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The mysterious multi-modal repellency of DEET

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DEET is the most effective insect repellent available and has been widely used for more than half a century. Here, I review what is known about the olfactory and contact mechanisms of DEET repellency. For mosquitoes, DEET has at least two molecular targets: Odorant Receptors (ORs) mediate the effect of DEET at a distance, while unknown chemoreceptors mediate repellency upon contact. Additionally, the ionotropic receptor Ir40a has recently been identified as a putative DEET chemosensor in *Drosophila*. The mechanism of how DEET manipulates these molecular targets to induce insect avoidance in the vapor phase is also contested. Two hypotheses are the most likely: DEET activates an innate olfactory neural circuit leading to avoidance of hosts (smell and avoid hypothesis) or DEET has no behavioral effect on its own, but instead acts cooperatively with host odors to drive repellency (confusant hypothesis). Resolving this mystery will inform the search for a new generation of insect repellents.

DEET is the most effective invertebrate repellent to prevent mosquitoes, flies, ticks, and even parasitic worms from feeding on humans.[1-4] How a single chemical can change the normal behavioral response to otherwise attractive stimuli is of considerable interest to neuroscientist, or anyone who seeks to prevent the transmission of vector-borne disease. DEET is not without its drawbacks, and understanding the molecular mechanism of DEET's action has great potential for the development of more effective repellents. Recent studies have suggested multiple modes of action for DEET repellency. Given the controversy in the field, I seek to provide a context to published results and suggest directions for future research.

The Discovery of DEET

In 1942, the United States Department of Agriculture (USDA), in collaboration with the US military, screened more than 7,000 compounds over a 5-year period to develop insecticides, miticides, and repellents.[5,6] Potential repellents against *Aedes aegypti* were identified by testing a diverse set of 6,241 compounds. *Aedes aegypti*, the vector for yellow and dengue fever as well as chikungunya, was selected because its behavior is easier to assay in the laboratory than that of other disease transmitting mosquitoes. During screening, 1 ml of each test compound was distributed on the forearms, and the hands were placed in cages containing 2,000–4,000 mosquitoes. Based on the protection time, 56% of 4,137 tested compounds were effective for less than 1 h, 28% for 1–2 h, 7% for 2–3 h, and 9% for more than 3 h. In separate experiments, cloths were impregnated with 3.6 mg / cm^2 of one of 3,239 compounds, and then placed on the forearms. 51% of the compounds were effective for less than 1 d, 16% for 1–5 d, 8% for 5–10 d, and 25% for more than 10 d. *N,N*-diethylbenzamide was among the most effective compounds found, repelling for more than 3 h when applied to skin and 10 d when applied on cloth. However, it also caused skin irritation.[7]

Determined to find a repellent that was not an irritant, 33 derivatives from *N,N*-diethylbenzamide were created.[7] All toluic acid derivatives, including *N,N*- diethyl-3-methylbenzamide (i.e., *N,N*-diethyl-*m*-toluamide) repelled mosquitoes when applied on skin or cloths.[7,8] LD 50 of *N,N*-diethyl-*m*-toluamide in rats was very low (2 g/kg), with no evidence of systemic toxicity upon frequent dermal application or inhalation.[9] Subsequent studies showed that *N,N*-diethyl-*m*-toluamide is safe for human use, but it was recommended that ingestion be avoided.[10] *N,N*-diethyl-*m*-toluamide was renamed DEET by the Committee on Insecticide Terminology of the Entomological Society of America, because of "numerous complaints that diethyltoluamide was too long for a common name."[11] DEET was registered in the United States for use by the general public in 1957, and reregistered in 1998 (US EPA document EPA 738-R-98–010). There are approximately 120 products currently on the market that contain DEET at concentrations from 4% to 100%.

Theories of Repellency

Early studies suggested that repellents target the central nervous system (CNS), the peripheral nervous system (PNS), or both. Five theoretical modes of action for insect repellents were proposed: 1) inhibiting the response of sensory neurons of host attractants, 2) activating a receptor system that mediates a competing or inappropriate behavior, 3) acting as attractant at low concentration, but as a repellant at high concentration 4) activating receptors linked to several behavioral programs to increase

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the noise/signal ratio in order to "jam" the relevant sensory circuit, and 5) activating unique avoidance/aversive receptor(s).[12] These hypotheses informed later studies but were conceived before olfactory receptors were identified. They provided a conceptual framework for the current proposed molecular mechanisms of DEET repellency.

An additional model for DEET repellency was based purely on its molecular structure. It suggested that DEET, which is nonpolar, interacts with lipids of the cell membrane of chemosensory neurons.[13] Here, DEET is proposed to have an indirect effect on olfactory receptor activation by altering membrane excitability or function. If the cell membrane is altered by DEET, the change must be extremely transient, as electrophysiological studies of insect sensilla have shown rapid recovery of olfactory receptor neurons to baseline spike activity after the application of DEET.[14-16] Studies uncovering DEET-insensitive mutants further challenged this model by showing that DEET had particular molecular targets and did not have a promiscuous effect on cell membrane function.[15,17,18] However, this lipid interaction model was prescient in suggesting that DEET could modulate the activity of multiple olfactory receptors and thereby disrupt the odor coding necessary for host detection.

Olfactory Mechanism of Action

Although the volatility of DEET is not particularly high (0.00167 mmHg at 25°C) compared to many host odors such as lactic acid (0.0813 mmHg at 25°C) or 1-octen-3-ol (0.53 mmHg at 25°C), it is effective in the vapor phase. Laboratory studies have focused on identifying the molecular target(s) of DEET using a combination of behavioral genetics and electrophysiological approaches. These studies have led to 3 main hypotheses to explain the mode of action of DEET: the inhibition of host odor detection hypothesis, the confusant hypothesis, and the smell and avoid hypothesis (**Fig. 1**).

Inhibition of lactic acid sensation

It is possible that a repellent like DEET could work by masking a host odor, thereby decreasing the ability of the insect to detect its feeding target. Lactic acid is a human odor that can attract mosquitoes particularly when co-presented with other kairomones, such as carbon dioxide.[19,20] Electrophysiological studies showed that DEET reduces the sensitivity of olfactory receptor neurons to odors by decreasing the responses of lactic acid-excited neurons and increasing the inhibition of lactic acid-inhibited neurons.[21] Behavioral assays in a repellometer confirmed that DEET inhibited the attraction of *Aedes aegypti* to lactic acid, but both compounds were also attractive to the mosquito.[22] Thus, the authors of these studies classified DEET as a behavioral inhibitor that reduced attraction, rather than activating avoidance behavior itself.[14,22] Additional electrophysiological and behavioral studies supported the inhibitory effect of DEET.[23,24] Whether DEET can directly inhibit the as yet unidentified lactic acid receptor has not been shown.

DEET requires insect ORs to repel in the vapor phase

From the 1970s onward, it was clear that DEET changed the olfactory responses of insects, but the molecular mechanism was unknown. Beginning in 1999, insect olfactory receptors were identified and the search for the molecular target(s) of DEET began.[25-30] Insect olfactory sensilla usually contain 2 or more olfactory receptor neurons that respond to distinct odors due to the different olfactory receptors they express. We now know that insects use at least 3 families of olfactory receptors to smell: odorant receptors (ORs), ionotropic receptors (IRs), and gustatory receptors (GRs).[31,32] A small number of GRs have been identified that respond to carbon dioxide,[20,28,33] but surprisingly, a few GRs known to detect sweet compounds in taste neurons were also found to be expressed in select olfactory receptor neurons.[34] IRs and ORs[25,26,29] respond to a broad spectrum of odorants. For example, in *Aedes aegypti* there are 131 odor-selective ORs.[30] In *Drosophila melanogaster* and *Aedes aegypti*, DEET repellency has been clearly shown to require Orco,[15,35] the obligate co-receptor for the OR family of odor-gated ion channels.[36-39] In addition, natural variation in *Drosophila* Or59B was shown to change the receptor's electrophysiological response to 1-octen-3-ol when co-presented with DEET. Loss of receptor sensitivity to DEET was mapped to a change in just one amino acid (valine 91 to alanine).[18] These genetic studies present strong evidence that both ORs and Orco are required for insects to sense DEET, but they do not reveal which of the OR(s) are the behaviorally relevant molecular DEET targets.

ORs and behavioral inhibition

ORs are molecular targets of DEET, but how DEET interacts with ORs to change insect behavior is an area of active investigation. The initial genetic analysis of *Drosophila* behavior suggested that DEET inhibited odor detection via the OR pathway.[15] Loss of Orco allowed flies to enter food-baited traps that are perfumed with 10% DEET that wild-type flies avoid. If food was absent from the traps, wild-type flies entered DEET perfumed traps. This result suggests that the presence of food odors is required for DEET's ability to repel flies. However, DEET could repel without food odors at high concentrations, particularly when flies were able to contact DEET. Electrophysiological studies in mosquito and fly sensilla and experiments with heterologously expressed receptors showed that DEET can inhibit the responses of a subset of ORs to their odor-ligands.[15] This inhibitory effect extended to non-selective cation channels, such as *Drosophila* Ether-a-go-go and mouse TRPM8. However, not all ORs or ion channels tested were inhibited by DEET, suggesting that DEET possesses some selectivity. Combining their electrophysiological and behavioral data, Ditzen et al.[15] concluded that DEET acts in the vapor phase to inhibit the detection of attractive odors. However, the observations made in these studies have since been reinterpreted.[18,35]

The recent development of genome editing techniques in *Aedes aegypti* allowed for the genetic analysis of the OR pathway in a mosquito.[35] *Aedes orco* mutants did not respond to host odor alone, but were still able to host-seek in the presence of carbon dioxide, demonstrating that redundant mechanisms exist for

mosquitoes to sense hosts.[35] Redundant mechanisms for mosquito host-seeking were also revealed by genetically ablating carbon dioxide detection and testing responses of the mutants to diverse attractive stimuli.[20] As in *Drosophila, orco* mutants were unaffected by the presence of DEET in the vapor phase, responding both to human odor and human skin treated with 10% DEET.[35] Given that *orco* mutants can host-seek, if DEET simply masks host odors by blocking OR activation, it would not be an effective repellent. Therefore, current evidence argues that repellency by DEET does not involve the global inhibition of olfactory receptors.

The confusant hypothesis

If DEET does not mask host odor detection by ORs, then another explanation is required. In early electrophysiological studies of *Aedes aegypti* mosquitoes, DEET increased the firing rate of olfactory receptor neurons in trichoid short and long A2 sensilla, but inhibited the spontaneous activity of medium-length sensilla.[40] Neurons in basiconic A3 sensilla either did not show any response,[14] or DEET inhibited neural spontaneous activity at low concentration, followed by excitation at a higher concentration.[40] In coeloconic A4 sensilla, DEET inhibited neural spontaneous activity.[40] These studies were based on the morphological and not the molecular identification of sensilla, (i.e., olfactory receptor neurons).[41] Later, molecular studies revealed that with some exceptions, neurons in trichoid and basiconic sensilla express ORs, whereas neurons in coeloconic sensilla express IRs.[42] Thus, DEET likely activated, inhibited or had no effect on OR-expressing neurons and inhibited IR-expressing neurons, underscoring the complex role of DEET in olfactory modulation. Yet again, the molecular players involved in these processes could only be inferred, but not specifically identified.

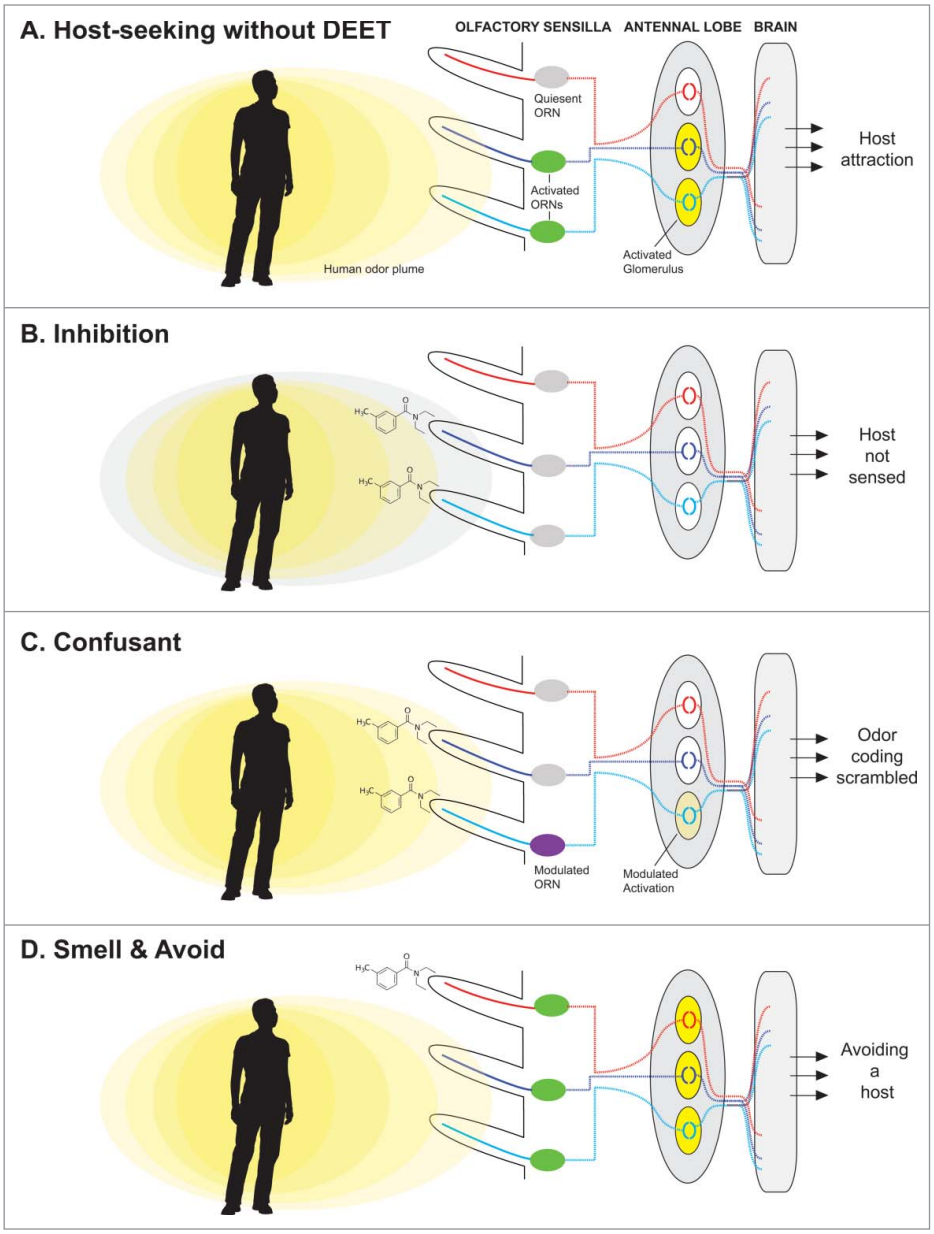

Figure 1. Proposed hypotheses for how insect behavior is modulated by DEET in the vapor phase. (**A**) Human odor (yellow) binds to specific olfactory receptors (blue and light blue, but not red), activating olfactory receptor neurons (ORNs) colored in green, which in turn activates glomeruli in the antennal lobe (bright yellow) leading to host attraction. (**B**) DEET inhibits the sensation of host odor by binding to olfactory receptors and blocking the activation of ORNs (gray). (**C**) DEET modulates olfactory receptor activation by human odor (blue and light blue, but not red), leading to changes in olfactory receptor neuron activation (gray and purple) that scramble odor coding by changing the normal activation pattern of glomeruli, and host attraction is blocked. (**D**) DEET binds to a specific olfactory receptor (red) that is expressed in an ORN that activates a neural circuit that causes aversion. The aversive signal overrides the neural activation pattern elicited by attractive cues sensed by other ORNs. Activated olfactory receptor neurons are green, inactivated are gray, and modulated are purple. Odor plumes from the human host are indicated in shades of yellow. The molecule depicted is DEET.

The promiscuous effects of DEET on olfaction have been supported by molecularly defined electrophysiological surveys of OR-expressing neurons[15,18] and heterologously expressed ORs.[16,43-45] In these studies, DEET administered with odors could activate, inhibit, or have no effect, depending on the olfactory receptor tested. Application of DEET itself had little to no effect on OR-expressing neurons in some studies,[15,18] but others have shown responses of ORs to DEET without odors.[16,40,46,47] As insects are unlikely to

experience DEET in the absence of host odors, the variation in its effect across different ORs suggested that DEET disrupts olfactory coding by confusing the normal activation pattern elicited by host odor, and hence the confusant hypothesis was born[18]; It posited that the sensitivity of multiple ORs to attractive odors was altered by DEET, but DEET had little to no behaviorally relevant effect on its own. In this model, if any inherent repellent activity of DEET exists, that activity needs to be enhanced by host odor. The confusant hypothesis recognizes that DEET can inhibit the activation of some neurons, but suggests that the overall effect is not masking the host. Rather, host kairomones are still sensed but their interpretation by the insect is dramatically altered.

The smell and avoid hypothesis

Contradicting the findings showing that DEET had no effect on its own or was attractive in several studies,[15,22,48-50] a competing mechanism for DEET action has been suggested. The smell and avoid hypothesis proposes that DEET is perceived as a noxious odor by the insect.[51] Labeled-line repellency of this kind has been clearly demonstrated for the microbial odorant geosmin in *Drosophila*.[52] Similar to geosmin, DEET may be sensed by an olfactory receptor that activates a neural circuit that elicits avoidance behavior. Two studies in *Culex quinquefasciatus* mosquitoes support this view.[51,53] In the absence of odor cues, mosquitoes avoid a sugar solution they would normally feed on when it was surrounded by a paper cylinder containing 1mg/cm^2 of DEET. In a similar assay mosquitoes also avoided an attractive heat source surrounded by a 10% DEET-treated paper ring.[51] Both of these behavioral assays did not prevent mosquitoes from contacting DEET; thus, it is difficult to discern whether the repellency observed was mediated by olfactory or gustatory cues. Recent electrophysiology studies with a *Culex* OR, CquiOR136, showed that it could be activated by DEET, as well as other insect repellents, such as PMD, Picaridin, and IR3535.[53] CquiOR136 was also responsive to methyl jasmonate, a naturally produced insect repellent. This opens the possibility that the CquiOR136 pathway evolved to respond to repellents. Reducing the function of CquiOR136 via RNAi injection allowed *Culex* mosquitoes to be attracted to a heated blood-feeder surrounded by a paper soaked with 0.1% DEET that mock injected mosquitoes avoided.[53] This intriguing result suggests that only a single OR is the behaviorally relevant DEET sensor in the vapor phase and that host odor may not be required for DEET to activate this receptor. However, this assay does not exclude the possibility of contact chemorepellency.

How volatile DEET interacts with ORs to alter olfactory sensitivity is not yet clear. What is clear, however, is that DEET does not simply inhibit the detection of host odors by ORs. Instead, DEET may be a confusant that alters OR sensitivity to host odors or a labeled-line repellent that activates an OR specific for noxious odors. Discriminating between these 2 competing hypotheses will be useful for designing the next generation of repellents and for expanding our understanding of how repulsive behavior is generated in insects. To do this, the role that host odors play in DEET repellency must be clearly shown. Previous studies have failed to adequately address this question for a number of reasons, including (i) the difficulty of quantifying behavior in the absence of an attractive stimulus, (ii) the inability of published behavioral assays to control for physical contact with DEET and (iii) the possibility of insect specific differences, and hence the intrinsic difficulty for direct comparison between studies using different insect species. In addition, these hypotheses posit very different numbers of ORs necessary for DEET repellency. The confusant hypothesis suggests that many ORs are modulated by DEET. The smell and avoid hypothesis suggests that only one OR may be activated by DEET. This controversy is not easy to resolve. The genetic basis of DEET detection was discovered using *orco* mutants, which ablate the function of all ORs at once. Conclusively determining the number of ORs required for DEET repellency would involve generating many new OR mutants. If multiple ORs are involved, insects that contain several OR mutations at once must be tested. Such herculean efforts may be worthwhile, as identifying the odor-selective ORs required for DEET repellency would provide new molecular targets for repellent design.

A role for IRs in DEET-driven repellency?

A recent study suggests that IRs may also be necessary for DEET repellency.[54] Kain et al.[54] showed that DEET activates Ir40a-expressing neurons in the *Drosophila* sacculus, a 3-chambered pit beneath the antenna's surface. In addition, RNAi knockdown of Ir40a allowed flies to enter a 50% DEET-perfumed trap that mock-treated flies avoid. Kain et al. also used chemical informatics to identify compounds that flies and mosquitoes avoided. These compounds activated Ir40a neurons. Tetanus toxin silencing of Ir40a neurons allowed flies to enter a trap perfumed by these compounds that control flies avoid. Taken together, these findings suggest that Ir40a can activate an aversive neural circuit in insects. The higher concentration of DEET used in these behavioral assays makes it difficult to compare them to behavioral studies examining the role of ORs in DEET repellency.[15,35] It would be interesting to test if Orco is still necessary for repellency when concentrations of DEET are increased from 10% to 50%. Furthermore, studies in *Culex* mosquitoes showed that knocking down Ir40a function using RNAi did not reduce DEET repellency.[53] If Ir40a is required for the behavioral response to DEET, one must contemplate that both Ir40a and Orco are necessary for DEET sensation, but that neither pathway is sufficient for repellency on its own. This opens the possibility that Ir40a is a lower affinity DEET receptor that responds to high concentrations of DEET at close range. Additional loss-of-function studies in mosquitoes and other insects will be necessary to determine the role of IRs in repellency.

Gustatory and contact modes of action

Most studies of DEET have focused on vapor repellency against flying insects. Tactile repellency has been studied mostly in crawling arthropods such as ticks.[55] DEET has been shown to be both an anti-feedant and a repellant on contact in insects. Whether the same molecular targets mediate these behaviors remains unclear. What is clear is that DEET can alter behavior by multiple chemosensory modalities.

DEET is an anti-feedant

Humans perceive DEET as bitter.[9] In *Drosophila*, GRs that sense bitter chemicals are necessary to avoid ingestion of DEET containing food.[56] This avoidance occurs even with 0.1% DEET, a significantly lower concentration than the chemical has been used for olfactory-based laboratory studies or in commercial products. Similarly, other studies have shown that the mosquitoes do not feed on blood that contains a very small amount of DEET (0.065%), even though some of them penetrated their proboscis into a membrane feeder.[57,58] As mosquitoes are unlikely to smell DEET in these assays, these results suggest that repellency occurs through labellar taste receptors, and not through an olfactory mechanism. Repellency was also observed when DEET was applied to the feeding membrane, suggesting a role for taste neurons located on both the legs and the proboscis.[58]

Evidence for gustatory receptors specifically detecting DEET was obtained from studies in *Drosophila*.[56] It was found that aversive taste neurons tuned to numerous bitter compounds also respond to DEET and that DEET-mediated activation of these neurons required 3 bitter taste receptors, including Gr66a. Moreover, behavioral experiments showed that the proboscis extension reflex response induced by sugar solutions applied to tarsi of flies is severely reduced when DEET was added to the sugar solution. These observations indicate that DEET has an inhibitory effect on a feeding response by activating bitter taste neurons that counteract the activity of sweet sensing neurons. Electrophysiology recordings in *Aedes aegypti* have also shown that DEET activates bitter taste neurons in the labellum that respond to many other bitter compounds.[59] Interestingly, many labellar gustatory receptor neurons also express *Aaeg*GR14, the putative ortholog of the *Drosophila* bitter receptor GR66a.[60] Whether the tarsi of *Aedes* respond to DEET remains to be seen, as *Aaeg*GR14, is not expressed in neurons of sensilla located in tarsi. However, they do express many GRs related to *Drosophila* bitter taste receptors and hence are likely to respond to bitter compounds.[60] Regardless, the current evidence strongly suggests that insects taste DEET and avoid ingesting it like other bitter compounds.

Human skin treated with DEET repels mosquitoes

The mechanism of DEET repulsion of mosquitoes when they land on skin differs from DEET repellency in the vapor phase. *Aedes orco* mutants are attracted to DEET-treated skin, but do not blood feed.[35] This result could be explained by 2 mechanisms: 1) that *orco* mutant mosquitoes need physical contact with DEET-treated skin in order to be repulsed or 2) that there are low-affinity olfactory receptors that sense DEET when the mosquito is in close proximity to skin. To test this, video recordings of *orco* mutant mosquitoes and wild-type controls documented host-seeking behavior at close-range. When DEET was applied to skin, *orco* mutants landed on the skin and then left without biting. This result demonstrates that contact mediates the repulsion in the absence of an intact olfactory system. Thus the *orco* mutant allows the separation of the contact and olfactory mechanisms of DEET in the mosquito, with the potential caveat that other olfactory receptors, including IRs, are intact in this mutant.[35] It remains to be determined whether landing on DEET-treated skin triggers a bitter taste response through GRs or contact disengagement involving a distinct molecular pathway. Identification of the contact receptor(s) would provide additional molecular target(s) for chemical screens to identify new topical repellents that could block mosquito blood-feeding.

Implications for the Next Generation of Insect Repellents

DEET is safe and effective, but has several drawbacks.[4] It has to be applied at relatively high concentrations (10% or more) to be effective. DEET needs to be reapplied to skin every few hours to ensure repellency. Pure DEET melts plastic and vinyl. It is also not very volatile. Because of the short-range spatial protection of DEET,[50] it needs to be applied either on skin or cloths to effectively repel arthropods, whereas other application methods, such as wearing a DEET-impregnated wristband, do not work.[4] There is also evidence that mosquitoes can become resistant to DEET.[62] The limitations of DEET have fueled a search for alternatives.

The next generation of insect repellents are likely to be rationally discovered using molecular targets that enable insect host attraction such as olfactory receptors. This approach allows for high throughput screening of hundreds of thousands of compounds, many more than were screened to identify DEET.[5,6] ORs can be functionally expressed in cultured cells and their ion channel activity can be visualized.[63,64] This allows for screening of compounds that can directly activate ORs or change their sensitivity to odor-ligands. Recent screens have already identified an agonist of insect ORs, VUAA1.[63,65] This chemical activates all OR-Orco complexes tested and likely works by directly interacting with Orco. VUAA1's main drawback is that it is not very volatile. Continued effort will likely yield other compounds that can manipulate insect olfactory receptors and are volatile enough to become candidate insect repellents. Identified compounds should posses a number of qualities to overtake the current gold standard, DEET.[3] These include: efficacy in the vapor phase at low concentrations, specificity for the receptor it was designed to target, low cost, easy to impregnate into wearable items, such as wristbands, be long lasting, have a pleasant odor, and of course be non-toxic to humans and the environment.

Although the mechanism is not yet clear, DEET likely alters the activity of olfactory receptors either in the context of odors or on its own. This has several implications for any screening protocol. Chemical screens designed to isolate candidate repellents will need to seek chemicals that broadly inhibit multiple classes of insect odorant receptors due to the redundancy that exists in insect olfaction.[20,35] As an alternative, screens can seek to modulate specific classes of olfactory receptors such as ORs that have been associated with repellency. Understanding which ORs are modulated by DEET, and whether the changes are behaviorally relevant would do much to narrow the field of molecular targets to be screened. In other words, to find volatile chemicals that trigger repellency, it is necessary to connect insect olfactory

receptors with the behaviors they enable. Understanding how DEET works may lead us to these important molecular targets.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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