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Supporting Online Material for
**Insect Odorant Receptors Are Molecular Targets
of the Insect Repellent DEET**

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Supporting Materials and Methods.

Animals

w^{1118} and $Or83b^{-/-}$ (1) stocks were maintained on conventional fly food (cornmeal-agar-molasses medium) at 25 °C. Adult mosquitoes [*Anopheles gambiae* strain G3 strain; MRA-112; obtained from MR4 (www.mr4.org) through the Centers for Disease Control and Prevention, Atlanta, GA] were kindly provided by Paul Howell and Mark Q. Benedict and kept at 25 °C and 70% humidity on sucrose solution.

Chemicals

DEET was kindly provided by Moorflex. All odors were obtained from Sigma-Aldrich.

Drosophila behavior

Behavior was measured using a modification of a previously described trap assay (1). Two round holes (2.2 cm diameter) were punched along the midline into a 15 cm Petri dish at equal distance (3 cm) from the rim. Rigid cellulose acetate plugs perforated with pipet tips (1 ml) connected the Petri dish with two small shell vials (2.5 x 9.5 cm). To test repellency *per se*, empty shell vials were used. To investigate the effect of DEET on olfactory attraction, the vials either contained ~2 ml of fly food diluted 3:2 in water or a filter strip with 10 µl pure odorant. Small strips of filter paper (5 x 15 mm) lining the inner wall of the pipette tip were coated with 10 µl DEET (pure or diluted in ethanol) or 10 µl solvent (ethanol). For experiments with decreased direct contact, strips of 40 µm wire mesh or cut and perforated 200 µl pipet tips were used to cover the filter paper. All experiments were conducted in the dark at 25 °C and ~25% humidity with female flies

that were starved for 24 h in humidified shell vials prior to being introduced into the trap. Approximately 60 flies were released into the Petri dish per assay and scored after 24 h by calculating the percent of flies in each trap.

Single sensillum electrophysiology

Extracellular recordings were conducted as described previously (2, 3), using a 10x AC Probe connected to an IDAC-4 amplifier. Two filter strips (4 x 50 mm), one for the food/odor and the second for the DEET/solvent stimulation, were placed into a Pasteur pipet and presented for 1 s under the control of the CS-55 stimulus delivery system. For DEET and odorant stimulations 30 μ l of each substance were applied to the filter paper. For food stimulations, the entire filter strip was bathed in a 3:2 fly food/water solution. Odorants were diluted in paraffin oil. Pure DEET was used in all experiments. For CO₂ stimulation, pre-mixed formulations of 10% or 0.1% CO₂ were mixed with room air in a 20 ml plastic syringe to desired concentrations and then blown over the DEET/solvent-containing Pasteur pipette. In *Drosophila melanogaster*, sensilla were identified by size/location and responsiveness to their preferred ligands (4, 5). While we could attribute different spikes to specific OSNs for the basiconic sensilla ab1-ab4, we were unable to reliably do the same for the remaining basiconic and three of the trichoid sensilla. In these sensilla, we summed all spikes and calculated sensillar responses. In the absence of good ligands for the trichoid sensilla at2-at4 we recorded 29 individual sensilla. Clustering these sensilla according their responses to food odor, DEET, and food + DEET revealed three clusters, each of which likely represented one of the trichoid sensilla. In the absence of knowledge of which cluster represents which trichoid

sensillum, we named them at β , at γ and at δ . The *Anopheles gambiae* capitata peg sensillum was identified by its responsiveness to CO₂ (6).

Data were recorded using Autospike, exported to ASCII format, and subsequently analyzed using custom written software. Spike trains were separated into one second bins and responses were calculated by subtracting the average prestimulus bin from the one second bin during stimulation.

Oocyte expression and electrophysiology

Full length cDNAs of fruit fly ORs [*Or47a* (7) and *Or83b* (1)], mosquito ORs (*GPROR1*, *GPROR2*, *GPROR8*, and *GPROR7*) (6, 8, 9), mouse TRP channel (mTRPM8) (10), OR (MOR-EG) (11), were cloned into the *Xenopus laevis* oocyte expression vector pXpress-X and linearized with XbaI. Full length cDNAs of CFTR (12) and rat olfactory CNGs (CNGB1, CNGA2, and CNGA4) (13) were cloned into pGEMHE and linearized with NheI. Full length cDNA of the fruit fly K-channel *ether-a-go-go* (EAG) (14) was cloned into pGH19 and linearized with NotI. All plasmids were transcribed *in vitro* with mMessage mMachine.

Stock solutions of pentyl acetate (1 M), 2-methyl phenol (1 M), 4-methyl phenol (1 M), 1-octen-3-ol (1 M), forskolin (40 mM), and menthol (1 M) were prepared in DMSO, and then added to Ringer's solution. DEET was diluted directly to the extracellular solution. Odorants were applied to the recording chamber using a gravity driven perfusion system.

Two-electrode voltage clamp recording from *Xenopus laevis* oocytes was carried out as previously described (15). 25 ng of cRNA for the chosen receptor(s) were

microinjected in oocytes. Whole-cell currents were recorded in a two-electrode voltage clamp mode and amplified, low-pass filtered at 50 Hz, digitized at 1 KHz, and saved to a PC hard disk using pClamp 6.0 acquisition software. Data were analyzed using Clampfit 9.0 and Origin PRO 7. Subtracted I-V curves were acquired with a step protocol ranging from -80 mV to +40 mV (20 mV step), and the currents were normalized to the +40 mV data point in the presence of ligand only. Electrodes were filled with a 3 M KCl solution, while the extracellular oocyte Ringer's solution contained (in mM): 82.5 NaCl, 2 KCl, 1 MgCl₂, 5 HEPES (pH 7.5), 1.8 CaCl₂, except for mTRPM8 experiments where no CaCl₂ was added.

Statistics

All statistical analysis was done in R2.3.1 (<http://www.r-project.com>). The multiple comparisons in Fig. 4L were done using a one tailed Kruskal Wallis Test with posthoc multiple comparison correction against the CFTR control. All other comparisons were calculated using a Mann-Whitney Test. Clustering of trichoid sensilla was done using the Mclust algorithm. Dose-response curves in Fig. 1C and D and Fig. 3C and D were fitted with a logistic equation using Origin 7.0.

Supporting Figures.

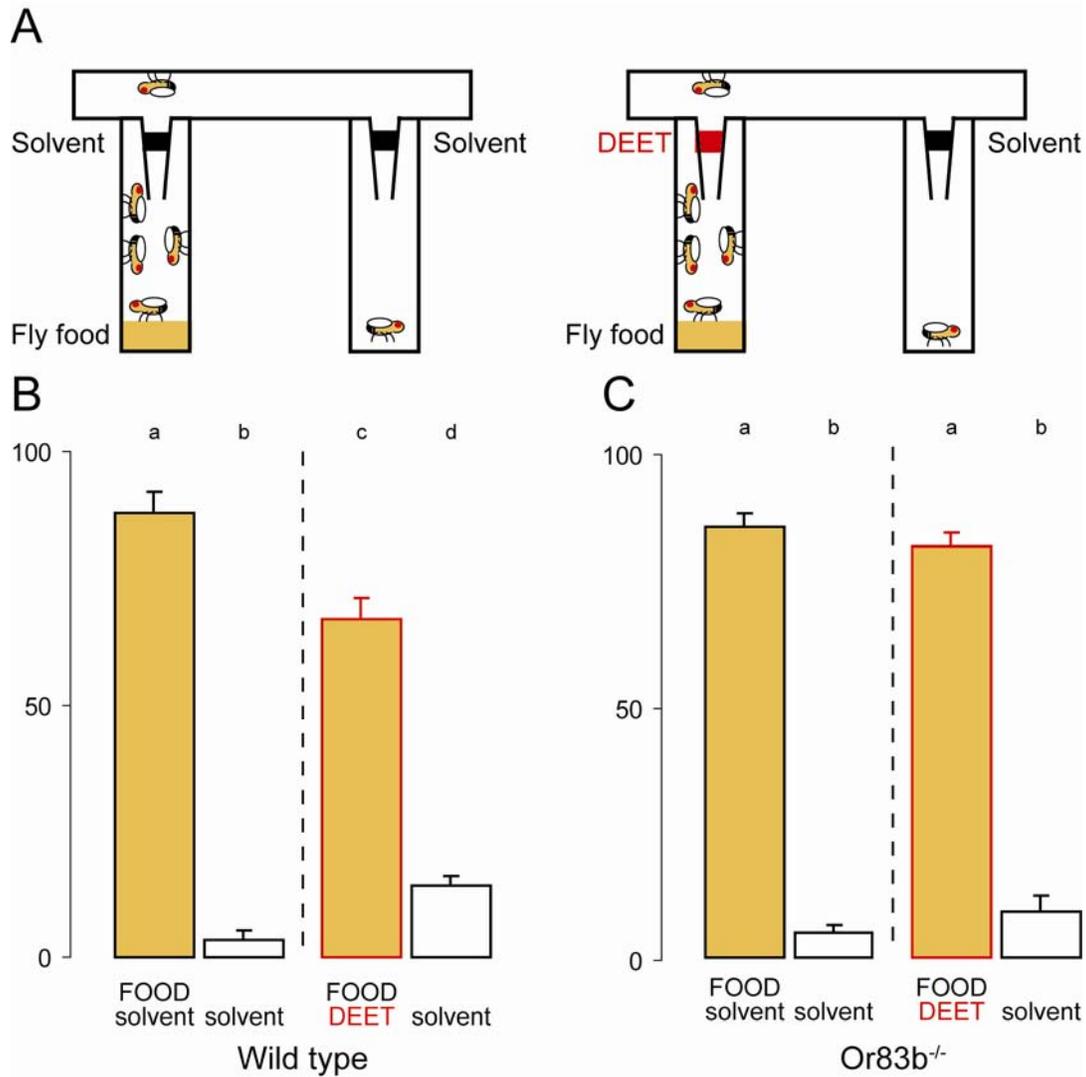


Fig. S1. Distribution of flies in traps baited with one food source. (A) Schematic of trap assay with one food bait. Entrance to trap is coated with solvent (black) or DEET (red). (B-C) Effect of DEET on distribution of wild type (B) or *Or83b^{-/-}* mutant (C) flies in traps with one food bait. Bars labeled with different letters are significantly different ($p < 0.05$, Mann-Whitney Tests with Bonferroni correction for multiple comparisons; mean \pm S.E.M., $n = 12$).

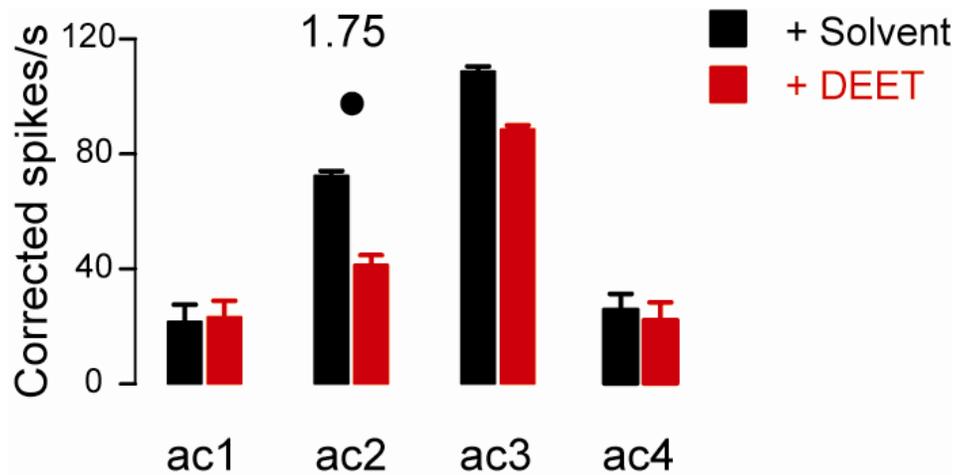


Fig. S2. Responses of antennal coeloconic sensilla to food odor. Single sensillum electrophysiology responses of antennal coeloconic (ac) sensilla stimulated with food + solvent (black bars) or food + DEET (red bars). With the exception of one cell in ac3, all other coeloconic neurons are *Or83b*-independent (16). Data are plotted as mean corrected spikes/s \pm S.E.M. (ac1: n = 5; ac2: n = 5; ac3: n = 11; ac4; n = 6). Filled circle above bar graph indicates the fold decrease in response in the presence of DEET.

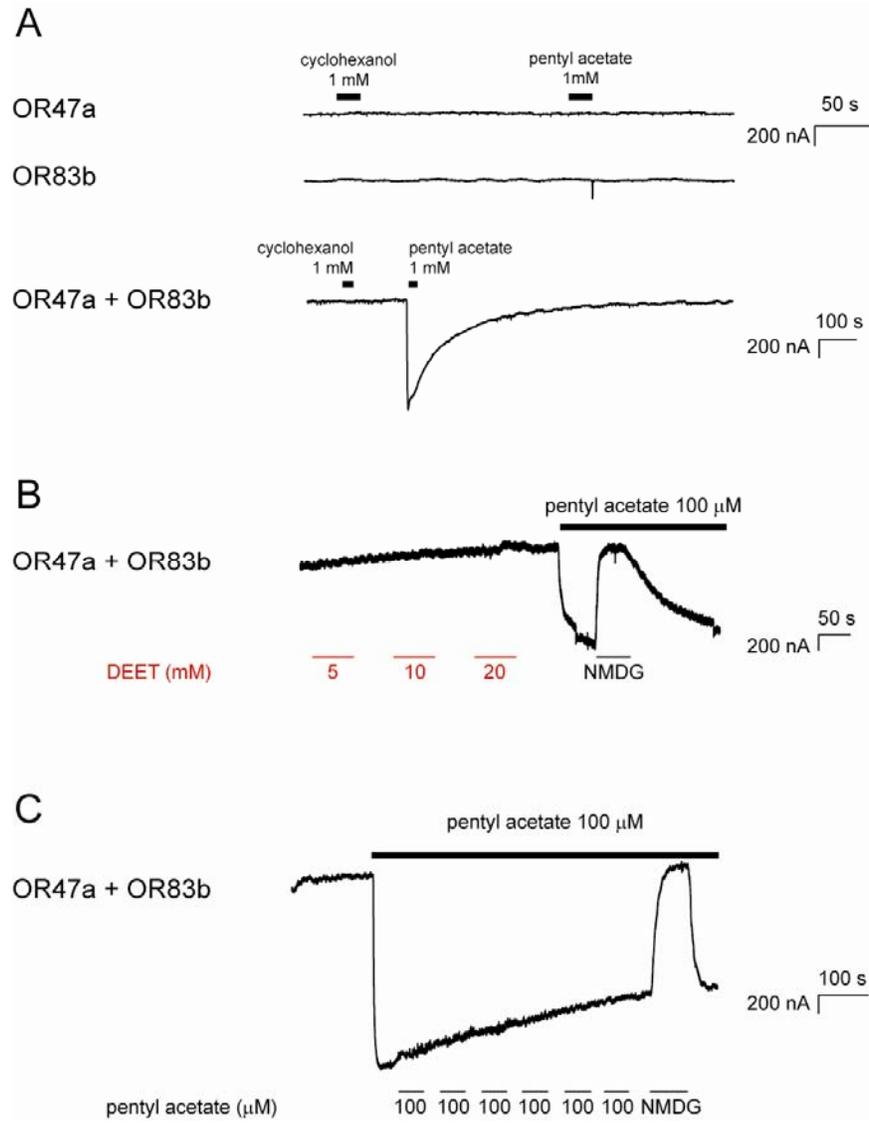


Fig. S3. Ligand- and ion-selectivity of insect odorant receptors reconstituted in oocytes. (A) Two-electrode voltage clamp recordings of odor-evoked macroscopic currents in oocytes expressing OR47a, OR83b, or OR47a + OR83b. Ligand application is indicated by black bars above the traces. (B-C) OR47a + OR83b odor-evoked currents measured after DEET pre-stimulation (B) and with repeated stimulation with ligand (C). Replacement of Na^+ with the impermeant cation N-methyl-D-glucamine (NMDG $^+$) in the extracellular solution is indicated by NMDG at the end of each trace.

Supporting References.

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