Insect Odorant Receptors Are Molecular Targets of the Insect Repellent DEET

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Mathias Ditzen, Maurizio Pellegrino, Leslie B. Vosshall*

DEET (*N,N*-diethyl-meta-toluamide) is the world's most widely used topical insect repellent, with broad effectiveness against most insects. Its mechanism of action and molecular target remain unknown. Here, we show that DEET blocks electrophysiological responses of olfactory sensory neurons to attractive odors in *Anopheles gambiae* and *Drosophila melanogaster*. DEET inhibits behavioral attraction to food odors in *Drosophila*, and this inhibition requires the highly conserved olfactory co-receptor OR83b. DEET inhibits odor-evoked currents mediated by the insect odorant receptor complex, comprising a ligand-binding subunit and OR83b. We conclude that DEET masks host odor by inhibiting subsets of heteromeric insect odorant receptors that require the OR83b co-receptor. The identification of candidate molecular targets for the action of DEET may aid in the design of safer and more effective insect repellents.

Blood-feeding insects transmit many of the world's deadliest diseases. Malaria alone infects an estimated 500 million people annually, leading to the deaths of ~1 million people per year (*1*). In addition to conventional measures of insect control, topically applied insect repellents play a crucial role in protecting humans from blood-feeding insects (*2*). The attraction of mosquitoes to human hosts is largely odor-mediated, with human body emanations such as CO_2, lactic acid, and 1-octen-3-ol acting as strong mosquito attractants (*3*).

Humans have used plant compounds such as eucalyptus and citronella, as well as smoke from incense or burning plant material, for thousands of years to ward off biting insects (*4*). In the 20th century, potent synthetic insect repellents were developed that repelled insects without a strong odor perceptible to humans (*5*, *6*). Among these, DEET is the most commonly used active ingredient of topically applied insect-repellent formulations. It is effective against a wide range of arthropods (*7*, *8*), but its exact mode of action and molecular target are unknown (*8–13*). DEET acts as a volatile agent to repel mosquitoes at distances of at least 38 cm from their host (*10*) and repels ticks in vapor phase (*7*). DEET blocks behavioral attraction to lactic acid, a component of human sweat (*11*), and strongly inhibits the electrophysiological activity of lactic acid–sensitive olfactory sensory neurons (OSNs) on the antennae of *Aedes aegyptii* (*12*). DEET also appears to have a deterrent effect on feeding (*8*) and exhibits insecticidal properties (*9*). We carried out behavioral and electrophysiological experiments in the malaria mosquito and the fruit fly to elucidate a molecular mechanism of action for the observed olfactory repellency of DEET.

CO_2 and 1-octen-3-ol, emitted in human breath, are potent olfactory attractants for *Anopheles gambiae* in the field (*3*, *14*). Two OSNs housed in capitate peg (cp) sensilla in the mosquito maxillary palp respond with high sensitivity to CO_2 and 1-octen-3-ol (*15*). The large-amplitude spiking cpA cell is tuned to CO_2 and expresses three gustatory receptors (GRs)—GPRGR22, GPRGR23, and GPRGR24—whereas the intermediate-amplitude spiking cpB cell is tuned to 1-octen-3-ol and expresses GPROR8 and the mosquito ortholog of the *Or83b* co-receptor, GPROR7 (*15*).

We performed extracellular electrophysiological recordings on the cp sensilla housing these OSNs to test whether DEET affects peripheral reception of these attractants (Fig. 1). CO_2-evoked responses of the cpA cell were unaffected by DEET (Fig. 1, A and C), suggesting that olfactory transduction mediated by the CO_2 receptor is not affected by the repellent. In contrast, DEET strongly inhibited 1-octen-3-ol–evoked responses mediated by GPROR8 + GPROR7 in the cpB cell (Fig. 1, B and D). The B cell responded to 1-octen-3-ol with a median effective concentration (EC_{50}) of 1.7×10^{-10}, which was shifted to 2.5×10^{-7} in the presence of DEET (Fig. 1D). Because the cpB neuron expresses GPROR7, the mosquito ortholog of the OR83b co-receptor, we carried out a series of experiments in *Drosophila* to determine if DEET acts on *Or83b*-dependent olfactory responses.

Fruit flies avoid a food-baited trap whose entrance is treated with DEET (*16*). To investigate the genetic basis of this repulsion, we adapted a previous two-choice olfactory trap assay (*17*, *18*) (Fig. 2A). In the absence of a food bait and DEET, 72% of the flies entered and distributed equally among the two trap vials, and the remaining flies were found in the starting chamber (Fig. 2B, right two bars). When a filter paper
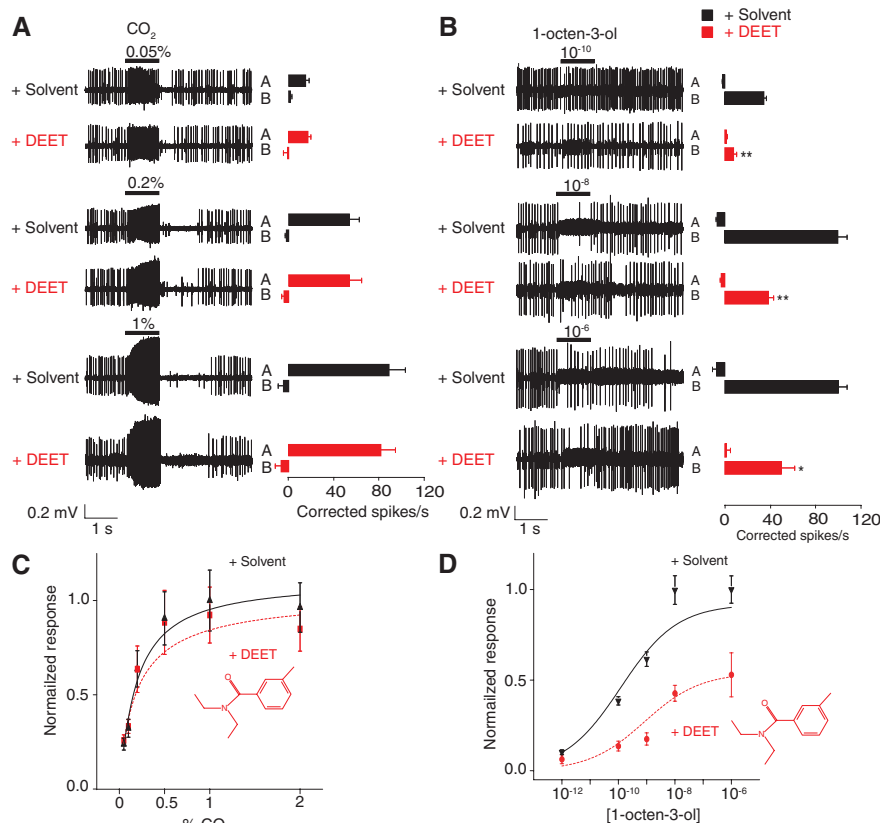

Fig. 1. DEET inhibits mosquito olfactory neuron responses to the attractant 1-octen-3-ol. (**A** and **B**) Recordings from the *A. gambiae* maxillary palp cp sensilla with varying concentrations of CO_2 and 1-octen-3-ol with or without pure DEET. (Left) Representative traces. (Right) Corrected responses of cpA (A) and cpB (B) cells (significance assessed with Mann-Whitney test: *$P < 0.05$, **$P < 0.01$, unlabeled bars not significantly different; mean ± SEM, $n = 5$ to 7). (**C** and **D**) Dose-response curves of cpA and cpB cell to CO_2 and 1-octen-3-ol with or without DEET (mean ± SEM, $n = 7$ to 13). The chemical structure of DEET is depicted in red.

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lining the entry of one of the two traps was treated with 100% DEET, flies strongly avoided entering this vial (Fig. 2B). If the flies were partially shielded from direct contact with the DEET-treated filter paper by a wire mesh or a perforated polypropylene barrier, avoidance of the DEET-treated trap was reduced (Fig. 2B), and this effect was eliminated when DEET was diluted to 10%, a typical concentration used in insect repellent formulations. Because DEET has been shown to repel airborne mosquitoes (8, 10), we carried out all subsequent behavioral experiments under conditions in which the contact repellent effects of DEET were eliminated by covering 10% DEET with a perforated polypropylene barrier.

To investigate the effect of DEET on food-seeking behavior of D. melanogaster, we baited first one and then both traps in the assay with fly food (Fig. 2C). When only one trap contained food (fig. S1A), 85% of flies entered the food vial, 3% entered the empty control vial, and the remaining flies were found in the starting chamber (fig. S1B). When both traps contained food, 93% of flies entered and distributed roughly equally between the two food vials (Fig. 2D, left). However, when the entrance to one baited food vial was treated with 10% DEET, significantly more flies entered the untreated food vial (Fig. 2D, middle), even though DEET was not repellent per se at this concentration (Fig. 2B). When both entrances were treated with 10% DEET, the distribution was again equalized between the two food traps (Fig. 2D, right).

We then asked whether DEET inhibits food attraction by acting peripherally on the olfactory system. Whereas intact flies and those retaining one antenna continued to avoid the DEET-treated food vial (Fig. 2E, left and middle), flies lacking both antennae but retaining secondary olfactory organs, the maxillary palps, entered both food vials, with a slight preference for the DEET-treated side (Fig. 2E, right). We conclude that DEET avoidance requires antennae, which in Drosophila house OSNs as well as sensory neurons tuned to humidity, temperature, and mechanical stimuli (19, 20). To investigate whether DEET acts directly on antennal OSNs, we tested the effect of DEET on flies lacking Or83b, an essential co-receptor required for the proper trafficking and function of insect odorant receptors in ~80% of antennal OSNs (17, 18). Whereas wild-type flies avoided the DEET-treated food vial, Or83b$^{-/-}$ mutants were insensitive to DEET and distributed equally in the two food vials (Fig. 2F). We presume that Or83b mutants continue to detect the food bait because CO_2-sensitive neurons and a class of coeloconic OSNs sensing acids and humidity remain functional in Or83b mutants (17, 18, 21) (fig. S2C). Our observation that Or83b mutants are DEET-resistant suggests that DEET acts on the olfactory system and requires Or83b function for repellency.

To investigate whether DEET blocks all or only a subset of olfactory responses, we recorded food-evoked electrophysiological responses of all Or83b-dependent antennal OSNs (Fig. 3A) as well as coeloconic OSNs (fig. S2). Extracellular spiking activity of OSNs in identified basiconic and trichoid sensilla in response to fly food odor was recorded and compared to the same response when food odor was delivered together with DEET. The odor of fly food causes a range of responses in a large number of different OSNs (Fig. 3A, black bars). Although food-evoked responses in most OSNs were not affected by DEET, a subset of neurons showed potentiation (ab1A, ab3B, ab7, ab8) or inhibition (ab1B, ab5, atδ) in the presence of DEET (Fig. 3A, red bars). These data in Drosophila are in accord with previous experiments in mosquitoes showing complex effects of DEET on odor-evoked responses in OSNs (14).

The strongest inhibition of food odor by DEET was observed in the ab5 sensillum, which houses two OSNs: ab5A, which expresses the odorant receptor (OR) Or82a, and ab5B, which expresses Or47a (22). Both ab5A and ab5B OSNs coexpress and require Or83b for function (17, 18). To relate the electrophysiological inhibition of ab5 OSNs to behavioral effects of DEET, we examined DEET-mediated inhibition of ab5 OSNs in more detail by using cognate ligands to distinguish ab5A and ab5B. Whereas methyl acetate–induced responses in the ab2 sensillum were not affected by DEET (Fig. 3B, top), both geranyl acetate–induced ab5A responses (Fig. 3B, middle) and 3-methylthio-1-propanol–induced ab5B responses (Fig. 3B, bottom) were strongly inhibited by DEET. The inhibition by DEET of odor-evoked spiking in ab5B was seen across a range of ligand concentrations for both 3-methylthio-1-propanol (Fig. 3C) and pentyl acetate, another ligand of the OR47a + OR83b receptor expressed in ab5B neurons (22, 23) (Fig. 3D).
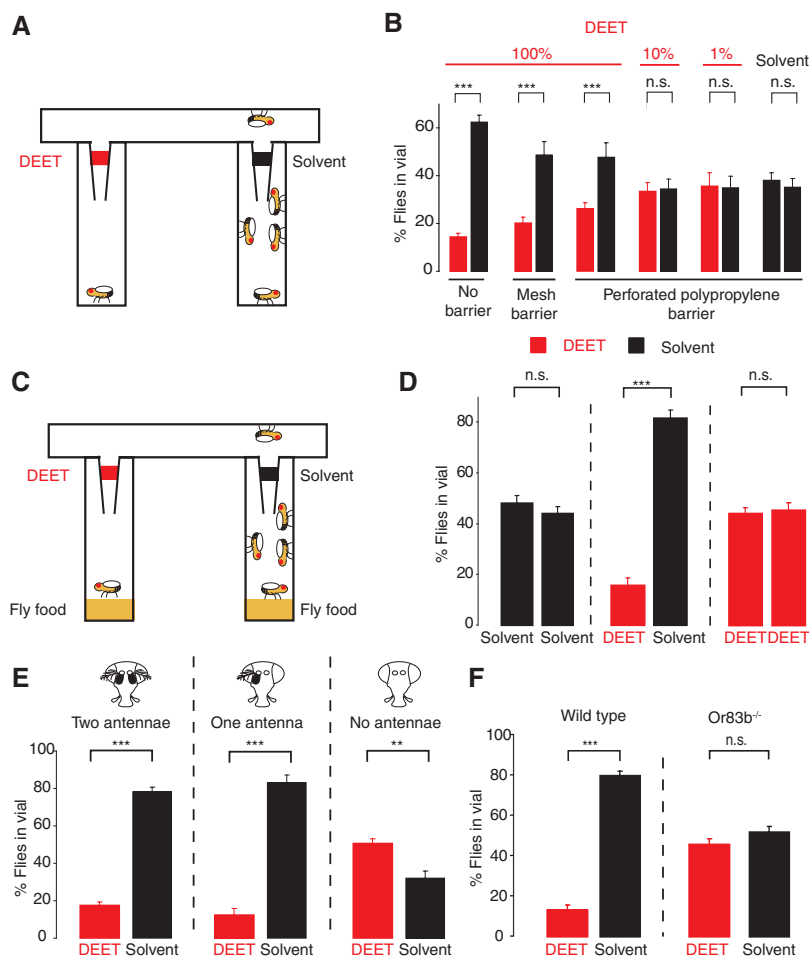

Fig. 2. DEET repellency requires Drosophila OR83b (**A**) Schematic of trap assay without food bait. Entrance to trap is coated with DEET (red) or solvent (black). (**B**) Repellency of varying concentrations of DEET in the trap assay without food bait, with different barriers to impede direct contact with DEET (***P < 0.001, n.s., not significant, Mann-Whitney test; mean ± SEM, n = 11 to 12). (**C**) Schematic of trap assay with fly food bait (yellow). (**D**) Repellency of 10% DEET with perforated polypropylene barrier in the trap assay with food bait (***P < 0.001, n.s., not significant, Mann-Whitney test; mean ± SEM, n = 12, 22, 12). (**E**) Same assay as (D) with surgically de-antennated flies (**P < 0.01, ***P < 0.001, Mann-Whitney test; mean ± SEM, n = 12). (**F**) Same assay as (D) with wild-type and Or83b$^{-/-}$ flies (*** P < 0.001, n.s., not significant, Mann-Whitney test; mean ± SEM, n = 13, 46).

To investigate whether DEET inhibition of electrophysiological responses of ab5B translated to an effect on behavior, we carried out trap assays with 3-methylthio-1-propanol. Flies showed potent attraction to 3-methylthio-1-propanol (Fig. 3E, left), and this attraction was significantly decreased by DEET (Fig. 3E, right). Odor-evoked activity in the *Or47a-* and *Or83b*-expressing ab5B neuron thus contributes to attractive behavior that can be inhibited by DEET and allows us to correlate a selective electrophysiological effect of DEET with a behavioral phenotype. Consistent with the failure of DEET to inhibit methyl acetate responses of ab2 (Fig. 3B, top), DEET did not alter behavioral responses to methyl acetate in the trap assay (Fig. 3F). We were unable to test the behavioral relevance of ab5A inhibition by DEET because flies were not attracted to geranyl acetate in our trap assays.

We next carried out heterologous expression experiments in which odor-evoked responses of different insect ORs were examined in *Xenopus* oocytes. The functional insect OR is a heteromeric complex of a variable ligand-binding OR subunit and the constant OR83b receptor (*18, 23, 24*). Both subunits adopt an atypical topology and share no homology with G protein–coupled receptors (*18, 25, 26*). When expressed in heterologous cells, insect ORs mediate odor-evoked increases in intracellular calcium and inward nonselective cation currents (*23, 24*).

In two-electrode voltage-clamp recordings in oocytes, we confirmed previous results that odor-evoked inward cation currents required coexpression of both OR and OR83b subunits and application of the cognate ligand (*23*) (fig. S2A). Pretreatment of OR47a- and OR83b-expressing oocytes with high concentrations of DEET alone did not generate currents in the oocyte membrane and did not prevent subsequent pentyl acetate–evoked currents in the same oocyte (fig. S2B, upper trace). This suggests that DEET does not have nonspecific effects on biological membranes or membrane proteins. When oocytes stimulated with pentyl acetate were challenged with additional pentyl acetate, no effect was seen on the slow inactivation of the current (fig. S3C). However, when DEET was applied in combination with the cognate ligands of four different ORs, the evoked inward current decreased in a DEET dose-dependent and reversible manner (Fig. 4, A, C, E, and G). DEET inhibited the *Drosophila* OR47a + OR83b receptor expressed in ab5B OSNs (Fig. 4A), two *Anopheles* ORs tuned to human body-odor components (*27*) (Fig. 4, C and E), and the *Anopheles* 1-octen-3-ol receptor, GPROR8 + GPROR7 (*15*) (Fig. 4G). DEET inhibition of the 1-octen-3-ol receptor in heterologous cells is in accord with the in vivo inhibition observed in Fig. 1, B and D. Although DEET inhibited odor-evoked currents in oocytes expressing each of the four insect ORs tested in a dose-dependent manner, the extent of inhibition was dependent on OR + OR83b subunit composition (Fig. 4K).
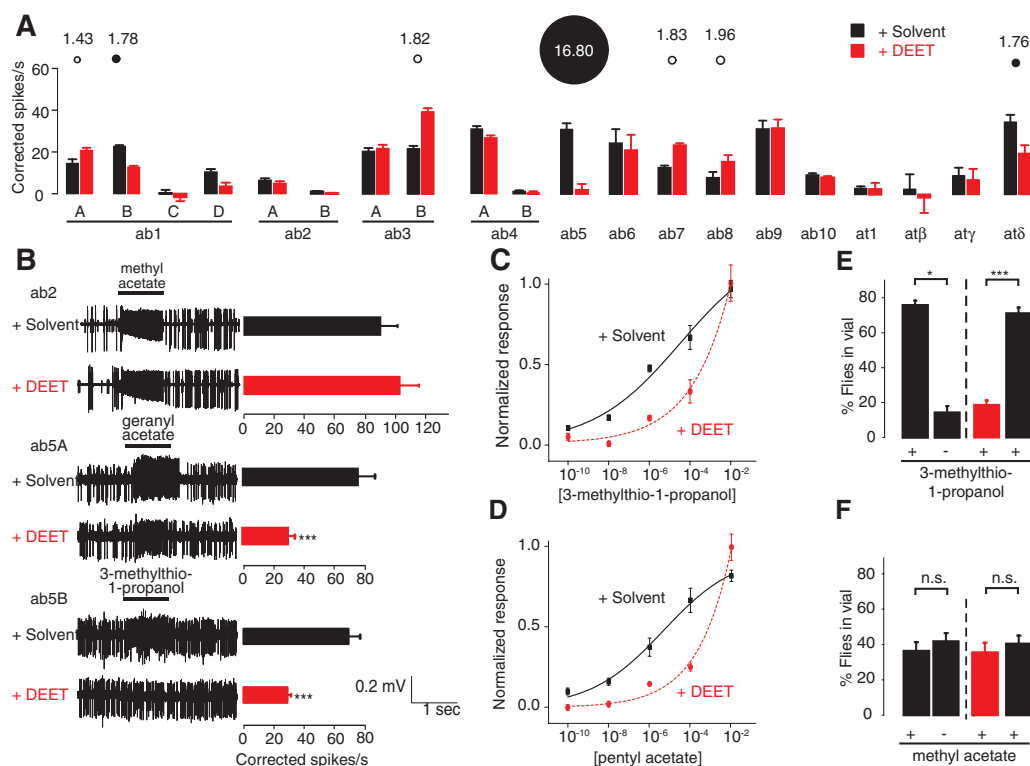
To determine whether the DEET-induced decrease in odor-evoked current was due to a change in ion permeability, we analyzed current-voltage (*I-V*) relation curves during ligand stimulation in the presence and absence of DEET. The effect of DEET on OR-evoked current was symmetric at positive and negative potentials, and no change in reversal potential was observed (Fig. 4, B, D, F, and H). This suggests a reduction in permeability of the channels affected by DEET, but not a change in ion selectivity.

DEET did not affect currents elicited by activation of the chloride channel CFTR by forskolin (Fig. 4, I and J). Moreover, odor-evoked increases in adenosine 3′,5′-monophosphate (cAMP) mediated through the mouse eugenol receptor (mOR-EG) and detected by activation of CFTR currents (*28*) were also not affected by DEET (Fig. 4L). DEET also inhibited three nonselective cation channels structurally unrelated to insect ORs: mouse TRPM8 (mTRPM8), the heterotrimeric rat olfactory cyclic-nucleotide gated (CNG) channel, and the *Drosophila* ether-a-go-go potassium channel (Fig. 4L). This suggests that DEET may interfere generally with the ionic permeability of a subset of cation channels. We conclude that DEET inhibits odor-evoked currents mediated by selected OR + OR83b complexes.

Electrophysiological and behavioral data presented here suggest that DEET inhibits odor-evoked activation of a subset of insect OR + OR83b complexes, thereby inhibiting the perception of food odors. Because DEET did not uniformly inhibit all *Or83b*-dependent responses in *Drosophila*, it is unlikely that the insect odor-

Fig. 3. DEET affects odor-evoked activity of *Drosophila* olfactory sensory neurons (**A**) Single-sensillum electrophysiology responses of *Or83b*-dependent antennal basiconic (ab) and trichoid (at) sensilla stimulated with food plus solvent (black bars) or food plus DEET (red bars). Data are plotted as mean corrected spikes/s ± SEM (n = 5 to 17 sensilla). Circles above bar graph indicate the fold change in response in the presence of DEET (filled circles, decrease; open circles, increase). (**B**) Representative single-sensillum traces (left) and population responses (right) of ab2 sensilla to methyl acetate at 10^{-5} (top), ab5A neurons to geranyl acetate at 10^{-8} (middle), and ab5B neurons to 3-methylthio-1-propanol at 10^{-5} (bottom) with solvent or DEET (significance assessed with Mann-Whitney test: *** P < 0.001, unlabeled bars not significantly different; mean corrected spikes/s ± SEM, n = 8, 8, 10). (**C** and **D**) Dose-response curves of ab5B stimulated with 3-methylthio-1-propanol (C) or pentyl acetate (D), with solvent (black) or DEET (red) (mean ± SEM, n=4). (**E**) (Left) Trap assay in which one vial is baited with pure 3-methylthio-1-propanol (*P < 0.05, Mann-Whitney test; mean ± SEM, n = 4). (Right) Repellency of 10% DEET with perforated polypropylene barrier in the trap assay with pure 3-methylthio-1-propanol as bait (***P < 0.001, Mann-Whitney test; mean ± SEM, n = 12). (**F**) Same experiment as (E) with pure methyl acetate as bait (n.s., not significant, Mann-Whitney test; mean ± SEM, n = 12, 12).
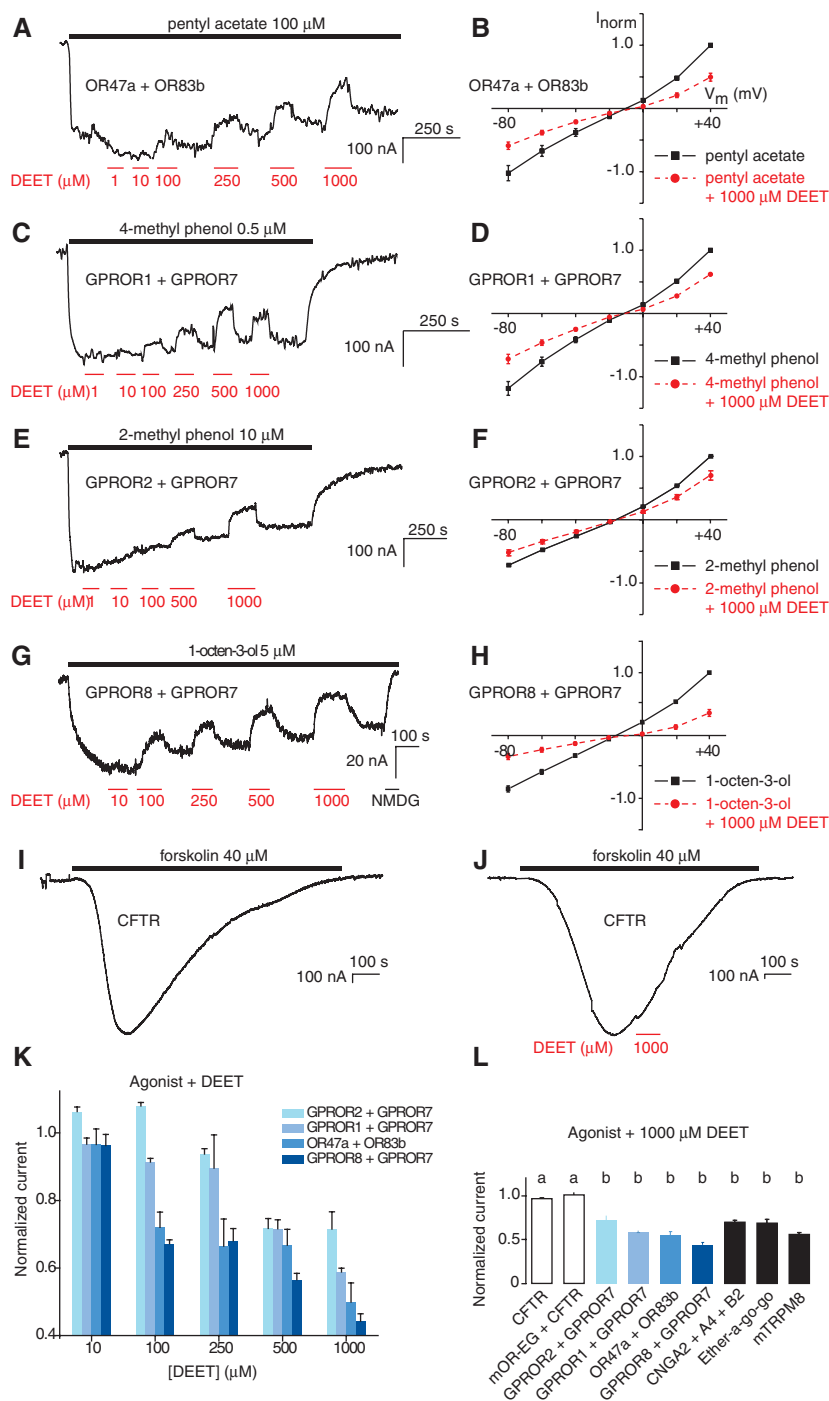
Fig. 4. DEET decreases currents mediated by insect ORs (**A** to **H**) Ligand-evoked currents in the presence of DEET in oocytes expressing OR47a + OR83b (A), GPROR1 + GPROR7 (C), GPROR2 + GPROR7 (E), and GPROR8 + GPROR7 (G). (B), (D), (F), and (H) show current-voltage (*I-V*) curves during ligand stimulation in the absence (black squares) or presence (red circles) of 1000 μM DEET. Current was normalized to the value of +40 mV in the absence of DEET (mean ± SEM, *n* = 3 to 6). (**I** and **J**) Forskolin-evoked currents in the absence (I) or presence (J) of 1000 μM DEET. (**K**) DEET effects on ligand-dependent currents of insect ORs (mean ± SEM, *n* = 3 to 5). Current was normalized to the value of the current in the absence of DEET. (**L**) Normalized stimulus-evoked currents in oocytes expressing various receptors or ion channels in the presence of 1000 μM DEET (CFTR: 40 μM forskolin; mOR-EG + CFTR: 50 μM eugenol; GPROR2 + GPROR7: 10 μM 2-methylphenol; GPROR1 + GPROR7: 0.5 μM 4-methyl phenol; OR47a + OR83b: 100 μM pentyl acetate; GPROR8 + GPROR7: 5 μM 1-octen-3-ol; CNGA2+A4+B2: 100 μM cAMP; Ether-a-go-go: voltage steps from −60 mV to +20 mV; mTRPM8: 50 μM menthol). Bars labeled with different letters are significantly different (*P* < 0.05, Kruskal Wallis test with posthoc multiple comparison correction against the CFTR control; mean ± SEM, *n* = 4 to 7).

ant co-receptor encoded by OR83b or GPROR7 alone is a direct target of DEET. Although the basis for this selective inhibition by DEET of certain OR + OR83b complexes remains to be investigated, we favor a model in which the subunit composition of the OR + OR83b complex governs both the sensitivity to DEET inhibition and the response properties of the receptor, as previously suggested for *Drosophila* ORs (*22*).

Past attempts to identify novel insect repellent compounds with improved efficacy compared to DEET have been hampered by the absence of a known molecular target for this insect repellent and have relied instead on computational chemistry to explore chemical space around the structure of DEET. We propose that DEET inhibits odor-evoked activation of a subset of OR + OR83b complexes. Accordingly, cell-based assays of behaviorally relevant OR + OR83b complexes could be exploited for high-throughput screening to identify new inhibitory compounds that could represent highly effective insect repellents for use in interrupting infectious disease transmission.

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29. We thank P. Rivkin for technical assistance; K. Lee, J. Klun, K. Touhara, and members of the Vosshall Lab for comments on the manuscript; and P. Howell and M. Q. Benedict of the Centers for Disease Control and Prevention and MR4 for mosquitoes. DNA clones were provided by T.-Y. Chen (CNGs), A. G. Kovacs (CFTR), A. Patapoutian (mTRPM8), G. Wilson (EAG), and L. Zwiebel (GPROR8). M.P. and L.B.V. thank D. Gadsby and Gadsby Lab members M. Mense, P. Artigas,

Aversive Learning Enhances Perceptual and Cortical Discrimination of Indiscriminable Odor Cues

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Learning to associate sensory cues with threats is critical for minimizing aversive experience. The ecological benefit of associative learning relies on accurate perception of predictive cues, but how aversive learning enhances perceptual acuity of sensory signals, particularly in humans, is unclear. We combined multivariate functional magnetic resonance imaging with olfactory psychophysics to show that initially indistinguishable odor enantiomers (mirror-image molecules) become discriminable after aversive conditioning, paralleling the spatial divergence of ensemble activity patterns in primary olfactory (piriform) cortex. Our findings indicate that aversive learning induces piriform plasticity with corresponding gains in odor enantiomer discrimination, underscoring the capacity of fear conditioning to update perceptual representation of predictive cues, over and above its well-recognized role in the acquisition of conditioned responses. That completely indiscriminable sensations can be transformed into discriminable percepts further accentuates the potency of associative learning to enhance sensory cue perception and support adaptive behavior.

The ability to minimize contact with aversive experience is a hallmark of adaptive behavior. Via mechanisms of associative learning, organisms can use sensory information in the environment to predict impending danger and initiate fight-or-flight responses. The behavioral efficacy of associative learning thus hinges on sensitive and accurate perceptual evaluation of sensory signals. In particular, the ability to discriminate between biologically meaningful cues (e.g., smell of a 175-kg lion) and similar but irrelevant stimuli (e.g., smell of a 3-kg housecat) maximizes an organism's response sensitivity while minimizing hypervigilant and impulsive behaviors.

However, models of associative learning have traditionally focused on delineating the formation of associations between a sensory cue [the conditioned stimulus (CS)] and a biologically salient event [the unconditioned stimulus (US)] (*1*, *2*), paying scant attention to perceptual changes in the CS itself. Several studies have considered how

associative learning modifies cue-related tuning profiles in sensory cortex (*3–8*), although none has provided concomitant measures of sensory perception. As a consequence, direct links relating learning-induced changes in sensory cortex to perceptual gains in cue discrimination are unavailable, such that the functional importance of these neural effects on behavior remains poorly characterized. To the extent that conditioning can transform indiscriminable sensations into distinct percepts, such a mechanism would constitute a unique and potent means of optimizing adaptive behavior.

We combined functional magnetic resonance imaging (fMRI) with multivariate analytical techniques to explore the impact of aversive olfactory conditioning on perceptual and neural discrimination of predictive odor cues. The use of perceptually identical odor enantiomers (mirror-image molecules differing only in their chiral properties) (*9*, *10*) enabled us to determine whether humans can acquire the ability to distinguish between odorous stimuli that initially smell the same. Twelve healthy human subjects (age range, 22 to 35 years; 8 female) were presented with four enantiomers (two different pairs), one of which (the target CS+, "tgCS+") was repetitively paired with an electric shock (US) during a conditioning phase, whereas its chiral counterpart ("chCS+") was not accompanied by shock (Fig. 1) (*10*). The second pair of odor enantiomers served as nonconditioned control stimuli ("CS–" and

"chCS–"). The central prediction was that associative learning would enhance behavioral discrimination of related CS+ odorants, in parallel with reorganization of neural coding in human primary olfactory (piriform) cortex.

We first examined the behavioral effects of aversive conditioning on perceptual discrimination between the conditioned cue (tgCS+) and its related enantiomer (chCS+). We administered a triangular (triple-forced-choice) odor discrimination test (*10*, *11*) to assess differences in perceived odor identity (i.e., the quality or character of a smell emanating from an odorous object). On each trial, subjects smelled sets of three bottles (two containing one odorant, the third containing its chiral opposite) and selected the odd stimulus. Before conditioning, discrimination accuracy was at chance (33%) for both CS+ and CS– enantiomer pairs, confirming that each pair was initially indistinguishable (Fig. 2A). After conditioning, behavioral accuracy for distinguishing between tgCS+ and chCS+ rose by more than a factor of 2, significantly exceeding both chance and preconditioning performance ($Ps \leq 0.01$; Wilcoxon test, two-tailed), without any improvement in distinguishing between CS– and chCS–. Subjective ratings of odor intensity, valence, or familiarity (*11*) did not vary across conditions ($Ps > 0.4$), ruling out confounds of the triangular test due to these extraneous variables and accentuating the change in perceived odor identity. Associative learning thus can enhance perceptual discriminability between initially indistinguishable odors, and these effects are specific for the CS+.

We next clarified the neural mechanisms underlying learning-induced perceptual enhancement of the predictive cue (*11*). Because neural representations of odor identity are maintained in posterior piriform cortex (*12–14*), and given the highly distributed spatial organization of afferent projections into the piriform region (*15–17*), we used multivariate fMRI (*18*, *19*) to test the hypothesis that spatially distributed patterns of neural activity in piriform cortex evoked by tgCS+ and chCS+ would be reorganized as a consequence of associative learning (fig. S1).

By extracting the raw fMRI signal intensity from every activated piriform voxel (fig. S2), we found that the spatial activity patterns in posterior piriform cortex strongly correlated for the CS+ pair (tgCS+:chCS+) and the control pair (CS–:chCS–) before odor-shock learning (Fig. 2B), corresponding to the high perceived similarity within each pair. However, after conditioning, these spatial correlations declined for the CS+ pair, particularly in

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