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Olfactory Neurons and Brain Centers Directing Oviposition Decisions in *Drosophila*

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SUMMARY

The sense of smell influences many behaviors, yet how odors are represented in the brain remains unclear. A major challenge to studying olfaction is the lack of methods allowing activation of specific types of olfactory neurons in an ethologically relevant setting. To address this, we developed a genetic method in *Drosophila* called olfactogenetics in which a narrowly tuned odorant receptor, Or56a, is ectopically expressed in different olfactory neuron types. Stimulation with geosmin (the only known Or56a ligand) in an *Or56a* mutant background leads to specific activation of only target olfactory neuron types. We used this approach to identify olfactory sensory neurons (OSNs) that directly guide oviposition decisions. We identify 5 OSN-types (Or71a, Or47b, Or49a, Or67b, and Or7a) that, when activated alone, suppress oviposition. Projection neurons partnering with these OSNs share a region of innervation in the lateral horn, suggesting that oviposition site selection might be encoded in this brain region.

Graphical Abstract

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AUTHOR CONTRIBUTIONS

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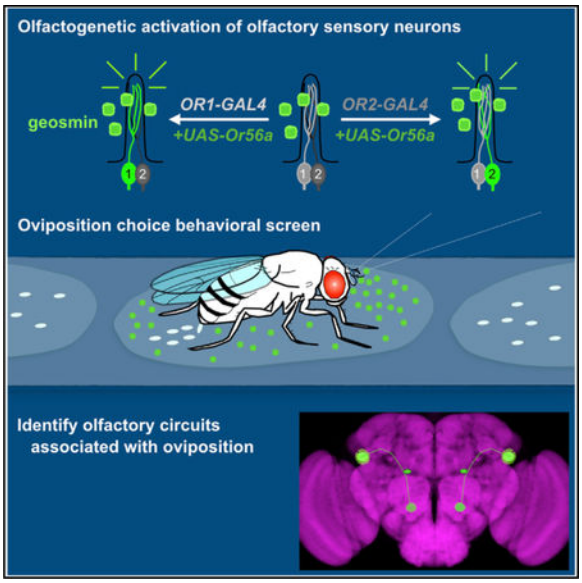
DECLARATION OF INTERESTS

The authors declare no competing interests.



In Brief

Linking olfactory neurons to discrete behaviors is challenging. To address this, Chin et al. develop a genetic method in Drosophila that uses an odor to selectively activate different olfactory neurons. From a behavioral screen, they identify olfactory neurons and brain regions that might underlie aversive egg-laying decisions.

INTRODUCTION

The sense of smell is an ancient and vital sensory system, but how olfactory information is processed in an animal's brain remains unclear. Organisms ranging from insects to humans use the olfactory system to detect volatile chemicals (odorants), and these odorants act as environmental sensory cues representing the physical objects that emit them. Upon detection of these volatile chemicals, the olfactory system must detect, interpret, and then guide behavioral actions based on these cues. Olfactory perception results from the activation of individual olfactory sensory neuron (OSN) types found in olfactory organs (such as the nose), which each express specific odorant receptors responsible for detecting a near-infinite variety of odorants. The number of OSN types, defined as expressing a specific olfactory receptor, differs among species, with humans having 388 OSN types, mice having 1,200 OSN types, and vinegar flies having 62 OSN types (Masse et al., 2009; Kaupp, 2010). Axons of OSNs that express the same olfactory receptor project directly to a specific region of the brain called a glomerulus, which is the primary organizing unit for the olfactory system. The collection of all glomeruli is called the olfactory bulb in mice and the antennal lobe in insects. Dendrites of second-order neurons (mitral or tufted cells in mammals and projection neurons in insects) typically innervate a single glomerulus. Thus, a glomerulus acts as an organizing center to match OSNs to their cognate second-order projection neuron partners. Projection neurons then send axons to higher brain centers for further olfactory processing (piriform cortex in mammals and lateral horn in insects). How does activation of individual OSN types contribute to a complex behavior? This question has been difficult to

study because most natural odors contain complex mixtures of odorants, and most odorants can activate several different olfactory receptor classes to varying degrees (Hallem and Carlson, 2006). This aspect of the olfactory system makes a precise, odor-driven experimental study of OSNs extremely challenging. A goal of this study was to develop genetic tools in an animal model that could be used to investigate the contributions of individual classes of OSNs to odor-guided behaviors.

The vinegar fly, *Drosophila melanogaster*, is a powerful genetic model for investigating sensory perception. As a highly olfaction-driven organism, the fly uses its sense of smell to direct all essential behaviors from locating food, navigating space, mating with the correct species, and finding locations to lay eggs (Mansourian and Stensmyr, 2015). Many of these olfactory behaviors occur on highly odiferous rotting fruits, which serve as both food source and oviposition substrate. The fly's olfactory system must filter through a complex sensory world in order to obtain ethologically relevant odor information and behave appropriately. Comprehensive screens have matched the identity of each odorant receptor type to the OSNs that express them, and the response profiles of many OSNs have been determined (de Bruyne et al., 2001; Hallem and Carlson, 2006; Grabe et al., 2016). Furthermore, the glomerular targets for each OSN type in flies have been genetically mapped (Couto et al., 2005; Fishilevich and Vosshall, 2005); this represents the most comprehensive olfactory map generated for any organism. Like the mammalian olfactory system, *Drosophila* OSNs expressing the same odorant receptor all target a single glomerulus. However, unlike in mammals, insect OSN targeting does not depend on which odorant receptor is expressed, and anatomical locations of individual glomeruli in the antennal lobe are highly stereotyped. The projection neurons innervating many glomeruli have been genetically identified and are also highly stereotyped in their anatomical targeting patterns in the lateral horn (Jefferis et al., 2007; Chiang et al., 2011). Based on these axonal innervation patterns into the lateral horn, it appears that projection neurons reorganize olfactory information from the antennal lobe to the lateral horn such that the different regions of the lateral horn represent biologically relevant stimuli such as food versus pheromone odors and possibly aversive odors (Jefferis et al., 2007). The identification of additional lateral horn domains has been hampered by the previously mentioned experimental challenge in linking individual OSN activities to discrete olfactory behaviors.

A critical behavior for species survival in most insects is determining where to lay eggs (oviposition) (Yang et al., 2008). Females that choose high-quality egg-laying substrates ensure the health of both eggs and the resulting larvae. Since larvae cannot fly and usually remain close to the oviposition site, their ability to survive depends heavily on the patch of food on which they hatch. Thus, a mated female fly must balance her own nutritional and safety needs with the needs of her future offspring to maximize nutrient intake and protection from parasites and disease-causing microorganisms (Lihoreau et al., 2016). Many sensory cues guide oviposition decisions: taste (gustation), texture, vision, and olfaction (Markow and O'Grady, 2008; Zhu et al., 2014). The chemical senses, in particular, strongly contribute to oviposition decision-making, and several individual olfactory receptors have indeed been associated with oviposition decisions (Bartelt et al., 1985; Stensmyr et al., 2012; Dweck et al., 2013; Ebrahim et al., 2015; Lin et al., 2015). Nonetheless, how the brain processes olfactory information to generate egg-laying behavior is not well understood.

To study how defined OSN types give rise to olfactory signals used by the brain to guide oviposition decisions, we generated a genetic tool that allows highly specific odorant-directed activation of individual OSN classes. We call this method “olfactogenetics” to reflect that it uses a volatile odorant to activate genetically defined types of olfactory neurons. This approach offers several advantages over existing genetic activation methods such as optogenetics (Klapoetke et al., 2014) and thermogenetics (Hamada et al., 2008); for example, induced olfactory activities will more closely mimic natural odorant stimulations and do not rely on sophisticated equipment designs. We used the olfactogenetic method to systematically identify OSNs that, when activated, mediate oviposition choices. By linking the glomerular targets of the OSNs to likely activated projection neurons, we identified a region of the lateral horn that might represent negative oviposition cues.

RESULTS

An Olfactogenetic Method to Activate Specific OSN Types

We developed an olfactogenetic method for specific OSN activation that takes advantage of an unusually specific odorant receptor to odorant pair identified in *Drosophila* (Stensmyr et al., 2012). In a screen of olfactory sensilla, the odorant geosmin was found to activate only a single olfactory neuron class (antennal basiconic 4B) expressing the Or56a receptor (Stensmyr et al., 2012). This suggests that Or56a is the only odorant receptor that is activated by geosmin, and deleting the Or56a receptor should result in a fly that is completely anosmic to geosmin. Furthermore, ectopically expressing Or56a in a different OSN type should then confer geosmin-induced responses to that olfactory neuron. The olfactogenetic approach thus requires three components: (1) *Or56a* mutant (*Or56a*$^{-/-}$) animals to eliminate wild-type responses to geosmin, (2) a *UAS-Or56a* transgene to drive *Or56a* in olfactory neurons, and (3) *OrX-GAL4* lines to direct which types of olfactory neurons express *UAS-Or56a* (Figure 1A). We generated *Or56a*$^{-/-}$animals and confirmed by single sensillum recordings (SSR) that responses to geosmin in the ab4 sensillum were abolished (Figure 1B). To verify the identity of *Or56a*$^{-/-}$mutant sensilla, we stimulated the sister olfactory neuron (ab4A) that expresses Or7a with the activating pheromone 9-tricosene (Lin et al., 2015) and found that ab4A neurons fired normally. This confirmed that in the *Or56a*$^{-/-}$mutant sensilla, only the large spiking amplitude A neuron fires (Or7a) while the B neuron (Or56a) is completely silenced, indicating the fly no longer expresses an olfactory receptor capable of detecting geosmin. Consistent with a previous study examining geosmin responses for all olfactory neurons in *Drosophila* (Stensmyr et al., 2012), we found ab4B (Or56a+) neurons were the only olfactory neurons activated by geosmin (see behavior results below; data not shown).

Ectopically Expressed Or56a Activates OSNs through Stimulation with Geosmin

Four types of sensillum, classified according to morphology, cover the fly’s olfactory organs: basiconic, intermediate, trichoid, and coeloconic (Lin and Potter, 2015; Grabe et al., 2016). The olfactory neurons in these sensilla tend to respond to different types of odorants: trichoid olfactory neurons respond to pheromones, intermediate olfactory neurons respond to kairomones (odorants from other species), basiconic olfactory neurons respond to a diverse set of environmental odorants, and coeloconic olfactory neurons respond to amines and acids

(Hallem and Carlson, 2006; Lin and Potter, 2015). Previous studies indicate that the intracellular molecular environment of different sensillum types may differ. For example, signaling in antennal trichoid (at) 1 has been shown to be enhanced by the presence of the odorant binding protein LUSH and sensory neuron membrane protein (SNMP1) (Benton et al., 2007; Laughlin et al., 2008), and a recent study deorphanizing Or83c in antennal intermediate (ai) 2 suggests intermediate sensilla may possess intra-sensillar components more similar to trichoids than basiconics (Ronderos et al., 2014). Or56a normally expresses in the antennal basiconic sensillum ab4 so it was important to validate that ectopic expression of this receptor in non-native sensillum types produces a physiological response. Thus, we used SSR to determine if ectopically expressed Or56a in non-ab4B neurons would be functional and activated by geosmin.

We sampled the olfactogenetic activation of neurons from both olfactory organs (antenna and palp) and three of the four major sensillum types. *UAS-Or56a* was expressed in ab3A (*Or22a-GAL4*), palp basiconic (pb) 1B (*Or71a-GAL4*), ai2B (*Or23a-GAL4*), at1 (*Or67d-GAL4*), and at4A (*Or47b-GAL4*). Since Or56a is natively expressed in a basiconic sensillum (ab4), it was expected that ectopic expression of Or56a in other basiconic sensilla would be able to confer a geosmin response. This was the case in both the antenna (ab3A) and maxillary palp (pb1B) (Figure 2). While primarily testing the response of ab3A (*Or22a-GAL4>UAS-Or56a*), we observed that geosmin stimulation also appeared to cause a change in firing rate of its neighboring B neuron (Figure S2), eliminating the B neuron's native response to the mineral oil control. Since neurons housed in the same sensillum share sensillar lymph, the firing of one neuron has the ability to inhibit firing in its neighbors through the phenomenon of ephaptic coupling (Su et al., 2012). Ephaptic coupling along with ab3B's native response to the vehicle mineral oil likely explains why presentation of geosmin to activate ab3A also silences ab3B.

The intermediate sensillum neuron ai2B also reliably increased firing, but only by <10 Δspikes/s (Figure 2). It is possible that non-basiconic intermediate sensilla contain different molecular components (e.g., odorant-binding proteins, co-receptors, or odorant degrading enzymes), which might be responsible for weak activation. Therefore, we also tested expressing *Or56a* in ai2A (*Or83c-GAL4*), the companion neuron to ai2B, in the same sensillum (Figure S2). The ai2A neuron was robustly activated by geosmin (30–50 Δspikes/s), suggesting that basiconic Ors can function normally in intermediate sensilla. Expression of Or56a in trichoid sensillum neurons at1 and at4A conferred activation by geosmin but only at the higher (10^{-4}) geosmin concentration (Figures 2A and2B). This suggests trichoid sensilla, like intermediate sensilla, may contain different molecular components that normally optimize responses to pheromones but may reduce the responses to non-pheromone odors (Benton et al., 2007; Laughlin et al., 2008). Nonetheless, the level of olfactogenetic activity induced in intermediate and trichoid sensilla should be sufficient to drive olfactory behaviors (Bell and Wilson, 2016). Olfactory sensilla most commonly express canonical Ors along with the co-receptor Orco (Larsson et al., 2004). However, the ab1C neuron expresses two gustatory receptors (Gr21a and Gr63a) that together detect CO_2 as well as many other odorants (Kwon et al., 2007; Turner and Ray, 2009; Tauxe et al., 2013; MacWilliam et al., 2018). The Or56a-geosmin olfactogenetic tool could potentially be useful in studying gustatory receptor neuron (GRN) functionality. GRNs do not express Ors or

Orco (Clyne et al., 2000; Scott et al., 2001). Given that the mechanism of activation and necessary intracellular signaling components of Grs are not well defined, it was unclear whether Or56a could be used to activate a GRN (also see Benton et al., 2006). Expressing both *Or56a* and *Orco* in ab1C (*Gr63a-Gal4 > UAS-Or56a, UAS-Orco*) (Suh et al., 2004, 2007) and stimulating with geosmin indicates that ab1C can be robustly activated using Or56a-geosmin olfactogenetics (Figures 2C and2D). Notably, 10^{-5} geosmin stimulation to ab1C elicited typical firing dynamics with a continuous burst of firing after odor presentation that gradually returned to baseline. Interestingly, this was not the case with 10^{-4} geosmin stimulation. In 5 of the 6 recordings using 10^{-4} geosmin, we observed a biphasic response where, upon initial presentation of the odor, the ab1C neuron fired rapidly, followed by a sudden decrement to no firing, and then a second burst of firing erupted shortly thereafter (Figure 2C). This exclusively occurred in response to high-concentration geosmin stimulation. While Ors likely make a complex with Orco to form ion channels, several studies have shown that second messengers are necessary for normal responses to odorants (Sato et al., 2008; Wicher et al., 2008). The difference in firing dynamics in ab1C suggests that Grs may have different downstream second messengers that act at a different timescale than those of Ors or that Gr-specific cellular machinery can act to temporarily repress Orco-Or56a receptor complex signaling. In addition, the odorant properties of geosmin (e.g., its volatility or stability in a different sensillar environment) might influence the response dynamics of the ectopically activated olfactory neuron.

T-Maze Olfactory Assays Using Olfactogenetics

A simple olfactory assay is the T-maze two-choice assay, in which flies are given a choice between vials containing either an odorant or a solvent control (Dudai et al., 1976; Tully and Quinn, 1985). T-maze assays have been used to assess innate attraction or aversion toward odorants (Suh et al., 2004; Stensmyr et al., 2012; MacWilliam et al., 2018). We performed a series of olfactogenetic experiments using a T-maze assay to activate olfactory neurons previously implicated in innate attraction, including Or42a (Mathew et al., 2013; Hernandez-Nunez et al., 2015; Jung et al., 2015; Bell and Wilson, 2016), Or71a (Dweck et al., 2015a), Or85a (Semmelhack and Wang, 2009; Knaden et al., 2012; Gao et al., 2015; Bell and Wilson, 2016), or innate repulsion, including Or85a (Semmelhack and Wang, 2009; Knaden et al., 2012; Gao et al., 2015; Bell and Wilson, 2016) and Gr63a/Gr21a (Suh et al., 2004, 2007; Turner and Ray, 2009; Bell and Wilson, 2016; MacWilliam et al., 2018) (Figure S3). In our hands, high variability in T-maze behavioral responses resulted in performance index scores that were not statistically different among the different olfactogenetics genotypes examined (Figure S3; also see Supplemental Experimental Procedures for additional information). We instead focused on using olfactogenetics with an olfactory oviposition choice assay that proved to be behaviorally robust in assaying innate olfactory decisions (Lin et al., 2015).

Two-Choice Oviposition Assay Relies on Olfactory Signaling

Many sensory cues, including olfactory cues, influence oviposition choice in female flies. We established an oviposition choice assay in which only olfaction was used as a differentiating cue. In this assay, three wells in a dissection spot glass were filled with odorless 1% agarose to serve as a suitable egg-laying substrate. Geosmin was mixed into the

agarose in one of the three wells. Egg-laying behaviors were conducted in a completely dark, humidified incubator for 23 hr. An oviposition index (OI) was calculated as the number of eggs laid in the odor well minus the average number of eggs laid in the other two wells, divided by the total number of eggs; an oviposition index = 1 indicates all eggs were laid in the odor well, whereas an oviposition index < 0 indicates flies avoided laying eggs in the odor well. In oviposition assays that used food as a background substrate, geosmin directed egg-laying avoidance (Stensmyr et al., 2012). We found that when agarose was used as a background substrate, wildtype flies slightly avoided laying eggs in a geosmin well, but this was not significantly different from *Or56a*$^{-/-}$mutant flies (Figure 3). We further tested oviposition responses of additional olfactory mutant flies: (1) an *Orco* mutant, which disrupts all Or-type signaling, and (2) flies mutant for ionotropic receptor co-receptors (*Ir8a* and *Ir25a*), *Orco*, and *Gr63a,* which disrupts almost all olfactory signaling. All olfactory mutant genotypes exhibited neutral oviposition indices to geosmin (Figure 3). Changing the position of the geosmin odorant well did not change oviposition scores (Figure 3C). Altogether, these data suggest there is no difference in the oviposition behavior of wild-type flies, *Or56a*$^{-/-}$mutants, and nearly anosmic flies when these flies are presented with the choice of ovipositing on agarose versus geosmin-laced agarose. As such, the odorant geosmin and the *Or56a*$^{-/-}$mutant background can be used with this oviposition assay to identify neurons directing oviposition decisions.

Single OSN Types Mediate Negative Oviposition Decisions

We systematically performed an olfactogenetic screen using 23 *OrX-Gal4* lines to identify types of olfactory neurons that, when activated, contributed to oviposition choices in females. These *OrX-GAL4* lines were chosen because they drive expression in OSNs with target glomeruli for which partnering projection neuron (PN) morphology is known. We reasoned this might help elucidate downstream olfactory circuit signaling. The results of the olfactogenetic screen are shown in Figure 4. A range of oviposition indices was observed among the different *OrX-GAL4* lines used. Our data indicate that activating just a single OSN type is, indeed, sufficient to elicit a reproducible oviposition behavior. While we verified olfactogenetic activities in many olfactory neurons (Figure 2) and used only previously validated *OrX-Gal4* lines (Lin and Potter, 2015), it remains possible that neutral behavioral responses might reflect olfactory neurons that were not activated using this approach. Nonetheless, it is notable that all statistically significant *OrX-GAL4* responses from the screen demonstrated negative oviposition decisions (Figure 4A). Many of the OrX-expressing olfactory neurons (e.g., Or71a, Or49a, and Or7a) have been previously shown to be involved in oviposition decisions (Table S1). To determine if the negative oviposition results reflected egg-laying site-selection decisions versus negative chemotaxis (laying fewer eggs in a well as a result of avoiding that location), we tracked and plotted the location of Or71a, Or47b, Or49a, and Or7a olfactogenetic flies during the 23-hr egg-laying assay (Figure S4). In general, flies explored the entire arena, showing slight preferences to edges and to regions between agarose well. Flies did not avoid the center geosmin well, suggesting behavioral results likely reflected oviposition decisions versus chemotaxis.

To determine if the olfactogenetic approach might recapitulate the behavioral responses of native odorant-to-Or responses, we examined the responses of three odorants (4-

ethylguiacol, 9-tricosene, methyl laurate) that are fairly specifically tuned toward their respective odorant receptors (Or71a, Or7a, and Or47b) (Dweck et al., 2015a, 2015b; Lin et al., 2015) (Figure 4B). The behaviors mediated by the olfactogenetic approach mimicked the oviposition odorant-induced responses toward 4-ethyl guiacol and 9-tricosene but not toward methyl laurate. The lack of recapitulation in the methyl laurate case may be due to the activation of other chemosensory neurons that simultaneously respond to methyl laurate (Dweck et al., 2015b; Lin et al., 2016) that could possibly modify oviposition behavioral decisions. Altogether, this highlights how an olfactogenetic approach can be used to investigate which behaviors are solely mediated by the targeted olfactory neurons.

A Region of the Lateral Horn May Mediate Negative Oviposition Decisions

The olfactogenetic screen took an unbiased approach toward identifying *OrX-Gal4* lines that could direct oviposition decisions. We next examined the projection patterns for PNs predicted to be the primary signaling partners for each of the identified OSNs (Figure 5A). Interestingly, the PNs predicted to guide negative oviposition decisions were found to be significantly more similar to one another than by chance based on their morphology in the lateral horn (Figures 5B and S5). Indeed, the axonal arbors of each negative oviposition PN inhabited an anterior-central region of the lateral horn (Figure 5B). Closer examination of these PN traces indicated that for VC2 and DL5, only the medial branches, rather than whole axonal pattern, shared morphological similarity to the other negative oviposition neurons. This implies that lateral horn regions underlying biologically relevant information may also be formed by the convergence of different PN axonal segments.

DISCUSSION

Chemosensation is considered one of the most primal senses, as all living organisms (from bacteria to humans) use chemical information to interact with their environments. The olfactory system has the ability to detect volatile odorants that drive integral survival behaviors such as finding nutritious food, identifying an attractive mate, avoiding ingestion of disease-causing microbes and toxins, and influencing oviposition, courtship, aggregation, flight, and aggressive behaviors (Vosshall, 2007; Dweck et al., 2013; Wasserman et al., 2013; Lone et al., 2015). The chemical world contains a large, diverse number of compounds. How does the brain make sense of it all?

A Genetic Olfactogenetic Approach for the Dissection of Olfactory Behaviors

The contribution of olfactory neuron types in guiding olfactory signaling has been technically challenging to investigate. In contrast to other sensory systems, such as audition or vision, which benefit from the ability to precisely control the experimental sensory input, the olfactory system is not as amenable to such systematic experimental investigations. This is because most olfactory neurons do not respond to a single odorant, and most odorants activate many olfactory neuron types, making it difficult to link which olfactory neuron activities in response to an odor are actually the main drivers for the resulting olfaction-guided behavior. To overcome this hurdle, we took advantage of an unusually specific odorant-odorant receptor pair (geosmin/Or56a) and developed an olfactogenetic approach that uses a natural odorant stimulus to activate an experimentally defined olfactory neuron

population. Using geosmin to naturalistically activate discrete OSN types preserves the dynamic features and structures of ethological olfactory stimuli such as plumes and gradients. This eliminates confounding factors in the interpretation of behavior often encountered by other experimental methods aimed at neuronal activation, such as optogenetics (which can lead to phototaxis) and thermogenetics (which can lead to thermotaxis). Being able to use the same odorant to compare results among behavioral assays also helps to eliminate effects of varying volatility between different odorants, and the system allows for the study of receptor neurons whose receptors have no known activating ligands.

In initial studies of OSNs and their firing rates, relatively high concentrations of odorants (1%) were used to elicit olfactory neuron firing. For example, in the first instances where OSNs were systematically screened to a panel of odorants (Hallem and Carlson, 2006; Schlief and Wilson, 2007), responses greater than 50 spikes/s were categorized as “hits,” and responses of ~150–200 spikes/s were considered as reflecting “real” odorant-to-Or matches. A recent study (Bell and Wilson, 2016) used an elaborate and sophisticated optogenetic setup to tightly control stimulus intensity toward individual moving flies. The study showed that high levels of OSN activity may not be required for generating behavior. In some cases, lower induced activity of the olfactory neuron of ~40–50 spikes/s generated stronger behavior. This supports the efficient coding hypothesis (Barlow, 1961) that postulates the level of a stimulus should match the level of neuronal firing in natural environments where an animal has evolved to survive, optimizing the neuron’s metabolic consumption and dynamic range. While, to our knowledge, extensive studies have not been conducted to quantify concentrations of natural odorants, natural odorants rarely come in the extremely high concentrations used in laboratory studies. Therefore, more likely than not, sparse coding is used in sensory systems, and weak activation of sensory neurons are significant to the animal’s perception of its environment. The Or56a-geosmin olfactogenetic approach leads to reproducible (20–60 spikes/s) increases in olfactory neuron signaling, thus likely reflecting activation of an olfactory neuron to ethologically relevant odorant concentrations. Interestingly, it was difficult to over-activate an olfactory neuron using this approach: maximal olfactory neuron responses plateaued at ~60 spikes/s even when geosmin levels were increased to 10^{-4} or more. This was an unexpected, yet advantageous, aspect of the olfactogenetics approach as experimental over-activation of an olfactory neuron by other genetic methods often leads to inhibition (Lin and Potter, 2015), which could confuse behavioral interpretations.

Oviposition Decisions Are Complex Sensory Choices

Drosophila lay eggs on their food substrate (rotting fruit), so chemosensation plays a large role in oviposition choice, since smell and taste provide essential information about the composition of a food source such as nutritional content and toxicity. Thus far, the primary studies on chemosensation in oviposition have involved Grs, which are found in many body regions that come in contact with food sites, such as the labellum, legs, and ovipositors. While flies are generally attracted to calorie-rich sugar substrates and avoid substrates that contain bitter compounds, oviposition sites can change based on the context of the decision. Laying eggs on a bitter substrate may confer survival benefits in the form of deterring

parasitic predators or protecting eggs from fungal or microbial infections. However, on larger and/or physically distant patches, larval foraging costs would be high, necessitating large energy expenditure in order to reach a sugar patch to eat. Therefore, under these conditions, it is more advantageous for the female fly to directly lay eggs on sweet, nutrient-rich substrates (Schwartz et al., 2012).

Olfaction also plays an important role in oviposition choices. However, it can often be difficult to distinguish whether a chemical functions solely as an odorant or also as a tastant. For example, a bitter volatile chemical could potentially be smelled by the olfactory system as well as tasted by the gustatory system. This seemingly semantic distinction is important to make because it appears that receptors that detect the same chemical on different body regions can mediate opposing behaviors. This is thought to be true in the case of bitter compounds eliciting different behavioral valences in oviposition. Gr66a, a bitter receptor that detects a compound commonly used in egg laying assays called lobeline, causes aversion when activated on the legs but egg-laying attraction when activated in the labellum (Joseph and Heberlein, 2012). A similar phenomenon has been observed with olfactory versus gustatory responses to acetic acid (Joseph et al., 2009). The integration of these two sensory modalities along with elements of the egg-laying environment such as patchiness of food resources illustrates that oviposition choice is a complex decision making task.

Oviposition Decisions Based on Single Olfactory Neuron Activities

Five olfactory receptors have been specifically associated with oviposition. Or19a and Or49a are implicated in avoidance of larval parasitization by wasps (Dweck et al., 2013; Ebrahim et al., 2015). Or19a mediates positive oviposition and responds to citrus volatiles repellent to wasps, and Or49a detects parasitoid wasp semiochemicals, which female flies should avoid during oviposition. Or56a and Or71a have been implicated in avoiding the negative effects of infection by microorganisms (Stensmyr et al., 2012; Dweck et al., 2015a). Or56a detects geosmin, which is emitted by harmful microorganisms (Gerber and Lechevalier, 1965; Mattheis and Roberts, 1992; Stensmyr et al., 2012), and Or71a promotes attractive oviposition because it is thought to detect antioxidants in food that can attenuate oxidative stress resulting from exposure to toxins (Vertuani et al., 2004; JimenezDel-Rio et al., 2010). Finally, Or7a has been shown to detect the social pheromone 9-tricosene and mediates geographical tagging of food sites by males used to attract females (Lin et al., 2015). 9-Tricosene has also been shown to positively stimulate oviposition through Or7a (Lin et al., 2015).

In order to systematically screen for and identify more olfactory inputs involved in female oviposition, we used the olfactogenetic approach to test 23 olfactory receptor Gal4 (*OrX-GAL4*) lines (Supplemental Experimental Procedures). The top five statistically significant hits ($p < 0.001$) correspond to neurons expressing Or71a, Or47b, Or49a, Or67b, and Or7a. The major commonality among these receptors is that they detect social chemical cues either from the same species or as hallmarks of other insects. Or47b and Or7a have both been shown to specifically respond to pheromones that male and female flies can use to influence individuals of the opposite sex, and Or49a is activated by chemicals that parasitic wasps deposit on substrates that they have visited (Dweck et al., 2015b; Ebrahim et al., 2015; Lin

et al., 2015). In the context of egg laying, the olfactogenetic results could indicate that cues such as density of both conspecifics and interspecifics and the presence or absence of parasites that infect larvae are most relevant to a female fly's oviposition decisions.

Interestingly, we only saw statistically significant negative oviposition behavior. Stimulating Or92a OSNs, neurons that contribute to attraction to apple cider vinegar (Semmelhack and Wang, 2009), is the only behavioral result that yielded a positive average oviposition index, but this result was not statistically significant. While Or71a, Or19a, and Or7a have been behaviorally shown to detect positive oviposition cues, our OSN activation screen produced no attractive oviposition when we olfactogenetically stimulated these classes of OSNs. This can be explained in several ways. First, the parameters of behavior assays, especially oviposition, can influence behavioral results. The assays used to identify Or71a, Or19a, Or49a, and Or56a as mediators of oviposition behavior were performed under conditions where odorants were presented with fly food. The chemical components of a naturalistic odor such as fly food can interact with each other in unpredictable and complicated ways. Insect studies show that background odor can indeed change behavior and physiology of olfactory neurons (Montague et al., 2011; Riffell, 2012; Su et al., 2012). We hypothesize that since our oviposition assay is agarose based rather than food based, we are likely minimizing the olfactory background and experimentally only getting low-level activation of single classes of OSNs that project to a single glomerulus. As such, the olfactogenetic screen may lead to the identification of those olfactory neuron classes that are sufficient to drive behaviors on their own. As an extension of this, an olfactory response that needed a specific combination of olfactory neuron activities would not be picked up in the screen. For oviposition, single olfactory neuron classes only had "negative" valences. This suggests that the major contributions for single olfactory neuron classes regarding oviposition may be toward avoidance, and it is possible that attraction requires the activation of several olfactory inputs and glomeruli rather than a single glomerulus.

Identification of a Negative Oviposition Region in the Lateral Horn

Our results support previous findings that the lateral horn functions as a categorizer of salient olfactory information. Previous studies defined lateral horn domains based on the entire axonal morphology of the PNs (Jefferis et al., 2007). Our analysis of PNs involved in negative oviposition suggests that information may be organized based on axonal subsegments. For each PN predicted to guide negative oviposition behaviors, the PN axons shared a dorsal posterior segment. The non-oviposition anterior branch of the U-shaped PN neuronal target regions may confer an as-yet-unknown, yet-shared biological significance in the lateral horn as they localize together.

Using Olfactogenetics to Investigate Odor Coding

There are many hypotheses about how the brain processes incoming olfactory information. The "labeled line" hypothesis supported by studies identifying dedicated Ors reacting to highly specific odorants operates under the assumption that highly biologically relevant stimuli are encoded as labeled lines of information. It is postulated that most receptors will have a "most relevant" ligand yet to be identified (Andersson et al., 2015). This extreme seems unlikely. The olfactory world of an animal like the vinegar fly contains more

important biologically relevant stimuli that it needs to respond to than olfactory neuron types. As evidenced by contradicting behaviors seen in different assays (Suh et al., 2004; Wasserman et al., 2013), even olfactory circuits previously thought to be labeled lines for attraction or repulsion do not absolutely produce the same behavior in all contexts. Alternatively, the “combinatorial code” hypothesis stipulates that odorant information is processed and acted upon by the combinatorial activity of many olfactory neurons (Malnic et al., 1999). The antennal lobe acts to linearly summate all inputs from activated and inhibited glomeruli and/or use coincidence detection of simultaneously activated OSNs to determine odor identity and direct behavioral responses (Wyatt, 2014; Badel et al., 2016; Bell and Wilson, 2016).

The olfactogenetics approach allows rigorous experimental testing of the labeled line hypothesis by enabling each olfactory neuron type to be activated and assayed for behaviors directed by activity of only that olfactory neuron. This strategy might help to distinguish behavioral situations guided by labeled lines (like negative oviposition) from those that require combinatorial signaling to drive behaviors. It is also possible that labeled lines exist primarily as modulators of combinatorial signaling. Combinatorial signaling may implicate the behavioral context of each odorant, with labeled lines modulating the overall response to each particular situation. Further study using the olfactogenetics approach could identify even more olfactory neuron types involved with imparting important olfactory information to strongly modulated olfactory circuits or influencing the activity of other OSNs in complex odor environments.

An optogenetics approach aimed at identifying olfactory neurons that guide attraction or repulsion supports this hypothesis. Experiments by Bell and Wilson (2016) involved low-level optogenetic activation of OSNs in a two-choice walking assay. The authors were able to obtain attractive and repulsive motor behavior upon stimulating eight OSN classes previously identified as attractive or repellent. The authors further examined the effects of stimulating two classes of OSNs on attractive or repulsive behaviors. These pairwise studies revealed that activation of certain OSNs resulted in behavioral output that summed linearly (were more attractive), but others did not. Pairing attractive OSNs with repellent OSNs did decrease attraction. Together, these results suggest that different OSNs can contribute different “weights” toward an output behavior, which is also consistent with olfactory coding strategies identified in *Drosophila* larvae (Kreher et al., 2008). The negative oviposition OSNs we identified in our study most likely add negative weight to an olfactory-guided oviposition choice (Badel et al., 2016). Furthermore, it has been shown that certain glomeruli have greater influence over others (Fişek and Wilson, 2014). This observation gives rise to the possibility that a class of OSNs (in this case, one mediating aversion) could act as a master switch and carry much more weight in the summation of antennal lobe inputs, giving that glomerulus the ability to “veto” other inputs. It is unclear if this would be the case for the negative oviposition OSNs, but it is possible that OSNs that are sufficient to drive a specific behavior alone may carry more weight.

The olfactogenetic method can be used to study an array of behaviors amenable to odor presentation, although olfactory conditions will need to be optimized to produce robust behaviors. Ectopic expression of Or56a and activation by geosmin could be used to singly

interrogate OSNs in many more olfactory contexts and allow for the widespread identification of putative receptors involved in behaviors including courtship, aggregation, or aggression. Furthermore, olfactogenetics may be a powerful tool used to interrogate how combinatorial OSN activities summate to produce any of these complex behaviors. With the stereotypic mapping of second-order neurons, identifying primary inputs could lead to conclusions about higher-order processing in olfactory cortex that ultimately elucidate how the brain evaluates and uses olfactory information to help animals survive.

EXPERIMENTAL PROCEDURES

Fly Stocks

Wild-type flies were *IsoD1 (w^{1118})*, and all lines used in behavioral experiments, including the two *Or56a* knockout lines, were backcrossed for five generations to wild-type. All *OrX-Gal4* lines were crossed into the outcrossed *$Or56a^{-/-}$* knockout background. *Gal4* lines used for this study are listed in Table 1 in Lin and Potter (2015). Flies used for *Orco* mutant experiments contained two different alleles as reported previously (Larsson et al., 2004).

Generation of the UAS-Or56a Fly Line

The *Or56a* coding region was PCR amplified from *IsoD1 (w^{1118})* genomic DNA using primers with 15 bp extensions appropriate for InFusion cloning (5′-GAAT AGGGAATTGGGAATTCATGTTTAAAGTTAAGGATCTGTTGC-3′ and 5′-ATCT GTTAACGAATTCCTAATACAAGTGGGAGCTACG-3′). InFusion cloning (Clontech Laboratories) was used to subclone *Or56a* into the EcoRI cut site in the multiple cloning region of *pUAST* (Brand and Perrimon, 1993). This vector was then injected into embryos for P-element insertion.

Generation of the Or56a Knockout through Accelerated Homologous Recombination

A deletion mutant was generated using accelerated gene targeting as reported previously (Baena-Lopez et al., 2013). Briefly, 4,559 bp of genomic sequence immediately upstream and 3,021 bp immediately downstream of *Or56a* were PCR amplified using primers designed for InFusion cloning to create a 5′ and 3′ homology arm, respectively, as follows: Or56a_5′homarm_REV 5′-AGTTGGGGCACTACGGTTAAACTGTTTAGCGTTAACCATATTC-3′, Or56a_5′homarm_FOR2 5′-CTAGCACATATGCAGCTCACAGCGCTTGTCGTA AT-3′; Or56a_3′homarm_FOR 5′-ACGAAGTTATCAAGGGAAAGCCTTTTCTTC AGG-3′, Or56a_3′homarm_REV2 5′-GATCTTTACTAGTTTTCCGCTTCTGCTC TACG-3′, where bolded nucleotides represent genomic sequence and non-bolded sequences indicate vector nucleotides. Sequentially, the 3′ homology arm was InFusion cloned into the *SpeI* restriction site of multi-cloning site (MCS) B in *pTV^{Cherry}* (vector from lab of J.P. Vincent), and the 5′ homology was cloned into the *NheI* site of the MCS A. This knockout construct was used to generate a “donor” line that was crossed to *hs-Flp, hs-SceI* (BS#25679). Flies were heat shocked at 37 C, 48 and 72 hr after egg-laying for 1 hr each. Female progeny of the heat-shocked flies were then screened for mottled eyes and crossed with *ubi-Gal4[pax-GFP]* to select against off-target recombination events. The *Or56aKI* was validated with

primers *GAL4-FOR* (5′-TCGATACCGTCGACTAAAGCC-3′) and *Or56a-TEST-REV2* (5′- AAAATCGAGGGGCTAAACAGTGTC-3′), as shown in Figure S1.

Chemicals

Geosmin in methanol was purchased from Sigma-Aldrich at highest available purity (≥97%; product G5908–1ML, 2 mg/mL; lot BCBP7178V). Chemical as received was dried to remove methanol and then diluted to 4 mg/mL in mineral oil (0.4% w/v geosmin; Sigma-Aldrich, product 330779–1L, lot MKBF6530V). 1% (4×10^{-5} w/v geosmin, simplified to 10^{-5} in text and figures) or 10% (4×10^{-4} w/v geosmin, simplified to 10^{-4} in text and figures) of the 4 mg/mL geosmin solution was used for SSR (see below). Methyl laurate (product 234591–2.5G, lot BCBQ6830V), (Z)-9-tricosene (product 859885–1G, lot 04706LDV), and farnesol (product F203–25G, lot MKBG0101V) were purchased from Sigma-Aldrich.

SSR

All sensilla were identified using fluorescence from either *10X-UAS-IVS-mCD8-GFP* (II) or *15X-UAS-IVS-mCD8-GFP* (III) recombined onto *OrX-Gal4* lines. These recombined lines were crossed to *UAS-Or56a* to test the efficacy of misexpressing Or56a in non-ab4 neurons. Single sensillum recordings from the *OrX-Gal4*'s cognate sensillum were obtained using methods and lines described previously (Lin and Potter, 2015).

Oviposition Assay

Equal numbers of female and male adult flies were collected within 24 hr of eclosion and group housed in fly food vials for 3 days. On day 4, all flies were transferred to a vial with only wet yeast paste to prime females for egg laying. 50 mL of 1% agarose in deionized distilled water was allowed to cool to precisely 65 °C. 1 μL odorant (for geosmin, 4×10^{-5} w/v geosmin in mineral oil) or vehicle was pipetted into the 50 mL of 1% agarose. This solution was dispensed into each well of a three-well spot plate (Corning, product 722334 [discontinued], 20 drops per well; Replica three-well spot plate printed in porcelain with matte black finish through Shapeways, 14 drops per well) using a pipet aid with a 10-mL serological pipette (Danville Scientific, part P7134). Flies were anesthetized on ice for 3–5 min, and males were removed. ~10 female flies were tapped onto each spot plate, and the lid of a 100 × 20 mm tissue culture dish (Corning, product 353003) was placed on top to cover the top of the assay. The lip on the spot plate allows room for the flies to walk on and between the three wells. All experiments were begun between 17:00 and 19:00, flies were incubated on the assay in a dark, humidified incubator at 25 °C and 89%–94% humidity for 22–23 hr, and the number of eggs on the agarose of each well was counted. Counts were normalized to the number of flies loaded into each assay (number of eggs in a well/number of flies). We discarded experiments in which flies laid fewer than 8 eggs/fly per day.

The oviposition index was calculated as follows:

$$\mathrm{OI} = \left(\mathrm{O} - \mathrm{NO}^{\mathrm{avg}}\right) / \left(\mathrm{O} + \mathrm{NO}^{\mathrm{avg}}\right),$$

where O is the number of eggs in well containing odorant and NO^{avg} is the average number of flies between two no-odorant vehicle control wells.

Statistics

Normality was determined using the Bartlett's test of homogeneity of variances ($p < 0.01$). Given that the data meet requirements for running parametric tests, an ANOVA shows that at least two of the means from the experimental groups are different from one another ($p = 2.76e\text{-}13$). The post hoc Dunnett's many-to-one multiple comparisons test, with each experimental group compared to the *Or56a* knockout control, indicates that 9 out of the 23 tested *OrX-Gal4* lines statistically significantly induce aversive behavior in our oviposition assay. These tests were all run using the native and "multcomp" statistics packages in R.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Olfactogenetics allows genetically defined neurons to be activated by an odorant
- Ectopic expression of Or56a in olfactory neurons confers sensitivity to geosmin
- Odor-guided oviposition screen identifies olfactory neuron types directing avoidance
- The lateral horn might contain a domain underlying oviposition avoidance decisions

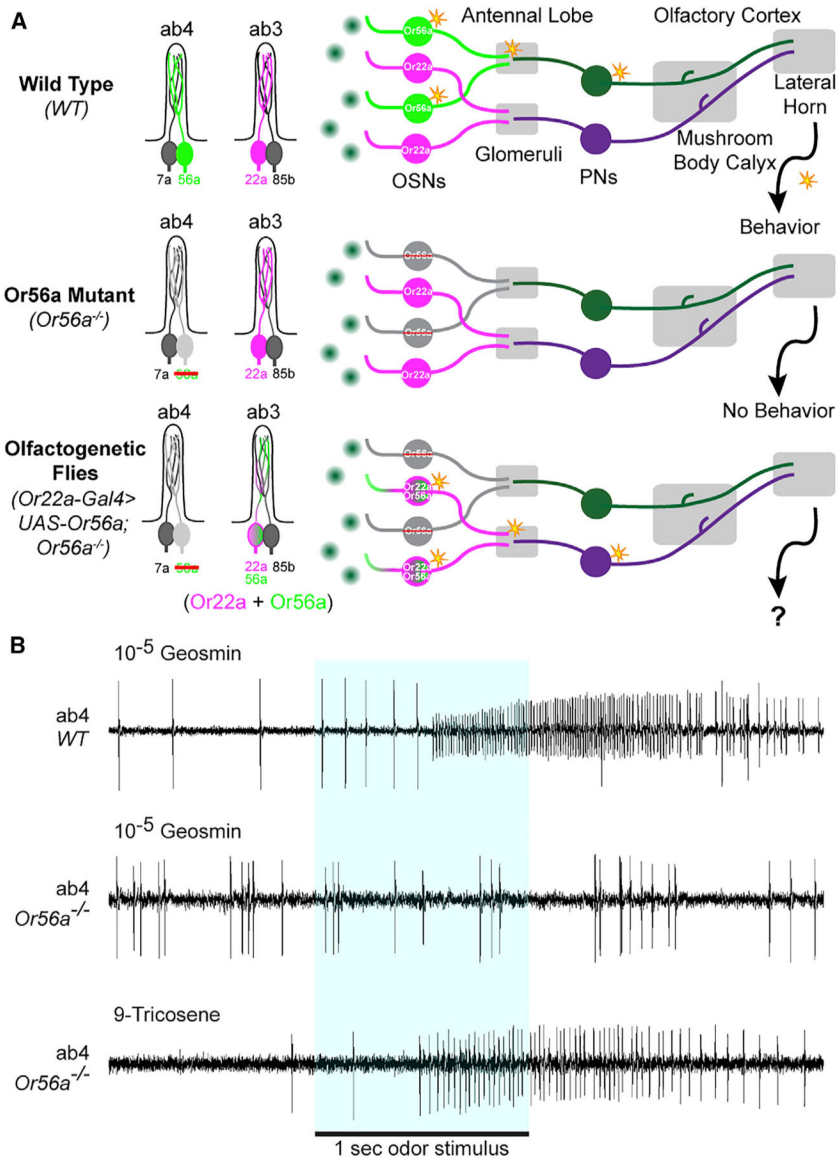

Figure 1. Or56a-Geosmin Olfactogenetic Method for Investigating Odor-Guided Behaviors

(A) Schematic of the olfactogenetic approach. In wild-type (WT) conditions, the ab4 sensillum contains 2 OSNs: the "A" neuron expresses Or7a (gray), and the "B" neuron expresses Or56a (green). The ab3 sensillum also contains 2 OSNs: the ab3A neuron expresses Or22a (magenta), and the ab3B neuron expresses Or85b (gray). Geosmin (dark green circles) activates (orange star) only Or56a-positive neurons and not other neurons. Mutating the *Or56a* receptor results in an olfactory system that does not detect nor behaviorally respond to geosmin. To create olfactogenetic flies, we use the GAL4/*UAS* system to ectopically express *UAS-Or56a* in a specific olfactory neuron (e.g., *Or22a-GAL4,* magenta plus green with magenta outline) in an *Or56a*$^{-/-}$mutant background. This allows the odorant geosmin to activate olfactory neurons with high specificity to drive olfactory behaviors.

(B) Single sensillum recordings (SSR) of ab4 sensilla. The B neuron in an *Or56a*$^{-/-}$mutant no longer responds to geosmin. The A neuron in an *Or56a*$^{-/-}$mutant responds normally to 9-tricosene. ab, antennal basiconic; PNs, projection neurons; OSNs, olfactory sensory neurons. See Figure S1 for details on generation of the *Or56*$^{-/-}$mutant.

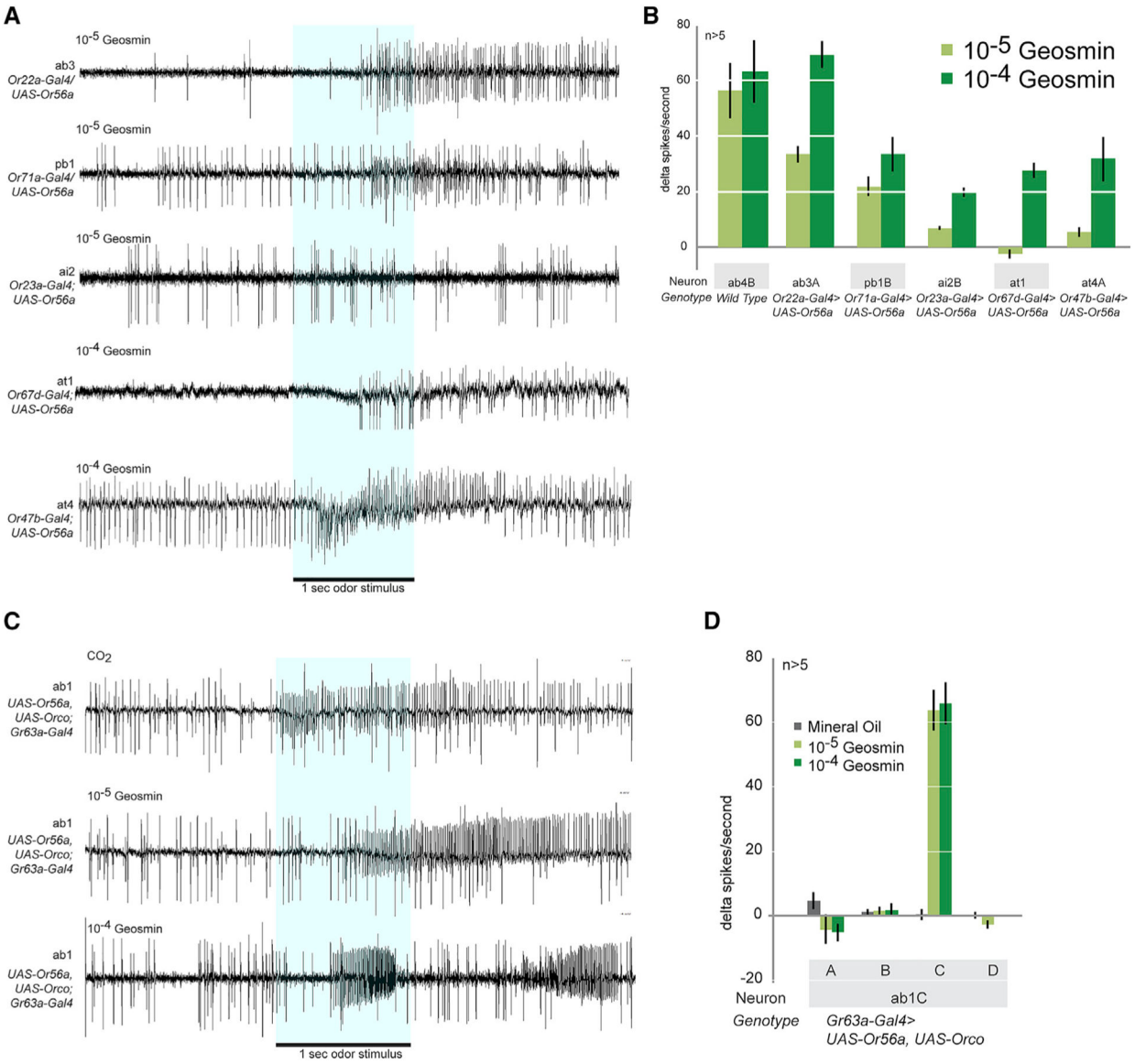

Figure 2. Ectopic Expression of Or56a in Olfactory Neurons Confers a Response to Geosmin

(A) Representative single sensillum recordings of basiconic, intermediate, and trichoid sensilla containing neurons expressing Or56a. The blue bar highlights the 1 s odor pulse.

(B) Quantification of recordings.

(C) Representative SSR of ab1 sensilla that contain 4 olfactory neurons. The “C” neuron expresses Gr21a/Gr63a and is sensitive to CO_2.

(D) Quantification of recordings.

Error bars represent SEM. See also Figure S2.

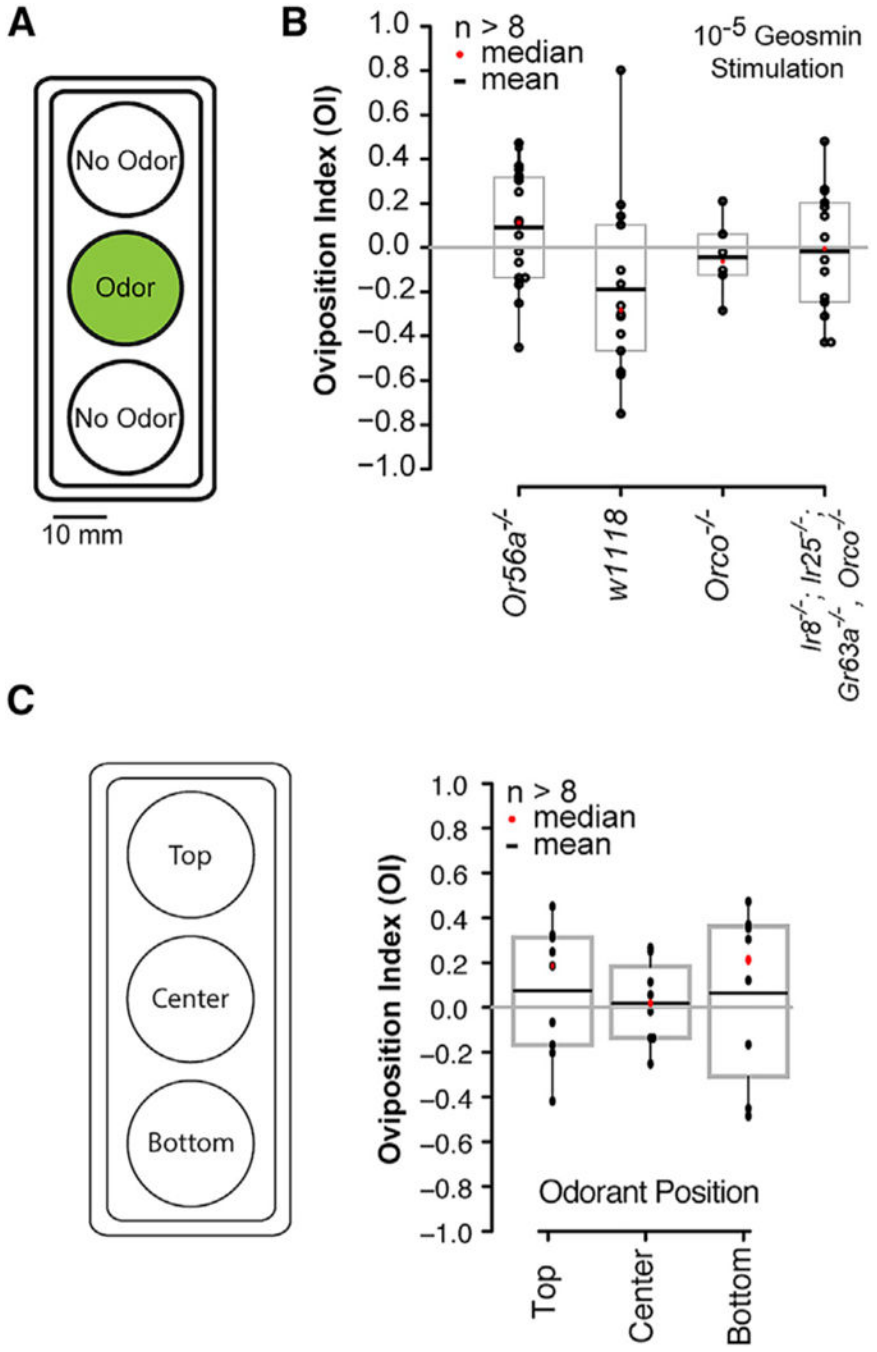

Figure 3. Behavioral Effects in the Two-Choice Oviposition Assay Using Geosmin Rely on Olfaction

(A) Schematic of oviposition assay.

(B) Olfactory mutant animals do not exhibit behavioral responses to geosmin. Control w^{1118} animals are slightly repelled by geosmin. The oviposition index is calculated as (the number of eggs laid in odor well the average number eggs laid in the no-odor wells)/the total number of eggs. The different genotypes are not statistically significant as determined by a Dunnett's many-to-one comparisons test.

(C) Positional controls for two-choice oviposition assay. The position of the odorant well in the three-well assay does not affect behavior. Differences are not statistically significant as determined by a Dunnett's many-to-one comparisons test.

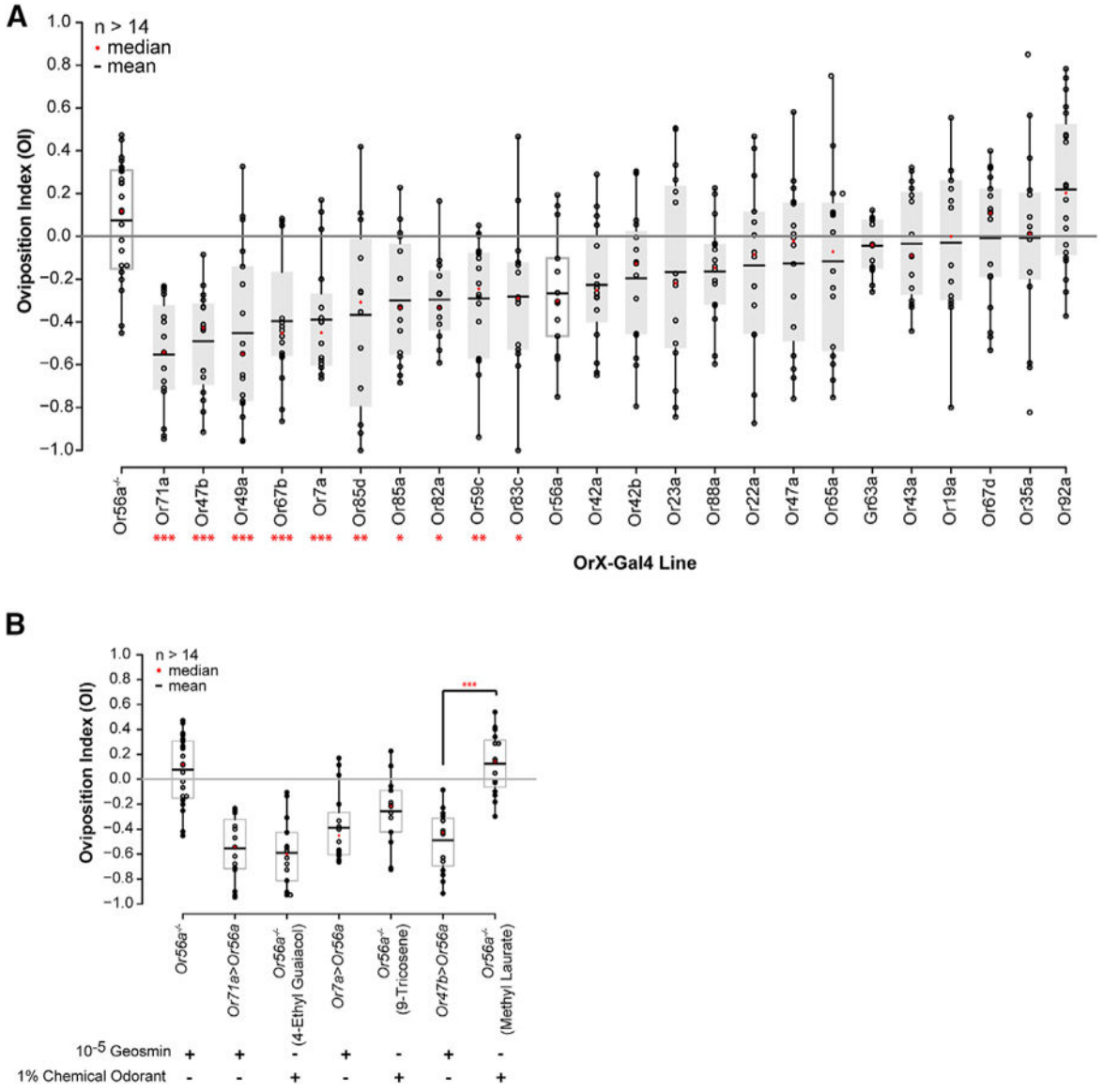

Figure 4. Olfactogenetic Activation of Specific OSN Types Mediates Negative Oviposition

(A) Oviposition assays using geosmin. *OrX-Gal4* lines were combined with *UAS-Or56a* in the *Or56a*$^{-/-}$mutant background (gray bars). *Gr63a-GAL4* combined with *UAS-Or56a and UAS-Orco* in the *Or56a*$^{-/-}$background (gray bar). Mutant *Or56*$^{-/-}$and wild-type (*Or56a*) responses denoted by white bars.

(B) Oviposition assays comparing results obtained from an olfactogenetic approach to those ob-tained using the indicated odorants. Statistics are a Dunnett's many-to-one comparisons test compared to *Or56a*$^{-/-}$. Asterisks indicate p values: *p < 0.1, **p < 0.05, and ***p < 0.001.

See also Figure S4 and Table S1.

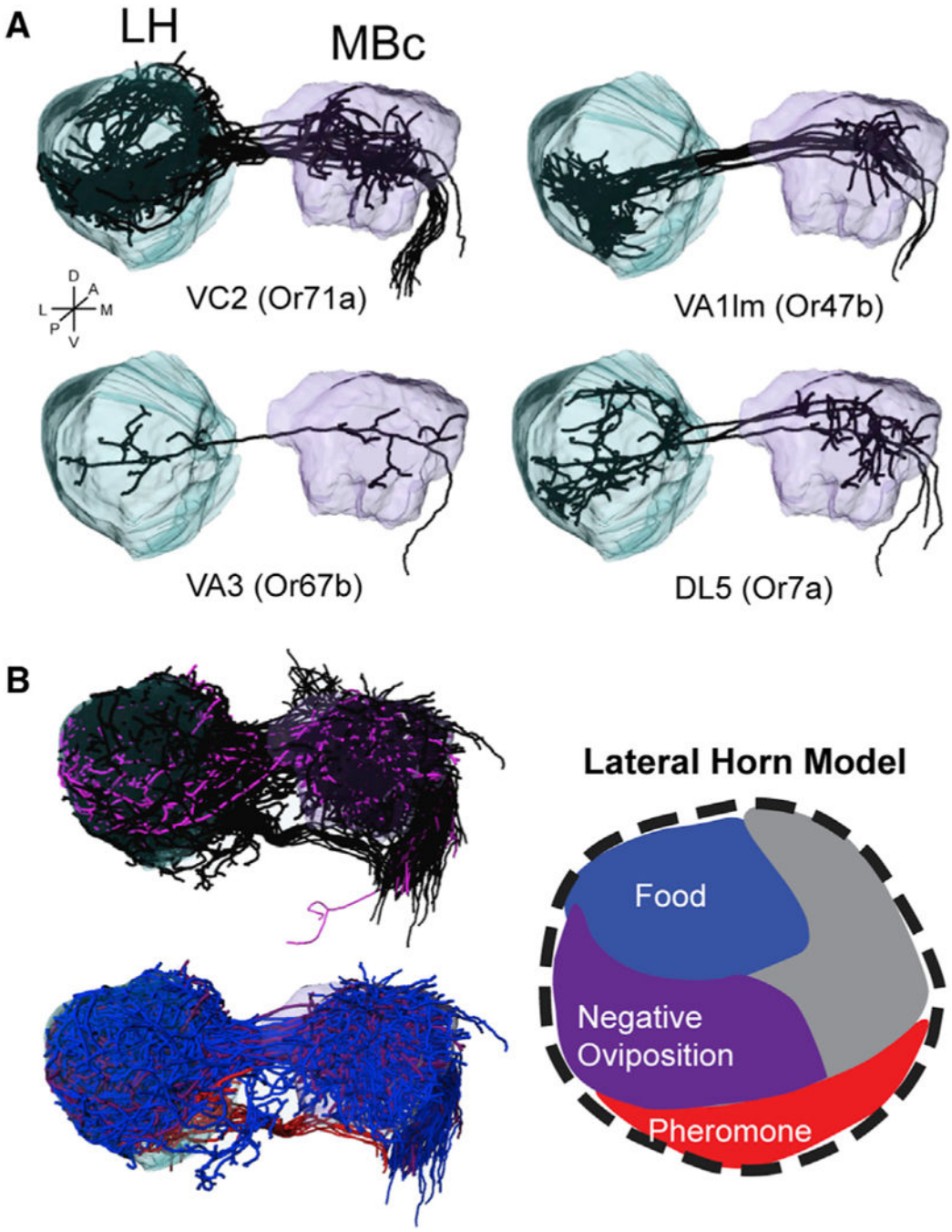

Figure 5. Representation of Negative Oviposition Olfactory Cues in the Lateral Horn

(A) PN traces corresponding to the listed OSN type for the most statistically significant responses in the olfactogenetic oviposition assay ($p < 0.001$). LH, lateral horn; MBc, mushroom body calyx; A, anterior; P, posterior; D, dorsal; V, ventral; L, lateral; M, medial. (B) Comparison of negative oviposition PNs (purple) to all other PN types (black in top trace, blue and red in categorized bottom).

See also Figure S5.