Valence opponency in peripheral olfactory processing

Shiuan-Tze Wu, Jen-Yung Chen, Vanessa Martin, Renny Ng, Ye Zhang, Dhruv Grover, Ralph J. Greenspan, Johnatan Aljadeff, and Chih-Ying Su

Neurobiology Section, Division of Biological Sciences, University of California San Diego, La Jolla, CA 92093; and Kavli Institute for Brain and Mind, University of California San Diego, La Jolla, CA 92093

Edited by John Carlson, Department of Molecular, Cellular and Developmental Biology, Yale University, New Haven, CT; received November 3, 2021; accepted December 21, 2021

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 centers. Furthermore, computational modeling suggests that this organization likely contributes to 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.

Valence opponency in peripheral olfactory processing

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 (1). In olfaction, however, it is unclear whether such a functional sensory map exists. In both rodents and flies, sensory neurons which project to nearbyglomeruli—processing units in the first olfactory relay center—do not necessarily respond to structurally similar odorants (2, 3), 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 olfactory receptor neurons [ORNs, (3–8)] (SI Appendix, Table S1). Each sensillum typically houses two and up to four ORNs, which are named “A,” “B,” “C,” or “D” in descending order of their stereotypical extracellular spike amplitudes (9). ORN pairing in a sensillum is also stereotyped—whereby a neuron expressing a particular receptor always neighbors an ORN expressing another specific receptor (4, 6, 8)—implying functional importance for such organization. Indeed, lateral inhibition broadly occurs between compartmentalized ORNs across sensillum types (10).

Interestingly, the sensillum is the only such place in the olfactory circuit where short-range lateral inhibition is commonly observed between specific input channels (10–12). Previous work has revealed that direct ephaptic interaction is sufficient to mediate lateral inhibition between electrically coupled ORNs (11). Furthermore, systematic morphological analysis of Drosophila antennal sensilla through serial block-face scanning electron microscopy shows that the basic anatomical features that support ephaptic coupling between ORNs—the close apposition of neuronal processes in a confined compartment (13, 14)—are conserved across sensillum types (15). 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.

Results

ORNs Are Likely Paired Based on Valence. We began by analyzing responses from ORN pairs to a large odor panel in a published dataset (3). The Euclidean distance between neighboring neurons or any two randomly selected ORNs was not significantly different (SI Appendix, Fig. S1), 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 antennal ORNs [ORNs, (3–8)] (SI Appendix, Table S1). Each sensillum typically houses two and up to four ORNs, which are named “A,” “B,” “C,” or “D” in descending order of their stereotypical extracellular spike amplitudes (9). ORN pairing in a sensillum is also stereotyped—whereby a neuron expressing a particular receptor always neighbors an ORN expressing another specific receptor (4, 6, 8)—implying functional importance for such organization. Indeed, lateral inhibition broadly occurs between compartmentalized ORNs across sensillum types (10).

Interestingly, the sensillum is the only such place in the olfactory circuit where short-range lateral inhibition is commonly observed between specific input channels (10–12). Previous work has revealed that direct ephaptic interaction is sufficient to mediate lateral inhibition between electrically coupled ORNs (11). Furthermore, systematic morphological analysis of Drosophila antennal sensilla through serial block-face scanning electron microscopy shows that the basic anatomical features that support ephaptic coupling between ORNs—the close apposition of neuronal processes in a confined compartment (13, 14)—are conserved across sensillum types (15). 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.

Significance

Are olfactory receptor neurons (ORNs) arranged in a functionally meaningful manner to facilitate information processing? Here, we address this long-standing question by uncovering a valence map in the olfactory periphery of Drosophila. Within sensory hairs, we find that neighboring ORNs antagonistically regulate behaviors: stereotypically compartmentalized large- and small-spike ORNs, recognized by their characteristic spike amplitudes, either promote or inhibit the same type of behavior, respectively. Systematic optogenetic and thermogenetic assays—covering the majority of antennal sensilla—highlight a valence-opponent organization. Critically, odor-mixture behavioral experiments show that lateral inhibition between antagonistic ORNs mediates robust behavioral decisions in response to countervailing cues. Computational modeling predicts that the robustness of behavioral output depends on odor mixture ratios.

Author contributions: S.-T.W., J.-Y.C., V.M., R.N., J.A., and C.-Y.S. designed research; S.-T.W., J.-Y.C., V.M., R.N., Y.Z., J.A., and C.-Y.S. performed research; D.G., R.J.G., J.A., and C.-Y.S. contributed new reagents/analytic tools; S.-T.W., J.-Y.C., R.N., and J.A. analyzed data; and S.-T.W., R.N., J.A., and C.-Y.S. wrote the paper. The authors declare no competing interest.

This article is a PNAS Direct Submission. This open access article is distributed under Creative Commons Attribution-NonCommercial-NoDerivatives License 4.0 (CC BY-NC-ND).

Published January 28, 2022.

PNAS 2022 Vol. 119 No. 5 e2120134119
https://doi.org/10.1073/pnas.2120134119 | 1 of 11
ORNs: activation of large-spike neurons tends to positively regulate odor-guided behaviors, such as mediating attraction, stimulating egg laying, or promoting courtship (16–26), while activation of small-spike ORNs tends to have antagonistic effects, such as avoidance or inhibiting egg laying (SI Appendix, Table S1) (16, 27–29). Moreover, some paired neurons play opposite roles in modulating the same behavior. For example, ab4A and its ab1-B neighbor promotes and suppresses oviposition, respectively (17, 28). 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 (SI Appendix, Table S1). Based on the receptor driver availability and labeling specificity (SI Appendix, Fig. S2), we examined 23 different ORNs, which represent paired neurons in 10 out of the 17 identified antennal sensillum types that house multiple ORNs (6, 30).

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) (31) in ab1C, aversion. In a proof-of-principle experiment, we expressed a sensilla which house ORNs known to mediate attraction 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.

We next assayed other preference-related sensillum types (SI Appendix, Table S1 and S2). Among the four previously characterized large-spike ORNs—ab5A, ai2A, ac3A, and ab9A (18–21)—all mediated attraction when optogenetically activated (Fig. 1 E–H). Conversely, we observed avoidance caused by activation of their respective small-spike neighbors, except for ab9B (Fig. 1 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 (SI Appendix, Fig. S3 A and B), in contrast to findings in Drosophila larvae (32). Taken together, valence opponency was broadly observed in the context of place preference.

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 (17, 28) (Fig. 2 A and B). We then extended our analysis to ab10 and ai3, the other two known oviposition-related sensilla (23, 29). Optogenetic activation of ab10B suppressed egg laying, concordant with the neuron’s reported role (29), whereas activation of ab10A enhanced oviposition as predicted (Fig. 2C). We observed a trend of increased, albeit statistically nonsignificant ($P = 0.07$), oviposition promotion with activation of ai3A (Fig. 2D), consistent with a previous report (23). Meanwhile, activation of the small-spike neighbor that expresses Or43a reduced oviposition (Fig. 2D). These results support our hypothesis and show that valence opponency also holds for oviposition-related sensilla.

Valence Opponency in Courtship. Does valence opponency also modulate courtship? In D melanogaster, male courtship is suppressed by the singly housed at1 (33) but promoted by ac4A and at4A (24–26). We began by examining at4A, which expresses the Or47b receptor (25, 26), in a thermogenetic assay where three males of different genotypes—Or47b-GAL4::UAS-TnpA1 and both parent-line controls—competed to mate with one wild-type female (Fig. 2E). Single-sensillum recordings verified at4A’s thermogenetic activation (Fig. 2E). Concordant with published reports (25, 26), the copulation rate of at4A-activated males was markedly higher than in controls (Fig. 2F). 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. 2 G and H).

A similar antagonistic relationship was observed between ORNs housed in the ac4 sensillum (Fig. 2I). Thermogenetic activation of ac4A increased its spike activity (Fig. 2I) and conferred a copulation advantage as reported (24) (Fig. 2I). In contrast, courtship suppression was observed upon activation of its small-spike neighbor that expresses the Ir76a receptor (Fig. 2K). We excluded the other small-spike ac4 ORN in our analysis because its receptor, Ir75d, is also expressed in other neurons (8), 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.

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 (SI Appendix, Fig. S4 A–C), indicating that a given ORN does not necessarily influence all behaviors. Further experiments showed valence opponency between ab4A and ab4B for courtship (SI Appendix, Fig. S4 D–F) and between at4A and at4C for place preference but not for oviposition (SI Appendix, Fig. S4 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 (SI Appendix, Fig. S4F).

In summary, antagonizing ORN pairs were identified in 9 of the 10 examined sensilla (Table 1). 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.

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 (10). When paired ORNs are concurrently activated by odor mixtures, lateral inhibition enables the dominant cue to be selectively propagated (10, 11). 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 (34, 35). Thus, antagonistic odorants are expected to influence behavior more effectively when detected by paired ORNs than by non-neighboring neurons.

Valence opponency in peripheral olfactory processing

Wu et al.

https://doi.org/10.1073/pnas.2120134119

Citation copy and paste

Contribution by guest on January 26, 2022

2 of 11 | PNAS
To test this prediction, we focused on two pheromone-sensing sensilla. The at1 sensillum houses a single ORN, which expresses Or67d and detects the antiaphrodisiac pheromone cis-vaccenyl acetate (cVA) (33, 36, 37). In the at4 sensillum, the “A” neuron expresses Or47b, responds to palmitoleic acid, and promotes courtship (25), while the small-spike “C” neuron expresses Or88a, responds to methyl palmitate (26), and suppresses courtship (Fig. 2H). Notably in the at4 sensillum, these
Fig. 2. Valence opponency in oviposition and courtship. (A) Optogenetic oviposition preference assay. (B–D) Violin plots showing oviposition preference indices (OI) 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: OI 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 Student’s \( t \) 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) Each circle denotes the end copulation rate of a given experiment with lines connecting data from the same experiments (\( n = 4 \) to 6 experiments, total 68 to 94 matches). (I–K) As in E–H, except that TrpA1 was expressed in ac4A (J) or its small-spike \( \text{ir}76a^- \) neighbor (K) (\( n = 5 \) experiments; total 83 to 85 matches). \( P \) values are indicated; one-sample z-test.
pheromone ligands only activate the receptors of the target ORNs (25, 26). Using their respective pheromone ligands, paired at4 ORNs were coactivated 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. 3A and SI Appendix, Fig. S5A), consistent with previous findings that ephaptic inhibition between grouped ORNs requires odor-induced excitation from both neurons (10). However, no significant inhibition of at4A was observed by the Or67d ligand cVA regardless of palmitoleic acid stimulation (Fig. 3B). Although cVA is reported to also activate at4B (36), we did not observe such responses (Fig. 3B and SI Appendix, Fig. S5B), in agreement with other studies (38, 39). Through lateral inhibition, methyl palmitate–evoked at4C activity, but not cVA–evoked at1 activity, can suppress at4A’s response to palmitoleic acid (Fig. 3 C and 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 (SI Appendix, Fig. S6 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. 3 E–G, and SI Appendix, Fig. S6 C and D). The modulation was also eliminated when synaptic transmission was blocked by expressing shibirets1 in at4C or at1 neurons (Fig. 3 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. 3 K–M), despite their similar antiaphrodisiac effects when applied alone (Fig. 3G). As negative controls, we performed similar odor-mixture experiments with receptor mutant flies (Or88a−/− or Or67d−/−), wherein courtship inhibition was eliminated as expected (SI Appendix, Fig. S6 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, 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 shibirets1 (Fig. 3H). This manipulation did not affect at4C’s spike responses or its ability to inhibit at4A (SI Appendix, Fig. S7). Strikingly, when palmitoleic acid was coapplied, methyl palmitate reduced courtship by 20% despite the absence of at4C feedforward output (Fig. 3 N and P). These results demonstrate that at4A’s output is indeed attenuated by at4C ephaptic inhibition. In comparison, the antiaphrodisiac effect of cVA was completely abolished in Or67d::shibirets1 males even when palmitoleic acid was coapplied (Fig. 3 O and P).

As negative controls, we performed similar odor-mixture experiments except that palmitoleic acid was replaced by a pheromone ligand for ab4A (17), an ORN type which we found also promotes courtship (SI Appendix, Fig. S4F). As expected, the ab4A ligand 9-tricosene promoted male courtship in a receptor-dependent manner (Fig. 4 A and B). Notably, we no longer observed differential antiaphrodisiac effects between methyl palmitate and cVA when either antiaphrodisiac pheromone was coapplied with 9-tricosene, regardless whether the males were wild type, Or67d::shibirets1, or Or88a::shibirets1 (Fig. 4 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. 5A; see Methods for modeling details, and SI Appendix, Figs. S11 and S12). Ephaptic inhibition increases the response difference between paired ORNs (Δy = x_A − x_B) relative to the difference between stimuli (Δs = 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 Δy/Δs was therefore defined as the degree of valence amplification (a) (Fig. 5B). Our mathematical analysis showed that the magnitude of amplification varies depending on the ratio of respective stimulus strength in a mixture (Fig. 5C). 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,
Fig. 3. 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, 10−3 vol/vol dilution), presented in the background (Right). The colored bars indicate odourant stimulations. MP: methyl palmitate (10−3). Parallel experiments, mean ± SEM (n = 12, from 3 to 4 male flies). (B) As with (A), except that recordings were performed with the at1 ligand, cis-vaccenyl acetate (cVA, 10−3), as the transient odorant. Parallel experiments, mean ± SEM (n = 8, from 3 to 4 male flies). (C) Peri-stimulus time histograms of at4A spikes from data shown in (A–B). Line width indicates SEM. (D) Quantification of at4A activity. Each data point represents the basal spike frequency of at4A before methyl palmitate (MP) or cis-vaccenyl acetate (cVA) were presented, as shown in (A and 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::Shits1) or in at1 (J, Or67d::Shits1). (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::Shits1) or in at1 (O, Or67d::Shits1). *P < 0.05, **P < 0.01, Wilcoxon rank-sum test. n = 5 to 6, 125 to 150 matches for individual conditions.
whose constituents typically activate compartmentalized ORNs, can most effectively trigger robust behavior when presented in narrowly defined ratios (40, 41). Therefore, the valence-opponent organization between ephaptically coupled ORNs may have evolved to process ethologically meaningful ratios of odor mixtures.

Discussion

Here, we identify a valence-opponent olfactory map in *D. melanogaster* (Table 1). This organization is likely conserved among insects, as other compartmentalized insect ORNs have been reported to detect antagonistic cues (41). However, 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 (41). As another example, 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 (42). In addition, the aversive CO₂ cue is detected by a small-spike ORN (ab1C) expressing Gr21a/Gr63a receptors in fruit flies (27, 43, 44); 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 (45).

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 (11)? 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...
Without ephaptic inhibition, a function of stimulus difference ($\Delta X$), is inhibited ephaptically in a manner that depends on the strength of both $S_A$ and $S_B$. (Fig. 5) Steady-state response of ORN $i$ ($X_A$) 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 $j$ is also activated by $S_A$, $X_A$ (green lines) is inhibited ephaptically in a manner that depends on the strength of both $S_A$ and $S_B$. (b) Relation between paired neurons ($\Delta X$) as a function of stimulus difference ($\Delta S$). Ephaptic inhibition amplifies the valence signal if $\Delta S = S_A - S_B$ have the same sign, and if $|\Delta S| > |\Delta X|$. The simulated responses fall almost entirely within the amplified region (pink-shaded area, see Methods for modeling details). (c) The degree of valence amplification ($\alpha$) 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 SI Appendix, Fig. S12 and Methods for modeling details).

**Materials and Methods**

**Flies.** All flies (*D. melanogaster*) were housed on standard cornmeal food containing molasses at 25°C, 60% humidity in an incubator with a 12-h 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 SI Appendix, Tables S2 and S3.

**Immunohistochemistry and Confocal Imaging.** Female flies expressing mCD8-GFP in the target ORNs were anesthetized on ice. The brains were dissected in phosphate-buffered saline (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 h at RT. The brains were then washed with rabbit $\alpha$-green fluorescent protein (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 h at 4°C. The brains were then stained with rabbit $\alpha$-goat secondary antibodies, Alexa 488 goat $\alpha$-rabbit 1:500 (A31628, Life Technologies), and Alexa 647 goat $\alpha$-mouse 1:500 (A21236, Life Technologies) in the dilution buffer for 24 h at 4°C. After three washes in 1% PBT at RT, the brains were mounted in FocusClear (CEL-prep) – mouse. Three samples were imaged on a Zeiss LSM 880 confocal microscope using 40x, N.A.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 × 35 × 3.5 mm with a sloped edge (32). In this assay, a custom light-emitting diode (LED) board was placed beneath the arena (SI Appendix, Fig. S8). The LED board was composed of interleaving 625-nm red LEDs (VLRM3434ABC-G508CT-ND, Digi-Key) and 850-nm infrared (IR) LEDs (VSMY3850-G508CT-ND, Digi-Key). The LEDs were powered by programmable direct current (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 Chrimson (31) 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 h 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-d-old) was tested. A group-fly assay was adopted because collective behavior is known to enhance the robustness of ORN-mediated preference (33). 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 (flight only) for 30 s. Subsequently, the red LEDs were turned on to illuminate the right half of the arena for 45 s. After another 30 s 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 μW/mm². In some experiments (ab1A and ac3A) where less robust preference was observed (0.05 < P < 0.01), higher irradiance (9.1 μW/mm²) 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 (Edmond Optics) from the top of the arena. The location of flies was determined by a custom tracking C++ code. The preference index (PI) of flies to the illuminated side was calculated every half second based on the positions of flies in the arena. The PI was defined as: PI = (Nlight − Ndark) / Ntotal, where Nlight indicates the number of flies on the illuminated half of the arena, Ndark indicates the number of flies on the dark half of the arena, and Ntotal indicates the total number of flies in the arena (10 flies). For each experiment, the average PI 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 flies’ place preference were also examined in preliminary experiments by employing single-sensillum, photostimulation, and paired light stimulation with a constant airflow (54). In pilot experiments, the preference behavior of Gr21a::CsChrimson flies was examined in the absence or presence of airflow (~25 cm/s). In the absence of airflow, the flies exhibited robust avoidance to light (SI Appendix, Fig. 59A), as expected from ab1C’s role in mediating CO2 avoidance (27). However, with constant airflow, both the control and experimental flies exhibited a strong tendency to walk upward, resulting in robust attraction toward the side of air input even when it was illuminated by red LEDs (SI Appendix, Fig. 59B). In another pilot experiment, pulsed red light (1, 5, 10, and 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 (55). 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 photostimulation, referencing electrical activity of target ORNs was recorded extracellularly by placing a sharp electrode in the hemolymph solution as described (59). Of note, smaller chambers were used for single-pair courtship experiments vs. the ones for the 3:1-male–female competition experiments described above in Thermogenetic Courtship Competition Assay. Courtship assays were conducted at either 25°C (Fig. 3 E–G and Fig. 4 A and B and SI Appendix, Fig. S6 A–D) or around 30°C (Fig. 3 H–P and Fig. 4 C–H and SI Appendix, Fig. S6 E and F) 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′(59, 60). Of note, the UAS-Shibire′ line used in this study contained a translational enhancer for robust expression (60). Each experiment contained 25 matches. The control and experimental groups were tested in parallel experiments. Copulation was visually confirmed, and the CCR was calculated for the 2-h period. The inhibition index was calculated as follows:

\[
\text{Inhibition index} = \frac{\text{CCR}_{\text{Control}} - \text{CCR}_{\text{Experimental}}}{\text{CCR}_{\text{Control}}}
\]

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 micropipette tube and a coverslip covered with double-sided tape. Extracellular single-sensillum recordings were performed as described (61). Briefly, electrical activity of target ORNs was recorded extracellularly by placing a sharp electrode filled with artificial hemolymph solution (62) in the at4 sensillum (Or47b ORN recordings). For recordings of other sensillum types, 0.6% sodium lymph Ringer solution (63) was used instead. A reference electrode filled with the same solution was placed in the eye of fresh food every other day as described (57). 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 (58). In brief, three naive males of different genotypes and one 3-d-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 different fluorescent dyes (UV420, Life Technologies; cyanine green, Molecular Probes). The location of flies on the illuminated half of the arena was determined by averaging the preference indices during periods of illumination over the three trials. 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 (25)—significantly enhanced courtship in 2-d-old males but not in 7-d old (not shown). The lack of courtship enhancement in the latter condition likely reflects a ceiling effect arising from the high 7-d male copulation around 30°C (total CCR: 82% from four 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-d-old males, likely reflecting a floor effect arising from the low copulation rate of 2-d-old males (total CCR: 47% from three experiments, total 60 matches; no significant difference between control and experimental groups). Of note, male age was found to influence only the manifestation or degree of courtship modulation by thermogenetic ORN activation, but never the polarity of the modulation. Therefore, to avoid ceiling or floor effects, 2-d-old males were used in experiments where at4A or ac4A was thermogenetic activated, while 7-d-old males were used for the at4B, at4C, or ac4C experiments.

Pheromone Perfuming Experiments. Briefly, 0.3 μl each individual pheromones or binary mixtures were directly applied to the abdomen of female flies essentially as described (33). Pheromones were diluted in acetone (vol/vol) prior to experiments (palmitoleic acid: 3 × 10−6, ~0.1 ng/μl; methyl palmitate: 3 × 10−4, ~87 ng/μl; cis-vaccenyl acetate: 1.5 × 10−4, ~38 ng/μl; (Z)-9-tricosene: 3 × 10−6, ~7 ng/μl). 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 1 h prior to experiments. For single-pair courtship assays, one 3-d-old naive male and one 3-d-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 (25). Of note, smaller chambers were used for single-pair courtship assays than the ones for the 3:1-male–female competition assays described above in Thermogenetic Courtship Competition Assay. Courtship assays were conducted at either 25°C (Fig. 3 E–G and Fig. 4 A and B and SI Appendix, Fig. S6 A–D) or around 30°C (Fig. 3 H–P and Fig. 4 C–H and SI Appendix, Fig. S6 E and F) 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′(59, 60). Of note, the UAS-Shibire′ line used in this study contained a translational enhancer for robust expression (60). Each experiment contained 25 matches. The control and experimental groups were tested in parallel experiments. Copulation was visually confirmed, and the CCR was calculated for the 2-h period. The inhibition index was calculated as follows:

\[
\text{Inhibition index} = \frac{\text{CCR}_{\text{Control}} - \text{CCR}_{\text{Experimental}}}{\text{CCR}_{\text{Control}}}
\]

Data were analyzed using custom MATLAB codes.

Valence opponency in peripheral olfactory processing

Valence opponency in peripheral olfactory processing

Valence opponency in peripheral olfactory processing

Valence opponency in peripheral olfactory processing

Valence opponency in peripheral olfactory processing
dyses. No more than three sensilla from the same antenna were recorded per fly. Alternating current signals (100 to 20,000 Hz) and DC signals were simultaneously recorded on an NPI EXT-02F amplifier (ALA Scientific Instruments) and digitized at 5 kHz with Digidata 1500 ( 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. Methyl palmitate (10⁻², Sigma P5177) or cis-vaccenyl acetate (Cayman 10010101, 10x dilution from 5% stock) was diluted in ethanol (vol/vol), applied as a 4.5-μL portion to a filter disk and delivered via a 500-ms air pulse at 250 mL/min directly to the antenna from close range as described (61). For recordings with a background odorant, palmitoleic acid (10⁻¹, Cayman 9001798) was applied as a 4.5-μL portion to a filter disk, which was placed in close proximity adjacent to the fly head.

For optogenetic stimulation, newly eclosed female flies expressing ChR2-GFP in targeted ORNs were reared in constant darkness for 4 d on fly food supplemented with 10 μM all trans-retinal (Sigma R25500). 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 to 660 nm) can activate ChR2-GFP (31). 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 be 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-808-500S-5) was directed to the antenna via a 1-mm optic fiber (Roithner, RLTM-5MA950-1000). 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 ~30 °C, which did not elicit any excitable 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:

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

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

For simplicity, we assumed that the response decays to the baseline linearly with the timescale $\tau = 1$. The parameter $\Delta w_{AB}$ describes the strength of ephaptic coupling from ORN$_{A}$ to ORN$_{B}$, and similarly for $\Delta w_{BA}$. 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 (11). Furthermore, ephaptic interaction from ORN$_{A}$ to ORN$_{B}$ scales nonlinearly with the activity level of ORN$_{A}$ ($w_{AB}$ scaling was exquisitely observed ($q > 1$) 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 ($\alpha$) in the stimulus space as a function of $S_A$ and $S_B$ (SI Appendix, Fig. S12). For certain $q$ and $n$ combinations, there were regions in the stimulus space where there is no amplification ($\alpha < 1$, white-shaded areas in SI Appendix, Fig. S12). For demonstration purposes, a specific combination of $n$ and $q$ was selected for Fig. 5C ($n = 2$ 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–Wilks normality test. For ovisposition preference, significance was determined by unpaired two-sample Student’s $t$ test, as all datasets passed the Shapiro–Wilks normality test. Courtship competing data were analyzed by one-sample z-test. All pheromone perceiving 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.

Data Availability. All study data are included in the article and/or supporting information.

ACKNOWLEDGMENTS. We thank Karen Menuez and Larry Squire for comments on the manuscript, Kalyani Cauwenberghs for assistance with statistical analysis, and Takeo Katsuki for manufacturing the chambers for place preference assays. We also thank the University of California San Diego (UCSD) School of Medicine Microscopy Core (NS047101) and the UCSD Qualcomm Innovation Institute Prototyping Lab. This work was supported by a Kavli Institute for Brain and Mind Innovative Research Grant (C.-Y.S.), NIH Grant Nos. R01NS087444 and R21DC008912 (C.-Y.S.), Air Force Office of Scientific Research Grant Nos. FA-9550-18-1-0051 (R.G.) and FA-9550-19-1-0280 (D.G.), and a Defense Advanced Research Projects Agency Young Faculty Award No. D21AP10162 (J.A.).
