited by virgin, but male-conditioned, females (containing only cuticular pheromones; Fig. 2c, d). Male-conditioned females suppressed courtship in control males, but not in *Gr32a*^{-/-} males. Increased courtship toward mated and male-conditioned females was fully and partially rescued by two *Gr32a* transgenes, respectively, indicating that cuticular pheromones are a major determinant of female mating status (Fig. 2c, d), but leaving open the possibility for compounds in seminal fluid as ligands for GR32a.

If GR32a is necessary for the detection of an inhibitory male pheromone, ablation of *Gr32a*-expressing neurons should lead to male-male courtship. Indeed, males with diphtheria toxin (DTI)-ablated *Gr32a*-expressing neurons showed similarly high levels of courtship as *Gr32a*^{-/-} males (Figs. 3a and 2b and Supplementary Table 2 online). In contrast, males expressing DTI in *Gr66a*- or *Gr22e*-expressing neurons and *UAS-DTI* control males showed low male-male courtship. These observations imply that chemosensory neurons residing outside of the labial palps mediate *Gr32a*-dependent courtship suppression, as all labial palp neurons expressing *Gr32a* also express *Gr66a* and *Gr22e*^{9,10}. Indeed, we found that *Gr32a* and *Gr66a/Gr22e* were expressed in distinct, nonoverlapping sets of gustatory receptor neurons in the foreleg (Fig. 3b and data not shown). A role for these neurons in courtship suppression is consistent with our observation of an increase in male-male courtship in *Gr32a*^{-/-} males immediately after the tapping step (Supplementary Table 1). Finally, none of the ablations had an effect on male courtship toward females (Fig. 3a and Supplementary Fig. 2).

All *Gr32a*-expressing neurons have been reported to project to a small area in the subesophageal ganglion (SOG)^{9,10}. However, we occasionally observed axons of *Gr32a*-

expressing neurons projecting into the ventrolateral protocerebrum. Thus, we reexamined neuronal projections using a more sensitive reporter (*UAS-mCD8::gfp*) and a stronger *Gr32a-Gal4VP16* driver (Fig. 3c and Supplementary Fig. 3 online). This analysis revealed that GFP-labeled afferents exited the SOG anterior into the ventrolateral protocerebrum (Fig. 3c). These projections strongly correlated with the presence of the forelegs, but axons from the mid- and hindlegs may also be found in this afferent (Supplementary Table 3 online). We noted that individual animals varied in route and endpoint of this afferent (Supplementary Fig. 3 and Supplementary Table 4 online), implying that either the location of these target cells varies or that the actual targets represent only a small subset of potential targets in the protocerebrum. We did not observe any *Gr32a* projection differences between sexes, albeit their targets may well show sex-specific properties (see below). To the best of our knowledge, *Gr32a*-expressing neurons represent the first instance of chemosensory neurons relaying sensory input directly to a higher-order brain structure.

Even though cellular expression of *Gr32a* has been determined indirectly using the *Gal/UAS* expression system, three lines of evidence suggest that this expression profile reflects largely endogenous *Gr32a* expression. First, several driver lines showed identical expression patterns⁹ (Supplementary Fig. 3). Second, neuronal ablation by *Gr32a-Gal4* driving diphtheria toxin mimicked the *Gr32a*^{-/-} phenotype. And third, expression of a *Gr32a* cDNA using the *Gr32a-Gal4* driver suppressed male-male courtship in *Gr32a*^{-/-} males (Supplementary Fig. 2). Thus, although we cannot positively affirm that the *Gr32a-Gal4* driver is active in every single neuron expressing endogenous *Gr32a* RNA, these experiments demonstrate that the *Gr32a-Gal4* driver is expressed in the cells that provide essential, endogenous *Gr32a* function.

One of the *Gr32a* phenotypes that we have described here is reminiscent of the phenotype recently reported for a mutation in the olfactory receptor gene *Or67d*⁴. *Or67d*^{-/-} males show an increase in male-male courtship, although they are still attracted to females. Hence, these two receptors appear to mediate general courtship suppression toward other males. However, there are clear differences between *Or67d*^{-/-} and *Gr32a*^{-/-} males. First, the two receptors mediate their role in courtship suppression along different sensory pathways. OR67d, which detects the volatile chemical cVA, is expressed in olfactory sensory neurons that project to a single glomerulus in the antennal lobe⁴, whereas GR32a-expressing neurons mediate sensory input via gustatory pathways to the SOG and higher brain centers in the ventrolateral protocerebrum. Second, we observed that *Gr32a*^{-/-} males, which have a functional *Or67d* gene and hence should respond normally to cVA, courted virgin and mated females equally well (Fig. 2a, c), suggesting that cVA/OR67d are not involved in courtship suppression toward mated females. However, to unequivocally determine whether *Or67d* has a role in courtship suppression toward mated females, respective mutant animals will have to be subjected to the assays presented here. Finally, OR67d⁴, but not GR32a (Supplementary Fig. 2), appears to be required in females for efficient mating.

How are pheromone cues from *Gr32a*-expressing neurons integrated into the neural courtship circuitry? Neuroanatomical studies have revealed that the ventrolateral protocerebrum, to which *Gr32a*-expressing neurons project, also receives input from visual, auditory and possibly olfactory channels^{11–13}. Thus, we propose that different sensory

courtship cues are integrated in this brain region. Notably, several neuronal cell clusters expressing male forms of the FRUITLESS protein (FRU^M)¹⁴ are located near the ventrolateral protocerebrum, even though immunohistochemical studies have not yet revealed direct contact between FRU^M- and *Gr32a*-expressing neurons (Supplementary Fig. 3).

In this study, we found that *Gr32a* is important for suppressing courtship toward males and mated females. Thus, GR32a is required for discriminating between the two sexes and between females of different mating status. We suggest that the latter role is especially relevant because the physical difference between mated and virgin females is restricted to chemosensory signals and mated females are ‘low-efficiency’ mating targets that show extensive rejection behavior.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Knockout of the *Gr32a* gene and courtship analysis of mutant males. **(a)** Representation of the *Gr32a* locus before (top) and after (bottom) homologous recombination. Gels show Southern and PCR analysis from wild-type and *Gr32a*^{-/-} DNA, respectively. B = *Bgl*II; X = *Xma*I. PCR1 and PCR2 indicate amplified fragments (black bars, 5' and 3' probe; white boxes, neighboring genes). **(b)** *Gr32a*^{-/-} males mated with females as efficiently as wild-type males in single pair courtship assays ($n > 30$). Fraction of non-maters (left) and time to copulation (right) were the same in both type of males. Error bars represent s.e.m. ($P > 0.05$, χ^2 test for non-maters, ANOVA for copulation latency). **(c)** Competition experiments (two males, one female). The bar shows the percentage of copulation success of either *Gr32a*^{-/-} males (left) or *Gr32a*^{-/-} males carrying two *Gr32a* 5.9 transgenes (right) competed with the wild-type males. (* $P < 0.05$, χ^2 test for non-maters, ANOVA for copulation latency, $n > 40$). **(d)** Still frame from a movie showing a *Gr32a*^{-/-} male (arrow) courting his competitor, who courts the female (see Supplementary Video 1).

Figure 2.

Gr32a mutant male shows high courtship activity toward males. (a–d) Courtship index of males toward decapitated females (a), males (b), mated females (c) or male-conditioned females (d). Male genotypes were wild type (left), *Gr32a*^{-/-} (middle) or *Gr32a*^{-/-} containing two *Gr32a*_{5.9} transgenes (right). Error bars represent s.e.m. Statistical significance was measured by ANOVA (* *P* < 0.05, ** *P* < 0.0001, *n* > 30).

Figure 3.

Expression of *Gr32a* in tarsal neurons and their role in male-male courtship suppression. **(a)** Courtship index of males (6–10 d old) with DTI-ablated neurons toward decapitated males. DTI was expressed in distinct neurons (indicated by *Gal4* drivers; 1 and 2 indicate different lines). Error bars represent s.e.m. Statistical significance was measured by ANOVA (* $P < 0.05$, ** $P < 0.0001$, $n > 30$). **(b)** Visualization of *Gr32a*- and *Gr66a*-expressing neurons in a fore tarsi; *mCD8::GFP* reporter was expressed by *Gr32a* (top), *Gr66a* (middle) or both drivers (bottom). **(c)** Projection pattern of *Gr32a*-expressing neurons in brain. Axons (antibody to GFP, green) and the neuropil (antibody to nc82, red) were visualized by performing antibody staining on whole brains. Outlined square is enlarged on the right. AL, antennal lobe; VP, ventro-lateral protocerebrum.