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Multiple channels of DEET repellency in *Drosophila*

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Abstract

Mosquitoes and flies transmit a number of serious human pathogens including malaria, African sleeping sickness, Zika and West Nile Virus. DEET (N,N-diethyl-m-toluamide) is the most widely used prophylactic insect repellent to inhibit insect bites and the subsequent vector-borne diseases. Despite its use since 1944, the mechanism for DEET repellency remains controversial. Both olfactory and gustatory mechanisms have been proposed. Here, we revisited the role of smell and taste in DEET repellence using *Drosophila* as a model. We observed a modest defect in DEET repellency in mutants defective for *ORCO*, a co-receptor mediating the majority of odorant responses. Analysis of the responses of individual olfactory receptor neuron (ORN) classes to DEET reveals none are strongly activated by this compound, and blocking individual ORN classes in the antenna by expressing tetanus toxin under odorant receptor (Or) promoters does not block DEET repellence. This argues against the existence of a specific ORN mediating DEET repellence in *Drosophila*. Activation of all ORCO-expressing neurons using red light activated channelrhodopsin favors attraction, not repellence, in behavioral valence. This result is not consistent with the hypothesis that DEET activates multiple ORN classes to act as a 'confusant' to block attraction. Examination of gustatory role of DEET revealed fruit flies are approximately 100 times more sensitive to DEET detected by the gustatory system compared to the olfactory neurons. We used RNAi to knock-down all known 34 Grs (gustatory receptors) expressed in inhibitory gustatory neurons in the labellum and legs. We also screened candidate receptors encoded by other gene families involved in the detection of bitter compounds including 14 Ionotropic receptors (Irs), 5 pick-pocket subunits (PPK), 3 transient receptor potential ion channels (TrpA, TrpL, Painless) and 1 metabotropic glutamate receptors gene (DmXR). Using an egg-laying preference assay, we saw striking defects in DEET avoidance when expression of either *Gr32a* or *Gr33a* were inhibited, confirming these receptors are necessary for DEET discrimination in this assay. No other receptors tested had a significant effect on DEET-induced gustatory behavior including Gr66a. Our findings confirm a multimodal mechanism for DEET detection in fruit flies and support a prominent role for taste detection mediating DEET repellence.

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Author Contributions:

HG performed the experiments, made the figures, wrote the manuscript and assisted with experimental design. KS assisted with the generation of *UAS-Gr32* line and the feeding assay. D.S. guided the studies. The authors have no competing financial interests with this work.

Introduction

Arthropods transmit a wide-range of infectious human pathogens that cause devastating effects on human health (Brouqui 2011). Flies transmit leishmaniasis and African sleeping sickness, ticks transmit Lyme disease and Rocky Mountain Spotted Fever, and mosquitoes spread malaria, dengue, yellow fever, West Nile and Zika viruses (Gubler 2009). Malaria alone is responsible for hundreds of thousands of human deaths annually, mostly young children (Murray et al. 2012). While advances in treatment for malaria and vaccines for some of these pathogens are progressing, the best way to reduce disease incidence is to prevent insect bites and control vector populations.

DEET (N,N-diethyl-m-toluamide) is the most commonly used tropical prophylactic tool to prevent insect bites due to its extended protection time and relatively low toxicity (Osimitz and Grothaus 1995; Goodyer and Behrens 1998; Fradin and Day 2002). However, DEET has drawbacks including unaffordability in some endemic populations, it dissolves plastics, and it has an unpleasant smell (Ray 2015). In some individuals, DEET is an irritant and can cause severe epidermal reactions (Brown and Hebert 1997). Despite its widespread use for almost 75 years, the mechanisms of action of DEET detection are still a subject of debate (Leal 2014; DeGennaro 2015).

Both *Drosophila* and mosquitoes are repelled by the vapor phase of DEET. The repellence is dependent on odorant receptor co-receptor (ORCO) (Ditzen et al. 2008; DeGennaro et al. 2013). Two hypotheses have been postulated to explain vapor phase avoidance. First, DEET could activate an olfactory receptor neuron (ORN) circuits that elicit an avoidance behavior (Xia et al. 2008; Stanczyk et al. 2010; Xu et al. 2014). Second, DEET could alter the response patterns of many ORNs, acting as an olfactory “confusant” (Pellegrino et al. 2011; DeGennaro 2015; Liu et al. 2017). In mosquitoes *Culex quinquefasciatus*, one odorant receptor (Or), CquiOr136 is activated by DEET and a naturally-derived repellent compound, methyl jasmonate (Xu et al. 2014). Knocking down CquiOr136 significantly reduces avoidance of DEET (Xu et al. 2014). Therefore, in mosquitoes, DEET appears to exert its vapor phase repellence through an inhibitory circuit. *Drosophila* lack a homolog of this receptor, and the mechanism of DEET repellence is unclear. Among the 60 classes of olfactory receptor neurons in *Drosophila*, only Or42 neurons have been reported to respond robustly to DEET (Syed et al. 2011). However, a role for DmOr42a in DEET avoidance has not been established.

DEET has also been reported to act as a contact repellent in *Drosophila* (Lee et al. 2010). Three gustatory receptors (Grs), *DmGr32a*, *DmGr33a* and *DmGr66a* have been reported as necessary but not sufficient for DEET detection (Lee et al. 2010). This suggests additional DEET receptor components might remain undiscovered. In addition to Grs, Ionotropic receptors, pick-pocket channel subunits, transient receptor potential ion channels and metabotropic glutamate receptors (DmXR) have been implicated in bitter sensation (Mitri et al. 2009; Freeman and Dahanukar 2015). However, their potential roles in the gustatory detection of DEET have not been explored.

Here, we systematically revisited the roles for *Drosophila* olfactory and gustatory neurons for DEET responses. We confirmed a modest role for ORCO in DEET repellence, but find the Drosophila gustatory system is much more sensitive to DEET. Our studies confirm roles for *Gr32a* and *Gr33a* in DEET avoidance behaviors and ruled out the involvement of a large set of candidate receptors in the detection of DEET.

Material and Method

Drosophila stocks

An isogenized strain of w^{1118} was used as a wild type control. *pORCO-GAL4* flies were a gift form Leslie Vosshall. Gr32a mutants, Gr33a mutants and Gr66a mutants were obtained from Craig Montell. NropA^{p24} flies were provided by William Park. The following stocks were obtained from the Bloomington Stock Center (Bloomington IN) Gr2a RNAi (BS65220), Gr22b RNAi (BS64902), Gr22d RNAi (BS38248), Gr22f RNAi (BS64880), Gr32a RNAi (BS65205), Gr33a RNAi (BS62940), Gr39b RNAi (BS62396), Gr57a RNAi (BS36738), Gr58c RNAi (BS62508), Gr59c RNAi (BS63673), Gr59d RNAi (BS64677), Gr66a RNAi (BS31284), Gr77a RNAi (BS38236), Gr89a RNAi (BS64023), Gr93b RNAi (BS61242), Gr93d RNAi (BS60481), Gr98b, Gr98c RNAi (BS36735), Gr8a mutant (BS40976), Gr10a mutant (BS29947), Gr23a mutant (BS19287), Gr28b mutant (BS24190), Gr36b mutant (BS24608), Gr36c mutant (BS26496), Gr47a mutant (BS65843), Gr58b mutant (BS29065), Gr92a mutant (BS61734), Gr93a mutant (BS35886), Gr94a mutant (BS17550), Gr97a mutant (BS56384), Gr98b mutant (BS42084), Ir11a RNAi (BS61898), Ir25a mutant (BS41737), Ir52c mutant (BS24580), Ir56a RNAi (BS60903), Ir56b mutant (BS27818), Ir56d RNAi (BS64617), Ir62a mutant (BS32713), Ir76a RNAi (BS34678), Ir76b mutant (BS51310), Ir94a RNAi (BS53703), Ir94f mutant (BS33095), Ir94h RNAi (BS53975), Ir100a (BS31853), PPK11 mutant (BS23781), PPK19 mutant (BS36434), PPK23 mutant (BS33300), PPK28 mutant (BS33559), PPK29 mutant (BS19016), Painless mutant (BS31432), TrpL mutant (BS31433), and UAS-Gr66a (BS24775). Gr22e RNAi (v9389), Gr28a RNAi (v31165), Gr36a RNAi (v48018), Gr98d RNAi (v4398), Gr39a mutant (v8686) were obtained from the Vienna Drosophila Resource Center (Vienna Austria).

Trap assay

The experimental procedures were previously described in (Guo et al. 2017). Briefly, traps consisting of 13×100mm borosilicate culture tubes (Fisher) containing 3 ml of 50% apple cider vinegar diluted with 3 ml of 1% agarose (Sigma) with an eppendorf centrifuge tube in the opening. A 1 mm hole was drilled through the bottom of the eppendorf tube to allow flies access to the trap. Forty flies were released in a 30 cm^3 chamber containing 4 DEET traps and 4 control traps. Flies were allowed to choose the traps overnight.

Single sensillum recordings

Single sensillum recordings were performed as described in (Laughlin et al. 2008; Pitts et al. 2016). Filtered AC signals (200Hz-3kHz) were recorded and digitized for analysis (Autospike 32). Briefly, flies were housed in fresh vials containing standard yeast molasses food in small groups prior to SSR recordings. For conventional stimulation in SSR, 30 μl of

pure DEET (Sigma-Aldrich) were placed on a small piece of filter paper (1.5 cm^2) inserted into a 5.75-inch Pasteur pipette and air was passed over this filter into a constant stream (30 ml/sec) of humidified air passing over the preparation. For close-up stimulation, 30 μl of pure DEET were placed on a small piece of filter paper (1.5 cm^2) inserted into a 5.75-inch Pasteur pipette and puffed directly at a distance of 0.5 cm away from the antennae.

Optogenetics experiment

The light source used for optogenetic stimulation was described in Inagaki et al. (Inagaki et al. 2014). New enclosed blind flies (*NorpA*^{P24};*pORCO-Gal4/UAS-ReaChR* and *NorpA* ^{P24};*pORCO-Gal4* and *NorpA* ^{P24};*UAS-ReaChR*) were collected and were housed in the dark on Nutri-Fly media for 6 days supplemented with 1% retinal stock solution (40 mM all-trans-retinal (Sigma)). High-power Rebel LEDs (627 416 nm, LUXEON) were placed at 5 cm distance from the traps. Forty flies were released in a 30 cm^3 chamber containing 4 traps in the light zone and 4 traps in darkness. Activation of the olfactory receptor neurons by the red-light conditions above was confirmed by SSR.

Transgenic flies

The coding sequence of Gr32a was cloned by high fidelity DNA polymerase (NEB) and sequenced to rule out PCR errors. Gr32a was subcloned into the pUAST vector (Brand and Perrimon 1993). Embryos were injected as described in (Spradling and Rubin 1981). Around 200 adults were collected and individually crossed to *w*^{1118}. Transgenic animals were identified by eye color.

Oviposition preference assay

Fifty percent apple cider vinegar (ACV) with or without DEET was dissolved in 1% agarose gel and poured into a 35 mm plate. The agarose plates were halved and the half plate containing DEET was swapped with control half and vice versa. Twenty female flies that were starved for 4 hours were released in the egg-laying chamber overnight and the number of eggs laid on each half was recorded. The preference index was calculated by ($^{\#}$eggs laid in control part) - ($^{\#}$eggs laid in DEET part) / total number of laid eggs.

Feeding assay

The experiment procedures were largely adapted from (Lee et al. 2010). Briefly, 10 μl of food dye (Green or Red) were added in 1 ml of 1% agarose gel containing 5 mM sucrose. Subsequently, 10 μl of DEET or paraffin oil control were added were added to reach the concentration of 1% before pouring a strip of 8 PCR-tube caps. Finally, 40 flies (20 male and 20 female) were starved for 2 hours were released into a chamber (10 cm × 10 cm × 3 cm) containing the food. The feeding preference was calculated by counting flies according to the color of the abdomen as $^{\#}$G (Green), $^{\#}$R (Red) and $^{\#}$Purple (P). The preference index was calculated by ($^{\#}$G + $^{\#}$0.5P) - ($^{\#}$R + $^{\#}$0.5P) / ($^{\#}$G + $^{\#}$R + $^{\#}$ P).

Experimental Design and Statistical Analysis

Two to six days-old flies were used for electrophysiological recording. For behavior, flies were isolated at least 24 hours before the test to avoid the anesthesia effects of CO_2. Two-

tailed Student test was used to test for statistical significance using Origin 8.0. Two-way ANOVA with Post Hoc Turkeys's multiple comparisons was used (SPSS package). All data were plotted as mean ± SEM.

Results

DEET avoidance requires ORCO

To confirm ORCO involvement in DEET repellence, we designed a food trap assay where the flies relied on olfaction. Wild type flies showed striking avoidance behavior to the food vials containing 30 μl of pure DEET or to a less extent 30 μl of 10% DEET, but were not repelled by 30 μl of 1% DEET (Supplementary figure 1A). In contrast, *ORCO* mutants were indifferent of the presence of 10% DEET (Supplementary figure 1B). This confirms previous studies indicating that ORCO olfactory neurons mediate DEET responses (Ditzen et al. 2008)

In order to identify specific Ors responsive to DEET, we conducted a comprehensive electrophysiological SSR analysis of antennal ORNs. Undirected application of DEET failed to elicit significant responses from ORNs in the antenna (Figure 1A). However, direct application of DEET vapor without dilution elicited responses in most of ORNs, including those in antennal basiconic sensilla ab2, ab3, ab4, ab6, ab8, ab9, ab10, intermediate sensilla ai3 and the neurons located in one basiconic sensilla on maxilla palp pb1 (Figure 1B). With the exception of ab1, ab2 and ab3, we did not distinguish the responses from different ORNs in the sensilla. The responses from ab4-ab10, ai3 and pb1 were a summation of spikes from all ORNs (Figure 1B). ORNs in ab3, ab8, ai3 and pb1 had more robust responses to DEET than other ORNs. Interestingly, the ORNs in ab5 and at4 were not activated by DEET, and in fact the spontaneous activity was inhibited by DEET in these neurons (Figure 1C).

Contribution of specific OR circuits to DEET avoidance

To identify the potential involvement of DEET sensitive ORNs in DEET repellence, we blocked the olfactory signal transmission of specific receptor inputs by driving tetanus toxin with individual *pOr-GAL4* drivers (Sweeney et al. 1995). Expression of tetanus toxin in these neurons blocks synaptic transmission in the antennal lobes, and is expected to produce a behavioral effect similar to a receptor gene mutation. Functional ablation of Or2a ORNs in ai3 or Or42a ORNs in pb1 led to a moderate defect in DEET avoidance in the food trap assay, but the differences between treatments and control were not significant (Figure 2). This result indicated that multiple ORNs, not the single one, were necessary for DEET repellence.

Global activation of ORCO ORNs leads to attraction

If *Drosophila* lacks inhibitory olfactory neurons tuned to DEET, an alternative possibility is that widespread activation of ORNs by DEET could result in inhibition. To test this hypothesis, we expressed red-shift channelrhodopsin in the all ORNs using *ORCO-GAL4* (Inagaki et al. 2014). We confirmed red light activation of the olfactory neurons by red light using SSR (data not shown). We assayed the ability to locate ACV sources with or without DEET. In order to avoid visual cues that might occur between light and no light conditions,

we used blind flies mutant for *norpA*^{P24} (Pearn et al. 1996). The blind flies carrying *ReaChR* showed a preference towards food traps illuminated by red light, while two controls, *UAS-ReaChR* and *ORCO-GAL4* were not statistically affected by the light (Figure 3). The red light caused a slight increase in chemotaxis behavior to traps in WT controls, presumably because of the phototaxis behavior of insects (data not shown). These results suggest that activation of ORCO expressing olfactory neurons enhances chemotaxis to ACV traps.

Taste responses mediated through Gr32 and Gr33a receptors are necessary but not sufficient for DEET sensitivity and avoidance

We tested the effects of the presence of DEET in an egg laying assay. 35 mm plates were produced with ACV on both halves, but a dilution of DEET only in one half. Remarkably, we found dilution of DEET down to 0.01% still inhibited females from laying eggs. These concentrations are 100-fold lower than the DEET concentrations required for olfactory avoidance. We confirmed the behavior was due to gustation by testing *ORCO* mutants without antennae. These flies were indistinguishable from wild type controls in the DEET egg laying assay (Figure 4). To identify the receptors for DEET detection, we used either RNAi or genetic mutants for corresponding genes that are involved in the detection of bitter compounds (Ishimoto and Tanimura 2004; Montell 2009; Weiss et al. 2011; Freeman and Dahanukar 2015) (Figure 5). We screened a large array of candidate receptors (Figure 5). First, we obtained and screened Gr mutants for abnormal DEET avoidance in egg laying assays (Figure 5A). When *Gr32a*, *Gr33a*, *Gr66a* or *Gr98b* were mutated, the females showed a strikingly diminished avoidance towards laying eggs on the DEET side of the plates. (Figure 5 A and B). However, mutants often have additional genetic lesions and genetic backgrounds that can affect behavior. Therefore, we also used RNAi to specific receptors to confirm the mutant phenotypes. RNAi to *Gr32a* and *Gr33a* produced defective egg laying behavior that was similar or worse than the mutants. However, RNAi to *GR66a* or *GR98b* failed to recapitulate the mutant phenotypes of *Gr66a* mutant or *Gr98b* mutant (Figure 5 D). Finally, using mutants or RNAi, we also screened 14 *Irs*, 5 *PPKs*, *TrpA*, *TrpL*, *Painless*, and *DmXR* (Figure 5C). No significant defects in DEET avoidance behavior were detected in the egg laying assay (Figure 5 C). Testing these receptors in the trap feeding assay also failed to show any defects in DEET avoidance. (Supplementary figure 2).

Expression of Gr32a and Gr33a in CO_2 detecting antennal neurons does not confer DEET sensitivity

Gr32a and Gr33a are both required to avoid laying eggs on DEET substrates. It is likely these receptors function together in a DEET receptor complex. To determine if these two receptors are sufficient as well as necessary, we expressed them together in the CO_2-sensing neurons, ab1c. These antennal neurons normally express Gr63a and Gr21a to form a CO_2 receptor (Kwon et al. 2007). Co-expression of *Gr32a* and *Gr33a* in the CO_2-sensing ab1c neurons using *Gr63a-GAL4* failed to confer DEET sensitivity to the ab1c neuron (Figure 6). Therefore, there must be one or more additional factors required for a functional DEET receptor that are lacking in these neurons.

Discussion

DEET is the most widely used insect repellent. The identification of the DEET receptor in different insects could greatly expedite the development of a new repellents. In this study, we took advantage of the model system, *Drosophila melanogaster*, to explore DEET detection. We found that high concentrations of DEET are detected by the olfactory system, and this detection is partly dependent on the obligatory Or subunit, ORCO. This is consistent with the conclusions drawn from previous work (Ditzen et al. 2008; DeGennaro et al. 2013). However, we find the fruit fly olfactory system is rather insensitive to DEET. Work in Culex mosquitoes implicates a specific tuning receptor, CquiOR136 in olfactory DEET detection (Xu et al. 2014). Indeed *Drosophila* lacks a conserved homolog of this receptor, that could explain the relative insensitivity of the fly olfactory system to DEET. Using SSR and concentrated DEET as a stimulus, ORNs located in ab3, ab8, ai3 and pb1 are DEET sensitive (Figure 1), and previous studies have implicated Or42a in pb1 sensilla as DEET sensitive neurons (Syed et al. 2011).

Whether there is an ORN specifically mediating DEET repellence in *Drosophila* is a matter of debate. In this study, we used tetanus toxin to block the signal transmission at the synapse in the antennae lobe for individual DEET sensitive ORNs. Functional ablation of Or2a or Or42a neurons had little effect on DEET avoidance, suggesting that there is no specific inhibitory ORN mediating DEET repellence in *Drosophila* (Figure 2). Although different behavior assays were utilized in these different studies, mosquitoes seem to have a higher olfactory sensitivity to DEET.

Intriguingly, the SSR recording revealed that most of OSNs were activated by concentrated DEET, albeit at variable degrees of sensitivity. Non-specific activation of multiple ORNs could account for the DEET avoidance. However, the global activation of ORCO-expressing ORNs using ReaChR activation by red light does not support this hypothesis (Figure 4). One possibility is that besides activation, the inhibition of ORNs, such as those residing in ab5 and at4, are also necessary for DEET repellence. It would be interesting to simultaneously simulate the activation and inhibition pattern of DEET by optogenetics activation and the simultaneous expression of TNT in ab5 and at4 neurons. We cannot rule out the possibility that DEET works as an “confusant” in which DEET caused activation and inhibition together to scramble the olfactory coding (Pellegrino et al. 2011). However, we can conclude that gustation also contributes to mediating DEET behavioral responses.

DEET has been reported to function as a feeding deterrent (Lee et al. 2010; Sanford et al. 2013; Dickens and Bohbot 2013; Lu et al. 2017). Gr32a, Gr33a and Gr66a are widely expressed in bitter-sensing neurons, and are thought to be necessary for DEET repellence (Lee et al. 2010). However, expression of these three Grs in sweet-sensing neurons failed to result in DEET sensing. This implied there are additional components remaining to be uncovered (Lee et al. 2010). We sought to identify additional genes for DEET detection by expanding the list to all Grs, PPKs, Trp channels and DmXR associated with bitter detection. We employed two assays, egg laying preference and a feeding assay to test for defective DEET avoidance behavior. We ruled out the involvement of other genes and we confirmed the necessity of *Gr32a* and *Gr33a* by both RNAi and mutation (Figure 5 and Supplementary

figure 2). However, *Gr66a* was previously implicated in DEET taste detection in *Drosophila*, and we were able to confirm this finding in flies mutant for this receptor. However, flies expressing RNAi to Gr66a were normal. The *Gr66a* mutant used in Lee et al, is a deletion mutation including two flanking genes in addition to *Gr66a*, *Sbp2* and *BI-1. Sbp2* (CG7066) is an mRNA binding protein that is reported to have neuroanatomical defects when mutated (Dumitrescu et al. 2005). *Bl-1* (CG7188) is a regulator of apoptosis (Chae et al. 2004). We suspect the loss of one or the combination of these other genes is responsible for the defects in DEET behavior. Similarly, the defective DEET avoidance in *Gr98b* mutants we identified here may result from other loci, as the RNAi failed to produce a similar phenotype. The Gr98b mutants are extremely unhealthy, suggesting they have additional defects that could account for abnormal egg laying behavior.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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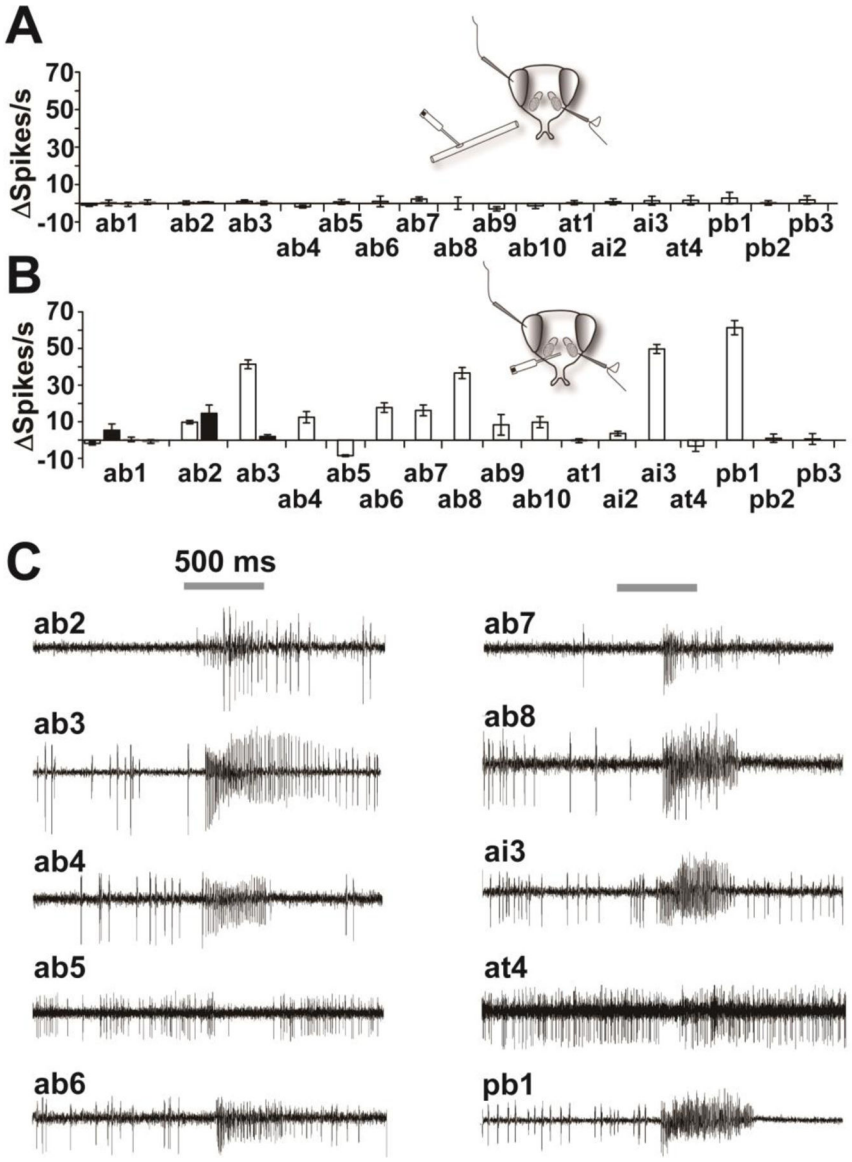

Figure 1. The olfactory responses to pure DEET.

(A) The olfactory response pattern of ORNs to pure DEET diluted in background air. No ORNs were activated by the diluted DEET. n= 5. **(B)** The ORN response pattern to the pure DEET that was directly puffed to antennae. DEET elicited olfactory responses from ab2, ab3, ab4, ab6, ab7, ab8, ai3 and pb1, while worked as an inhibitor of ab5 and at4. n=5. **(C)** the representative traces of activation and inhibition posed by DEET.

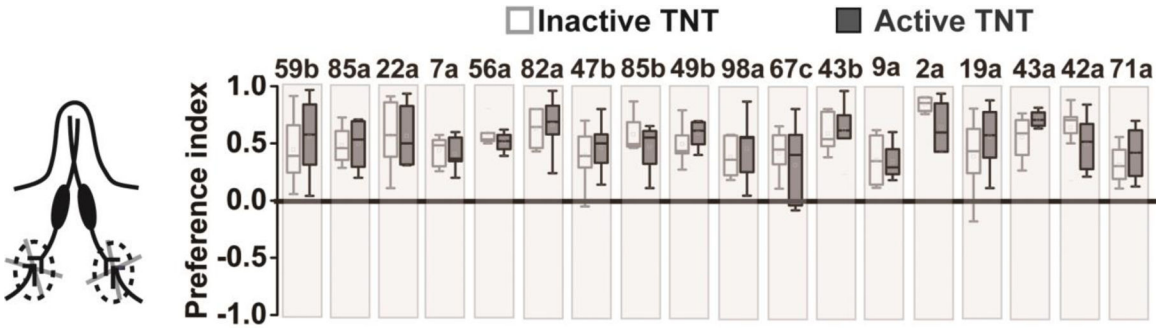

Figure 2. The avoidance behaviors of flies expressing TNT in ORNs to DEET.

When Individual ORN was functionally ablated by TNT, no significant DEE-avoidance defects were found, suggesting the non-existence of specific ORN to mediate DEET repellence. Two-tailed *t* tests were used to compare the differences between the groups of inactivated-TNT and active TNT. n=4.

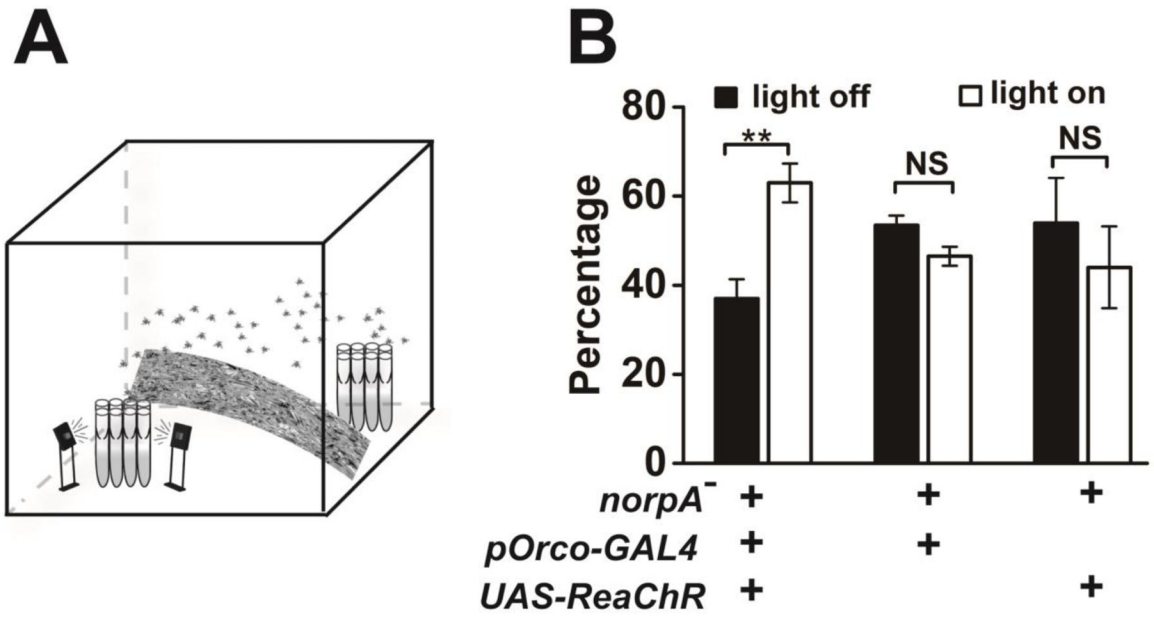

Figure 3. The global activation of ORNs favors attraction.

(A) the experimental schematic of trap assay. One stripe of foil was put in the middle line of the box to make the illumination zone and darkness zone. **(B)** The activation of ORNs by red light triggered attraction behavior, not avoidance. The driver line and UAS line were largely not influenced by light. ** $p<0.01$ by two-tailed t test. n=5. Two-way ANOVA analysis indicated the significant differences between *ORCO>ReaChR* group and *pORCO-GAL4* group ($F(1,12) = 22.987$. $p < 0.001$) as well as between *ORCO>ReaChR* group and *UAS-ReaChR* group ($F(1,12) = 6.190$. $p = 0.029$).

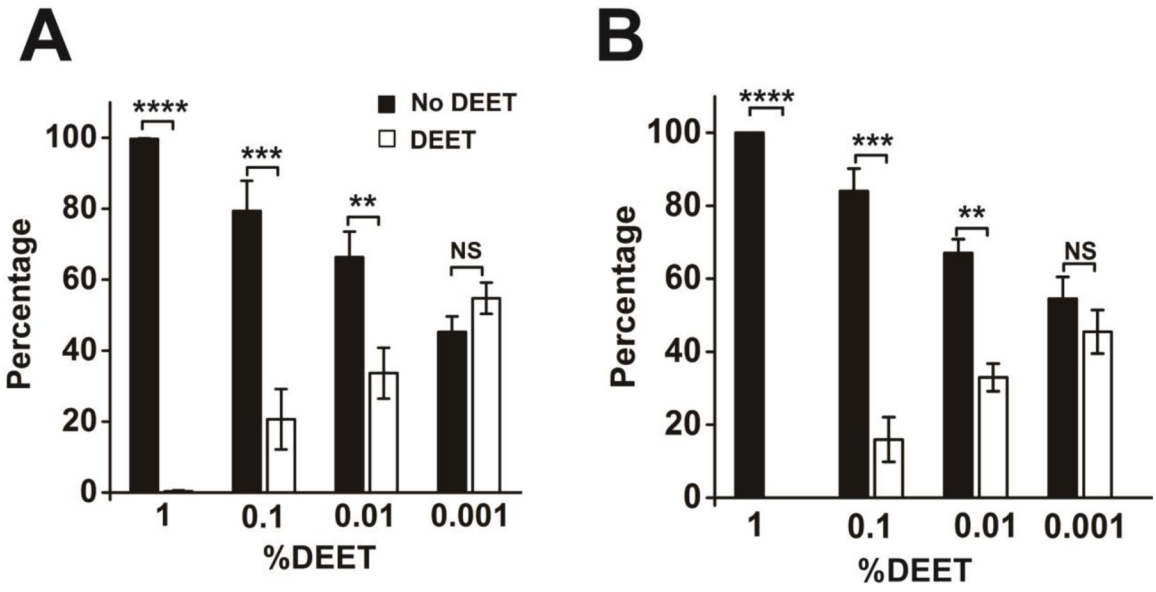

Figure 4. The feeding avoidance of wild type and ORCO mutant without antennae to different concentrations of DEET.

(A) the anti-feeding valence of DEET were detected down to 0.01% in wild type. **(B)** ORCO mutant without antennae showed similar feeding avoidance with wild type to DEET. n=5 for each treatment. ****p<0.0001, ***p<0.001, **p<0.01 by two-tailed t test.

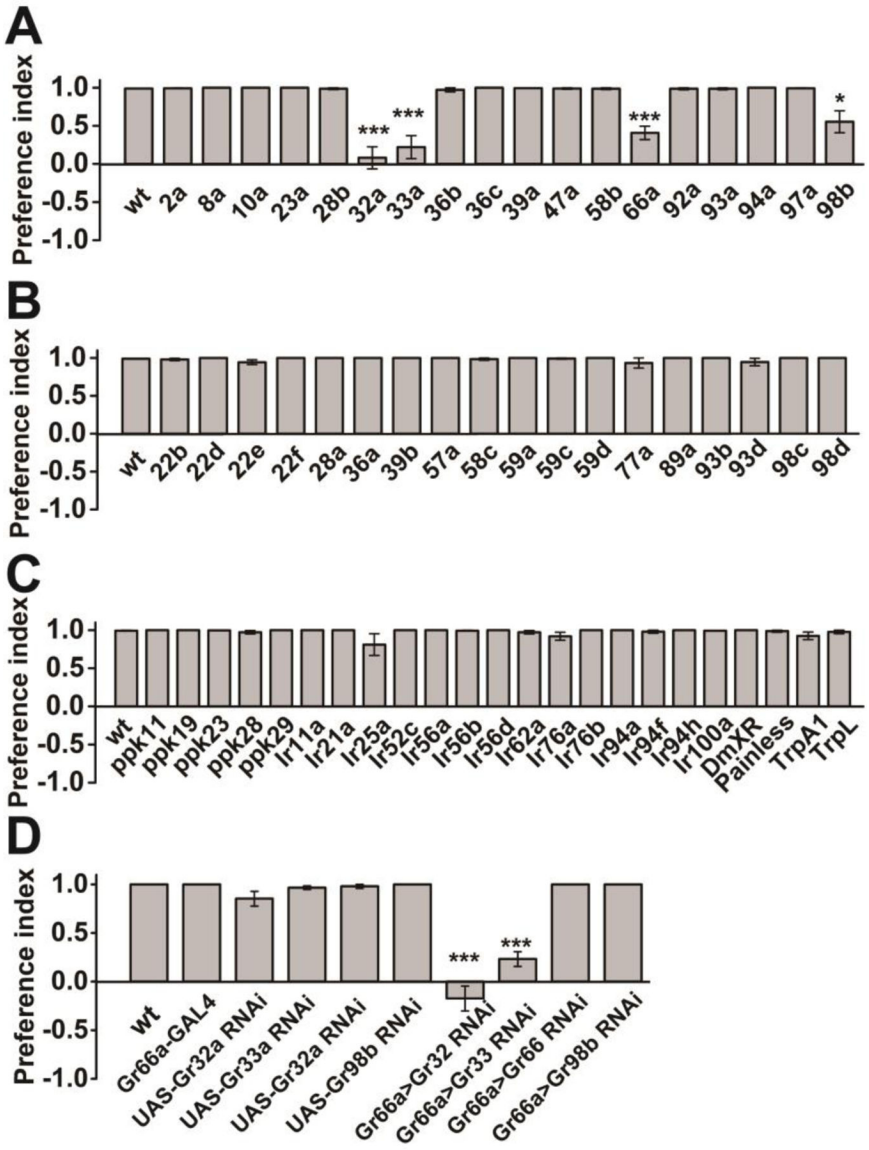

Figure 5. The screening of DEET receptors by oviposition preference assay.

(A) the screening of mutants of members of Gr family expressing in bitter responsive neurons. Mutants of Gr32a, Gr33a, Gr66a, Gr98b showed defective DEET avoidance behaviors. **(B)** the screening of members of Gr family expressing in bitter responsive neurons using RNAi. **(C)** the screening of members of PPK family, Ir family, Trp channel family and DmXR. No defective avoidance behavior was detected when knocking down or mutants of those genes. **(D)** The validation of defects of mutants of Gr32a, Gr33a, Gr66a and Gr98b using RNAi. The defects in Gr32a mutant and Gr33a mutant were confirmed by RNAi, however, the knowing down of *Gr66a* and *Gr98b* failed to resemble the defects detected in mutants. n=4. ***p<0.001 by two-tailed t test.

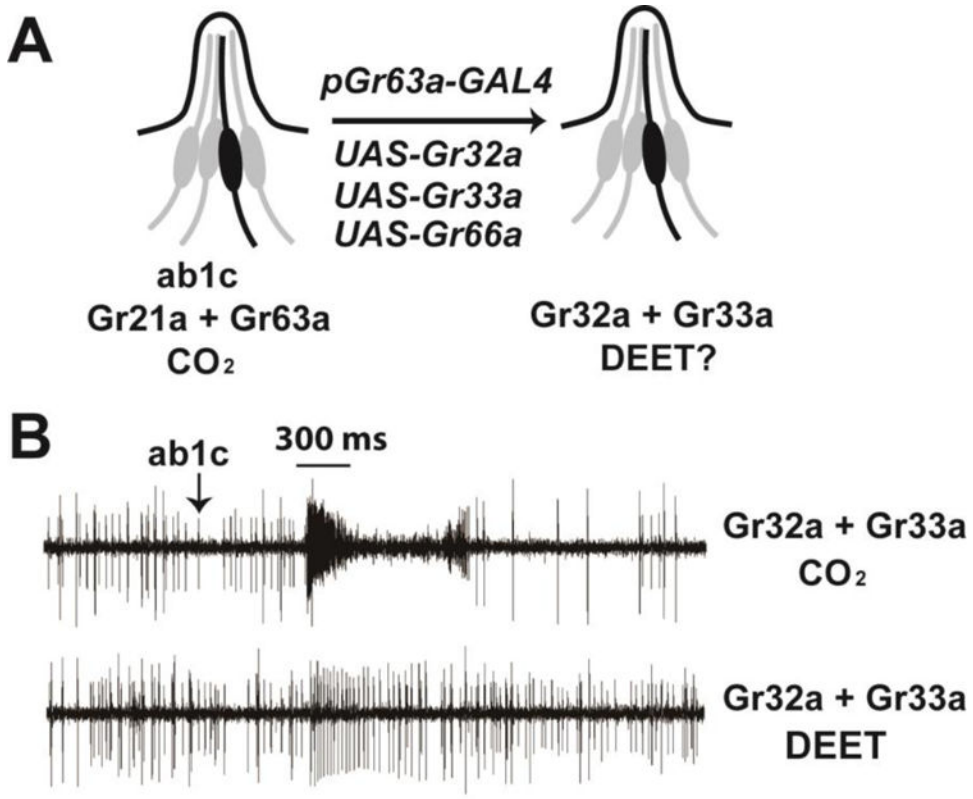

Figure 6. Ectopic expression of *Gr32a* and *Gr33a* in ab1c neuron failed to reconstitute of DEET sensitivity.

(A) the schematic showing the expression of *Gr32a* and *Gr33a* in ab1c neuron expressing CO2 sensing receptors, Gr21a and Gr63a. **(B)** The expression of *Gr32a* and Gr33a driven by *Gr63a* promotor could not produce DEET sensitivity in ab1c neuron. The upper trace showing the CO2 sensitivity. The lower trace showing no activation by DEET in ab1c neuron expressing *Gr32a* and *Gr33a*. The weak activation in ab1a by DEET was detected.