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Olfactory processing in the sensory periphery of *Drosophila*

A dissertation submitted in partial satisfaction of the requirements
for the degree Doctor of Philosophy

in

Biology

by

Shiuan-Tze Wu

Committee in charge:

Professor Chih-Ying Su, Chair
Professor Kenta Asahina
Professor William J. Joiner
Professor Michael W. Perry
Professor Jing W. Wang

2022

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University of California San Diego

2022

DEDICATION

This dissertation is dedicated to the memory of my grandmother, Su-Yun Luo, who taught me how to love unconditionally.

TABLE OF CONTENTS

Dissertation Approval Page.....	iii
Dedication.....	iv
Table of Contents.....	v
List of Figures.....	vi
List of Tables.....	vii
Acknowledgements.....	viii
Curriculum Vita.....	xi
Abstract of the Dissertation.....	xiii
Chapter 1: Introduction.....	1
1.1 Sensory neuron compartmentalization is broadly observed across animal species...2	
1.2 Sensory neuron compartmentalization in olfactory sensillum.....4	
1.3 Lateral inhibition in olfactory sensilla likely functions to process valence.....6	
1.4 Key findings and outlook.....8	
1.5 References.....9	
Chapter 2: Valence opponency in olfactory processing.....	13
2.1 Abstract.....	14
2.2 Introduction.....	14
2.3 Results.....	15
2.3.1 ORNs are likely paired based on valence.....	16
2.3.2 Valence opponency in place preference.....	17
2.3.3 Valence opponency in oviposition.....	18
2.3.4 Valence opponency in courtship.....	18
2.3.5 Valence opponency is context-dependent.....	19
2.3.6 A means to process countervailing cues.....	20
2.4 Discussion.....	23
2.5 Material and methods.....	26
2.6 References.....	64
Appendix 1: A peripheral mechanism underlying concentration-dependent valence switch in olfaction.....	68
Appendix 2: Amplification of Drosophila Olfactory Responses by a DEG/ENaC Channel.....	76
Appendix 3: An ammonium transporter is a non-canonical olfactory receptor for ammonia.....	91
Appendix 4: Neuronal Compartmentalization: A Means to Integrate Sensory Input at the Earliest Stage of Information Processing?.....	102

LIST OF FIGURES

Figure 1: Neighboring ORNs housed in the same sensillum do not necessarily exhibit similar odor response profiles.....	38
Figure 2: GAL4-drivers for grouped ORNs.....	39
Figure 3: Valence opponency in place preference.....	40
Figure 4: The influence of satiety state on ORN-mediated place preference.....	41
Figure 5: Valence opponency in oviposition and courtship.....	42
Figure 6: Valence opponency is context-dependent.....	44
Figure 7: Ephaptic lateral inhibition processes countervailing olfactory cues.....	46
Figure 8: at4 ORN spike responses to odor stimulation.....	48
Figure 9: Pheromonal modulation of courtship was abolished in receptor mutants.....	49
Figure 10: <i>shibire</i> -expressing at4C retains its peripheral spike responses.....	50
Figure 11: Countervailing cues detected by non-neighboring ORNs.....	51
Figure 12: Theoretical modeling of valence enhancement by ephaptic inhibition.....	52
Figure 13: Optogenetic place preference apparatus.....	53
Figure 14: Flies' tendency to walk upwind overrides ORN-mediated preference.....	54
Figure 15: Optogenetic oviposition preference apparatus.....	55
Figure 16: ORN spike responses follow a Poisson distribution.....	56
Figure 17: Simulation of valence amplification.....	57
Figure 18: Lateral excitation in the ab2 sensillum.....	73
Figure 19: Lateral excitation between at4 ORNs mediates concentration-dependent valence switch in courtship	74

LIST OF TABLES

Table 1: Compartmentalized <i>Drosophila</i> ORNs and their odor-guided behavioral outputs....	58
Table 2: Compartmentalized <i>Drosophila</i> ORNs and their behavioral outputs (including this study)	59
Table 3: Fly genotypes in figures.....	60
Table 4: Reagent or resource.....	62

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Chapter 1, in part and with modifications, is material as it appears in Neuronal Compartmentalization: A Means to Integrate Sensory Input at the Earliest Stage of Information Processing? 2020. Ng, Renny; Wu, Shiuan-Tze; Su, Chih-Ying, *Bioessays* 42: 2000026, 2020. The dissertation author and Renny Ng contributed equally to this paper as primary authors.

Chapter 2, in part, is material as it appears in Valence opponency in peripheral olfactory processing 2022. Wu, Shiuan-Tze; Chen, Jen-Yung; Martin, Vanessa; Ng, Renny; Zhang, Ye; Grover, Dhruv; Greenspan, Ralph J.; Aljadeff, Johnatan; Su, Chih-Ying, *Proc. Natl. Acad. Sci. USA*, 119 (5) : e2120134119, 2022. The dissertation author and Jen-Yung Chen contributed equally to this paper as primary researchers and authors.

Appendix 1, in part is currently being prepared for submission for publication, under the title “A peripheral mechanism underlying concentration-dependent valence switch in olfaction”. Wu, Shiuan-Tze; Martin, Vanessa; Su, Chih-Ying. The dissertation author is the primary researcher and author.

Appendix 2, in part, is a reprint of the material as it appears in Amplification of Drosophila Olfactory Responses by a DEG/ENaC Channel 2019. Ng, Renny; Salem, Secilia S.; Wu, Shiuan-Tze; Wu, Meilin; Lin; Hui-Hao; Shepherd, Andrew K.; Joiner, William J.; Wang, Jing W.; Su, Chih-Ying, *Neuron* 104: 947-959, 2019. The dissertation author is a co-author of this paper.

Appendix 3, in part, is a reprint of the material as it appears in An ammonium transporter is a non-canonical olfactory receptor for ammonia 2021. Vulpe, Alina; Kim, Hyong S.; Ballou, Sydney; Wu, Shiuan-Tze; Grabe, Veit; Nava Gonzales, Cesar; Liang, Tiffany;

Sachse, Silke; Jeanne, James M.; Su, Chih-Ying; Menuz, Karen, *Current Biology* 31, 1-9, 2021. The dissertation author is a co-author of this paper.

Appendix 4, in part, is a reprint of the material as it appears in Neuronal Compartmentalization: A Means to Integrate Sensory Input at the Earliest Stage of Information Processing? 2020. Ng, Renny; Wu, Shiu-an-Tze; Su, Chih-Ying, *Bioessays* 42: 2000026, 2020. The dissertation author and Renny Ng contributed equally to this paper as primary authors.

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ABSTRACT OF THE DISSERTATION

Olfactory processing in the sensory periphery of *Drosophila*

By

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Doctor of Philosophy in Biology

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In most sensory systems, neurons are organized into topographic maps which allow for comparison of adjacent inputs via lateral inhibition to selectively propagate salient information. However, in olfaction, it is unclear whether a sensory map exists. Here we address this question by examining the organizing principle underlying the stereotyped pairing of olfactory receptor neurons (ORNs) in *Drosophila* sensory hairs (sensilla), where the compartmentalized ORNs can inhibit each other via non-synaptic lateral inhibition. By selectively activating individual ORN types, we found that paired neurons in the same sensillum regulate the same behavior but mediate opposing hedonic value—large-spike neurons convey positive valence whereas small-spike neurons convey negative valence. Remarkably, our behavioral assays reveal that the valence-opponent organization is broadly observed between different sensillum types and is relevant in critical behaviors including place preference, egg-laying, and courtship. Additionally, pheromone perfuming experiments show that antagonistic cues can more profoundly modulate courtship when they are detected by neighboring rather

than by non-neighboring ORNs. Therefore, lateral inhibition between the antagonistic ORNs can evaluate countervailing olfactory inputs and thereby facilitate behavioral decisions. Our study uncovers a valence map in olfaction, whereby environmental cues are processed by simple circuits at the earliest stage of olfactory processing.

Chapter 1: Introduction

Encountered in nature as combinations of chemical and physical cues, environmental stimuli require sophisticated mechanisms for detection and integration. To that end, animals rely on dedicated sensory systems employing processing at multiple layers of nodes. While such processes in the central nervous system often form the focus of studies on signal integration, a more complete understanding of sensory neurobiology requires clarifying how inputs are transformed at every node of the circuit, including neurons in the peripheral nervous system. In numerous sensory organs, external cues are detected by receptor cells compartmentalized within specialized structures consisting of cuticular or epithelial tissues. Within each compartment, multiple neurons share a private microenvironment in which regulation of ionic composition, peri-receptor secretions, or paracrine signaling molecules occurs separately from other sensory units. Furthermore, in certain compartments, adjacent neurons are directly connected via ephaptic couplings, gap junctions, or chemical synapses. The interactions between compartmentalized neurons likely pre-process the mixed sensory cues and pass on the most salient information to higher brain regions. Using the *Drosophila* olfactory peripheral system as a model, my PhD study investigated how olfactory receptor neurons (ORNs) are organized in the periphery and how this feature regulates odor-guided behaviors in fruit flies.

1.1 Sensory neuron compartmentalization is broadly observed across animal species

Sensory neuron compartmentalization is prevalent across species. In the nematode *C. elegans*, for example, the phasmid sensillum houses two chemosensory neurons, whereas the inner labial sensillum houses one chemo- and one mechanosensory cell (Bargmann, 2006). Compartmentalization is particularly prominent in arthropods and has been broadly observed in studies on ultrastructure of their sensory organs. In arachnids, the pectine sensilla of the scorpion *E. italicus* groups 11 chemosensory neurons together with one mechanosensory cell (Foelix and Müller-Vorholt, 1983). In average, the spiny lobster *P. argus* houses ~300 ORNs per sensillum (Steullet et al., 2000); similarly large numbers of grouped ORNs are also observed in other

crustaceans (Hallberg et al., 1997). Most prominently in insects, primary sensory neurons are compartmentalized across nearly all modalities. A well-known example is the trichoid sensillum of the silk moth *B. mori*, which encases the dendrites of two ORNs that detect female pheromones (Gnatzy et al., 1984; Steinbrecht and Gnatzy, 1984).

Sensory neuron compartmentalization is not unique to invertebrate receptor neurons. Found in all fish, the lateral line detects water motions and pressure gradients (Bleckmann and Zelick, 2009). This mechanosensory system's functional unit is the neuromast, which houses upwards of 30 hair cells that detect flow along either direction of the lateral line (Pujol-Martí and López-Schier, 2013). Some fish species, such as weakly electric fish, can sense electrical signals to communicate and navigate. Interestingly, the electrosensory cells are also compartmentalized into specialized electroreceptive organs located in the epidermis throughout the body (Tricas and Carlson, 2012).

Although not as prevalent, some sensory cells in mammals are also compartmentalized. The best known example is the taste buds. On the surfaces of the tongue and palate, taste buds are ovoid structures comprising 50–100 taste receptor cells, which can be categorized into five distinct functional classes: sweet, bitter, umami, sour and salt. (Liman et al., 2014; Yarmolinsky et al., 2009). Their microvilli are oriented towards the taste pore, allowing tastants to initiate gustatory transduction. The signaling cascades lead to depolarization at the basal cellular domain, where neurotransmitters are released and detected by gustatory afferent fibers (Liman et al., 2014; Roper and Chaudhari, 2017; Yarmolinsky et al., 2009).

Interestingly, many types of grouped receptor neurons share a common progenitor cell (Lai and Orgogozo, 2004; Li et al., 2018), and their compartmentalization has been generally taken to reflect developmental constraints. However, such organization likely also contributes to information processing enabled by private communication between grouped neurons, through a shared microenvironment enveloping their dendrites and somata, and/or through the proximity of their axons projecting from the same peripheral unit. Consistent with this idea, synaptic or gap

junction connections are observed between subsets of grouped receptor neurons in scorpions, ants, fruitflies, and bumblebees (Gaffin, 2002; Miriyala et al., 2018; Schnaitmann et al., 2018; Takeichi et al., 2018). On the other hand, a direct electric field effect, known as ephaptic coupling, occurs between ORNs in the same olfactory sensillum of *D. melanogaster* (Su et al., 2012; Zhang et al., 2019).

Despite these interesting observations, the contribution of such organization to sensory processing remains largely underexplored. How do the structural features of sensory compartment influence the outputs of receptor neurons? What functional advantages can compartmentalization confer? How does the organization affect sensory coding, such as stimulus intensity and identity? These questions can be better informed by a close examination on insect's olfactory periphery. Many of their compartmentalized ORNs are accessible for *in vivo* electrophysiological recordings and ultrastructural characterization through electron microscopy (EM). Furthermore, some insect species are genetically tractable, which provide a causal link between molecules to function, morphology, and behavior.

1.2 Sensory neuron compartmentalization in olfactory sensillum

The olfactory system detects and discriminates a wide variety of environmental volatiles to guide most aspects of insect behavior including foraging, feeding, mating, egg-laying and aggression. There are two major olfactory organs: the maxillary palp and the antenna, which contains the majority of olfactory sensilla (de Bruyne and Baker, 2008). In Coleoptera, Lepidoptera, and Diptera, each multiporous sensillum typically encapsulates the dendrites of two to four ORNs that exhibit distinct extracellular spike amplitudes and are correspondingly named “A”, “B”, “C” or “D” by descending spike size. Serial block-face scanning electron microscopy (SBEM) of genetically identified ORNs (Tsang et al., 2018) demonstrates that neuronal size differences reflect the disparity in spike amplitude — the large-spike “A” ORN is generally larger than its small-spike neighbor(s) (Zhang et al., 2019); thus, grouped ORNs are almost always

morphologically inequivalent. At the molecular level, compartmentalized ORNs express distinct receptors, which define the unique odorant specificities for each cell. In nearly all characterized insect species, ORNs are grouped in stereotyped combinations, such that an ORN with a particular response profile is paired with ORNs of different tuning properties (De Bruyne et al., 2001; Chang et al., 2016; Kaissling, 1996; Krieger et al., 2009; Lu et al., 2007; Nakagawa et al., 2005). In all, the conserved pairing of anatomically and functionally distinct ORNs suggests a critical role for this prevalent neuronal arrangement.

Given the stereotypy of ORN grouping, can neurons interact within a sensillum? Beetles offer examples whereby an olfactory stimulus activates one ORN and inhibits its neighbor (Andersson et al., 2009; Nikonov and Leal, 2002). Additionally in *D. melanogaster*, the compartmentalized ORNs can inhibit each other in multiple sensillum types (Su et al., 2012). Despite the lack of direct synaptic connections (Shanbhag et al., 1999, 2000), strong activation of one ORN suppresses the activity of its neighbor (Su et al., 2012). Based on Vermeulen and Rospars' electric circuit modeling (Vermeulen and Rospars, 2004), it was predicted that ephaptic interaction underlies the crosstalk between grouped ORNs (Su et al., 2012). Indeed, by recording from pairs of olfactory sensilla impaled by the same tungsten electrode, a following study provides direct experimental evidence to demonstrate that ephaptic interaction alone is sufficient to drive lateral inhibition between ORNs that share the same electric field (Zhang et al., 2019). Remarkably, unequal cell sizes confer disparate circuit functions for compartmentalized neurons: most large-spike "A" ORNs exert greater ephaptic influence and are also less susceptible to electric field changes induced by neighbors (Zhang et al., 2019), allowing for asymmetric lateral inhibition. Importantly, a misexpression experiment shows that the asymmetry is contributed by the electrotonic properties of ORN rather than the receptor types. Unequal ephaptic interactions provide a potential peripheral mechanism to evaluate parallel inputs and selectively favor the propagation of signals carried by the dominant ORNs (Zhang et al., 2019). One possible advantage of ephaptic coupling is its low energy cost in that metabolically expensive synaptic

connections are not required for circuit interaction. Furthermore, before entering higher brain centers, inputs are already filtered and constrained at the olfactory periphery, which in principle allows salient information to be more efficiently processed at subsequent nodes. Critically, this lateral inhibition also modulates behavior in response to odor mixtures (Su et al., 2012), highlighting the capability of peripheral processing in biasing olfactory-driven choices.

The olfactory sensilla of ants, bees, and wasps house over a dozen receptor cells (Getz and Akers, 1994; Martini, 1986b, 1986a; Takeichi et al., 2018), and these exceptionally numerous neurons raise the possibility that grouped Hymenopteran ORNs can potentially employ another type of functional interaction. Indeed in Japanese carpenter ants, Ozaki and colleagues identified gap junction-like adhesions between the dendrites of the 100 or more compartmentalized ORNs. These cells presumably form an electrically coupled neural network, in which weak olfactory inputs have a low probability of generating spike responses (Takeichi et al., 2018). This network computation may underlie the sensillum's remarkable selectivity to cuticular hydrocarbons (CHCs) produced by non-nestmates (Ozaki et al., 2005). Input currents in response to nestmate CHCs are expected to be small due to desensitization from chronic exposure, and such inputs should dissipate within a gap junction-coupled neural network. Conversely, input currents elicited by novel CHCs are likely larger and thus have a higher probability to induce strong spike responses (Takeichi et al., 2018). It will be interesting to determine if such computation applies strictly to pheromone-sensing sensilla and, more generally, whether it is applicable for other Hymenopteran sensilla that house remarkably large numbers of ORNs.

Taken together, functional communication between compartmentalized ORNs may enable individual sensilla to serve as processing units. The olfactory information can be computed either by enhancing stimulus contrast via ephaptic inhibition or by filtering out weak stimuli through gap junction-coupled neural networks.

1.3 Lateral inhibition in olfactory sensilla likely functions to process valence

If lateral inhibition is expected to enhance contrast between parallel inputs, what then is accentuated by ephaptic coupling between grouped ORNs? Valence information is a probable answer. Across insects, a striking pairing pattern emerges: ORNs housed in the same sensillum respond to odorants from ecologically-related sources but elicit opposing behaviors. In cotton bollworm moths, a large-spike “A” ORN senses a behavioral antagonist released by immature females, while the small-spike “B” ORN responds to a pheromone that is attractive at low levels (Chang et al., 2017). Antagonism also occurs in other insects but overwhelmingly, attractive odorants are detected by large-spike ORNs, whereas their small-spike neighbors detect aversive volatiles. When presented with a blend of sex pheromones, silk moths detect attractive bombykol with a large-spike ORN, while its small-spike neighbor responds to aversive bombykal (Kaissling et al., 1978). In spruce bark beetles, the attractive aggregation pheromone excites an “A” ORN while an aversive cue released by damaged host plants is detected by the paired “B” neuron (Andersson, 2012).

Moreover in Japanese beetles, an attractive conspecific sex pheromone is detected by the “A” ORN whereas a behavioral antagonist released by the sympatric Osaka beetle excites the “B” ORN housed in the same sensillum (Nikonov and Leal, 2002). This pairing principle — in which an ORN sensing conspecific odors is grouped with another neuron responsive to sympatric antagonistic volatiles — is observed in multiple insect species (Hansen, 1984; Hansson and Löfstedt, 1987; Mustaparta and Berg, 1995). The prevalence of this antagonistic organization together with its ability to discriminate sympatric from conspecific pheromones suggest an important role for peripheral processing in establishing reproductive isolation (Todd and Baker, 1999).

Such isolation is also achieved through the antagonistic detection of host odors by pairing one ORN that responds to attractive host volatiles with a neighbor that detects aversive, non-host odors. Two subgroups of *R. pomonella* prefer either hawthorns or apples. As shown by Olsson and colleagues, the hawthorn flies sense an attractive host odorant (3-methyl-1-butanol) with an

“A” ORN, which is paired with a small-spike neuron responding to butyl hexanoate, a behavioral antagonist emitted by apples. Conversely for the apple flies, butyl hexanoate is attractive and detected by an “A” ORN, whereas 3-methyl-1-butanol is aversive and detected by a small-spike “B” cell in the same sensillum (Tait et al., 2016). This switch between these neurons’ response profiles, such that the dominant large-spike “A” cell responds to different attractive cues, likely contribute to different host preferences between the two closely related *R. pomonella* subgroups.

Beyond the contexts mentioned above, antagonism is also observed in sensilla that inform other behaviors critical for survival, such as foraging and egg-laying behaviors in *D. melanogaster*. Specifically in the ab1 sensillum, the large-spike and small-spike ORNs detect attractive vinegar odors (Sammelhack and Wang, 2009) and aversive CO₂ (Suh et al., 2004) respectively, both of which are component odorants of fermented fruit. Moreover in the ab4 sensillum, the “A” neuron detects an aggregation pheromone to promote egg-laying (Lin et al., 2015), while the neighboring “B” neuron responds to an aversive mold volatile to inhibit oviposition (Stensmyr et al., 2012). Given the ephaptic interactions known to occur in *Drosophila* ORNs (Su et al., 2012; Zhang et al., 2019), antagonistic inputs can be pre-processed in a sensillum. Therefore, this enhanced valence contrast of sensillar outputs is expected to facilitate behavioral decisions.

Although the examples above support a valence-opponent organization in *Drosophila* olfaction, it remains unclear whether this organizing principle is generalizable across sensillum types. A comprehensive survey of fly’s olfaction periphery would be important to determine the generality of valence opponency.

1.4 Key findings and outlook

In Chapter 2, we addressed several key questions: is there an olfactory map, how might it be organized, and what is the underlying functional significance? We revealed the first functional sensory map for the sense of smell in a complex organism and characterized its valence-opponent organization. Specifically, in *D. melanogaster*, we found that ORNs housed in the same

sensillum antagonistically regulate the same behavior—large-spike neurons convey positive valence while small-spike neurons convey negative valence. Furthermore, we uncovered the map’s function by considering lateral inhibition between compartmentalized ORNs. A previous study shows many natural odors are mixtures with both attractive and aversive components (Zhang et al., 2019), and these countervailing cues can activate paired ORNs that signal both positive and negative valence. We found that the lateral inhibition enhances signal contrast between compartmentalized ORNs, propagates salient cues to higher brain regions, and thereby facilitates animal’s behavioral decisions.

Given that this valence olfactory map likely exists in other insects besides *Drosophila*, we believe this organizing principle provides insights into how to effectively manipulate insect odor-driven behavior. For example, mosquito disease vectors and agricultural pests may be controlled by activating large-spike neurons for traps or activating small-spike neurons for aversive repellants.

Moreover, our study carries implications for odor classification. It will be interesting to examine whether and how this antagonistic ORN pairing shapes odor recognition. A recently proposed mathematical model shows that ephaptic interaction between compartmentalized ORNs facilitates clustered odor representation in downstream circuits (Puri et al., in preparation), raising the possibility that the peripheral neuronal organization can also impact odor recognition and olfactory memory.

Chapter 1, in part and with modifications, is material as it appears in Neuronal Compartmentalization: A Means to Integrate Sensory Input at the Earliest Stage of Information Processing? 2020. Ng, Renny; Wu, Shiuan-Tze; Su, Chih-Ying, *Bioessays* 42: 2000026, 2020. The dissertation author and Renny Ng contributed equally to this paper as primary authors.

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Chapter 2: Valence opponency in olfactory processing

2.1 Abstract

A hallmark of complex sensory systems is the organization of neurons into functionally meaningful maps, which allow for comparison and contrast of parallel inputs via lateral inhibition. However, it is unclear whether such a map exists in olfaction. Here we address this question by determining the organizing principle underlying the stereotyped pairing of olfactory receptor neurons (ORNs) in *Drosophila* sensory hairs, wherein compartmentalized neurons inhibit each other via ephaptic coupling. Systematic behavioral assays reveal that most paired ORNs antagonistically regulate the same type of behavior. Such valence opponency is relevant in critical behavioral contexts including place preference, egg-laying, and courtship. Odor-mixture experiments show that ephaptic inhibition provides a peripheral means for evaluating and shaping countervailing cues relayed to higher brain regions. Furthermore, computational modeling suggests that this organization likely provides a novel mechanism for processing ratio information in odor mixtures. This olfactory valence map may have evolved to swiftly process ethologically meaningful odor blends without involving costly synaptic computation.

2.2 Introduction

In complex sensory systems, neurons are typically organized into functionally meaningful maps. This arrangement allows specific stimulus attributes, such as color or spatial contrast, to be computed via lateral inhibition (van Hateren, 1992). In olfaction, however, it is unclear whether such a functional sensory map exists. In both rodents and flies, sensory neurons which project to nearby glomeruli—processing units in the first olfactory relay center—do not necessarily respond to structurally similar odorants (Hallem and Carlson, 2006; Soucy et al., 2009), suggesting an absence of chemotopic organization at this circuit level.

Might a functional olfactory map instead exist in the periphery? *Drosophila melanogaster* provides a unique opportunity to address this question, as the receptors, ligands, and behavioral outputs have been characterized for many ORNs (Benton et al., 2009; Couto et al., 2005; Fishilevich and Vosshall, 2005; Hallem and Carlson, 2006; Hallem et al., 2004; Silbering et al., 2011) (Table 1). Each sensillum typically houses two or up to four ORNs, which are named “A”, “B”, “C” or “D” in descending order of their stereotypical extracellular spike amplitudes (de Bruyne et al., 2001). ORN pairing in a sensillum is also stereotyped—whereby a neuron expressing a particular receptor always neighbors an ORN expressing another specific receptor (Benton et al., 2009; Couto et al., 2005; Hallem et al., 2004)—implying functional importance for such organization. Indeed, lateral inhibition broadly occurs between compartmentalized ORNs across sensillum types (Su et al., 2012).

Interestingly, the sensillum is the only such place in the olfactory circuit where short-range lateral inhibition is commonly observed between specific input channels (Su et al., 2012; Wilson, 2013; Zhang et al., 2019). Previous work has revealed that direct ephaptic interaction is sufficient to mediate lateral inhibition between electrically coupled ORNs (Zhang et al., 2019). Furthermore, systematic morphological analysis of *Drosophila* antennal sensilla through serial block-face scanning electron microscopy (SBEM) shows that the basic anatomical features that support ephaptic coupling between ORNs—the close apposition of neuronal processes in a confined compartment (Faber and Korn, 1989; Jefferys, 1995)—are conserved across sensillum types (Nava Gonzales et al., 2021). Taken together, these studies consistently support the notion that ephaptic coupling occurs broadly across olfactory sensilla. Each sensillum can thus be considered a processing unit for olfactory computation, and understanding the organizing principle of ORN pairing will elucidate whether a functional olfactory map exists and how it is arranged.

2.3 Results

2.3.1 ORNs are likely paired based on valence

We began by analyzing responses from ORN pairs to a large odor panel in a published dataset (Hallem and Carlson, 2006). The Euclidean distance between neighboring neurons or any two randomly selected ORNs was not significantly different (Fig. 1), arguing against the presence of a peripheral chemotopic map. If sensilla are not processing units for odorant identity, what stimulus attribute might be processed instead? Behavioral valence is a likely possibility. While an odor's valence can be determined by the combinatorial ORN activation and neuromodulatory mechanisms, fly ORNs themselves appear to carry intrinsic valence. A striking pattern emerged from surveying the function of *Drosophila* ORNs: activation of large-spike neurons tends to positively regulate odor-guided behaviors, such as mediating attraction, stimulating egg laying or promoting courtship (Dweck et al., 2013, 2015; Grosjean et al., 2011; Lebreton et al., 2017; Lin et al., 2015, 2016; Min et al., 2013; Prieto-Godino et al., 2017; Ronderos et al., 2014; Schlieff and Wilson, 2007; Semmelhack and Wang, 2009), while activation of small-spike ORNs tends to have antagonistic effects, such as avoidance or inhibiting egg laying (Table 1) (Ebrahim et al., 2015; Semmelhack and Wang, 2009; Stensmyr et al., 2012; Suh et al., 2004). Moreover, some paired neurons play opposite roles in modulating the same behavior. For example, ab4A and its neighboring ab4B promotes and suppresses oviposition, respectively (Lin et al., 2015; Stensmyr et al., 2012). On the basis of animals' behavioral output, we defined positive or negative valence of a given ORN as promotion or inhibition of a certain behavior upon activation of the neuron. We therefore hypothesized that ORNs housed in the same sensillum antagonistically regulate the same behavior.

To test this hypothesis, we conducted systematic behavioral assays with fictive odors. Given that individual ORNs may not mediate innate behaviors in all contexts, we focused on sensillum types in which odor-induced behavior of at least one ORN has been characterized. This

list of published reports—identifying ethologically relevant odor-guided behaviors for individual ORNs—served as the ground truth to direct our choices of sensilla and behavioral contexts in this study (Table 1). Based on receptor-driver availability and labeling specificity (Fig. 2), we examined 23 different ORNs, which represent paired neurons in 10 out of the 17 identified antennal sensillum types that house multiple ORNs (Couto et al., 2005; Lin and Potter, 2015).

2.3.2 Valence opponency in place preference

We first examined the sensilla which house ORNs known to mediate attraction or aversion. In a proof-of-principle experiment, we expressed a red-shifted channelrhodopsin (*CsChrimson*) (Klapoetke et al., 2014) in *ab1C*, which underlies flies' aversion to CO₂ (Suh et al., 2004). When fed with the chromophore all *trans*-retinal, *ab1C::CsChrimson* flies showed robust avoidance to light as expected (Fig. 3 A–C). Using this validated assay, we further examined *ab1A* and *ab1B*, which both mediate attraction to vinegar (Sammelhack and Wang, 2009), and found that their optogenetic activation resulted in the expected preferences (Fig. 3D). For *ab1D*, whose hedonic value was hitherto uncharacterized, we observed aversion upon its activation (Fig. 3D). These results indicate that in the *ab1* sensillum, both large-spike ORNs mediate attraction, whereas both small-spike neurons underlie aversion.

We next assayed other preference-related sensillum types (Table 1 and Fig. 2). Among the four previously characterized large-spike ORNs—*ab5A*, *ai2A*, *ac3IA* and *ab9A* (Lebreton et al., 2017; Prieto-Godino et al., 2017; Ronderos et al., 2014; Schlieff and Wilson, 2007)—all mediated attraction when optogenetically activated (Fig. 3 E–H). Conversely, we observed avoidance caused by activation of their respective small-spike neighbors, except for *ab9B* (Fig. 3 E–H). Of note, we tested both sated and starved flies, and found that satiety state did not switch the hedonic value of target neurons (Fig. 4 A and B), in contrast to findings in *Drosophila* larvae

(Vogt et al., 2021). Taken together, valence opponency was broadly observed in the context of place preference.

2.3.3 Valence opponency in oviposition

Next, we tested our hypothesis in the context of oviposition. Our optogenetic assay recapitulated the antagonistic roles of ab4A and ab4B in regulating egg laying (Lin et al., 2015; Stensmyr et al., 2012) (Fig. 5 A and B). We then extended our analysis to ab10 and ai3, the other two known oviposition-related sensilla (Dweck et al., 2013; Ebrahim et al., 2015). Optogenetic activation of ab10B suppressed egg laying, concordant with the neuron's reported role (Ebrahim et al., 2015), whereas activation of ab10A enhanced oviposition as predicted (Fig. 5C). We observed a trend of increased, albeit statistically non-significant ($p=0.07$), oviposition promotion with activation of ai3A (Fig. 5D), consistent with a previous report (Dweck et al., 2013). Meanwhile, activation of the small-spike neighbor that expresses Or43a reduced oviposition (Fig. 5D). These results support our hypothesis and show that valence opponency also holds for oviposition-related sensilla.

2.3.4 Valence opponency in courtship

Does valence opponency also modulate courtship? In *D. melanogaster*, male courtship is suppressed by the singly housed at1 (Kurtovic et al., 2007), but promoted by ac4A and at4A (Dweck et al., 2015; Grosjean et al., 2011; Lin et al., 2016). We began by examining at4A, which expresses the Or47b receptor (Dweck et al., 2015; Lin et al., 2016), in a thermogenetic assay where three males of different genotypes—*Or47b-GAL4::UAS-TrpA1* and both parent-line controls—competed to mate with one wildtype female (Fig. 5E). Single-sensillum recordings verified at4A's thermogenetic activation (Fig. 5E). Concordant with published reports (Dweck et

al., 2015; Lin et al., 2016), the copulation rate of at4A-activated males was markedly higher than in controls (Fig. 5F). Having validated the assay, we further tested the small-spike at4B and at4C and found that their thermogenetic activation indeed suppressed courtship as predicted (Fig. 5 G and H).

A similar antagonistic relationship was observed between ORNs housed in the ac4 sensillum (Fig. 5I). Thermogenetic activation of ac4A increased its spike activity (Fig. 5I) and conferred a copulation advantage as reported (Grosjean et al., 2011) (Fig. 5J). In contrast, courtship suppression was observed upon activation of its small-spike neighbor that expresses the Ir76a receptor (Fig. 5K). We excluded the other small-spike ac4 ORN in our analysis because its receptor, Ir75d, is also expressed in other neurons (Benton et al., 2009), thus preventing specific manipulation of the neuron. Together, these results indicate that valence opponency also underlies ORN pairing in a courtship context, further supporting our hypothesis that ORNs housed in the same sensillum can mediate related but opposite behaviors.

2.3.5 Valence opponency is context-dependent

Does valence opponency hold in all behavioral contexts for a given sensillum? To address this question, the ab1, ab4 and at4 sensilla—which we characterized in place preference, oviposition and courtship, respectively—were further examined under additional contexts. In the ab1 sensillum, activation of ab1A did not influence oviposition or courtship, while ab1C suppressed oviposition but not courtship (Fig. 6 A–C), indicating that a given ORN does not necessarily influence all behaviors. Further experiments showed valence opponency between ab4A and ab4B for courtship (Fig. 6 D–F), and between at4A and at4C for place preference but not for oviposition (Fig. 6 G–I). Collectively, these results suggest that ORNs differentially regulate distinct behaviors, and that valence opponency in a given sensillum likely holds only in ethologically relevant behavioral contexts (Fig. 6J).

In summary, antagonizing ORN pairs were identified in 9 of the 10 examined sensilla (Table 2). Valence opponency is thus broadly observed between compartmentalized ORNs across sensillum types in multiple behavioral contexts. Our results thereby uncover a behavioral valence map in the *Drosophila* peripheral olfactory system.

2.3.6 A means to process countervailing cues

What is the functional significance of valence opponency in olfactory processing? We previously showed that lateral inhibition between a pair of ab1 ORNs biases preference to an odor mixture to influence behavior (Su et al., 2012). When paired ORNs are concurrently activated by odor mixtures, lateral inhibition enables the dominant cue to be selectively propagated (Su et al., 2012; Zhang et al., 2019). As such, opposing constituents in a mixture are no longer represented in their original proportions; rather, the salient cue is favorably transmitted. Consequently, countervailing cues may no longer be sent as countervailing signals that could mitigate each other at higher olfactory centers (Mohamed et al., 2019; Qiu et al., 2021). Antagonistic odorants are expected thus to influence behavior more effectively when detected by paired ORNs than by non-neighboring neurons.

To test this prediction, we focused on two pheromone-sensing sensilla. The at1 sensillum houses a single ORN, which expresses Or67d and detects the anti-aphrodisiac pheromone *cis*-vaccenyl acetate (cVA) (van der Goes van Naters and Carlson, 2007; Kurtovic et al., 2007; Tal and Smith, 2006). In the at4 sensillum, the “A” neuron expresses Or47b, responds to palmitoleic acid, and promotes courtship (Lin et al., 2016), while the small-spike “C” neuron expresses Or88a, responds to methyl palmitate (Dweck et al., 2015), and suppresses courtship (Fig. 5H). Notably in the at4 sensillum, these pheromone ligands only activate the receptors of the target ORNs (Dweck et al., 2015; Lin et al., 2016). Using their respective pheromone ligands, paired at4 ORNs

were co-activated with a mixture of palmitoleic acid and methyl palmitate, or unpaired at4A and at1 neurons with palmitoleic acid mixed with cVA.

We first verified lateral inhibition of at4A by at4C via single-sensillum recording. We found that strong activation of at4C markedly inhibited at4A only when at4A was also stimulated by palmitoleic acid (Fig. 7A, Fig. 8A), consistent with previous findings that ephaptic inhibition between grouped ORNs requires odor-induced excitation from both neurons (Su et al., 2012). However, no significant inhibition of at4A was observed by the Or67d ligand cVA regardless of palmitoleic acid stimulation (Fig. 7B, Fig. 8B). Although cVA is reported to also activate at4B (van der Goes van Naters and Carlson, 2007), we did not observe such responses (Fig. 7B), in agreement with other studies (Gomez-Diaz et al., 2013; Pitts et al., 2016). Through lateral inhibition, methyl palmitate-evoked at4C activity, but not cVA-evoked at1 activity, can suppress at4A's response to palmitoleic acid (Fig. 7 C–D).

Next, we examined pheromone-mediated courtship modulation. When applied directly on females, low doses of palmitoleic acid enhanced copulation in a manner dependent on the male Or47b receptor (Fig. 9 A and B). Conversely, perfuming females with higher doses of methyl palmitate or cVA resulted in comparable courtship inhibition in a receptor-dependent manner (methyl palmitate: 27%; cVA: 24%) (Fig. 7 E–G, and Fig. 9 C and D). The modulation was also eliminated when synaptic transmission was blocked by expressing *shibire^{ts1}* in at4C or at1 neurons (Fig. 7 H–J), thus demonstrating the specificity of these pheromone ligands.

Interestingly, when methyl palmitate or cVA was presented with palmitoleic acid in a mixture, palmitoleic acid-induced courtship was markedly attenuated by methyl palmitate (27%) but to a lesser extent by cVA (13%) (Fig. 7 K–M), despite their similar anti-aphrodisiac effects when applied alone (Fig. 7G). As negative controls, we performed similar odor-mixture experiments with receptor mutant flies (*Or88a^{-/-}* or *Or67d^{-/-}*), wherein courtship inhibition was eliminated as expected (Fig. 9 E and F). A parsimonious interpretation of these results is that

through ephaptic inhibition, at4C activation attenuates at4A output, thereby selectively propagating at4C signal to suppress courtship. In contrast, at1 activation does not influence at4A peripheral responses (Fig. 8B), resulting in propagation of countervailing pheromone signals that may mitigate each other at higher brain centers and thus attenuate their efficacy in modulating behavior.

To test whether peripheral lateral inhibition is sufficient to modulate behavior, we blocked synaptic transmission from Or88a ORNs with *shibire^{ts1}* (Fig. 7H). This manipulation did not affect at4C's spike responses or its ability to inhibit at4A (Fig. 10). Strikingly when palmitoleic acid was co-applied, methyl palmitate reduced courtship by 20% despite the absence of at4C feedforward output (Fig. 7 N and P). These results demonstrate that at4A's output is indeed attenuated by at4C ephaptic inhibition. In comparison, the anti-aphrodisiac effect of cVA was completely abolished in *Or67d::shibire^{ts1}* males even when palmitoleic acid was co-applied (Fig. 7 O and P).

As negative controls, we performed similar odor-mixture experiments except that palmitoleic acid was replaced by a pheromone ligand for ab4A (Lin et al., 2015), an ORN type which we found also promotes courtship (Fig. 6F). As expected, the ab4A ligand 9-tricosene promoted male courtship in a receptor-dependent manner (Fig. 11 A and B). Notably, we no longer observed differential anti-aphrodisiac effects between methyl palmitate and cVA when either anti-aphrodisiac pheromone was co-presented with 9-tricosene, regardless whether the males were wildtype, *Or67d::shibire^{ts1}* or *Or88a::shibire^{ts1}* (Fig. 11 C–H). These results demonstrate that methyl palmitate in a mixture is not inherently more effective than cVA in suppressing courtship. Taken together, these results highlight a critical role of ephaptic inhibition in processing countervailing olfactory cues detected by compartmentalized neurons.

To further investigate the impact of ephaptic coupling on valence contrast, we modeled paired ORNs' responses to binary mixtures of their respective ligands. In our model, activation of one neuron ephaptically inhibits its neighbor, and the degree of inhibition scales nonlinearly with

each neuron's response, which is determined by the stimulus strength (S_A or S_B , Fig. 12A; see SI Methods for modeling details). Ephaptic inhibition increases the response difference between paired ORNs ($\Delta_X = X_A - X_B$) relative to the difference between stimuli ($\Delta_S = S_A - S_B$). Given that paired ORNs mediate antagonistic behaviors, the net behavioral output is more robust when valence contrast is enhanced by ephaptic inhibition. The ratio Δ_X/Δ_S was therefore defined as the degree of valence amplification (α) (Fig. 12B). Our mathematical analysis showed that the magnitude of amplification varies depending on the ratio of respective stimulus strength in a mixture (Fig. 12C). In other words, certain S_A to S_B ratios are expected to more effectively elicit valence amplification, and thus more effectively trigger robust behavioral response. This finding may explain why certain insect pheromone blends, whose constituents typically activate compartmentalized ORNs, can most effectively trigger robust behavior when presented in narrowly defined ratios (Löfstedt, 1990; Ng et al., 2020). Therefore, the valence-opponent organization between ephaptically coupled ORNs may have evolved to process ethologically meaningful ratios of odor mixtures.

2.4 Discussion

Here we identify a valence-opponent olfactory map in *D. melanogaster* (Table 2). This organization is likely conserved among insects, as other compartmentalized insect ORNs have been reported to detect antagonistic cues (Ng et al., 2020). But whereas previous studies reported valence opponency in a small number of select ORN pairs, our study demonstrates that this principle holds broadly across antennal sensilla. Our findings also suggest that the hedonic value of an insect ORN can be predicted based on its relative extracellular spike size. For example, in moths and beetles, attractive sex or aggregation pheromones are typically detected by large-spike ORNs, whose small-spike neighbors instead respond to behavioral antagonists (Ng et al., 2020). Interestingly, this organizing pattern is also observed in maggot flies. Two subgroups of *R.*

pomonella prefer either hawthorns or apples. The hawthorn flies sense an attractive host plant odorant (3-methyl-1-butanol) with an “A” ORN, which is paired with a small-spike neuron responding to a behavioral antagonist emitted by apples (butyl hexanoate). Conversely for the apple flies, butyl hexanoate is attractive and detected by an “A” ORN, whereas 3-methyl-1-butanol is aversive and detected by a neighboring small-spike neuron (Tait et al., 2016). In addition, the aversive CO₂ cue is detected by a small-spike ORN (ab1C) expressing Gr21a/Gr63a receptors in fruitflies (Jones et al., 2007; Kwon et al., 2007; Suh et al., 2004); in mosquitoes, however, CO₂ is an attractive arousal cue which is in turn sensed by a large-spike ORN (cpA), despite its expression of Gr21a/Gr63a’s orthologous receptors (Lu et al., 2007).

Our observations also raise many interesting questions for future research. Why do large- and small-spike ORNs signal behaviors of positive and negative valence, respectively? In the context of valence opponency, how does the asymmetric nature of ephaptic interaction between compartmentalized ORNs influence its circuit function, given that large-spike ORNs can exert greater ephaptic influence on their small-spike neighbors (Zhang et al., 2019)? Why do some sensory hairs, such as the ab1 and at4 sensilla, house more than one large- or small-spike ORNs? How is the expression of OR genes coordinated for compartmentalized neurons, and what could be the evolutionary driving force for such an arrangement? Moreover, at the circuit level, it will be interesting to determine how paired ORNs are wired to mediate antagonistic behaviors. It is hoped that our study will lay the foundation for future work to address these intriguing and important questions.

Natural odors are notoriously complex, as they often contain countervailing cues. For example, many fruit odors include components that simultaneously activate large- and small-spike ORNs within the same fruitfly sensillum (Zhang et al., 2019). Moreover, in most insect species examined, paired ORNs detect antagonistic components of pheromone blends (Ng et al., 2020). Furthermore, mixtures of odors with opposing valence elicit strong inhibition in certain attractant-

responsive input channels, a phenomenon mediated by GABAergic interneurons in the antennal lobe (Mohamed et al., 2019). Such complexity poses a unique challenge to the olfactory system's ability to evaluate natural odors and guide behaviors, as antagonistic components in an odor mixture may mitigate each other at higher olfactory centers (Mohamed et al., 2019; Qiu et al., 2021). This challenge can be met through the operation of lateral inhibition, which is known to enhance the signal-to-noise ratio of salient cues among concurrent inputs (van Hateren, 1992). The valence-opponent olfactory map may address this ethologically critical demand by allowing complex olfactory inputs to be filtered for simplification through ephaptic inhibition between grouped ORNs at the first level of the sensory circuit. This arrangement provides a means to both evaluate and shape the countervailing sensory signals relayed to higher brain centers for further processing.

Olfactory coding in both insects and mammals is believed to be distributive, whereby odor identity and hedonic value are determined by unique combinatorial activation patterns of broadly tuned ORNs, which are not held to individually convey valence information (Ramdya and Benton, 2010). In contrast, a small minority of specialist ORNs follows labeled line coding: these neurons respond to a limited number of ethologically salient odors, such as pheromones or predator odors, to mediate robust innate behaviors (Dewan et al., 2013; Haverkamp et al., 2018). However, the distinction between generalists and specialists may not apply in insect olfaction. Given that the majority of ORNs examined by others (Dweck et al., 2013, 2015; Ebrahim et al., 2015; Grosjean et al., 2011; Lebreton et al., 2017; Lin et al., 2015, 2016; Min et al., 2013; Prieto-Godino et al., 2017; Ronderos et al., 2014; Schliep and Wilson, 2007; Semmelhack and Wang, 2009; Stensmyr et al., 2012; Suh et al., 2004) and in this study exhibited inherent valence, we hereby propose a hybrid model that combines the valence labeled line (Liman et al., 2014) and distributive models. Our hybrid model, which accounts for the inherent hedonic values and valence-opponent organization of ORNs, remains compatible with combinatorial coding for odor identities and

context-dependent (Badel et al., 2016) or learning-mediated modulation of odor valence (Davis, 2005). But importantly, as demonstrated in our synaptic silencing experiments, our model reveals that the entire central activation pattern is not necessarily required to define the overall valence of an odor mixture. Therefore, the peripheral mechanism may have evolved to lessen the computational burden in the central brain by swiftly processing antagonistic cues at the first neurons of the olfactory circuit.

2.5 Material and methods

Flies

All flies (*Drosophila melanogaster*) were housed on standard cornmeal food containing molasses at 25°C, ~60% humidity in an incubator with a 12-hr light/dark cycle unless noted otherwise. Flies were collected upon eclosion, separated by sex and used for experiments at indicated ages. The genotypes of fly lines used in this study are listed in Tables 3 and 4.

Immunohistochemistry and confocal imaging

Two to four day-old female flies expressing mCD8-GFP in the target ORNs were anesthetized on ice. The brains were dissected in PBS, fixed in PBS with 4% paraformaldehyde (MPX00553, Fisher Scientific) for 20 min at room temperature (RT). The samples were then washed three times in 0.3% PBT (PBS with 0.3% Triton X-100), and transferred to 0.3% PBT with 10% normal goat serum (NGS) for 2 hr at room temperature. The brains were then stained with rabbit α -GFP 1:400 (A11122, Life Technologies) and nc82 monoclonal antibody 1:50 (Developmental Studies Hybridoma Bank) in a dilution buffer (PBS with 1% NGS and 0.3% Triton X-100) for 48 hr at 4 °C. Samples were washed three times with 0.3% PBT at room temperature before being stained with secondary antibodies, Alexa 488 goat α -rabbit 1:500 (A31628, Life

Technologies) and Alexa 647 goat α -mouse 1:500 (A21236, Life Technologies) in the dilution buffer for 24 hr at 4 °C. After three washes in 1% PBT at room temperature, the brains were mounted in FocusClear (CelExplorer). The samples were imaged on a Zeiss LSM 880 confocal microscope using 40x, N.A.1.2 C-Apochromat water-immersion objective lens. Airyscan images were processed with ZEN (Zeiss) and Fiji (<https://imagej.net/Fiji>).

Optogenetic place preference assay

A custom-designed behavioral assay was used to evaluate the place preference of flies when select ORNs were optogenetically activated. The dimensions of the arena were 80 mm x 35 mm x 3.5 mm with a sloped edge (Simon and Dickinson, 2010). In this assay, a custom LED board was placed beneath the arena (Fig. 13). The LED board was composed of interleaving 625-nm red LEDs (VLMR334BACB-GS08CT-ND, Digi-Key) and 850-nm IR LEDs (VSMY3850-GS08CT-ND, Digi-Key). The LEDs were powered by programmable DC switching power supplies (Model 1697, B&K Precision) and controlled by an Arduino microcontroller with a custom program. An opaque plastic partition (4-cm height) was used to restrict light to one half of the arena. A diffuser (Rosco) was placed immediately underneath the area for homogeneous illumination.

Female flies expressing the red-shifted channelrhodopsin CsChrimson (Klapoetke et al., 2014) were collected daily, group housed in 15 per vial, and reared in constant darkness on standard cornmeal medium supplemented with 0.2 mM all *trans*-retinal (Sigma-Aldrich R2500). Negative controls were age-matched siblings without the retinal supplement. Sated and starved flies were tested separately. For starvation, 24 hr prior to experiments, flies were transferred to empty vials containing dampened Kimwipes with either 1 ml 0.2 mM all *trans*-retinal (experimental groups) or distilled water (control groups). For each experiment, a group of 10 female flies (4 days old) was tested. A group-fly assay was adopted because collective behavior is known to enhance the robustness of ORN-mediated preference (Ramdya et al., 2014). The IR LEDs remained on

constantly on both sides of the arena. At the beginning of each trial, flies were acclimated to the arena under darkness (IR only) for 30 sec. Subsequently, the red LEDs were turned on to illuminate the right half of the arena for 45 sec. After another 30 sec of darkness, the red LEDs were switched on at the left side. Each experiment consisted of three trials with a total of six illuminating periods. Red light irradiance was $1.2 \mu\text{W}/\text{mm}^2$. In some experiments (ab1A and ac3A) where less robust preference was observed ($0.05 > p > 0.01$), higher irradiance ($9.1 \mu\text{W}/\text{mm}^2$) was used.

Flies in the arena were recorded at 30 frames/s using a Flea3 camera (Point Grey Research) with fixed lens (Computar) and a long-pass filter (Edmund Optics) from the top of the arena. The location of flies was determined by a custom tracking C++ code. The preference index of flies to the illuminated side was calculated every half a second based on the positions of flies in the arena. The preference index (PI) was defined as: $PI = (N_{light} - N_{dark}) / N_{total}$ where N_{light} indicates the number of flies on the illuminated half of the arena, N_{dark} indicates the number of flies on the dark half of the arena, and N_{total} indicates the total number of flies in the arena (10 flies). For each experiment, the average preference index was determined by averaging the preference indices during periods of illumination over the three trials. Data were analyzed offline using custom MATLAB codes.

Although our assay did not involve pulsed light or airflow, their respective effects on fly's place preference were also examined in preliminary experiments. A previous study employing single-fly optogenetic reference assays paired light stimulation with a constant airflow (Bell and Wilson, 2016). In pilot experiments, the preference behavior of *Gr21a::CsChrimson* flies was examined in the absence or presence of airflow ($\sim 25 \text{ cm/s}$). In the absence of airflow, the flies exhibited robust aversion to light (Fig. 14A), as expected from ab1C's role in mediating CO₂ aversion (Suh et al., 2004). However with constant air flow, both the control and experimental flies exhibited a strong tendency to walk upwind, resulting in robust attraction towards the side of air

input even when it was illuminated by red LEDs (Fig. 14B). In another pilot experiment, pulsed red light (1, 5, 10, 20 Hz, 50% duty cycle) was tested for *Gr21a::CsChrimson* flies in the absence of airflow. No significant preference difference between pulsed and static light was observed, except in the case of 20 Hz pulsed light where avoidance was attenuated (data not shown). Although varying lighting conditions may influence the degree of preference, it does not alter the polarity of an ORN's valence (Tumkaya et al., 2019). Therefore, for simplicity and consistency, static red light was used throughout the optogenetic assays for all ORN types.

Optogenetic oviposition assay

A custom-designed behavioral assay was used to evaluate the oviposition preference of flies when select ORNs were optogenetically activated. This egg-laying assay employed a custom-built array of transparent acrylic that comprised five individual chambers, each measuring 110 mm x 50 mm x 5 mm. Placed at the bottom of both sides of each chamber was a 40 mm diameter plastic dish, filled with 1% agarose with 5 mM sucrose (Schwartz et al., 2012). A plastic sheet placed on top of the chamber prevented escape by flies, which freely moved between either side of the chamber and laid eggs on the agar dishes. An LED board with 625-nm red LEDs (VLMR334BACB-GS08CT-ND, Digi-Key) was placed beneath the array, while an opaque plastic partition restricted the illuminated field to one side of each chamber (Fig. S15). Data collected from each chamber constituted one experiment, while results from each array of five chambers constituted one trial.

Flies expressing *CsChrimson* were collected daily, separated by sex, group housed in 15 per vial, and reared in constant darkness on standard cornmeal medium supplemented with 0.2 mM all *trans*-retinal (Sigma-Aldrich R2500). Negative controls were age-matched siblings without the retinal supplement. For mating, 15 pairs of 3-day-old females and males were transferred to a new vial containing either the regular (control) or retinal fly food (experimental group)

supplemented with diluted yeast paste; mating was allowed to take place over a 24-hr period. Subsequently, 15 females were loaded into an individual chamber. The side of illumination was alternated between trials (5 experiments per trial, and 4 trials for each condition). Experiments were conducted in a dark incubator at 25 °C for ~24 hours. Red light irradiance was 0.3 $\mu\text{W}/\text{mm}^2$ for all ORN types except for ab10A, which was examined with higher irradiance (2.1 $\mu\text{W}/\text{mm}^2$). Following each experiment, eggs were counted, and the oviposition preference to the illuminated chamber was calculated as: $\text{Oviposition index} = (N_{\text{light}} - N_{\text{dark}}) / N_{\text{total}}$ where N_{light} indicates the number of eggs on the illuminated chamber, N_{dark} is the number of eggs on the other chamber, and N_{total} indicates the total number of eggs. Data were analyzed offline using custom MATLAB codes.

Thermogenetic courtship competition assay

Male flies expressing TrpA1 in the target ORNs and corresponding parental controls were raised at 21°C. Naïve males were raised in groups of ten and transferred to new vials with fresh food every other day as described (Sethi et al., 2019). Prior to courtship assays, flies were acclimated in the behavioral room, which was heated to ~30°C, for 30 min. The courtship competition assays were conducted essentially as described (Ng et al., 2019). In brief, three naïve males of different genotypes and one 3-day-old virgin female (*Canton-S*) were loaded in a mating chamber (4 cm in diameter and 1 cm in height) positioned atop a Petri dish containing diluted fly food (50% water). The base of the chamber comprises a piece of gauze to allow flies access to food odors. To facilitate fly identification, two males of different genotypes were dusted with fluorescent dyes (UVXPBB and UVXPBR, Llewellyn Data Processing LLC) ~20 hours prior to experiments. Dye application was alternated among genotypes across experiments to minimize dye-induced behavioral bias. Courtship competition assays were conducted at ~30°C, under 660-nm red light. For each experiment, 30 matches were set up and only trials in which copulation

occurred within 2 hours were included in the analysis. Courtship was visually confirmed, and the identity of the mated males and the time of copulation were recorded during the 2-hr period. Cumulative copulation rates were normalized to the total copulation rate of all tested males in the same experiments. Data were analyzed using custom MATLAB codes.

Pilot experiments testing males of different ages showed that thermogenetic activation of *at4A*—known to promote courtship in an age-dependent manner (Lin et al., 2016)—significantly enhanced courtship in 2-day-old males, but not in 7 day old (not shown). The lack of courtship enhancement in the latter condition likely reflects a ceiling effect arising from the high 7-day male copulation around 30°C (total cumulative copulation rate: 82% from 4 experiments, total 120 matches; no significant difference was observed between control and experimental groups). Conversely, pilot experiments testing *at4C* showed significant courtship inhibition only with 7-day-old males, likely reflecting a floor effect arising from the low copulation rate of 2-day-old males (total cumulative copulation rate: 47% from 3 experiments, total 90 matches; no significant difference between control and experimental groups). Of note, male age was found to influence only the manifestation or degrees of courtship modulation by thermogenetic ORN activation, but never the polarity of the modulation. Therefore, to avoid ceiling or floor effects, 2-day-old males were used in experiments where *at4A* or *ac4A* was thermogenetic activated, while 7-day-old males were used for the *at4B*, *at4C*, or *ac4C* experiments.

Pheromone perfuming experiments

Briefly, 0.3 μ l of either individual pheromones or binary mixtures were directly applied to the abdomen of female flies essentially as described (Kurtovic et al., 2007). Pheromones were diluted in acetone (v/v) prior to experiments (palmitoleic acid: 3×10^{-7} , ~0.1 ng/fly; methyl palmitate: 3×10^{-4} , ~87 ng/fly; *cis*-vacacenyl acetate: 1.5×10^{-4} , ~38 ng/fly; (Z)-9- tricosene: 3×10^{-6} , ~7 ng/fly). Pilot dosage experiments were conducted to determine appropriate pheromone dilutions such

that individual pheromones enhanced or inhibited courtship to similar degrees. After the pheromone dilution (experimental group) or acetone (negative control) was applied to females, the solvent was allowed to evaporate for one hour prior to experiments. For single-pair courtship assays, one 7-day-old naïve male and one 3-day-old virgin CS female were loaded into a mating chamber (2 cm in diameter and 1 cm in height) placed atop a food patch as described (Lin et al., 2016). Of note, smaller chambers were used for single-pair courtship assays than the ones for the 3-male-1-female competition assays described above. Courtship assays were conducted at either 25°C (Fig 7 E–G, Fig 9 A–D, Fig 11 A and B) or around 30°C (Fig 7 H–P, Fig 9 E and F, Fig 11 C–H) under 660-nm red light. Synaptic transmission in select ORNs was blocked around 30°C when the neurons expressed the temperature-sensitive dynamin mutant *shi^{ts1}* (Kitamoto, 2001; Pfeiffer et al., 2012). Of note, the *UAS-Shibire^{ts1}* line used in this study contained a translational enhancer for robust expression (Pfeiffer et al., 2012). Each experiment contained 25 matches. The control and experimental groups were tested in parallel experiments. Copulation was visually confirmed, and the cumulative copulation rate (CCR) was calculated for the 2-hr period. The inhibition index was calculated as:

$$Inhibition\ index = \left(\frac{CCR_{control} - CCR_{experimental}}{CCR_{control}} \right)$$

Data were analyzed using custom MATLAB codes.

Single-sensillum recordings and stimuli

To prepare an antenna for recording, a fly was wedged into the narrow end of a truncated 200-μl pipette tip to expose the antenna, which was subsequently stabilized between a tapered glass microcapillary tube and a coverslip covered with double-sided tape. Extracellular single-sensillum recordings were performed essentially as described (Ng et al., 2017). Briefly, electrical activity of target ORNs was recorded extracellularly by placing a sharp electrode filled with

artificial hemolymph solution (Wang et al., 2003) in the at4 sensillum (Or47b ORN recordings). For recordings of other sensillum types, 0.6X sensillum lymph Ringer solution (Kaisling, K. E., Thorson, 1980) was used instead. A reference electrode filled with the same solution was placed in the eye or clypeus. No more than three sensilla from the same antenna were recorded per fly. AC signals (100-20k Hz) and DC signals were simultaneously recorded on an NPI EXT-02F amplifier (ALA Scientific Instruments) and digitized at 5 kHz with Digidata 1550 (Molecular Devices). ORN spikes were sorted and analyzed offline using Clampfit 10 (Molecular Devices) and Igor Pro (WaveMetrics). Spike responses were averaged, binned at 50 ms, and smoothed using a binomial algorithm to obtain peri-stimulus time histograms (PSTHs). Methyl palmitate (10^{-2} , Sigma P5177) or *cis*-vacacenyl acetate (Cayman 10010101, 10x dilution from 5% stock) was diluted in ethanol (v/v), applied as a 4.5- μ l portion to a filter disc and delivered via a 500-ms air pulse at 250 ml/min directly to the antenna from close range as described (Ng et al., 2017). For recordings with a background odorant, palmitoleic acid (10^{-1} , Cayman 9001798) was applied as a 4.5- μ l portion to a filter disc, which was placed in close proximity adjacent to the fly head.

For optogenetic stimulation, newly eclosed female flies expressing *CsChrimson* in targeted ORNs were reared in constant darkness for 4 days on fly food supplemented with 10 μ M all *trans*-retinal (Sigma R2500). A light stimulus was generated via a LED (635 nm, Universal LED Illumination System, pE-4000, Cool LED). Light pulses (500-ms duration) were controlled by Clampex 10.4 (Molecular Devices). Of note, a wide wavelength range of light (470–660 nm) can activate *CsChrimson* (Klapoetke et al., 2014). Consequently, in the process of identifying target sensilla for recording, *CsChrimson*-expressing ORNs were already strongly activated by microscopic light. Such strong and prolonged activation of target ORNs could markedly reduce their spike amplitude to a degree that spikes become too small to be visualized above the level of noise. Therefore, in order to observe 635-nm elicited *CsChrimson* responses, the ORNs

needed to first be dark adapted, and recordings had to be performed with the microscopic light off.

For thermogenetic stimulation, an infrared laser (Roithner, 808 nm, 500 mW, RLTM DL-808-500-5) was directed to the antenna via a 1-mm optic fiber (Roithner, RLTMxL SMA905-1000). The laser power and stimulation duration were controlled by Clampex 10.4 (Molecular Devices). Temperature around the position of the recorded antenna was measured with a K-type thermocouple (Gain Express Holdings 68022) and the stimulus was set at $\sim 30^{\circ}\text{C}$, which did not elicit any excitatory responses in ORNs without TrpA1 expression (data not shown).

Computational modeling

We modeled time-dependent activation of paired ORNs, A and B, as $X_A(t)$ and $X_B(t)$, respectively. These activation variables obey the following coupled nonlinear ordinary differential equations:

$$\frac{d}{dt}X_A = -X_A - X_A w_{AB} X_B^n + S_A,$$

$$\frac{d}{dt}X_B = -X_B - X_B w_{BA} X_A^n + S_B.$$

For simplicity, we assumed that the response decays to the baseline linearly with the timescale $\tau = 1$. The parameter w_{AB} describes the strength of ephaptic coupling from ORN_B to ORN_A, and similarly for w_{BA} . Note that the overall input into ORN_A is scaled by the activity level of both neurons (i.e., the second term on the right-hand side of the above equations is proportional to X_A and X_B), consistent with our previous circuit modeling of ephaptic interactions (Zhang et al., 2019). Furthermore, ephaptic interaction from ORN_A to ORN_B scales nonlinearly with the activity level of ORN_B (scaling exponent, n). We considered the scaling to be nonlinear ($n > 1$) because

ephaptic inhibition occurs between paired neurons only when neuronal activity exceeds a certain threshold (Su et al., 2012).

Since odor stimuli in our behavioral experiments were sustained, we analyzed the differential equations at the steady state (i.e., $\frac{d}{dt}X_A = \frac{d}{dt}X_B = 0$). We reduced the model complexity by assuming that ephaptic interactions are measured in units of w_{BA} (i.e., $w_{BA} = 1$, $w_{AB} = q$). By convention, ORN_A refers to the larger ORN, so $0 < q \leq 1$. S_A and S_B indicate the stimulus strength for ORN_A and ORN_B , respectively (in arbitrary units). In this model, in the absence of ephaptic coupling ($w_{AB} = w_{BA} = 0$), ORN activity is defined to be equal to the stimulus strength at the steady state: $X_A = S_A$, $X_B = S_B$. With ephaptic inhibition, ORN responses are expected to be attenuated: $X_A \leq S_A$, $X_B \leq S_B$. To determine the degree of valence amplification by ephaptic inhibition, we considered the response and stimulus differences: $\Delta_X = X_A - X_B$, $\Delta_S = S_A - S_B$. Amplification takes place when Δ_X and Δ_S have the same sign and when the response difference (Δ_X) is larger than stimulus difference (Δ_S). We therefore defined the valence amplification index (α) as follows.

$$\alpha = \frac{\Delta_X}{\Delta_S}$$

Before determining the value of α , we first considered regions in stimulus space where valence amplification is not expected to be meaningful. Note that the stimulus space is defined by S_A and S_B . We excluded the region where $S_A \approx S_B$ (or $\Delta_S \approx 0$) because within this ambiguous region, behavioral output may fluctuate between positive and negative valence, making it difficult to accurately predict the valence polarity of the behavioral response. To mathematically define the excluded region, we began by testing whether ORN spike responses follow a Poisson distribution. If so, the response standard deviation (σ) is expected to be equal to the square-root of the mean (μ), i.e. $\sigma = \mu^{0.5}$. We therefore analyzed spike responses from ten different ORN types based on our published data (Zhang et al., 2019) by fitting individual dosage responses to

the function $\sigma = \beta\mu^m$, where β and m are fitting parameters. Indeed, we found that the average scaling constant (β) and exponent (m) were close to 1 and 0.5, respectively, supporting the notion that ORN spike responses follow a Poisson distribution ($\beta = 1.3 \pm 0.6$; $m = 0.58 \pm 0.19$, Fig. 16). Having determined that the ORN response distribution is consistent with Poisson, we could then define the meaningful amplification region to be where $S_A > S_B + \sqrt{S_B}$ and where $S_B > S_A + \sqrt{S_A}$ (outside the gray-shaded area in Fig. 12C and Fig. S17).

Next, we determined α within the meaningful domains of stimulus space by solving the steady-state equations. We considered different degrees of nonlinearity ($n = 2, 2.5$ or 3) and a range of ephaptic coupling parameter ($q = 0.1-1$). Amplification was broadly observed ($\alpha > 1$) for all combinations of n and q , suggesting that the validity of our analysis was not affected by the exact value of n or q . However, these two parameters influence the degree of amplification (α) in the stimulus space as a function of S_A and S_B (Fig. S17). For certain n and q combinations, there were regions in the stimulus space where there is no amplification ($\alpha < 1$, white-shaded areas in Fig. S17). For demonstration purposes, a specific combination of n and q was selected for Fig. 12C ($n = 2$ and $q = 0.9$), where amplification was found broadly for a wide range of S_A and S_B combinations within the meaningful domain of stimulus space.

Quantification and statistical analysis

Place-preference data were analyzed by Wilcoxon rank-sum test because not all ORN datasets passed the Shapiro-Wilk normality test. For oviposition preference, significance was determined by unpaired two-sample t-test, as all datasets passed the Shapiro-Wilk normality test. Courtship competing data were analyzed by one-sample z-test. All pheromone perfuming data collected from parallel experiments were analyzed by Wilcoxon rank-sum test. Courtship inhibition

indices between different experiments were analyzed by Wilcoxon rank-sum test. Statistical analysis was performed in MATLAB and RStudio.

Chapter 2, in part, is material as it appears in Valence opponency in peripheral olfactory processing 2022. Wu, Shiuan-Tze; Chen, Jen-Yung; Martin, Vanessa; Ng, Renny; Zhang, Ye; Grover, Dhruv; Greenspan, Ralph J.; Aljadeff, Johnatan, Su, Chih-Ying, *Proc. Natl. Acad. Sci. USA*, 119 (5) : e2120134119, 2022. The dissertation author and Jen-Yung Chen contributed equally to this paper as primary researches and authors.

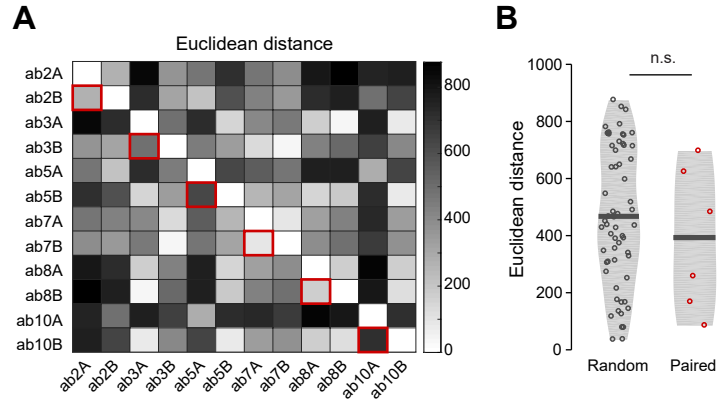


Figure 1. Neighboring ORNs housed in the same sensillum do not necessarily exhibit similar odor response profiles.

(A) Heat map of the Euclidean distance between any two of the six pairs of ORNs based on a published dataset describing the response profiles of 24 antennal odorant receptors (Hallem and Carlson, 2006). According to the spike responses of these ORNs to 110 odorants, the Euclidean distances between any two ORNs were determined. A shorter distance means that the two neurons have more similar response profiles. The distances between neurons housed in the same sensillum are outlined in red. (B) Euclidean distance between any two randomly paired neurons (gray circles) or between paired ORNs in the same sensillum (red circles). Horizontal bars indicate average distances. Wilcoxon rank-sum exact test, $p=0.43$.

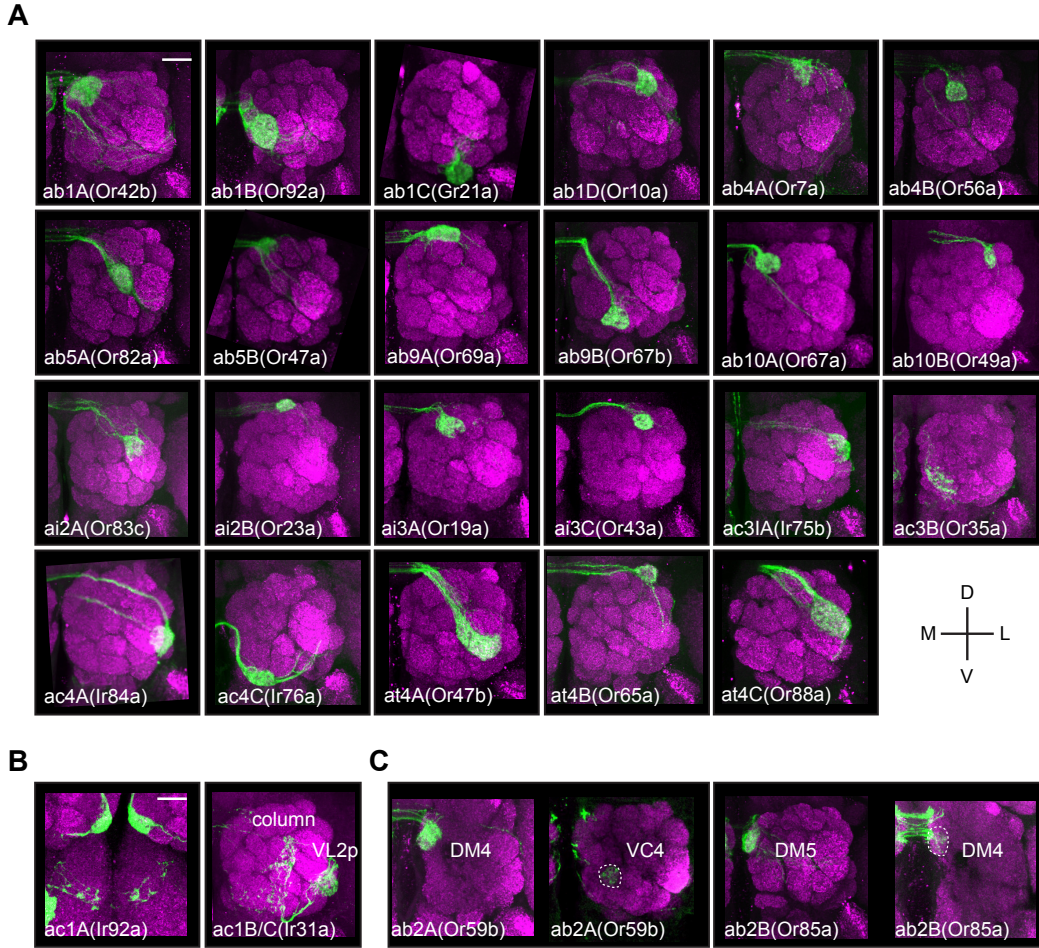


Figure 2. GAL4-drivers for grouped ORNs.

Antennal lobe images of receptor-GAL4>mCD8::GFP flies from z-projections of multiple adjacent confocal sections. The ORN axons were stained with anti-GFP (green), and counterstained with nc82 to visualize the glomerular structure (magenta). All driver lines were backcrossed to w 1118 or Berlin, and were identical to the ones used for optogenetic or thermogenetic behavioral assays (see Tables 3 and Table 4). (A) Specific GAL4 drivers for grouped ORNs housed in 10 different antennal sensillum types (ab1, ab4, ab5, ab9, ab10, ai2, ai3, ac3, ac4, and at4). Only a single glomerulus in each antennal lobe was labeled by each driver. The positions of labeled glomeruli were as reported (Fishilevich and Vosshall, 2005; Couto et al., 2005). (B) The GAL4 drivers for the ac1 ORNs labeled additional neurons. Left: apart from the reported VM1 glomerulus (Silbering et al., 2011), the Ir92a-GAL4 driver also labeled neurons projecting to the subesophageal zone. Right: the Ir31a-GAL4 driver labeled two glomeruli: the reported VL2p glomerulus (Silbering et al., 2011), as well as the column/VP1 glomerulus receiving input from hygro-sensory neurons (Enjin et al., 2016; Knecht et al., 2017). The ac1 ORNs were therefore excluded in our behavioral assay. (C) The GAL4 drivers for the ab2 ORNs also labeled additional neurons. Left: apart from the reported DM4 glomerulus (Fishilevich and Vosshall, 2005; Couto et al., 2005), the Or59b-GAL4 driver also labeled neurons which likely project to the VC4 glomerulus (outlined). Right: the Or85a-GAL4 driver labeled two glomeruli: the reported DM5 (Fishilevich and Vosshall, 2005; Couto et al., 2005) as well as DM4 (outlined). Multi-glomerular labeling was also reported with the other available Or85a-GAL4 driver (Task et al., 2020). The ab2 ORNs were therefore excluded in our behavioral assay. Scale bars, 20 μ m.

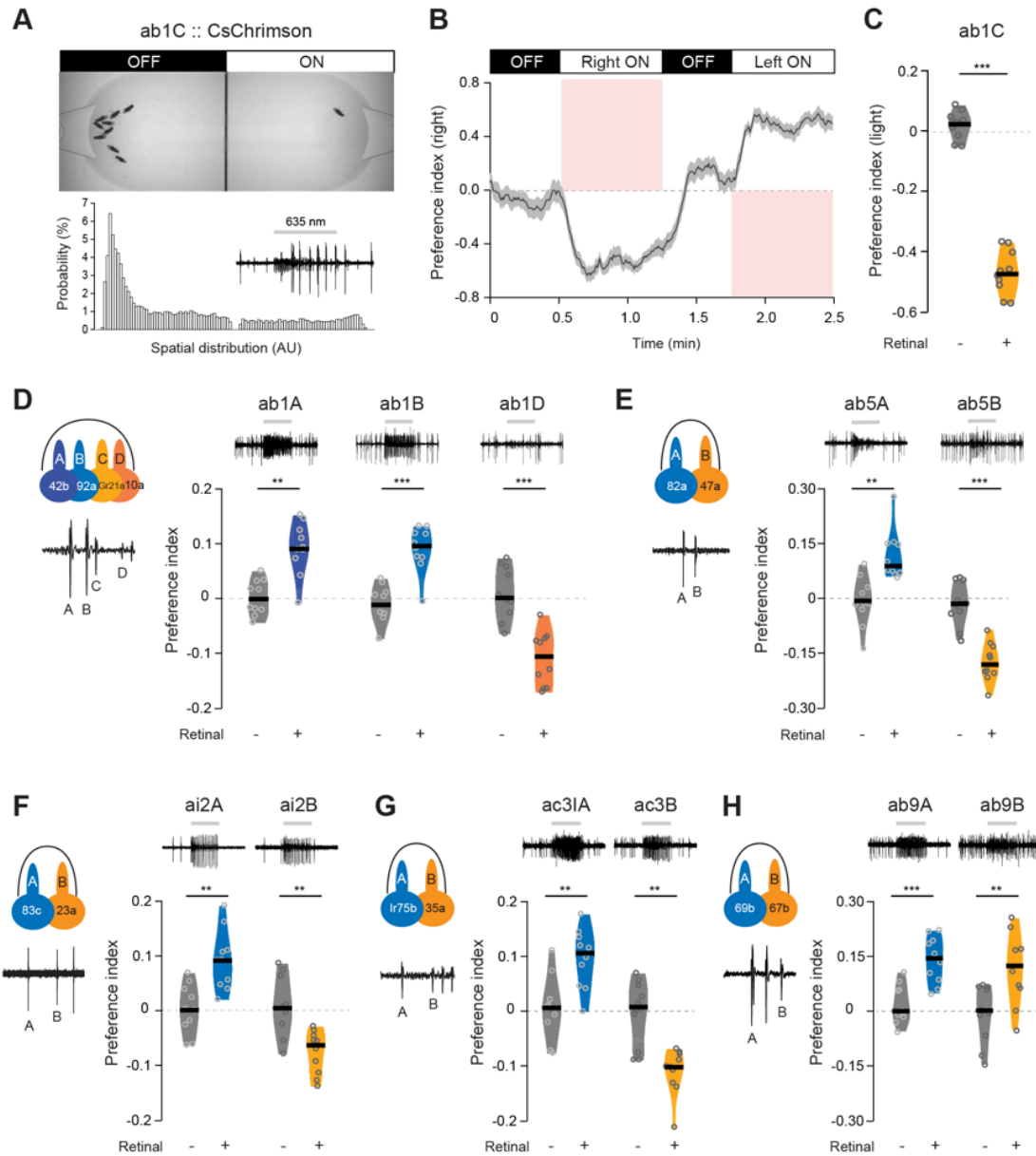


Figure 3. Valence opponency in place preference.

(A) Top: Optogenetic place preference assay. Inset: Single-sensillum recording; ab1C responded to a 500-ms pulse of 635-nm light. Bottom: Collective distribution probability along the arena; the illuminated side was adjusted to be on the right ($n=10$). (B) Average place preference over time when ab1C was optogenetically activated. Shaded area, s.e.m. (C–H) Violin plots showing preference indices to the illuminated side when CsChrimson was expressed in the indicated ORN types. Sample traces of single-sensillum recordings were shown to demonstrate optogenetic activation of target ORNs. Circle: average preference index from three trials of each experiment; horizontal line: median preference. Negative controls were age-matched siblings without retinal feeding (gray). Results are from sated flies. $n=10$ for each condition, ** $p < 0.01$, *** $p < 0.001$, Wilcoxon rank-sum test.

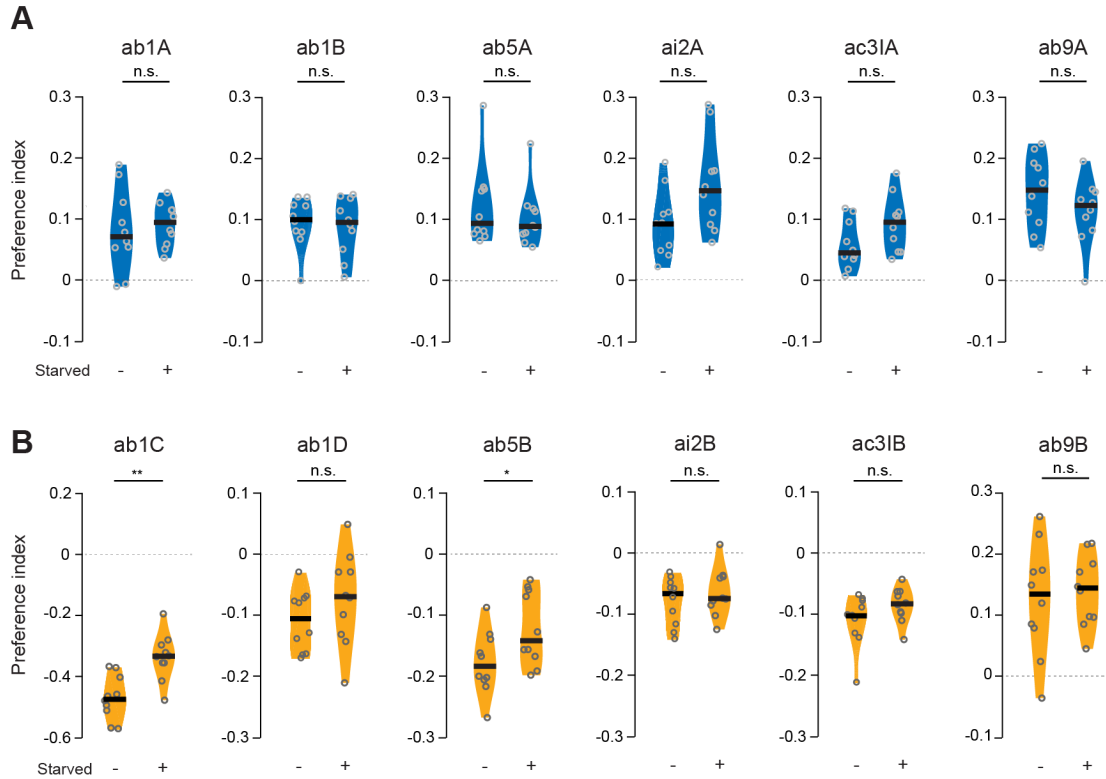


Figure 4. The influence of satiety state on ORN-mediated place preference.

(A-B) Violin plots showing preference indices to the illuminated side when CsChrimson was expressed in the indicated ORN types. The flies were either starved for 24 hr or sated. (A) Optogenetic activation of large-spike ORNs. (B) Optogenetic activation of small-spike ORNs. Circle: average preference index from three trials of each experiment; horizontal line: median preference. $n=10$ for each condition, * $p<0.05$, ** $p<0.01$, Wilcoxon rank-sum test. Irradiance was $1.2 \mu\text{W}/\text{mm}^2$ for all ORN types.

Figure 5. Valence opponency in oviposition and courtship.

(A) Optogenetic oviposition preference assay. (B–D) Violin plots showing oviposition preference indices to the illuminated side when CsChrimson was expressed in the indicated ORN types. Sample traces of single-sensillum recordings were shown to demonstrate optogenetic activation of target ORNs. Circle: preference index for each trial; horizontal line: median preference. Negative controls were age-matched siblings without retinal feeding (gray). $n=20$ trials, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, unpaired t-test. (E) Top: at4 ORNs and their spikes. Bottom left: Courtship competition assay. Bottom right: single-sensillum recording of at4A responses to heat generated by an IR laser. (F–H) Normalized cumulative copulation rates of males whose at4A (F), at4B (G) or at4C (H) was thermogenetically activated, when competing with the respective parental controls (black and gray). Cumulative copulation rates were normalized to the total copulation rate of all tested males in the same experiments. Right panels: each circle denotes the end copulation rate of a given experiment with lines connecting data from the same experiments ($n=4–6$ experiments, total 68–94 matches). (I–K) As in (E–H), except that TrpA1 was expressed in ac4A (J) or its small-spike Ir76a + neighbor (K) ($n=5$ experiments; total 83–85 matches). p values are indicated; one-sample z-test.

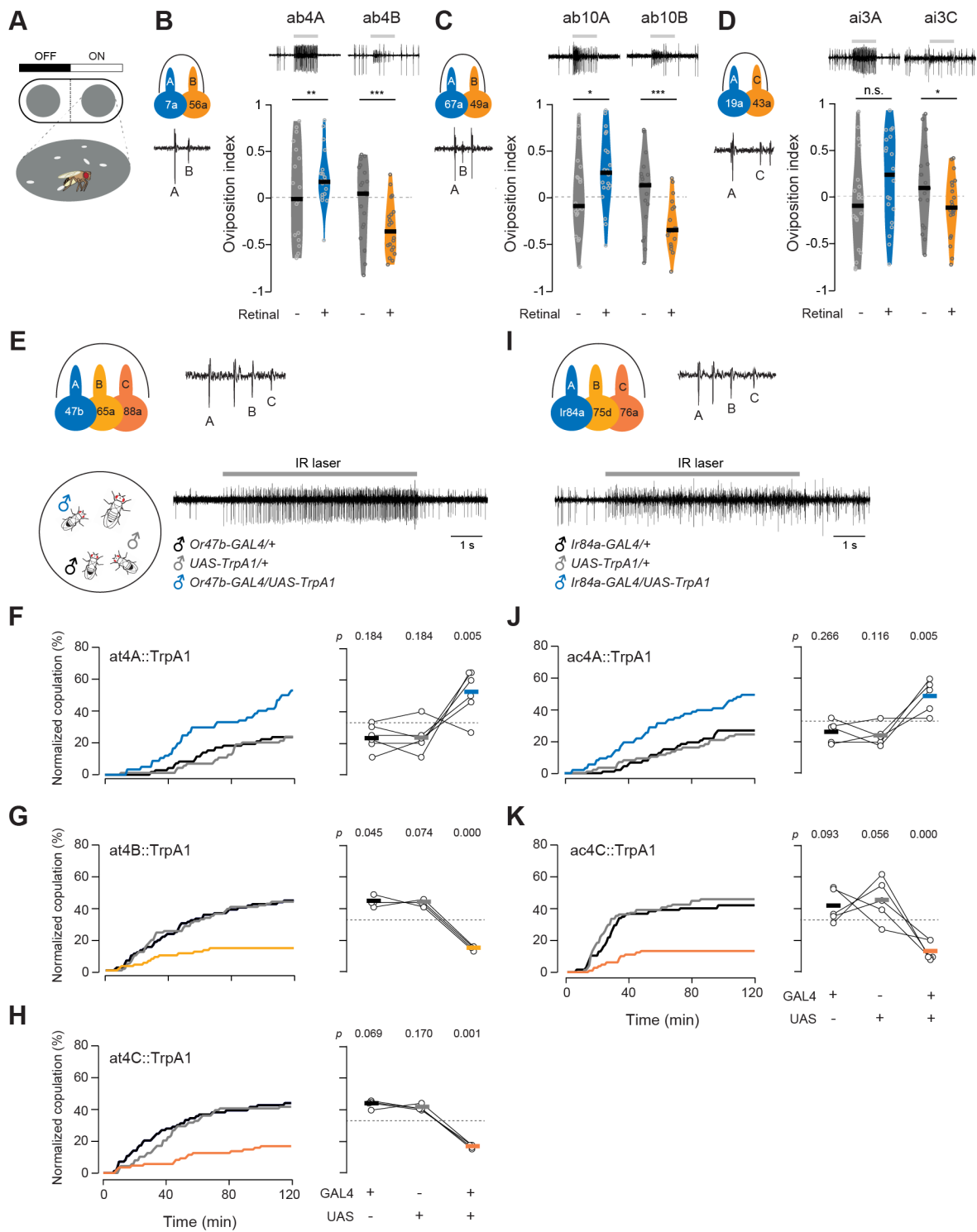
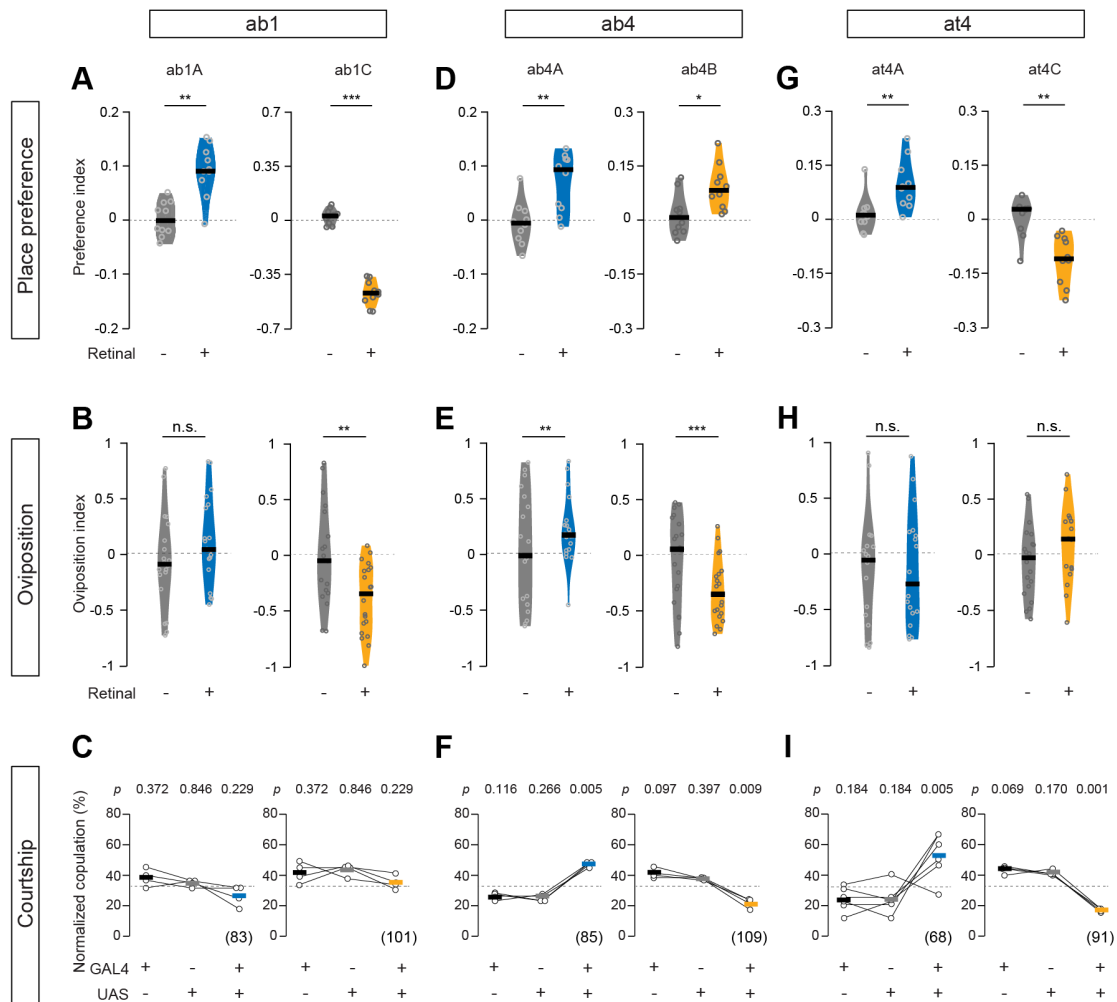


Figure 6. Valence opponency is context-dependent.

The hedonic value of paired ORNs housed in the ab1 (A–C), ab4 (D–F), and at4 (G–I) was determined in three different behavioral contexts: place preference (top panels), oviposition (middle panels) and courtship (bottom panels). (A) Optogenetic place preference assay. Violin plots show preference indices to the illuminated side when CsChrimson was expressed in ab1A or ab1C. Circle: average preference index from three trials of each experiment; horizontal line: median preference. Negative controls were flies without retinal feeding (gray). Results are from sated flies. Irradiance was 9.1 $\mu\text{W}/\text{mm}^2$ for ab1A and 1.2 $\mu\text{W}/\text{mm}^2$ for ab1C. $n=10$ for each condition, $*p<0.05$, $**p<0.01$, $***p<0.001$, Wilcoxon rank-sum test. (B) Optogenetic oviposition assay. Violin plots showing oviposition preference indices to the illuminated side over ~24 hr when CsChrimson was expressed in the indicated ORN types. Circle: preference index for each trial; horizontal line: median preference. Negative controls were age-matched siblings without retinal feeding (gray). Irradiance was 0.3 $\mu\text{W}/\text{mm}^2$ for both ORN types. $n=20$ for each condition, $**p<0.01$, $***p<0.001$, unpaired t-test. (C) Thermogenetic courtship competition assay. Each circle denotes the normalized copulation rate of a given experiment with lines connecting data from the same experiments. The numbers of successful matches where copulation occurred were shown in the parentheses. p values are indicated; one-sample z-test. (D–F) As with (A–C) except that ab4 ORNs were tested. Irradiance was 1.2 $\mu\text{W}/\text{mm}^2$ for both ORNs in (D). (G–I) As with (D–F) except that at4 ORNs were tested. Data shown in (A) were the same as in Figure 1C–D, (E) as in Figure 2B, and (I) as in Figure 2F and 2H. (J) Summary table.

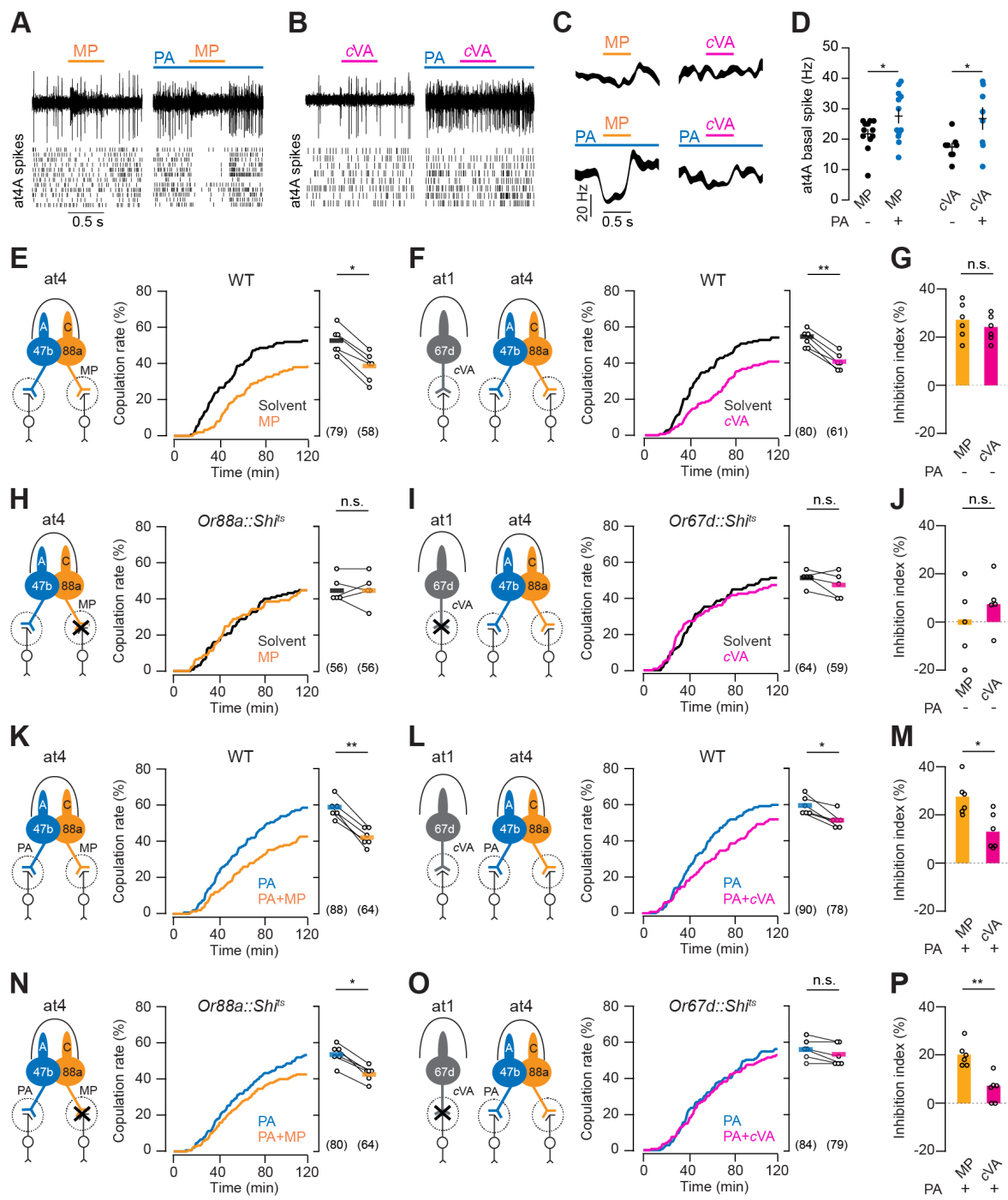


J

	ab1		ab4		at4	
	A	C	A	B	A	C
Place Preference	+	-	+	+	+	-
Oviposition	no effect	-	+	-	no effect	no effect
Courtship	no effect	no effect	+	-	+	-

Figure 7. Ephaptic lateral inhibition processes countervailing olfactory cues.

(A) Single-sensillum recording. Representative trace (top) and raster plot (bottom) are shown for at4A spikes without (left) or with its ligand, palmitoleic acid (PA, 10^{-1} v/v dilution), presented in the background (right). Colored bars indicate odorant stimulations. MP: methyl palmitate (10^{-2}). Parallel experiments, mean $\pm$ s.e.m. (n=12, from 3–4 male flies). (B) As with (A) except that recordings were performed with the at1 ligand, cis-vaccenyl acetate (cVA, 5×10^{-3}), as the transient odorant. Parallel experiments, mean $\pm$ s.e.m. (n=8, from 3–4 male flies). (C) Peri-stimulus time histograms of at4A spikes from data shown in (A–B). Line width indicates s.e.m. (D) Quantification of at4A activity. Each data point represents the basal spike frequency of at4A before methyl palmitate (MP) or cisvaccenyl acetate (cVA) was presented as shown in (A–B). (E) Single-pair courtship assays. Cumulative and final copulation rates. Females were perfumed with solvent (acetone) or methyl palmitate (~ 87 ng/fly). Lines connect results from parallel experiments. (F) As with (E) except that females were perfumed with cVA (~ 38 ng/fly). Numbers of copulated males are indicated in parentheses. (G) Inhibition indices from results in (E) and (F). (H–J) As with (E–G) except that synaptic transmission was blocked in at4C (H, Or88a::Shi^{ts1}) or in at1 (I, Or67d::Shi^{ts1}). (K–M) As with (E–G) except that females were perfumed with palmitoleic acid alone (~ 0.1 ng/fly) or with a binary mixture of palmitoleic acid and methyl palmitate (K) or palmitoleic acid and cVA (L). (N–P) As with (K–M) except that synaptic transmission was blocked in at4C (N, Or88a::Shi^{ts1}) or in at1 (O, Or67d::Shi^{ts1}). * $p < 0.05$, ** $p < 0.01$, Wilcoxon rank-sum test. n=5–6, 125–150 matches for individual conditions.



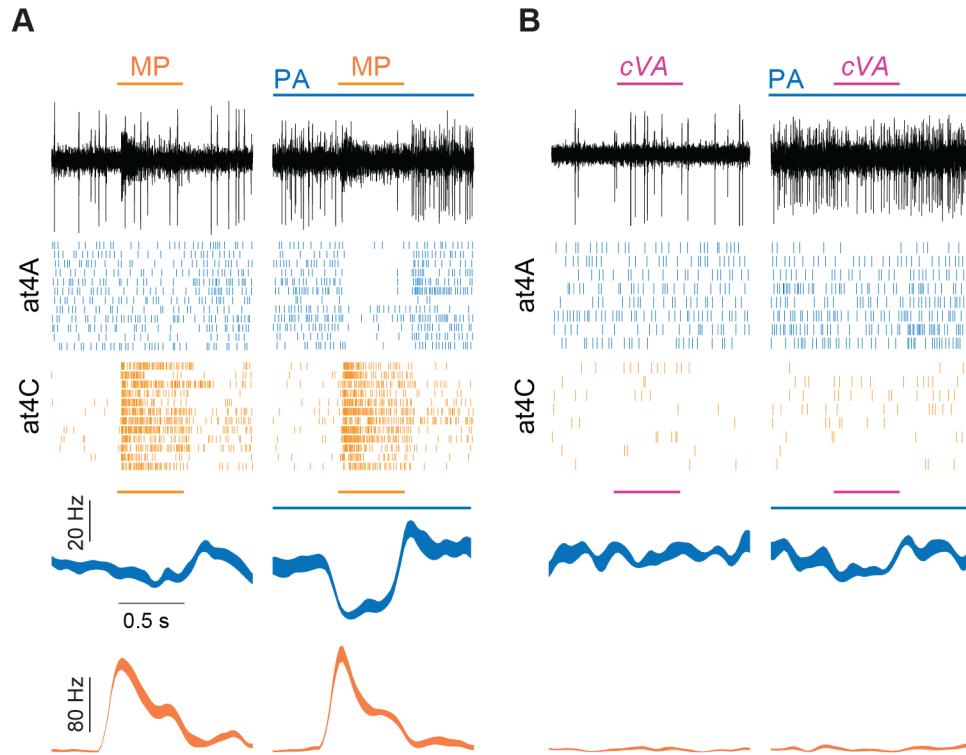


Figure 8. *at4* ORN spike responses to odor stimulation.

Single-sensillum recordings of spike responses from *at4A*/Or47b (blue) and *at4C*/Or88a (orange) during odorant stimulation with or without palmitoleic acid background odorant. (A) Representative trace (top), raster plot (middle), and peri-stimulus time histogram (bottom, PSTH binned at 50 ms) are shown for *at4A* (blue) and *at4C* (orange) in response to a 500-ms pulse of methyl palmitate (MP, 10^{-2} , v/v dilution) with (left) or without chronic stimulation of *at4A* with palmitoleic acid background odorant (PA, 10^{-1} , v/v dilution, right). Colored bars indicate odorant stimulations. Transient activation of *at4C* by methyl palmitate inhibited *at4A*'s response to PA. PSTH line width indicates s.e.m. (B) As with A except that cVA was applied as the transient odorant (10^{-1} , v/v dilution). None of the *at4* ORNs showed spike responses to cVA pulse stimulation.

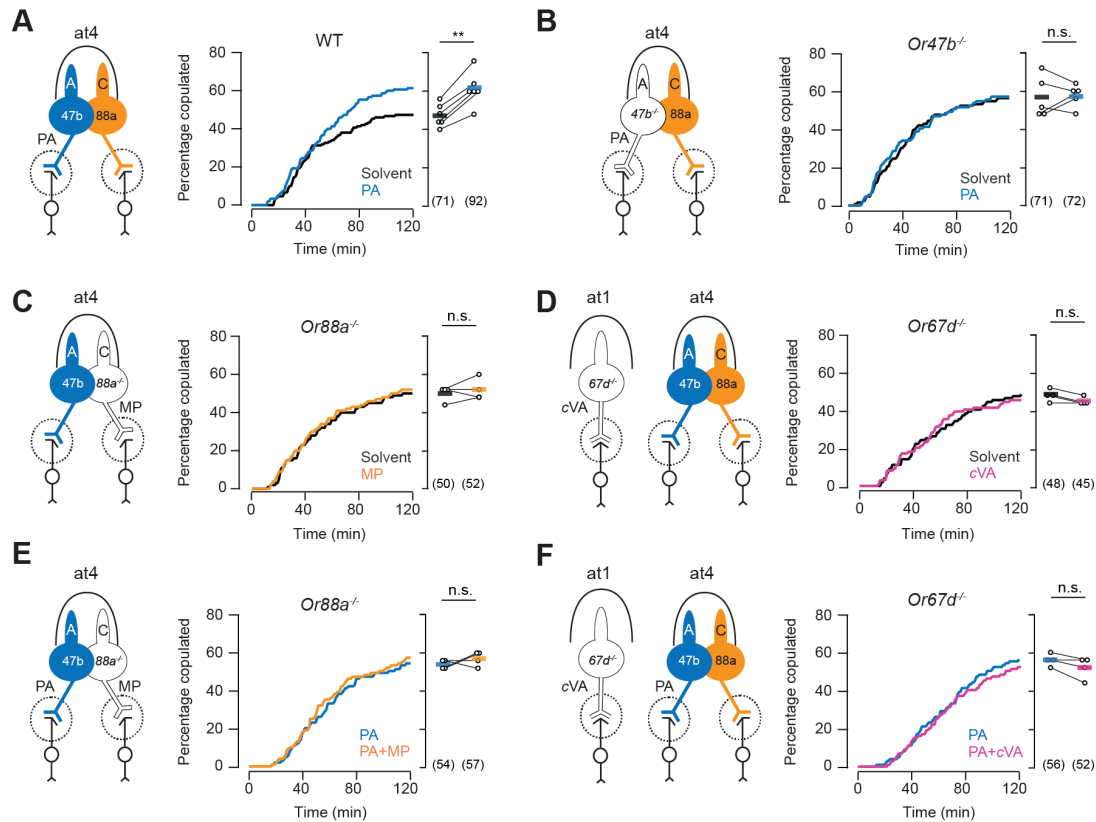


Figure 9. Phomonal modulation of courtship was abolished in receptor mutants.

Single-pair courtship assays with a 7-day-old male and a 3-day-old female perflumed with either solvent (acetone) or indicated pheromone(s). (A) Cumulative copulation rates of wildtype males when the females were perflumed with either solvent (acetone) or palmitoleic acid (~ 0.1 ng/fly); the final copulation rates are shown on the right. Lines connect results from parallel experiments. The total numbers of copulated males are indicated in the parentheses. (B) As with (A) except that *Or47b* mutant males were tested. ** $p < 0.01$, Wilcoxon rank-sum test. $n=5-6$, total 125–150 matches, experiments conducted at $\sim 25^\circ\text{C}$. (C) Cumulative copulation rates of *Or88a* mutant males when the wildtype females were perflumed with either solvent or methyl palmitate (~ 87 ng/fly); the final copulation rates are shown on the right. Lines connect results from parallel experiments. The total numbers of copulated males are indicated in the parentheses. (D) As with (C) except that *Or67d* mutant males were tested and the females were perflumed with cVA (~ 38 ng/fly). (E) As with (C) except that females were perflumed with palmitoleic acid (~ 0.1 ng/fly) or a binary mixture of palmitoleic acid (~ 0.1 ng/fly) and methyl palmitate (~ 87 ng/fly). (F) As with (E) except that *Or67d* mutant males were tested and the females were perflumed with palmitoleic acid (~ 0.1 ng/fly) or a binary mixture of palmitoleic acid (~ 0.1 ng/fly) and cVA (~ 38 ng/fly). n.s., not significant, Wilcoxon rank-sum test. $n=4$, total 100 matches.

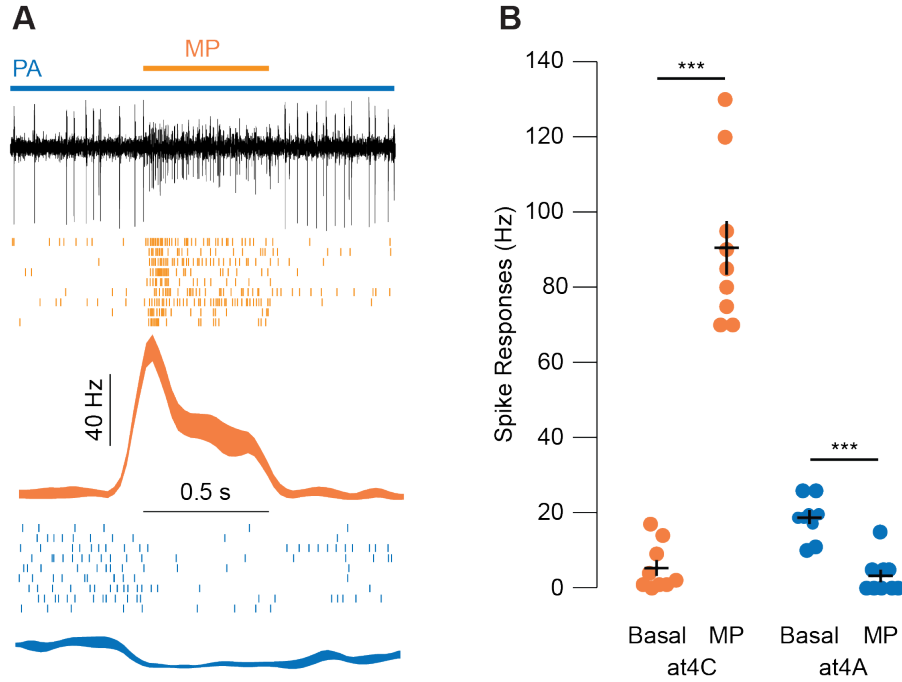


Figure 10. shibire-expressing at4C retains its peripheral spike responses.

Single-sensillum recording performed from Or88a::Shi^{ts1} flies at restrictive temperatures (>30°C). (A) Representative trace (top), raster plot (middle), and peri-stimulus time histogram (bottom, PSTH binned at 50 ms) are shown for at4C (orange) and at4A spikes (blue) in response to a 500-ms pulse of methyl palmitate (MP, 10⁻², v/v dilution). The at4A neuron was chronically stimulated by its ligand, palmitoleic acid (PA, 10⁻¹), presented in the background. Colored bars indicate odorant stimulations. Transient activation of at4C by methyl palmitate inhibited at4A's response to PA. PSTH line width indicates s.e.m. (B) Quantification of at4C and at4A spike activity before (basal) and during methyl palmitate stimulation (MP). (n=9, from 5 male flies).

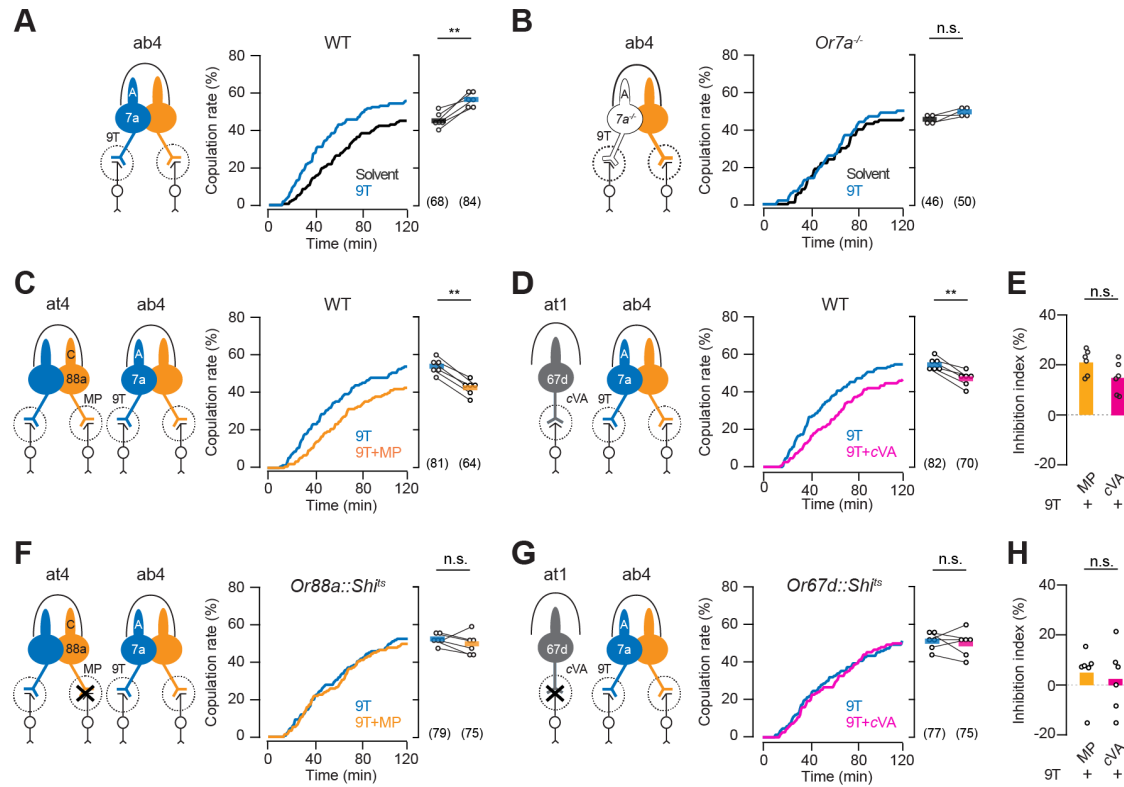


Figure 11. Countervailing cues detected by non-neighboring ORNs.

(A) Single-pair courtship assays with a 7-day-old male and a 3-day-old female perfumed with either solvent (acetone) or 9-tricosene (~7 ng/fly). Cumulative copulation rates of wildtype males; the final copulation rates are shown on the right. Lines connect results from parallel experiments. The total numbers of copulated males are indicated in the parentheses. (B) As with (A) except that *Or7a* mutant males were tested. (C–D) As with (A) except that females were perfumed with 9-tricosene alone (~7 ng/fly) or with a binary mixture of 9-tricosene and methyl palmitate (C) or 9-tricosene and cVA (D). (E) Inhibition indices from results in (C) and (D). (F–H) As with (C–E) except that synaptic transmission was blocked in at4C (F, *Or88a::Shi^{ts1}*) or in at1 (G, *Or67d::Shi^{ts1}*). ** $p < 0.01$, Wilcoxon rank-sum test. $n=4-6$, 100–150 matches for individual conditions.

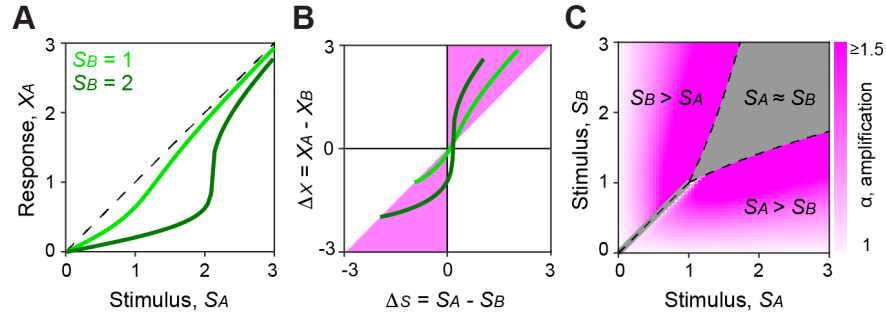


Figure 12. Theoretical modeling of valence enhancement by ephaptic inhibition.

(A) Steady-state response of ORN_A (X_A) as a function of odor stimulus S_A . Without ephaptic inhibition, X_A is defined to be equal to S_A (dashed line, in arbitrary units). When the neighboring ORN_B is also activated by S_B , X_A (green lines) is inhibited ephaptically in a manner that depends on the strength of both S_A and S_B . (B) Response difference between paired neurons (ΔX) as a function of stimulus difference (ΔS). Ephaptic inhibition amplifies the valence signal if ΔX and ΔS have the same sign, and if $|\Delta X| > |\Delta S|$. The simulated responses fall almost entirely within the amplified region (pink-shaded area, see Material and Methods for modeling details). (C) The degree of valence amplification (α) as a function of S_A and S_B . Ephaptic inhibition amplifies valence in a manner that depends on the ratio of S_A and S_B . Gray-shaded region: $S_A \approx S_B$, where the response polarity is likely ambiguous and is thus excluded in the analysis (see Material and Methods for modeling details).

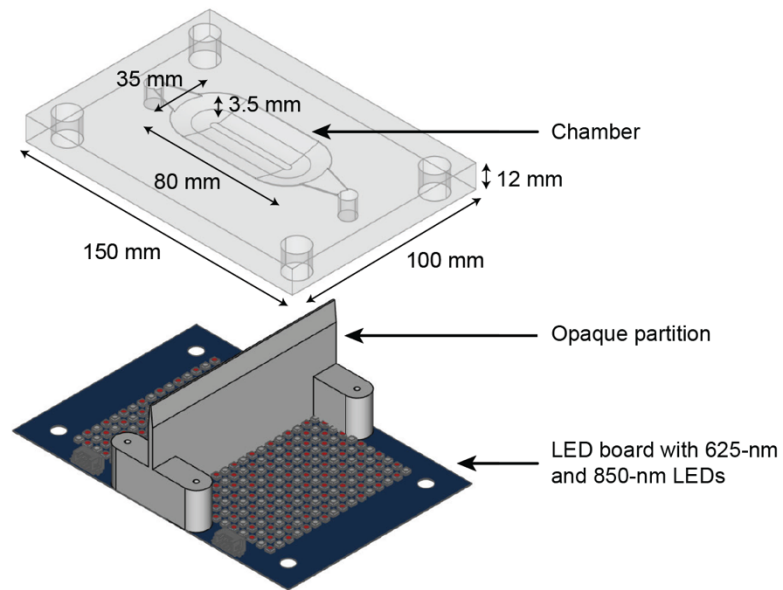
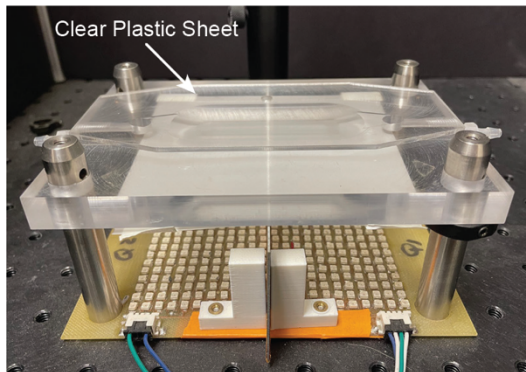
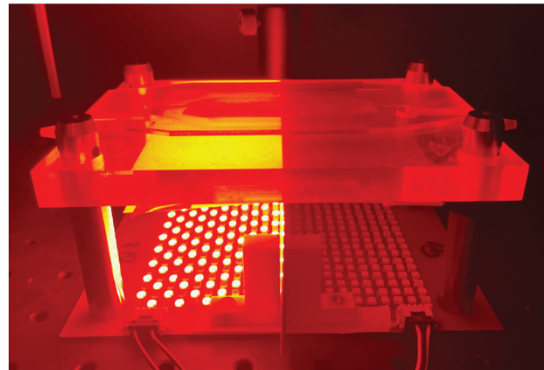
A**B****C**

Figure 13. Optogenetic place preference apparatus.

(A) Schematic diagram of the apparatus. Flies were contained within a shallow, transparent chamber during assays. Below the chamber was a custom LED board composed of interleaving 625-nm and 850-nm LEDs. An opaque partition restricted light to one half of the chamber. (B) The place preference apparatus, consisting of the chamber aligned with the LED board. A clear plastic sheet placed atop the chamber prevented flies from escaping. (C) Place preference apparatus with illumination on half of the chamber.

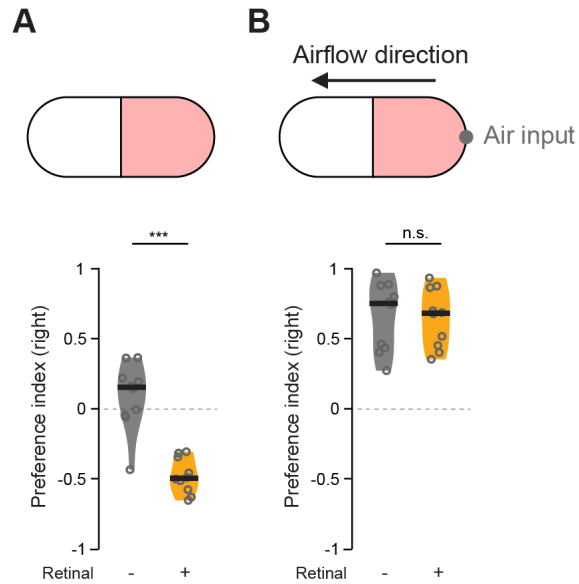


Figure 14. Flies' tendency to walk upwind overrides ORN-mediated preference.

Violin plots showing preference indices to the illuminated side (right, $10 \mu\text{W}/\text{mm}^2$) when CsChrimson was expressed in the ab1C ORNs. The assays were conducted in the absence (A) or presence of a constant air flow (B, $\sim 25 \text{ cm}/\text{sec}$). Circle: average preference index from three trials of each experiment; horizontal line: median preference. Negative controls were flies without retinal feeding (gray). $n=10$ for each condition, *** $p<0.001$, unpaired t-test.

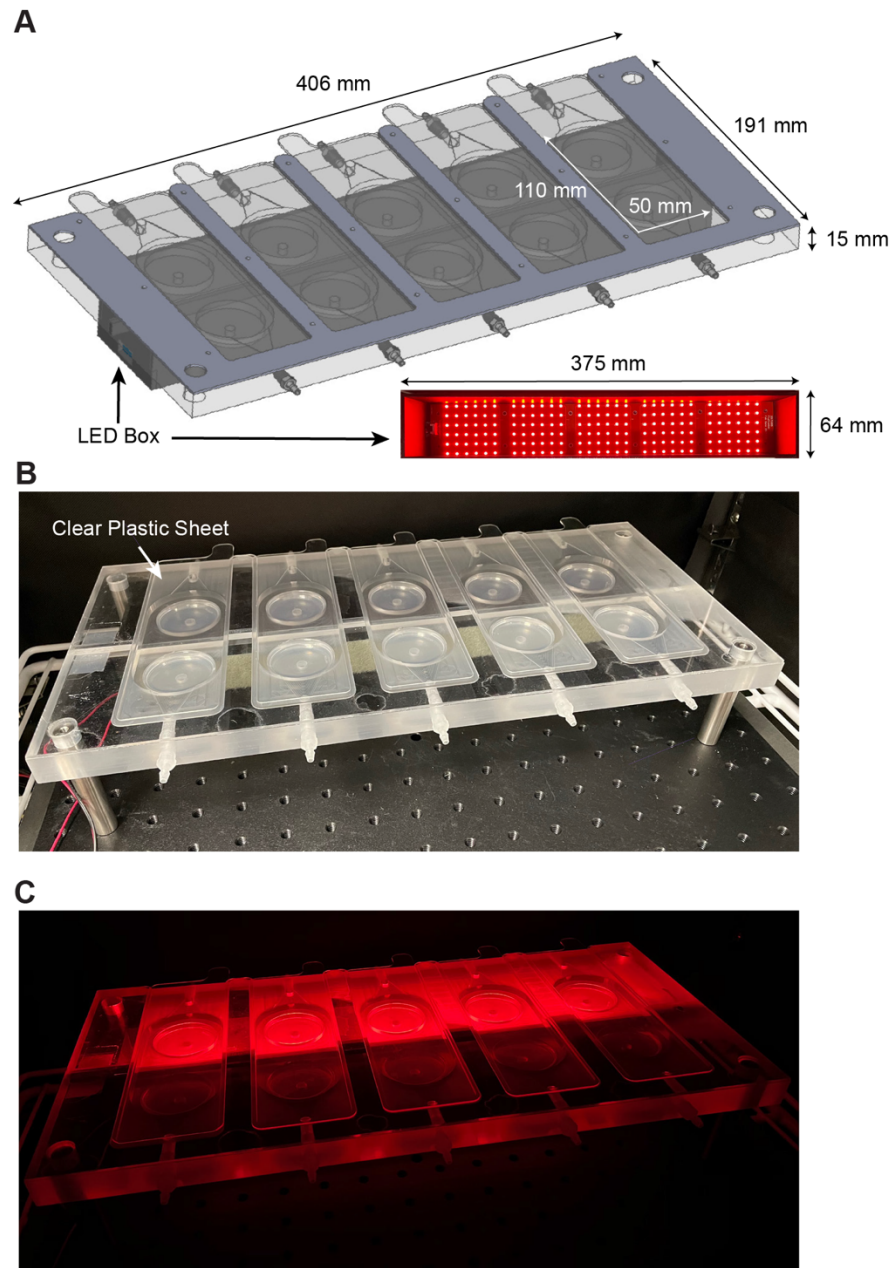


Figure 15. Optogenetic oviposition preference apparatus.

(A) Schematic diagram of the apparatus. The acrylic array comprised five transparent chambers. Each chamber contained two plastic dishes filled with 1% agarose/5 mM sucrose. Inset: top view of the LE box, placed underneath one side of the array to illuminate a single side of each chamber. (B) Oviposition apparatus. A clear plastic sheet placed atop each chamber prevented flies from escaping. (C) Oviposition apparatus with illumination on one side of the chambers.

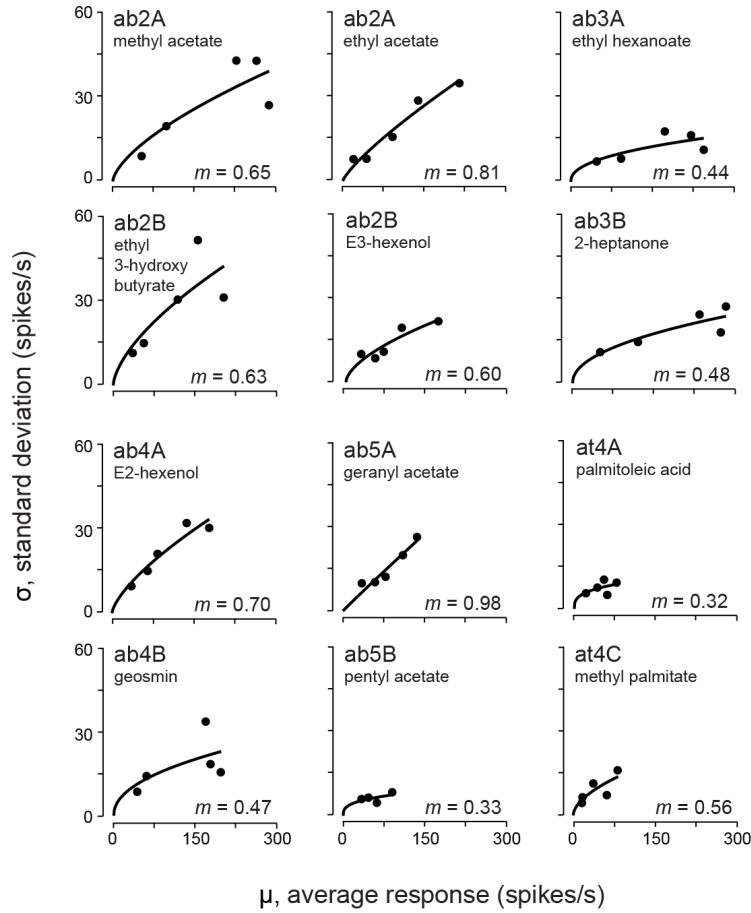


Figure 16. ORN spike responses follow a Poisson distribution.

The standard deviation of ORN responses (σ) is plotted as a function of the mean (μ) for twelve ORN-odorant combinations based on published data (Zhang et al., 2019). Curves are fits to the function $\sigma = \beta \mu^m$. The ORN type, tested odorant, and exponent are indicated in each panel. $\beta = 1.3 \pm 0.6$; $m = 0.58 \pm 0.19$ (mean $\pm$ s.d. .)

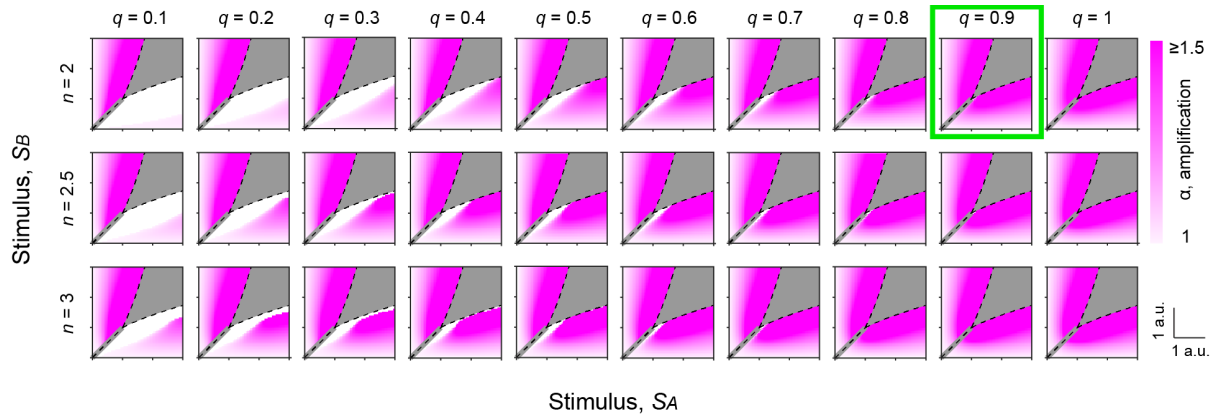


Figure 17. Simulation of valence amplification.

The degree of valence amplification (α) is plotted as a function of stimulus strength (S_A and S_B , in arbitrary units) with n indicating the degree of nonlinearity and q the ephaptic coupling parameter (see Materials and Methods for details). Individual panels indicate different combinations of n and q . White-shaded region: $\alpha < 1$; gray-shaded region: $S_A \approx S_B$, where the response polarity is likely ambiguous and is thus excluded in the analysis. Outlined in green is the panel shown in Figure 12.

Table 1. Compartmentalized *Drosophila* ORNs and their characterized odor-guided behavioral outputs.

Antennal ORNs are named based on their relative spike size and sensillum identity (b: basiconic; c: coeloconic; i: intermediate; t: trichoid). The receptors are indicated in parentheses.

Context	Sensillum	Large-spike ORNs		Small-spike ORNs	
Place preference	ab1	A (Or42b)	Attraction to vinegar	C (Gr21a/63a)	Aversion to CO ₂
		B (Or92a)	Attraction to vinegar	D (Or10a)	Unknown
	ab2	A (Or59b)	Unknown	B (Or85a)	Aversion to high vinegar
	ab5	A (Or82a)	Attraction to geranyl acetate	B (Or47a)	Unknown
	ab9	A (Or69aA/B)	Attraction to a female odor component	B (Or67b)	Unknown
	ai2	A (Or83c)	Attraction to fruit rind odors	B (Or23a)	Unknown
	ac1	A/B (Ir92a)	Attraction to amines	C/D (Ir31a)	Unknown
	ac3	A (Ir75b)	Attraction to short-chain acids	B (Or35a)	Unknown
Oviposition	ab4	A (Or7a)	Promoting egg laying by a fly odor	B (Or56a)	Suppressing egg laying by a mold odor
	ab10	A (Or67a)	Unknown	B (Or49a)	Suppressing egg laying wasp pheromones
	ai3	A (Or19a)	Promoting egg laying by citrus odors	C (Or43a)	Unknown
Courtship	ac4	A (Ir84a)	Stimulating courtship by food odors	B/C (Ir76a)	Unknown
	at4	A (Or47b)	Stimulating courtship by fly pheromones	B (Or65a)	Unknown
				C (Or88a)	Unknown

Table 2. Compartmentalized *Drosophila* ORNs and their behavioral outputs described in this study.

The table summarizes the reported ORN-mediated behaviors that were recapitulated by our optogenetic or thermogenetic assays, as well as the behavioral outputs of hitherto uncharacterized neighboring ORNs. Antennal ORNs are named based on their relative spike size and sensillum identity (b: basiconic; c: coeloconic; i: intermediate; t: trichoid). The receptors are indicated in parentheses.

Context	Sensillum	Large-spike ORNs		Small-spike ORNs	
Place preference	ab1	A (Or42b)	Attraction to vinegar	C (Gr21a/63a)	Aversion to CO ₂
		B (Or92a)	Attraction to vinegar	D (Or10a)	Aversion (this study)
	ab5	A (Or82a)	Attraction to geranyl acetate	B (Or47a)	Aversion (this study)
	ab9	A (Or69aA/B)	Attraction to a female odor component	B (Or67b)	Attraction (this study)
	ai2	A (Or83c)	Attraction to fruit rind odors	B (Or23a)	Aversion (this study)
	ac3	A (Ir75b)	Attraction to short-chain acids	B (Or35a)	Aversion (this study)
Oviposition	ab4	A (Or7a)	Promoting egg laying by a fly odor	B (Or56a)	Suppressing egg laying by a mold odor
	ab10	A (Or67a)	Promoting egg laying (this study)	B (Or49a)	Suppressing egg laying by wasp pheromones
	ai3	A (Or19a)	Promoting egg laying by citrus odors	C (Or43a)	Suppressing egg laying (this study)
Courtship	ac4	A (Ir84a)	Stimulating courtship by food odors	B/C (Ir76a)	Inhibiting courtship (this study)
	at4	A (Or47b)	Stimulating courtship by fly pheromones	B (Or65a)	Inhibiting courtship (this study)
				C (Or88a)	Inhibiting courtship (this study)

Table 3. Fly genotypes in figures.

Figure	Short genotype	Full genotype
2A	Or7a-GAL4 > UAS-mCD8::GFP	<i>Or7a^{GAL4}/w-; UAS-mCD8::GFP/+; UAS-mCD8::GFP/+</i>
2A	Or10a-GAL4 > UAS-mCD8::GFP	<i>w-; Or10a-GAL4/UAS-mCD8::GFP; UAS-mCD8::GFP/+</i>
2A	Or19a-GAL4 > UAS-mCD8::GFP	<i>w-; Or19a-GAL4/UAS-mCD8::GFP; UAS-mCD8::GFP/+</i>
2A	Gr21a-GAL4 > UAS-mCD8::GFP	<i>w-; Gr21a-GAL4/UAS-mCD8::GFP; UAS-mCD8::GFP/+</i>
2A	Or23a-GAL4 > UAS-mCD8::GFP	<i>w-; Or23a-GAL4/UAS-mCD8::GFP; UAS-mCD8::GFP/+</i>
2A	Or35a-GAL4 > UAS-mCD8::GFP	<i>w-; UAS-mCD8::GFP/+; Or35a-GAL4/UAS-mCD8::GFP</i>
2A	Or42b-GAL4 > UAS-mCD8::GFP	<i>w-; UAS-mCD8::GFP/+; Or42b-GAL4/UAS-mCD8::GFP</i>
2A	Or43a-GAL4 > UAS-mCD8::GFP	<i>w-; Or43a-GAL4/UAS-mCD8::GFP; UAS-mCD8::GFP/+</i>
2A	Or47a-GAL4 > UAS-mCD8::GFP	<i>w-; Or47a-GAL4/UAS-mCD8::GFP; UAS-mCD8::GFP/+</i>
2A	Or47b-GAL4 > UAS-mCD8::GFP	<i>w-; UAS-mCD8::GFP/+; Or47b-GAL4/UAS-mCD8::GFP</i>
2A	Or49a-GAL4 > UAS-mCD8::GFP	<i>w-; Or49a-GAL4/UAS-mCD8::GFP; UAS-mCD8::GFP/+</i>
2A	Or56a-GAL4 > UAS-mCD8::GFP	<i>w-; UAS-mCD8::GFP/+; Or56a-GAL4/UAS-mCD8::GFP</i>
2A	Or65a-GAL4 > UAS-mCD8::GFP	<i>w-; Or65a-GAL4/UAS-mCD8::GFP; UAS-mCD8::GFP/+</i>
2A	Or67a-GAL4 > UAS-mCD8::GFP	<i>w-; Or67a-GAL4/UAS-mCD8::GFP; UAS-mCD8::GFP/+</i>
2A	Or67b-GAL4 > UAS-mCD8::GFP	<i>w-; UAS-mCD8::GFP/+; Or67b-GAL4/UAS-mCD8::GFP</i>
2A	Or69a-GAL4 > UAS-mCD8::GFP	<i>w-; Or69a-GAL4/UAS-mCD8::GFP; UAS-mCD8::GFP/+</i>
2A	Ir75b-GAL4 > UAS-mCD8::GFP	<i>w-; Ir75b-GAL4/UAS-mCD8::GFP; UAS-mCD8::GFP/+</i>
2A	Ir76a-GAL4 > UAS-mCD8::GFP	<i>w-; Ir76a-GAL4/UAS-mCD8::GFP; UAS-mCD8::GFP/+</i>
2A	Or82a-GAL4 > UAS-mCD8::GFP	<i>w-; Or82a-GAL4/UAS-mCD8::GFP; UAS-mCD8::GFP/+</i>
2A	Or83c-GAL4 > UAS-mCD8::GFP	<i>w-; Or83c-GAL4/UAS-mCD8::GFP; UAS-mCD8::GFP/+</i>
2A	Ir84a-GAL4 > UAS-mCD8::GFP	<i>w-; Ir84a-GAL4/UAS-mCD8::GFP; UAS-mCD8::GFP/+</i>
2A	Or88a-GAL4 > UAS-mCD8::GFP	<i>w-; Or88a-GAL4/UAS-mCD8::GFP; UAS-mCD8::GFP/+</i>
2A	Or92a-GAL4 > UAS-mCD8::GFP	<i>w-; UAS-mCD8::GFP/+; Or92a-GAL4/UAS-mCD8::GFP</i>
2B	Ir31a-GAL4 > UAS-mCD8::GFP	<i>w-; Ir31a-GAL4/UAS-mCD8::GFP; UAS-mCD8::GFP/+</i>
2B	Ir92a-GAL4 > UAS-mCD8::GFP	<i>w-; UAS-mCD8::GFP/+; Ir92a-GAL4/UAS-mCD8::GFP</i>
2C	Or59b-GAL4 > UAS-mCD8::GFP	<i>w-; Or59b-GAL4/UAS-mCD8::GFP; UAS-mCD8::GFP/+</i>
2C	Or85a-GAL4 > UAS-mCD8::GFP	<i>w-; UAS-mCD8::GFP/+; Or85a-GAL4/UAS-mCD8::GFP</i>
3A to C	<i>Gr21a-GAL4 > UAS-CsChrimson</i>	<i>w-; Gr21a-GAL4/+; 20xUAS-IVS-CsChrimson-mVenus in attP2/+</i>
3D	<i>Or42b-GAL4 > UAS-CsChrimson</i>	<i>w-; +; Or42b-GAL4/20xUAS-IVS-CsChrimson-mVenus in attP2</i>
3D	<i>Or92a-GAL4 > UAS-CsChrimson</i>	<i>w-; +; Or92a-GAL4/20xUAS-IVS-CsChrimson-mVenus in attP2</i>
3D	<i>Or10a-GAL4 > UAS-CsChrimson</i>	<i>w-; Gr10a-GAL4/+; 20xUAS-IVS-CsChrimson-mVenus in attP2/+</i>
3E	<i>Or82a-GAL4 > UAS-CsChrimson</i>	<i>w-; Or82a-GAL4/+; 20xUAS-IVS-CsChrimson-mVenus in attP2/+</i>
3E	<i>Or47a-GAL4 > UAS-CsChrimson</i>	<i>w-; Or47a-GAL4/+; 20xUAS-IVS-CsChrimson-mVenus in attP2/+</i>
3F	<i>Or83c-GAL4 > UAS-CsChrimson</i>	<i>w-; Or83c-GAL4/+; 20xUAS-IVS-CsChrimson-mVenus in attP2/+</i>
3F	<i>Or23a-GAL4 > UAS-CsChrimson</i>	<i>w-; Or23a-GAL4/+; 20xUAS-IVS-CsChrimson-mVenus in attP2/+</i>
3G	<i>Ir75b-GAL4 > UAS-CsChrimson</i>	<i>w-; Ir75b-GAL4/+; 20xUAS-IVS-CsChrimson-mVenus in attP2/+</i>
3G	<i>Or35a-GAL4 > UAS-CsChrimson</i>	<i>w-; +; Or35a-GAL4/20xUAS-IVS-CsChrimson-mVenus in attP2</i>
3H	<i>Or69a-GAL4 > UAS-CsChrimson</i>	<i>w-; Or69a-GAL4/+; 20xUAS-IVS-CsChrimson-mVenus in attP2/+</i>
3H	<i>Or67b-GAL4 > UAS-CsChrimson</i>	<i>w-; +; Or67b-GAL4/20xUAS-IVS-CsChrimson-mVenus in attP2</i>
4A	<i>Or42b-GAL4 > UAS-CsChrimson</i>	<i>w-; +; Or42b-GAL4/20xUAS-IVS-CsChrimson-mVenus in attP2</i>
4A	<i>Or92a-GAL4 > UAS-CsChrimson</i>	<i>w-; +; Or92a-GAL4/20xUAS-IVS-CsChrimson-mVenus in attP2</i>
4A	<i>Or82a-GAL4 > UAS-CsChrimson</i>	<i>w-; Or82a-GAL4/+; 20xUAS-IVS-CsChrimson-mVenus in attP2/+</i>
4A	<i>Or83c-GAL4 > UAS-CsChrimson</i>	<i>w-; Or83c-GAL4/+; 20xUAS-IVS-CsChrimson-mVenus in attP2/+</i>
4A	<i>Ir75b-GAL4 > UAS-CsChrimson</i>	<i>w-; Ir75b-GAL4/+; 20xUAS-IVS-CsChrimson-mVenus in attP2/+</i>
4A	<i>Or69a-GAL4 > UAS-CsChrimson</i>	<i>w-; Or69a-GAL4/+; 20xUAS-IVS-CsChrimson-mVenus in attP2/+</i>
4B	<i>Gr21a-GAL4 > UAS-CsChrimson</i>	<i>w-; Gr21a-GAL4/+; 20xUAS-IVS-CsChrimson-mVenus in attP2/+</i>
4B	<i>Or10a-GAL4 > UAS-CsChrimson</i>	<i>w-; Gr10a-GAL4/+; 20xUAS-IVS-CsChrimson-mVenus in attP2/+</i>
4B	<i>Or47a-GAL4 > UAS-CsChrimson</i>	<i>w-; Or47a-GAL4/+; 20xUAS-IVS-CsChrimson-mVenus in attP2/+</i>
4B	<i>Or23a-GAL4 > UAS-mCD8::GFP</i>	<i>w-; Or23a-GAL4/UAS-mCD8::GFP; UAS-mCD8::GFP/+</i>
4B	<i>Or35a-GAL4 > UAS-CsChrimson</i>	<i>w-; +; Or35a-GAL4/20xUAS-IVS-CsChrimson-mVenus in attP2</i>
4B	<i>Or67b-GAL4 > UAS-CsChrimson</i>	<i>w-; +; Or67b-GAL4/20xUAS-IVS-CsChrimson-mVenus in attP2</i>
5B	<i>Or7a-GAL4 > UAS-CsChrimson</i>	<i>Or7a^{GAL4}/w-; +; 20xUAS-IVS-CsChrimson-mVenus in attP2/+</i>
5B	<i>Or56a-GAL4 > UAS-CsChrimson</i>	<i>w-; +; Or56a-GAL4/20xUAS-IVS-CsChrimson-mVenus in attP2</i>
5C	<i>Or67a-GAL4 > UAS-CsChrimson</i>	<i>w-; Or67a-GAL4/+; 20xUAS-IVS-CsChrimson-mVenus in attP2/+</i>
5C	<i>Or49a-GAL4 > UAS-CsChrimson</i>	<i>w-; Or49a-GAL4/+; 20xUAS-IVS-CsChrimson-mVenus in attP2/+</i>
5D	<i>Or19a-GAL4 > UAS-CsChrimson</i>	<i>w-; Or19a-GAL4/+; 20xUAS-IVS-CsChrimson-mVenus in attP2/+</i>
5D	<i>Or43a-GAL4 > UAS-CsChrimson</i>	<i>w-; Or43a-GAL4/+; 20xUAS-IVS-CsChrimson-mVenus in attP2/+</i>
5E	<i>Or47b-GAL4 > UAS-TrpA1</i>	<i>w-; UAS-TrpA1 in attP16/+; Or47b-GAL4/+</i>
5F	<i>UAS-TrpA1 (control for Or47b-GAL4 > UAS-TrpA1)</i>	<i>w-; UAS-TrpA1 in attP16/+; +</i>
5F	<i>Or47b-GAL4</i>	<i>w-; +; Or47b-GAL4/+</i>
5F	<i>Or47b-GAL4 > UAS-TrpA1</i>	<i>w-; UAS-TrpA1 in attP16/+; Or47b-GAL4/+</i>
5G	<i>UAS-TrpA1 (control for Or65a-GAL4 > UAS-TrpA1)</i>	<i>w+; UAS-TrpA1 in attP16/+; +</i>

Table 3. Fly genotypes in figures (continued).

5G	<i>Or65a-GAL4</i>	<i>w⁺; Or65a-GAL4/+</i>
5G	<i>Or65a-GAL4 > UAS-TrpA1</i>	<i>w⁻; Or65a-GAL4/UAS-TrpA1 in attP16; +</i>
5H	<i>UAS-TrpA1</i> (control for <i>Or88a-GAL4 > UAS-TrpA1</i>)	<i>w⁺; UAS-TrpA1 in attP16/+; +</i>
5H	<i>Or88a-GAL4</i>	<i>w⁺; Or88a-GAL4/+</i>
5H	<i>Or88a-GAL4 > UAS-TrpA1</i>	<i>w⁻; Or88a-GAL4/UAS-TrpA1 in attP16; +</i>
5I	<i>Ir84a-GAL4 > UAS-TrpA1</i>	<i>w⁻; Ir84a-GAL4/UAS-TrpA1 in attP16; +</i>
5J	<i>UAS-TrpA1</i> (control for <i>Or47b-GAL4 > UAS-TrpA1</i>)	<i>w⁻; UAS-TrpA1 in attP16/+; +</i>
5J	<i>Ir84a-GAL4</i>	<i>w⁻; Ir84a-GAL4/+</i>
5J	<i>Ir84a-GAL4 > UAS-TrpA1</i>	<i>w⁻; Ir84a-GAL4/UAS-TrpA1 in attP16; +</i>
5K	<i>UAS-TrpA1</i> (control for <i>Ir76a-GAL4 > UAS-TrpA1</i>)	<i>w⁺; UAS-TrpA1 in attP16/+; +</i>
5K	<i>Ir76a-GAL4</i>	<i>w⁺; Ir76a-GAL4/+</i>
5K	<i>Ir76a-GAL4 > UAS-TrpA1</i>	<i>w⁻; Ir76a-GAL4/UAS-TrpA1 in attP16; +</i>
5F, G, H, J, K	<i>Canton-S</i> (female)	<i>Canton-S</i>
6A and B	<i>Or42b-GAL4 > UAS-CsChrimson</i>	<i>w⁻; +; Or42b-GAL4/20xUAS-IVS-CsChrimson-mVenus in attP2</i>
6A and B	<i>Gr21a-GAL4 > UAS-CsChrimson</i>	<i>w⁻; Gr21a-GAL4/+; 20xUAS-IVS-CsChrimson-mVenus in attP2/+</i>
6C	<i>UAS-TrpA1</i> (control for <i>Or42b-GAL4 > UAS-TrpA1</i>)	<i>w⁻; UAS-TrpA1 in attP16/+; +</i>
6C	<i>Or42b-GAL4</i>	<i>w⁻; +; Or42b-GAL4/+</i>
6C	<i>Or42b-GAL4 > UAS-TrpA1</i>	<i>w⁻; UAS-TrpA1 in attP16/+; Or42b-GAL4/+</i>
6C	<i>UAS-TrpA1</i> (control for <i>Gr21a-GAL4 > UAS-TrpA1</i>)	<i>w⁺; UAS-TrpA1 in attP16/+; +</i>
6C	<i>Gr21a-GAL4</i>	<i>w⁺; Gr21-GAL4/+</i>
6C	<i>Gr21a-GAL4 > UAS-TrpA1</i>	<i>w⁻; Gr21a-GAL4/UAS-TrpA1 in attP16; +</i>
6D and E	<i>Or7a-GAL4 > UAS-CsChrimson</i>	<i>Or7a^{GAL4} /w⁻; +; 20xUAS-IVS-CsChrimson-mVenus in attP2/+</i>
6D and E	<i>Or56a-GAL4 > UAS-CsChrimson</i>	<i>w⁻; +; Or56a-GAL4/20xUAS-IVS-CsChrimson-mVenus in attP2</i>
6F	<i>UAS-TrpA1</i> (control for <i>Or7a-GAL4 > UAS-TrpA1</i>)	<i>w⁻; UAS-TrpA1 in attP16/+; +</i>
6F	<i>Or7a-GAL4</i>	<i>Or7a^{GAL4}</i>
6F	<i>Or7a-GAL4 > UAS-TrpA1</i>	<i>Or7a^{GAL4} ; UAS-TrpA1 in attP16/+</i>
6F	<i>UAS-TrpA1</i> (control for <i>Or56a-GAL4 > UAS-TrpA1</i>)	<i>w⁺; UAS-TrpA1 in attP16/+; +</i>
6F	<i>Or56a-GAL4</i>	<i>w⁺; +; Or56a-GAL4/+</i>
6F	<i>Or56a-GAL4 > UAS-TrpA1</i>	<i>w⁻; UAS-TrpA1 in attP16/+; Or56a-GAL4/+</i>
6G and H	<i>Or47b-GAL4 > UAS-CsChrimson</i>	<i>w⁻; +; Or47b-GAL4/20xUAS-IVS-CsChrimson-mVenus in attP2</i>
6G and H	<i>Or88a-GAL4 > UAS-CsChrimson</i>	<i>w⁻; Or88a-GAL4/+; 20xUAS-IVS-CsChrimson-mVenus in attP2/+</i>
6I	<i>UAS-TrpA1</i> (control for <i>Or47b-GAL4 > UAS-TrpA1</i>)	<i>w⁻; UAS-TrpA1 in attP16/+; +</i>
6I	<i>Or47b-GAL4</i>	<i>w⁻; +; Or47b-GAL4/+</i>
6I	<i>Or47b-GAL4 > UAS-TrpA1</i>	<i>w⁻; UAS-TrpA1 in attP16/+; Or47b-GAL4/+</i>
6I	<i>UAS-TrpA1</i> (control for <i>Or88a-GAL4 > UAS-TrpA1</i>)	<i>w⁺; UAS-TrpA1 in attP16/+; +</i>
6I	<i>Or88a-GAL4</i>	<i>w⁺; Or88a-GAL4/+</i>
6I	<i>Or88a-GAL4 > UAS-TrpA1</i>	<i>w⁻; Or88a-GAL4/UAS-TrpA1 in attP16; +</i>
6C, F, I	<i>Canton-S</i> (female)	<i>Canton-S</i>
7A to D, E, F, K, L	<i>Berlin(W⁺)</i>	<i>Berlin(W⁺)</i>
7H,N	<i>Or88a-GAL4 > UAS-Shibire^{ts1}</i>	<i>w⁻; Or88a-GAL4/+; 20XUAS-IVS-Shibire^{ts1} in VK00005/+</i>
7I,O	<i>Or67d-GAL4 > UAS-Shibire^{ts1}</i>	<i>w⁺/w⁻; Or67d^{GAL4} /+; 20XUAS-IVS-Shibire^{ts1} in VK00005/+</i>
7E, F, H, I, K, L, N, O	<i>Canton-S</i> (female)	<i>Canton-S</i>
7A, C, D	<i>Berlin(W⁺)</i>	<i>Berlin(W⁺)</i>
7B	<i>Or7a^{-/-}</i>	<i>Or7a^{GAL4} ;+;+</i>
7F	<i>Or88a-GAL4 > UAS-Shibire^{ts1}</i>	<i>w⁻; Or88a-GAL4/+; 20XUAS-IVS-Shibire^{ts1} in VK00005/+</i>
7G	<i>Or67d-GAL4 > UAS-Shibire^{ts1}</i>	<i>w⁺/w⁻; Or67d^{GAL4} /+; 20XUAS-IVS-Shibire^{ts1} in VK00005/+</i>
7A to D, F to G	<i>Canton-S</i> (female)	<i>Canton-S</i>
8A and B	<i>Berlin(W⁺)</i>	<i>Berlin(W⁺)</i>
9A	<i>Berlin(W⁺)</i>	<i>Berlin(W⁺)</i>
9B	<i>Or47b^{-/-}</i>	<i>w⁻; Or47b^{2/2}</i>
9C and E	<i>Or88a^{-/-}</i>	<i>w⁺; Or88a^{-/-}</i>
9D and F	<i>Or67d^{-/-}</i>	<i>w⁺; Or67d^{GAL4}</i>
9	<i>Canton-S</i> (female)	<i>Canton-S</i>
10	<i>Or88a-GAL4 > UAS-Shibire^{ts1}</i>	<i>w⁻; Or88a-GAL4/+; 20XUAS-IVS-Shibire^{ts1} in VK00005/+</i>
14	<i>Gr21a-GAL4 > UAS-CsChrimson</i>	<i>w⁻; Gr21a-GAL4/+; 20xUAS-IVS-CsChrimson-mVenus in attP2/+</i>

Table 4. Reagent or Resource.

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Rabbit α -GFP	Invitrogen	RRID:AB_221569
Bruchpilot (nc82) Mouse mAB	Developmental Studies Hybridoma Bank	RRID:AB_2314866
Goat anti-Rabbit IgG (H+L), Superclonal Recombinant Secondary Antibody, Alexa Fluor 488	Invitrogen	RRID:AB_2536097
Goat anti-Rabbit IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 647	Invitrogen	RRID:AB_2535812
Chemicals, peptides, and recombinant proteins		
<i>trans</i> -Palmitoleic acid	Cayman Chemical	CAS:10030-73-6
11- <i>cis</i> Vaccenyl Acetate	Cayman Chemical	CAS:6186-98-7
(Z)-9-Tricosene	Sigma-Aldrich	CAS:27519-02-4
Methyl palmitate	Sigma-Aldrich	CAS:112-39-0
Experimental models: Organisms/strains		
<i>Drosophila</i> : <i>w¹¹¹⁸</i>	10.1016/j.neuron.2016.05.004	N/A
<i>Drosophila</i> : <i>Berlin (W⁺)</i>	10.1016/j.neuron.2016.05.004	N/A
<i>Drosophila</i> : <i>Canton-S</i>	10.1016/j.neuron.2016.05.004	N/A
<i>Drosophila</i> : <i>Or47b^{2/2}</i>		RRID:BDSC_51306
<i>Drosophila</i> : <i>Or88a^{-/-}</i>	10.1073/pnas.1504527112	N/A
<i>Drosophila</i> : <i>Or7a^{GAL4}</i>	10.7554/eLife.08688	N/A
<i>Drosophila</i> : <i>Or67d^{GAL4}</i>	10.1038/nature05672	N/A
<i>Drosophila</i> : <i>UAS-mCD8::GFP</i>		RRID:BDSC_5137
<i>Drosophila</i> : <i>UAS-mCD8::GFP</i>		RRID:BDSC_5130
<i>Drosophila</i> : <i>UAS-CsChrimson</i>		RRID:BDSC_55136
<i>Drosophila</i> : <i>UAS-Shibire^{ts1}</i>		RRID:BDSC_66599
<i>Drosophila</i> : <i>UAS-TrpA1</i>		RRID:BDSC_26263
<i>Drosophila</i> : <i>Or10a-GAL4</i>		RRID:BDSC_9944
<i>Drosophila</i> : <i>Or19a-GAL4</i>		RRID:BDSC_24617
<i>Drosophila</i> : <i>Gr21a-GAL4</i>	10.1038/nature02980	N/A
<i>Drosophila</i> : <i>Or23a-GAL4</i>		RRID:BDSC_9956
<i>Drosophila</i> : <i>Ir31a-GAL4</i>		RRID:BDSC_41726
<i>Drosophila</i> : <i>Or35a-GAL4</i>	10.1523/JNEUROSCI.2432-05.2005	N/A
<i>Drosophila</i> : <i>Or42b-GAL4</i>		RRID:BDSC_9972
<i>Drosophila</i> : <i>Or43a-GAL4</i>		RRID:BDSC_9973
<i>Drosophila</i> : <i>Or47a-GAL4</i>		RRID:BDSC_9981
<i>Drosophila</i> : <i>Or47b-GAL4</i>		RRID:BDSC_9984
<i>Drosophila</i> : <i>Or49a-GAL4</i>		RRID:BDSC_9985
<i>Drosophila</i> : <i>Or56a-GAL4</i>		RRID:BDSC_23896
<i>Drosophila</i> : <i>Or59b-GAL4</i>		RRID:BDSC_23897
<i>Drosophila</i> : <i>Or65a-GAL4</i>		RRID:BDSC_9993
<i>Drosophila</i> : <i>Or67a-GAL4</i>		RRID:BDSC_23904
<i>Drosophila</i> : <i>Or67b-GAL4</i>		RRID:BDSC_9995
<i>Drosophila</i> : <i>Or69a-GAL4</i>		RRID:BDSC_9999
<i>Drosophila</i> : <i>Ir75b-GAL4</i>	10.1016/j.cell.2008.12.001	N/A
<i>Drosophila</i> : <i>Ir76a-GAL4</i>		RRID:BDSC_41735
<i>Drosophila</i> : <i>Or82a-GAL4</i>		RRID:BDSC_23125
<i>Drosophila</i> : <i>Or83c-GAL4</i>		RRID:BDSC_23131

Table 4. Reagent or Resource (continued).

<i>Drosophila: Ir84a-GAL4</i>		RRID:BDSC_41734
<i>Drosophila: Or85a-GAL4</i>		RRID:BDSC_24461
<i>Drosophila: Or88a-GAL4</i>		RRID:BDSC_23137
<i>Drosophila: Ir92a-GAL4</i>		RRID:BDSC_41733
<i>Drosophila: Or92a-GAL4</i>		RRID:BDSC_23139
Software and algorithms		
Fiji	NIH	RRID: SCR_002285
MATLAB R2019a	MathWorks	http://www.mathworks.com/
Igor Pro 6.32A	WaveMetrics	RRID: SCR_000325
Clampfit 10.7	Molecular Devices	https://www.moleculardevices.com

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Appendix 1: A peripheral mechanism underlying concentration-dependent valence switch in olfaction

Animals respond differentially to environmental cues based on stimulus intensity. For example, *C. elegans* shows preference to food-derived odorants at lower concentrations but rejects the same chemicals at higher concentrations (Choi et al., 2022; Yoshida et al., 2012). This concentration-dependent olfactory valence switch has been observed in many other species including humans (Horio et al., 2019; Laing et al., 1978; Semmelhack and Wang, 2009; Stensmyr et al., 2003). Although the neuronal substrates that detect different concentrations of the same odorants have been discussed (Choi et al., 2022; Semmelhack and Wang, 2009; Yoshida et al., 2012), where and how these behaviorally antagonistic signals are processed are not fully understood. Here, we identified a peripheral mechanism underlying concentration-dependent valence switch in fly's olfaction. Specifically, we found that within the at4 sensillum, the courtship-inhibiting Or88a neuron can be passively activated by its courtship-promoting neighbor expressing the Or47b receptor. As such, the Or47b pheromone ligand, palmitoleic acid, can promote courtship at low levels but instead inhibit courtship at high concentrations. The concentration-dependent valence switch of palmitoleic acid was abolished in transgenic flies whose Or88a neuronal transmission was silenced. Our results explain how the hedonic value of an odorant is concentration-dependent.

Lateral excitation in an olfactory sensillum

Ephaptic coupling between olfactory receptor neurons (ORNs) was observed across sensillum types (Su et al., 2012; Zhang et al., 2019). Lateral inhibition occurs when both olfactory receptor neurons are activated by odor mixture. Interestingly, when the large-spike neuron is not activated by its cognate odorant, strong activation of small-spike ORN can instead elicit excitatory response in its large-spike neighbor, suggesting that ephaptic interaction can be either excitatory or inhibitory depending on activity (Su et al., 2012). Given the asymmetric nature of ephaptic interaction in a sensillum, that is, the physically larger "A" ORN has greater ephaptic influence on its small-spike "B" partner (Zhang et al., 2019), we expect to see more substantial lateral excitation

from large-spike ORN to small-spike ORN in a sensillum than contrariwise. To test this hypothesis, we first examined lateral excitation in the ab2 sensillum, where the “A” ORN is markedly larger than the neighboring “B” ORN (Gonzales et al., 2021).

Within the ab2 sensillum, the individual receptors can be selectively activated by their specific odorants. Methyl acetate can only activate the “A” neuron receptor Or59b, but not the “B” neuron receptor Or85a. Similarly, ethyl 3-hydroxy butyrate only targets the “B” receptor (Fig. 18A). To examine whether the ab2B ORN can be passively activated by its large-spike neighbor, we expressed a calcium indicator in the ab2B ORN. We found that the ab2B neuron showed significant response to the ab2A neuron odorant in addition to its own private odorant (Fig. 18A). To determine whether the excitatory response of “B” neuron to methyl acetate is mediated by its large-spike neighbor through lateral excitation, we examined the response of ab2B neuron in mutant flies whose ab2A neuron receptor, Or59b, was mutated by CRISPR deletion. Interestingly, in *Or59b* mutants, the ab2B neuron no longer showed response to the ab2A neuron odorant (Fig. 18B), suggesting that the ab2B neuron’s response to methyl acetate resulted from the strong activation of the ab2A neuron. As a control, we examined the activity in the receptor knockout ab2B neuron in mutant flies whose B neuron receptor, Or85a, was mutated by CRISPR deletion. As expected, Or85a mutation abolished ab2B ORN’s response to its cognate odorant, ethyl 3-hydroxy butyrate (Fig. 18C). Strikingly, the mutant ab2B neuron maintained significant excitatory response to the ab2A neuron odorant (Fig. 18C), suggesting that the excitatory response was not mediated cell-autonomously but instead through the neighboring neuron’s activation. Together, these results indicate that the small-spike ORN can be activated by excitation of its large-spike neighbor in a sensillum.

Lateral excitation between at4 ORNs mediates concentration-dependent valence switch in courtship

Can lateral excitation in a sensillum influence behavior? Given that paired ORNs typically antagonistically modulate the same behavior (Wu et al., 2022), we hypothesized that lateral excitation from large-spike ORN to small-spike ORN can reverse the hedonic value of the same odorant from positive to negative. To test our hypothesis, we focused on the at4 sensillum where the large-spike at4A ORN promotes courtship, while the small-spike at4B and at4C ORNs both inhibit courtship (Lin et al., 2016; Wu et al., 2022). We first confirmed that the “A” neuron ligand, palmitoleic acid (PA), does not activate at4B or at4C neurons directly by performing single-sensillum recording from Or47b mutants (Lin et al., 2016) (Fig. 19A). Interestingly, in the presence of functional Or47b receptor, we found that the at4A ligand, PA, can instead elicit marked excitatory response in at4C ORNs (Fig. 19B), raising the possibility that strong activation of at4A can excite the co-housed at4C ORN. As such, lateral excitation of the courtship-inhibiting at4C neuron by at4A can in principle attenuate the aphrodisiac effect of PA when the concentration of PA is high.

To test this hypothesis, we performed single-pair courtship assays (Wu et al., 2022) while perfuming different concentrations of PA on females (Fig. 19C). We found that a low level of PA (~0.1 ng) promotes male courtship. However, when higher concentrations of PA (1 ng or 10 ng) were applied, anti-aphrodisiac effects were instead observed (Fig. 19C). Interestingly, we found that both the courtship enhancing and inhibitory effects of PA were abolished in male Or47b mutants, suggesting that at4A response is indispensable for both PA-evoked courtship promotion and inhibition (Fig. 19D).

To test whether the at4C input is required for the courtship inhibition in the presence of higher concentrations of PA, we blocked feedforward transmission of the at4C neuron. In support of our hypothesis, this manipulation abolished the courtship inhibitory effect of PA; high concentrations of PA instead promoted courtship (Fig. 19E). A similar phenotype was observed when the synaptic transmission of both small-spike ORNs were blocked (Fig. 19F). Together, these results suggest that lateral excitation from courtship-promoting “A” neuron to courtship-

inhibiting “C” neuron in the at4 sensillum mediates the concentration-dependent valence switch in courtship.

Future works and outlook

In this study, we reported that lateral excitation within the at4 sensillum can mediate concentration-dependent valence switch in courtship. Nevertheless, whether lateral excitation occurs in other sensillum types and whether this peripheral mechanism applies to other behavioral contexts remain unclear. A comprehensive investigation is required to address these questions. Given that no gap junction or synaptic connection has been observed between compartmentalized ORNs in prior EM studies (Shanbhag et al., 1999, 2000), it will be interesting to determine whether lateral excitation in a sensillum is also mediated by ephaptic coupling.

For male flies, excessive aphrodisiac substance can be anti-aphrodisiac (Fig. 19C). Interestingly, we found that this switch of valence is mediated, at least in part, by compartmentalized ORNs at the earliest stage of olfactory system. Our results thus provide a novel peripheral mechanism which mediates concentration-dependent valence switch in olfaction.

Appendix 1, in part is currently being prepared for submission for publication, under the title “A peripheral mechanism underlying concentration-dependent valence switch in olfaction”. Wu, Shiuan-Tze; Martin, Vanessa; Su, Chih-Ying. The dissertation author is the primary researcher and author.

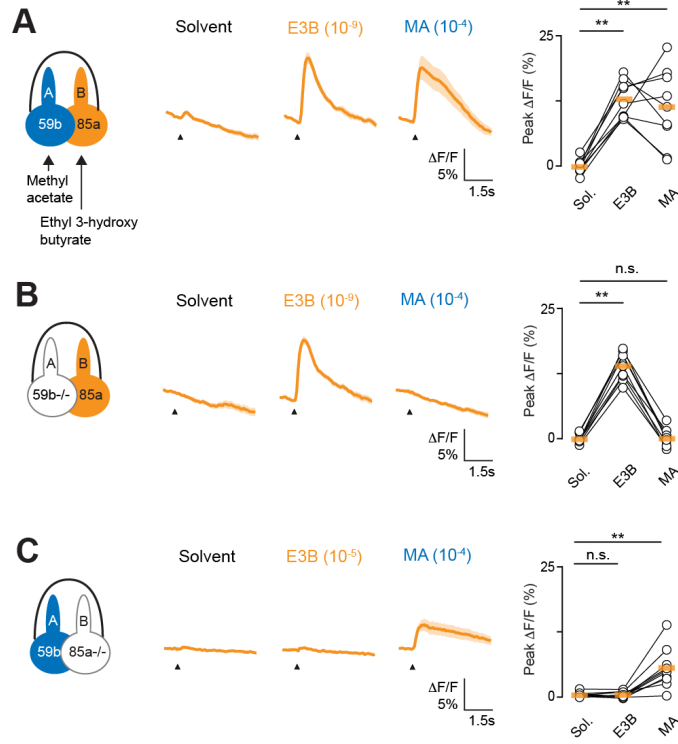


Figure 18. Lateral excitation in the ab2 sensillum.

Calcium activity of ab2B ORNs to the solvent (paraffin oil), ethyl 3-hydroxy butyrate (E3B), or methyl acetate (MA) in *Or85a::GCaMP7c* flies. (A) wildtype flies. (B) *Or59b*^{-/-} mutants. (C) *Or85a*^{-/-} mutants. Mean $\Delta F/F$ is plotted against time. Right panels: each circle denotes the peak $\Delta F/F$ of a given antenna with lines connecting data from the same sample ($n=9-10$ flies, one antenna per fly); horizontal lines indicate mean peak calcium responses. ** $p < 0.01$, Wilcoxon signed-rank test.

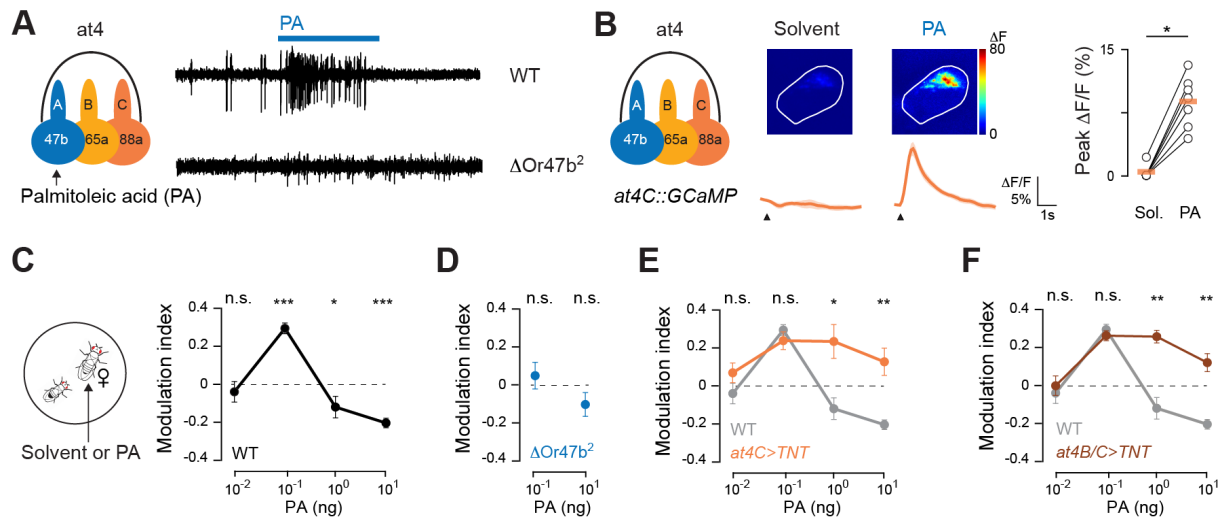


Figure 19. Lateral excitation between *at4* ORNs mediates concentration-dependent valence switch in courtship (A) Sample traces of single-unit recording of *at4* sensillum in WT and *at4A* mutant ($\Delta Or47b^2$) males. Horizontal line: 500-ms palmitoleic acid (PA) pulse. (B) Calcium activity of *at4C* ORNs in *Or88a::GCaMP6m* flies. Pseudocolored images show representative ORN responses to solvent (ethanol) or PA. Antennae are outlined. Mean $\Delta F/F$ is plotted against time. Right panels: each circle denotes the peak $\Delta F/F$ of an antenna with lines connecting data from the same sample ($n=7$); horizontal lines indicate mean peak calcium responses. * $p < 0.05$, Wilcoxon signed-rank test. (C-F) Single-pair courtship assays. Each female was perfumed with solvent (acetone) or palmitoleic acid. Copulation rates (CR) were calculated at the end of 2-h assays. Copulation modulation index: $(CR_{PA} - CR_{solvent}) / CR_{solvent}$. (C-D) PA-elicited copulation modulation in WT and *at4A* mutant ($\Delta Or47b^2$) males. One-sample z-test was used to determine whether PA applications impact copulation rate, * $p < 0.05$, *** $p < 0.001$. (E-F) PA-elicited copulation modulation in *at4C>TNT* and *at4B/C>TNT* males. Wilcoxon rank-sum test was used to determine whether the synaptic transmission blocked males had different courtship phenotypes compared to WT, * $p < 0.05$, ** $p < 0.01$.

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Appendix 2: Amplification of *Drosophila* Olfactory Responses by a DEG/ENaC Channel

Significance: This study illustrates how ionotropic inputs are amplified in sensory neurons. Using *Drosophila* olfactory receptor neurons (ORNs) as a model, we found that receptor-mediated calcium influx serves as a secondary messenger to activate a sodium channel, Pickpocket 25 (PPK25), thereby amplifying ionotropic input. Interestingly, the expression of PPK25 is upregulated by a reproductive hormone in courtship-promoting ORNs, and PPK25 in these neurons is required for courtship. Together, our findings suggest that reproductive behavior can be dynamically regulated by hormone at olfactory periphery.

Note of contribution: To directly compare the courtship drive of male flies of different genotypes, I developed a three-male competition courtship assay and found that the expression of PPK25 in male Or47b ORNs is critical for mating success (Figure 2A-B, page 82). I further analyzed the detailed behavioral sequence of individual male flies using video recording to confirm that PPK25 indeed impacts courtship drive (Figure 2C-D, page 82). My results demonstrate the functional significance of the PPK25-mediated signal amplification of olfactory response.

Appendix 2, in part, is a reprint of the material as it appears in Amplification of *Drosophila* Olfactory Responses by a DEG/ENaC Channel 2019. Ng, Renny; Salem, Secilia S.; Wu, Shiuan-Tze; Wu, Meilin; Lin; Hui-Hao; Shepherd, Andrew K.; Joiner, William J.; Wang, Jing W.; Su, Chih-Ying, *Neuron* 104: 947-959, 2019. The dissertation author is a co-author of this paper.

Amplification of *Drosophila* Olfactory Responses by a DEG/ENaC Channel

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SUMMARY

Insect olfactory receptors operate as ligand-gated ion channels that directly transduce odor stimuli into electrical signals. However, in the absence of any known intermediate transduction steps, it remains unclear whether and how these ionotropic inputs are amplified in olfactory receptor neurons (ORNs). Here, we find that amplification occurs in the *Drosophila* courtship-promoting ORNs through Pickpocket 25 (PPK25), a member of the degenerin/epithelial sodium channel family (DEG/ENaC). Pharmacological and genetic manipulations indicate that, in Or47b and Ir84a ORNs, PPK25 mediates Ca²⁺-dependent signal amplification via an intracellular calmodulin-binding motif. Additionally, hormonal signaling upregulates PPK25 expression to determine the degree of amplification, with striking effects on male courtship. Together, these findings advance our understanding of sensory neurobiology by identifying an amplification mechanism compatible with ionotropic signaling. Moreover, this study offers new insights into DEG/ENaC activation by highlighting a novel means of regulation that is likely conserved across species.

INTRODUCTION

Vertebrates detect odorants with G-protein-coupled receptors (GPCRs), the activation of which triggers subsequent metabolic signaling cascades in the olfactory receptor neurons (ORNs) to transduce chemical stimuli into electrical signals (Kaupp, 2010; Reisert and Zhao, 2011). These series of transduction events also provide opportunities to amplify input signals (Kaupp, 2010; Reisert and Zhao, 2011). In contrast, insect olfaction is initiated by ligand-gated receptor channels that lack canonical G protein interacting domains (Abuin et al., 2011; Butterwick et al., 2018; Nakagawa and Vossahl, 2009; Sato et al., 2008; Wicher et al., 2008). Although various G proteins and effectors have been implicated in the function of insect

ORNs (Nakagawa and Vossahl, 2009; Wicher, 2015), it remains an open question whether those molecules play a specific role in olfactory transduction or a regulatory role in neuronal development, maintenance, and neuromodulation. Given that ligand-gated receptor channels can directly convert sensory stimuli to neuronal depolarization (Julius and Nathans, 2012), it is unclear whether and how ionotropic inputs can be amplified in the absence of any known intermediate transduction steps.

In earlier work, we found that the responses of Or47b ORNs, which detect aphrodisiac fly odors in *D. melanogaster*, increase with age in male flies (Lin et al., 2016), pointing to the possibility of signal amplification downstream of insect olfactory receptors. This age-dependent plasticity therefore presents an opportunity to investigate the mechanisms by which ionotropic sensory inputs can be amplified. Intriguingly, Or47b ORNs express a degenerin/epithelial sodium channel (DEG/ENaC) subunit named Pickpocket 25 (PPK25) (Starostina et al., 2012). Within invertebrate genomes, DEG/ENaCs constitute one of the largest ion channel families (Ben-Shahar, 2011). In mechanosensory and gustatory neurons, PPK subunits are involved in touch, proprioception, nociception, salt taste, water sensation, and recognition of contact pheromones (Adams et al., 1998; Ainsley et al., 2003; Cameron et al., 2010; Chen et al., 2010; Guo et al., 2014; Lin et al., 2005; Liu et al., 2003a, 2012; Lu et al., 2012; Starostina et al., 2012; Thistle et al., 2012; Toda et al., 2012; Vijayan et al., 2014; Zhong et al., 2010). However, the functional role of PPK25 in olfaction remains unknown.

If PPK25 amplifies olfactory signals, how then is its activity regulated? Can it function as a transduction channel activated by intracellular second messengers downstream of receptor activation? Members of the DEG/ENaC superfamily, including the mammalian nonvoltage-gated sodium channels (SCNNs) and acid-sensing sodium ion channels (ASICs), are known to open in response to mechanical stimuli, extracellular ligands, or are otherwise constitutively active (Cameron et al., 2010; Eastwood and Goodman, 2012; Kellenberger and Schild, 2002; Younger et al., 2013). In cultured cell lines, various intracellular signaling mechanisms can influence DEG/ENaC currents by regulating channel transcription, endocytosis, degradation, or translocation (Alli et al., 2015; Malik et al., 2001; Shi et al., 2002; Tokuda et al., 2002; Yu et al., 2013; Zentner et al., 1998). Post-translational modification is also known to modulate DEG/ENaC function; for example, constitutive channel activity

can be regulated by CaMKII-mediated phosphorylation (Sun et al., 2013) or by protease-mediated cleavage of the extracellular domain (Vallet et al., 1997). However, the possibility of DEG/ENaC activation by direct interaction with an intracellular ligand has not been explored.

In this study, we show that signal amplification can occur downstream of ligand-gated receptor channels. We find that the age-dependent response plasticity of Or47b ORNs arises through PPK25-mediated amplification. Additionally, this mechanism is employed in another type of courtship-promoting ORN expressing the Ir84a receptor. Interestingly, the degree of amplification is determined by PPK25 expression levels, which are in turn upregulated by a reproductive hormone. Thus, a common hormone regulates these two parallel olfactory pathways to coordinate courtship behavior. Mechanistically, PPK25 operates as a transduction channel: its activation requires odor-induced Ca^{2+} influx and a calmodulin binding motif (CBM) in the N-terminal intracellular domain. This result therefore highlights a novel mechanism whereby DEG/ENaCs can be activated by second messengers, a critical feature common to all transduction channels. Moreover, similar intracellular CBMs are predicted in multiple DEG/ENaCs across animal species, suggesting an evolutionarily conserved regulatory mechanism for channels in this superfamily.

RESULTS

A DEG/ENaC Channel for Olfaction

We first tested whether the age-dependent response increase in the Or47b ORNs (Figures 1A and 1B) is mediated by mechanisms that amplify signals downstream of the Or47b ligand-gated receptor channel. We focused on PPK25, a DEG/ENaC that is expressed in these neurons (Starostina et al., 2012; Figure 1C), where it could function as a transduction channel to amplify inputs from the Or47b receptors. We performed single-sensillum recording from *ppk25* mutant males (*ppk25*⁴⁵⁻²², deletion of the first three exons of *ppk25*; Lin et al., 2005) and found that the responses of their Or47b ORNs did not increase with age (Figures 1D and 1E) but rather remained low, at a similar level as 2-day-old control males (Figure 1F). The lack of age-dependent sensitization phenotype was also observed in another *ppk25* mutant allele (*Δppk25*^{LexA}), which was generated by knocking in LexA after the start codon of *ppk25* (Figure 1F). Importantly, deleting *ppk25* eliminated the age-dependent plasticity but did not abolish Or47b ORN responses. This result suggests that PPK25 amplifies, but not directly mediates, the responses of these neurons. In agreement with this idea, genetic rescue of *ppk25* in the Or47b ORNs restored amplification of their responses (Figure 1F). We note that *ppk25* did not appear to function in female Or47b ORNs, the responses of which did not increase with age (Figures S1A and S1B) or decrease with *ppk25* mutation (Figures S1C–S1E). Together, these experiments establish that amplification of ionotropic inputs occurs downstream of the Or47b receptor and that age-dependent response plasticity in the Or47b ORNs results from this amplification.

In a complementary experiment, we knocked down *ppk25* with RNAi selectively in the Or47b ORNs, which markedly

reduced the responses in 7-day-old males (Figures 1G and 1H) to the level of 2-day-old controls (Figure 1I). We note that, in 2-day-old males, *ppk25* knockdown resulted in a small but significant reduction in their responses (Figure 1I), suggesting a subtle function of PPK25 in the Or47b ORNs of young males. As a control for possible off-target effects, we expressed *ppk25* RNAi in the Or22a ORNs, which do not express *ppk25* or exhibit any age-dependent plasticity, and found no changes in their responses (Figure S2).

ppk25 in ORNs Impacts Courtship

Using this RNAi manipulation (*Or47b-GAL4>UAS-ppk25-RNAi*), we further tested whether *ppk25* influences olfactory behavior. In a courtship competition assay, we pitted *ppk25* knockdown males against the *GAL4* and *UAS* controls and found that the knockdown males exhibited lower copulation rates than controls (Figure 2A). Among the males that copulated with females, only ~20% of them were *ppk25* knockdown flies (Figure 2B). Additionally, in single-pair courtship assays, *ppk25* knockdown males exhibited prolonged courtship latency (Figure 2C), as well as a lower courtship index (Figure 2D). These results highlight the functional importance of PPK25, whereby the loss of a single channel in one subset of ORNs can profoundly compromise courtship success.

Together, our electrophysiological recordings and behavioral experiments show that *ppk25* is necessary for the amplified Or47b ORN responses in older males and that this plasticity in the primary sensory neurons significantly influences behavior.

PPK25 Levels and Amplification

We next investigated the relationship between PPK25 expression levels and signal amplification. Given the age-dependent increase of Or47b ORN responses (Figure 1B), we tested whether the antennal expression of *ppk25* also increases with age. Through qRT-PCR, we observed a subtle but significant increase in the *ppk25* transcript between 2- and 7-day-old males (Figure 3A), but not in females (not shown). We note that *ppk25* was already expressed in the antennae of 2-day-old males, consistent with the observation that *ppk25* knockdown further reduced the Or47b ORN responses of young males (Figure 1I). Interestingly, exposing newly eclosed males for 2 days to a juvenile hormone analog, methoprene (Jindra et al., 2013), significantly increased their *ppk25* expression (Figure 3A). In earlier work, we found that age modulates Or47b ORN responses via juvenile hormone (Lin et al., 2016), a pleiotropic reproductive hormone that regulates male fertility and courtship behavior (Flatt et al., 2005). Specifically, methoprene treatment markedly increased the Or47b ORN responses in 2-day-old males (Lin et al., 2016). Thus, the qRT-PCR result suggests that age likely upregulates *ppk25* expression by means of juvenile hormone signaling. In support of this hypothesis, methoprene treatment of *ppk25* mutant males did not increase their Or47b ORN responses (Figure S3), suggesting that *ppk25* upregulation is downstream of juvenile hormone signaling. Based on these results, we predicted that increasing *ppk25* expression in the Or47b ORNs would heighten their olfactory responses.

To test this hypothesis, we used the *GAL4*-upstream activating sequence (*UAS*) system to increase *ppk25* expression in

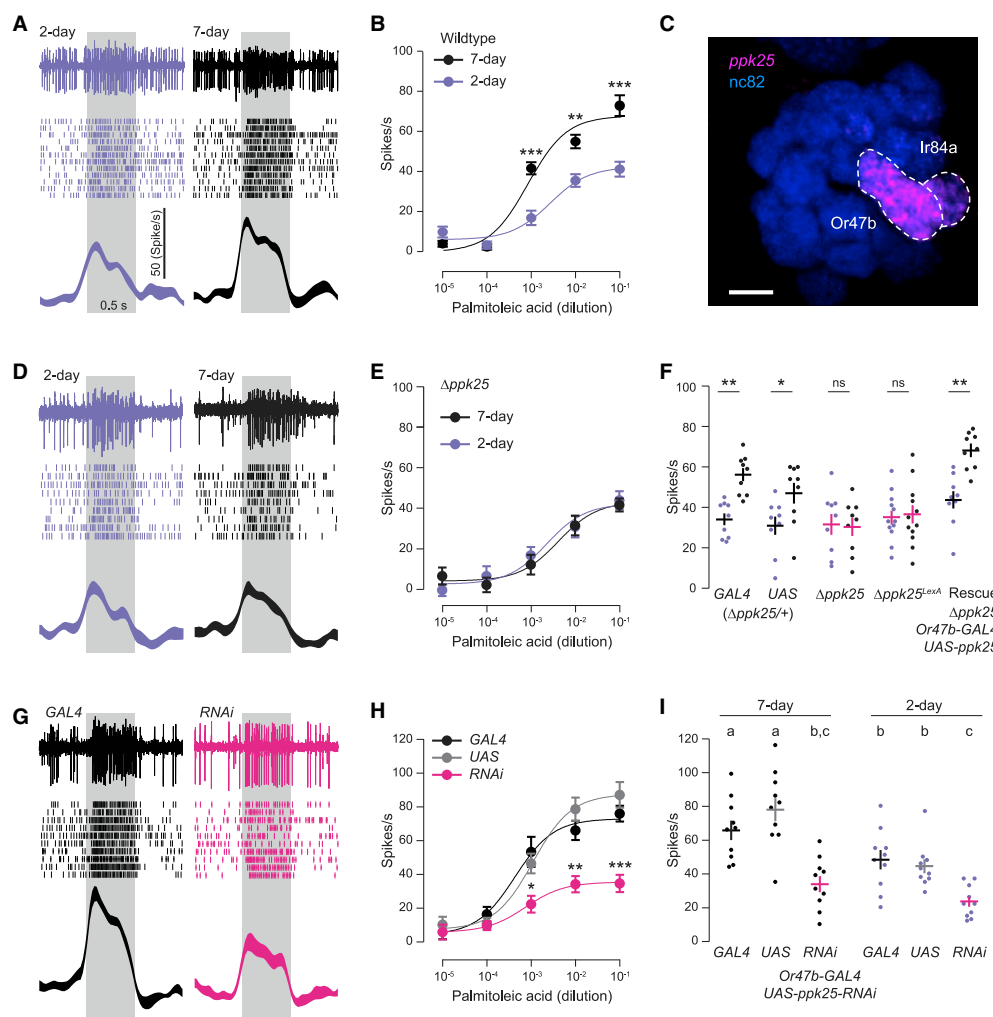


Figure 1. *ppk25* Is Required for the Amplification of Or47b ORN Responses

(A) Single-sensillum recording. Representative traces (top), raster plots (middle), and peri-stimulus time histograms (PSTHs; bottom) are shown for Or47b ORN responses in wild-type males (palmitoleic acid: 10^{-2}). Line width, SEM; gray bar, stimulus duration.

(B) Dosage curves of Or47b spike responses from 2-day and 7-day males. Adjusted peak responses (pre-stimulus baseline activity subtracted from the peak response; see STAR Methods). Parallel experiments, mean $\pm$ SEM ($n = 12$, from 6–7 flies). * $p < 0.05$; t test.

(C) Confocal image of the antennal lobe. *ppk25*-GAL4 labeled Or47b and Ir84a ORNs in 7-day males (magenta: VA1Im and VL2a glomeruli, respectively). nc82, neuropil marker (blue). Scale bar: $10 \mu\text{m}$.

(D and E) As in (A) and (B) except that recordings were performed in *ppk25* mutant males (*ppk25*⁴⁵⁻²²). Single-sensillum recording (D) and dosage curves (E) are shown.

(F) Quantification of Or47b ORN responses to palmitoleic acid (10^{-2}). *Or47b*-GAL4 and *UAS*-*ppk25* heterozygous controls (*Δppk25*/+, *Δppk25*, another *ppk25* mutant allele (*Δppk25*^{LexA}), and genetic rescue of *ppk25* in the Or47b ORNs are shown. Mean $\pm$ SEM ($n = 9$, from 4–6 flies).

(G and H) RNAi knockdown of *ppk25* reduced Or47b ORN responses in 7-day males. Sample traces (G) and dosage curves (H) are shown. * $p < 0.05$; ** $p < 0.005$; *** $p < 0.0005$; t test.

(I) Quantification of Or47b ORN responses to palmitoleic acid (10^{-2}). Parallel experiments, mean $\pm$ SEM ($n = 10$, from 6–8 flies). ANOVA followed by Tukey's test is shown. Significant differences ($p < 0.05$) are indicated by different letters, for example, between the 7-day controls (a) and the RNAi group (b and c). No significant differences were observed between groups indicated by the same letters.

See also Figures S1 and S2. Table S1 lists genotypes for all experiments.

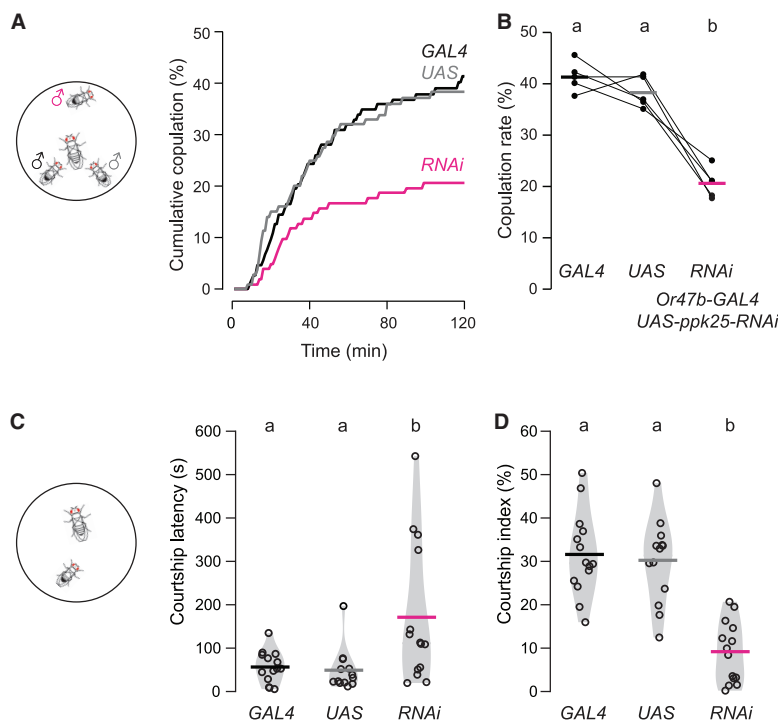


Figure 2. *ppk25* in Or47b ORNs Impacts Courtship

(A) Courtship competition assays were conducted with one 3-day wild-type female and three 7-day males of different genotypes: *Or47b-GAL4/+*; *UAS-ppk25-RNAi/+*; and *Or47b-GAL4/UAS-ppk25-RNAi*. Cumulative copulation rates over time are shown for each genotype. (B) Copulation rates at the end of the 2-h assays. Lines connect results from the same experiments ($n = 5$; total 102 matches). (C and D) Single-pair courtship assays were conducted with one 3-day wild-type female and one 7-day male of different genotypes as indicated in (A) and (B). Courtship latency (C) and courtship index (D) are shown for each genotype ($n = 13$ –14). Horizontal bars, mean. Significant differences ($p < 0.05$) are indicated by different letters; ANOVA followed by Tukey's test.

the Or47b ORNs and found that this overexpression markedly increased the Or47b ORN responses in 2-day-old males (Figures 3B–3D). In addition, Or47b ORN responses could be further increased in 7-day-old males overexpressing *ppk25* (not shown), indicating that *ppk25* levels determine the degree of amplification. Furthermore, $\Delta ppk25/+$ heterozygous males exhibited an intermediate phenotype: their Or47b ORN responses were significantly lower than the wild-type controls but higher than the $\Delta ppk25$ homozygous mutants (Figure 3E). This haploinsufficient phenotype (Roote and Russell, 2012) further supports the notion that the level of *ppk25* expression is the key determinant of Or47b ORN response amplification. Taken together, by regulating PPK25 expression levels, juvenile hormone modulates Or47b ORN responses through flexible degree of amplification.

To determine whether PPK25 alone can amplify the responses of ORNs, which do not show age-dependent plasticity, we overexpressed *ppk25* in female Or47b ORNs (Figures S4A and S4B) and Or22a ORNs (Figures S4C and S4D) and found no response increase. These results suggest that PPK25 function requires additional components, likely other PPK subunits, which are selectively expressed in the male Or47b ORNs. Consistent with this notion, heterologous expression of PPK25 alone in HEK293 cells failed to yield any stable, detectable protein in the absence of protease inhibitors (not shown).

Having established that PPK25 expression determines the degree of amplification in Or47b ORNs, we next examined the role of Or47b receptor level in this amplification. Given that Or47b receptors are ligand-gated ion channels, elevating their expression levels could in principle also contribute to the age-dependent

response increase in the Or47b ORNs. To test this possibility, we first examined male Or47b mRNA expression levels and found that they did not change with age or methoprene treatment (Figure S5A). Furthermore, overexpression of the receptor in the Or47b ORNs along with the obligatory co-receptor, Orco (Larsson et al., 2004), did not increase their responses (Figures S5B and S5C), although the exogenous Or47b receptor was detected in the sensory cilia where olfactory transduction occurs (Figure S5D). This result suggests that endogenous Or47b expression is already near saturation, making receptor upregulation an unlikely mechanism for age-dependent plasticity. It also argues against the possibility that PPK25 functions to regulate Or47b expression in a receptor-specific manner.

In all, our data show that Or47b ORN responses are heightened in older males as a result of elevated expression of a DEG/ENaC channel, PPK25, and not as a result of increased Or47b receptor level. This finding establishes that transduction-mediated amplification underlies the plasticity in Or47b ORN responses.

Ca²⁺-Mediated Signal Amplification

What is the mechanism by which PPK25 amplifies Or47b ORN responses? All DEG/ENaC channels have two transmembrane helices, a long extracellular loop and two short intracellular domains (Kellenberger and Schild, 2002; Zelle et al., 2013). In the N-terminal intracellular domain of PPK25, we identified a putative calmodulin (CaM) binding motif (CBM) (Rhoads and Friedberg, 1997; Figure 4A). The CBM is highly conserved among PPK25 orthologs in multiple *Drosophila* species (Figure 4B), suggesting that this motif is critical for PPK25 function. Additionally, insect odorant receptors (ORs) are ligand-gated cation channels permeable to calcium (Butterwick et al., 2018; Sato et al., 2008; Smart et al., 2008; Wicher et al., 2008), which led us to hypothesize that receptor-mediated Ca²⁺ influx serves as a second

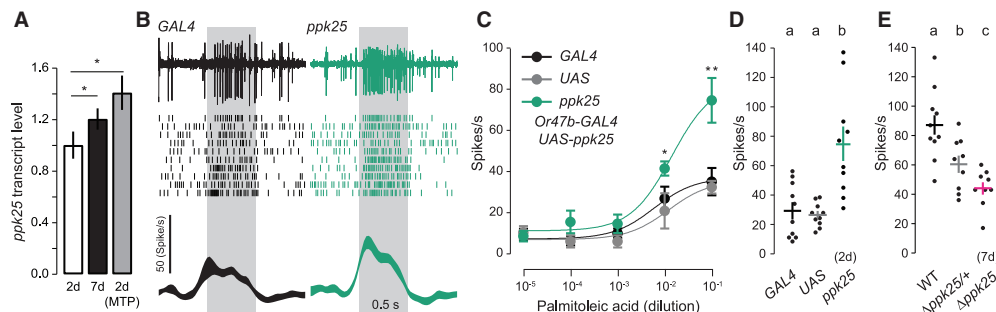


Figure 3. Overexpression of *ppk25* Increases Or47b ORN Responses in Males

(A) qRT-PCR of *ppk25* from male antennal mRNA. MTP, methoprene-treated. Expression level is normalized to that of 2-day males ($n = 12$; $p < 0.05$; t test). (B and C) Overexpression of *ppk25* elevated Or47b ORN responses in 2-day males. Sample traces, raster, PSTH (palmitoleic acid: 10⁻²; B) and dosage curves (C) are shown. Adjusted peak responses are shown. $p < 0.05$; $p < 0.005$; t test. (D) Quantification of Or47b ORN responses to palmitoleic acid (10⁻¹) in 2-day males. Parallel experiments, mean $\pm$ SEM ($n = 10$, from 6–8 flies). (E) Comparison of Or47b ORN responses to palmitoleic acid (10⁻¹) in 7-day wild-type, $\Delta ppk25/+$ heterozygous, and $\Delta ppk25$ homozygous mutant males. Mean $\pm$ SEM ($n = 9$ –12, from 7–9 flies). Significant differences between any two groups ($p < 0.05$) are indicated by different letters; ANOVA followed by Tukey's test. See also Figures S3, S4, and S5.

messenger that, together with CaM, activates PPK25 by binding to its N-terminal CBM.

To test this possibility, we first employed a pharmacological approach by introducing a Ca²⁺ chelator, EGTA, in the sensillum to reduce Ca²⁺ in the extracellular lymph. We limited the duration of EGTA application to 5 min to study the acute effect of this manipulation. From single-sensillum recordings, we measured local field potential (LFP) responses as well as corresponding spike responses. The LFP response originates in the sensory cilia and thus directly reflects transduction events (Guillet and Bernard, 1972; Nagel and Wilson, 2011). We note that, in the antennal trichoid sensillum of type 4 (at4), palmitoleic acid selectively activates the Or47b ORN, but not the neighboring Or88a or Or65a ORNs (Lin et al., 2016), and is therefore an ideal odorant for studying the LFP responses of Or47b ORNs.

As expected, EGTA attenuated odor-evoked LFP in the Or47b ORNs of 7-day-old wild-type males (Figure 4C, upper left panel), and this reduction resulted in a lower spike response (Figure 4C, lower left panel). Compared to wild-type controls, EGTA did not reduce the Or47b responses of *ppk25* mutants (Figure 4C, right panels) or those of females (Figure S6A, left panels). Additionally, EGTA did not reduce the responses of Or22a ORNs that do not express PPK25 (Figure S6B, left panels), suggesting that the attenuation was PPK25-dependent. We noticed that in *ppk25* mutants, Or47b LFP responses were slightly increased by EGTA (Figure 4C, upper right panel), likely due to its effect on Ca²⁺-mediated adaptation (Cao et al., 2016) or on possible Ca²⁺-mediated feedback regulation of Or47b-Orco channel conductance. Together, these results show that Ca²⁺ influx from the sensillum lymph is required for PPK25-mediated amplification in the Or47b ORNs, indicating that Ca²⁺ functions as a second messenger for signal amplification in these neurons.

To investigate the role of CaM in PPK25 function, we blocked CaM in the sensory cilia with W-7 (Hidaka et al., 1981), a membrane-permeable antagonist, and found that 5-min application of the drug also attenuated odor-evoked LFP responses in the

Or47b ORNs of control males (Figure 4D). As with EGTA, this attenuation was not observed in *ppk25* mutants (Figure 4D), female Or47b ORNs (Figure S6A, right panels), or in Or22a ORNs (Figure S6B, right panels). These results indicate that functional PPK25 is required for the inhibitory effect of W-7. In addition, genetic rescue of *ppk25* in Or47b ORNs restored their sensitivity to W-7 (Figure 4D). The simplest interpretation is that W-7 blocks CaM in the sensory cilia, which prevents PPK25 from being activated by odor-induced Ca²⁺ influx, thereby abolishing signal amplification in the Or47b ORNs.

Furthermore, to directly test whether PPK25 functions as a subunit of a DEG/ENaC channel, we applied benzamil hydrochloride, an effective DEG/ENaC antagonist for *Drosophila* PPK channels (Liu et al., 2003b; Younger et al., 2013). We found that benzamil hydrochloride significantly attenuated Or47b ORN responses in 7-day-old wild-type males, but not *ppk25* mutants (Figures S7A and S7B), indicating that the DEG/ENaC blocker reduces response amplification in Or47b ORNs and that its inhibitory effect depends on PPK25. Furthermore, this supports our model in which a PPK25-containing DEG/ENaC channel functions downstream of Or47b receptor to amplify olfactory input.

Next, we addressed whether the putative CBM of PPK25 interacts with CaM. We synthesized a PPK25 CBM peptide and its mutant counterpart, in which the signature 1–8–14 hydrophobic residues were replaced with glutamate (L29E, W36E, and W42E; Figure 4E), a common strategy to abolish CBM function (Vocke et al., 2013). *In vitro* binding assay showed that CaM was pulled down by the wild-type CBM peptide, but not by the mutant (Figure 4E). These results indicate that the PPK25 CBM can indeed interact with CaM in a manner that depends on the signature 1–8–14 residues.

We then asked whether the CBM is required for PPK25 function *in vivo*. To address this question, we expressed either wild-type or CBM-mutated PPK25 in the Or47b ORNs of *ppk25* mutants. In the positive control, expressing wild-type *ppk25* conferred an amplified response, which could then be attenuated by W-7

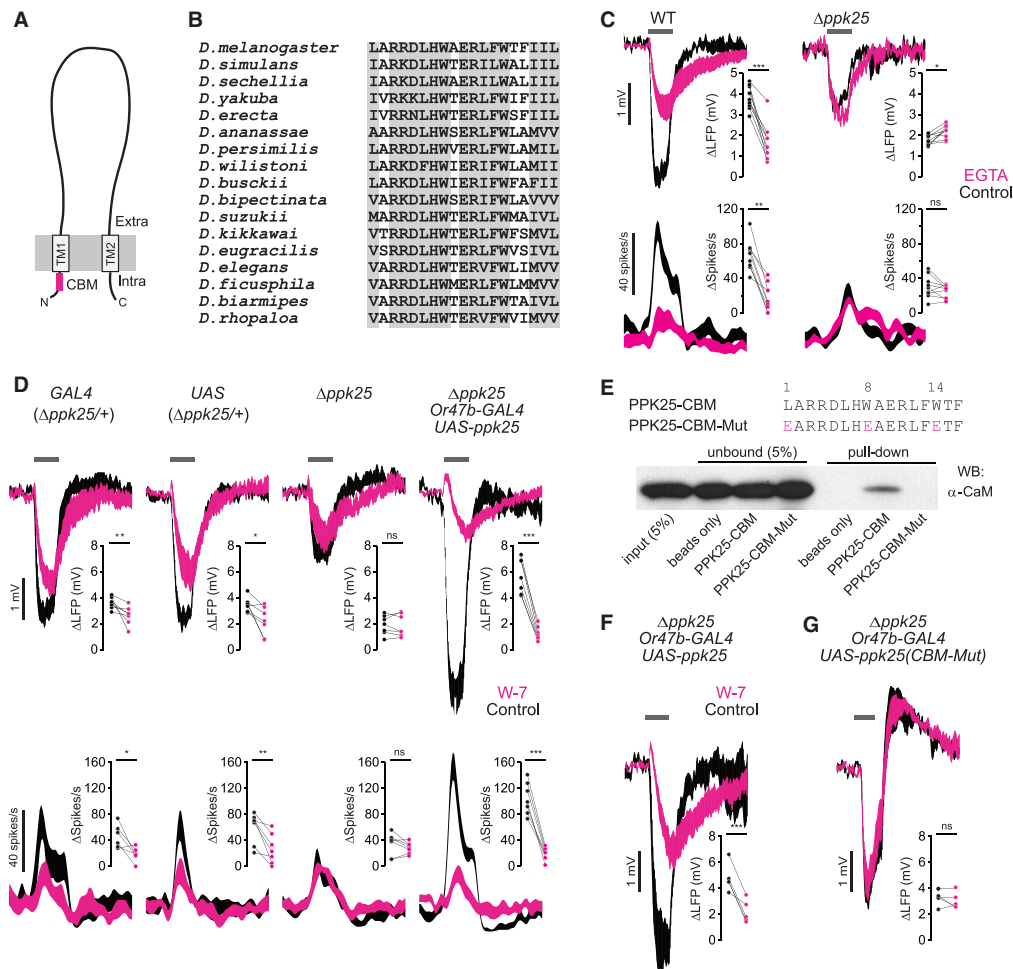


Figure 4. PPK25 Is a Ca^{2+} -Activated Transduction Channel in Or47b ORNs

(A) Topology of PPK25. CBM, calmodulin binding motif. (B) Multiple sequence alignment. Conserved PPK25 CBM residues are shaded in gray. (C) Reducing extracellular Ca^{2+} with EGTA attenuated Or47b responses in wild-type (left panels), but not $\Delta ppk25$ males (right panels). Top and bottom traces indicate LFP and the corresponding spike responses. Black, control responses; magenta, responses after 5-min application of 5 mM EGTA. Horizontal bar, 0.5 s palmitoleic acid (10^{-2}). Parallel experiments, line width indicates SEM ($n = 9$, from 5–7 flies). Lines connect results from the same neurons. (D) Similar to (C) except that 500 μM W-7 was used to block CaM ($n = 7$, from 4–6 flies). (E) CaM binding assay with wild-type or mutant CBM peptides. Numbers are signature hydrophobic residues (top panel). Western blotting with CaM antibody is shown (bottom panel). The input, unbound, and pull-down fractions are shown. (F) Rescuing $ppk25$ mutation with wild-type PPK25 and W-7 was used to block CaM ($n = 5$, from 5 flies; right panel). (G) As in (F) except that PPK25 with 1–8–14 CBM mutations was expressed. * $p < 0.05$; ** $p < 0.005$; *** $p < 0.0005$; paired t test. See also Figures S6 and S7.

(Figure 4F). In contrast, CBM-mutated PPK25 yielded a smaller LFP response, which was insensitive to W-7 inhibition (Figure 4G). These results suggest a critical role for the CBM in the normal function of PPK25. Of note, the LFP kinetics of the CBM mutant had a faster onset and a pronounced off response (Figure 4G), suggesting that these CBM mutations may have introduced gain-of-function abnormalities by altering the response kinetics.

Taken together, results from our pharmacological, biochemical, and genetic experiments suggest that PPK25 functions as Ca^{2+} -activated transduction channel, which amplifies ionotropic input responses in the sensory cilia of Or47b ORNs. If this model was correct, PPK25 is expected to amplify ionotropic input from other Ca^{2+} -permeable receptor channels, such as channelrhodopsin-2 (ChR2) (Nagel et al., 2003). To test this hypothesis, we expressed ChR2 in Or47b ORNs and used W-7 to block any potential

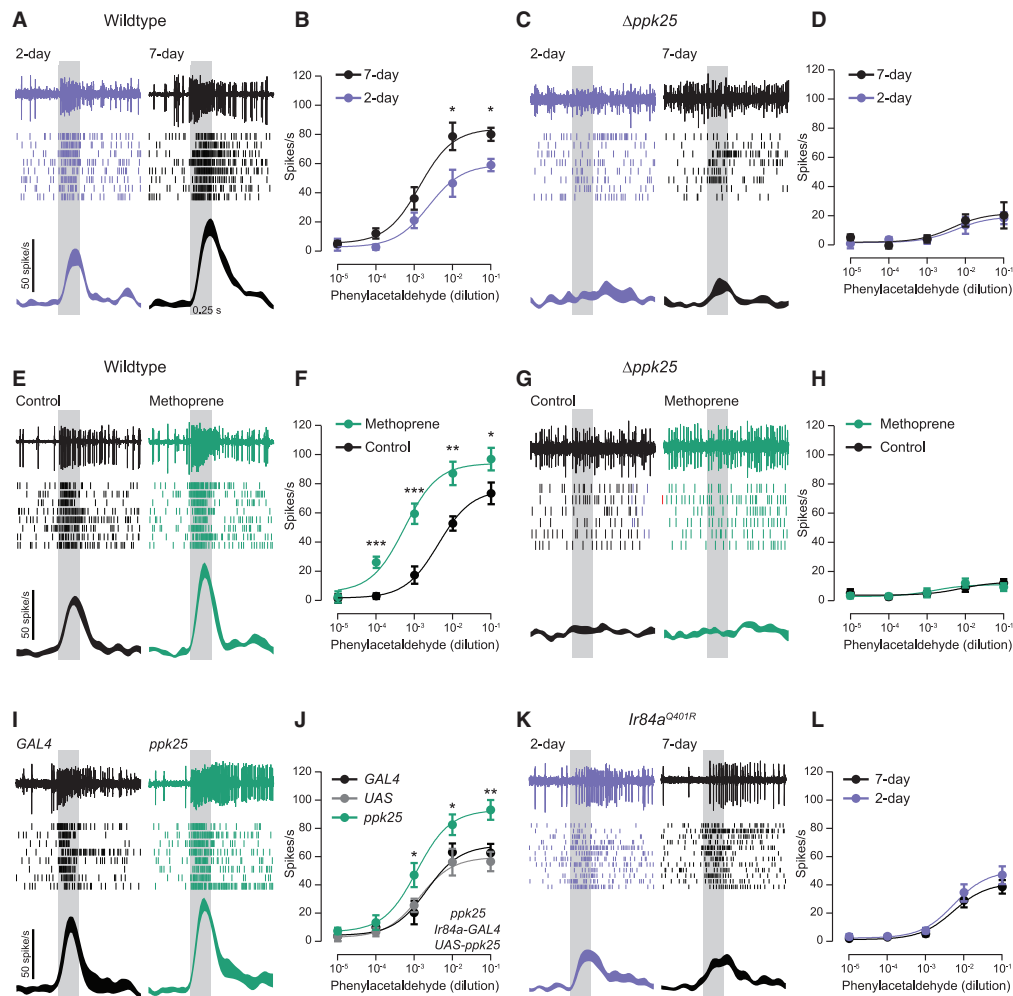


Figure 5. Age-Dependent Response Plasticity in Ir84a ORNs

(A) Single-sensillum recording. Representative traces (top), raster plots (middle), and PSTHs (bottom) are shown for Ir84a ORN responses (phenylacetaldehyde; 10^{-2}). Line width, SEM; gray bar, stimulus duration.
 (B) Dosage curves of Ir84a spike responses (adjusted peak responses).
 (C and D) As in (A) and (B) except that recordings were performed in $\Delta ppk25$ males ($n = 8$, from 4–6 flies). Single-sensillum recording (C) and dosage curves (D) are shown.
 (E) Ir84a responses from 2-day males treated for 2 days with solvent (ethanol, control) or methoprene, a synthetic juvenile hormone analog.
 (F) Dosage curves of Ir84a spike responses (adjusted peak responses). Parallel experiments, mean $\pm$ SEM ($n = 8$, from 4–6 flies).
 (G and H) As in (E) and (F) except that 2-day $ppk25$ mutant males were examined. Parallel experiments, mean $\pm$ SEM ($n = 6$, from 4–6 flies). Single-sensillum recording (G) and dosage curves (H) are shown.
 (I and J) Overexpression of $ppk25$ elevated Ir84a ORN responses in 2-day males. Parallel experiments, mean $\pm$ SEM ($n = 8$, from 4–6 flies). Single-sensillum recording (I) and dosage curves (J) are shown.
 (K and L) As in (A) and (B) except that recordings were performed in $Ir84a^{Q401R}$ males. Parallel experiments, mean $\pm$ SEM ($n = 12$, from 8–10 flies). * $p < 0.05$; ** $p < 0.005$; *** $p < 0.0005$; t test. Single-sensillum recording (K) and dosage curves (L) are shown.

PPK25-mediated amplification. As a negative control, we performed the same optogenetic and pharmacological experiment in Or22a ORNs that do not express $ppk25$. In support of our model, we found that W-7 attenuated ChR2-induced responses in Or47b neurons (Figure S7C), but not in Or22a ORNs (Figure S7D), sug-

gesting that PPK25 is also capable of amplifying responses downstream of other Ca^{2+} -permeable receptor channels. Overall, these observations suggest that PPK25 does not increase olfactory responses by modulating ligand responsiveness of Or47b but rather through amplification of the ionotropic input.

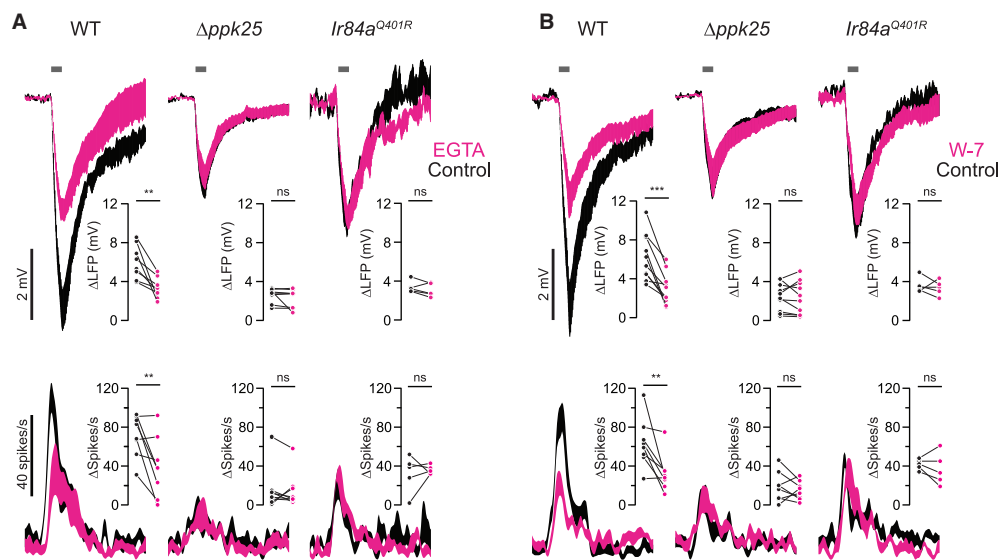


Figure 6. PPK25 Also Functions as a Ca^{2+} -Activated Transduction Channel in Ir84a ORNs

(A and B) LFP and the corresponding spike responses from wild-type, $\Delta ppk25$, and *Ir84a*^{Q401R} males (7 days old). Black, control responses; magenta, responses after 5-min application of 5 mM EGTA (A) or 500 μM W-7 (B). Horizontal bar, 0.25 s phenylacetaldehyde (10^{-2} ; n = 5–10, from 4–10 flies). **p < 0.005; ***p < 0.0005; paired t test.

A Common Transduction Channel for IR and OR

Aside from the Or47b ORNs, the *ppk25* driver labeled the other type of courtship-promoting ORNs expressing the Ir84a receptor (Starostina et al., 2012; Figure 1C), which belongs to the ionotropic receptor (IR) family (Benton et al., 2009). In addition to having distinct evolutionary origins and topologies (Benton et al., 2009; Butterwick et al., 2018; Croset et al., 2010), ORs and IRs are generally believed to involve different mechanisms when mediating olfactory responses (Cao et al., 2016; Wicher, 2015). It is therefore unclear whether PPK25 can operate downstream of Ir84a in the same manner as with Or47b. To test this possibility, we performed single-sensillum recording to determine whether PPK25 also functions as a transduction channel in the Ir84a ORNs.

Similar to the Or47b ORNs, the responses of Ir84a ORNs to their ligand, phenylacetaldehyde (Grosjean et al., 2011; Yao et al., 2005), increased with age (Figures 5A and 5B), indicating that amplification also occurs in this ORN subset. This age-dependent plasticity in Ir84a olfactory response is both sexually dimorphic (Figure S8) and similarly requires *ppk25*: in *ppk25* mutants, the Ir84a ORN responses were indistinguishable between 2- and 7-day-old males (Figures 5C and 5D). As with Or47b ORNs, Ir84a ORN responses were elevated with methoprene treatment in 2-day-old wild-type, but not *ppk25* mutant males (Figures 5E–5H). Interestingly, and in contrast to the situation in the Or47b ORNs (Figure 1), the Ir84a ORN responses of *ppk25* mutants were markedly smaller than those in 2-day-old wild-type males (Figures 5B and 5D), suggesting that *ppk25* expression was already substantial in the Ir84a ORNs of young males.

Despite this possibility, overexpression of *ppk25* in Ir84a ORNs further elevated olfactory responses in 2-day-old males (Figures 5I and 5J; please see the following paragraph for Figures 5K and

5L), indicating that PPK25 levels also determine the responses of this neuronal subset. Furthermore, the LFP responses of Ir84a ORNs were likewise sensitive to inhibition by the Ca^{2+} chelator EGTA and the CaM antagonist W-7 in a PPK25-dependent manner (Figures 6A and 6B, left and middle panels). We note that LFP responses to the same concentration of phenylacetaldehyde were nearly abolished in *Ir84a* mutant males (0.58 ± 0.15 mV; n = 10; traces not shown). Taken together, these results indicate that PPK25 functions as a Ca^{2+} -activated transduction channel in the Ir84a ORNs to mediate signal amplification, mirroring its role in the Or47b ORNs.

Lastly, we investigated whether the receptor is a key source of the Ca^{2+} that activates PPK25 in the Ir84a ORNs. The Ir84a receptor complex is permeable to Ca^{2+} , and importantly, this permeability depends on a glutamate residue in the pore domain of the receptor (Q401; Abuin et al., 2011). Using CRISPR/Cas9-mediated genome editing (Kondo and Ueda, 2013), we engineered flies bearing the *Ir84a*^{Q401R} mutation, which has been shown to abolish Ca^{2+} permeability (Abuin et al., 2011). Similar to the situation in *ppk25* mutants, the LFP responses of *Ir84a*^{Q401R} mutants were reduced compared to wild-type responses and were insensitive to EGTA or W-7 (Figures 6A and 6B, right panels), suggesting that Ir84a-mediated Ca^{2+} influx is the key link between PPK25 activation and the age-dependent response increase in those neurons. In support of this notion, the Ir84a ORN responses were indistinguishable between 2- and 7-day-old males carrying the *Ir84a*^{Q401R} mutation (Figures 5K and 5L).

Age-Dependent Plasticity in Tarsal *ppk25*⁺ GRNs

In addition to Or47b and Ir84a ORNs, PPK25 is expressed in gustatory receptor neurons (GRNs) in the forelegs for the detection

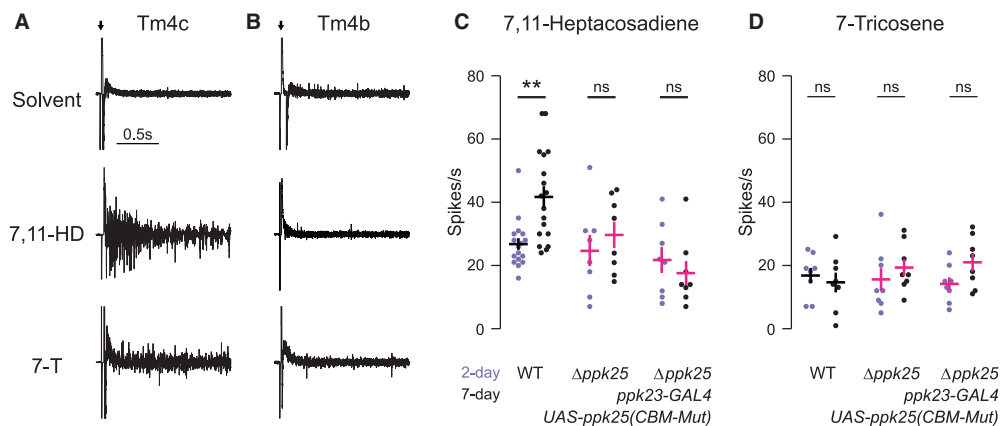


Figure 7. Age-Dependent Response Plasticity in Tarsal *ppk25*⁺ GRNs

(A and B) Tip recording of foreleg gustatory sensilla. Representative traces are shown for Tm4c (A) and Tm4b (B) sensilla in response to solvent (1 mM KCl), 7,11-heptacosadiene (7,11-HD) (20 ng/ μ L) or 7-tricosene (7-T) (10 ng/ μ L) from 7-day wild-type males.

(C) Quantification of Tm4c GRN spike responses to 7,11-HD from wild-type, $\Delta ppk25$, and *ppk25*-CBM mutant males ($n = 8-18$, from 6-13 flies). Average spike responses for the first second following tastant presentation are shown. ** $p < 0.005$; t test.

(D) As in (C) except that the spike responses to 7-T were recorded ($n = 8$, from 6-8 flies).

of female contact pheromones that promote male courtship (Lin et al., 2005; Liu et al., 2012, 2018; Lu et al., 2012; Starostina et al., 2012; Thistle et al., 2012; Toda et al., 2012; Vijayan et al., 2014). However, it is uncertain whether gustatory PPK25 is a pheromone receptor as suggested (Liu et al., 2018) or a transduction channel as described for olfaction. Given that all three types of *ppk25*⁺ sensory neurons promote courtship, we hypothesized that the responses of tarsal *ppk25*⁺ GRNs also increase with age, such that sensitivity to these aphrodisiac cues is coordinated with male fertility (Lin et al., 2016), regardless of sensory modality.

To test this hypothesis, we performed tip recording from Tm4c tarsal sensillum (Figure 7A), which houses a *ppk25*⁺ GRN (Toda et al., 2012). As a negative control, we recorded from the adjacent Tm4b sensillum and observed no pheromone responses (Figure 7B). In support of our hypothesis, we found that the responses of *ppk25*⁺ GRNs to their ligand, 7,11-heptacosadiene (7,11-HD) (Pikielny, 2012; Thistle et al., 2012; Toda et al., 2012) increased with age in males (Figure 7C, left). This age-dependent plasticity was abolished in *ppk25* mutants (Figure 7C, middle) and mutants expressing PPK25 without a functional CaM-binding motif (Figure 7C, right). In comparison, the response from the neighboring *ppk25*⁻ GRN to the male contact pheromone, 7-tricosene (7-T), was not affected by age or *ppk25* mutation (Figure 7D). We note that *ppk25* mutants still responded to 7,11-HD, albeit at a lower level. This result argues that, as in Or47b and Ir84a ORNs, PPK25 also functions as an amplification channel in this subset of GRNs.

DISCUSSION

Here, we demonstrate that ionotropic sensory inputs can be amplified in select *Drosophila* ORNs whose receptors are ligand-gated cation channels (Abuin et al., 2011; Butterwick

et al., 2018; Nakagawa and Vosshall, 2009; Sato et al., 2008; Wicher et al., 2008). Pharmacological and genetic experiments reveal a simple and elegant mechanism for this amplification. Upon odor stimulation, receptor excitation allows for direct influx of Ca^{2+} , which serves as a second messenger to activate a DEG/ENaC channel, PPK25, and thereby amplify ORN responses.

Ionotropic signal amplification, as described, affords remarkable versatility in sensory signaling when compared against G-protein-mediated metabotropic mechanisms. In vertebrate olfaction, separate families of metabotropic receptors typically couple to different G proteins, each engaging a unique downstream signaling cascade (Kaupp, 2010). In contrast, activation of specific G proteins is not required for Ca^{2+} -mediated amplification, making it compatible with a wide variety of ionotropic receptor channels, so long as these receptors are permeable to Ca^{2+} . As evidenced in this study, PPK25 can function downstream of Or47b, Ir84a, and ChR2, despite their low sequence similarity and distinct topologies (Benton et al., 2009; Butterwick et al., 2018; Nagel et al., 2003) because these receptors can flux Ca^{2+} .

Our findings highlight striking differences and commonalities between insect and vertebrate olfactory transduction. We observed surprising heterogeneity within insect olfactory transduction: PPK25 is neither expressed nor functional in another ORN type expressing Or22a, which belongs to the same receptor family as Or47b. It is unclear whether input signals in Or22a ORNs are amplified. If so, it is likely through a different mechanism. This finding indicates that insect ORNs expressing the same family of receptors do not necessarily employ the same mechanism for amplification, in contrast with vertebrate olfaction where receptors from the same family share a common signaling pathway (Kaupp, 2010). Despite this difference, the activation mechanism and function of PPK25 are remarkably similar to those of Anoctamin 2 (ANO2), a key transduction

Table 1. Predicted Calmodulin Binding Motifs (CBMs) in the N-Terminal Intracellular Domains of Members in the DEG/ENaC Superfamily

DEG/ENaC Subunits	Predicted CBMs	Residue Numbers
C. elegans		
DEG-1 (CE24854)	AKRVLIAIRNSFSKL	78–81
DEGT-1 (CE45781)	LHTRYKTWFRVLW	56–68
ASIC-1 (CE45595)	PRAYVSTGWRRYMWLLCF	27–44
DEL-8 (CE43303)	SQALMSKTLRMRIFWWIV	42–59
D. melanogaster		
PPK2 (CG1058)	GIDLTFRRRRKAGSVAC	13–29
PPK6 (CG11209)	MRRNRTYGLSRFI	53–65
PPK7 (CG9499)	IHGLDRLLSAKASR	44–57
PPK15 (CG14239)	QVLKKRGFGFVTN	10–23
PPK16 (CG34059)	TRQDISRHERWFVLV	72–87
PPK23 (CG8527)	FLETLVIFRRSLIQ	16–30
PPK24 (CG15555)	SGRRIQERFFWFV	79–91
PPK25 (CG33349)	LARRDLHWAERLFWTFIIL	29–47
PPK27 (CG10858)	KTSLNGFGLLYFIRKRR	18–34
PPK28 (CG4805)	NTICSRAAIKRSVYYLKN	28–46
A. aegypti		
PPK06258 (AAEL006258)	TSRKYHYTERLFW	31–43
PPK00926 (AAEL000926; PPK28-like)	CRERSICEKFWWWVV	34–48
PPK10995 (AAEL010995; PPK28-like)	HLANRRLLTLE	26–36
PPK14009 (AAEL014009; PPK23-like)	PFGTVRLKGSLLYQTK	43–59
M. musculus		
SCNN1G (NP_035456.1)	VSRGRLRRLW	46–56
ASIC2, isoform MDEG2 (NP_031410.1)	RTAAGGSFQRRALWVL	75–91
ASIC5 (NP_067345.1)	HAEGLLGKIKRYLS	10–24
H. sapiens		
SCNN1G (AIC49684.1)	VSRGRLRRLWI	46–57
ASIC5 (NP_059115.1)	VQNRSKIRRVLWLV	54–66

channel in vertebrate ORNs (Reisert and Zhao, 2011; Stephan et al., 2009). Specifically, both PPK25 and ANO2 are activated by calcium to amplify olfactory inputs. Although the sources of Ca^{2+} may differ—ligand-gated ion channels in insects or cyclic nucleotide-gated cation channels in vertebrates— Ca^{2+} -mediated amplification may represent a shared signaling motif between the two olfactory systems.

Interestingly, the impact of signal amplification on spike output differs between insect and vertebrate ORNs. Consistent with its role as a transduction channel, *Ano2* mutation markedly reduces odor-evoked currents (Billig et al., 2011). However, the spike output of *Ano2* knockout ORNs is higher than that of wild-type neurons (Pietra et al., 2016; Zak et al., 2018). Vertebrate ORN spike number peaks when the transduction current is largely carried by ANO2 at intermediate odor concentrations (Reisert and Matthews, 1999), suggesting a strong depolarization block

whereby amplification provides negative feedback to clamp total spike output (Zak et al., 2018). In contrast, the LFP and spike responses of insect ORNs both peak at saturating odor concentrations (Zhang et al., 2019). As such, blocking PPK25-mediated amplification not only reduces LFP response but also total spike number (Figures 4 and 6). Therefore, signal amplification in insect ORNs may predominantly serve to modulate the gain of neuronal output.

What is the functional significance of PPK25 in the Or47b and Ir84a ORNs? Notably, these are the only ORN types known to promote courtship in *D. melanogaster* males, whose fertility and courtship drive increase and peak at about 7 days of age (Dweck et al., 2015; Grosjean et al., 2011; Lin et al., 2016; Long et al., 1980). Male mating drive is highly influenced by external olfactory cues, including the availability of mates and food signaled by Or47b and Ir84a ORNs, respectively (Grosjean et al., 2011; Lin et al., 2016). Remarkably, the responses of both ORN types exhibit age-dependent plasticity (Figures 1 and 5), which is coordinated by the same reproductive hormone—juvenile hormone—through upregulation of PPK25 expression in older males (Lin et al., 2016; Figures 3, 5, and S3). The expression level of PPK25 in turn determines the ORN response magnitude, with striking impacts on courtship (Figure 2). Therefore, flexibility over this biologically salient behavior is afforded by the dynamic regulation of PPK25 expression, which heightens males' sensitivity to food and mate odors at their age of peak fertility. This upregulation of PPK25 provides a molecular mechanism for how sex-specific refinements of olfactory circuits are achieved via hormonal signaling (Knoedler and Shah, 2018).

The critical role of the intracellular CBM in PPK25 function (Figures 4E and 4G) argues that Ca^{2+} /CaM activates the channel by directly interacting with this motif. Such regulation contrasts sharply with previously reported mechanisms, in which Ca^{2+} /CaM indirectly modulates ENaC activity through intermediate proteins. For example, in cultured *Xenopus* cells, Ca^{2+} /CaM can inhibit ENaC currents by interacting with MARCKS (myristoylated alanine-rich C kinase substrate) to modulate channel open probability and also by activating CaMKII to regulate ENaC apical trafficking (Ali et al., 2015). Together, these findings highlight the complexity of interactions between Ca^{2+} /CaM and neuronal DEG/ENaC.

The results described in this study further advance our understanding of DEG/ENaC activation. The gating mechanisms for this family of sodium channels are known to be highly diverse: some open in response to mechanical stimuli; others to extracellular ligands; and still others are constitutively active (Cameron et al., 2010; Eastwood and Goodman, 2012; Kellenberger and Schild, 2002; Younger et al., 2013). In light of our findings, it is possible that other members of the DEG/ENaC superfamily may also be directly activated by intracellular second messengers, allowing them to function as transduction channels to amplify sensory inputs. In support of this notion, similar N-terminal intracellular CBMs were bioinformatically identified in multiple members of the DEG/ENaC superfamily across species—including worm, fruit fly, mosquito, mouse, and human (Table 1)—suggesting that those channels have the potential to function as Ca^{2+} -activated transduction channels.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

- KEY RESOURCES TABLE
- LEAD CONTACT AND MATERIALS AVAILABILITY
- EXPERIMENTAL MODEL AND SUBJECT DETAILS
- METHOD DETAILS
 - Single-sensillum recording
 - Odor stimuli
 - Optogenetic stimulation
 - Tip recording of tarsal taste sensilla
 - Courtship assays
 - Quantitative RT-PCR
 - Bioinformatics
 - DNA constructs and *Drosophila* transgenesis
 - Immunohistochemistry
 - Pharmacology
 - Methoprene treatment
 - CaM binding assay
- QUANTIFICATION AND STATISTICAL ANALYSIS
- DATA AND CODE AVAILABILITY

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at <https://doi.org/10.1016/j.neuron.2019.08.041>.

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AUTHOR CONTRIBUTIONS

R.N. performed single sensillum recordings, tip recordings, pharmacological experiments, immunostaining, bioinformatics analysis, molecular cloning, and data analysis. S.S.S. performed coeloconic sensillum recordings. S.-T.W. performed courtship assays. H.-H.L. performed antennal lobe immunostaining. M.W. and W.J.J. performed CaM binding assays and western blots, and A.K.S. conducted qRT-PCR. R.N., J.W.W., and C.-Y.S. wrote the manuscript with input from all authors.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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Appendix 3: An ammonium transporter is a non-canonical olfactory receptor for ammonia

Significance: This study uncovers a highly conserved ammonium transporter, Amt, as an odorant receptor detecting ammonia in *Drosophila*. This is the first transporter known to function as an odorant receptor in a complex organism. Importantly, we identified olfactory receptor neurons expressing Amt and found that these neurons mediate fly's attraction to ammonia. Our findings also suggest that the function of this ammonium transporter may be conserved in other insect species, including the disease vector *Anopheles* mosquitos.

Note of contribution: I first demonstrated Amt is expressed in two populations of olfactory receptor neurons using confocal microscopy: one population is housed in ac1 sensilla and another population is encased in the sacculus sensilla (Figure 3A, page 97). The spike responses of ac1 sensilla were recorded by Karen Menuz's lab, however, the sacculus Amt⁺ neurons were inaccessible to electrophysiological recordings. To examine the olfactory response of sacculus neurons, I developed a transcuticular imaging assay and measured the calcium activity of sacculus Amt⁺ neurons. My results show that both ac1 and sacculus Amt⁺ neurons have dose-dependent responses to ammonia, suggesting that the two neuronal populations are functionally similar (Figure 3E-J, page 97).

Appendix 3, in part, is a reprint of the material as it appears in An ammonium transporter is a non-canonical olfactory receptor for ammonia 2021. Vulpe, Alina; Kim, Hyong S.; Ballou, Sydney; Wu, Shiuan-Tze; Grabe, Veit; Nava Gonzales, Cesar; Liang, Tiffany; Sachse, Silke; Jeanne, James M.; Su, Chih-Ying; Menuz, Karen, *Current Biology* 31, 1-9, 2021. The dissertation author is a co-author of this paper.

Report

An ammonium transporter is a non-canonical olfactory receptor for ammonia

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SUMMARY

Numerous hematophagous insects are attracted to ammonia, a volatile released in human sweat and breath.^{1–3} Low levels of ammonia also attract non-biting insects such as the genetic model organism *Drosophila melanogaster* and several species of agricultural pests.^{4,5} Two families of ligand-gated ion channels function as olfactory receptors in insects,^{6–10} and studies have linked ammonia sensitivity to a particular olfactory receptor in *Drosophila*.^{5,11,12} Given the widespread importance of ammonia to insect behavior, it is surprising that the genomes of most insects lack an ortholog of this gene.⁶ Here, we show that canonical olfactory receptors are not necessary for responses to ammonia in *Drosophila*. Instead, we demonstrate that a member of the ancient electrogenic ammonium transporter family, Amt, is likely a new type of olfactory receptor. We report two hitherto unidentified olfactory neuron populations that mediate neuronal and behavioral responses to ammonia in *Drosophila*. Their endogenous ammonia responses are lost in Amt mutant flies, and ectopic expression of either *Drosophila* or *Anopheles* Amt confers ammonia sensitivity. These results suggest that Amt is the first transporter known to function as an olfactory receptor in animals and that its function may be conserved across insect species.

RESULTS

Ammonium transporters in a new ORN class

In all insect species examined, ammonia activates olfactory receptor neurons (ORNs) housed in sensilla with a grooved peg morphology,^{2,13} also known as coeloconic sensilla. Olfactory receptors define the odor tuning of individual ORNs, and the stereotyped receptor combinations expressed by neighboring ORNs define functional subtypes of sensilla. In *Drosophila*, robust responses to low levels of ammonia (NH₃) are observed in the ac1 subtype of coeloconic sensilla.^{11,13,14} Ammonia detection by ac1 ORNs depends on Amt,¹⁴ a member of the conserved ammonium transporter family.^{15,16} The *Anopheles* ortholog AgAmt can restore ammonia responses in Amt mutant flies¹⁴ and produces the typical ammonium-selective inward current when members of this transporter family are heterologously expressed.¹⁷ In *Drosophila*, Amt is exclusively expressed in chemosensory tissues including the antenna and labellum.^{14,18,19} Its expression is most abundant in the antenna, where it is only found in ac1 sensilla and the third chamber of the sacculus,¹⁴ a three-chambered cavity invaginated from the antennal surface.²⁰ The precise reason why Amt is indispensable for ammonia detection remains

unclear given that Amt expression was only detected in sensillar support cells.¹⁴

Insect genomes also contain a second ammonium transporter, Rh50, which has widespread expression in multiple tissues.^{15,17,19} Within the antenna, Rh50 transcript level is reduced ~10-fold in atonal flies that fail to develop coeloconic sensilla (Figure S1A),¹⁴ suggesting a potential connection between Rh50 and ammonia detection. We therefore generated an Rh50 reporter line to identify Rh50⁺ cells within the antenna. The scattered population of labeled cells had a neuronal morphology and was positive for the neuronal marker elav (Figures 1A and 1B). The reporter line was faithful because all antennal GFP⁺ cells were labeled by an Rh50 anti-sense probe (Figure S1B). Thus, Rh50 expression in the antenna is found solely in neurons.

Might Amt likewise be expressed in ORNs in addition to its known expression in support cells? ORNs are enwrapped by several support cells, and the strong Amt expression in these cells could potentially obscure visualization of Amt⁺ ORNs. Close examination of antennal sections from *Amt-GAL4; UAS-GFP* flies revealed weak GFP⁺ axons emerging from strongly labeled support cells in ac1 sensilla (Figure 1C). A similar antennal expression pattern of an AgAmt reporter line was recently

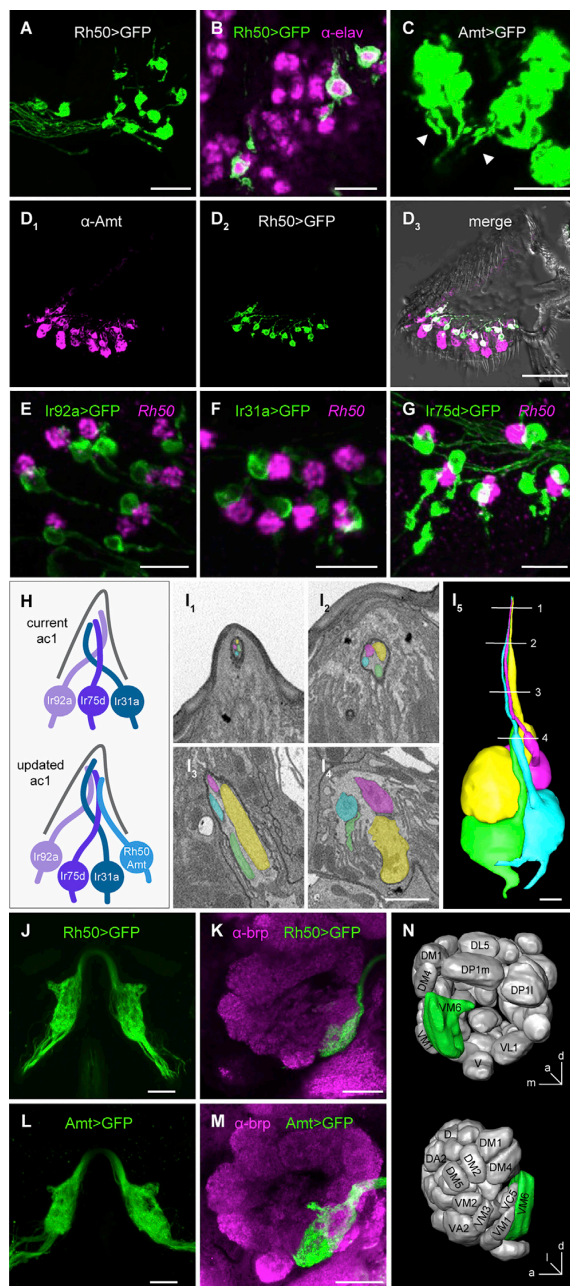


Figure 1. Ammonium transporters label a previously unidentified ac1 ORN

(A) Antennal section from an *Rh50 > GFP* fly stained with anti-GFP. Scale bar, 15 μ m.
(B) *Rh50 > GFP* fly antennal section stained with anti-GFP (green) and anti-elav (magenta), a neuronal marker. Scale bar, 10 μ m.
(C) High-gain confocal image of an *Amt > GFP* antennal section stained with anti-GFP. Labeled axons (arrowheads) emerge from clusters of GFP⁺ cells. Scale bar, 10 μ m.
(D) Immunostaining with anti-Amt (magenta, D₁) and anti-GFP (green, D₂) on an antennal section from an *Rh50 > GFP* fly. D₃, merged image. Scale bar, 40 μ m.

reported in mosquitos.²² Interestingly, anti-Amt staining revealed that Amt is co-expressed in every Rh50⁺ neuron (Figures 1D and S1C). Consistent with the reported expression pattern of Amt,¹⁴ Rh50⁺ ORNs were found in ac1 sensilla, but not in ac2, ac3, or ac4 coeloconic sensilla (Figures S1D–S1G).

The three reported neurons in ac1 sensilla express *Ir92a*, *Ir31a*, and *Ir75d* receptors, respectively.¹² To determine which neuron expresses the transporters, we examined Rh50 expression in antennal sections from flies in which one of the three ac1 neurons was genetically labeled. Surprisingly, the Rh50⁺ neurons did not co-localize with any of the three known neurons, but instead always neighbored the labeled ac1 ORNs (Figures 1E–1G). Similarly, these three ac1 ORNs were not labeled with the anti-Amt antibody, although they were surrounded by Amt⁺ cells (Figures S1H–S1J). Together, these results indicated that Rh50 and Amt are co-expressed in a previously undetected fourth neuron in ac1 sensilla (Figure 1H).

Early electron microscopy work only identified coeloconic sensilla that house two or three neurons.²³ By examining recent antennal serial block-face scanning EM (SBEM) datasets,^{24,25} we found that approximately one in four coeloconic sensilla indeed contain four ORNs (Figures 1I and S1K). Further, sensilla with two, three, and four neurons are unevenly distributed over datasets acquired from distinct antennal regions (Figure S1K), which could explain why coeloconic sensilla with four neurons were overlooked previously.

The axonal projections of all ORNs expressing the same receptor coalesce into a glomerulus in each antennal lobe. Examination of *Rh50 > GFP* and *Amt > GFP* flies revealed that the terminals of these ORNs both converge to a large ventromedial glomerulus (Figures 1J–1N). This glomerulus corresponds to the “orphan” glomerulus VM6,^{10,26} recently shown to be targeted by neurons sharing a similar developmental origin as the other ac1 ORNs.^{26–28} Because VM6 is difficult to discern with neuropil staining, it has been the source of confusion in recent antennal lobe atlases, where it was merged with VP1²⁹ or listed as VC5.^{30,31}

Amt/Rh50⁺ ORNs respond selectively to NH₃

The identification of the previously undetected Amt/Rh50⁺ ac1 ORN raised the question of its odor response profile. Although ac1 sensillar responses to ammonia and many amines were

(E–G) Antennal sections from *Ir92a > GFP* (E), *Ir31a > GFP* (F), and *Ir75d > GFP* (G) flies labeled with an *in situ* hybridization probe for Rh50 (magenta) and anti-GFP (green). Scale bars, 10 μ m.

(H) Models of neurons and receptors in ac1 sensilla.

(I) SBEM images of a coeloconic sensillum with four ORNs. 3D reconstructions of the ORNs are shown in (I₅). Numbered lines indicate the locations of individual sections shown in (I_{1–4}). Scale bars, 1 μ m.

(J) Two-photon *in vivo* image of the bilateral antennal lobe glomeruli innervated by Rh50⁺ axons in an *Rh50 > GFP* fly. Scale bar, 20 μ m.

(K) Confocal image of an antennal lobe from an *Rh50 > GFP* fly brain immunolabeled with antibodies targeting GFP (green) and brp (nc82, magenta), a neuropil marker used to delineate glomeruli. Scale bar, 20 μ m.

In (J) and (K), glial GFP expression driven by *Rh50-GAL4* was suppressed with *repo-GAL80*²¹ to improve visualization of the ORN projections.

(L and M) Similar to (J) and (K), but with *Amt > GFP*.

(N) Diagram of the glomerulus innervated by Amt/Rh50⁺ ORNs, corresponding to VM6.

See also Figure S1.

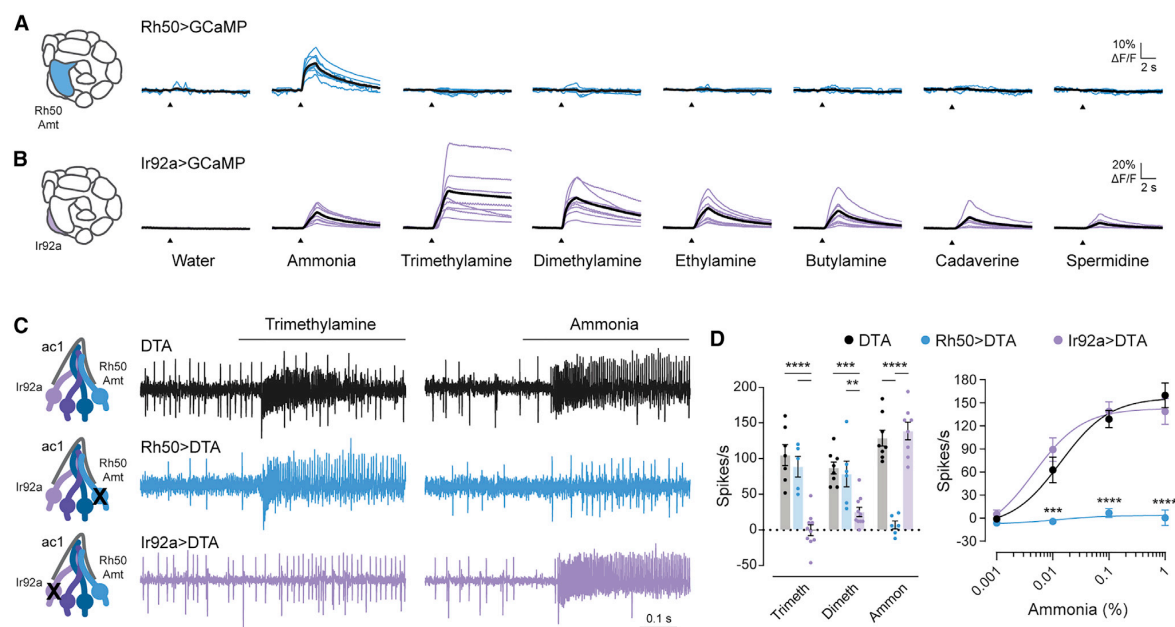


Figure 2. Amt/Rh50⁺ ORNs selectively respond to ammonia

(A and B) Antennal lobe calcium responses to water, ammonia, and several amines in axonal projections labeled in *Rh50 > GCaMP7s* (A) and *Ir92a > GCaMP7s* (B) flies. Blue lines (A) and purple lines (B) are responses in individual flies. Black lines are mean responses. (C) Representative traces of extracellular recordings of action potentials elicited by 1% trimethylamine and 0.1% ammonia in ac1 sensilla in which diphtheria toxin (DTA) was used to ablate Rh50⁺ ORNs (blue) or Ir92a⁺ ORNs (purple). UAS-DTA flies were used as a control (black). (D) Left: quantification of odor responses in UAS-DTA (black), *Rh50 > DTA* (blue), and *Ir92a > DTA* (purple) flies (n = 5–10 sensilla). Right: dose-response curves of responses to increasing concentrations of ammonia (n = 6–8 sensilla per genotype). The dose-response data for 0.1% ammonia are replotted in the bar graph to show individual data points.

Bar graphs depict the mean $\pm$ SEM overlaid with the individual data points. Dose response curves show the mean $\pm$ SEM and the curve fit to the Hill equation. Statistical significance is presented as *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. Other comparisons were not significant (p > 0.05). See also Figure S2.

previously ascribed to its Ir92a⁺ ORN,^{5,11,12} the fourth ac1 ORN may mediate a portion of those responses. We specifically examined its odor responses through antennal lobe calcium imaging in *Rh50 > GCaMP7s* flies. Ammonia evoked robust calcium responses, whereas there was no response to water or amines (Figure 2A). In contrast, Ir92a⁺ ORNs responded broadly to ammonia and to amines (Figure 2B), consistent with previous reports.^{5,11} Neither of these neurons is likely to detect alkaline pH, which may result from ammonia application, because ac1 neurons were unresponsive to two basic amines at 1% concentration, butylamine (pKa 10.8) and isoamylamine (pKa 10.6), even though 0.01% ammonia (pKa 9.4) induced robust responses (Figure S2A). Thus, Amt/Rh50⁺ ORNs are selectively tuned to ammonia.

Amt/Rh50⁺ ORNs mediate spiking responses to NH₃

We next examined the relative contributions of Amt/Rh50⁺ and Ir92a⁺ neurons to ammonia-induced spiking in ac1 sensilla. There is a nonlinear relationship between GCaMP7s responses and action potential firing because this highly sensitive sensor can detect single action potentials but saturates at low firing frequencies.³² Although the Ir92a receptor and associated ORNs have been implicated in mediating spike responses to ammonia, this was inferred indirectly from GCaMP imaging data

demonstrating the ammonia sensitivity of Ir92a⁺ ORNs and the ammonia insensitivity of Ir31a⁺ and Ir75d⁺ ORNs.^{5,11}

We recorded from flies in which either the Ir92a⁺ or Amt/Rh50⁺ neurons were ablated genetically by diphtheria toxin. Ablation of the Amt/Rh50⁺ ORNs abolished the large amplitude action potential responses in ac1 sensilla over a broad range of ammonia concentrations, whereas loss of Ir92a⁺ ORNs had no significant effect (Figures 2C and 2D). In contrast, spiking responses to trimethylamine and dimethylamine, odorants that strongly activate Ir92a⁺ ORNs in calcium imaging^{5,11} (Figure 2B), were unaffected by the loss of Amt/Rh50⁺ ORNs but were nearly eliminated by Ir92a⁺ ORN ablation (Figures 2C and 2D). An Ir92a mutant had similar effects on ac1 odor responses as Ir92a⁺ ORN ablation (Figures S2B–S2F). Thus, Ir92a⁺ ORNs function primarily as amine detectors, whereas the Amt/Rh50⁺ ac1 ORNs mediate the robust spiking observed in response to ammonia.

Sacculus Amt/Rh50⁺ ORNs also respond to NH₃

Examination of *Rh50 > GCaMP7s* antennae revealed a second population of Rh50⁺ neurons in the sacculus (Figure 3A). This was reminiscent of Amt expression, which is found in sacculus chamber III in addition to ac1 sensilla.¹⁴ There were 26 ± 3.6 (SD, n = 10) Rh50⁺ ORNs in the sacculus, suggesting one Rh50⁺ ORN is likely housed in each of the ~22–26 chamber

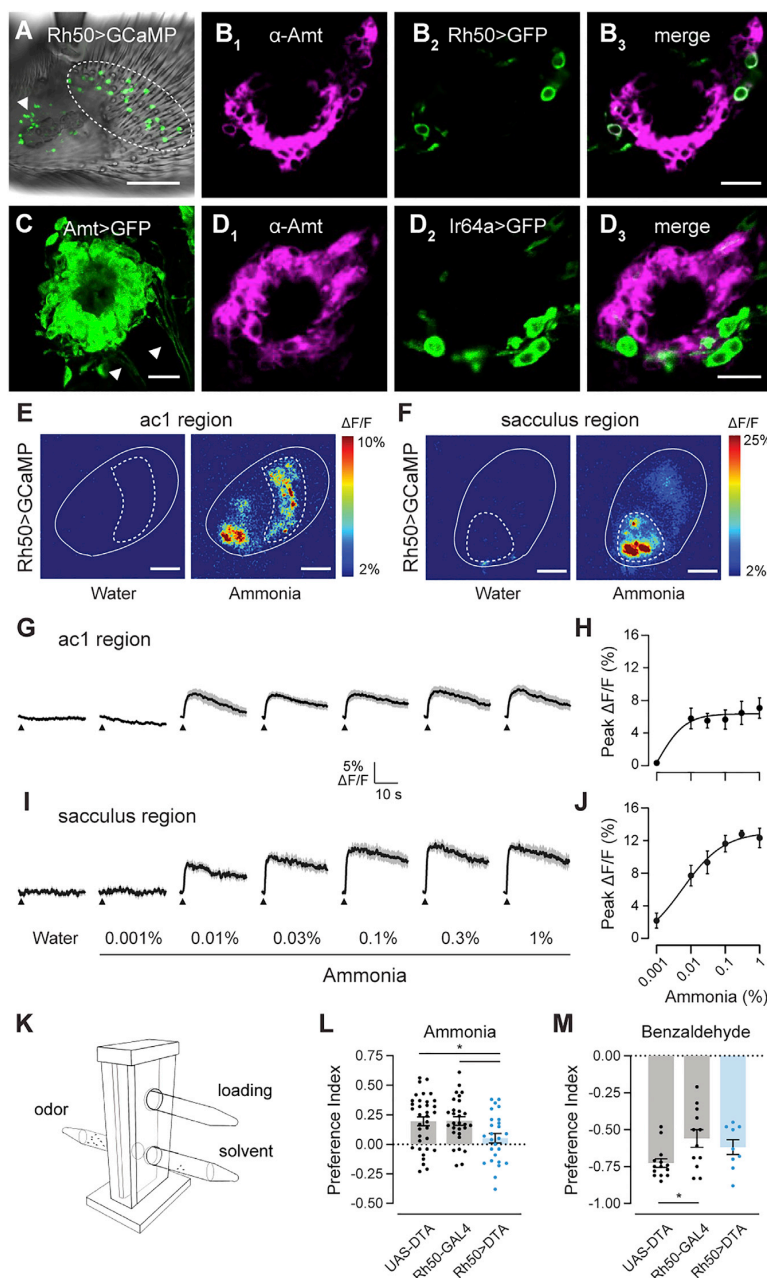


Figure 3. Two populations of Amt/Rh50⁺ ORNs mediate ammonia sensing

(A) Whole-mount image of an antenna from an *Rh50 > GCaMP7s* fly. *Rh50*⁺ neurons (green) are found on the ac1 region of the antennal surface (dotted circles) and surrounding the sacculus (arrowhead). Scale bar, 30 μ m.

(B) Close-up view of sacculus chamber III in an antennal section from an *Rh50 > GFP* fly stained with anti-Amt (magenta, B₁) and anti-GFP (green, B₂). B₃, merged image. Scale bar, 10 μ m.

(C) High-gain confocal image of sacculus chamber III in an antennal section from an *Amt > GFP* fly stained with anti-GFP. Labeled axons (arrowheads) emerge from clusters of GFP⁺ cells. Scale bar, 10 μ m.

(D) Immunostaining for Amt (magenta, D₁) and GFP (green, D₂) on antennal sections from *Ir64a > GFP* flies. D₃, merged image. Scale bar, 10 μ m.

(E) Pseudocolored heatmaps of calcium responses in the ac1 region (dotted circle) of the antenna (solid outline) of *Rh50 > GCaMP7s* flies to either water or 0.1% ammonia. Scale bars, 30 μ m.

(F) Similar to (E) but acquired at a different depth and location to focus on the sacculus region (dotted circle). Scale bars, 30 μ m.

(G) Traces of the mean calcium responses (black) $\pm$ SEM (gray) in the ac1 region. Arrowheads indicate time when the 250 ms odor stimulus was applied ($n = 6-7$ flies).

(H) Dose-response curve of the peak ac1 calcium responses.

(I and J) Similar to (G) and (H), except for the sacculus region.

(K) T-maze assay schematic showing the elevator in the lower position with flies moving between the odor and solvent arms. The loading tube is above and is accessible with the elevator in the upper position.

(L and M) Preference indices of *Rh50 > DTA*, *UAS-DTA*, and *Rh50-GAL4* flies when given the choice between ammonia and water (L) or between benzaldehyde and paraffin oil (M). Each dot represents one assay ($n = 26-35$ ammonia, $n = 9-12$ benzaldehyde).

Bar graphs depict the mean $\pm$ SEM overlaid with the individual data points. Dose response curves show the mean $\pm$ SEM and the curve fit to the Hill equation. Statistical significance is presented as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. Other comparisons were not significant ($p > 0.05$). See also Figure S2.

III sensilla reported previously.²⁰ As in ac1 sensilla, we detected Amt in both *Rh50*⁺ neurons and support cells in the sacculus (Figures 3B and 3C). Sensilla in sacculus chamber III house two neurons.²⁰ One expresses *Ir64a* and responds to acids,³³ whereas markers for the second have not been reported, precluding its functional analysis. We found that *Ir64a*⁺ ORNs do not express Amt (Figure 3D), suggesting that sacculus III *Amt/Rh50*⁺ and *Ir64a*⁺ neurons are distinct. Consistent with

this finding, *Ir64a*⁺ ORNs are known to project to DC4 and DP1m antennal lobe glomeruli,³³ whereas all *Amt/Rh50*⁺ ORNs project to VM6 (Figures 1J–1N). Our data do not exclude the possibility that ac1 and sacculus *Amt/Rh50*⁺ ORNs target distinct regions within VM6.

Chamber III sensilla are proposed to contain only one olfactory neuron because the dendrite of the second neuron does not fully extend into the sensillum lumen.²⁰ This raised the question of whether the sacculus *Amt/Rh50*⁺ neurons are ammonia sensitive like their ac1 counterparts. Sacculus sensilla are inaccessible for

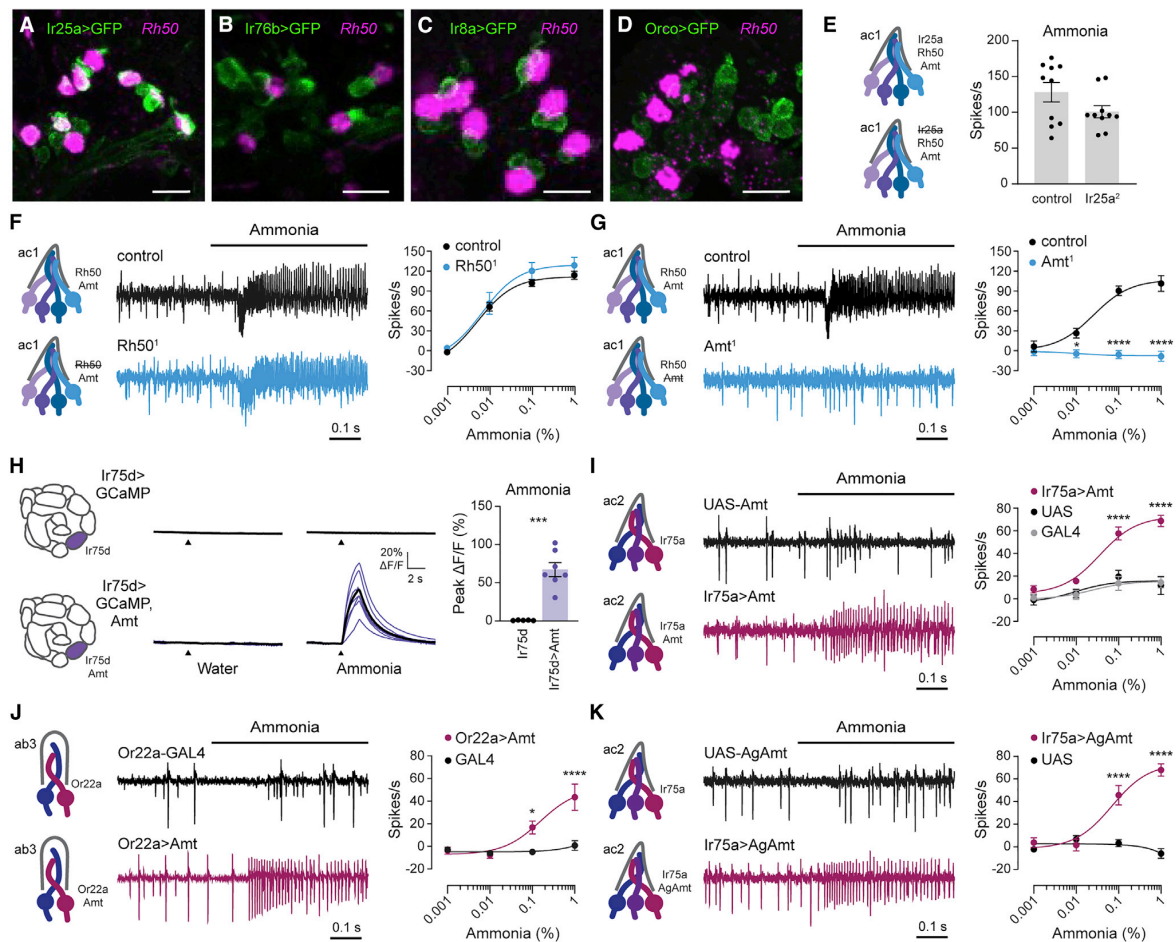


Figure 4. Amt transporter serves as an olfactory receptor for ammonia

(A–D) Confocal images of antennal sections labeled with an antisense probe for Rh50 (magenta) and an antibody against GFP (green) driven by *Ir25a-GAL4* (A), *Ir76b-GAL4* (B), *Ir8a-GAL4* (C), or *Orco-GAL4* (D). Scale bars, 10 μ m.

(E) Action potential responses to 0.1% ammonia in ac1 sensilla in control flies and *Ir25a²* mutants ($n = 10$ sensilla).

(F and G) Action potential responses to ammonia in ac1 sensilla in *Rh50¹* (F) and *Amt¹* (G) mutants (blue) and control flies (black). Left: representative traces of response to 0.1% ammonia. Right: dose-response curves ($n = 8$ –10 sensilla).

(H) Left: antennal lobe calcium responses to water and 0.01% ammonia in axon termini of *Ir75d > GCaMP6s* flies, with and without ectopic expression of Amt. Purple lines are responses in individual flies, and black lines are mean responses. Right: quantification of peak responses ($n = 5$ and 7 flies).

(I) Action potentials elicited by ammonia in ac2 sensilla in *Ir75a > Amt* (crimson), *Ir75a-GAL4* (gray), and *UAS-Amt* (black) flies. Left: sample traces of 1% ammonia responses. Right: dose-response curves ($n = 7$ –9 sensilla).

(J) Action potentials elicited by ammonia in ab3 sensilla in *Or22a > Amt* (crimson) and *Or22a-GAL4* (black) flies. Left: sample traces of 1% ammonia responses. Right: dose-response curves ($n = 9$ –11 sensilla).

(K) Similar to (I), except *Ir75a > AgAmt* (crimson) and *UAS-AgAmt* (black) flies ($n = 7$ –8 sensilla).

Bar graphs depict the mean $\pm$ SEM overlaid with the individual data points. Dose response curves show the mean $\pm$ SEM and the curve fit to the Hill equation. Statistical significance is presented as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. Other comparisons were not significant ($p > 0.05$). See also Figure S3.

electrophysiological recordings but can be assayed using calcium imaging. We therefore turned to transcuticular imaging of *Rh50 > GCaMP7s* fly antennae, where the two neuron populations can be segregated by location (Figures 3E and 3F). Similar to ac1 neurons, sacculus neurons showed dose-dependent responses to ammonia (Figures 3G–3J), providing evidence that the two neuron populations are both molecularly and functionally similar.

Amt/Rh50⁺ ORNs contribute to NH₃ attraction

Like many insects, *Drosophila* are attracted to low levels of ammonia.⁵ We examined the contribution of Amt/Rh50⁺ ORNs to this behavior using a T-maze two-choice assay in which naive flies are given a short time to navigate toward or away from an odorant (Figure 3K). For these assays, we used flies in which Amt/Rh50⁺ ORNs were ablated by diphtheria toxin, the same

genotype used for electrophysiological recordings (Figures 2C and 2D). Parental control lines showed a preference for ammonia, and this attraction was significantly reduced in flies in which Amt/Rh50⁺ ORNs were eliminated (Figure 3L). As controls, we tested the flies' general odor responsiveness and locomotor ability using the aversive odor benzaldehyde, which remained repulsive to the control and ablated flies (Figure 3M). Together, these data indicate the importance of Amt/Rh50⁺ ORNs in *Drosophila* ammonia attraction. Consistent with our electrophysiological data, the behavioral responses of *Ir92a*¹ mutants were similar to controls (Figures S2G and S2H).

NH₃ reception does not involve IRs/ORs

What is the ammonia receptor in Amt/Rh50⁺ neurons? Nearly all insect olfactory neurons utilize a member of either the ionotropic receptor (IR) or odorant receptor (OR) families.^{6,10} Individual tuning receptors that bind odorants rely on a co-receptor for function, with odor responses absent in co-receptor mutants.^{7,34,35} OR receptors depend on Orco, whereas IR receptors require Ir25a, Ir8a, or Ir76b.^{7,34,35} We therefore asked whether any of these co-receptors are expressed in Amt/Rh50⁺ ORNs and found that only Ir25a was co-expressed with Rh50 in ac1 sensilla (Figures 4A–4D). However, ammonia-induced spiking responses were unchanged in *Ir25a*² mutants (Figure 4E), in agreement with a previous study.⁷ As expected, ac1 ammonia responses were also not reduced in mutants for any of the three other co-receptors (data not shown). Thus, neither the OR nor IR co-receptors are required for ac1 ammonia responses.

Amt as an ammonia receptor

This observation raised the possibility that Amt/Rh50⁺ ORNs utilize a non-canonical receptor to detect ammonia. We wondered whether ammonium transporters might fulfill this role. Ammonium transporters in other species are highly selective for ammonium, with little to no transport of larger amines,^{36,37} in accordance with the narrow tuning of Amt/Rh50⁺ ORNs (Figure 2A). Further, ammonium transporters are electrogenic, mediating inward NH₄⁺ currents.^{16,17,37,38} Given that NH₃ gas is expected to form ammonium ions (NH₄⁺) in the sensillum lymph, these cations could be transported through either Amt or Rh50 and depolarize the ORNs. In this manner, the transporters could act as ammonia-selective receptors.

If this model is correct, ammonia responses should require either Amt or Rh50. We first generated an Rh50 mutant that eliminated Rh50 expression (Figure S3). Spike responses to all concentrations of ammonia persisted in the *Rh50*¹ ac1 sensilla—if anything, trending slightly higher than controls (Figure 4F). In contrast, *Amt*¹ mutants entirely lack ammonia responses at all concentrations (Figure 4G).¹⁴

To determine whether Amt operates as an ammonia receptor, we ectopically expressed Amt in ammonia-insensitive neurons and found that expression of Amt in Ir75d⁺ neurons conferred robust antennal lobe calcium responses to ammonia that were absent in controls (Figure 4H). Further, ectopic expression of Amt in Ir75a⁺ ORNs, found in ac2 coeloconic sensilla, produced dose-dependent spiking responses to ammonia (Figure 4I). Finally, Amt misexpression conferred a similar ammonia sensitivity to basiconic Or22a⁺ ORNs (Figure 4J), demonstrating that Amt function requires neither morphological nor molecular

features specific to coeloconic sensilla. Together, these data indicate that Amt is an olfactory receptor for ammonia in *Drosophila*.

Amt is evolutionarily conserved, with orthologs found in multiple insect species, including the malaria vector *Anopheles* mosquitoes.^{15,17} Misexpression of AgAmt in Ir75a⁺ ORNs produced dose-dependent responses to ammonia (Figure 4K), similar to those induced by *Drosophila* Amt (Figure 4L). This raises the possibility that ammonium transporters also operate as receptors in other insect species.

DISCUSSION

Our identification of two previously unstudied populations of olfactory neurons expressing Amt and Rh50 transporters has paved the way to a new understanding of ammonia detection in *Drosophila*. In insects, ORNs detect odors using IR and OR olfactory receptors, ligand-gated cation channels that are generally tuned to multiple structurally related odorants. Our loss- and gain-of-function experiments reveal that the newly identified Amt/Rh50⁺ ac1 ORNs instead use an ammonium transporter as their receptor (Figures 4G–4K); the molecularly and functionally similar sacculus ORNs likely operate similarly. The narrowly tuned ammonia response in these ORNs (Figure 2A) is consistent with the strict selectivity of ammonium transporters.^{36,37} Amts also distinguish NH₄⁺ ions from similarly sized K⁺ ions,^{17,37,38} an advantage considering the presence of >100 mM K⁺ in the sensillum lymph bathing ORN dendrites. We propose that a simple electrogenic influx of ammonium ions leads to ORN depolarization, but we cannot rule out an ammonium-initiated signaling cascade analogous to the mechanism for vertebrate sour sensing, in which H⁺ influx closes acid-sensitive K⁺ channels.³⁹ However, an ammonium signaling cascade is unlikely because it would require the components to be widely expressed in ammonia-insensitive neurons in both basiconic and coeloconic sensilla.

The ammonia-sensing ORNs in *Drosophila* express both Amt and Rh50, members of the two main branches of the AMT/MEP/Rh ammonium transporter family. Ammonium transporters in plants, bacteria, and fungi serve to uptake ammonium for biosynthesis of nitrogenous molecules, whereas animal Rh proteins play a role in ammonia excretion and acid-base homeostasis.^{15,16} Our findings indicate a novel role for Amt ammonium transporters as chemosensory receptors in animals. In contrast, we did not find a significant role for Rh50 in ammonia sensing, perhaps due to its >20-fold lower expression in antennae than Amt.¹⁴ Rh50 may also be unable to function as an ammonia receptor because most Amts transport ammonium at low micromolar concentrations whereas Rh proteins require millimolar levels.⁴⁰ The role of Amt proteins in support cells is likewise unclear; Amt misexpression into neurons in coeloconic and basiconic sensilla that lack Amt in support cells is sufficient to induce ammonia sensitivity (Figures 4I and 4J). Support cell Amt may affect the magnitude of the ORN response, a possibility suggested by the somewhat smaller ammonia responses in ac2 versus ac1 ORNs (Figures 4G and 4I).

Ammonia-sensitive ORNs are found in every insect species examined, and many insects are attracted to low levels of ammonia. Its effectiveness as an attractant has spurred the

use of ammonium-containing solutions in commercial insect traps.^{2–4} Several lines of evidence suggest that Amt orthologs are widely used as ammonia receptors and therefore likely mediate this behavior. First, Amt proteins are highly conserved, with, for example, *Drosophila* and *Anopheles* orthologs sharing 83% amino acid identity in the transmembrane regions. Accordingly, expression of *Anopheles* AgAmt conferred ammonia sensitivity to ORNs (Figure 4K), and a recent study detected Amt⁺ neurons in ammonia-sensitive olfactory sensilla in *Anopheles* mosquitoes.²² Additionally, Amt orthologs can be found in antennal transcriptomes from multiple, distantly related insect species.¹⁴ Together, these similarities suggest Amt transporters have taken on a new function as ammonia receptors in insects.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

- KEY RESOURCES TABLE
- RESOURCE AVAILABILITY
 - Lead Contact
 - Materials Availability
 - Data and Code Availability
- EXPERIMENTAL MODEL AND SUBJECT DETAILS
- METHOD DETAILS
 - Reporter line generation
 - Generation of Ir92a and Rh50 mutants
 - Quantitative reverse transcriptase-PCR
 - FISH and antennal immunocytochemistry
 - 3D reconstruction of labeled glomeruli
 - *In vivo* structural imaging of antennal lobes
 - Electron microscopy analysis
 - Whole-mount Confocal Imaging
 - *Ex vivo* ORN somata counts
 - Behavioral assay
 - Odorants
 - Electrophysiology
 - Ca²⁺ imaging in the antennal lobe
 - Transcuticular antennal calcium imaging
- QUANTIFICATION AND STATISTICAL ANALYSIS

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at <https://doi.org/10.1016/j.cub.2021.05.025>.

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AUTHOR CONTRIBUTIONS

K.M. conceived the project, with A.V. and C.-Y.S. providing conceptual contributions. All authors contributed to experimental design, analysis, and interpretation of results. A.V. generated transgenic *Drosophila* lines and performed electrophysiological recordings. A.V., S.B., and V.G. carried out histological studies. A.V., S.B., and T.L. performed behavioral experiments. H.S.K. and S.-T.W. performed GCaMP imaging. S.B. carried out qPCR analysis. C.N.V. analyzed SBEM data. A.V. and K.M. wrote the paper with input from all other authors.

DECLARATION OF INTERESTS

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Appendix 4: Neuronal Compartmentalization: A Means to Integrate Sensory Input at the Earliest Stage of Information Processing?

Significance: This review article illustrates the prevalence of neuronal compartmentalization in peripheral sensory systems. We described the characteristics of grouped sensory neurons across animal species and discussed the possible interactions between these neurons. We particularly focused on the studies of insect peripheral olfactory system since their morphology and physiology are extensively examined. Critically, the neuronal interactions within each sensillum can impact the coding of olfactory information. We hope this article can stimulate future research that determines whether the communication between compartmentalized neurons can also impact signal processing in other sensory systems.

Note of contribution: I drafted following sections: 2.1. Ommatidium: A Visual Receptive Unit; 2.3. Proprioceptive Neurons and their Structural Compartments; 2.5. Sensilla for Hygro- and Thermosensation. I was also involved in the writing of the first section: “Introduction” and the third section: “Information Processing Can Take Place at the Sensory Periphery”.

Appendix 4, in part, is a reprint of the material as it appears in Neuronal Compartmentalization: A Means to Integrate Sensory Input at the Earliest Stage of Information Processing? 2020. Ng, Renny; Wu, Shiuan-Tze; Su, Chih-Ying, *Bioessays* 42: 2000026, 2020. The dissertation author and Renny Ng contributed equally to this paper as primary authors.

Neuronal Compartmentalization: A Means to Integrate Sensory Input at the Earliest Stage of Information Processing?

Renny Ng, Shiuan-Tze Wu, and Chih-Ying Su*

In numerous peripheral sense organs, external stimuli are detected by primary sensory neurons compartmentalized within specialized structures composed of cuticular or epithelial tissue. Beyond reflecting developmental constraints, such compartmentalization also provides opportunities for grouped neurons to functionally interact. Here, the authors review and illustrate the prevalence of these structural units, describe characteristics of compartmentalized neurons, and consider possible interactions between these cells. This article discusses instances of neuronal crosstalk, examples of which are observed in the vertebrate tastebuds and multiple types of arthropod chemosensory hairs. Particular attention is paid to insect olfaction, which presents especially well-characterized mechanisms of functional, cross-neuronal interactions. These examples highlight the potential impact of peripheral processing, which likely contributes more to signal integration than previously considered. In surveying a wide variety of structural units, it is hoped that this article will stimulate future research that determines whether grouped neurons in other sensory systems can also communicate to impact information processing.

sense organs, external cues are detected by receptor cells compartmentalized within specialized structures consisting of cuticular or epithelial tissues. Within each compartment, multiple neurons share a private microenvironment in which regulation of ionic composition, peri-receptor secretions, or paracrine signaling molecules occurs separately from other sensory units. Furthermore in certain compartments, adjacent neurons are directly connected via gap junctions or chemical synapses. As discussed below, each of these aforementioned features has been shown to enable functional interaction between compartmentalized sensory neurons. Anatomical organization thus provides opportunities for private neuronal crosstalk, which in principle can allow structural compartments to serve as the first processing units in sensory integration.

1. Introduction

Encountered in nature as combinations of chemical and physical cues, environmental stimuli require sophisticated mechanisms for detection and integration. To that end, animals rely on dedicated sensory systems employing processing at multiple layers of nodes. While such processes in the central nervous system often form the focus of studies on signal integration, a more complete understanding of sensory neurobiology requires revealing how inputs are transformed at every node of the circuit, including neurons in the peripheral nervous system. In numerous

1.1. Sensory Neuron Compartmentalization is Broadly Observed across Animal Species

Sensory neuron compartmentalization is prevalent across species (Figure 1). In the nematode *C. elegans*, sensory hairs, also known as sensilla, of the phasmid variety each houses two chemosensory neurons, while inner labial sensilla contain one chemosensory and one mechanosensory cell^[1] (Figure 1a). Compartmentalization is particularly prominent in arthropods, and has been broadly observed in studies on sensory organ ultrastructure. In arachnids, the pectine sensilla of the scorpion *E. italicus* groups 11 chemosensory neurons together with one mechanosensory cell^[2] (Figure 1b). On average, the spiny lobster *P. argus* houses ≈ 300 olfactory receptor neurons (ORNs) per sensillum^[3] (Figure 1c); similarly large numbers of grouped ORNs are also observed in other crustaceans.^[4] Most prominently in insects, primary sensory neurons are compartmentalized across nearly all modalities (see sections below). A well-known example is the trichoid sensillum of the silk moth *B. mori*, which encases the dendrites of two ORNs that detect female pheromones^[5,6] (Figure 1d).

Compartmentalization is not unique to invertebrate receptor neurons. Found in all fish, the lateral line detects water motions and pressure gradients.^[7] This mechanosensory system's functional unit is the neuromast, which houses upwards of 30 hair

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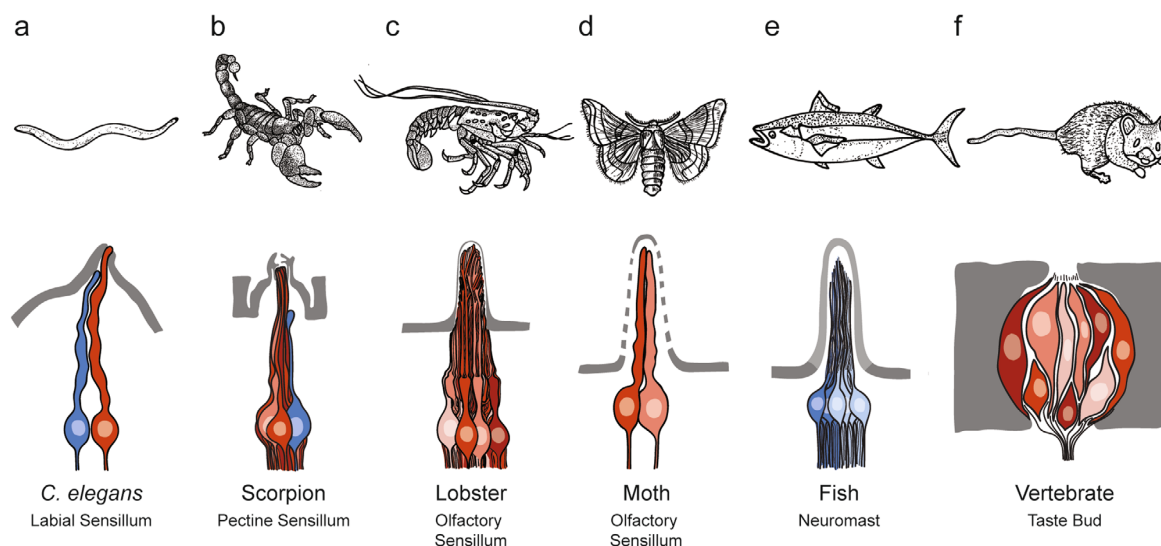


Figure 1. Sensory neuron compartmentalization is broadly observed across animal species. a) Inner labial sensillum of the nematode *C. elegans* with one mechano- and one chemosensory neuron in a uniporous cuticular hair. b) Pectine sensillum of the scorpion *E. italicus* with a mechanosensory neuron and typically 11 chemosensory neurons in a uniporous cuticular hair. c) Olfactory sensillum of the spiny lobster *P. argus* with ≈ 300 chemosensory neurons in a hair with thin, permeable cuticle. d) Olfactory sensillum of the silk moth *B. mori* with typically two chemosensory neurons in a multiporous cuticular hair. e) Neuromast of the fish lateral line with upwards of 30 mechanosensory hair cells whose tips are encased in a jellylike cupula. f) Vertebrate taste bud with 50–100 chemoreceptor cells in an ovoid structure embedded within gustatory epithelia. Compartmentalized cells are color coded based on their sensory modality: chemosensory (red) and mechanosensory (blue). Neurons with different shades of one color indicate detection of different stimulus types from the same sensory modality.

cells that detect flow along either direction of the lateral line^[8] (Figure 1e). In weakly electric fish, electrosensory cells are also grouped within specialized electroreceptive organs located in the epidermis throughout the body.^[9]

While also found in mammals, known examples of sensory compartmentalization are restricted to the gustatory system. On the surfaces of the tongue and palate, taste buds are ovoid structures comprising groups of 50–100 taste receptor cells categorized into five functional classes: sweet, bitter, umami, sour, and salt^[10,11] (Figure 1f). Their microvilli are oriented towards the taste pore, allowing tastants to initiate gustatory transduction, which leads to depolarization at the basal cellular domain, where neurotransmitters are released and detected by gustatory afferent fibers.^[10–12]

1.2. Examples of Communication between Neurons Sharing a Sensory Compartment

Many types of grouped receptor neurons share a common progenitor cell,^[13,14] and their compartmentalization has been generally taken to reflect developmental constraints. However, such organization likely also contributes to information processing enabled by private communication between grouped neurons, through a shared microenvironment enveloping their dendrites and somata, and/or through the proximity of their axons projecting from the same peripheral unit. Consistent with this idea, synaptic or gap junction connections are observed between subsets of grouped receptor neurons in scorpions, ants, fruitflies, and bumblebees.^[15–18] On the other hand a direct electric field

effect, known as ephaptic coupling, occurs between ORNs in the same olfactory sensillum of *D. melanogaster*.^[19,20]

Notably upon taste reception, extensive cell-to-cell signaling occurs within the taste bud via neurotransmitter-mediated crosstalk, a topic which has been thoroughly reviewed.^[12,21] Briefly, activation of Type II cells—which express the metabotropic sweet, bitter, or umami receptors—triggers the secretion of ATP from these cells; meanwhile, sour tastants stimulate the ionotropic receptors^[22] in Type III cells, which in turn release serotonin. In addition to acting on their respective gustatory afferent fibers, these two neurotransmitters also target neighboring cells through paracrine signaling. ATP excites Type III cells through activation of P2X receptors; in contrast, serotonin inhibits Type II cells via 5-HT receptors.^[12,21] Consequently, lateral purinergic excitation broadens the receptive range of sour-sensing Type III cells to sweet, bitter, and umami,^[12,21] while lateral serotonergic inhibition provides opportunities for sour tastants to suppress taste modalities sensed by Type II cells.^[12,21,23] Taken together, it appears that neuronal interaction within a compartment can occur regardless of whether the sensory neurons express metabotropic or ionotropic receptors. These interactions may enhance the perceptual contrast of tastants in a mixture, or influence the temporal dynamics of afferent signaling. While the exact functional significance of such extensive crosstalk awaits further clarification, short-range paracrine signaling is evidently permitted by the proximity of cells within the taste bud.^[21] Taken together, these observations highlight notable examples of intra-compartment communication, mediated by the close apposition of grouped neurons, which can in turn shape afferent outputs.

1.3. Sensory Neuron Compartmentalization: An Underexplored Means for Sensory Processing

Despite these characterized interactions between grouped receptor cells, neuronal compartmentalization's contribution to signal processing remains largely underexplored and continue to pose open questions. How can such organization affect sensory coding? Does it alter characteristics of stimuli, such as their intensity and identity? What functional benefits might compartmentalization confer? These questions can be better informed through close examination of insects, whose sensory periphery offers several advantages for the purpose. Anatomically, their primary receptor neurons are typically compartmentalized. Many of their sense organs are accessible for in vivo electrophysiological recordings. In addition, the morphology and ultrastructure of insect sense organs have been extensively characterized through electron microscopy (EM), and are described in excellent reviews.^[24,25] Furthermore, the genetic tractability of select species has aided studies in causally linking molecules to morphology, function, and behavior. As such, insects afford unique opportunities for understanding how external stimuli are integrated at the earliest stage of sensory processing.

In the remainder of this article, we focus on compartmentalized receptor cells observed across the major sense organs of insects. We note that studies demonstrating unequivocal neuronal crosstalk remain relatively scant when compared to the anatomical and functional characterizations, compiled over decades, of compartmentalized receptor cells. Therefore by calling attention to the anatomy, response profiles, and interactivity of grouped neurons, we hope to stimulate deeper consideration and exploration of these prevalent organizational schemes. We focus on the following themes: that primary receptor neurons may be compartmentalized; that these cells may demonstrate distinct tuning properties or even confer multimodality within a functional unit; and that functional interactions may occur between grouped cells. Particular attention is paid to olfaction, as this sense has been extensively characterized. Finally, we discuss the functional significance of compartmentalized ORNs by considering how their organization enables olfactory sensilla to serve as processing units at the sensory periphery.

2. Receptor Neurons are Compartmentalized across the Major Sense Organs of Insects

2.1. Ommatidium: A Visual Receptive Unit

Insect compound eyes are made up of hundreds to thousands of receptive units named ommatidia, each typically containing eight elongated photoreceptor neurons (R1–R8)^[26] (Table 1). Some insects, such as bees and butterflies, have an additional photoreceptor (R9) at the compartment base.^[27] In *D. melanogaster*, R1–R6 cells occupy the outer regions of the ommatidium and are immediately surrounded by pigment cells, while R7 and R8 are positioned centrally, with R7 atop R8 (Figure 2a).^[28] R1–R6 photoreceptors express the same Rh1 rhodopsin and thus have identical spectral sensitivity. In contrast, R7 cells express a UV-sensitive rhodopsin, Rh3 or Rh4, and R8 cells express either the blue-sensitive Rh5 or the green-sensitive Rh6.^[29,30]

There is evidence to suggest that functional interactions occur between photoreceptors in the same ommatidial column. Within the first optic neuropil of locusts and butterflies, strong activation of one chromatic photoreceptor inhibits surrounding neighbors with differing spectral sensitivity.^[66,67] This lateral inhibition is likely mediated by a direct electric field effect, known as ephaptic coupling, which takes place between closely apposed neuronal structures in local environments exhibiting high extracellular resistance,^[68] such as within an ommatidial column.^[66] Briefly, current outflow from the axon terminal of the strongly activated cell enters the counterparts of neighboring photoreceptors, and thereby hyperpolarizes their presynaptic terminals.^[66,67]

Besides electric field effects, grouped photoreceptors can also communicate via chemical synapses. In the medulla of *D. melanogaster*, chemical synapses are found between the axons of the two chromatic photoreceptors from the same ommatidium. Specifically, both R7 and R8 express histamine-gated chloride channels, allowing these two histamine-releasing photoreceptors to inhibit each other through direct synaptic connections.^[17] Although it is unclear whether this direct lateral inhibition can take place across different ommatidia, R7, and R8 synaptic connections appear to occur only between axons that project from the same ommatidium.^[69] Importantly, lateral inhibition between chromatically distinct photoreceptors is a hallmark of color opponency. Therefore, an interesting direction for future research will be to determine how such lateral inhibition influences color perception to regulate vision-guided behavior.

2.2. A Peripheral Taste-Processing Unit

Insect gustation plays a critical role for evaluating the quality of food and suitability of mates. Two main gustatory organs, the legs, and the labella (Table 1), are covered with taste sensilla that mediate gustation through contact chemosensation.^[10] Regardless of organ, these sensory hairs are uniporous cuticular structures that ensheath multiple bipolar receptor cells^[31] (Figure 2b,c). Each structural unit is typically innervated by four gustatory receptor neurons (GRNs), the dendrites of which extend into the sensillar lumen, together with one mechanosensory cell, whose dendritic tip terminates at the sensillum base (Figure 2c). Well-conserved among insects, this organization is prevalent in mosquitoes,^[70,71] honeybees,^[72] and Dipteran flies.^[73,74,32]

Grouped GRNs typically detect different stimuli from primary tastant classes including sugars, low- or high-salt levels, bitter compounds and water.^[10] Such organization potentially allows each gustatory sensillum to integrate information from foods containing all major tastant types. Although some organizational schemes diverge in the numbers and types of GRNs, it appears that each sensillum type processes specific kinds of taste mixtures. The ability to detect antagonistic taste modalities, such as sweet and bitter, is well-demonstrated in a dedicated subset of *Drosophila* labellar taste hairs (i-type) that only houses two GRNs that sense sugars or bitter compounds.^[32–34] Conversely in another sensillum type (l-type), its fourth GRN does not respond to bitter compounds.^[35] These examples illustrate how certain subtypes of taste hairs may diverge from the prevalent organizational scheme.

Table 1. Functional units and compartmentalized neurons in the external sensory organs of *D. melanogaster*. The numbers of sensory neurons housed in individual functional units are indicated in parentheses.

Main sensory organs	Functional units	Compartmentalized sensory neurons	Sensory stimuli	References
Vision				
Eye	Ommatidium	Achromatic photoreceptors (6) Chromatic photoreceptors (2) Rh3/Rh5 or Rh4/Rh6	Light, $\lambda_{\max}^*$: 478 nm, Rh1 Light, $\lambda_{\max}^*$: 345 nm, Rh3 375 nm, Rh4 437 nm, Rh5 508 nm, Rh6 * $\lambda_{\max}$ indicates the peak spectral sensitivity of the visual pigment, which can absorb photons with varying probabilities from a wide band of wavelengths.	[30]
Gustation				
Labellum	Gustatory sensillum	Gustatory receptor neurons, GRNs (2–4) Mechanosensory neuron, MN (1)	Tastants Food texture	[31–40]
Leg	Gustatory sensillum	GRNs (4) MN (1)	Tastants ?	[32,33,41]
	Pheromone sensillum	Pheromone-sensitive GRNs (2) Other GRNs (2) MN (1)	Contact pheromones ? ?	[42–46]
Audition				
Johnston's organ (2nd antennal segment connecting to the 3rd segment)	Scolopidium	MNs (2–3)	Sound, vibrations, deflection, and gravity	[47–50]
Hygro- and Thermosensation				
Arista (3rd antennal segment)	Thermosensitive sensillum	Cold or cooling cell (1) Warm cell (1)	Cold/decreasing temperature Warm temperature	[51–54]
Sacculus (3rd antennal segment)	Triads	Cold cell (1) Dry cell (1) Moist cell (1)	Cold temperature Aridity Humidity	[55–59]
Olfaction				
Funiculus (3rd antennal segment)	Olfactory sensillum	Olfactory receptor neurons, ORNs (1–4)	Odorants	
	Pheromone sensillum	Pheromone-sensitive ORNs (1–3)	Airborne pheromones	
Maxillary palp	Olfactory sensillum	ORNs (2)	Odorants	[60–65]

Why is an individual mechanosensory neuron (MN) compartmentalized with multiple chemosensory cells? During feeding, this multimodality may permit integration of texture and taste.^[36,37] For instance, strong activation of these MNs can inhibit sweet-sensing GRNs through GABAergic inhibition of their axon terminals, which mediates *D. melanogaster*'s preference for softer foods over sweeter, but harder foods.^[37] It remains an open question whether inhibitory synapses are formed exclusively between individual MN and sweet-sensing GRN from the same taste hair or broadly between the two neuronal populations across different sensilla. Interestingly, a non-compartmentalized MN is also found in each labellar lobe, where a single multidendritic md-L neuron extends its dendritic arbors to the bases of taste hairs and, in contrast with grouped MNs, appears to report absolute hardness of food substrates. Intriguingly, the ideal food texture—soft but not too soft—is encoded by the inter-

mediate levels of md-L neuronal activity.^[75] These studies raise the possibility of functional distinctions between compartmentalized MNs and standalone md-L neurons, and furthermore suggest a unique role for the former, likely in the multimodal integration of texture and taste at the first synapses of the taste circuit.

In addition to evaluating food quality, taste sensilla mediate pheromonal responses: GRNs in fruitflies' tarsal hairs detect pheromones to regulate sexual behavior.^[76] Notably within a specific sensillum subset, two co-housed chemosensory neurons detect pheromones specific to opposite sexes, which either promote or inhibit courtship behavior.^[77] This pair of cells—expressing different combinations of the DEG/ENaC subunits PPK23, 29, and/or 25—inhibits male-male courtship through the detection of male pheromones, while its neighbor promotes male-female courtship via responses to female pheromones.^[42–46,78] That is,

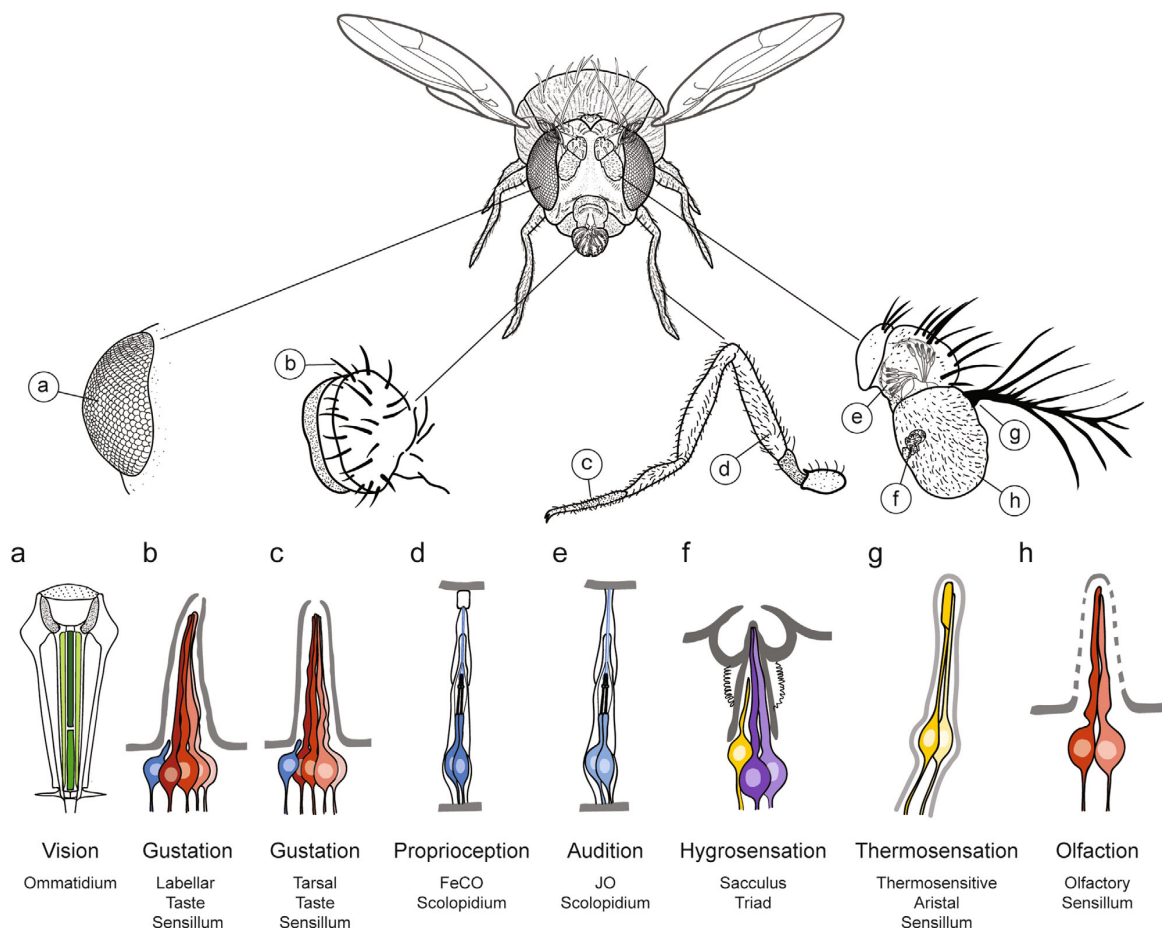


Figure 2. Compartmentalized neurons in major sensory organs of *D. melanogaster*. a) Ommatidium of the compound eye with eight photoreceptor neurons. b) Taste sensillum of the labellum with four GRNs and one mechanosensory neuron in a uniporous cuticular hair. c) Taste sensillum of the foreleg tarsi with four GRNs and one mechanosensory neuron in a uniporous cuticular hair. d) Scolopidium of the femoral chordotonal organ (FeCO) with two mechanosensory neurons. e) Scolopidium of the Johnston's organ (JO), located in the second and connected to the third antennal segment, with two mechanosensory neurons. f) One cold-sensing and two hygrosensory neurons (triad) in a poreless sensillum within the sacculus. g) Temperature-sensitive sensillum of the arista with one warm- and one cold-sensing cells in a non-porous structure. h) Antennal olfactory sensillum with two ORNs in a multiporous cuticular hair. Compartmentalized cells are color coded based on their sensory modality: chemosensory (red), mechanosensory (blue), photoreceptor (green), thermosensory (yellow), and hygrosensory (purple). Neurons with different shades of one color indicate detection of different stimulus types from the same sensory modality.

compartmentalized “male” and “female” cells exert opposing influences on courtship behavior.

The grouping of GRNs responsive to stimuli of different hedonic values suggests that taste sensilla may serve as functional processing units to integrate valence information at the sensory periphery. If so, these cells are expected to demonstrate cross-modality interaction and indeed, there is evidence for crosstalk between compartmentalized GRNs. Tarsal sensilla of the grasshopper *S. americana* house two neurons that respectively detect salt or bitter alkaloids: spiking of the first cell inhibits the tastant-evoked firing of its neighbor, resulting in disruption of uniform spike trains.^[79] A similar example is observed in the galeal sensilla of bumblebees, wherein the spiking of a GRN interrupts the high-frequency spike trains of its

compartmentalized neighbor, whose sustained sucrose-induced responses are transformed into bursts of spikes.^[16]

Besides neuronal interactions, crosstalk between taste modalities can occur through other means. In *D. melanogaster*, bitter compounds suppress the response of sugar-sensing GRNs to prevent flies from ingesting food contaminated with bitter, probably toxic substances. This suppression is achieved through direct inhibition of sugar receptors by the bitter compounds themselves,^[80] or through the action of an odorant binding protein (OBP)—OBP49a—enriched in the sensillum lumen.^[81] Notably, the proboscis extension reflex (PER) is reduced when co-compartmentalized sugar- and bitter-sensing GRNs are simultaneously activated by a mixture, which in principle allows for integration of inputs at both peripheral

and higher brain centers. However as elegantly demonstrated by Marion-Poll and colleagues, PER is unaffected when non-neighbor sugar- and bitter-sensing GRNs are concurrently stimulated in a manipulation that precludes peripheral processing but retains central computation.^[80] Thus, the gustatory periphery is critical for integrating opposing attractive and aversive inputs, and cannot be substituted by central mechanisms for informing feeding decisions. In summary, whether through neuronal or non-neuronal interactions, compartmentalized GRNs can engage in inter-modal crosstalk to provide the sensillum with the means to function as a peripheral taste processing unit.

2.3. Proprioceptive Neurons and their Structural Compartments

Insects detect the position and motion of their body parts through proprioceptive organs, consisting of mechanosensory neurons that provide feedback for coordinating movement.^[47,82] Found in all insects, the femoral chordotonal organ (FeCO) is a well-characterized proprioceptive sensor that monitors movement of the tibia in relation to femur^[82] (Figure 2d). The organs' functional units—named scolopidia—typically compartmentalize two proprioceptive neurons,^[47,83,84] unlike other sensory organs in which proprioceptive cells are singly-housed.^[82,85] In *Drosophila*, FeCO scolopidia and their neurons are known to respond to different stimuli, including vibration, position, or directional movement of the leg.^[86] However, it awaits future experimentation to determine whether paired FeCO neurons within each individual scolopidium exhibit identical tuning properties, or if not, what the organizing principle underlying neuronal pairing might be.

Do compartmentalized FeCO neurons functionally interact? While this remains an open question, desmosome-like junctions have been shown to connect their proximal dendrites, thus physically coupling both neurons.^[84] It is plausible that desmosome structures are required for the structural integrity of the scolopodial unit. However, it is also possible that they serve to increase the displacement threshold of these coupled neurons, such that only sufficiently strong mechanical input can elicit neuronal outputs. Therefore, it remains to be determined whether the desmosome-like junctions are integral to the function of individual FeCO neurons, or if they also facilitate sensory processing by filtering out weaker mechanical stimuli.

2.4. Auditory Neurons of Johnston's Organ

Finding mates, locating prey, and avoiding predators all require audition. Across Insecta, detection of near-field sound is mediated by the Johnston's organ (JO), a specialized exteroceptive chordotonal organ consisting of a scolopodial array located in the second antennal segment^[47,48] (Figure 2 and Table 1). Two to three bipolar mechanosensory cells, known as Johnston's organ neurons (JONs), are compartmentalized within each auditory scolopidium, in contrast with tympanal and subgenual organs—which detect far-field sound and vibrations borne through solid substrates, respectively—but whose scolopidia each contain only one sensory neuron.^[47,48,87,88] The JO connects the second and

third antennal segments; when the distal segment(s) vibrates in response to acoustic stimuli, these movements are detected and transduced into electrical signals by the JONs.^[89]

In *D. melanogaster*, each JO scolopidium typically compartmentalizes two morphologically similar neurons^[49] (Figure 2e). In addition to vibration-sensitive JONs, which detect sound, JO neurons may also act as gravity and wind sensors by detecting static deflections.^[88] Notably, JONs with distinct tuning properties appear to be grouped together in stereotypical combinations: a recent histological study suggests that a vibration-sensitive JON is paired with a deflection-sensitive neuron within the same scolopodial compartment.^[90] In principle, this pairing allows an individual scolopidium to mediate hearing in addition to sensing wind and gravity, and to potentially integrate these stimuli at the sensory periphery.

Functional distinction between JONs within a scolopidium is also likely present in mosquitoes. A recent study in male *C. pipiens* provides evidence of acoustic opponency in the response profiles between compartmentalized JONs, which are shown to respond to either the phase or anti-phase component of the same sound stimulus.^[91] Detection of opponent auditory stimuli by pairwise-organized JONs housed in the same structural unit could potentially permit weaker vibratory signals, such as low-frequency deflection by wind currents, to be filtered out at the level of the sensory periphery.^[91,92] If so, a potential benefit conferred by this organization may be improved signal-to-noise ratios of auditory inputs at the initial stage of auditory processing.

2.5. Sensilla for Hygro- and Thermosensation

Given their small size and relatively large surface area, insects are especially vulnerable to desiccation, making their ability to detect temperature and humidity essential for survival. These sensory modalities are largely mediated by two types of poreless hairs: bimodal sensilla responsive to both temperature and humidity, and unimodal sensilla that sense temperature alone. The former of these, known as “triads”, houses three neurons activated by either temperature, moisture, or dryness (Table 1). Although the compartmentalization scheme is highly conserved for triads, their sensillar morphology varies greatly, including campaniform sensilla of ground beetles; mound-shaped sensilla of stick insects; coelocapitular sensilla of honeybees; capitulum sensilla of cockroaches; styliform complex sensilla of moths; and coeloconic sensilla of mosquitoes.^[93–100] While these hairs are all located on the antennal surface, the triads of fruitflies are instead housed in coeloconic sensilla contained within a specialized, pit-like structure known as the sacculus^[55] (Figure 2f). The diversity of triad morphology raises questions about whether their form influences function, and whether their anatomical structure is in turn informed by the ecological requirements of individual insect species.

Why might a temperature-sensing cell be paired with two hygro-sensory neurons? At the molecular level in *D. melanogaster*, three ionotropic receptors—IR25a, IR40a, and IR93a—are expressed in the dry-sensing cell and are necessary for sensing reductions in humidity.^[56,57] On the other hand, IR68a is required for responses in the neighboring moist cell.^[58,59] Surprisingly, genetic silencing of IR40a neurons, which include

the temperature- and dry-sensing cells, affects humidity but not temperature preference behavior.^[56] This phenotype suggests an unanticipated role for the temperature-sensing neuron in modulating humidity preference; as such, it appears that all three cells within the triad inform the hygroregulatory behavior of fruitflies. It will be informative to determine whether this characteristic likewise applies to triads in other insect species.

Interestingly, dedicated thermosensitive sensilla sensing warmth and cold are found in Diptera, for whom thermosensory inputs are central in informing behavior. For mosquitoes, which rely on temperature cues to detect hosts,^[101] thermosensitive sensilla contain warm- and cold-sensing neurons and are located at the distal ends of their antennae.^[102,103] In *D. melanogaster*, similar sensilla are found in the arista, a specialized bristle located on the third antennal segment (Figure 2g and Table 1). The two grouped cells detect antagonistic temperature cues and are morphologically distinct—having either lamellated or unbranched outer dendrites.^[51] In contrast with the thermosensory cells in sacculus triads, the arista neurons influence temperature-guided behavior.^[52–54]

Close apposition between neurons is characteristic of thermo- and hygro-sensory sensilla, whose receptor dendrites completely fill the luminal volume;^[95–97,99,100,104] their proximity might also allow for intercellular interactions. Although gap junctions and other physical connections have not been reported, their absence does not preclude other forms of intra-sensillum signaling, such as ephaptic coupling. Rather unexpectedly, an OBP—Obp59a—is expressed in hygro-sensory sensilla in the sacculus and is required for normal humidity preference in *D. melanogaster*,^[105] thus raising the possibility of OBP-mediated crosstalk similar to that observed between bitter- and sweet-sensing labellar GRNs.^[81] An intriguing direction for future research will be to establish whether functional interactions indeed occur.

2.6. A Peripheral Olfactory Processing Unit

The olfactory system detects and discriminates a wide variety of environmental volatiles to guide most aspects of insect behavior including foraging, feeding, mating, egg-laying, and aggression. There are two major olfactory organs: the maxillary palp and the antenna, which contains the majority of olfactory sensilla^[106] (Table 1). In Coleoptera, Lepidoptera, and Diptera, each multiporous sensillum typically encapsulates the dendrites of two to four ORNs (Figure 2h) that exhibit distinct extracellular spike amplitudes, and are correspondingly named “A”, “B”, “C”, or “D” by descending spike size. Serial block-face scanning electron microscopy (SBEM) of genetically identified ORNs^[107] demonstrates that neuronal size differences reflect the disparity in spike amplitude, in that the large-spike “A” ORN is generally larger than its small-spike neighbor(s);^[20] thus, grouped ORNs are almost always morphologically inequivalent. At the molecular level, compartmentalized ORNs express distinct receptors, which define the unique odorant specificities for each cell. In nearly all characterized insect species, ORNs are grouped in stereotyped combinations, such that an ORN with a particular response profile is paired with ORNs of different tuning properties.^[60,108–112] In all, the conserved pairing of anatomically and functionally dis-

tinct ORNs is highly prevalent, and suggests a critical role for such neuronal arrangement.

Given the stereotypy of ORN grouping, can neurons interact within a sensillum? Beetles offer examples whereby an olfactory stimulus activates one ORN and inhibits its neighbor.^[113,114] Additionally in *D. melanogaster*, Su et al. showed that compartmentalized ORNs can inhibit each other in multiple sensillum types.^[19] Despite the lack of direct synaptic connections,^[61,62] strong activation of one ORN suppresses the activity of its neighbor.^[19] Based on Vermeulen and Rospars’ electric circuit modeling,^[115] it was predicted that ephaptic interaction underlies the crosstalk between grouped ORNs.^[19] Indeed, by recording from pairs of olfactory sensilla impaled by the same tungsten electrode, Zhang et al. provide direct experimental evidence to demonstrate that ephaptic interaction alone is sufficient to drive lateral inhibition between ORNs that share the same electric field.^[20] Remarkably, unequal cell sizes confer disparate circuit functions for compartmentalized neurons: most large-spike “A” ORNs exert greater ephaptic influence and are also less susceptible to electric field changes induced by neighbors,^[20] allowing for asymmetric lateral inhibition. Unequal ephaptic interactions provide a potential peripheral mechanism to evaluate parallel inputs and selectively favor the propagation of signals carried by the dominant ORNs.^[20] One possible advantage of ephaptic coupling is its low energy cost, in that physical, metabolically expensive synaptic connections are not required for circuit interaction. Furthermore, before entering higher brain centers, inputs are already filtered and constrained at the olfactory periphery, which in principle allows salient information to be more efficiently processed at subsequent nodes. Critically, this lateral inhibition also modulates behavior in response to odor mixtures,^[19] highlighting the capability of peripheral processing in biasing olfactory-driven choices.

The olfactory sensilla of ants, bees, and wasps house over a dozen receptor cells,^[18,116–118] and these exceptionally numerous neurons raise the possibility that grouped Hymenopteran ORNs can potentially employ another type of functional interaction. Indeed in Japanese carpenter ants, Ozaki and colleagues identified gap junction-like adhesions between the dendrites of the 100 or more compartmentalized ORNs. These cells presumably form an electrically coupled neural network, in which weak olfactory inputs have a low probability of generating spike responses.^[18] This network computation may underlie the sensillum’s remarkable selectivity to cuticular hydrocarbons (CHCs) produced by non-nestmates.^[119] Input currents in response to nestmate CHCs are expected to be small due to desensitization from chronic exposure, and such inputs should dissipate within a gap junction-coupled neural network. Conversely, input currents elicited by novel CHCs are likely larger and thus have a higher probability to induce strong spike responses.^[18] It will be interesting to determine if such computation applies strictly to pheromone-sensing sensilla and, more generally, whether it is applicable for other Hymenopteran sensilla that house remarkably large numbers of ORNs.

Taken together, functional communication between compartmentalized ORNs may enable individual sensilla to serve as processing units in olfactory computation—either by enhancing stimulus contrast via ephaptic inhibition, or by filtering out weak stimuli through gap junction-coupled neural networks.

3. Information Processing Can Take Place at the Sensory Periphery

3.1. Means of Neuronal Interaction between Compartmentalized Sensory Cells

From the examples described, it emerges that three major types of neuronal interaction may occur between compartmentalized primary sensory cells. First, gap junction-coupled networking allows sensilla to function as intensity filters.^[18] Second, gap junction-mediated inhibitory coupling can affect the temporal response dynamics of compartmentalized neurons, as exemplified by the bumblebee galeal sensilla.^[16] Such inhibition underlies the burst spiking responses, which prevents adaptation from prolonged stimulation; the sugar-sensing GRN therefore maintains sustained responses for longer periods of time, a feature likely critical for feeding regulation.^[16] Third, lateral inhibition through ephaptic or synaptic interaction can influence the coding of stimulus identity. For instance, crosstalk between R7 and R8 photoreceptors likely permits color-opponent processing of light to support chromatic vision.^[17] As discussed below, ephaptic inhibition between grouped ORNs also potentially provide a peripheral mechanism for evaluating concurrent, countervailing olfactory cues.

3.2. Lateral Inhibition in Olfactory Sensilla Likely Functions to Process Valence Information

If lateral inhibition is expected to enhance contrast between parallel inputs, what then is accentuated by ephaptic coupling between grouped ORNs? Valence information is a probable answer. Across insects, a striking pairing pattern emerges: ORNs housed in the same sensillum appear to respond to odorants from ecologically-related sources, but which elicit opposing behaviors (Table 2). In cotton bollworm moths, a large-spike “A” ORN senses a behavioral antagonist released by immature females, while the small-spike “B” ORN responds to a pheromone that is attractive at low levels.^[120] Antagonism also occurs in other insects but overwhelmingly, attractive odorants are detected by large-spike ORNs, whereas their small-spike neighbors detect aversive volatiles (Table 2). When presented with a blend of sex pheromones, silk moths detect attractive bombykol with a large-spike ORN, while its small-spike neighbor responds to aversive bombykal.^[121] In spruce bark beetles, the attractive aggregation pheromone excites an “A” ORN while an aversive cue released by damaged host plants is detected by the paired “B” neuron.^[122]

Moreover in Japanese beetles, an attractive conspecific sex pheromone is detected by the “A” ORN whereas a behavioral antagonist released by the sympatric Osaka beetle, excites the “B” ORN housed in the same sensillum.^[114] This pairing principle—in which an ORN, sensing conspecific odors, is grouped with another neuron responsive to sympatric antagonistic volatiles—is observed in multiple insect species^[123,125,126] (Table 2). The prevalence of this antagonistic organization, together with its ability to discriminate sympatric from conspecific pheromones, both suggest an important role for peripheral processing in establishing reproductive isolation.^[134]

Such isolation is also achieved through the antagonistic detection of host odors; namely, by pairing one ORN that responds to attractive host volatiles with a neighbor that detects aversive, non-host odors. Two subgroups of *R. pomonella* prefer either hawthorns or apples. As shown by Olsson and colleagues, the hawthorn flies sense an attractive host odorant (3-methyl-1-butanol) with an “A” ORN, which is paired with a small-spike neuron responding to butyl hexanoate, a behavioral antagonist emitted by apples. Conversely for the apple flies, butyl hexanoate is attractive and detected by an “A” ORN, whereas 3-methyl-1-butanol is aversive and detected by a small-spike “B” cell in the same sensillum^[127] (Table 2). This switch between these neurons’ response profiles, such that the dominant large-spike “A” cell responds to different attractive cues, likely contribute to the different host preferences between the two closely related *R. pomonella* subgroups.

Beyond the contexts mentioned above, antagonism is also observed in sensilla that inform other behaviors critical for survival, such as foraging and egg-laying behaviors in *D. melanogaster* (Table 2). Specifically in the ab1 sensillum, the large-spike and small-spike ORNs detect attractive vinegar odors^[128] and aversive CO₂^[129] respectively, both of which are component odorants of fermented fruit. Moreover in the ab4 sensillum, the “A” neuron detects an aggregation pheromone to promote egg-laying,^[132] while the neighboring “B” neuron responds to an aversive mold volatile to inhibit oviposition.^[133] Given the ephaptic interactions known to occur in *Drosophila* ORNs,^[19,20] parallel countervailing inputs may be selectively propagated. This enhanced valence contrast of sensillar outputs is expected to facilitate behavioral decisions, a hypothesis that future research should test experimentally. To understand the meaning of asymmetric inhibition, it will also be important to address why dominant large-spike neurons typically convey inputs of positive valence.

Although the above examples support a valence-opponent organization in *Drosophila* olfaction, caution is warranted regarding whether this organizing principle is generalizable across sensillum types. Might there be grouped ORNs that all mediate the same behavioral outcome? Conversely, what is the functional significance of singly-housed ORNs, such as the pheromone-sensing Or67d neurons?^[63,135] Through future research, it will be important to determine the generality of valence opponency, perhaps through a comprehensive survey of olfaction that leverages the genetic tools and the wealth of information applicable to the fruitfly’s odorant receptors, their ligands and ecological significance.^[106]

4. Conclusion and Outlook

While far from exhaustive, the examples examined shed light on the prevalence and sophistication of compartmentalization, an underexplored aspect of sensory signaling. Here, we describe several characteristics of primary receptor neurons that, in numerous peripheral sense organs, are grouped within structural units. An advantage of such organization lies in the close apposition imposed between neurons, an arrangement which is conducive for neural communication via ephaptic coupling, gap junctions, or synaptic connections. Furthermore, receptor cells are grouped in stereotypical combinations that frequently allows

Table 2. Ligands and their behavioral significance for neighboring olfactory receptor neurons (ORNs) in select insect species. Compartmentalized ORNs are named “A”, “B”, or “C” based on their relative spike amplitudes in descending order. Odorant receptors, if known, are indicated in parentheses.

Insect orders and species	Sensilla	ORNs	Ligands	Behavioral significance	References
Coleoptera					
Japanese beetle (<i>Popillia japonica</i>)	Type B sensillum	A B	(R)-japonilure (S)-japonilure	Attractive sex pheromone released by conspecific females Behavioral antagonist, a sex pheromone released by a sympatric beetle species	[114]
Spruce bark beetle (<i>Ips typographus</i>)	Trichoid sensillum	A B	cis-verbenol 1,8-cineole	Attractive aggregation pheromone released by pioneering males Behavioral antagonist, released by damaged host plants	[122]
Lepidoptera					
European corn borer (<i>Ostrinia nubilalis</i>) E strain	Trichoid sensillum	A B	E11-14:OAc Z11-14:OAc	Attractive, major sex pheromone component Antagonistic, minor sex pheromone component & major pheromone component released by Z strain	[123]
European corn borer (<i>Ostrinia nubilalis</i>) Z strain	Trichoid sensillum	A B	Z11-14:OAc E11-14:OAc	Attractive, major sex pheromone component Antagonistic, minor sex pheromone component & major pheromone component released by E strain	[123]
Silk moth (<i>Bombyx mori</i>)	Trichoid sensillum	A (BmOR-1) B (BmOR-3)	Bombykol Bombykal	Attractive, major sex pheromone component Antagonistic, minor sex pheromone component	[112,121,124]
Cotton bollworm moth (<i>Helicoverpa armigera</i>)	Type C Trichoid sensillum	A (HarmOr16) B (HarmOr6)	Z11-16:OH Z9-14:Ald	Behavioral antagonist, released by immature females Attractive sex pheromone component at low concentrations	[110,120]
Oriental tobacco budworm moth (<i>Helicoverpa assulta</i>)	Trichoid sensillum	A B	Z9-16:Ald Z9-14:Ald	Attractive, major sex pheromone component Behavioral antagonist, a sex pheromone released by a sympatric moth species	[125]
Gypsy moth (<i>Lymantria dispar</i> L)	Trichoid sensillum	A B	(+)-disparlure (-)-disparlure	Attractive, major sex pheromone component Behavioral antagonist, a sex pheromone released by a sympatric moth species	[126]
Diptera					
Hawthorn fly (<i>Rhagoletis pomonella</i>)	Basiconic sensillum (b9)	A B	3-methyl-1-butanol Butyl hexanoate	Attractive volatile from downy hawthorns Behavioral antagonist, a volatile from apples	[127]
Apple fly (<i>Rhagoletis pomonella</i>)	Basiconic sensillum (b7)	A B	Butyl hexanoate 3-methyl-1-butanol	Attractive volatile from apples Behavioral antagonist, a volatile from hawthorns	[127]
Fruit fly (<i>Drosophila melanogaster</i>)	Basiconic sensillum (ab1)	A (Or42b) B* (Or92a)	Vinegar Vinegar	Attractive food odor Attractive food odor	[128] [129–131]
*ab1A and ab1B are both large-spike ORNs exhibiting similar spike amplitudes. ^[20]	Basiconic sensillum (ab4)	C (Gr21a/63a) A (Or7a) B (Or56a)	CO ₂ 9-tricosene Geosmin	Aversive odor Aggregation pheromone released by males, promoting female egg laying Aversive mold odor released by fungi, inhibiting female egg laying	[132] [133]

for the detection of multiple modalities—shown by gustatory and mechanical inputs detected by taste hairs—or multiple stimulus types from the same modality, such as aversive and attractive olfactory cues. Thus, the compartmentalization of multimodal or distinctly-tuned neurons is remarkable in its ability to confer a broad range of responses to individual structural units. In addition, the functional interactions between these neurons can enable cross-modal interactivity. Such intra-compartmental processes distinguish the sensory periphery by its ability to shape input signals—including their intensity and kinetics—when grouped receptor neurons functionally interact.

Taken together, these examples of interactions suggest that compartmentalized neurons can constitute more than the sum

of their parts. They also highlight the diversity of signal computation mediated by the peripheral nervous system. However, much remains unknown about whether and how neuronal interactions occur between compartmentalized neurons in other sensory systems, such as in the insect auditory scolopidia, hygro-sensory triads, or thermosensory sensilla. Also unclear is how such peripheral integration influences perception which, in all likelihood, would be very different without processing by compartmentalized primary sensory neurons. For instance, how might the flavors of food change if our taste receptor cells are not compartmentalized in taste buds? In surveying a wide variety of sensory units, we hope this article will stimulate future research that addresses these fascinating questions.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords

ephaptic interaction, lateral inhibition, neuronal compartmentalization, ommatidium, sensillum, sensory processing, taste buds

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