UNIVERSITY OF CALIFORNIA
RIVERSIDE

An Investigation Into Novel Mechanisms of Sensory Detection and Neural Processing of Repellent Odorant Information

A Dissertation submitted in partial satisfaction
of the requirements for the degree of

Doctor of Philosophy

in

Neuroscience

by

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June 2020

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Acknowledgements

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> Clark, J.T. & ray, A. Olfactory Mechanisms for Discovery of Odorants to Reduced Insect-Host Contact. *J Chem Ecol* **42**, 919-930, doi:10.1007/s10886-016-0770-3 (2016)
>
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As is typical with a work of this magnitude, I the author am deeply indebted to several individuals who have contributed to the scientific data and editorial synthesis of this dissertation:

Dr. Anandasankar Ray supervised and directed the research on which this dissertation is based. Tom Guda Ogata conducted behavioral experiments found in Figs 2.1, 2.8, and 2.9, while Matthew Luy performed the mosquito landing assays found in Fig 2.1F. Jadrian Ejercito generated the calcium imaging data in Figs 2.4, 2.8, 2.11, 3.4, and 3.5. Rogelio Nunez-Flores assisted me in generating some of the data in Fig 2.7, and Dr. Iliano Coutinho-Abreu performed the mosquito experiments in Fig 4.2.

I also thank Dr. Michael Adams and Dr. Naoki Yamanaka for provided insights during our weekly lab meetings.

Personal Acknowledgements:

I must first thank my advisor, Dr. Anandasankar Ray. You are a man of innumerable talents, among which the ability to excite others, young scientists included, to see the wonders of cutting-edge biological research perhaps ranks first. Watching as you juggled the responsibilities of running a private company, teaching courses, writing and reviewing scientific articles, planning experiments, and raising a daughter was an invaluable inspiration for me, and I thank you.

I thank Dr. Anupama Dahanukar for in the beginning being the one to take the risk and accept me as a naïve first-year undergrad into her laboratory, teach me how to think and act like a scientist, and open the doorway to all that has happened during the last decade of my professional career. Special thanks is also due to Tom Guda, who's generous heart and perennially-cheerful mood acted as a much needed theriac to what was oftentimes a stressful journey. I thank Matthew Luy, Rogelio Nunez-Flores, and Dr. Christi Ann Scott for their companionship over the years. To my office mate Joel Kowalewski: the many discussions and laughs we have had, covering by this point nearly every topic under the sun, I will always remember with fondness.

To my parents, for their unfailing support and love that were absolutely essential not just during the time of my work detailed here, but also throughout my life to get me to this position in the first place, I thank you. To my extended family, Aunt Janie, Alayna, Julian, and the kids, the Swain clan going 32(?) strong: you have all been instrumental in making this journey much easier.

I thank my friend and fellow songwriter Alex Bernal for helping to bring my music to life when I needed an artistic outlet, and for hosting over the years so many pool parties, soirées, and other social events that provided a critical respite from the daily grind. To Spencer Lake and Mason Vigil, whose 29 and 17-year-long friendships, respectively, have kept me grounded, I look forward to many more years of gaming, jamming, golfing, skiing, kayaking, trap shooting, horseback riding, and all the other activities that constitute our unusually eclectic group of interests and hobbies. I also thank my roommate and colleague, Andy Tseng, for his companionship and all the free rides to and from campus so that I didn't need to always ride the RTA bus…

Finally, I must give a hearty thanks to Billy Joel, who scored and penned the soundtrack to this seven-year journey.

ABSTRACT OF THE DISSERTATION

An Investigation Into Novel Mechanisms of Sensory Detection and Neural Processing of Repellent Odorant Information

by

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Doctor of Philosophy, Graduate Program in Neuroscience
University of California, Riverside, June 2020
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Because of their diversity, abundance, and nigh ubiquity on land, insects represent the largest group of animal competitors to humanity. Insect vectors carry diseases such as malaria, yellow fever, and Zika that are responsible for hundreds of thousands of deaths each year, and agricultural loss to insect pests reaches billions of dollars annually. Because insects rely principally on olfactory cues to target hosts and food sources, their olfactory system is of great import for scientific study. The fruit fly *Drosophila* provides a genetically tractable and numerically simple nervous system well suited as a model for studying the detection and processing of odor cues. By developing in the fruit fly a technique for studying the

detection of common insect repellants, we have discovered a novel mechanism of the detection of DEET involving widespread neuronal activation and inhibition, and find that this mechanism is conserved across mosquito species. We show that DEET induces either neuronal activation or inhibition in a manner dependent upon the identity of the odorant receptor expressed in the detecting neuron. A similar widespread effect is also found in response to noxious concentrations of basic amine compounds. Exposure to high concentrations of amines inhibits neurons across several sensillar classes and this inhibition renders them unresponsive to other odor ligands. We also find that certain amine compounds are able to reduce humidity sensing by the neurons in the coeloconic sensilla of *Drosophila* and that these compounds are able to reduce oviposition preference in mosquitoes. In summary, our research identifies several novel mechanisms of repellent detection and can potentially inform efforts in repellent development and pest management.

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CHAPTER 1

AN INTRODUCTION TO OLFACTORY MECHANISMS FOR DISCOVERY OF ODORANTS TO REDUCE INSECT-HOST CONTACT

INTRODUCTION

Volatile chemicals have been used to repel insects since ancient times, when certain plant or animal extracts were found to ward off biting insects, and were used for personal protection. Thus, people burned plant extracts to create smoke (Charlwood 2003) or anointed themselves with vinegar or oil. The primary path to discovery of effective insect repellents was trial-and-error. This type of empirical testing approach continued into the 20th century, and was expanded with the development of chemical synthesis techniques, which enabled the production of potential repellents on a large scale. Many commonly used repellents, including DEET and IR3535, were the result of these concerted efforts to synthesize and screen large numbers of compounds as potential repellents (Mccabe, Barthel et al. 1954).

The 1950s brought the advent of analytical tools that could greatly expedite insect olfaction research. The modern gas chromatograph, developed in 1952, provided a means to rapidly separate complex mixtures of volatiles into individual compounds (James and Martin 1952). This meant that biologically active molecules could be isolated from their sources and tested individually. Electrophysiological developments such as the electroantennogram (Jefferis, Marin et al.) led to another great leap forward, allowing neural activity in insects to

be recorded in real time while the insect was exposed to individual compounds (Schneider and Kaissling 1957). The power of this method was increased still further by the coupling of gas-chromatography with electroantennogram detection (GC-EAD), providing a method of separating a complex odor blend into its individual components while simultaneously testing the antennal responses to each component. This technique greatly accelerated the identification of biologically active compounds in complex mixtures such as plant or insect extracts. The development of molecular modeling in the latter half of the 20th century allowed for more efficient compound discovery, leading to the identification of the repellent Icaridin in the 1980s. Despite these major advances in analytical methods, physiological recording methods, and molecular modeling, discovery and development of new insect repellents for applications in human health and agriculture has not yet followed. Effective and affordable repellents are largely unavailable in tropical countries that bear the brunt of vector-borne diseases, and use of repellents in agriculture is almost non-existent worldwide. However, the advent of modern genomics and molecular biology tools has led to a much better understanding of the insect olfactory system, and consequently, a deeper knowledge of the mechanisms by which repellents affect insect behavior. More recently, chemical informatics can even screen and predict active compounds, so that bioassays can be focused on the subsets of compounds that are most likely to be active (Boyle, Guda et al. 2016).

OVERVIEW OF THE INSECT OLFACTORY SYSTEM

Volatile chemicals that repel insects typically act on the olfactory system. The principal olfactory organs in insects are the antennae and maxillary palps. These organs are covered with hair-like projections called sensilla that house a small number of olfactory receptor neurons (ORNs), the exact number varying by sensillar type, as well as various support cells (Shanbhag, Singh et al. 1995, Shanbhag, Muller et al. 1999). The sensillar sheath is perforated with pores that allow compounds to make contact with the inner lymph bathing the neurons. Each ORN expresses a small number of olfactory receptor proteins, which pass ion current through the membrane upon binding odorant molecules (Sato, Pellegrino et al. 2008, Benton, Vannice et al. 2009). The olfactory receptor proteins are encoded by 3 different families of genes: the Odorant Receptor (Or), the Ionotropic Receptor (Ir) and a few members of the Gustatory Receptor (Gr) family (Clyne, Warr et al. 1999, Vosshall, Amrein et al. 1999, Clyne, Warr et al. 2000, Benton, Vannice et al. 2009). The ORNs send projections to the antennal lobes (AL) where they synapse with the dendrites of second order projection neurons (PNs) (Fig 1.1). ORNs expressing the same receptor(s) will synapse in the same neurite conglomeration, called a glomerulus (Vosshall, Wong et al. 2000). From the antennal lobe, the information is carried to the mushroom bodies and lateral horns for higher processing (Jefferis, Marin et al. 2001). Both excitatory and inhibitory interneurons provide input to the olfactory circuit (Ng, Roorda et al. 2002, Wilson, Turner et al. 2004, Wilson and Mainen 2006). In *Drosophila melanogaster*, after

detection in the antenna or maxillary palps, and processing in the antennal lobes, information about most innate olfactory behaviors are thought to be routed through the lateral horn (Strutz, Soelter et al. 2014). The behavioral outcome of odor-induced neural activation therefore depends on which ORNs are activated and to what extent. This can also, in principle, be manipulated using odorants that affect the activity of relevant olfactory receptors and ORNs.

Although thousands of odorants can potentially interact with an insect's olfactory system, there are only four general mechanisms that have been shown so far to reduce an insect's contact with hosts: 1) the activation of olfactory receptors dedicated to aversion, 2) activation of pheromone receptors that cause aversion, 3) the inhibition of odorant receptors dedicated to attraction, and 4) prolonged activation of odorant receptors participating in attraction. In this review we discuss findings that support these four mechanisms and suggest ways to exploit these pathways in developing methods for reduction of host-seeking behavior using odorants. We also discuss how the internal physiological state of the insect such as hunger, thirst, mating, and egg-laying can influence neural activity, and be targeted to alter host-seeking behavior.

ACTIVATION OF OLFACTORY RECEPTORS DEDICATED TO AVERSION

Identification of dedicated receptors and ORNs for aversion in a target insect species can serve as an excellent way to identify powerful species-specific insect repellents. The idea of such labeled line codes – that is, the existence of

separate neural circuits dedicated to the processing of specific and fixed sensations – has been a staple of theory in sensory neuroscience since Johannes Peter Muller first proposed its less-refined predecessor in the mid-nineteenth century. Evidence from the model species *Drosophila melanogaster* suggests that in insects a labeled line code exists for some aversive odors (Suh, Wong et al. 2004, Stensmyr, Dweck et al. 2012). These odors can originate from a variety of sources such as plants, microbes, or, in the case of pheromones or carbon dioxide, other flies.

CO_2

One of the first olfactory circuits identified as causing innate aversion is that which detects the gas CO_2 in *Drosophila melanogaster* (Suh, Wong et al. 2004). This pathway is unusual among antennal lobe inputs in that its primary sensory neurons express members of the Gustatory Receptor (*Gr*) family *Gr63a* and *Gr21a*, instead of members of the more typical Odorant Receptor (*Or*) family (Suh, Wong et al. 2004, Jones, Cayirlioglu et al. 2007). Genetically silencing the *Gr21a/Gr63a*+ neurons abolished aversion to CO_2. Artificial activation by optogenetics of the CO_2 circuit using channelrhodopsin (ChR2) is also sufficient to cause aversion (Suh, Ben-Tabou de Leon et al. 2007), further corroborating its role in innate aversion behavior in *Drosophila*. When channelrhodopsin (ChR2) was expressed in Gr21a-expressing neurons, flies avoided the illuminated arm of a T-maze. Since the gaseous CO_2 is difficult to formulate for delivery, other odorants

that also activate this aversive labeled line could serve as effective repellents. Odorants that activate the CO_2 receptor (Gr21a+Gr63a) such as pyridine, cyclopentanone, and (*E*)-2-methylbut-2-enal were identified using electrophysiological recording techniques (Lu, Qiu et al. 2007, Tauxe, MacWilliam et al. 2013). As expected, when pyridine was tested in a T-maze with *Drosophila melanogaster*, it showed a strong repellent effect similar to CO_2 (Pham and Ray 2015). Comparative analysis across 5 related *Drosophila* species showed that the CO_2 receptor is conserved as a sensitive detector of CO_2 and pyridine; however, its role in aversive behavior was species-specific. Two of the 5 species tested with CO_2 and with pyridine, *Drosophila suzukii* and *Drosophila virilis*, showed little avoidance in a T-maze assay (Pham and Ray 2015). In fact, more distantly related dipterans like mosquitoes are attracted to CO_2 plumes, suggesting that the aversion response is not well conserved among Diptera (Cardé and Gibson 2010, Ray 2015).

The T-maze assays used in the laboratory test walking flies in a narrow tube, and when flying *Drosophila* were tested for longer-term behaviors using a 2-choice trap assay, *D. melanogaster* did not show significant avoidance of the pyridine-treated trap, suggesting that the aversion is dependent on the context of the assay design and duration of the assay. Thus, CO_2-receptor-activating odorants such as pyridine are unlikely to act as broad-spectrum repellents for *Drosophila* species, particularly for the agriculturally important pest *D. suzukii.* Free-flying *Drosophila* have not been tested for instantaneous responses to CO_2,

but tethered *D. melanogaster* that can flap their wings and turn directionally did not turn away from a puff of 100% CO_2 (Wasserman, Salomon et al. 2013).

Because species-specificity of the labeled aversive line was observed, one of the questions that emerges is why *D. melanogaster* would be averse to low concentrations of CO_2 in the first place, particularly since CO_2 is ever-present at low levels in the atmosphere (Gillies 1980, Turner and Ray 2009, Pham and Ray 2015), and is emitted by food sources such as fermenting fruit on which flies feed and oviposit. While stressed flies emit a small amount of CO_2 as part of their stress response (Suh, Wong et al. 2004), it is released at much higher levels by ripening fruit (Faucher, Forstreuter et al. 2006). One possibility is that this ability equips flies to avoid less ripe fruit, which emit higher levels of CO_2, in favor of fruits further along in the decaying process (Turner and Ray 2009, Pham and Ray 2015).

Organic acids

Calcium imaging of neuronal activity in the antennal lobe of *Drosophila* revealed that the DC4 glomeruli, innervated by coeloconic neurons expressing the ionotropic receptor *IR64a*, showed increased activity in response to carbonic acid and other short-chain organic acids (Ai, Min et al. 2010). The IR64a protein was determined to be necessary, but not sufficient, for acid detection, but activity of the IR64a-expressing neurons was required to achieve a strong aversion to acids.

Geosmin

A third labeled-line aversive pathway was discovered in *D. melanogaster* that comprises ORNs expressing the *Or56a* receptor, which is activated by the microbe-produced bicyclic compound geosmin (Stensmyr, Dweck et al. 2012). In addition to being narrowly tuned so as to be dedicated to geosmin detection, these neurons were shown to be sensitive to low, ecologically relevant concentrations and to confer aversion. For example, addition of geosmin to balsamic vinegar caused approximately a one-third reduction in the attraction index (Stensmyr, Dweck et al. 2012).

Multiple receptors

As qualified as these circuits are to be considered labeled lines, a more prominent form of odor detection is combinatorial coding, where one odor can elicit varying responses from multiple receptors and glomeruli (de Bruyne, Clyne et al. 1999, Galizia, Sachse et al. 1999, de Bruyne, Foster et al. 2001, Wang, Wong et al. 2003, Semmelhack and Wang 2009, Hallem and Carlson 2006). This means that a single odor may activate both attractive and repellent pathways, and it is the integration of such activity that determines the odor quality and subsequent behavior. In general, adult flies are averse to high concentrations of odorants, even if those odorants are attractive at lower concentrations (Semmelhack and Wang 2009). One way this can occur is via recruitment of additional neural populations with increasing odor concentration. *Drosophila melanogaster* is attracted to apple

cider vinegar due to activation of the DM1 and VA2 glomeruli, innervated by ORNs expressing *Or42b* and *Or92a*, respectively. However, these flies avoid higher concentrations of vinegar by recruitment of additional activated neurons, including those innervating the DM5 glomerulus, which express *Or85a*. This study indicated that DM5 is an innately aversive glomerulus, and its activation can override the attractive input of other glomeruli like DM1 and VA2 (Semmelhack and Wang 2009). In order to test whether this basic discovery can be utilized to identify novel repellents, odorants that were identified as activators of this neuron were used in behavioral tests. Indeed, an odorant that activates the Or85a receptor, such as ethyl 3-hydroxybutyrate (Hallem and Carlson 2006) causes aversion in *D. melanogaster*, although the behavior is not conserved across other *Drosophila* species such as *D. suzukii, D. yakuba, D. pseudoobscura,* and *D. virilis* (Pham and Ray 2015).

Using optogenetics to identify aversive neurons

A recent paper used optogenetics and systematically expressed a UV-light- sensitive Channelrhodopsin-2 protein in specific ORN types and tested behavior activated by light (Bell 2016). Surprisingly, activation of the *Or56a+, Or85a+* and the *Ir64a+* ORNs led to attraction instead of aversion as would have been expected from previous studies. The *Gr63a+* neurons showed aversion as before. Interestingly this study also reported that airflow within the behavior arena was required for a behavioral response (Bell 2016). These results suggest that

the design of laboratory assays can impact the behavioral outcome and several lines of experimentation are necessary for identifying aversive ORN lines.

Citronellal

Another widely known insect repellent is the monoterpenoid citronellal. The olfactory receptor activated by citronellal is not known; however, electrophysiological studies show both the ab11a and ab12a neurons are activated by citronellal (Kwon, Kim et al. 2010), and citronellal apparently does not activate other neurons in the antenna. In further screening of mutant flies, activation of ab11a was shown to be independent of *Orco,* whereas activation of ab12a was dependent on *Orco*. TrpA1, a cation channel that activates at temperatures over ~26 degrees C, was implicated in mediating the aversive response in one of the neurons in the mutant analyses, which showed a defect in the temporal kinetics of the response.

Orco ligand that activates broadly

Because each insect species will have its own repellent olfactory receptor pathways that are challenging to identify, an interesting alternative approach to identify repellents would be to find chemicals that show widespread activation of olfactory receptors in general. That is, these chemicals would probably non-specifically activate at least some repellent pathways as well. One group of such molecules, VUAA1 and its analogs, were identified as activators of the co-receptor

Orco using large-scale small molecule screening from a synthetic chemistry library (Jones, Pask et al. 2011). However, these molecules are nonvolatile, and behavioral testing to evaluate reduction in attraction to hosts has been difficult. Identification of volatile variants of these compounds in the future could have great potential for testing whether host seeking is reduced, and also whether the widespread activation acts as a confusing cue to an insect.

As expected, aversion to odorants is blocked when synaptic transmission is blocked in all ORNs by expressing shibire^{ts1} (Gao, Clandinin et al. 2015). However, selectively rescuing only a small subset of these silenced ORNs – as few as one – was sufficient to restore aversive behavior. Furthermore, the authors discovered that aversion was unaffected by silencing GH146+ excitatory projection neurons (ePNs), which are thought to form 2/3 of the total ePN population. This indicates that, like the primary ORNs, relatively few antennal lobe output neurons are required to convey aversion information to higher brain centers. These experiments indicate that aversive channels are possibly widespread.

DEET

The aversive mechanism that has received a great deal of interest recently is that for the repellent with the most widespread commercial use, N,N-Diethyl-*meta*-toluamide (DEET). It is a weak repellent, likely due to its low volatility, showing almost no repellency in *Drosophila melanogaster* when tested in a T-maze and requiring special close-range assays. Even in mosquitoes, DEET repels

only at close range and is used in formulations at high concentrations (5%-100%). It has very limited use in tropical countries where there is a great need for effective repellents, and it is not used at all in crop protection. Nevertheless, the main advantages of DEET include long-lasting repellency for 2 or more hours, perhaps due to the low volatility and high concentrations used, its non-specific action across many insect species including Diptera (Pham and Ray 2015). These two attributes make it one of the most commonly used insect repellents in the developed world. However, DEET has also been shown to dissolve nylons and plastics, blocks human acetylcholinesterase, activates human M3 muscarinic GPCR causing anti-angiogenesis, inhibits mammalian TRPM8 and inhibits mammalian CNGA2 channels, the health consequences of none of which are well understood (Ditzen, Pellegrino et al. 2008, Corbel, Stankiewicz et al. 2009, Swale, Sun et al. 2014, Abd-Ella, Stankiewicz et al. 2015, Legeay, Clere et al. 2016) (Swale 2015, Legeay 2016). The notion that identification of the mechanisms of action of DEET could help in the development of improved broad-spectrum repellents has resulted in much recent research in this area (Ray 2015). However, no mechanism uncovered so far fully explains its properties and conservation of those repellent properties across numerous species.

There is some evidence that DEET acts in part through *Or* family members in an *Orco*-dependent manner (Ditzen, Pellegrino et al. 2008, DeGennaro, McBride et al. 2013). There is also data from *Culex quinquefasciatus* that shows it activates specific ORNs directly (Syed and Leal 2008, Xia, Wang et al. 2008). Other lines of

evidence suggest that DEET may also inhibit responses to attractive odors (Bohbot and Dickens 2012). The *C. quinquifasciatus* receptor CquiOr136 was shown to impart response to DEET and other repellents when expressed in *Xenopus* oocytes (Xu, Choo et al. 2014). The RNAi knockdown of this one receptor led to mosquitoes with significantly reduced repellency to DEET, suggesting that a sole receptor type may be responsible for repellency in this species. The *CquiOr136* receptor does not have orthologs in other mosquito or insect species that also avoid DEET, raising the possibility that different insects have evolved different receptor pathways to detect this molecule and convey aversion (Xu, Choo et al. 2014). In another study, authors co-expressed the *Aedes aegypti* Ors AaOR2 or AaOR8 together with the co-receptor AaOR7 (now called Orco) in *Xenopus* oocytes and exposed them to four different insect repellents, including DEET (Bohbot and Dickens 2010). They found that when presented alone, DEET activated AaOR2 but not AaOR8. However, when presented as a mixture, DEET inhibited the activity elicited by 1-octen-3-ol, a ligand known to activate AaOR8. This effect was also observed when a DEET/indole mixture was presented to AaOR2. Two other repellents, IR3535 and picaridine, inhibited the activity of indole and 1-octen-3-ol on their cognate Ors but induced no activity themselves. Given these confusing results, clearly more research is needed to clarify the mechanism of DEET detection across species.

Genetic approaches using CRISPR-Cas9 have also been useful in addressing mechanistic questions about DEET repellency. For example, *Orco*

mutant *Aedes aegypti* no longer avoid a human arm covered with DEET placed outside a cage full of mosquitoes (DeGennaro, McBride et al. 2013). Interestingly, when a 37ºC heat source is used as an attractant instead of an arm, *Orco* mutants continue to avoid DEET, suggesting that repellent mechanisms other than Or receptors are also active in aversion to DEET (Guda, Kain et al. 2015). An alternative receptor pathway such as the ionotropic pathway could be involved. Hopefully, further experimentation to identify the non-Or receptor pathways as well as Or pathways that detect DEET will help to solve some of these conundrums.

The variety of different effects seen with DEET suggests that DEET could be a broadly tuned ligand that has binding sites on multiple olfactory receptors, albeit with lower affinity than those of cognate odors. One variation of this model was proposed as an unusual “odorant scramble” hypothesis which posited that DEET is not itself detected by the insect olfactory receptors but, when presented alongside weak ligands, causes differences in levels of activation and inhibition of multiple Or/ORNs, leading to a “scrambling” of the odor responses and the resulting disorientation (Pellegrino, Steinbach et al. 2011). This hypothesis was developed based on electrophysiology analyses in large basiconic sensilla of the adult *D. melanogaster* antennae when exposed to a mixture of DEET and various odorants. While 1-octen-3-ol normally suppresses activity of the ab2A neuron, a DEET + 1-octen-3-ol mixture activated the ab2A neuron. These effects were observed with other odorants that were weak ligands as well. Further experimentation revealed that only the inhibition of the Or59b receptor by odorants

was impaired; the activation by compounds that stimulate the neuron was not affected. This hypothesis has not yet been tested with behavioral bioassays and it is still possible that DEET's repellency is caused by its activation of one or a few critical, aversive pathways.

Computational approaches to repellent discovery

Given that DEET's repellent effect could work through several receptor pathways, it is extremely challenging to devise a screening method to identify improved DEET. To circumvent this hurdle, a computational approach has been tested and proven to be very effective (Katritzky, Wang et al. 2008, Katritzky, Wang et al. 2010, Oliferenko, Oliferenko et al. 2013, Boyle, Guda et al. 2016). These approaches generally rely on identifying physicochemical descriptors that can be predictive of repellent behavioral activity from previously identified compounds like DEET and picaridin, among others. Some form of learning such as neural networks or machine learning is then used so that the computer can learn to discriminate between repellents and non-repellents. In one instance this chemical informatics method was utilized to screen hundreds of thousands of chemicals, and several hundred compounds were predicted to be novel repellents. What made this approach particularly useful was the ability to screen a set of natural compounds to identify repellents with excellent safety properties. Behavioral testing revealed that many of the predicted compounds are indeed strong repellents and have potential in protecting against mosquitoes (Boyle, McInally et al. 2016). Since

many of these chemicals are non-toxic and already used in food they also were tested as potential repellents for use in agriculture. It was found that indeed they might offer protection against agricultural pests like the spotted wing Drosophila, *Drosophila suzukii* (Pham and Ray 2015).

Taken together, these discoveries show that members of all three olfactory receptor gene families, *Ors, Irs,* and *Grs*, can act as labeled-line aversive receptors. These studies also highlight that discovery of labeled aversive receptors and neurons require a significant number of genetic experiments that are extremely challenging with vector insects. However, the recently developed CRISPR-Cas9 mutagenesis method potentially enables these types of experiments to be carried out in a variety of different insect species. The conclusions of these studies also reveal that very few inputs, as little as a single class of ORNs, can be sufficient to cause aversion even if the ligands that activate them also activate other ORN classes. These *Or* pathways and ORNs will likely be different across species, and their identification could lead to development of species-specific repellents.

Larvae

A special case to consider for repellent research is that of insect larvae. Many herbivorous insects damage their host plants in the larval stage, so it is important to understand olfactory repellent pathways in this life stage as well. The *D. melanogaster* larval olfactory system is simpler than that of the adult, and thus

has provided a window into understanding hardwired olfactory processing (Fishilevich, Domingos et al. 2005, Kreher, Kwon et al. 2005, Ramaekers, Magnenat et al. 2005, Kreher, Mathew et al. 2008). Efforts to discover the valence encoded by individual larval ORNs were encumbered by the fact that most odorants activate multiple ORNs (Kreher, Mathew et al. 2008). A number of odorants that activate Ors in a combinatorial manner have been identified in larvae using large-scale deorphanization efforts (Kreher, Kwon et al. 2005, Kreher, Mathew et al. 2008, Mathew, Martelli et al. 2013). Knowledge of how odorants activate nearly the entire larval repertoire of odorant receptors allowed for modeling of behavior. Based on these foundational studies, it was shown that the vast majority of odorants and Ors mediate attraction, with only a few imparting weak to moderate repellency. Modeling the experimentally observed behavior to the electrophysiologically determined activities of each receptor suggested that Or74a and Or82a may contribute to aversion, as geranyl acetate, an aversive compound, only activates Or82a at the concentration tested, and 1-nonanol, another aversive compound, acts most strongly on Or74a (Kreher, Mathew et al. 2008). In a complimentary approach, transgenic larvae were created that selectively expressed either the light-sensitive channel channelrhodopsin-2 or adenylyl cyclase Pac-alpha in specific ORN types via the control of Or-specific drivers. Their behavior was evaluated upon activation of these selected neurons using blue light (Bellmann, Richardt et al. 2010). This study highlighted two important points: First, most larval ORNs likely carry a positive valence, because

ten of the twelve lines had reduced aversion to light. Second, the ORN types expressing Or33b and Or45a are much weaker conveyers of attractive valence, being either indifferent or possibly conveying repulsive behavior (Bellmann, Richardt et al. 2010). To determine if the Or33b and Or45a expressing ORNs were indeed mediators of repulsion, larvae were placed in an arena with blue light in two quadrants and the Or45a ligand octyl acetate in the other two quadrants. Wild-type larvae avoided the octyl acetate quadrants in favor of the illuminated quadrants, indicating that octyl acetate is even more repulsive than light. However, transgenic lines expressing channelrhodopsin-2 in both Or33b and Or45a avoided the light quadrants, indicating that the Or33b and Or45a neurons indeed convey a negative valence. Interestingly, when ChR2 or Pac-alpha was expressed in all ORNs simultaneously, larvae became attracted to blue light (Bellmann, Richardt et al. 2010). This indicates that activity of Or33b and Or45a can be overridden by attractive input of the other ORNs. These studies suggest that identifying repellents for insect larvae may pose a challenging problem, and if other insect larvae behave like those of *D. melanogaster*, pursuing dedicated aversive olfactory receptors may not be the most promising route to repellent discovery.

ACTIVATION OF PHEROMONE RECEPTORS DEDICATED TO AVERSION

An interesting target to exploit in the future for behavior modification could be pheromone receptors typically involved in mate location. Both heterospecific and intrasexual courtship in insects are often prevented through chemically

established barriers (Coyne, Crittenden et al. 1994, Savarit, Sureau et al. 1999, Billeter, Atallah et al. 2009). For example, males of other *Drosophila* species will not court *D. melanogaster* females because 7,11-heptacosadiene, a cuticular hydrocarbon excreted by the female's oenocytes, is aversive. If the oenocytes are ablated, these males will court *D. melanogaster* females. Additionally, these males will not court females of their own species if the females are treated with 7,11-heptacosadiene (Billeter, Atallah et al. 2009).

cVA

One of the best-studied volatile pheromones in *Drosophila* is the long-chain lipid *cis*-vaccenyl acetate (Datta, Vasconcelos et al.), which is produced in the ejaculatory bulb of the male and transferred to females during copulation (Butterworth 1969, Brieger and Butterworth 1970). This compound is detected by two receptor proteins, Or67d and Or65a (Ha and Smith 2006, van der Goes van Naters and Carlson 2007), and elicits different responses depending on the context, dosage, and sex of the detecting fly. Males will not court other males upon which cVA has accumulated, but *Or67d* mutants will do so at a rate three times higher (Kurtovic, Widmer et al. 2007). cVA also acts as an aphrodisiac in female flies (Kurtovic, Widmer et al. 2007). How can cVA bind to the same receptors in the same neurons of both males and females, and yet lead to entirely opposite behaviors? The Or67d-expressing neurons are the first components of a sexually dimorphic circuit that is characterized by the *fruitless* transcription factor (Datta,

Vasconcelos et al. 2008, Ruta, Datta et al. 2010). The Or67d-expressing ORNs project to the DA1 glomerulus and synapse with projection neurons (PNs). These PNs project to the lateral horn where they overlap with dendrites of aSP-f neurons in males, but not females, and aSP-g neurons in females, but not males (Cachero, Ostrovsky et al. 2010). Functional characterization of these dimorphic neuronal populations found that aSP-f neurons responded to cVA in males, but not in females, whereas aSP-g neurons responded to cVA in females, but not in males (Kohl, Ostrovsky et al. 2013). These neurons did not respond to cVA in *Or67d*-mutants, indicating that Or65a does not contribute to their input. The *fruitless* protein product, Fru^M, was found to specify the circuit dimorphism, feminizing the circuit in males such that aSP-f neurons no longer overlapped with DA1 PNs when Fru^M was knocked out, and masculinizing the circuit in females such that aSP-f neurons overlapped with DA1 PNs when Fru^M was expressed. This emerging understanding of pheromone detection and processing could in principle lead to development of sex-specific repellents for insects using pheromones or compounds that mimic their activities.

Other pheromones

Interestingly, both *D. melanogaster* larvae and adults can smell and avoid semiochemicals emitted by another insect species, the parasitic wasp *Leptopilina,* that can parasitize up to 80% of fly larvae in nature (Ebrahim et al. 2015). Both larvae and adults use the Or49a receptor to detect iridomyrmecin, whereas adult

flies use Or85f to detect the wasp odors actinidine and nepetalactol. Identification of aversive receptors for semiochemicals might also have enormous value in reducing contact of insect larvae with hosts.

INHIBITION OF ODORANT RECEPTORS DEDICATED TO ATTRACTION

Or

Many insects rely on their olfactory system to find hosts, thus volatile chemicals that inhibit receptors that detect host odorants may offer a potential strategy to reduce host-seeking behavior. In an electrophysiological study with *D. melanogaster*, 11% of tested odorants inhibited the spontaneous activity of Odorant Receptors (Or), acting as inverse agonists (Hallem and Carlson 2006). However, when such odorants were tested in mixtures with an activating odorant, they are unable to overcome the activity to a sufficient extent to be considered candidates for preventing attraction (Su, Martelli et al. 2011).

Orco

However, artificial antagonists for the co-receptor Orco have been identified through high-throughput screening of compounds (Jones, Pask et al. 2012, Pask, Bobkov et al. 2013). These compounds, VUANT1 and amiloride derivatives can inhibit responses to odorants when applied to cells expressing odorant receptors. In principle these chemicals should inhibit Orco-dependent receptors efficiently across most insect species because insects have a relatively well-conserved Orco.

Presently these chemicals are not volatile enough for use in behavioral studies with hosts, but one would expect to observe a decrease in attraction of insects to odor sources that are detected primarily using *Or*-family receptors.

Gr

One of the few receptors that can be efficiently inhibited by volatile odorants is the heteromeric CO_2-receptor (Gr1,2,3). The effects of inhibitors on behavior have been tested in mosquitoes where CO_2 is one of the few known strong attraction cues to humans (Turner and Ray 2009, Turner, Li et al. 2011). For example, an inhibitory odorant like ethyl pyruvate significantly reduced the entry of mosquitoes into a CO_2-baited trap tested overnight in a greenhouse. The same Gr1,2,3 receptor also detects other odorants from skin, and ethyl pyruvate inhibited this response and also reduced the number of mosquitoes attracted to a human arm as odor source (Tauxe, MacWilliam et al. 2013). However, in order to block attraction to a host completely one needs to find inhibitors for most of the attractive neuronal pathways that an insect uses, making this approach somewhat practically difficult compared to repellents. One possibility would be to identify volatile compounds that have effects like VUAANT1 for all three classes of odor receptors (Ors, Irs, and Grs) to mask the host-odor detection completely.

PROLONGED ACTIVATION OF ODORANT RECEPTORS PARTICIPATING IN ATTRACTION

A few odorants can cause prolonged tonic responses in olfactory neurons expressing *Gr* receptors for CO_2 (Turner, Li et al. 2011, Tauxe, MacWilliam et al. 2013) or *Or* receptors (Martelli, Carlson et al. 2013, Boyle, Guda et al. 2016). Thus, the neurons are unable to respond strongly to subsequent exposure to odorant activators (Turner, Li et al. 2011, Tauxe, MacWilliam et al. 2013, Boyle, Guda et al. 2016). Elevated activity in the olfactory receptor neuron is also likely to cause a depression in synaptic transmission to the projection neurons (PNs) (Bhandawat, Olsen et al. 2007). Both these effects would substantially undermine the ability of the neurons to accurately convey detection of subsequent activators, and for the insect to behave appropriately. It was shown that pre-exposure to a prolonged activator blend for the CO_2 receptor (Gr1,2,3) can significantly reduce the navigation of *A. gambiae, C. quinquefasciatus, and A. aegypti* mosquitoes towards a CO_2 source in a wind-tunnel. In semi-field greenhouse trials, this blend also significantly reduced the entry of mosquitoes into a fake hut with a CO_2-trap placed inside (Turner, Li et al. 2011). In experiments with *D. melanogaster* larvae, pre-exposure to a prolonged activator odorant for the Or42b receptor significantly reduced the navigation of the larvae to ethyl acetate or apple cider vinegar (Boyle, Guda et al. 2016).

For blocking odorant receptor detection pathways, prolonged activators are more likely to produce behavior modifications than inhibitory odorants, which so

far are unable to override activators. There also appears to be structural similarities between prolonged activators of a given receptor because a chemical informatics method can learn to identify new prolonged activators from structures of known prolonged activators (Boyle, Guda et al. 2016). Moreover, identifying prolonged activators may be more straightforward in non-model insects that are important disease vectors.

EFFECTS OF INTERNAL STATE ON AVERSION

Mating

In some cases, an odorant can elicit different behaviors depending on the internal physiological state of the insect. For example, preference of *D. melanogaster* for food rich in amino acids varies in accordance with their nutritional state (Toshima and Tanimura 2012). Also, virgin female *Drosophila* prefer sugars to yeast extract, but this preference is reversed once they have mated (Ribeiro and Dickson 2010, Vargas, Luo et al. 2010). This preference reversal appears to be mediated by the seminal fluid protein sex peptide (SP), because mutant flies lacking functional SP did not show the preference switch upon mating (Ribeiro and Dickson). However, exactly how the peripheral or central processing mechanisms are altered upon mating is unknown. Similarly, female mosquitos show host-seeking behavior only in the window between mating and consumption of a blood meal, after which they are primarily interested in finding an oviposition site (Takken, van Loon et al. 2001). As the primary means by which insects detect food

sources, the olfactory system is a potent mediator of state-dependent valence changes to odor signals (Root, Ko et al. 2011, Beshel and Zhong 2013, Bracker, Siju et al. 2013).

Starvation

It has been demonstrated that starved fruit flies exhibit a reduced avoidance of CO_2 when it is presented along with vinegar as a mimic of food odor (Bracker, Siju et al. 2013). Interestingly, this reduction in aversion was not generalizable to another aversive odor, 3-octanol, suggesting the CO_2 detection was processed differently. Electrophysiological recordings revealed that the CO_2-detecting ORNs did not function differently upon starvation, meaning that the processing difference may occur downstream of the primary sensory neurons. Indeed, blocking the output of the a'/B' neurons of the mushroom body abolished CO_2 aversion in starved, but not fed, flies. This indicates that the a'/B' neurons of the mushroom body are involved in context-dependent odor processing in addition to olfactory learning. A combination of genetic and imaging techniques led to the discovery that a bilateral ventral projection neuron was required for the state-dependent CO_2 avoidance: blocking its output abolished CO_2 aversion in starved, but not fed, flies. However, in another study it was argued that this bilateral PN, which the authors referred to as PN_v-1, was dependent on CO_2 concentration, as it responded to lower concentrations than other PNs of the V-glomerulus (Lin, Chu et al. 2013). While not directly contradictory, this finding warrants a more careful investigation

into the purposes of these differential-processing pathways in the brains of insects that could influence behaviors. A follow-up study found that dopamine and specific projection neuron circuits were involved in context-dependent behavior differences (Bracker, Siju et al. 2013, Siju, Bracker et al. 2014). Blocking dopaminergic signaling caused starved flies to be as repelled by CO_2 as fed flies, while artificially activating dopaminergic signaling caused fed flies to have a similarly reduced aversion as starved flies. Calcium imaging observations in the mushroom body were consistent with the behavior, as dopamine application reduced responses of a'/B' neuron to CO_2.

Another example of starvation increasing tolerance for aversive odors is the role of tachykinin (DTK) in suppressing the activity of the DM5 glomerulus in *D. melanogaster* (Ko, Root et al. 2015). Knocking down DTK reduced attraction to medium concentrations of apple cider vinegar in starved flies, but not fed flies. Two-photon microscopy revealed that the aversive DM5 glomerulus, which is required for aversion to high concentrations of vinegar (Semmelhack and Wang 2009), became reduced in sensitivity and required higher concentrations of vinegar to become active in starved flies than in fed flies. This modulation co-occurred with the effect of starvation-induced increases of short neuropeptide F (Ko, Root et al.) signaling in the innately attractive DM1 glomerulus (Root, Ko et al. 2011, Ko, Root et al. 2015). This suggests a dual mechanism for starvation-dependent reduction in aversion.

If the physiological state of an insect can modulate attractiveness of odors, can it cause a complete valence reversal? Evidence from the haematophagous assassin bug *Rhodnius prolixus* indicates that such valence reversals do occur. It was found that during a window of a few days immediately after feeding, the insects would show no behavioral response to CO_2, an odorant to which they were attracted when seeking hosts (Bodin, Vinauger et al. 2009). This period was followed by another period also lasting a few days in which the insects would actively avoid CO_2. This effect was observed in both larvae and adult females, and is not attributable to differences in amount of ambulation between the different periods post-feeding. Although the exact mechanisms of this valence reversal are not known, it seems that components in the blood of the host are necessary, as a saline meal could not recapitulate the effect (Bodin, Vinauger et al. 2009).

SUMMARY AND FUTURE DIRECTIONS

In conclusion, the olfactory system continues to provide a fascinating template to understand behavior and its modulation using odorants. As complex as it may be to unravel, we have discussed a few basic mechanisms of odor detection that can be targeted for odor-mediated behavior disruption (Fig 2.1). While all these approaches have promise, the ability to use chemical informatics to rapidly screen large numbers of compounds may also prove very effective in identifying and developing novel repellents. Prolonged activators for olfactory neurons may also have considerable promise in disrupting behaviors by masking

detection of attractive cues from the host. However, the most effective repellents will likely be discovered by identification of the dedicated aversive receptors in insect species. A better understanding of basic odor detection and processing mechanisms along with advances in computational techniques will further accelerate efforts to identify odorants that can repel or mask attraction effectively in low doses and in an affordable and environmentally safe manner.

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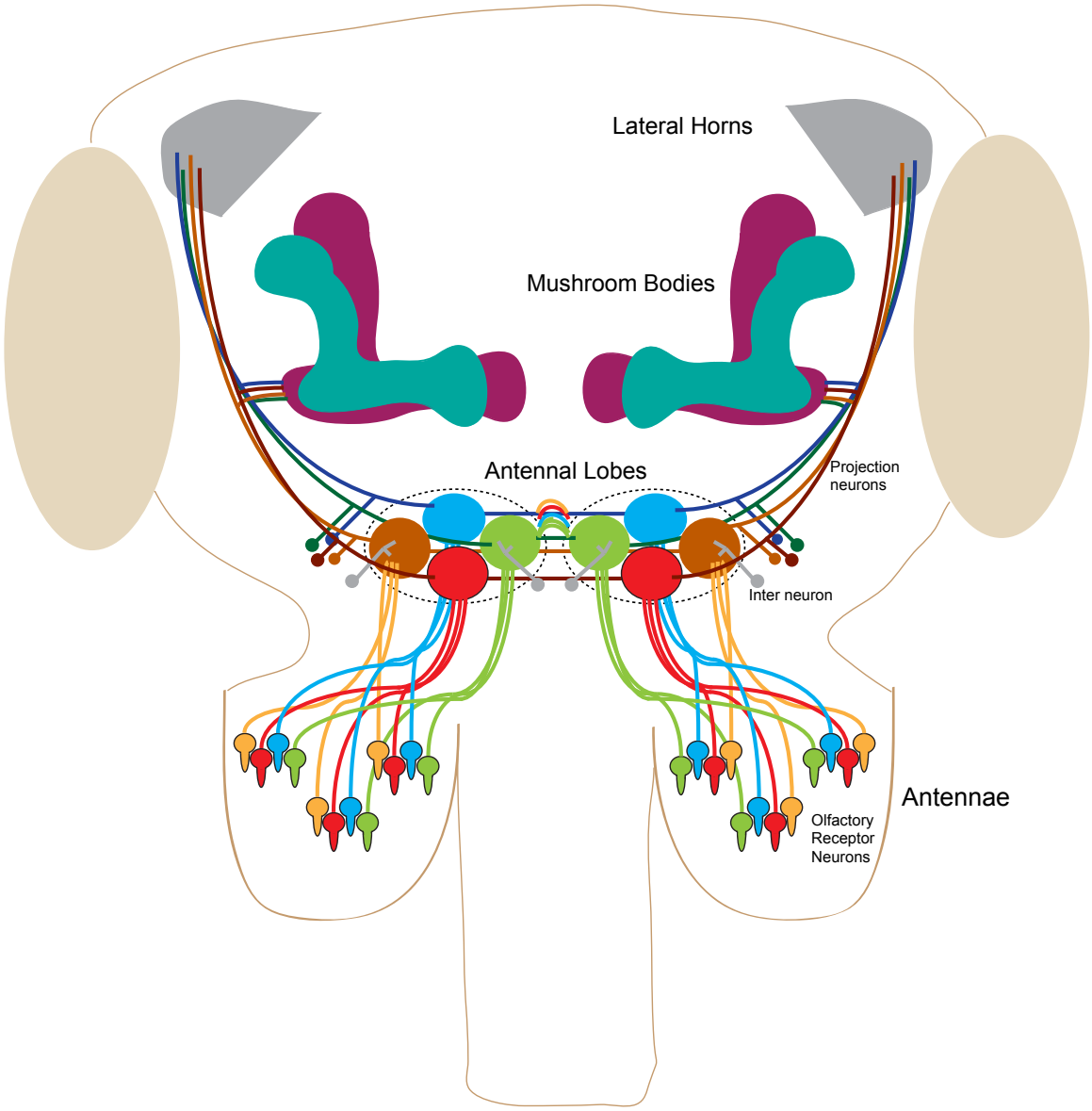

Figure 1.1. An overview of the stereotypic connections in the insect olfactory system.

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Olfactory processing begins with transduction in the olfactory receptor neurons, which are grouped in sensilla on the antennae and maxillary palps (not shown). All ORNs expressing the same olfactory receptors send an axon which fasciculates with other axons of the same ORN type. Each fasciculation terminates in a specific glomerulus of the antennal lobe, where it synapses with the dendrites of specific second order projection neurons. Excitatory and inhibitory interneurons also innervate the antennal lobes. The projection neurons in turn connect to the mushroom bodies, which are involved in olfactory learning and memory, and the lateral horns, which primarily route information instructing innate behaviors.

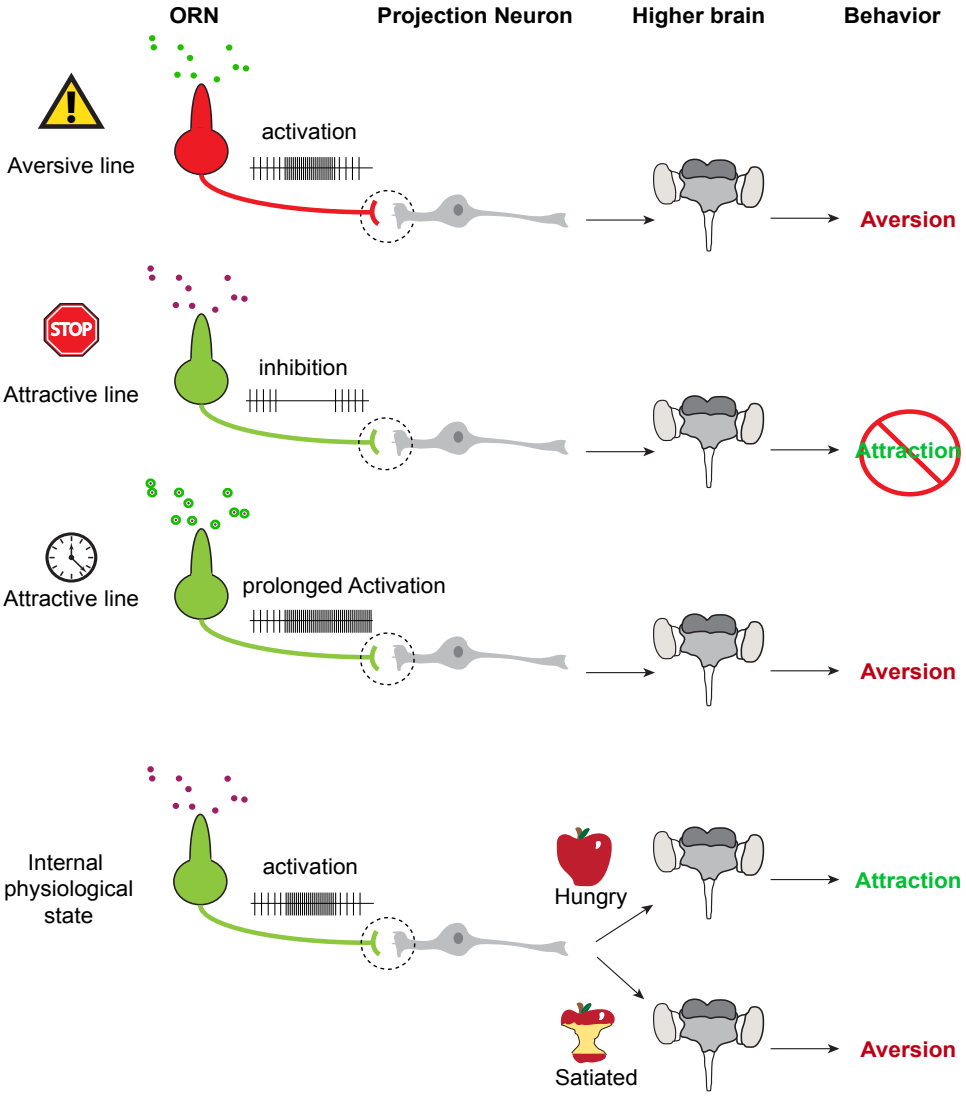

Figure 1.2. Olfactory mechanisms that can reduce attraction of insects to hosts.

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Activation of labeled-line aversive circuits will lead to innate aversive behavior**.** Some odorants can inhibit ORNs mediating attractive behavior and therefore "mask" the target odor source. Other compounds can act as prolonged activators of ORNs, meaning that they increase the baseline neural firing rate for extended periods of time. This elevated activity reduces the ability of the neuron to detect attractive odorant stimuli. The internal state of an insect can affect how it responds to attractive host-odors. Starved insects will often process the same olfactory signal differently than satiated insects, leading to differences in behavior. The mechanisms underlying this processing can potentially be used to discover ways to reduce host-seeking behavior in insect vectors.

CHAPTER 2

INSECT OLFACTORY NEURONS AND RECEPTORS DETECT DEET THROUGH WIDESPREAD ACTIVATION AND SELECTIVE INHIBITION

OVERVIEW

DEET is applied on skin at high concentrations and repels mosquitoes at close distances. A high-resolution behavioral analysis suggests that during the first approach of a mosquito to a DEET-treated surface, repellency is mediated by both non-contact detection of the volatile phase, as well as contact-based detection upon touching the surface. Repellency during subsequent approaches is largely non-contact in nature and dependent on the *Or*-coreceptor. By using a DEET dose that is representative of insect repellents, we identify three separate pathways for its detection in the volatile phase by olfactory neurons: direct activation and inhibition of Orco+ olfactory neurons, and inhibition of humidity-sensing Ir+ sensory neurons in the olfactory organs. The activation is observed in several classes of olfactory neurons, albeit to different levels. Both the co-receptor (*Orco*) and the *Or* are necessary. The broad activation and inhibition is conserved in *Aedes* and *Anopheles* mosquitoes as seen using electrophysiology and calcium imaging. Using an ectopic expression system in transgenic *Drosophila* we identify *Anopheles* odorant receptors that are activated, and one which is inhibited by DEET. Since DEET is also known to physically interact with and suppress evaporation of attractive skin odorants, we tested aversion where physical mixing is prevented and still find strong repellency. Therefore, a direct sensory detection

of DEET is also critical for repellency. In summary we identify multiple DEET-sensing neurons and odorant receptors that act at the repellent-relevant concentrations, with detection involving activation as well as inhibition of different pathways.

INTRODUCTION

The synthetic amide N,N-diethyl-*meta*-toluamide, commonly known as DEET, has for decades been used as a repellent of mosquitoes and acts on several other species of biting insects. DEET requires relatively high concentrations to be effective, with products as high as 98%, though typically lower (7%-30%), available on the market. Due to its long-lasting effect, it remains the market leader in the US despite various health concerns that have emerged recently (Corbel, Stankiewicz et al. 2009, Swale, Sun et al. 2014, Abd-Ella, Stankiewicz et al. 2015, Legeay, Clere et al. 2016). Use of personal topical repellents is limited in tropical countries that are most affected by mosquito-borne diseases such as malaria, Dengue, and Zika, all of which lack effective vaccines. As such, there remains a pressing need to find new repellents that are more effective and appealing for use.

Extensive investigations in the laboratory with DEET have revealed several possible mechanisms by which it may be detected, although a conserved receptor that detects it across numerous insect species remains elusive (Ditzen, Pellegrino et al. 2008, Syed and Leal 2008, Xia, Wang et al. 2008, Bohbot and Dickens 2010,

Liu, Pitts et al. 2010, Bohbot and Dickens 2012, Xu, Choo et al. 2014). DEET has a fixative effect that dramatically reduces the release of some volatiles emitted from skin and will therefore differentially affect the odor blend compositions of individuals (Syed and Leal 2008), and a recent study goes so far as to propose that DEET acts primarily by suppressing evaporation of skin odorants based on the study's inability to observe a direct detection of DEET (Afify, Betz et al. 2019). Conversely, other studies advocate a model where the aversive effect of DEET acts only when reaching an insect's antenna along with other odorants as a "confusant" (Pellegrino, Steinbach et al. 2011, DeGennaro, McBride et al. 2013), or as an antagonist (Ditzen, Pellegrino et al. 2008, Bohbot and Dickens 2010). Still others have shown that insects can directly detect DEET and avoid it without the need for other odorants (Syed and Leal 2008, Xu, Choo et al. 2014). There is evidence that after landing DEET aversion is mediated through a gustatory mechanism (Lee, Kim et al. 2010) via the tarsi (Dennis, Goldman et al. 2019).

RESULTS

Because DEET can act in a variety of ways on the chemosensory system of insects, it is important to understand the sensory detection of and behavior towards DEET in the context of how a mosquito would encounter a topical repellent. DEET is applied onto the human skin surface, which produces a complex blend of stimuli by itself that varies across individuals and comprises both attractive (heat, humidity, blend of several attractive odorants from skin) and aversive cues

(blend of aversive odorants from skin) (Fig 2.1A). We initiated an analysis of *Aedes aegypti* behavioral interactions with DEET using experimental paradigms selected to evaluate repellency in the context of different attractive cues emitted by a human. We tested DEET repellency in female *Aedes aegypti* mosquitoes in the context of human hand and then individual attractive cues emitted by a hand: heat, humidity and skin odor.

First, we tested a human hand as a complex cue to estimate the spatial range of DEET aversion, since it has been reported anecdotally that DEET repels insects from only a close range (Boyle, Guda et al. 2016). In order to measure the approximate distance at which DEET repels mosquitoes, we set up a behavior paradigm using an arm-in-cage assay with a net window through which human skin odor and warmth attracts mosquitoes, and where they can fly, navigate, land and probe as they would on a human arm. Half the net was treated with 10% DEET, which is in the range of concentrations for a number of commercially available formulations, while the other half was treated with acetone solvent (which was allowed to evaporate before the assay) in a manner that prevented DEET spreading across the boundary to the untreated half. The female *A. aegypti* mosquitoes landed and probed the control solvent side readily (Fig 2.1B), but avoided the DEET treated surface. By measuring the distance of the closest mosquitoes from the DEET boundary observed on a Z-stack of 5-minutes of video we found a mean distance of 4.2 mm from the DEET boundary on the treated side (Fig 2.1C). In this population-based assay we are unable to tell whether

mosquitoes have ever made brief contact with tarsi at the DEET boundary, or control the complex skin chemistry that varies from person to person and over time.

In order to evaluate the role of the olfactory system in this close-range behavior, we compared wild type *Aedes aegypti* with *Orco* mutants, that lack responses from all Or receptors and have been shown to lose aversion to DEET (Ditzen, Pellegrino et al. 2008). We used a heat block with a surface at body temperature (~37˚C) as a simple attractant and performed a video analysis of wild type and *orco- A. aegypti* females approaching a DEET-treated net placed above the block. In the wild type controls of the participating mosquitoes, 66.6% avoided the DEET-treated net without touching it, as determined from slow-motion videos (Fig 2.1D, E). The remaining 33.3% made momentary contact with the DEET-treated net with the tarsi before flying away (Fig 2.1E, F). Interestingly, during a second attempt at landing, none of the mosquitoes made contact, avoiding DEET by smell alone (Fig 2.1E). In the *Orco* mutant *A. aegypti* females, the percentage of participating mosquitoes that were repelled without touching the DEET net was 44%, lower than the 66% seen in wild type, with 77% being repelled only after contacting the treatment with their tarsi. During the second approach *Orco-* behavior contrasts with wild type mosquitoes even more, with 71% of the mosquitoes contacting the DEET surface before being repelled by it, as compared to 0% for wild-type (Fig 2.1E). A second observation that we make is that a robust aversion to volatile DEET occurs in the absence of skin odorants, or other attractive odorants.

To determine if DEET avoidance is similar across taxa, we also tested *Drosophila melanogaster* behavior. In a T-maze assay where flies were offered for one minute a choice between tubes with DEET vs control, we did not see an avoidance of the DEET tube of the maze under these conditions (Fig 2.9A). However, when we volatilized the DEET by warming the tube to 80˚C, and tested upon cooling to room temperature, the flies showed aversion to the DEET side (Fig 2.9A). Observations with both these species are consistent with the low volatility of DEET (5.6×10^{-3} mmHg vapor pressure at 20 °C, National Pesticide Information Center), presumably leading to a close-range repellency, which can be bypassed if the DEET is volatilized by warming.

As mosquitoes approach a DEET-treated human skin surface or heat-block they typically encounter a large surface area so that they fly through a wide concentrated plume of the chemical. Based on the behavioral observations, we hypothesized that mosquito and *Drosophila* sensory neurons that detect DEET at this proximity to an emitting surface, are those that likely mediate aversion to volatile DEET. In order to measure the electrophysiological responses of the insect olfactory sensilla to a repellent-relevant aversive direct undiluted plume of stimulus, we placed the outlet of the odor delivery cartridge at ~5 mm distance from the antenna and performed single-sensillum recordings (SSR) from the well-studied ab2 class of sensilla on the antenna of *D. melanogaster* (Fig 2.1G, H). We adjusted the quantity of pure DEET in the delivery cartridge to equal the quantity of WHO recommendations on skin-testing amounts equivalent to ~15%, 30%, and

90% (0.5, 1 and 3 micro L on filter paper, see Methods section), which are similar to concentrations found in commercially available repellents. The traditionally used methods (1 and 2) did not register much activity. Interestingly, this “direct plume” of DEET activated the ab2 class of sensilla in the *Drosophila* antenna strongly, but not when applied by the conventional method through diluting the plume in a carrier airflow as has been previously used from injecting it from a midway point or close by (Fig. 2.1G, H, method configuration 1 and 2). Other recent studies in *Drosophila* have also demonstrated that compounds of low volatility conferred a neural response when applied via a close-range delivery system puffing a direct plume of compound headspace directly onto the antenna, but did not respond much when applied in the conventional manner through a carrier air stream where the odor plume gets mixed and diluted before hitting the antenna (Xu, Choo et al. 2014, Ng, Lin et al. 2017). The mixing of a carrier humidified airflow with the stimulus airflow causes a 66% decrease in concentration of DEET molecules. Due to the small size of a sensilla (~0.015 mm) and antenna (~0.1mm) only a small fraction of the DEET molecules in the plume emitted from the exhaust port is sampled by them. From this perspective, the cross-section area of the exhaust plume depends on the port of the traditional delivery tube port (d=5mm) which is ~250x (2500%) greater than that of the delivery cartridge port (d=1mm) in the direct method, thus further diluting the flux of DEET molecules sampled by the antenna (Fig 2.13). Adding the dilution factor and the diameter difference of the exhaust port, we can estimate the number

of DEET molecules sampled by the antenna for the direct plume method is ~750 times more than the diluted method.

Since the humid air from the carrier tube is useful to keep the insect preparation healthy for multiple recordings; therefore, we tested directing it over the insect and delivering the direct plume DEET exhaust in front of it, pointed directly at the insect head (Fig 2.1G, H method configuration 4, S2A). This method showed a similar activity and therefore was used subsequently. Briefly pre-heating the DEET cartridge, as done previously (Pask, Slone et al. 2017), volatilizes a larger amount of DEET. This stimulus, when delivered thorough the traditional humidified airstream, also elicited significant increases in spike frequency from the ab2 sensilla (Fig 2.1G, method configuration 5). No significant activity was seen with control cartridges in all 5 methods. The simplest interpretations of these results are that DEET can activate some olfactory neurons strongly when delivered as a direct plume, and that lack of activity previously observed in airstream-based delivery methods is likely due to an inadequate quantity of volatile DEET molecules reaching the insect antenna. The modified electrophysiology method can be used to re-evaluate the detection of DEET in a variety of sensory pathways. Potentially the behavioral analyses point to at least 3 different pathways: a large non-contact (*orco* dependent), a smaller non-contact (*orco* independent), and one that is tarsal-mediated and contact-dependent (Fig 2.1I).

In order to fully understand how widespread, the activation by DEET is, and in particular to test whether known aversive ORN classes are so activated, we

conducted a systematic survey of a variety of ORN classes in *Drosophila*. Diagnostic features such as morphology and diagnostic odorant response can be used to identify different sensillar types expressing Odorant Receptor (Or), Ionotropic Receptor (Ir) or Gustatory Receptor (Gr) family proteins. All three types of large basiconic sensilla when tested with the “direct plume” method (ab1, ab2 and ab3) showed a response to DEET (Fig 2.1G, 2.2A). These include the known repellent Or10a+ ab1D ORNs (Mohamed, Retzke et al. 2019). We next tested a representative small basiconic sensillum type, ab4, and found that DEET elicited strong activity from it as well (Fig 2.2A). Both ORN types in this sensillum, the Or7a+ (ab4A) and Or56a+ (ab4B), are known to feed into strongly aversive pathways (Stensmyr, Dweck et al. 2012, Mohamed, Retzke et al. 2019) and in particular Or7a activation through the DL5 glomerulus has been shown to overpower input from attractive ORNs by laterally inhibiting the attractive ORN pathways via the LN interneurons (Stensmyr, Dweck et al. 2012, Mohamed, Retzke et al. 2019). The activation of the aversive ORNs by DEET is not seen when tested in the traditional airstream “mixed plume” method. We also identified a less common neuron class which could be an ab11 or ab12 with a high sensitivity to DEET, such that it showed some responses even in the traditional diluted plume method (Fig 2.2A). The maxillary palps hold six classes of neurons paired in three types of sensilla; in pb1 both neurons responded to DEET while in pb2 and 3 at least one of the neurons was DEET sensitive (Fig 2.2G).

To test whether the widespread activation is only specific to DEET or if it can occur with other odorants tested at comparably high doses, we decided to repeat the “direct plume” recordings using odorants that are known non-activators under standard application paradigms. We tested 0.5 uL of pure methyl salicylate and geranyl acetate on ab3 sensilla, which have been reported to not respond to these compounds in the traditional method (de Bruyne, Foster et al. 2001). Even with the “direct plume” method, these odorants did not cause activation of ORNs in ab3 sensilla (Fig 2.10B). This suggests that the activation is a result of DEET’s action and not a non-specific effect of volatiles in this delivery system. We also ruled out possible contaminations due to storage and handling during preparations when we obtained similar responses (Fig 2.10C) with high purity DEET (99.8%) from a freshly opened bottle. In order to rule out the possibility that the observed neural activation was caused by more volatile contaminants, we flushed the DEET cartridge with a continuous airstream at 5 mL/sec for 10 minutes. If the flushed cartridge could no longer elicit a response in the neuron, then the response could be attributable to a volatile contaminant which was washed away during the flush. However, after flushing, the DEET activation response was still present, in both samples tested using the “direct plume” delivery (Fig 2.10C).

The large basiconic sensilla are present mostly in the dorso-medial zone of the third antennal segment and house ORNs with Ors that are broadly-tuned to numerous odorants (de Bruyne, Foster et al. 2001, Hallem and Carlson 2006). The trichoid sensilla on the other hand, are localized to a more ventro-lateral zone of

the antenna and contain ORNs that express Ors narrowly tuned and highly specialized to detect pheromones (Kurtovic, Widmer et al. 2007, van der Goes van Naters and Carlson 2007). The at1 sensillum, which expresses Or67d and normally detects the male pheromone *cis*-vaccenyl acetate (CVA), is also activated by DEET in the “direct plume” method (Fig 2.2B).

Compared to the at1 sensilla, the at3 sensilla are present more distally along the antenna and house 3 ORNs, which respectively express Or47b, Or88a, and Or65a/b/c. These receptors are also narrowly tuned to pheromones (van der Goes van Naters and Carlson 2007, Dweck, Ebrahim et al. 2015). Interestingly, the activity of the neurons in at3 sensilla were strongly inhibited by DEET at all three quantities tested, confirming that DEET can also act to inhibit some *Or*-expressing ORNS (Fig 2.2B). Thus we have demonstrated that DEET detection can occur via Orco+ ORNs that express members of the Or family, by both direct activation and inhibition of spontaneous activity of different ORNs.

The ab1 sensillum of the large basiconic class is present on the dorso-medial region of the antenna and contains 4 ORNs, one of which, the ab1C, expresses the Gr family members Gr21a and Gr63a and detects CO_2 and a number of other odorants (Jones, Cayirlioglu et al. 2007, Kwon, Dahanukar et al. 2007, MacWilliam, Kowalewski et al. 2018). Because it does not express Ors, ab1C is the only neuron in the ab1 sensillum with activity in *orco-/-* mutant flies. Interestingly, DEET did not cause activation of the ab1C neuron and instead

caused a small but significant decrease in the spike frequency at the highest dose in the “direct plume” method (Fig 2.2C).

In order to test whether the ORNs that express receptors of the Ir family respond to DEET, we performed SSRs from the coeloconic sensilla on the antenna. Unlike the Or and Gr-expressing ORNs, these did not show activation or inhibition of the spontaneous activity (Fig 2.2D). The ac1 ORN responds to humid air, as does another IR-expressing type called ac3 (Yao, Ignell et al. 2005). Interestingly, in both sensillar types DEET in the “direct plume” delivery caused significant inhibition when delivered on top of a humidified airflow (Fig 2.2E, F). Thus, DEET acts as an antagonist of humidity detection, but is not an inverse agonist of the ac1 and ac3 Ir -expressing neurons. Thus, we find that DEET is also detectable, as an antagonist, by these *Ir*+ *Orco*- ORNs.

Since the “direct plume” DEET stimulus demonstrated broad effects that had not been seen previously, the key to finding the repellent-relevant receptors and pathways would be to identify those sensing DEET in this paradigm. In order to test whether Orco is necessary for DEET response in the ORNS in which it is expressed, electroantennogram recordings measuring the receptor potential changes were performed with the “pre-heated” method. The responses to DEET could be seen in wild type flies but were abolished in *orco-/-* flies, which is consistent with the behavioral observations (Fig 2.3A, B). In order to further test whether the Orco co-receptor subunit is necessary for DEET detection, we repeated SSR experiments in *orco* mutants. DEET failed to elicit any action

potentials in these mutants despite recording from multiple sensilla on 6 flies, indicating that Orco is also required for DEET activation in these neurons.

The Or co-receptor Orco has been demonstrated to form a functional homomeric receptor complex that can be activated by VUAA1 (Jones, Pask et al. 2011, Pask, Bobkov et al. 2013, Butterwick, Del Marmol et al. 2018). While there is a strong correlation between Orco+ ORNs and DEET sensitivity, different ORN types express different canonical tuning OrXs. To test whether the tuning OrX subunit is necessary for detection of DEET, we recorded action potentials from the at1 sensilla in *Or67d-/-* mutant flies, and found a lack of action potentials and no response to the "direct plume" DEET or the positive control cVA (Fig 2.3C). The simplest interpretation of this result is that the canonical tuning Or is also required for activation of the DEET response. This result also indicates that Orco is necessary for the Or+ ORNs to be activated by DEET, but is not sufficient to impart a response.

In order to corroborate our findings in mosquitoes, we performed SSRs in the sbtl sensilla of female *A. aegypti* to see if they also respond to DEET in the topical repellent-relevant "direct plume" method. First, we recorded activities from short blunt types of sensilla, which express Or genes, and observed that in every sensillum tested, a "direct plume" stimulus of DEET showed an activation response, while the negative controls and DEET in the traditional "diluted plume" method did not (Fig 2.4A).

Next, we recorded from the maxillary palp where another type of sensilla, the capitate pegs, are found. These contain a Gr-expressing cpA neuron (*Gr1, Gr2, Gr3*) that is normally responsive to CO_2 and a range of general odorants. It also contains two Or-expressing cells, cpB (*AaOr49, Orco*) and cpC (*AaOr8,Orco*) that are tuned to other discrete subsets of general odorants (Lu, Qiu et al. 2007). Interestingly, we find that the cpA neuron in *Aedes* is activated mildly by DEET (Fig 2.4B), whereas in contrast the *D. melanogaster* ab1C neuron expressing its orthologs (*Gr21a/Gr63a*) is unresponsive or mildly inhibited by it (Fig 2.2C). The cpA neuron spikes are lost in the *Gr3-/-* mutant *A. aegypti* indicating that it is required for the response. The mutant also allowed us to visualize and quantify the activities of the smaller amplitude cpB and cpC ORNs (Or-expressing) to activation by DEET (Fig 2.4C), similar to what has been seen in *D. melanogaster.*

Female mosquitoes have thousands of sensilla on their antenna and a receptor-ORN map has not yet been constructed as in *Drosophila*. Thus, it is very difficult to survey them with SSRs systematically to understand detection as we did in *Drosophila*. Based on the numbers of *Or* and *Ir* genes expressed by transcriptome studies (Matthews, McBride et al. 2016), we anticipate nearly a hundred different classes of ORNs with differing odor sensitivities on the antenna, in addition to those ORNs detecting heat and humidity. Ca^{2+} imaging can overcome this limitation to some degree since it can be used to survey multiple cells simultaneously. Using a recently developed Ubi-GCaMP6 *A. aegypti* line, we determined that a number of cells expressing GCaMP6 can be identified on the

antenna of the female mosquitoes (Fig 2.4D) (Bui, Shyong et al. 2018, Bui, Shyong et al. 2019, Lahondère C., Vinauger C. et al. 2019). We used two odorants emitted from human skin that have been previously implicated in host seeking behavior, albeit with opposing valence, nonanal (Syed and Leal 2009) and 6-methyl-5-hepten-2-one (Logan, Stanczyk et al. 2010, McBride, Baier et al. 2014). Nonanal acts as an attractant cue in various mosquito species, and at least some ORNs activated by it can be assumed to convey attraction (Syed and Leal 2009). The 6-methyl-5-hepten-2-one has been shown to be repellent to *Aedes aegypti* mosquitoes across multiple concentrations (Logan, Stanczyk et al. 2010, McBride, Baier et al. 2014), and at least some ORNs that it activates can be assumed to convey aversion. Several fluorescent cell-shaped regions show activity to either one or both of the odorants (Fig 2.8; Fig 2.4D, E). As with the SSR results, we find that a “direct plume” DEET stimulus activates a number of cells including cells that are activated by 6-methyl-5-hepten-2-one or nonanal (Fig 2.8, Fig 2.4E, F). Thus, the widespread activation potentially includes some repellent ORNs as well, as was the case in *Drosophila*. A fraction of cells also showed responses to DEET, but not to either of the two human odorants. A fraction of cells did not respond to any stimulus and showed inhibition (Fig 2.8, Fig 2.4E), which is also consistent with what we observed in *Drosophila*. The two odorants activate a small subset of the cells, with some overlap. The “direct plume” DEET stimulus on the other hand, alters Ca^{2+} responses more broadly than the host odorants, which is in agreement with the single-unit electrophysiological results.

Our findings with *D. melanogaster* and *A. aegypti* appear at first different from observation in a recent study on *A. gambiae* where *Orco-GCaMP6* transgenics found no activity with DEET (Afify, Betz et al. 2019). However, that study relied on a traditional DEET stimulus method of injection into a carrier airflow, similar to our "diluted plume" method which does not show responses. Therefore, in order to investigate whether *Anopheles gambiae* are fundamentally different, we performed the gold standard method of single-sensillum electrophysiology using the updated odor delivery methods. We systematically tested trichoid sensilla on female *A. gambiae* antenna and demonstrated that the "direct plume" method gave robust activation in all but one antennal sensilla tested, which showed inhibition (Fig 2.5A). The two previously used "diluted plume" methods (Syed and Leal 2008, Afify, Betz et al. 2019), at 135 mm and 5mm did not show activity (Fig 2.5B).

In order to identify members of the Or gene family in mosquitoes that detect DEET, either though activation or inhibition, we decided to use the *Drosophila* Or22a-Gal4 knock-in "empty neuron" system, which is considered the gold-standard in deorphanizing insect odorant receptors (Chahda, Soni et al. 2019). As a positive control we drove functional expression of *CquiOr136,* a *C. quinquefasciatus* receptor that has been shown to respond to DEET in cell culture (Xu, Choo et al. 2014). We confirmed that CquiOr136 does indeed respond to DEET in the *Drosophila* decoder system (Fig 2.6A).

However, *CquiOr136* does not have orthologs in the two other major vector species, *A. gambiae* and *A. aegypti*, raising the possibility that other members of

the Or family detect DEET in these species. In order to test this possibility, we turned our attention to *A. gambiae,* where the majority of AgOrs have been deorphanized in a previous version of the transgenic *D. melanogaster* "empty neuron" system, and in *Xenopus* oocytes (Carey, Wang et al. 2010, Wang, Carey et al. 2010). Based on data from these studies, we selected a small number of AgOrs that responded to toluene, which is a substructure of DEET. We tested whether any of these receptors respond to DEET in the "direct plume" application method, using the newly developed *Drosophila* "empty neuron" system. The AgOr9 receptor showed robust activation, whereas AgOr12 showed a weaker response to DEET.

Although CquiOr136, AgOr9 and AgOr12 do not show high amino acid % identity, they respond to DEET, raising the possibility that they may have similar response. The responses of the *A. gambiae* receptors to activating odorants was extracted from the published study (Carey, Wang et al. 2010, Wang, Carey et al. 2010), and the panel of compounds tested on the *CquiOr136* in the "empty neuron" system (Fig 2.6C). The receptor responded moderately to many odorants in the panel, like a broadly-tuned receptor. However, the odorant response profiles of the three receptors were distinct, as would be expected from divergent receptors (Fig 2.6C).

Interestingly, one of the receptors tested, AgOr20, showed an inhibitory response to DEET, silencing the spontaneous activity of the decoder ab3A neuron (Fig 2.7A, left). The neighboring ab3B neuron is activated as usual. The control

ab3A “empty neuron” which lacks *Or22a* and *Or22b* does not show an A neuron spike, or activity to DEET (Fig 2.7A, right). The AgOr20 is also known to respond to a human odorant 1-octen-3-ol which has been implicated as an attractive cue (Carey, Wang et al. 2010, Wang, Carey et al. 2010). In order to test whether DEET can also act as an antagonist, we delivered a 1-sec stimulus of DEET over a longer 3-sec stimulus of 1-octen-3-ol. The 1-octen-3-ol response was significantly inhibited, when compared to overlaid control (Fig 2.7C, D). A similar inhibitory effect is also seen with picaridin, another repellent which is not a toluene, but shares structural similarities to DEET (Fig 2.7C, D). The simplest interpretation is that AgOr20 is a detector of DEET through an antagonistic effect, while AgOr9 and AgOr12 detects it as an agonist.

We also evaluated an *Or* from the DEET-inhibited *Drosophila* at3A neurons, Or47b in the ab3A “empty neuron”. While a mild inhibition was observed to DEET, it did not appear robust or dose-dependent (Fig 2.12A). Expressing the Or47b in the at1 trichoid sensillum in the *Or67d{gal4}* mutant background (Kurtovic, Widmer et al. 2007), showed activation of the neuron (Fig 2.12B). We were therefore unable to conclude anything definitive from these contradictory results, except that it suggests that trichoid sensilla may have differences with basiconics such as different odorant binding proteins (OBPs) that can contribute to responses (McKenna et al., 1994;(Pikielny, Hasan et al. 1994, Hekmat-Scafe, Scafe et al. 2002, Larter, Sun et al. 2016, Sun, Xiao et al. 2018).

Next we wanted to test more broadly the effect of DEET in *IR*-expressing ORNs of mosquitoes since they are known to express many more IRs in the antenna compared to *Drosophila* (Benton, Vannice et al. 2009, Matthews, McBride et al. 2016). The *Ir*-expressing ORNs have not been mapped in the *Aedes* or *Anopheles* antenna, although they are expected to be in grooved peg sensilla, which respond to acids, amines and other IR-like ligands (Brock and Otter 2000, Qiu, van Loon et al. 2006). These sensilla proved fragile and difficult to record activity using SSRs in mosquitoes. However, we can evaluate the response properties of non-Or-expressing ORNs, many of which will presumably express *IRs*, by crossing the *Aedes Ubi-GCaMP6* transgene into the *orco-/-* genetic background where we expect only the non-Or expressing ORNs to be active. In doing so we observed that the Ca^{2+} responses to nonanal and 6-methyl-5-hepten-2-one stimuli were largely absent in these antennae, corroborating the fact that they are detected by members of the Or family (Fig 2.8A, B). We also found a large reduction in total number of cells responding to DEET in each antenna as expected, given its role as an activator of multiple Or-expressing ORNs (Fig 2.8B). Interestingly, a small number of DEET-responsive cells remained in the mutant, suggesting that other cells, presumably those expressing Irs or other uncharacterized receptors, can also be activated by DEET in the *A. aegypti* antenna (Fig 2.8C). Based on the SSR and Ca^{2+} imaging experiments, we conclude that repellent-relevant doses of DEET in the “direct plume” are detected broadly by both widespread activation of cells, and inhibition of fewer cells, across

multiple sensory neuron types in the three species tested *D. melanogaster, A. aegypti* and *A. gambiae.* In these species they appear to activate aversive ORN pathways as well as some attractive pathways. However, there are some differences across species: the CO_2-sensitive cpA GRN in *Aedes* is activated by DEET, but its orthologous CO_2-sensitive ab1C neuron in *Drosophila,* expressing Gr21a and Gr63a, is inhibited.

DEET has long been shown to have a fixative effect by suppressing volatilization of skin-odorants when physically mixed with them as occurs when it is applied on the human skin directly or mixed on the same filter paper (Syed and Leal 2008, Afify, Betz et al. 2019). This suppression is not visible when DEET is physically separated from the odorant (Syed and Leal 2008, Afify, Betz et al. 2019). Afify et al. have proposed that one of the primary modes of action for DEET is due to masking of attractive skin-odorants, since they could not find response to DEET in Orco+ neurons. Since we now find that DEET is in fact directly detected by the insect antenna, we wanted to test whether it causes aversion in paradigms where skin odor is not suppressed. Skin odor was collected on polyester netting placed in a sock covered foot and tested in a 2-choice behavioral paradigm that keeps DEET physically apart from skin odor (Syed and Leal 2008). We tested 5 human volunteers since individuals' skin odors vary in degrees of attractiveness (Logan, Birkett et al. 2008). Wild type *A. aegypti* females avoided the DEET treatment side of skin odor from all 5 individuals demonstrating that strong aversion to DEET occurs even when the attractive skin odorants are not suppressed by the fixative

effect (Fig 2.8D, E, F). However, when *orco*^{16} mutant females were tested, significant avoidance of the DEET-treated side was observed for only 1 of the individuals (P358). Even so, the aversion observed was less than in wildtype, as would be expected if Ors strongly contribute to the overall aversion behavior. For skin odor from one individual (P419), the avoidance to DEET is completely lost in the mutant. For 3 individuals (P937, P510 and P265) the *orco*^{16} mosquitoes showed a low participation rate as measured by attractiveness to the control side and were not analyzed.

We repeated the experiments with the trans-heterozygote *orco*$^{5/16}$ mutant females and retested the odors from two individuals that were most reliably attractive to wild type mosquitoes in this assay: P358 and P937. We found that the *orco* mutant mosquitoes were still able to avoid the DEET side, albeit at a reduced level than wildtype (Fig 2.8F). The simplest interpretation of these results is that olfactory avoidance of DEET in *Aedes* has a significant Or-receptor component, as well as a non-Or component. Taken together, these behavioral results are consistent with the heat-based behavior (Fig 2.1) and congruent with the electrophysiology / imaging activities that show that the Or-pathway is a major contributor to the detection and avoidance of DEET in a non-contact manner. However, we find that differences across individuals' skin odor milieu can lead to different behavioral results with the *orco* mutants, and non-Orco pathways also exist for aversion.

DISCUSSION

The insect olfactory system is extremely sophisticated, with transmembrane receptors from multiple pathways contributing to detection of volatiles in sensory neurons like *Or*, *Gr*, *Ir*, *Trp* channels, as well as expressing other transmembrane receptors, other GPCRs and Ion Channels. More often than not, single compounds are detected combinatorially by multiple receptors. Our electrophysiology and imaging results with DEET are consistent with the view that it is affecting multiple receptor pathways including *Or*, *Gr*, *Irs* and possibly others. Presenting a direct plume of DEET in a repellent relevant context showed direct detection through activation and inhibition which should not be confused with a previously proposed confusant hypothesis that was invoked when DEET, presented traditionally, showed no activity. However, in binary blend with a weak agonist of an ORN, it could marginally enhance or reduce activity (Pellegrino, Steinbach et al. 2011).

A few *Or* family receptors are indicated by *ex vivo* cell-based assays as being either activated or inhibited by DEET in a solution, but none have been tested with a volatile DEET stimulus as in insect olfactory neurons (Ditzen, Pellegrino et al. 2008, Syed and Leal 2008, Xia, Wang et al. 2008, Liu, Pitts et al. 2010, Stanczyk, Brookfield et al. 2010, Pellegrino, Steinbach et al. 2011, Bohbot and Dickens 2012, Xu, Choo et al. 2014). Here we identify AgOr20 from *Anopheles gambiae* that is inhibited by DEET and picaridin in the transgenic *Drosophila* empty neuron system. The inhibition also overcomes the activation of a human odorant

agonist, suggesting a potential role for this type of receptor inhibition in the reduction of attraction.

Our results also suggest a novel principle, that the most sensitive receptors and neurons that detect DEET at low concentrations, and active at >4-5mm away are less likely to contribute to repellency. It is more likely that lower affinity receptors/neurons activated by a high dose of DEET seen only upon the close approach of insects is required for widespread activity that causes aversion (Fig 2.7G). The model emerging from our experiments suggests that when an insect is at a distance its neurons/receptors sensing human-odor, warmth and humidity are active and cause attraction. At these distances, sensitive neurons/receptors that detect DEET, if they exist, likely do not contribute substantially to aversion as seen in our experiments. Only upon close approach to ~4-5 mm does the insect encounter sufficient quantities of DEET to cause the direct, widespread, and multimodal effects on numerous sensory neurons. And it is within these subsets of low-affinity effects that we should search for the mechanisms of aversion.

In taking a topical repellent-relevant stimulus alongside a reductionist approach to understanding the role of different sensing pathways, we employed several assays using heat, humidity as a non-olfactory attraction cues and simple resting behavior as a proxy for no cue assays. Our results unambiguously demonstrate that both *Or* and non-*Or* receptors play a role in non-contact repellency to DEET. Our model suggests several receptor pathways *Ors*, *Irs, Grs* or others yet to be uncovered, contribute to repellency, and this widespread effect

is what leads to its conservation across arthropods as a repellent. Further studies shifting the focus to low affinity receptors in mosquitoes will be required to completely describe the underlying molecular mechanisms of DEET repellency and to find new targets for identifying safer and more effective repellents. The repellency is multimodal, but a majority of mosquitoes avoid DEET prior to contact by acting on olfactory, heat, and humidity sensing in our analysis (Fig 2.7H). Our findings are also consistent with previous studies which have also shown involvement of the taste modality in aversion (Lee, Kim et al. 2010). Our identification of multimodal effects across multiple receptor classes might explain why some arthropods are repelled by DEET, such as ticks (Ogawa, Komagata et al. 2016), which lack the *Or* family in the genome.

MATERIALS AND METHODS

Drosophila stocks were maintained on standard media at 25C.

Electrophysiology

For *Drosophila*, adult male flies were tested at 3-7 days post-eclosion and inserted into a 1.0 mL pipette tip and secured with molding clay. A glass cover slip was placed underneath the head and a glass pipette was used as a rod to secure an antenna in place. A reference electrode filled with SLR solution was inserted into the eye. For *Aedes*, adult females were used at 2-4 days post-eclosion, before the cuticle became difficult to penetrate, and the preparation was otherwise similar.

All odorant compounds other than water and DEET were dissolved in paraffin oil, with 10 µL added to each cartridge except where noted. A 3 X 35 mm filter paper strip was placed in each 5¾" glass Pasteur pipette to form odor cartridges. Cartridges were allowed to sit for 15 minutes before use, each cartridge was used no more than twice before being discarded. A glass tube was used to maintain a humidified airstream of 10 mL/sec on the preparation, provided by a gas tank routed through a water-filled beaker. For "diluted" plume, the odor cartridge was inserted into the side of a glass tube through an opening at indicated distance from the end of the tube. The end of the glass tube was placed 5 mm away from the prep, for a total of 135 mm separating the preparation from the point in which odorant entered the airstream. For "direct" stimulus the Pasteur pipette tip was and pointed directly at the insect head placed ~5mm away. The comparison of stimulus exhaust sizes is drawn to scale in Fig 2.13.

A puff of air was released through the odor cartridge with a Syntech device for 1 second. Spikes were counted during the 1 s application window in the case of 5 mm application and for 0.25 s to 1.25 s after the start of the application window in the case of 135 mm application, owing to a slight delay caused by the increased distance to the antennae. The baseline activity from one second before the stimulus onset was subtracted from the total activity to obtain a net increase in spikes.

The quantity of pure DEET in the delivery cartridge was adjusted to the quantity recommended by WHO in the "Guidelines for efficacy testing of mosquito

repellents for human skin recommendations" (https://apps.who.int/iris/bitstream/handle/10665/70072/WHO_HTM_NTD_WHOPES_2009.4_eng.pdf) for DEET testing (1mL of 20% DEET in ethanol/~600 cm^2 skin = 200µl pure DEET/~600 cm^2 skin). The solvent evaporates depositing the DEET on the surface before testing the arm. We assumed that the approximate surface area of DEET-treated net encountered by an approaching mosquito causing repellency for 90% mosquitoes based on Figure 1K as a circle of radius 0.8cm, and therefore an area of $2cm^2$. Based on this we adjust the electrophysiology testing DEET cartridge volume 0.5µl=15%, 1µl=30%, and 3 µl =90% to approximate on-skin testing amount % equivalents.

Statistics were done with two-tailed unpaired Mann-Whitney tests, except figures 2.2D, E, and F in which paired T-tests were used to compare firing rates of stimuli and baseline for each trial, and 2.1N, 2.9B, 2.10, and 2.11, in which ordinary one-way ANOVAs were performed. Tukey's honest significance test was used post hoc when significance was found in ANOVAs.

For co-application assays, a second Syntech CS-55 stimulator was mounted above the first, each connected to an odor cartridge. In order to reduce variability, the Syntechs were routed through same channel on the controller, such that both could be activated by the same foot pedal. For Figures 1.6D-E, the 1-octene-3-ol stimulus was applied for three seconds, while the 1-second DEET stimulus was programmed to commence after a 1-second delay. Thus, the DEET stimulus began 1 second after and ended 1 second before the 1-octene-3-ol

stimulus. To account for natural response decay, the same 1-second block was counted for both the DEET and blank cartridge controls and counts compared.

Chemicals

Chemicals were obtained from Sigma-Aldrich and were >99% pure, except where indicated otherwise. Chiral compounds were racemic unless otherwise indicated.

Drosophila T-maze

Experiments were performed as before with a few modifications. For each test, 40 *D. melanogaster* that were starved for 24 hrs, but provided with a moist tissue, were used in a 1:1 male:female ratio. For the pre-heated test, both the odor and control tubes were capped and warmed on an 80^{o}C hot plate for ~10 mins and then allowed to return to room temperature by waiting and measuring temperature using an Infra-red thermometer. Assays lasted 1 min and were performed in the dark. The treatment sides were alternated. The avoidance response was calculated as a Preference Index (PI) = (number of flies in test arm - number of flies in control arm)/(total number of flies in assay).

Mosquito rearing and colony maintenance

A. gambiae wild-type*, A. aegypti* wild-type (Orlando strain), *orco*^{16} and *orco*$^{5/16}$ mutant strains (generated from *orco*^{5}*, orco*^{16}) were used in behavior experiments. Colonies were maintained in an insectary at ~27^{o}C, 70-80% humidity on a 14:10 hr (Light: Dark) photoperiod. Lights on at 0700hrs. Adults were held in the insectary in 30 cm cube Bugdorm cages and given access to 10% sucrose

solution. Females were offered blood using artificial blood feeding system and allowed to lay eggs. Upon hatching, larvae were thinned into 6qt size (5.7L) plastic containers filled with about 900mL deionized water and fed on Tetramin® fish food tablets until pupation.

Mosquito behavior

Experiments for DEET repellency with *Aedes aegypti* were conducted between 1400-1700hrs at 27±1^{o}C and 50-55% relative humidity under dim fluorescent lighting.

Single-mosquito repellency assays

Two female *Aedes aegypti* mosquitoes are introduced into a cage with an attractive heat stimulus provided by a VWR benchtop digital heating block (9.5 x 7.5 cm) held constant at 40C, placed directly below the cage. A landing pad assembly was placed on cage floor, directly above heating block to allow the landing surface to reach 37C. A piece of polyester netting (14 x 9.5cm) was treated with 500ul of test solution either solvent acetone alone or 10% DEET and allowed to dry in the fume hood for 30 min to evaporate solvent. The net was secured on plastic platform (9.5 x 7.5 x .9 cm) with scotch tape to create a uniform treated landing surface (9.5 x 7.5 cm). Assembled pads were stored in airtight containers for no more than two hours. Pads were placed into the trial cages 5 min prior to testing to allow temperature stabilization.

The experiments were performed blind to genotype of mosquitoes, either wild-type (control Orlando strain) or *orco*^{16}. Behavior was recorded by the

observer, and recorded by two video recorders for 6 min after mosquito introduction. CO_2 stimulus was provided by aspirator for 2 seconds, 2 minutes after mosquito introduction. A higher-resolution video focusing on the pad landing area was recorded with a Samsung Galaxy 10SE in slow motion setting. A second recorder (Go-Pro) was used to record the entire cage area to keep track of mosquito ID. Mosquito participation was defined as making a clear directed change in flight path towards the pad assembly; landing was defined as any tarsi touching the treated net surface. Participation and landing events were noted by observer and verified by blind video analysis.

Slow motion videos were slowed 3x. From these videos, frames were extracted at 10 frames/second using FFMPEG. Images had resolutions adjusted to 300X169 image using bulk resize photos. The images made up of stacks were then imported into FIJI and analyzed using the Mosaic 2D/3D particle tracker plugin to create traces of flight paths.

Humidity behavior assay

In the humidity-attraction repellency assay, mosquito responses in presence of DEET or solvent were tested under 'humid stimulus only' (with moist filter paper) and 'no humid stimulus' (with dry filter paper) conditions in 20x12x10 inch aquarium cages. Female mosquitoes (10) were held in cylindrical acrylic cages (7cm diameter x 5 cm high) covered with insect screen mesh on one end and sealed with clean manila paper on the open end. The cages were exposed to 500 uL of either 10% DEET-treated or acetone (solvent)-treated net held 5mm

above the screened top end of the acrylic cage and allowed to air dry for 30 minutes. This 5mm gap ensured no contact between the mosquitoes and the treated net. A 55mm diameter filter paper disc (Whatman #1) moistened with 400µL of distilled water held directly above the net provided humidity. Mosquito behavior around the top screen of the acrylic cage was recorded by video. The number probing or sitting on the top screen mesh for 5-sec or more for the 5-minute duration of the assay was counted at every 30-sec interval.

Arm-in-cage assays

Behavior assays were done with 40 mated, non-blood fed *Aedes aegypti* females of ages between 5 and 10 days in 30 cm cube Bugdorm cages fitted with 31cm x 31cm x 2.5mm clear glass tops. Female mosquitoes were sugar-starved for 24 hours prior to experiments but were provided with distilled water.

Non-contact arm-in-cage repellency assays were performed as described in the literature (Boyle, McInally et al. 2016). To treatment nets, 500uL of either 10% DEET or solvent (acetone) was added and the nets allowed to air dry for 30 minutes to evaporate solvent. A set of five 1.5mm thick magnetic window frames cut from flexible magnet strips (McMaster-carr #477K24) were used to secure the treated net over the glove window and a second untreated netting 6mm above the treated net. A hand was inserted into the glove and placed inside the test cage containing 40 female mosquitoes for 5 minutes. Each cage of test mosquitoes was only used for one trial.

Modified contact arm-in-cage half net spatial repellency

To observe the specific spatial range of DEET repellency, mosquitoes were presented with half DEET–half solvent treated net on a window cut out on a Sol-vex nitrile glove. One piece of test net was cut into equal sized halves One half was treated with 250uL 10% DEET while the other half was treated with 250 uL acetone. The two pieces were air dried and then set side by side to cover the glove window. A hand was inserted in the glove and then placed inside test cage containing 40 female mosquitoes for 5 minutes. Mosquito landing activity behavior and recorded on HD video. Videos of landing activity on the glove window were converted to multiple single frame images at 0.2 fps and all frames for the duration of the assay were stacked into a single using ImageJ software. The stacked images showed how close mosquito landings to the edge of the DEET-treated net were. We then used the images to measure the distance the mosquitoes closest to the edge of DEET-treated net.

Skin odor attraction repellency

Informed consent was sought from adult volunteers to participate in this experiment following approval by the Institutional Review Board (IRB). Skin odor collected from 5 of the enrolled individuals P265, P358, P419, P510 and P937 was used in testing *Aedes* skin odor attraction-DEET repellency. For each assay run, the volunteers were provided with a pair of polyester nettings, one for each foot and clean pair of nylon socks and gloves for handling the nets. Participants were instructed to wear the nets under each sole and put the nylon socks provided over their feet on the morning of each experiment day, after bath with fragrance-free or

odorless soap also provided. They were also requested to refrain from using cosmetics on feet on odor collection and experimentation day.

After six hours, the (skin odor) nets were retrieved from the volunteers and used to assemble a DEET-Skin odor and Control-Skin odor stimulus for a two-choice assays in this manner: first, the skin odor net was sandwiched between two flexible magnets with cut out window frames followed by three spacer magnet frames. Next, either a 10% DEET net (TEST) or solvent treated net (CONTROL) was air dried for 30 minutes and added, followed by another three spacer magnet frames. This ensured that the skin odor net and the DEET nets were never in contact. The whole assembly was then secured by an extra magnet placed from inside on either opposite sides of the cage containing test mosquitoes. All materials were handled with gloved hands. In this set up, mosquitoes responding to either side with DEET plus skin odor or solvent plus skin odor across the wall were not able to make contact either of the treatment nets. The position of each stimulus was switched to opposite side in subsequent runs and no same cage was tested twice. Mosquito behavior and choice between the test and control in the cage was recorded for 5 minutes by a pair of webcams recording on either side of the cage. Data was analyzed using ImageJ software by creating z-stacks and automated counting of image captures frame by frame.

Calcium Imaging

Mated female *A. aegypti* between 3-8 days age with the homozygous Ubi-GCaMP6 transgene were prepared for imaging on a glass slide. Imaging was

performed on an Olympus BX51WI fluorescence microscope using a 20X LMPLANFL N air objective with a Hamamatsu Orca-flash 4.0 camera and Metamorph software. Analysis was performed offline using ImageJ/FIJI software. A custom Macro was used to assist in the analysis which included image stabilization (if needed), partitioning the antenna into grids for cell numbering by region, and background control stimulus subtraction before calculating the delta F values. The controlled odor stimulus was delivered using a Syntech CS-55 unit from odor cartridges put together from disposable glass Pasteur pipettes containing a piece of filter paper, as is used in single-unit electrophysiology recordings. The placement of the cartridge was similar to that of the SSR recordings at direct plume distance from the preparation.

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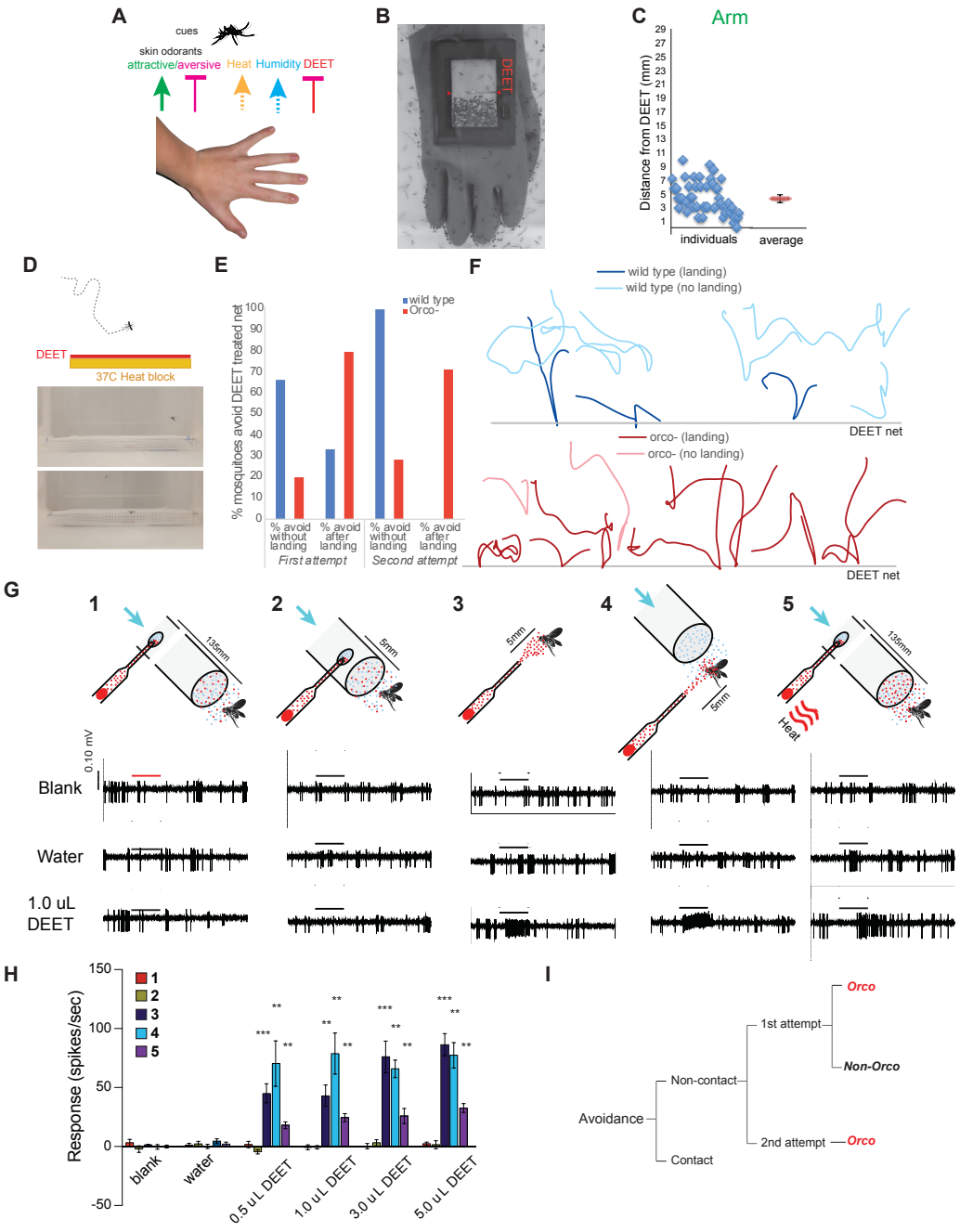

Figure 2.1. DEET acts at close range behaviorally and physiologically.

Figure 2.1. DEET acts at close range behaviorally and physiologically.

(A) Schematic of the various attractive and aversive cues emitted from a human arm. The skin odorant blend is complex, numbering in the hundreds, and behaviorally active components are divided into 2 classes: attractive and aversive. **(B)** Representative Z-stack of a 5-minute assay using a modified Arm-in cage assay to measure spatial zone of repellency to 10% DEET applied on half the net showing that most female *Aedes aegypti* land only on the control side and approach the treated side closely. **(C)** Distance from the DEET treated net edge to the closest mosquitoes across the length of the boundary. (**D)** Representative images of mosquito landing assays on a 37C net covered heat pad treated with 10% DEET showing an example in each genotype of a mosquito that avoided treated pad without landing and another that landed before avoidance. **(E)** Percentage of female *A. aegypti* of wild-type and $orco^{16}$ genotypes that avoided the DEET treated pad, without landing or after landing, in their first attempt and second attempt. N=9 and 10. **(F)** Flight tracks of individual mosquitoes around the time period of assay that they landed and did not land after approach. **(G)** A schematic of the different odor delivery systems in single-sensillum electrophysiology: 1: diluted plume, 2: diluted plume (5mm), 3: direct plume (without humid air), 4: direct plume, 5: heated diluted plume. Shown underneath are representative traces of ab2 DEET response in *Drosophila*, and **(H)** the average response in spike frequency of ab2 sensillar neurons in response to pure DEET at 3 doses in these methods (*$p< 0.05$, **$p< 0.01$; n = 6-12 sensilla, one fly

per sensillum). **(I)** Schematic showing the various behavioral components of repellency of DEET based on the heat landing experiments.

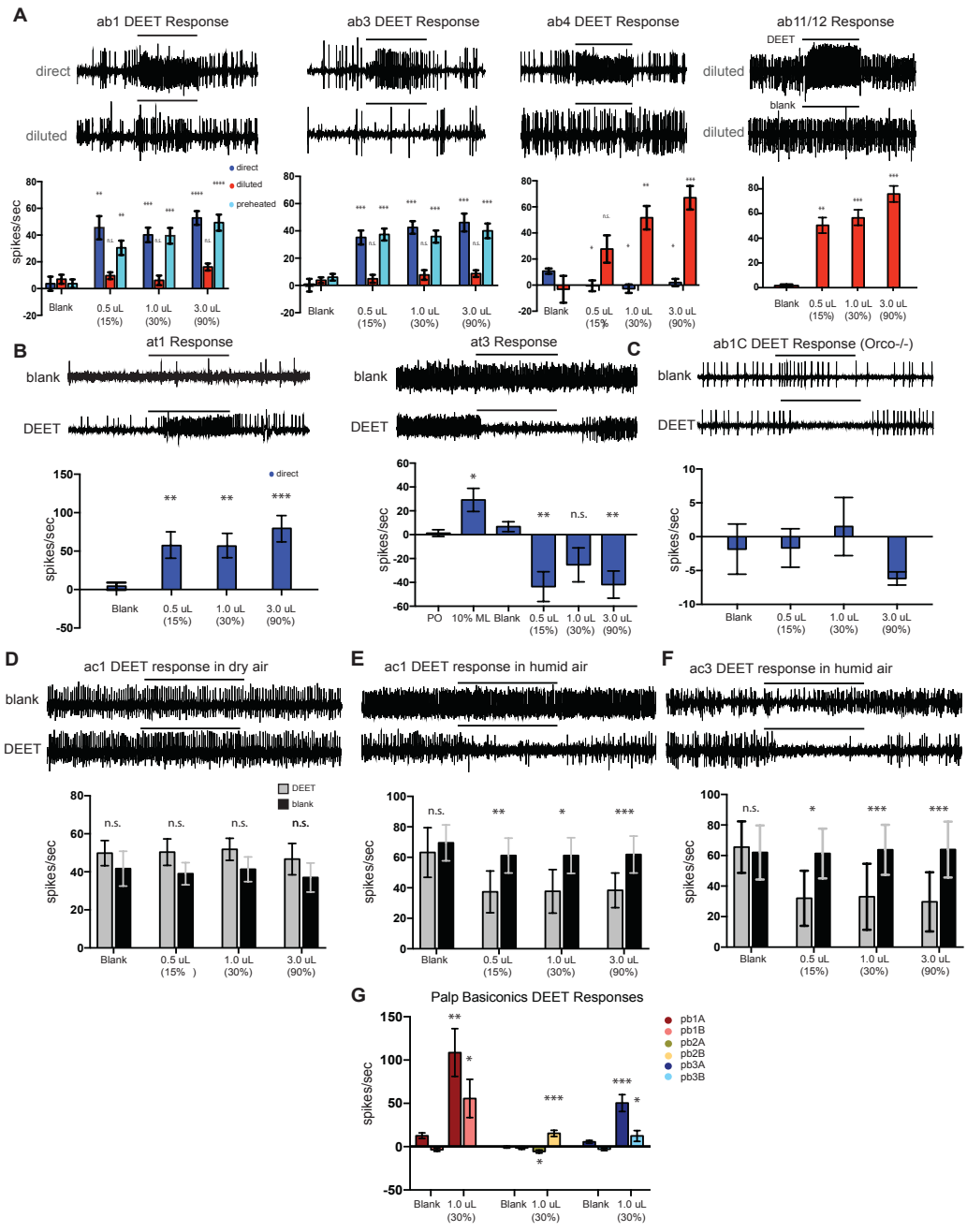

Figure 2.2. DEET is a broad activator or inhibitor of ORNs, GRN and IRNs in *Drosophila*.

Figure 2.2. DEET is a broad activator or inhibitor of ORNs, GRN and IRNs in *Drosophila*.

(A) Representative traces and averaged summed spike frequency of general odor-sensing, Or-expressing neurons in ab1 (left), ab3, ab4 and ab11/12(?) (right) respond to DEET using the indicated methods (error bars = s.e.m., *p< 0.05, **p< 0.01; n = 4-7). **(B)** Representative traces and averaged summed spike frequency of pheromone-sensing at1 (left) and at3 (right) sensillar neurons in response to DEET (*p< 0.05; n = 7-12). **(C)** Representative traces and averaged summed spike frequency of CO_2-sensing ab1C neuron in response to DEET and controls in 5mm odor delivery methods in *Orco-/-* flies (*p< 0.05; n = 6). **(D)** Representative traces and averaged summed spike frequency of ac1 sensillar neurons in response to DEET when tested in dry room air or **(E)** response in humidified air for an ac1, or **(F)** ac3 sensilla (*p< 0.05, **p<0.01, ***p<0.001; n = 6). **(G)** Averaged summed spike frequency of general odor-sensing, Or-expressing neurons in the maxillary palp sensilla response to DEET using the heated plume method (error bars = s.e.m., *p< 0.05, **p< 0.01, ***p<0.001; n = 8).

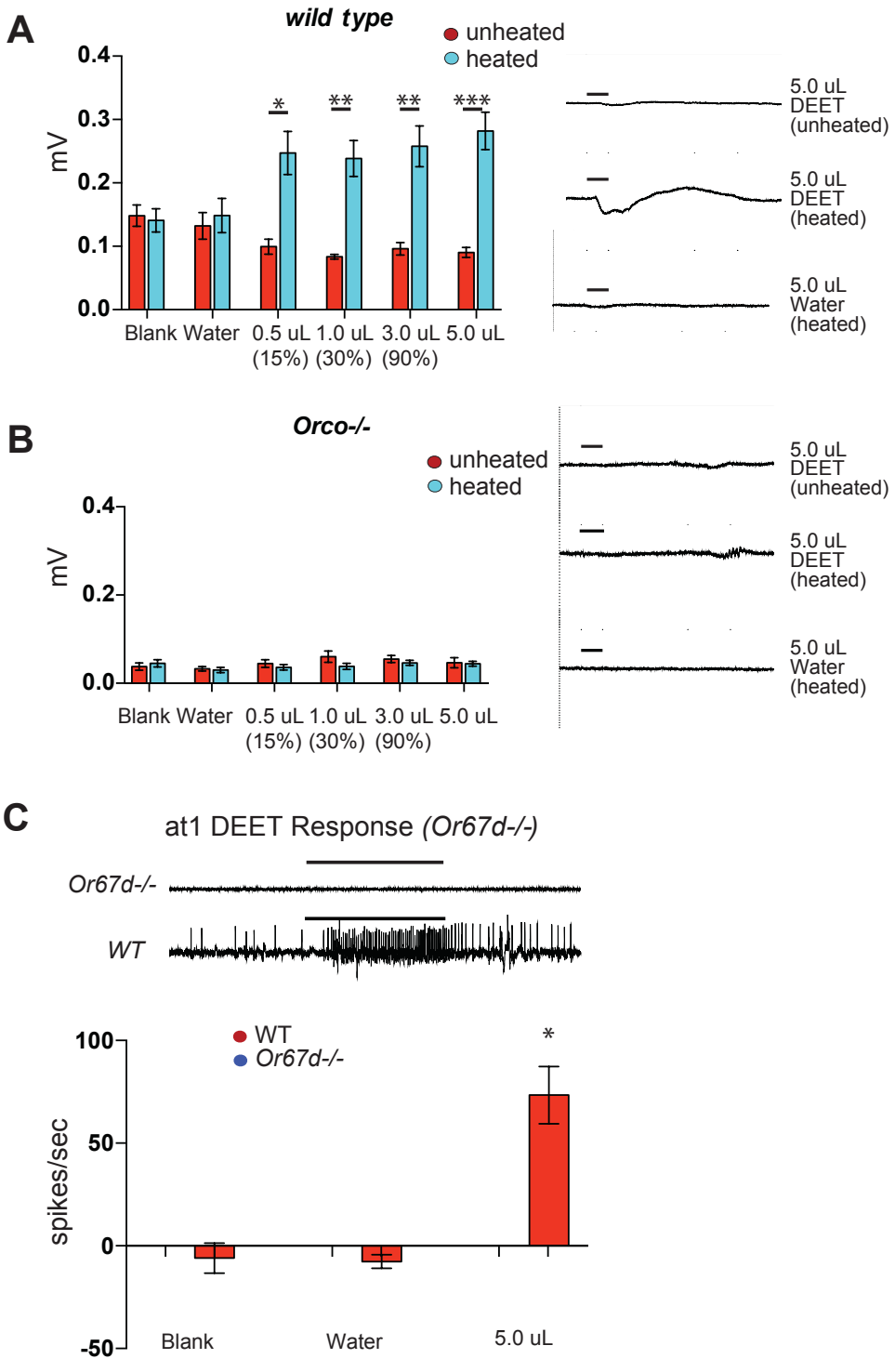

Figure 2.3. Odorant receptors required for DEET response.

Figure 2.3. Odorant receptors required for DEET response.

(A) DEET elicits an electroantennogram response that is abolished in the *orco-/-* mutant Electroantennogram of wild type flies to unheated (red) and heated (light blue) DEET. Representative traces of unheated (top) and heated (middle) DEET, as well as to heated water control (bottom), are to the right of the graph. N = 7-11. *p< 0.05, **p<0.01, ***p<0.001. Error bars represent standard error of the mean. **(B)** Electroantennogram of *orco-/-* flies to unheated (blue) and heated (magenta) DEET. Bars represent the mean peak response of the deflection. Representative traces of unheated (top) and heated (middle) DEET, as well as to heated water control (bottom), are to the right of the graph. N = 6-7 for all traces. *p< 0.05, **p<0.01, ***p<0.001. Error bars represent standard error of the mean. **(C)** A comparison of the at1 wildtype response with that of the *Or67d* mutant flies to various odors, including DEET. Responses are abolished in the mutant, as well as baseline activity (*p< 0.05, **p< 0.01; n = 3-15).

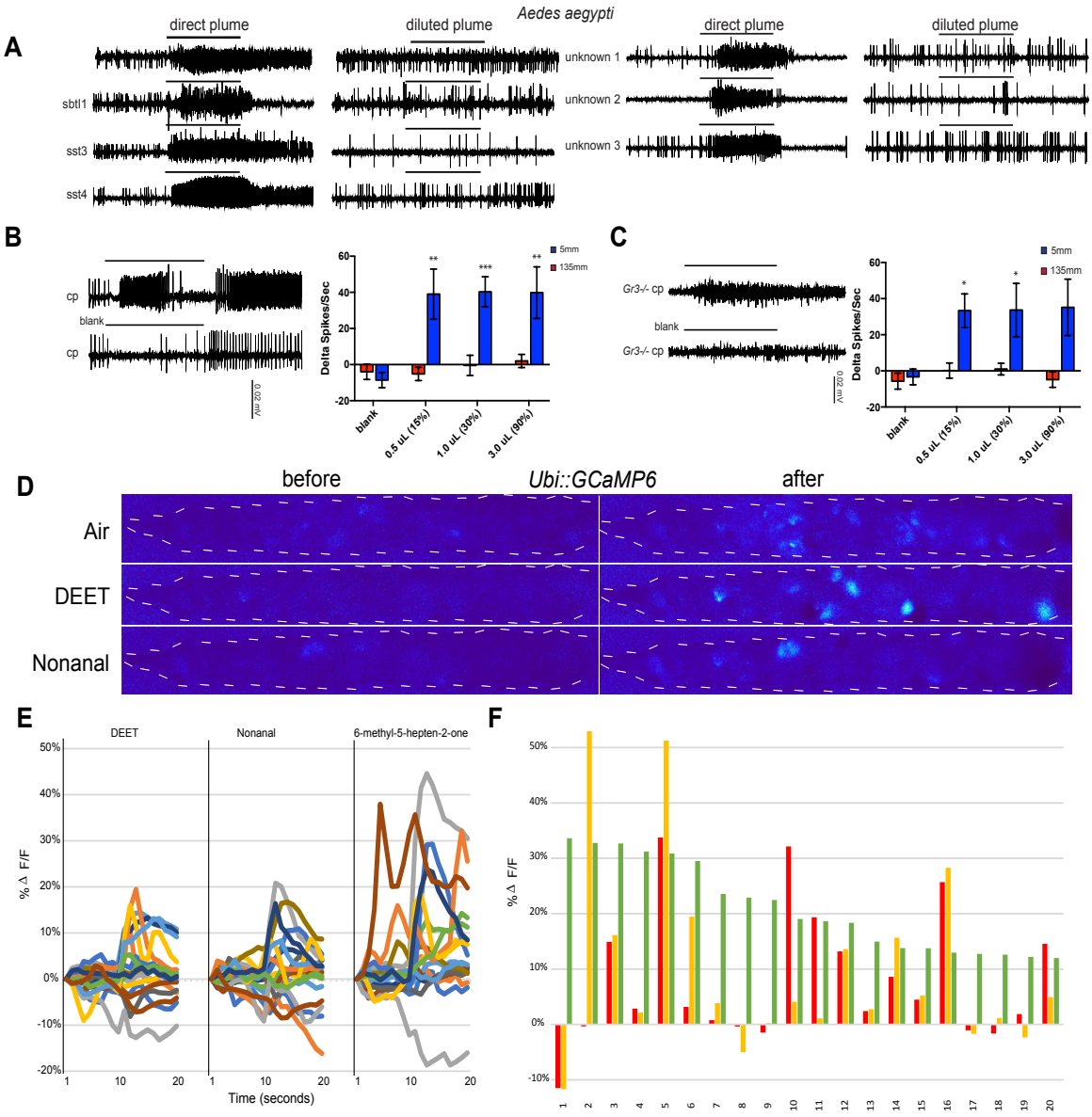

Figure 2.4. Olfactory neurons in *Aedes* detect DEET.

Figure 2.4. Olfactory neurons in *Aedes* detect DEET.

(A) Traces of response to DEET (15% dose) in single-sensillum recordings from sensilla on the surface of female *Aedes aegypti* antenna using the “direct plume” or “mixed plume” delivery systems. **(B)** Representative traces and mean response of the female *Aedes* maxillary palp capitate peg sensillum (cp) A neuron with larger spike amplitude to DEET in the 2 delivery methods. (**p< 0.01; ***p<0.001; n = 6-7). **(C)** Representative traces and mean response of female *Gr3*- mutant *Aedes* maxillary palp capitate peg sensillum B and C neurons to DEET in the 2 delivery methods. In the *Gr3*- mutant the large spike of the A neuron is absent and the smaller B and C neuron can be visualized and quantified. (*p< 0.05, **p< 0.01; n = 6). **(D)** Fluorescent micrographs of a representative antenna from transgenic *Aedes aegypti* carrying *Ubi:GCamp6* showing before and after the 3 indicated stimuli: air, DEET (10%) and nonanal (1%). **(E)** Temporal kinetics of the calcium response for individual cells from a single antenna. Each cell is identified from fluorescence intensity change to the stimuli indicated. **(F)** The quantification of activity of the top 20 DEET responsive cells (out of N=178 cells, 10 antennae) shown as the change for each cell after subtracting the blank control. N=20.

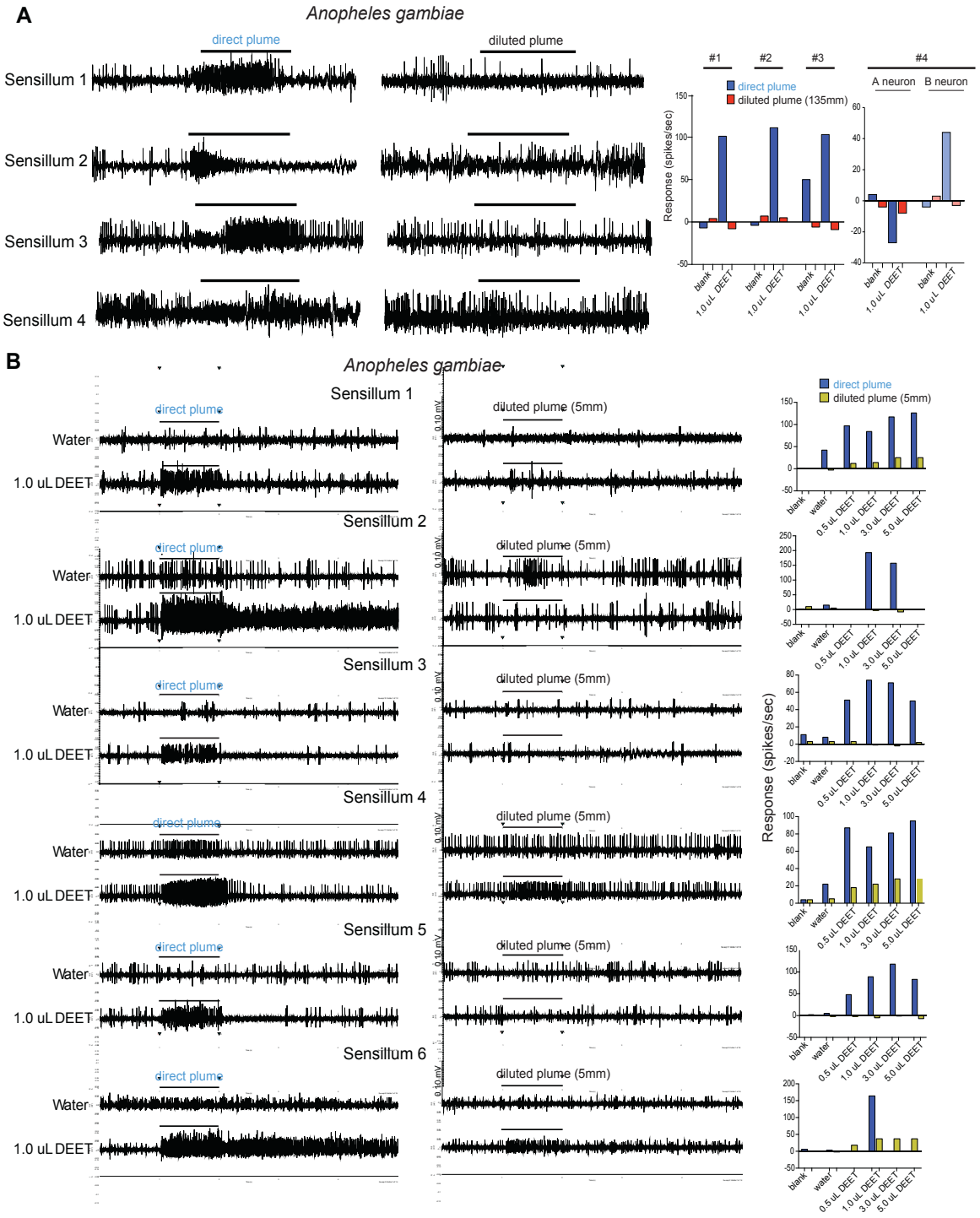

Figure 2.5. "Direct Plume" DEET-induced activity is conserved in *Anopheles*.

Figure 2.5. "Direct Plume" DEET-induced activity is conserved in *Anopheles.* **(A)** Traces of four female *Anopheles* trichoid sensilla responding to DEET applied in "direct plume" and "diluted (ie: traditional) plume" methods. DEET in the "direct plume method increased neuronal activity in three of the four sensillum and inhibited it in the other. N=1 for each of the four sensilla. **(B)** Traces of six female *Anopheles* trichoid sensilla responding to DEET applied in "direct plume" and a diluted plume where the odor cartridge is inserted into a hole located 5 mm away from the end of the glass tube carrying the humidified airstream. All six sensilla are located on the 11^{th} antennal segment. N = 1 for each of the six sensilla.

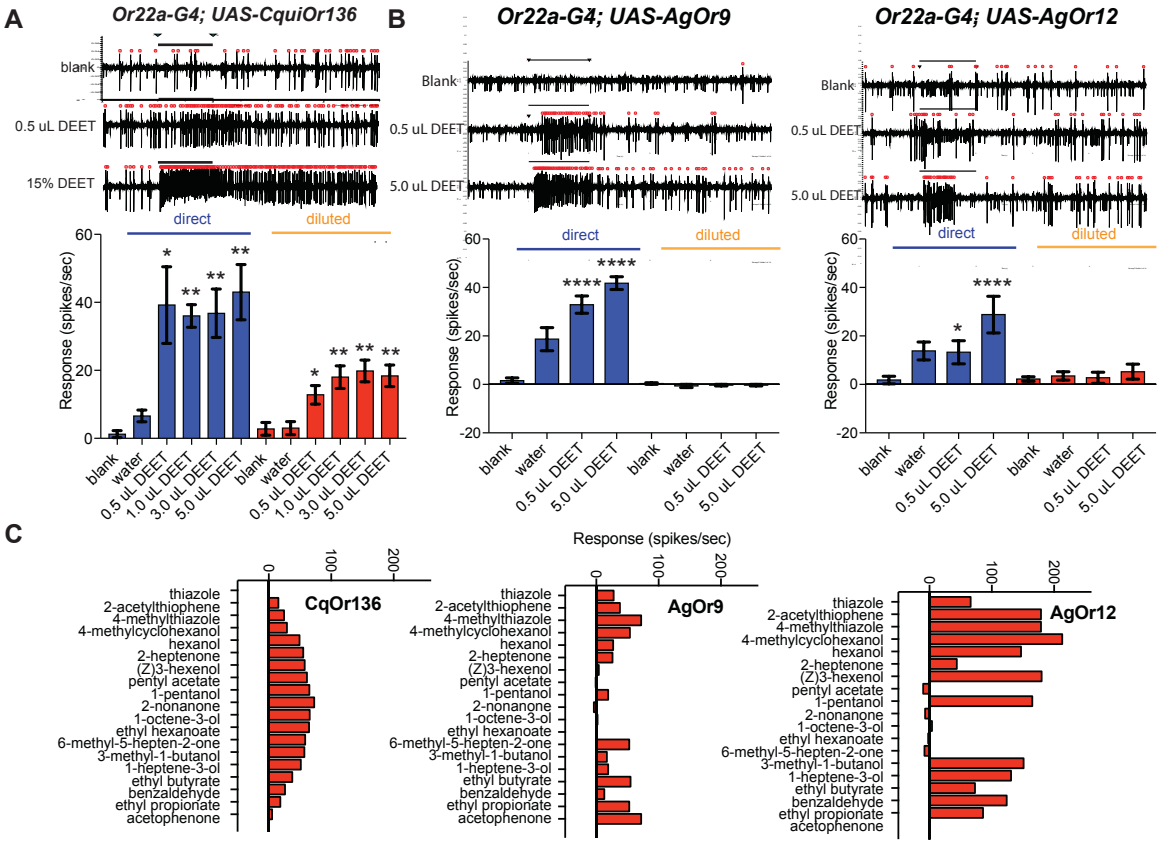

Figure 2.6. Identification of *Anopheles* odorant receptors that detect DEET.

Figure 2.6. Identification of *Anopheles* odorant receptors that detect DEET.

(A) Representative traces and mean responses of the ab3A neuron in *UAS-CquiOr136/TM3; Or22a*$^{<Gal4>}$ flies to indicated stimuli (error bars = s.e.m., *p< 0.05, **p< 0.01; N = 5). Red dots above traces indicate ab3A neuron spikes. **(B)** Representative traces and mean response in spike frequency of the ab3A neuron in transgenic lines *Or22a-G4; UAS-AgOr9* (left), and *Or22a-G4; UAS-AgOr12* (right), to indicated odorants (1% on filter paper), or DEET applied via the indicated methods (error bars = s.e.m., *p< 0.05, **p< 0.01, ***p<0.001, ****p<.0001; N = 3-14). Red dots above traces indicate ab3A neuron spikes. **(C)** Odor response profiles of the CquiOr136, AgOr9, and AgOr12 receptors to 19 odorants. The graphs are arranged based on the CquiOr136 receptor, so that the strongest stimuli are placed in the center while weaker stimuli are the sides.

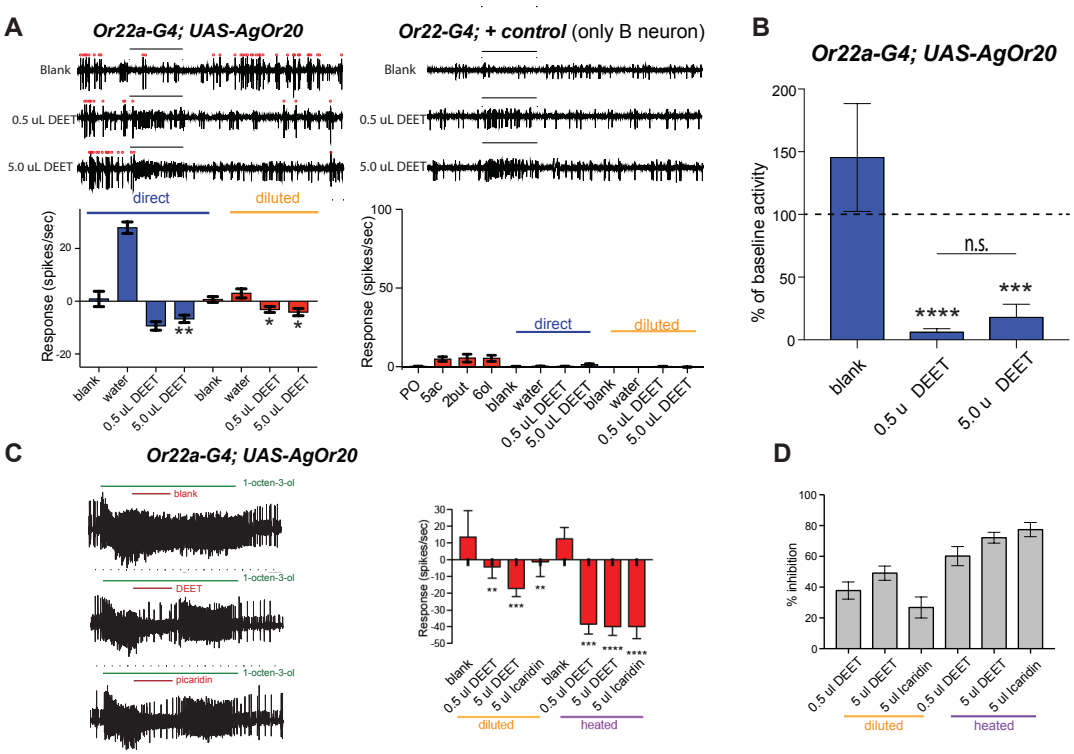

Figure 2.7. AgOr20 is inhibited by DEET in the empty neuron system

Figure 2.7. AgOr20 is inhibited by DEET in the empty neuron system

(A) Representative traces and mean response in spike frequency of the ab3A neuron in the *Or22a-G4; UAS-AgOr20 (left) and Or22a-G4; +/+* control line (right) (error bars = s.e.m., N = 8). The A neuron has been eliminated in the control line, leaving the B neuron to account for all spikes shown. **(B)** Responses of the ab3A neuron in transgenic line *Or22a-G4; UAS-AgOr20* as a percentage of baseline activity measured in the 1-sec prior to stimulus. (error bars = s.e.m.; ***p<0.001, ****p<0.0001; N = 9-12). **(C)** Representative traces and averaged summed spike frequency of the ab3 sensillum in the transgenic line *Or22a-G4; UAS-AgOr20* to a co-application of a 3-second 1-octene-3-ol (1% on filter paper) activating odor stimulus over-layed with a 1-sec stimulus of (pre-heated) DEET or icaridin. (error bars = s.e.m.; **p< 0.01, ***p<0.001, ****p<.0001; N = 10). **(D)** Percent inhibition of the the ab3A neuron in transgenic line *Or22a-G4; UAS-AgOr20* to (pre-heated) DEET or icaridin compared to the responses to the (heated) blank control cartridge co-application. (error bars = s.e.m.; N = 10).

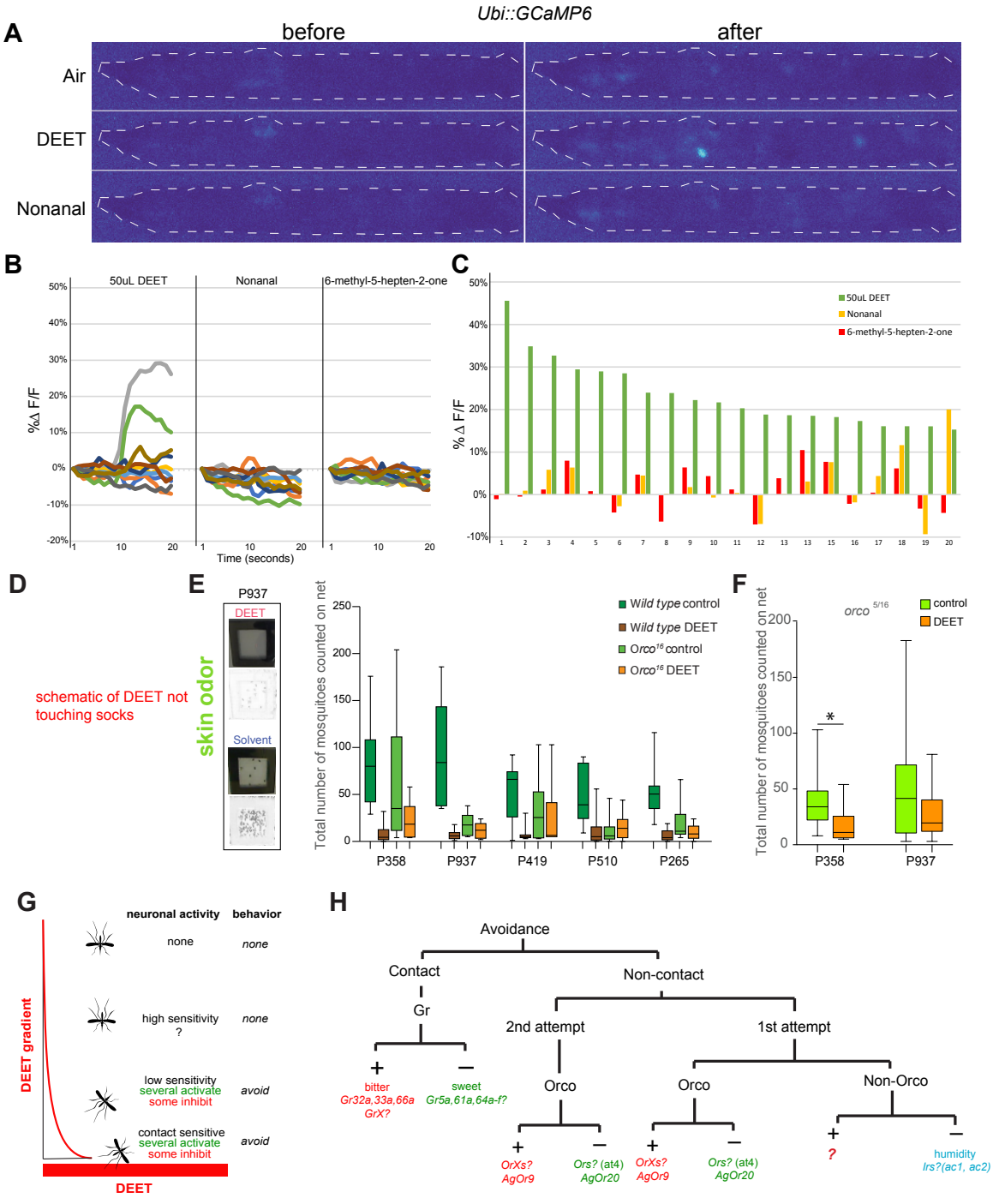

Figure 2.8. *Orco* mutant *Aedes* show greatly reduced detection and aversion to DEET.

Figure 2.8. *Orco* mutant *Aedes* show greatly reduced detection and aversion to DEET.

(A) Fluorescent micrographs of a representative antenna from *Orco*^{16} mutant *Aedes aegypti* carrying the *Ubi:GCamp6* transgene showing before and after images of the 3 indicated stimuli: air, DEET (10%) and nonanal (1%). **(B)** Temporal kinetics of the Ca response for individual cells from a single antenna. Each cell is identified from fluorescence intensity change to the stimuli. **(C)** The quantification of activity of the top 20 DEET responsive cells (out of N=174 cells, from 14 antennae) shown as the change for each cell after subtracting the blank control. (p=n.s., N=20 trials. ~40 mosquitoes/trial). **(D)** Schematic of 2-choice assay set up with human odor and DEET. **(E)** Representative photographs of the 2-choice non-contact skin odor assay to test DEET avoidance, and mean counts of numbers of female *A. aegypti* of *orco*^{16}, or **(F)** *orco*$^{5/16}$ present over each assay area. (N= 12 trials for person, ~20 mosquitoes/trial, * p=0.05). **(G)** Model of aversion to DEET for an approaching mosquito indicating observations of neuronal activity and observed behavior. The vertical y-axis is distance from surface and the horizontal x-axis graph indicates amount of volatile DEET. **(H)** Schematic of DEET detection pathways, indicating molecular receptors where known. Red font indicate known or proposed aversive pathways, and green and blue font indicates proposed attractive pathways.

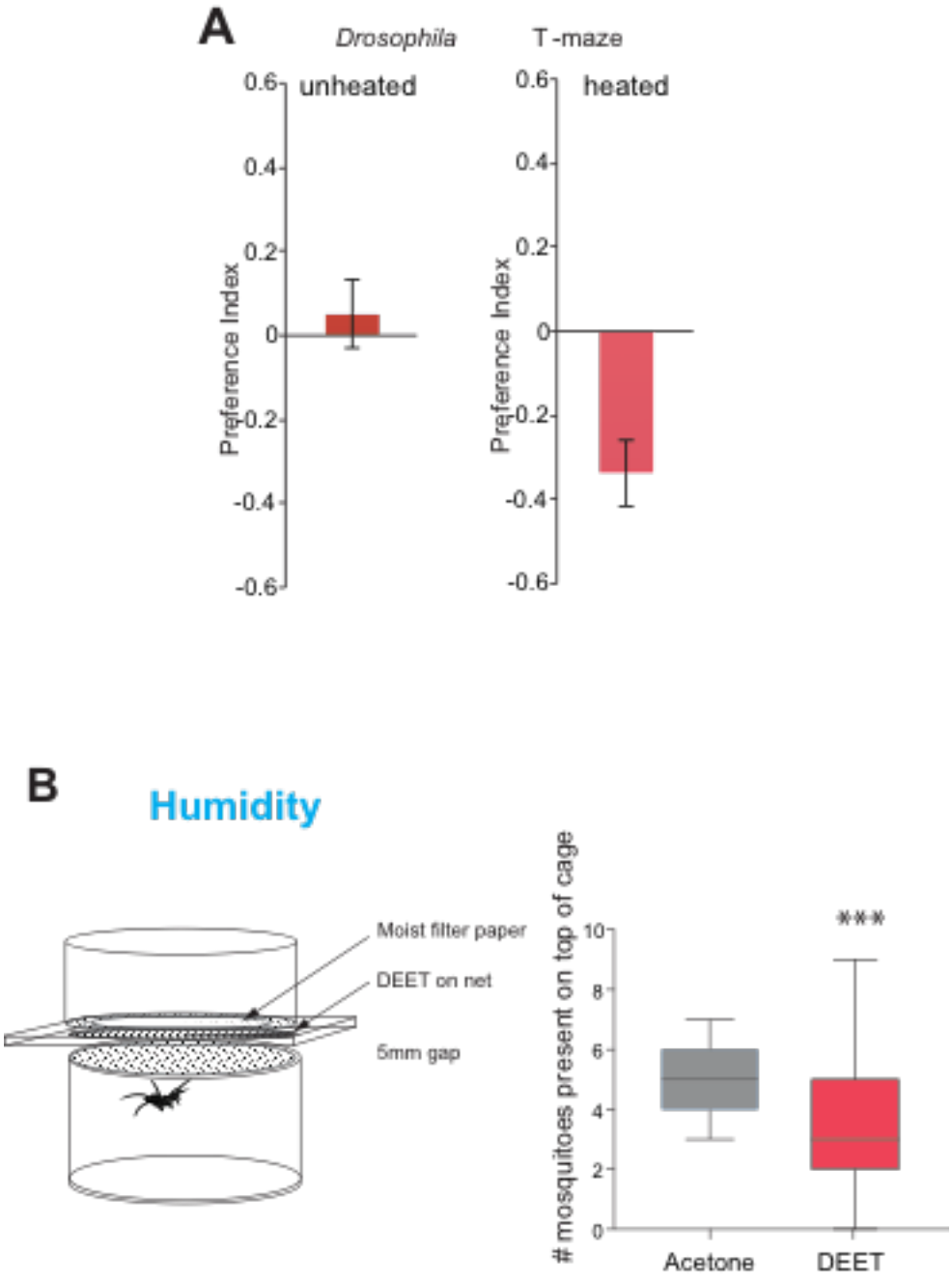

Figure 2.9. DEET causes avoidance in flies and mosquitoes.

Figure 2.9. DEET causes avoidance in flies and mosquitoes.

(A) A T-maze assay was used to measure avoidance of *Drosophila melanogaster* to test compounds and mean Preference Index to the tube containing 10uL DEET on a filter paper disc at room temperature, or at room temperature after pre-heating both tubes for 10 mins at 80 degrees Celsius. N = 8 and 11 trials (~40 flies/trial). **(B)** Schematic of the non-contact humidity attraction assay, and mean number of female *A. aegypti* sitting on the top net facing humid filter paper and non-contact DEET or control treatment (***p<0.001; unpaired t-test; n = 5 trials).

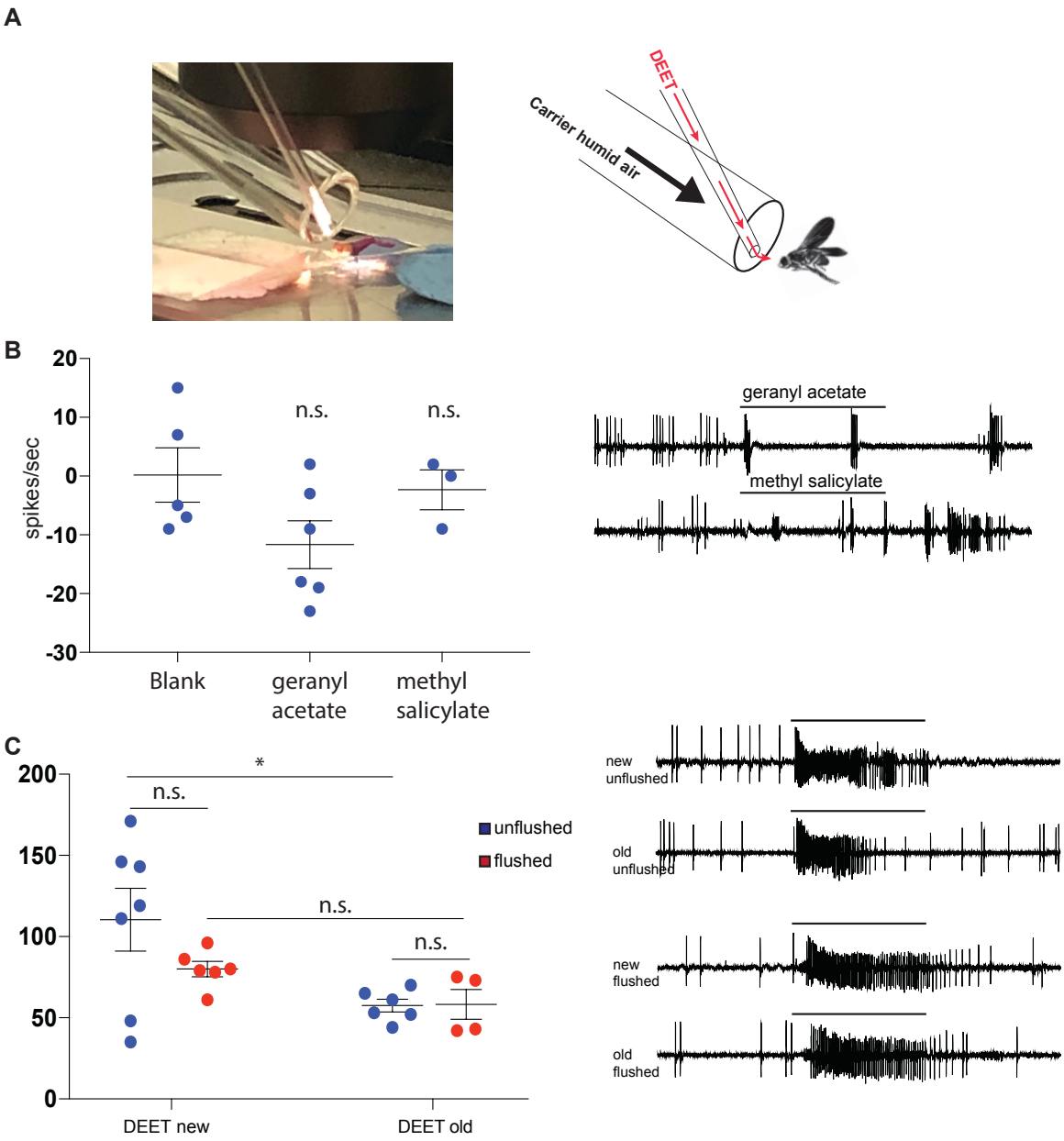

Figure 2.10. DEET activation is a specific effect.

Figure 2.10. DEET activation is a specific effect.

(A) Tip of odor cartridge is placed “5 mm” in front of insect and between carrier airflow tube opening. **(B)** Response of ab3 neurons to methyl salicylate and geranyl acetate. Bars represent total spike frequency of both neurons, with the baseline activity subtracted. Error bars represent standard error of the mean. Example traces (inset) of ab3 response to 5.0 uL of pure geranyl acetate (top) and methyl salicylate (bottom). N = 3-6 for all traces. *p<0.05; **p<0.01; ***p<0.001. **(C)** Response of ab2 neurons to new (left) and old (right) DEET samples, as well as flushed (red) or unflushed (blue) cartridges. N = 4-7. *p<0.05; **p<0.01; ***p<0.001. Error bars represent standard error of the mean.

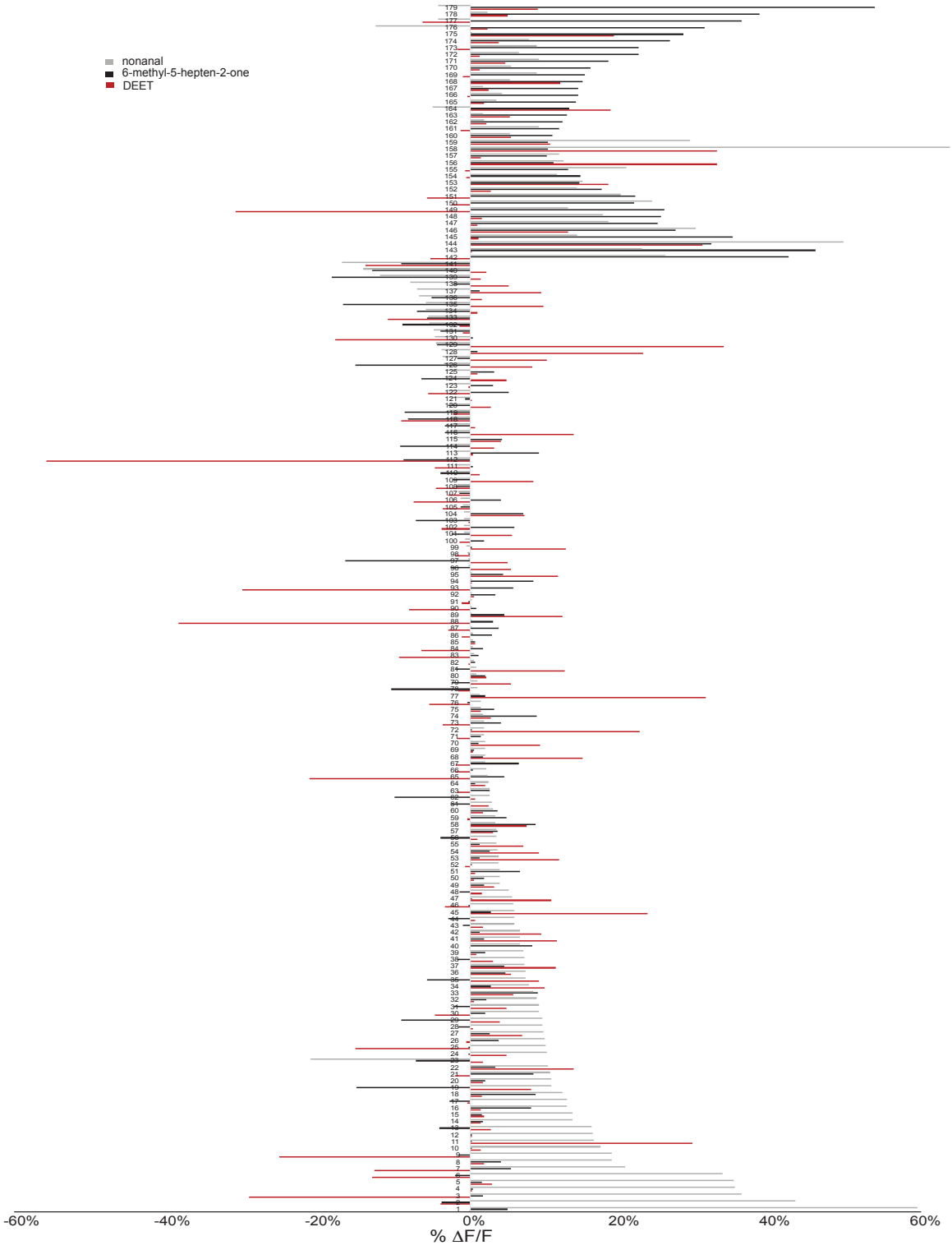

Figure 2.11. Calcium response in individual *Aedes* antennal neurons

Figure 2.11. Calcium response in individual *Aedes* antennal neurons

Percent change in fluorescence intensity of several individual cells from a large number of Pub-GCaMP6 female *Aedes aegypti* antennae to indicated stimuli (0.5 sec), after subtracting appropriate control (paraffin oil or blank cartridge).

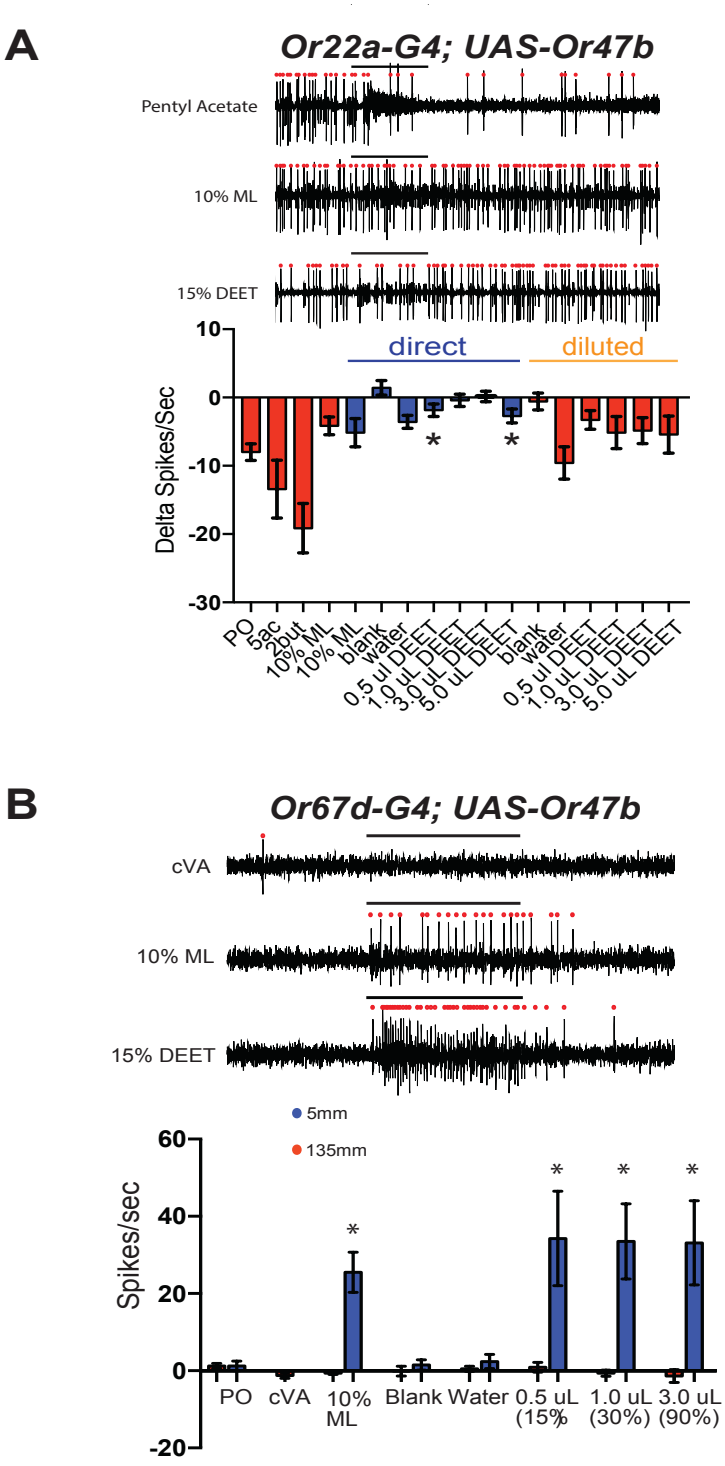

Figure 2.12. Effects of DEET on Or47b is dependent upon the identity of the neuron in which Or47b is expressed.

Figure 2.12. Effects of DEET on Or47b is dependent upon the identity of the neuron in which Or47b is expressed.

(A) Representative traces (top) and mean responses (middle) of the ab3A neuron in UAS-Or47b?TM3; Or22a<Gal4> flies to indicated stimuli (2but = ethyl butyrate, 5ac = pentyl acetate; error bars represent s.e.m.; *p<0.05; N = 7). Percent inhibition (bottom) of the ab3A neuron to DEET compared to the responses to the blank control cartridge at both 5mm and 135mm distances. (error bars = s.e.m.; N = 7). **(B)** Representative traces and mean responses of the at1 neuron in UAS-Or47b/+; Or67d<Gal4> flies to indicated stimuli (ML = methyl laurate; error bars = s.e.m.; *p<0.05; N = 4-8).

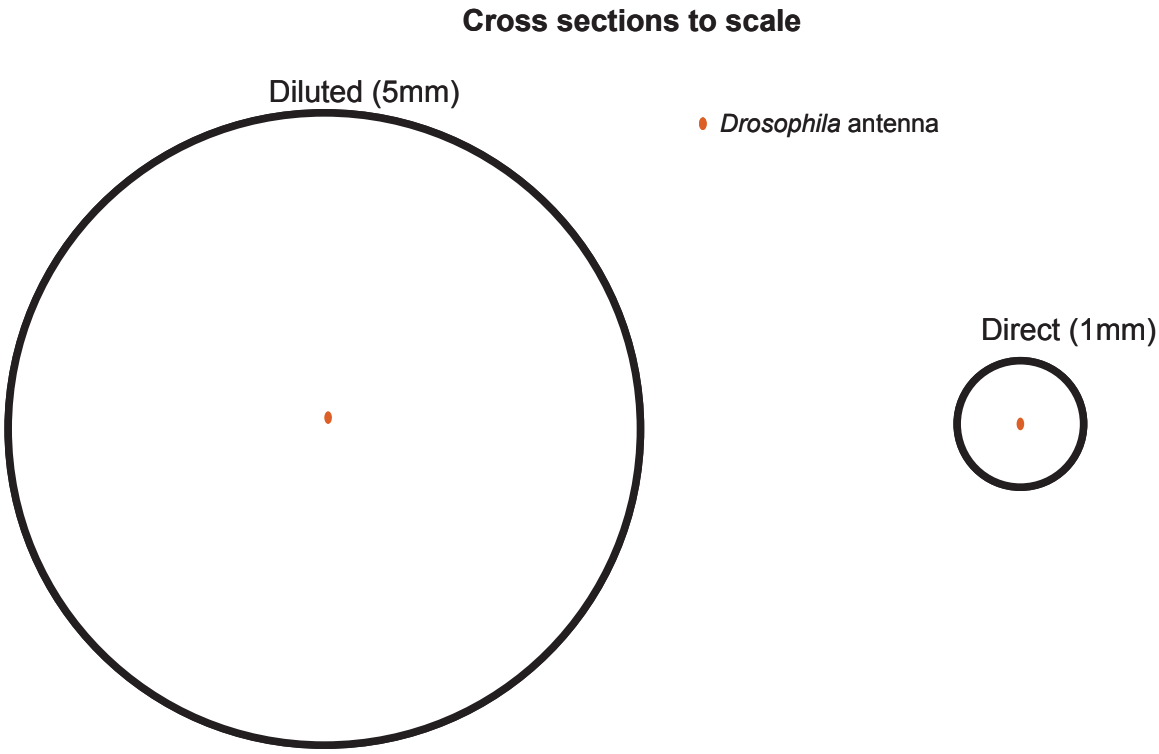

Figure 2.13. Cross-section of odor delivery tubes and *Drosophila* antenna

CHAPTER 3

DETECTION OF NOXIOUS CONCENTRATIONS OF AMMONIA IN INSECTS BY OLFACTORY NEURONS

OVERVIEW

Ammonia is commonly found in nature and is detected as a chemosensory cue across a wide range of taxa. Here, we investigate the chemosensory detection of noxious repellent levels of ammonia in the genetic model system *Drosophila melanogaster*. Repulsion caused by high concentrations coincides with an atypical action potential burst, followed by inhibition of several classes of antennal olfactory receptor neurons (ORNs). During the prolonged period of inhibition following ammonia exposure, ORNs are unable to fire action potentials to their cognate ligands, but remarkably still display receptor potentials. Further, high concentrations of ammonia increase calcium levels in antennal ORNs, and this effect is conserved in the yellow fever mosquito *Aedes aegypti*. We test other basic amines and find that they mimic the inhibitory effect of ammonia in olfactory neurons, and further, that their effects are correlated with their basicity. Our characterization of this receptor non-specific effect of basic compounds on the chemosensory system reveals a novel coding mechanism that can override detection of other ligands and therefore has potential in discovery of new types of insect repellents.

INTRODUCTION

Ammonia occurs commonly throughout different ecosystems at a wide range of concentrations. Low concentrations of ammonia present in human sweat act as attractive cues for disease vectors like *Anopheles gambiae* (Meijerink, Braks et al. 2001, Semmelhack and Wang 2009) and *Aedes aegypti* (Geier, Bosch et al. 1999), which can sense ammonia through grooved peg sensilla present on their antennae. Ammonia emitted by *Rafflesia* can also act as an attractive cue for certain insects facilitating pollination (Davis, Latvis et al. 2007). Outside insects, ammonia has been shown to act as an important cue in prey-finding and homing behavior of sea birds such as petrels, albatrosses and shearwaters (O'Dwyer and Nevitt 2009). It is also sensed by olfactory systems of other vertebrates like fish (Barimo and Walsh 2006) and mammals, including humans (Wallrabenstein, Kuklan et al. 2013) and mice (Liberles and Buck 2006). Amines present in mouse urine can act as social cues or pheromones and are detected by the olfactory epithelium (Liberles and Buck 2006). In other organisms, such as *C. elegans (Frokjaer-Jensen, Ailion et al. 2008)*, ammonium acetate can be detected by both olfactory and gustatory systems. Hence ammonia plays a chemosensory role in a vast range of invertebrate and vertebrate animals.

A conserved class of receptors known as trace amine associated receptors (TAAR) have been identified in mice to be responsible for sensing ammonia and amines (Liberles and Buck 2006). Attraction towards ammonia appears to be fairly conserved in insects too, since it is known that mosquitoes, *Drosophila*

melanogaster, as well as a related species, *Drosophila simulans,* display attraction to it in olfactory behavior assays. A recent study identified Ir92a, an ionotropic receptor, to be necessary for olfactory detection of ammonia and certain amines (Min, Ai et al. 2013). The same study mapped ammonia-elicited activity to VM1 glomeruli in the antennal lobes, and to VM1-PNs projecting to the lateral horns. Another study by Menuz *et al.* reported the role of an ammonia transporter gene *Amt* in olfactory responses to ammonia (Menuz, Larter et al. 2014). *Amt* is expressed in the auxiliary cells surrounding *Ir92a*-expressing ac1 neurons. Loss of *Amt* function greatly decrease ac1 firing frequency upon stimulation with ammonia. Nonetheless, how the two pathways contribute to ammonia-driven attractive behaviors is not well understood.

Even less is known about mechanisms underlying detection of higher concentrations of ammonia, which do not always result in attractive behaviors. The higher end of the naturally-occurring concentration range (40mmol/kg) can be found in biological excrement (Dai and Karring 2014), and rotting biomass in fruit and other plant parts that produce ammonia and other amines (Ough, Daudt et al. 1981, Kiss, Korbasz et al. 2006, Davis, Latvis et al. 2007). Ammonia is also present in the excreta of various animals, present directly or as a metabolite of urea, leading to high concentrations (Mensua and Moya 1983, Borash, Pierce et al. 2000). Finally, ammonia is also excreted by fly larvae and can build up to considerable levels in over-crowded cultures (Mensua and Moya 1983, Borash, Pierce et al. 2000). Although the majority of past studies were directed at

investigating olfactory detection of ammonia, a recent study reports physiological as well as behavioral responses to ammonium salts in the *Drosophila* gustatory system (Delventhal, Menuz et al. 2017). Ammonium salts evoke strong neuronal responses from Gr66a neurons in S-type sensilla of the labellum, which results in behavioral aversion in feeding experiments.

Here we test how higher concentrations of ammonia are encoded by chemosensory neurons in the model insect *Drosophila melanogaster.* We find that ammonia shows a strong phasic activation followed by an atypical inhibitory response in several different classes of olfactory neurons. In both olfactory and taste neurons the tonic inhibition can completely abolish the subsequent detection of other olfactory and gustatory cues. We attribute the burst-inhibition response to a high pH effect and identify several amines that also cause strong neuronal inhibition. The observed mechanisms appear to be conserved in mosquitoes and shed light on how at higher concentrations this attractive cue becomes less so.

RESULTS

To test whether ammonia follows a similar behavioral valence trajectory as other odorants, we tested wild type *Drosophila* with various concentrations of ammonia in a simple T-maze behavior assay (Fig 3.1A). We found that flies were repelled when the sample placed on the filter paper was at a concentration of 2% or higher, whereas they showed no preference for either side at lower

concentrations. This indicates that ammonia has a negative valence starting at a concentration around 2%.

To investigate the response of olfactory neurons to aversive concentrations of ammonia we performed single-sensillum recordings (SSRs) on the large basiconic sensilla of *Drosophila*. We began by testing 3% ammonia, which would elicit aversion in the T-maze assay. We found that in every case, a stimulus of 3% ammonia caused a brief strong burst in spikes before silencing the neuron (Fig 3.1B). This effect was consistent across all three large basiconic sensillar classes tested: ab1, ab2 and ab3. To determine the concentration threshold at which this unusual effect occurs, we tested ammonia across a range of concentrations. At 0.16% and 0.33%, ammonia did not cause the burst-silencing effect. At 0.66%, ammonia resulted in burst-silencing one-third of the time, and at 1.0% it always did so, indicating that the effect is concentration-dependent (Fig 3.1C). The length of the silencing period varied between neurons, lasting from 45 seconds to over 3 minutes. These results show that the baseline activity of olfactory neurons is suppressed after exposure to high concentrations of ammonia.

To test whether odorants detected by ORNs could still elicit neural activity during the post-ammonia silencing period, we tested responses of neurons in the ab2 sensillum to known activators (1-hexanol, geranyl acetate, and ethyl butyrate) both before and 20-60 seconds after exposure to 3% ammonia. As observed earlier, the ORNs were silenced following an initial burst upon ammonia exposure, and subsequent stimulation with the three odorant ligands failed to produce spiking

activity (Fig 3.2). In a complementary electrophysiological assay, we recorded the receptor potential of the ab2 sensillum to odorant stimuli both before and after exposure to 3% ammonia. Remarkably, we found that the odor-induced sensillar receptor potentials were not decreased after exposure to ammonia, although the action potentials were completely abolished (Fig 3.3). This indicates that olfactory receptors are still functional and presumably allowing odor-mediated cation flux, whereas downstream signaling pathways are affected in a manner that prohibits the generation of action potentials.

In order to measure calcium flux in response to ammonia in ORNs of the *Drosophila* antenna, we expressed GCaMP6 under the control of an *Orco-Gal4* driver that is broadly expressed in all *Or*-expressing neurons, which account for ~70% of the ORNs in the antenna. By monitoring GCaMP6 fluorescence in several cells, we were able to survey the activity of a number of ORNs simultaneously. We found that ammonia led to an increase of calcium activity in a dose-dependent manner. While 3.33% and 1% concentrations produced significant increases in signal, the 0.233% concentration did not, which is aligned with the initial strong burst of action potentials, as well as the receptor potentials observed in single-sensillar electrophysiology recordings.

We then tested whether the broad ammonia-induced increase in calcium levels is conserved by imaging in the yellow fever mosquito *Aedes aegypti.* Using a recently developed *Ubi-GCaMP6 Ae. aegypti* line, we determined that a number of cells expressing GCaMP6 can be identified in the antenna of female mosquitoes

(Fig 3.4C) (Bui, Shyong et al. 2018, Bui, Shyong et al. 2019, Lahondère C., Vinauger C. et al. 2019). Of the cell-like regions showing GCAMP6 expression, most showed increases in fluorescence in response to ammonia, many in a dose-dependent manner (Fig 3.4B, 3.5). By plotting the fluorescence increases of all cells that showed an increase in Ca^{2+} signal in response to at least one of stimulus, we found that a large fraction of fluorescent cell-like regions responded to ammonia at concentrations of 3.33% and 1%. This widespread activation is similar to that we observed in *Drosophila*.

To test if the inhibitory effect of the ammonia can be generalized to other compounds, we tested a panel of five amines using single sensillum electrophysiology. We found that three of the five amines – butylamine, dimethylamine, and 4-methylpiperidine – each showed the burst-silencing effect observed with ammonia exposure (Fig 3.6A). Comparison of effects across concentrations (Fig 3.6B) showed that 1.66% of butylamine mimicked the burst-silencing effect seen with ammonia, whereas at concentrations lower that 0.33% it briefly inhibited the ab2A neuron while activating the ab2B neuron. Dimethylamine at 3.33% produced the burst-silencing effect; at lower concentrations it activated the ab2B neuron and inhibited the ab2A neuron. Two of the tested compounds did not cause burst-silencing – phenethylamine completely silenced the ab2A neuron but did not affect the ab2B neuron, whereas pyridine did not cause inhibition and instead led to a strong increase in spike frequency (Fig 3.6A). The activation of ab2 neurons in response to pyridine exhibited dose-dependence similar to that of

a typical activating ligand and caused strong pinching at concentrations of 1.66% and higher (Fig 3.6B). Phenethylamine strongly inhibited the ab2A neuron at all concentrations tested while leaving the ab2B neuron unaffected.

DISCUSSION

We find that ammonia and amines, both of which have high pH, can cause a burst of activation followed by a prolonged period that lacks action potentials in the olfactory neurons. The mechanisms underlying the observed burst and subsequent inhibition are not known. The finding that odor-induced receptor potentials appear to be unaffected suggests that ion flux through odorant receptors is not drastically altered if at all, and therefore the resting membrane potential of the neuron must likewise be within its typical range. One possibility is that voltage-gated sodium channels required for action potentials are fixed in an inactive state during the period of inhibition. This would allow ions to flux across the membrane via odorant receptors, but the resulting receptor potential would fail to generate action potentials upon reaching the normal spiking threshold.

The variable effects of the different amines tested also raise interesting questions about the contribution of factors other than pH for each odorant-neuron interaction. However, it is notable that pyridine, which is the least basic of the five compounds, acts in a manner typical of an activating ligand, causing a robust increase in spike frequency in a dose-dependent manner. By contrast, 4-methylpiperidine, the most basic of the five compounds tested, acted most like

ammonia. Thus, the burst-silencing effect first seen with relatively high concentrations of ammonia and amines may be a consequence of high pH rather than the steric structure or functional groups of the ligand *per se*, similar to implications of gustatory neuron inhibition by high pH, and not the molecular identity, of the selected tastants.

Overall, our study offers a strategy to create new insect repellents that act on the olfactory system by blocking the ability to detect attractive volatile cues. The finding that compounds with high pH can independently serve both functions expands the scope of identifying newer compounds that could become the next generation of insect repellents.

MATERIALS AND METHODS

Fly stocks and Mosquito rearing

Wild type flies used in the experiments were Canton-S and reared on standard media at 22-25°C. *Ae. aegypti* wild-type (Orlando strain) were maintained in an insectary at ~27^{o}C, 70-80% humidity on a 14:10 hr (Light: Dark) photoperiod. Lights on at 0700hrs. Adults were held in the insectary in 30 cm^{3} Bugdorm cages and given access to 10% sucrose solution. Females were offered blood using artificial blood feeding system and allowed to lay eggs. Upon hatching, larvae were thinned into 6qt size (5.7L) plastic containers filled with about 900mL deionized water and fed on Tetramin® fish food tablets until pupation.

Electrophysiology

Olfaction

Adult male flies were tested at 3-7 days post-eclosion. Individual flies were inserted into a 1.0 mL pipette tip and secured with molding clay. A glass cover slip was placed underneath the head and a glass pipette was used as a rod to secure an antenna in place. A reference electrode filled with SLR solution was inserted into the eye. All compounds were dissolved in water to the designated dilutions. A 3 X 35 mm filter paper strip was placed in each 5¾" glass Pasteur pipette to form odor cartridges. Cartridges were allowed to sit and volatilize for 15 minutes before use. Each cartridge was used no more than twice before being discarded. A glass tube was used to maintain a humidified airstream of 10 mL/sec on the preparation, provided by a gas tank routed through a water-filled beaker. The odor cartridge was inserted into the side of a glass tube through an opening 130 mm distant from the end of the tube. The end of the tube was placed 5 mm away from the prep, for a total of 135 mm separating the preparation from the point in which DEET entered the airstream.

A puff of air was released through the odor cartridge with a Syntech device for 1 second. Spikes were counted during the 1 s application window in the case of 5 mm application and for 0.25 s to 1.25 s after the start of the application window in the case of 135 mm application, owing to a slight delay caused by the increased distance to the antennae. The baseline activity from one second before the stimulus onset was subtracted from the total activity to obtain a net increase in

spikes. While the cartridge contained a filter paper with ammonium hydroxide solution at a higher concentration, it should be noted that the injection of the cartridge headspace (5 ml/sec) into a humidified airstream (10 ml/sec) reduces concentrations by two-thirds. Thus, the concentration of ammonia fluxing over the antennae is equivalent to a 3% solution stimulus if a 9% solution is added to the filter paper. All stimulus percentages listed represent the two-thirds reduction fluxing over the antennae. To perform receptor potential recordings, the high pass filter was increased by one order of magnitude but recordings were otherwise obtained in the same fashion.

Calcium Imaging

Male *Drosophila* and mated female *A. aegypti* between 3-8 days age with the homozygous Ubi-GCaMP6 transgene were prepared for imaging on a glass slide. Imaging was performed on an Olympus BX51WI fluorescence microscope using a 20X LMPLANFL N air objective with a Hamamatsu Orca-flash 4.0 camera and Metamorph software. Analysis was performed offline using ImageJ/FIJI software. A custom Macro was used to assist in the analysis which included partitioning the mosquito antenna into grids for cell numbering by region, and background control stimulus subtraction before calculating the deltaF values. The controlled odor stimulus was delivered using a Syntech CS-55 unit from odor cartridges put together from disposable glass Pasteur pipettes containing a piece

of filter paper, as is used in single-unit electrophysiology recordings. The placement of the cartridge was similar to that of the SSR recordings.

Behavior

T-maze

Experiments were performed as before (Turner and Ray 2009) with a few modifications. For each test, 40 *D. melanogaster* that were starved for 24 hrs, but provided with a moist tissue, were used in a 1:1 male:female ratio. Assays lasted 1 min and were performed in the dark. Control and treatment sides were alternated. The avoidance response was calculated as a Preference Index (PI) = (number of flies in test arm - number of flies in control arm)/(total number of flies in assay).

Statistical Methods

Student's T test was conducted in Excel while Graphpad Prsim was used for the Mann-Whitney tests. Olfactory recordings were analyzed with two-tailed unpaired Mann-Whitney tests. In all the figures, error bars represent s.e.m.

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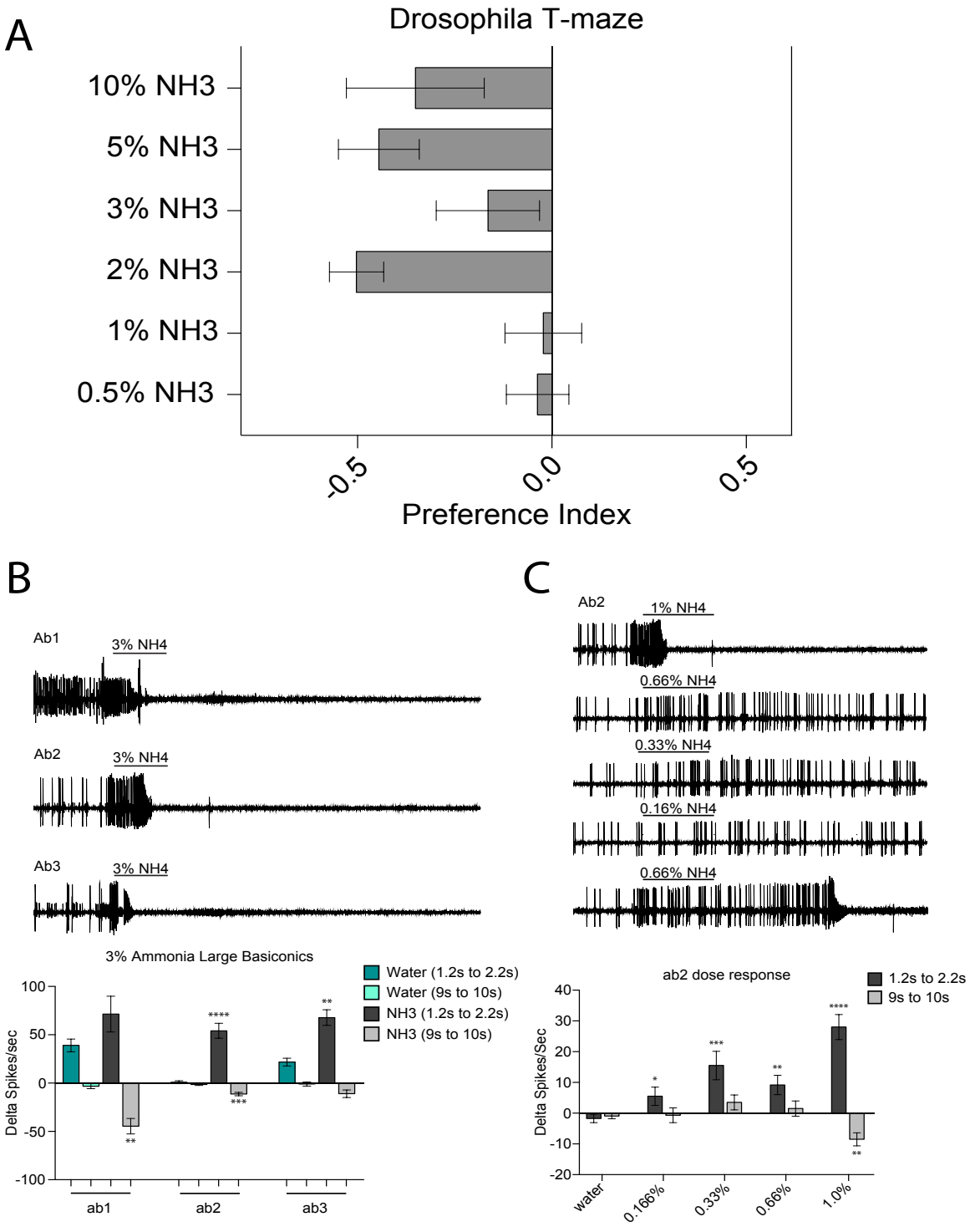

Figure 3.1. High concentrations of ammonia are aversive to *Drosophila* and inhibit neuronal activity.

Figure 3.1. High concentrations of ammonia are aversive to *Drosophila* and inhibit neuronal activity

(A) Behavioral preference index of wildtype *Drosophila* to ammonia at the indicated concentrations vs. water. All results were obtained from 3-5 day old flies. Preference index was calculated as (#flies on ammonia side) – (#flies on water side) / (total #flies). Only trials with at least 40% participation were included in the analysis. n = 10 trials of 40 flies (20 male + 20 female) for each concentration. Error bars indicate s.e.m. **(B)** Representative traces (top) and mean responses (bottom) for a 1-sec period of stimulation from each sensillum in response to ammonia. The 1-sec stimulation period is indicated by the black bar above each trace. Counting for the responses marked “1.2s to 2.2s” was begun at the start of the increase in spike frequency (~1.2 second mark on the recording). Counting for the responses marked “9s to 10s” was measured during the last second (from 9s to 10s) on each recording. All recordings were obtained from 3-5 day old wildtype (CS) flies. n = 5-13 sensilla from 6 flies. Error bars indicate s.e.m. *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001. **(C)** Representative traces (top) and mean responses (bottom) of the ab2 sensillar neurons to a 1-sec stimulus of indicated concentration of ammonia. The 1-sec stimulation period is indicated by the black bar above each trace. Counting for the responses marked “1.2s to 2.2s” was begun at the start of the increase in spike frequency (~1.2 second mark on the recording). Counting for the responses marked “9s to 10s” was measured during the last second (from 9s to 10s) on each recording. All recordings were obtained from 3-5

day old wildtype (CS) flies. n = 6-14 sensilla from 6 flies. Error bars indicate s.e.m. *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.

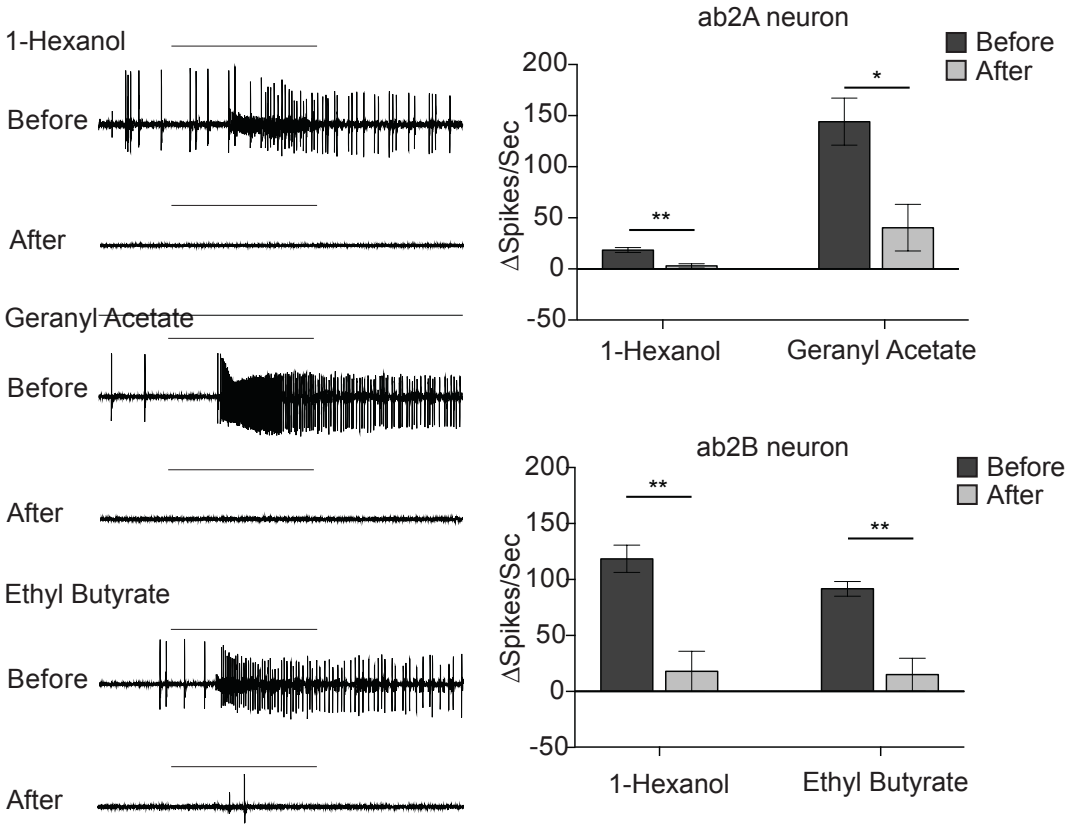

Figure 3.2. Exposure to ammonia completely blocks ability to detect cognate odorants.

Representative traces (left) and mean responses (right) for the ab2A and ab2B neurons to different stimulating odorants. The 1-sec stimulation period is indicated by the black bar above each trace. “Before” denotes that the recording precedes exposure to 3% ammonia, while “after” denotes that the recording follows exposure to 3% ammonia. All recordings were obtained from 3-5 day old wildtype (CS) flies. n = 5-6 sensilla from 6 flies. Error bars indicate s.e.m. **p<0.01.

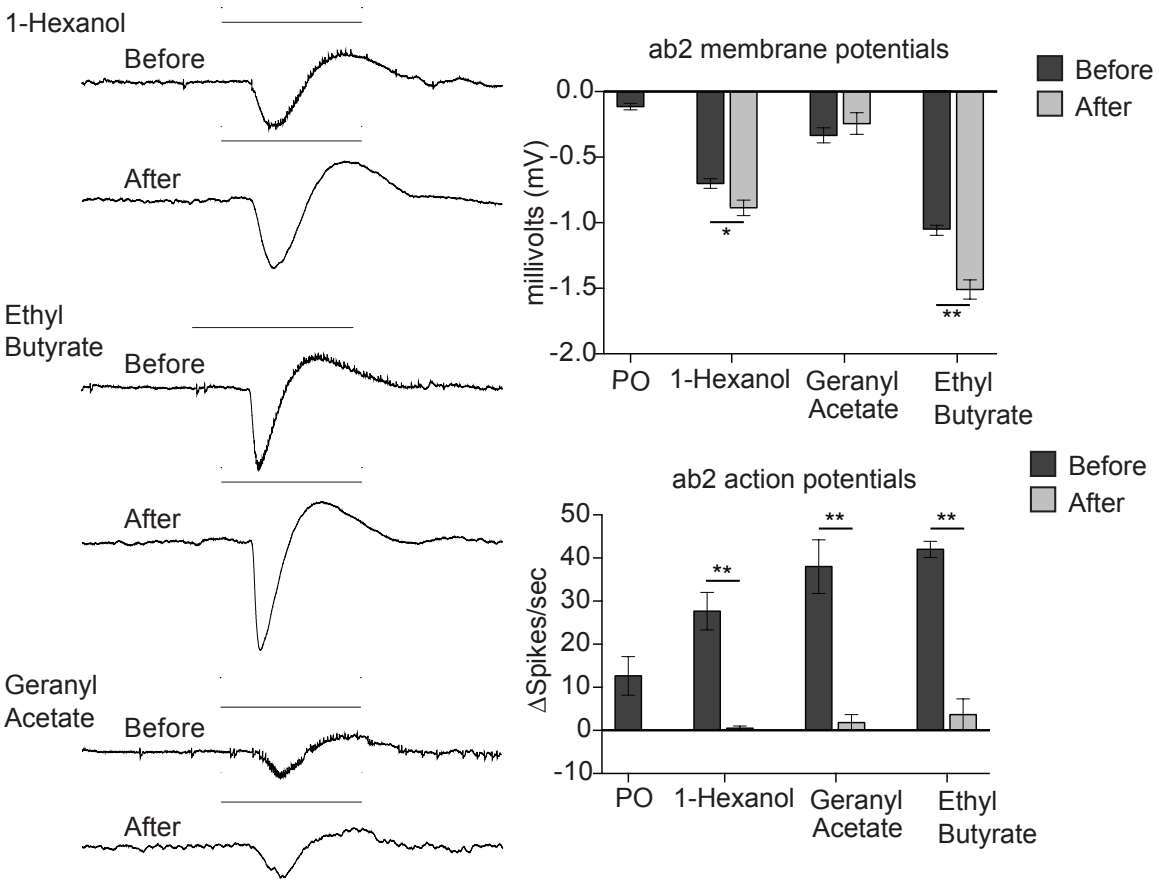

Figure 3.3. Exposure to ammonia does not abolish odorant-induced receptor potential.

Representative traces (left) and mean responses (right) for receptor potentials in the ab2 sensillum both before and after exposure to 3% ammonia. All recordings were obtained from 3-5 day old wildtype (CS) flies. n = 6 sensilla from 6 flies. Error bars indicate s.e.m. *p<0.05; **p<0.01.

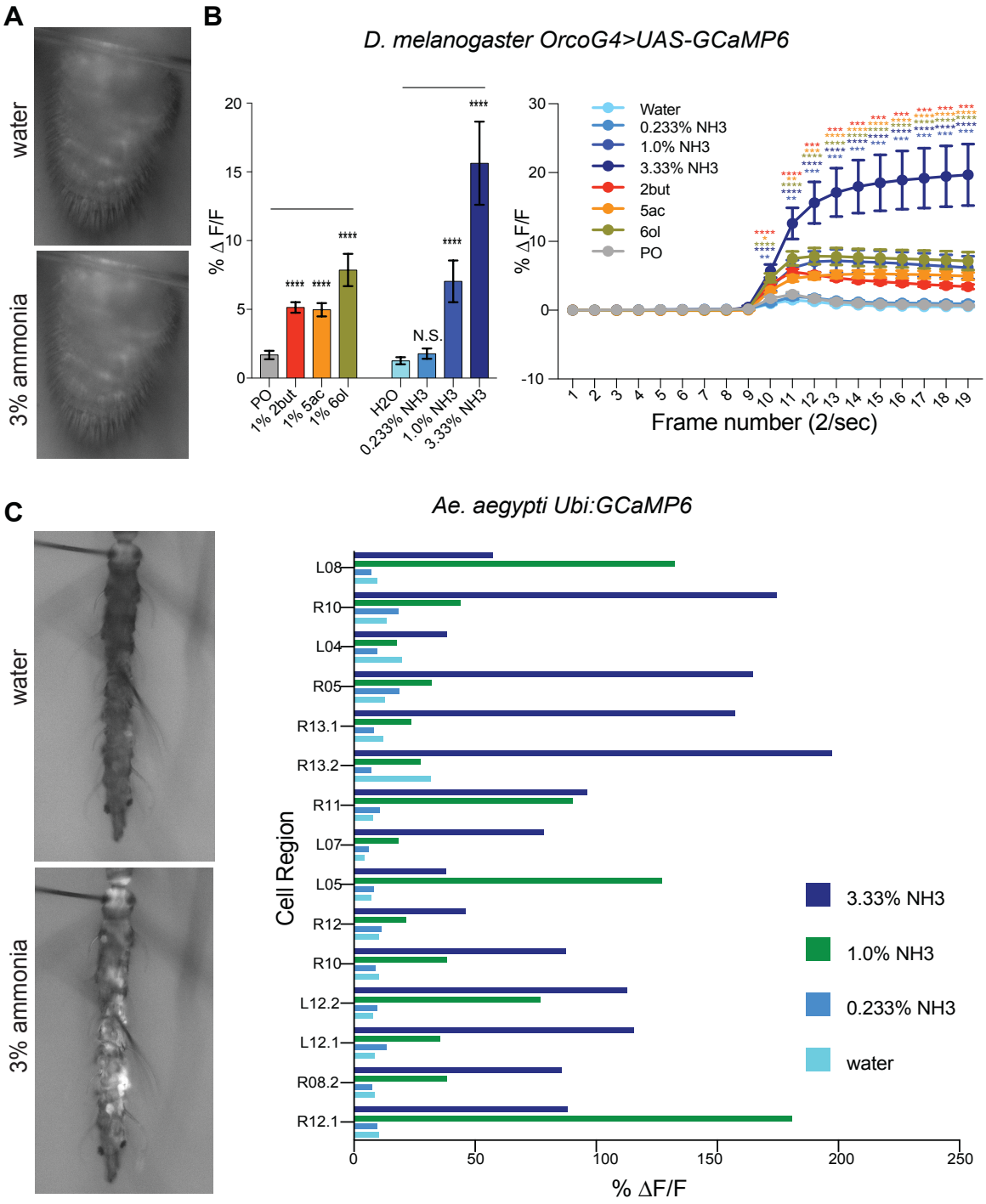

Figure 3.4. Ammonia causes widespread increase in calcium in antennal neurons.

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(A) Representative fluorescence micrograph of antenna exposed to indicated stimuli in *Orco-Gal4;UAS-GCaMP6 Drosophila melanogaster.* **(B)** Mean response to indicated stimulus at *(left)* 1.5 sec after stimulus (frame 12) for the indicated stimuli; and *(right)* averaged temporal kinetics of the calcium response for cells from *Drosophila* antennae to the indicated odorants. All recordings were obtained from 3-5 day old flies. n = 18 sensilla from 11 flies. Error bars indicate s.e.m. **p<0.01, ***p<0.01, ****p<0.001. **(C)** Representative fluorescence micrograph of antenna exposed to indicated stimuli and mean percentage change in fluorescence intensity of several individual cells from a large number of *PUb-GCaMP* female *Aedes aegypti* antennae to indicated stimulus (0.5 seconds). Each bar represents response of a single cell. Cells were measured from multiple mosquitoes and all cell are shown in Supplementary Figure 1.

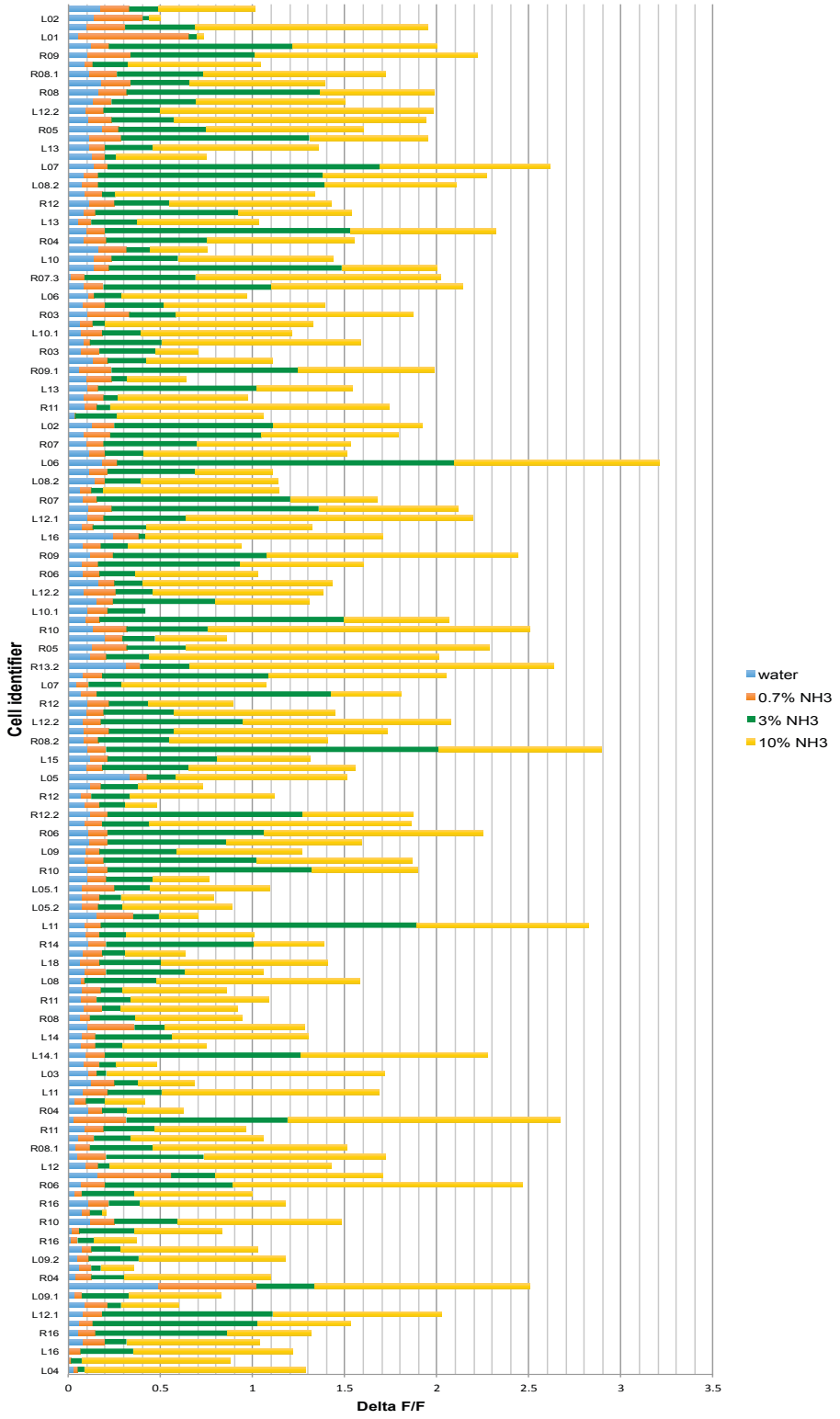

Figure 3.5. Ammonia-induced increase in calcium is conserved in *Aedes*.

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Percentage change in fluorescence intensity of several individual cells from a large number of *PUb-GCaMP* female *Aedes aegypti* antennae to indicated stimulus (0.5 seconds). Each bar represents response of a single cell. Cells were measured from multiple mosquitoes and all cells measured are shown.

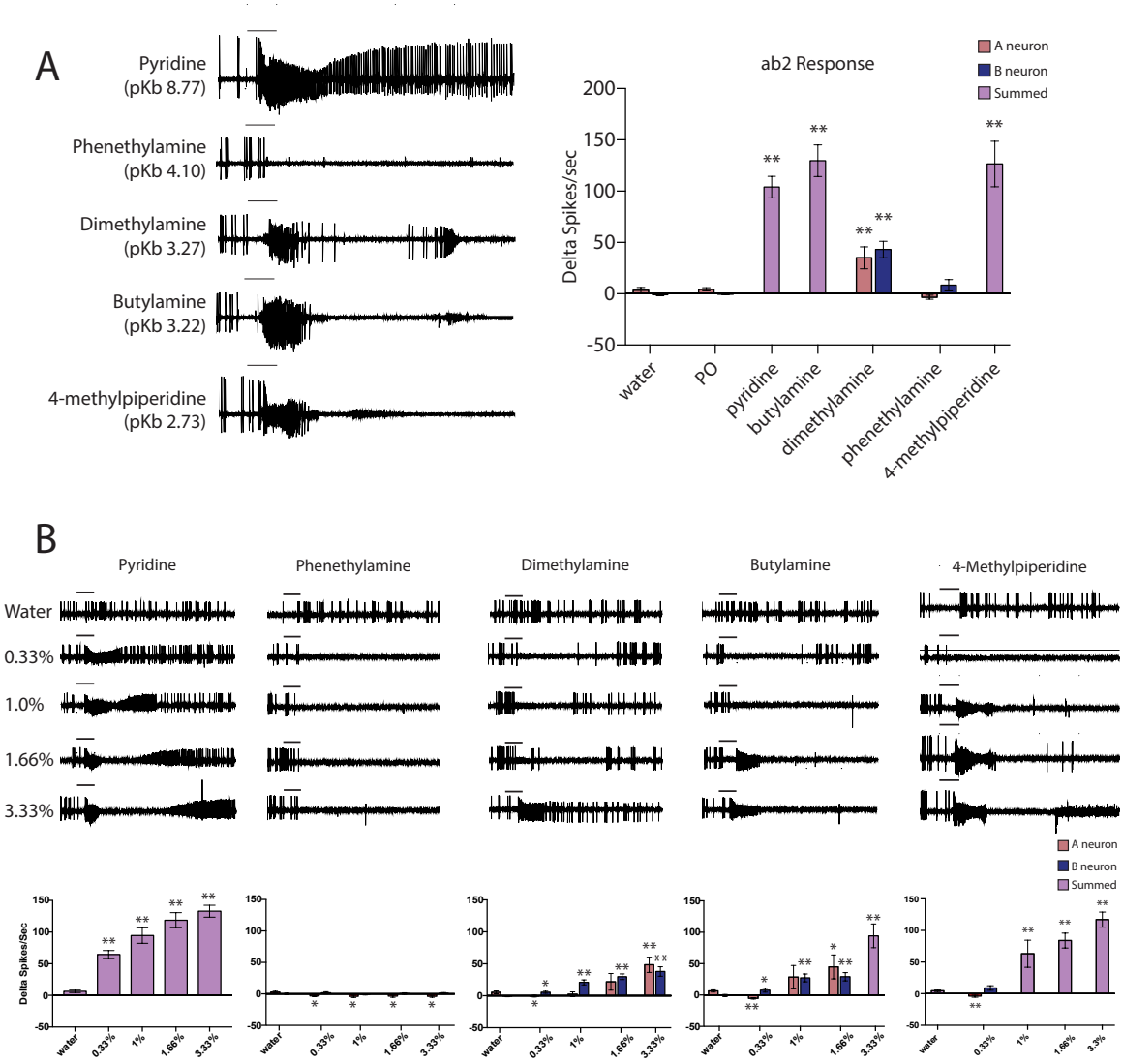

Figure 3.6. Amines also inhibit olfactory neurons in *Drosophila*.

Figure 3.6. Amines also inhibit olfactory neurons in *Drosophila*.

(A) Representative traces (left) and mean responses (right) for a 1-sec period of stimulation from each of the five basic compounds. Each count was begun at the start of the increase in spike frequency. All compounds tested at 3.33% concentration in water, except phenethylamine which was dissolved in paraffin oil. All recordings were obtained from 3-5 day old wildtype (CS) flies. n = 4-6 sensilla from 6 flies. Error bars indicate s.e.m. **p<0.01. **(B)** Representative traces (top) and mean responses (bottom) for a 1-sec period of stimulation from each concentration of the five basic compounds. Each count was begun at the start of the increase in spike frequency. All compounds were dissolved in water, except phenethylamine which was dissolved in paraffin oil. All recordings were obtained from 3-5 day old wildtype (CS) flies. n = 6 sensilla from 6 flies. Error bars indicate s.e.m. *p<0.05; **p<0.01.

CHAPTER 4

NATURAL ODORANTS THAT INHIBIT HUMIDITY SENSING IN *DROSOPHILA* AND MODIFY BEHAVIOR IN MOSQUITO VECTORS OF HUMAN DISEASE

OVERVIEW

Insects house humidity-sensing neurons in the antenna, which are presumed to be important for a variety of survival behaviors as water is a crucial environmental cue. We identify a class of natural odorants that significantly inhibits humidity-induced activation of antennal neurons. The inhibition is present in the humidity-sensing coeloconic neurons of dipteran *Drosophila melanogaster* that express specific members of the *Ionotropic receptor* family know to detect humidity, is dose-dependent, and is not seen in other coeloconic neurons that are not sensitive to humidity. Dipteran mosquitoes *Aedes aegypti* and *Anopheles gambiae* oviposit in water, and the addition of the humidity-inhibiting odorants in a two-choice oviposition assay significantly reduced oviposition in a dose-dependent manner. Our results demonstrate that these natural odorants effectively “mask” detection of an important environmental cue and behavior in important vectors of human disease. Odorants targeting the conserved humidity system therefore offer a novel strategy for mitigating the impact of dangerous insects.

INTRODUCTION

Humidity represents an important sensory modality for many animal species (Shelford 1918, Sayeed and Benzer 1996, Okal, Francis et al. 2013, Filingeri 2015). In addition to acting as a simple cue for detecting water sources, especially critical for small insects prone to desiccation due to large surface area-to-volume ratios (Gibbs, Fukuzato et al. 2003, Chown, Sorensen et al. 2011, Stinziano, Sove et al. 2015), humidity has been demonstrated to serve an astoundingly diverse range of functions across many species of insects. Foraging hawkmoths have been shown to use humidity as a means of assessing nectar availability in flowers (von Arx, Goyret et al. 2012), and ladybird beetles rely on a range of humidity for tarsal adhesion (Heepe, Wolff et al. 2016).

Of particular interest is the role that humidity plays in the behavior of destructive species such as disease vectors and agricultural pests. The female mosquito, which transmits diseases responsible for hundreds of thousands of deaths each year, uses humidity in both host-seeking behavior (Brown 1966, Bowen 1991, Zwiebel and Takken 2004) and as an oviposition cue (Okal, Francis et al. 2013). To detect hosts, mosquitoes often rely on complex blends of odorants, the components of which act synergistically by potentiating several attractive olfactory neurons (Dekker, Geier et al. 2005). The importance of humidity in insect behavior raises the interesting possibility of using “masking” compounds that can block activation of the insect neurons mediating humidity-detection.

RESULTS

Experiments from our lab determined that amine compounds act block humidity detection in *Diaphorina citri* (Asian citrus psyllid). To test whether amines act as antagonists on humidity-sensing neurons in the psyllid antenna was occurring via Ionotropic receptor (Ir) pathways that are conserved, we turned to the well characterized *Drosophila melanogaster* system. *Drosophila* detects humidity with olfactory neurons that are present in coeloconic sensilla on the antenna and express members of the *Ionotropic receptor* family (Yao, Ignell et al. 2005, Enjin, Zaharieva et al. 2016, Knecht, Silbering et al. 2017, Sun, Larter et al. 2018), including those in the antennal coeloconic sensilla ac1 and ac2 (Yao, Ignell et al. 2005). We performed SSRs in the ac1 sensillum while it was exposed to varying concentrations of hexylamine in both humidified and dry air backgrounds. We found that at the highest concentration tested (headspace from 1%), hexylamine significantly reduced baseline activity in both the dry and humidified airstream (Fig 1A). That the inhibitory effect occurs even in a dry airstream suggests that at concentrations of 1%, hexylamine acts at some level as an inverse agonist on the Ir-expressing ac1 neurons.

We also performed SSRs in the ac3 sensillum, which is not involved in humidity-sensing (Yao, Ignell et al. 2005). We found that hexylamine did not inhibit the baseline activity of these neurons at any concentration tested, and there was no significant difference at any given concentration between the responses in the humidified vs dry airstream (Fig 1B). Further, 1% hexylamine significantly activated

the ac3 neurons in the dry airstream when compared to paraffin oil. This indicates that the inhibitory effect of hexylamine is not generalizable to all olfactory neurons, or even to those housed in coeloconic sensilla.

An example of attraction behavior towards water is seen in gravid female mosquitoes that oviposit in standing water. It is presumed that humidity-sensing could play a critical role in their ability to detect and assess potential oviposition sites (Okal, Francis et al. 2013) along with other species-specific oviposition pheromones. We were interested in whether the compounds that antagonize humidity-sensing in ACP and *Drosophila* could also block the water-sensing behavior of the mosquito.

We tested oviposition preference in female *A. aegypti* mosquitoes, which transmit Dengue and Zika, using a two-choice oviposition assay where the test odorant could be presented as a volatile from a glass vial placed in a water-filled petridish. We found that mosquitoes avoided the water dish when the humidity-neuron inhibitor, hexylamine, was present (Fig 2A). In this assay with pentylamine, we similarly found that the two highest concentrations drove strong negative preference (Fig 2A). In both instances the mosquitoes laid their eggs in the control water dish. The simplest interpretation of these results is that hexylamine and pentylamine “mask” the humidity as an oviposition cue.

In order to test whether inhibition of oviposition behavior is conserved in the malaria mosquito *Anopheles gambiae,* we repeated the 2-choice oviposition assay with both hexylamine and pentylamine. Similar to what was observed in *Aedes*,

Anopheles females strongly avoided ovipositing on the odorant side in a dose-dependent manner (Fig 2B), although the avoidance was only statistically significant at the highest doses tested. Taken together, the humidity-sensing pathways and their inhibition with volatile compounds offer promise in blocking oviposition in *Aedes* and *Anopheles* mosquitoes.

DISCUSSION

The concept of chemical “masking” is, along with repulsion and luring, one of three major strategies of using chemicals to reduce vector-host interactions. Here we have demonstrated a class of chemicals that is effective at masking detection of and behavior toward a crucial environmental cue across a number of insect species. We demonstrate that hexylamine is able to antagonize humidity-sensing neurons in *Drosophila* and that this is dose-dependent. We also find an essential role for humidity in the oviposition behavior of mosquito vectors and show that these crucial behaviors can be modified or blocked by the use of these compounds.

As the compounds tested here are all similar in their chemical structure, there is promise that a chemical informatics approach will be able generate predicted compounds that are safe and effective alternatives to current pesticides and insect repellents.

MATERIALS AND METHODS

Stocks

Wild type *Drosophila melanogaster* used in the experiments were of the Canton-S strain and reared on standard cornmeal–dextrose media at 22-25°C. The M form of *Anopheles gambiae* (Herein *Anopheles coluzzi*, Ngousso strain, Cameroon) and *Aedes aegypti* (Orlando strain) were maintained in a 12:12 (Light:Dark) photocycle at 27°C and 70% RH.

Mosquito Oviposition

Oviposition behavior was carried out in bugdorm style (30 x 30 x 30 cm) mosquito cages under 14-10 light cycle, 60% relative humidity, and at 27°C. Six to seven days after blood feeding, fifteen female mosquitoes where transferred to each bugdorms where two oviposition sites (plastic petri dishes 50mm) were placed at opposite sides of the cage. The positions of odor-treated and control petri dishes were alternated in the different experimental replicates. In non-contact assays, a glass vial (2 mL total volume) containing 1mL of either the odor solution or water was placed onto each petri dish, which were filled with 13 mL of autoclaved tap water. In contact assay, the experimental set up was modified such that no glass vial was placed onto the petri dishes. In these experiments, 15 mL of either odor solution or water was poured onto the petri dished. A little cup containing sugar solution wicked through a cotton plug was placed in between the petri dishes. The experiments were carried out for 24 hours. The egg-containing

water was drained through Kimwipe tissues and counted under a scope with the help of a cell counting device. The number of eggs found in each oviposition chamber was counted at the end of the experiment.

Electrophysiology

Adult male flies (*D. melanogaster*) were tested at 3-7 days post-eclosion. Individual flies were inserted into a 1.0 mL pipette tip and secured with molding clay. A glass cover slip was placed underneath the head and a glass pipette was used as a rod to secure an antenna in place. A reference electrode filled with SLR solution was inserted into the eye. Hexylamine was dissolved in paraffin oil to the designated dilutions. A 3 X 35 mm filter paper strip was placed in each 5¾" glass Pasteur pipette to form odor cartridges. Cartridges were allowed to sit and volatilize for 15 minutes before use. Each cartridge was used no more than twice before being discarded. In the humidified setup, a glass tube was used to maintain a humidified airstream of 10 mL/sec on the preparation, provided by a gas tank routed through a water-filled beaker. The beaker was empty in the dry setup. The odor cartridge was inserted into the side of a glass tube through an opening 130 mm distant from the end of the tube. The end of the tube was placed 5 mm away from the prep, for a total of 135 mm separating the preparation from the point in which odors entered the airstream.

A puff of air was released through the odor cartridge with a Syntech device for 1 second. Spikes were counted during the 0.25 s to 1.25 s after the start of the

application window, owing to a slight delay caused by the increased distance to the antennae. The baseline activity from one second before the stimulus onset was subtracted from the total activity to obtain a net increase in spikes.

Statistics

All statistics were performed with two-tailed, nonparametric Mann-Whitney tests

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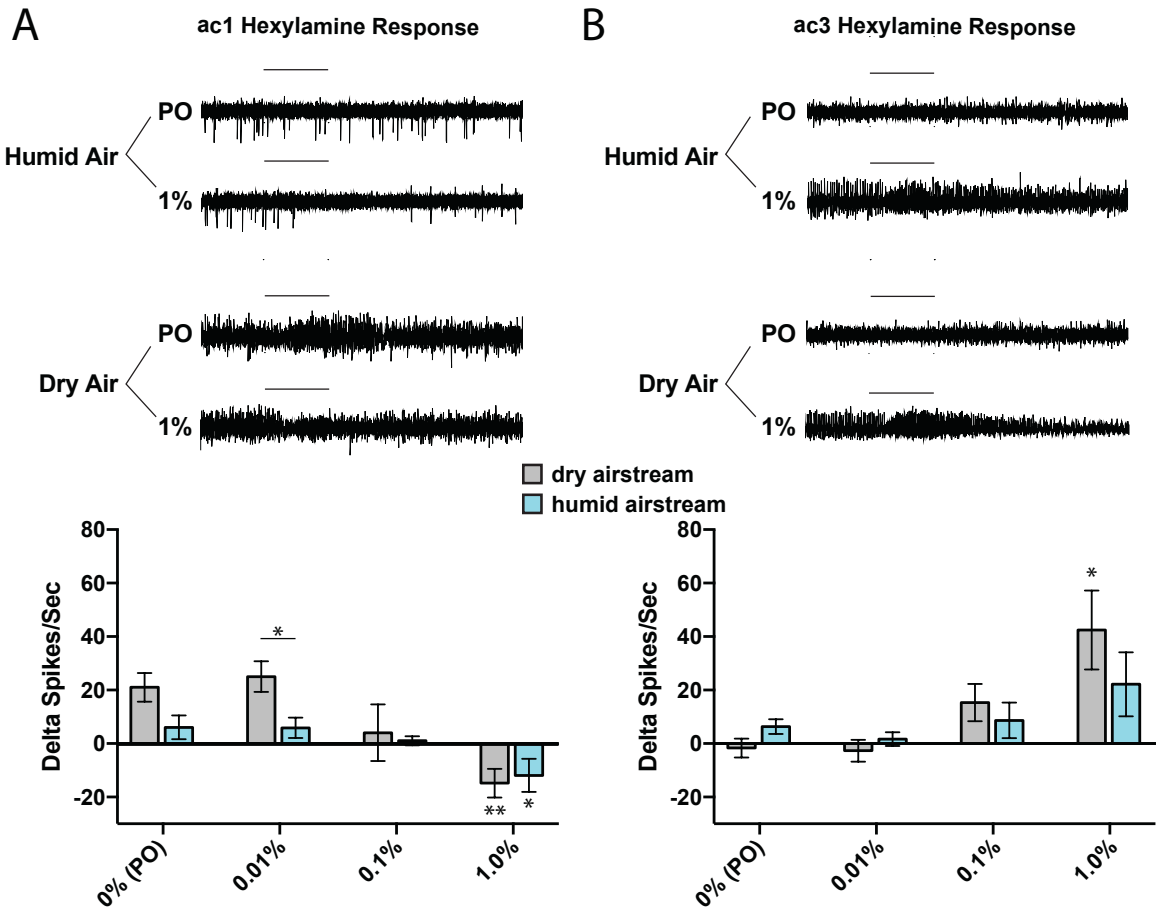

Figure 4.1. Inhibition is conserved in conserved IR-expressing olfactory neurons in the Drosophila antenna

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(A) Representative traces (bottom) and mean responses (top) from the ac1 neurons for a 1-sec period of stimulation of hexylamine at the indicated concentrations. Each count was begun at the start of the increase in spike frequency. All compounds were dissolved in paraffin oil. All recordings were obtained from 3-5 day old wildtype (CS) flies. n = 5-6 sensilla from 5-6 flies. Error bars indicate s.e.m. *p<0.05; **p<0.01. **(B)** Representative traces (bottom) and mean responses (top) from the ac3 neurons for a 1-sec period of stimulation of hexylamine at the indicated concentrations. Each count was begun at the start of the increase in spike frequency. All compounds were dissolved in paraffin oil. All recordings were obtained from 3-5 day old wildtype (CS) flies. n = 6 sensilla from 6 flies. Error bars indicate s.e.m. *p<0.05.

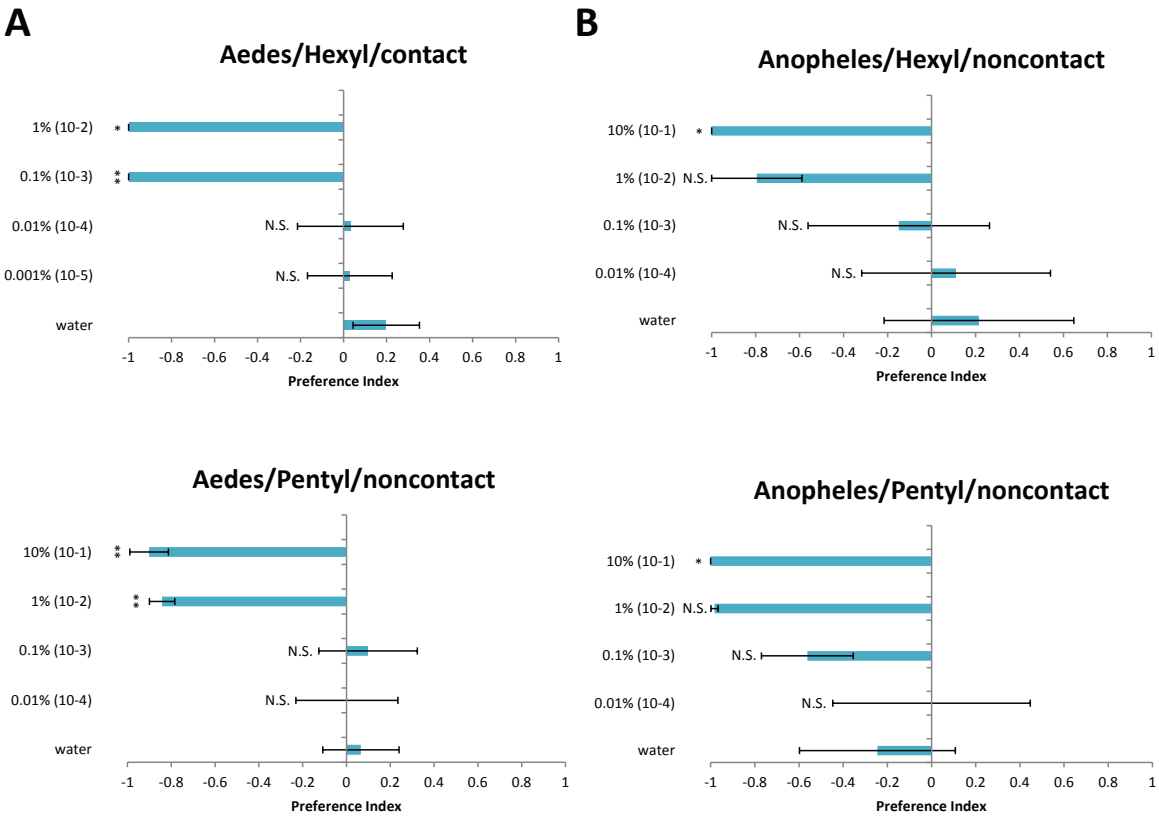

Figure 4.2. Humidity-mediated behavior of ACP and oviposition behavior of mosquitoes is reduced by antagonists

(A) Oviposition preference index in *Aedes aegypti* mosquitoes to the indicated concentration of hexylamine (top) and pentylamine (bottom) vs water alone. N = 4-6 trials with 15 mosquitoes per trial. Error bars represent s.e.m. *p<0.05; **p<0.01; N.S. = not significant. **(B)** Oviposition preference index in *Anopheles* mosquitoes to the indicated concentration of hexylamine (top) and pentylamine (bottom) vs water alone. N = 4 and 6 trials respectively, with 15 mosquitoes/trial. Error bars represent s.e.m. *p<0.05; N.S. = not significant.

CHAPTER 5

CONCLUDING REMARKS AND FUTURE DIRECTIONS

A recapitulation of the importance of olfactory repellency research

Insect vectors are responsible for millions of deaths and hundreds of billions of dollars in damage each year. They carry diseases including malaria, Zika, and dengue that ravage tropical regions. Malaria, just one of the many diseases spread by insect vectors, is responsible for 1,000,000 deaths every year. In addition to the appalling adversity certain insects present to human health, the impact they have on agriculture should not be understated. It is estimated that pests contribute up to 10% loss of total crop production worldwide. The cost of loss was estimated to exceed $17 billion in Brazil alone (Oliveira, Auad et al. 2014). Because of their diversity, abundance, and nigh ubiquity on land, insects represent the largest group of animal competitors of humanity.

One of the most ancient and effective tools for combating insect pests is chemical repellency. The denizens of early societies observed that certain plant extracts would ward off biting insects and applied them in oils or burned plant matter to generate smoke (Charlwood 2003). By the twentieth century, improved chemical synthesis methods allowed for a systematic, albeit trial-and-error, testing of synthetic compounds for repellent activity. The most effective product of these screening efforts, N,N-diethyl-meta-toluamide (DEET) (Mccabe, Barthel et al. 1954), is to this day the most successful repellent on the market (Leal 2014). Understanding how this molecule is detected and processed by the insect brain is

therefore useful in the development of low cost or otherwise more desirable alternatives. However, because of the lack of genetic tools available in mosquitoes, very little is currently understood about how these vectors detect and avoid DEET. Fortunately, repellency to DEET is conserved across many species of insects including the fruit fly *Drosophila*.

DEET elicits diffuse activation and selective inhibition and has a universal repellent effect

Here we have shown that DEET activates a wide range of ORNs and that this broad activation is conserved among flies and mosquitoes. Further, it differentially acts upon individual receptors, as ectopically-expressing individual receptors in the ab3A neuron alters the DEET response. Currently, there exist several models, not necessarily mutually exclusive, for how DEET results in repellency. The “smell-and-avoid” model (Syed and Leal 2008, Xu, Choo et al. 2014) states that DEET acts as a normal aversive volatile compound, activating labeled-line circuits hardwired to elicity an avoidance response. Contrastingly, the “confusant” model (Pellegrino, Steinbach et al. 2011, DeGennaro, McBride et al. 2013) and the “sequestering” model (Afify, Betz et al. 2019) require that DEET be combined with other odors in order to have its impact on behavioral output.

Our results suggest that some combination of the above is the most accurate interpretation of the evidence. Like the “smell-and-avoid” model, we show that DEET is capable of repelling insects without the presence of other odorant

compounds (Fig 2.1D-F). However, the wide-ranging effect of DEET on the ORNs may indeed serve as a kind of “confusant” that does not act in a manner consistent with the traditional understanding of labeled-lines. It is unlikely that a single ORN is responsible for the aversion to DEET, as it repels too broad a range of arthropods, including non-insect arthropods like ticks which lack completely the *Or* family in their genomes (Penalva-Arana, Lynch et al. 2009, Gulia-Nuss, Nuss et al. 2016, Eyun, Soh et al. 2017).

A word on the evolutionary significance of repellency due to broad ORN activity modulation

The universality of DEET’s repellency is a topic that warrants some discussion. While ammonia is a naturally-occurring compound common in many natural sources, DEET is of entirely xenobiotic (ie: synthetic) origin, and has thus never played a role in the evolution and ecology of any organism on Earth. Why such a compound should induce a common behavioral response across all insect species that detect it is therefore a fascinating question. How any response to sensory stimuli is effected by a nervous system is ultimately a question of molecular mechanisms in any species, and the current mechanisms by which DEET is transduced remain largely unknown. To answer the question of how the behavioral response to DEET is so ubiquitous, these mechanisms must first be discovered in many species and then cross-analyzed to see if an evolutionary picture emerges.

How DEET interacts with its molecular targets in insect chemosensory systems to produce widespread activity and why widespread neural activity leads to aversion are two separate questions. The answer to the latter seems relatively straight-forward. Extremely high concentrations of most chemicals are toxic, and ORNs are more broadly tuned at high odorant concentrations than at low odorant concentrations (Hallem and Carlson 2006). Therefore, a situation where a large number of ORNs were strongly effected at any given time would, in its natural context, signify that dangerously high concentrations of the specific chemical were present, and thus organisms that responded aversively would be favored by natural selection. While having broad scope and deep power, this explanatory story still does not explain how DEET repels arthropods that lack Ors completely.

One possibility could be that DEET mimics a natural compound, present in the ecological niches in which a multitude of insect genera evolved, that acted as a noxious stimulus (perhaps a toxin such as a plant alkaloid). Some precedent for this has been proposed in *Culex quinquefasciatus*, in which DEET triggers activity of the same neurons that detect the aversive plant volatile methyl jasmonate (Xu, Choo et al. 2014). However, the receptor responsible for this detection, CqOr136, does not have any homologs in other insects known to detect and avoid DEET, including other mosquito species. If a commonly occurring noxious chemical, on whose transduction mechanisms DEET is “piggybacking”, explains the ubiquity of DEET’s repellent effect, then the origins of its ecological relevance must be found in a stage of insect evolution vastly more primordial than those of methyl

jasmonate, or for that matter those of any species-specific chemical interaction. Such a compound remains elusive.

Another possibility is that DEET mimics not an environmental cue of any kind but instead acts as a disruptor of cellular activity of some type. While this hypothesis appears at first glance to be contradicted by the fact that *Orco-/-* mutant flies and mosquitoes have severely reduced ability to detect and avoid DEET, it is not necessarily so. Insects are covered in protective exoskeleton made of chitin, the hydrophobicity of which is enhanced by cuticular hydrocarbons. This would prevent most contact chemicals from immediately entering into the soft tissue and disrupting cellular function. However, because the primary olfactory neurons are specifically designed to interact with chemicals in the environment, they would be among those most susceptible to these effects. Because *Orco* is necessary for most of the spontaneous activity of olfactory neurons (Dobritsa, van der Goes van Naters et al. 2003, Olsen, Bhandawat et al. 2007), and because the *Orco/OrX* complex is both directly and indirectly responsible for proper wiring during olfactory system development (Brochtrup and Hummel 2011), it follows that DEET-induced disruption of olfactory neuron function would not be as impactful on behavior in *Orco-/-* mutants as compared to wild type insects. More cellular experiments, analyzing a wide range of parameters, will need to be done to solve this question.

The hunt for the molecular transduction mechanisms of DEET and ammonia

The evidence for the molecular target of DEET in ORNs is mixed. The ability of an ectopically-expressed *Or* to recapitulate its response to DEET depends on the identity of the neuron in which it is expressed, as, for example, Or47b, which is unresponsive to DEET in its natural at3 sensillum (Fig 2.2B), is still unresponsive to it when expressed in the ab3A neuron (Fig 2.12A) but confers responsiveness when expressed in the at1 sensillum (Fig 2.12B). One possibility for these contradictory observations is that cells of the different sensilla produce different "assisting" compounds, such as odorant binding proteins, that play a role in the transduction mechanisms.

The fact that Orco is necessary to for proper localization of the receptor complex in the dendritic membrane (Benton, Sachse et al. 2006) further complicates the effort, as it renders ambiguous the observation that Orco mutants do not respond behaviorally or electrophysiologically to DEET. Do mutants not respond because DEET binds the complex directly, or does it bind something else that is also altered in Orco mutants? Several Grs are known to be expressed in the *Drosophila* antenna (Scott, Brady et al. 2001). However, mutational analysis of each has thus far shown no significant difference in the electrophysiological responses of ORNs in wildtype flies vs flies mutant for individual Grs. A similar negative result has been found with TrpA1 mutants.

Like DEET, the mechanism by which ammonia is broadly activating and then silencing antennal neurons remains unknown. However, there is reason to

think this puzzle will be easier to solve. Because the odorant-induced receptor potentials are intact during the post-exposure silencing period, the membrane potential must be largely unaffected. It is therefore unlikely that potassium channels are blocked, as is the case when neurons are exposed to the similarly-structured tetraethylammonium, as this would prevent cation efflux and drive the membrane potential to a much more positive voltage. One possibility is that the positively-charged ammonium ion diffuses through the cell rapidly, causing a sharp depolarization and resulting action potential burst, and then effects the voltage-gated sodium channels in such a way that they undergo a prolonged inactivation that outlasts the period required for the resting potential of the neuron to return to a normal range.

Because this effect is recapitulated by other basic amines, and because altering the *pH* of the solution by any means can also inhibit activity of taste neurons, it is possible that the basicity of ammonia is what would be affecting the voltage-gated sodium channels. Voltage-gated sodium channels are known to be effected by *p*H levels (Khan, Romantseva et al. 2002, Jones, Peters et al. 2013, Ghovanloo, Peters et al. 2018), and the fact that the burst-silencing effect that we observe in olfactory neurons correlates with the basicity of the compounds lends credence to this hypothesis.

To those who carry on

Further molecular work must be done to discover the mechanisms through which DEET and ammonia exert their incredibly broad effects on olfactory and gustatory neurons in insects. Once their targets are identified, the chemical structures by which they bind to DEET can be focused on in chemical informatics searches for safer and more efficacious compounds. This work is already being done and its results have yielded substantial promise (Boyle, Guda et al. 2016). We hope that this work will lay the foundation for successful efforts in discovery of new repellents of insect vectors of both human and agricultural disease.

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APPENDIX

The following is an excerpt of my work from an unpublished manuscript on which I am a contributing author.

We examined responses to (E)-2-hexenal in the larval dome sensillum. Responses to (E)-2-hexenal at 10^{-4} were substantially reduced in the mutant compared with the control line and a reduction in response was observed even with the 10^{-2} concentration (Figure A1A, B).

While multiple attractive Or channels could be involved in the attraction to the higher concentrations of (E)-2-hexenal in combination, which would be difficult to test, we picked one receptor that showed greatest activity. In order to test whether Or42a is necessary for the valence reversal, we presented *Or42a-/-* mutant flies with (E)-2-hexenal alone at 10^{-2} and 10^{-3} concentrations, which are attractive to wild type flies. If Or42a alone necessary for the attractive response to high concentrations of (E)-2-hexenal, then we would expect the attractive response to be reduced in these mutants. However, we found that there was no significant difference in the attractive preference indices of wild type and *Or42a-/-* mutant flies at the 10^{-2} and 10^{-3} concentrations (Fig A2C), indicating that although Or42a is sufficient to induce the valence switch for other odorants presented in a mixture, it is not necessary to do so and its absence is likely compensated by one or more of the other receptors that detect this compound. Or42a mutant was verified with 4-hexene-3-one which showed a defect (Fig A3B)

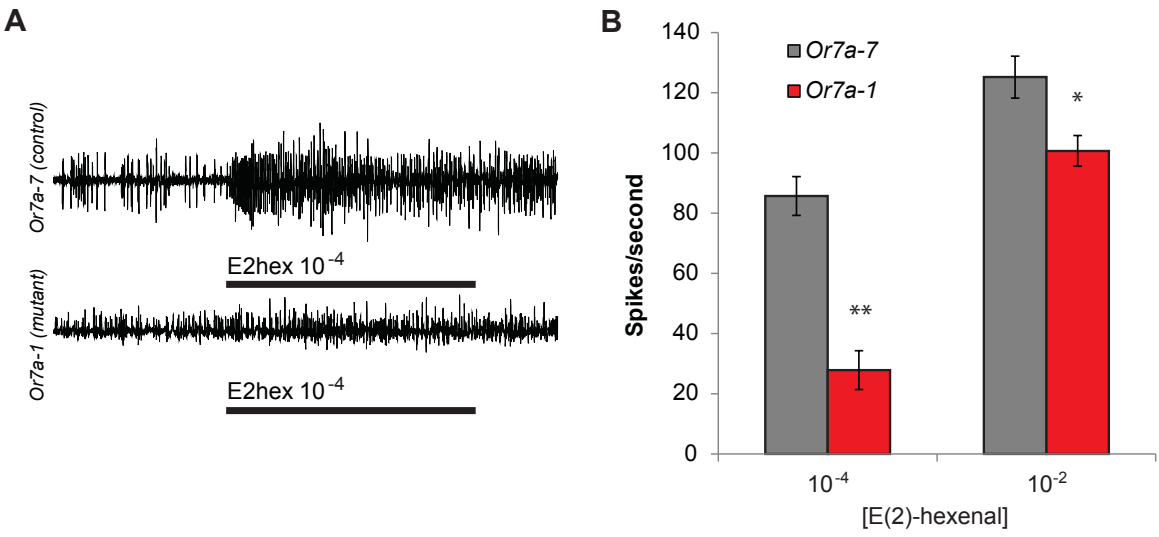

Figure A1. Or7a is necessary for electrophysiological response to low concentrations of (E)-2-hexenal in larvae.

(A) Representative traces from single-unit electrophysiological recordings from larval dome sensilla and **(B)** mean responses showing the response to (E)-2-hexenal at 10^{-4} in control and Or7a mutant animals. N=6-7. Unpaired T-test p*<0.05, p**<0.0001. Error bars represent +- s.e.m. Benz= Benzaldehyde, Bomb= bombykol, E2hex= (E)-2-hexenal.

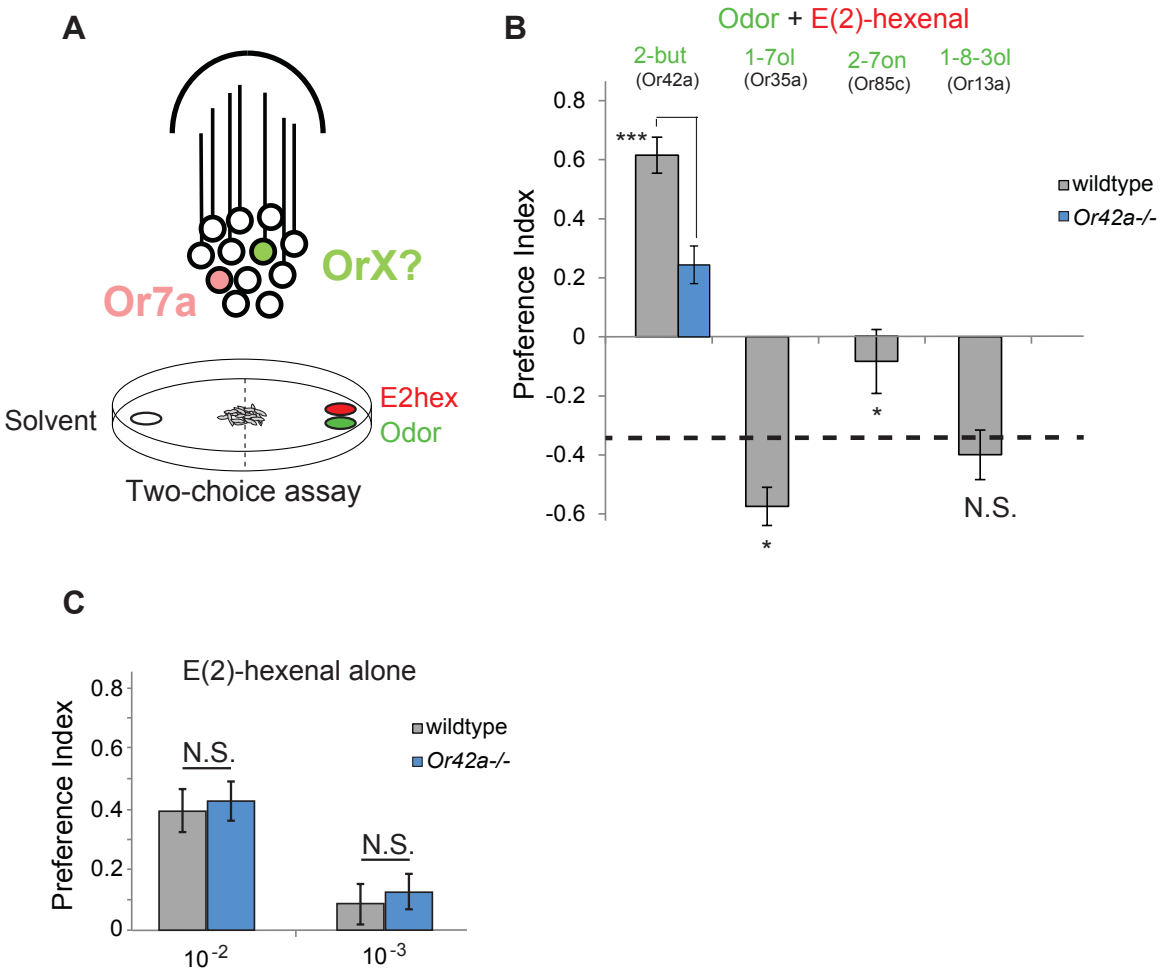

Figure A2. Or7a-driven repellency can be overridden by Or42a or Or42b.

Figure A2. Or7a-driven repellency can be overridden by Or42a or Or42b.

A) Schematic showing larval chemotaxis assay for activating Or7a and one or two additional receptors**. B)** Larval preference indices to odors (0.01%) in addition to (E)-2-hexenal. Known receptor activities to the odors are represented above the data points. Response to (E)-2-hexenal alone is shown with a dotted line. (N = 6-10) Pairwise comparisons between Odor+E2hex and E2hex alone were performed using a 2-tailed Student's T-test: **p <= 0.01 **C)** Larval preference indices to (E)-2-hexenal at 1% and 0.1% concentrations in wild type and Or42a mutant flies. (N = 10) 2-tailed Student's T-test: N.S. = not significant. Error bars represent +- s.e.m.

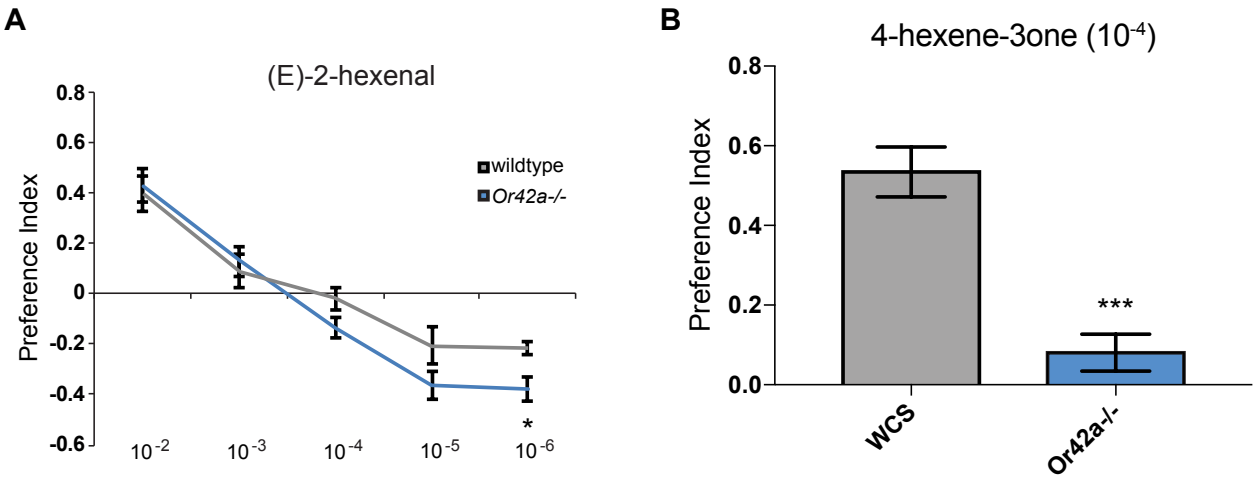

Figure A3. Or42a is not necessary for attraction to high concentrations of (E)-2-hexenal.

A) Dose response curve to (E)-2-hexenal alone in wild type and *Or42a-/-* larvae. Statistical analysis was performed using a 2-tailed Mann-Whitney U test. (N = 10). *p<0.05. Concentrations lacking asterisks are not significant. Error bars represent s.e.m. **B)** Response to 4-hexene-3-one (10^{-4}) in wild type and *Or42a-/-* larvae. Statistical analysis was performed using a 2-tailed Mann-Whitney U test. (N = 10). NS = p>0.05. Error bars represent s.e.m.