OLFACTORY PROCESSING IN OVIPOSITION BEHAVIOR OF *DROSOPHILA MELANOGASTER*

by

Sonia Grace Chin

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ABSTRACT

How does the brain make sense of complex mixtures of smells? An animal's olfactory system is capable of detecting and distinguishing hundreds (perhaps thousands) of chemicals, and translating those inputs into useful behaviors. We study olfaction in the vinegar fly *Drosophila melanogaster*, an animal that uses its sense of smell in nearly all aspects of its life.

The study of olfactory perception at its primary level of sensation is complicated by the possibility that monomolecular odorants typically activate many different olfactory receptors. This lack of a simple odorant-OSN match has made deciphering olfactory coding challenging. We have developed a novel chemogenetic approach to specifically activate genetically defined subsets of OSNs using a highly tuned odorant-receptor pair (geosmin and *Or56a*). Using this tool, we systematically tested how the individual activations of 23 of the fly's 62 OSN types each contribute to egg laying, an ethologically important behavior for flies. We found that in six of the 23 cases, flies perceived OSN activation as a negative oviposition cue.

Previous studies have demonstrated that the lateral horn, a higher order olfactory brain region innervated by secondary projection neurons (PNs), seems to sort information into two categories: food and pheromone. Examining the morphology of the PNs that synaptically partner with each class of negative oviposition OSNs revealed a novel finding that female fly brains use segments of, rather than whole, PNs to encode a negative oviposition domain in the lateral horn.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Chapter 1: Introduction

Chapter 2: Chemogenetics

Chapter 3: Behavior

Chapter 4: Anatomical Tracing

LIST OF ABBRIEVIATIONS

GFP	Green Fluorescent Protein
Gr	Gustatory Receptor
GRN	Gustatory Neuron Receptor
Ir	Ionotropic Receptor
LH	Lateral Horn
LN	Local Interneuron
MBC	Mushroom Body Calyx
OI	Oviposition Index
Or	Olfactory Receptor
OSN	Olfactory Sensory Neuron
PN	Projection Neuron
SSR	Single Sensillum Recording

1. Introduction

1.1 General Olfaction

The detection of environmental volatile chemicals contributes to crucial survival behaviors in organisms ranging from bacteria to humans [1-3] making olfaction one of the most primal of the senses. An animal's olfactory system must filter through a complex, smelly world in order to obtain ethologically relevant odor information [4]. Sensory receptors are the proteins that directly detect environmental stimuli, and the cloning and identification of olfactory receptors in many model organisms has driven a shift from mostly descriptive studies to studies that aim to form a mechanistic understanding of how this important sensory system handles such a complex set of stimuli [5, 6]. The front end of the olfactory system relating to the primary, direct sensing of odorants has been well studied. For instance, many olfactory receptors (Ors) in *Drosophila* have been odor-matched, the olfactory sensory neurons (OSNs) that house them characterized, and the first synapses identified in olfactory circuits [7]. However, how the olfactory system processes information and generates appropriate behavioral response remains to be illuminated.

1.2 Olfaction in *Drosophila melanogaster*

As its name suggests, the vinegar fly, *Drosophila melanogaster*, is a highly olfaction-driven organism that uses its sense of smell to do everything from locating food, navigating space, mating with the correct species, and laying eggs [8-11]. All these olfactory behaviors occur on highly odiferous rotting fruits, which serve as a food source

and oviposition substrate.

Drosophila melanogaster has ~1200 OSNs that express a complement of ~50 Ors, with most OSNs expressing a single Or [12]. The antennae and maxillary palps, the fly's olfactory organs, are covered in sensory hairs called sensilla. These sensilla house the dendrites from between one to four different types of OSNs [7, 13], and strong activation of one OSN in a sensillum can lead to silencing of its neighbors in a phenomenon called ephaptic coupling [14]. In order to function, Ors must complex with the Or-coreceptor Orco to form a transmembrane ion channel complex [15]. This is very different from the mammalian Ors, which are G-protein coupled receptors [16]. However, some studies have shown that olfactory signaling in the fly may also require second messengers, complicating our understanding of Or activation intracellular signaling mechanisms [17]. OSNs in the antenna and maxillary palp mostly express Ors, with one unusual OSN in an antennal sensillum that expresses two Grs (Gr21/Gr63a) [13].

The most recently discovered class of receptor involved in olfaction is the ionotropic glutamate receptor (Ir) [18]. Evolutionary studies comparing known chemosensory receptors indicate that Irs are more ancestral compared to Ors and Grs [19]. There are also many fewer Irs (17 antennal) than Ors (60 genes coding for 62 predicted proteins) and Grs (60 genes coding for 68 predicted proteins) [20]. Some hypothesize that Irs, being more ancestral and conserved across insect species, encode very specific essential olfactory information whose importance is common across many species of insects, and that the more expanded Ors and Grs are responsible for chemicals involved in speciation or adding complexity to existing sensory stimuli [21]. This hypothesis is somewhat born out in the current literature on Irs in that they have been shown to detect amines and acids

[22, 23], both molecules basic to survival in many organisms. In the interest of understanding how the brain uses olfactory codes and information in order to generate behavior, we have focused our investigations on the Or family of receptors because, since it includes a larger number of genes, Ors likely affect more complex and nuanced behaviors.

Functional mapping of Ors indicates that both broadly and narrowly tuned Ors exist. Narrowly tuned Ors are specialists that fire in response to a single odorant, or a highly related set of a few odorant [24, 25]. Most Ors are generalists and can be activated by a diverse set of odorants, and this has obscured easy ecoethological associations. Nonetheless, specialist receptors have been linked to highly relevant biologically odors and behavioral responses [26-29].

1.2.1 Olfactory Circuit of the Fly

Olfaction begins when odorants bind odorant receptors in the dendrites of olfactory sensory neurons (OSNs). In flies, these dendrites innervate olfactory sensillum, sensory hairs located on the third segment of the antenna and on the maxillary palps [20]. Axons of OSNs expressing the same odorant receptor project to a focal point in the fly's antennal lobe called a glomerulus, and the positions of each of the ~50 glomeruli in the antennal lobe are highly stereotyped [30, 31]. In each glomerulus, OSN axons synapse with second order projection neurons (PNs) that then project to two higher order brain regions: the mushroom body calyx (MBC) and lateral horn (LH) (Figure 1.1) [32]. The mushroom body calyx is thought to be analogous to the mammalian hippocampus and contributes to learning and memory [33]. By contrast, the lateral horn (LH), like the mammalian amygdala, is thought to mediate innate olfactory behaviors [34]. When we

refer to a ‘glomerulus’, it is as a functional unit of the antennal lobe. While a glomerulus is technically a group of synapses between OSNs and their cognate PNs, we use the

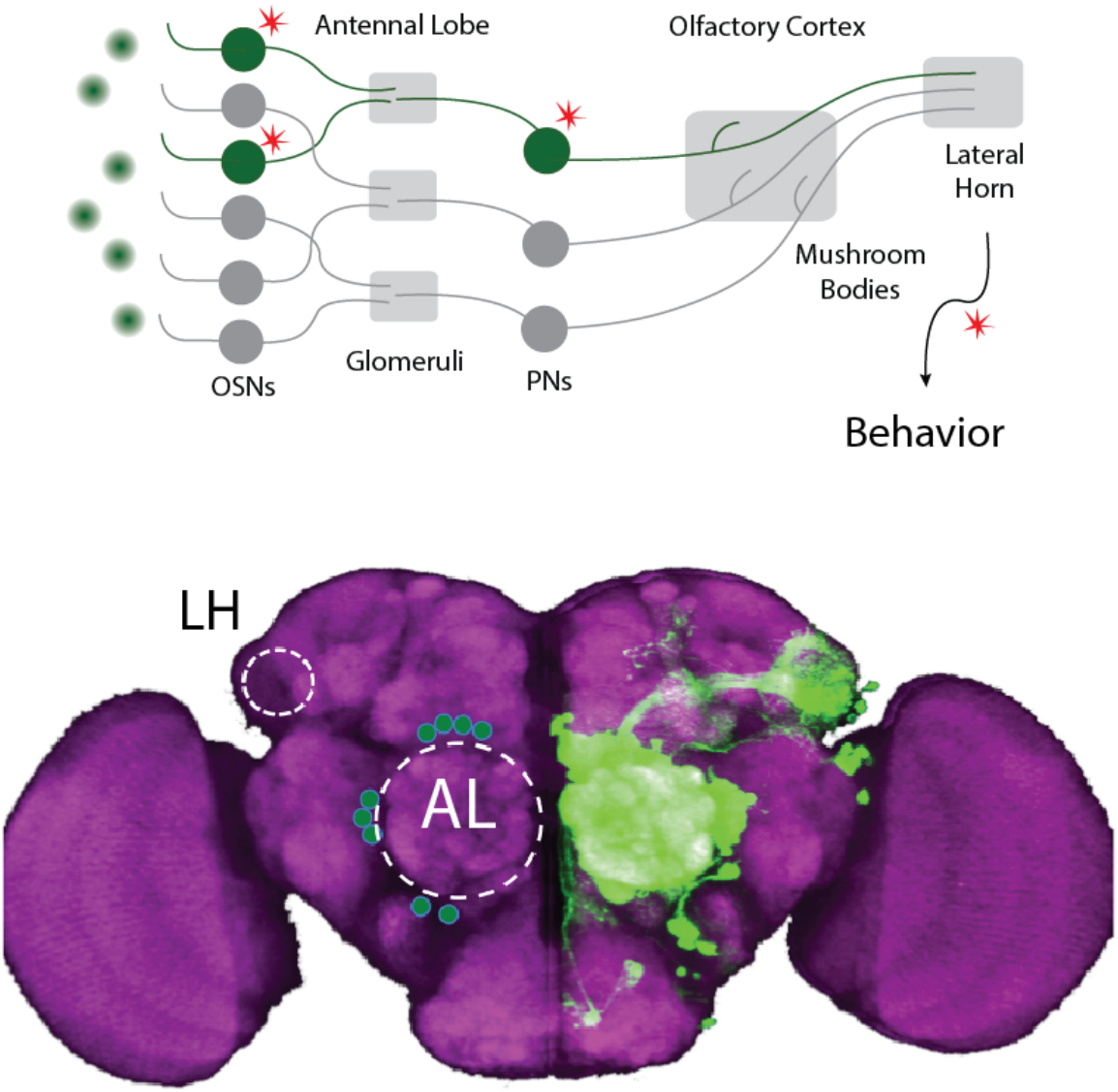


Figure 1. 1 The *Drosophila* Olfactory Circuit.

Top: Linear cartoon schematic. Bottom: Drosophila brain. Right side is PN-labeling GH146-Gal4 expression pattern.

language ‘activating a glomerulus’ describe the occurrence of an OSN innervating that glomerulus and synapsing with, and activating, the PNs that also innervate that same glomerulus. From glomerular organization to the general functions of brain regions where secondary projection neurons contact, organization of the olfactory circuit is preserved across many taxa [35]. Differences in receptor mechanism (ionotropic vs. metabotropic), developmental sequence, and OSN differentiation mechanisms among taxa suggest that the common motif for olfactory circuitry is a case of convergent evolution, and that this organizational structure may be the most efficient way to sort and process complex olfactory stimuli. Therefore, principles of organization found in *Drosophila* olfaction may be generalizable to other animals with larger nervous systems. In search of a theory for how olfactory processing works, strategies employed by other sensory systems, such as vision and audition, may also inform the study of olfaction because these different systems seem to use common processing strategies such as lateral inhibition [36].

1.3 Tools for Working with Drosophila

The long history of study, paired with a relatively small nervous system (~100,000 central brain neurons), makes flies an ideal model organism for understanding olfaction [6]. Fly research has a long and close relationship with genetics, and the 1933 Nobel Prize in Medicine was awarded to Thomas Hunt Morgan for discovering the relationship between chromosomes and heredity. Morgan used chemical mutagenesis to isolate mutants in the *white* eye color gene and was able to infer allelic segregation patterns for a sex-linked gene, demonstrating that physical traits and mutations are heritable [37]. This launched modern fly genetics, which has had far-reaching influences from the discovery

of the role of patterning genes in development [38] to foundational work on the modern evolutionary synthesis [39].

Over a hundred years of continued improvement on genetic methods places *Drosophila melanogaster* as an unmatched state-of-the-art experimental animal system in which behavior can be studied from all angles – genes to whole organism [6].

1.3.1 Binary Expression Systems

The ability to modularly manipulate gene expression in specific, genetically defined cell and tissue types is one of the most powerful features of working with flies. This feat is achieved through the use of two workhorse binary expression systems. The Gal4/UAS and Q systems pair elements found originally in the fungi *Saccharomyces serviciae* and *Neurospora crassa*, respectively [40, 41]. Promoter-driven transcriptional activators (*Promoter-Gal4* and *Promoter-QF*) bind Upstream Activation Sequences (*UAS-Effector* and *QUAS-Effector*) to express effectors in cells of interest. Each transactivator binds specifically to its corresponding upstream activation sequence (Gal4/UAS and QF/QUAS). Downstream effectors can be a wide range of molecules including fluorescent reporters to study anatomy (e,g., GFP) [42], ion channels in neurons for activation (e.g., dTRPA1, Channelrhodopsin) [43], and RNAi to selectively silence expression of target genes in target tissues [44]. Each of the 50 olfactory receptors (Ors), 13 olfactory ionotropic receptors (Irs), and 2 olfactory gustatory receptors (Grs) in flies has a corresponding OrX promoter driven Gal4 line (*OrX-Gal4*). Both the Gal4/UAS and Q systems also have specific repressible elements. Gal4 can be repressed by Gal80, a protein that binds to and blocks the Gal4 activation domain [45]. There is also a temperature sensitive Gal80 variant that allows for temporal control of Gal4 activation [46]. This can be

useful for situations where constitutively expressing an effector throughout development leads to lethality or major physiologically disruptive changes. The repressor for QF is QS, which can be inhibited by the small molecule quinic acid.

Different permutations of transgenic components (transactivator, UAS, repressor) of each binary system used together can form 'logic gates' (Figure 1.2) [41]. An example of this is used in Chapter 4 to study the anatomy of the CO_2-sensing projection neuron. The PN of interest overlaps in the expression pattern of two different driver lines: a Gal4 line (*NP7273-Gal4*) and a QF line (*12B-QF*), so the genotype reported is an 'AND' logic gate that only labels neurons in common to both expression patterns. We used this approach for anatomical tracing in order to achieve directed sparse labeling.

Another way to accomplish sparse labeling through repressible binary expression system genetics is through a technique called Mosaic Analysis with a Repressible Marker (MARCM) [47]. MARCM uses heat-shock Flippase-induced site-specific recombination in order to generate homozygous cells lacking a transcription factor repressor (e.g., Gal80) in an otherwise heterozygous animal. These homozygous cells are the only cells that are labeled (sample crossing scheme also shown in Experimental Procedures of Chapter 4).

1.3.2 Measuring Neuronal Activation

Neurogenetics is a term used to describe the study of the interaction between genes and behavior [3]. The first direct link of a genetic mutation with a behavior was made by Robert Konopka and Seymour Benzer [48] when they identified mutations in genes that changed the circadian rhythms of flies. Since then, modern neuroscience has embraced this way of studying behavior. This is particularly true in the field of olfaction.

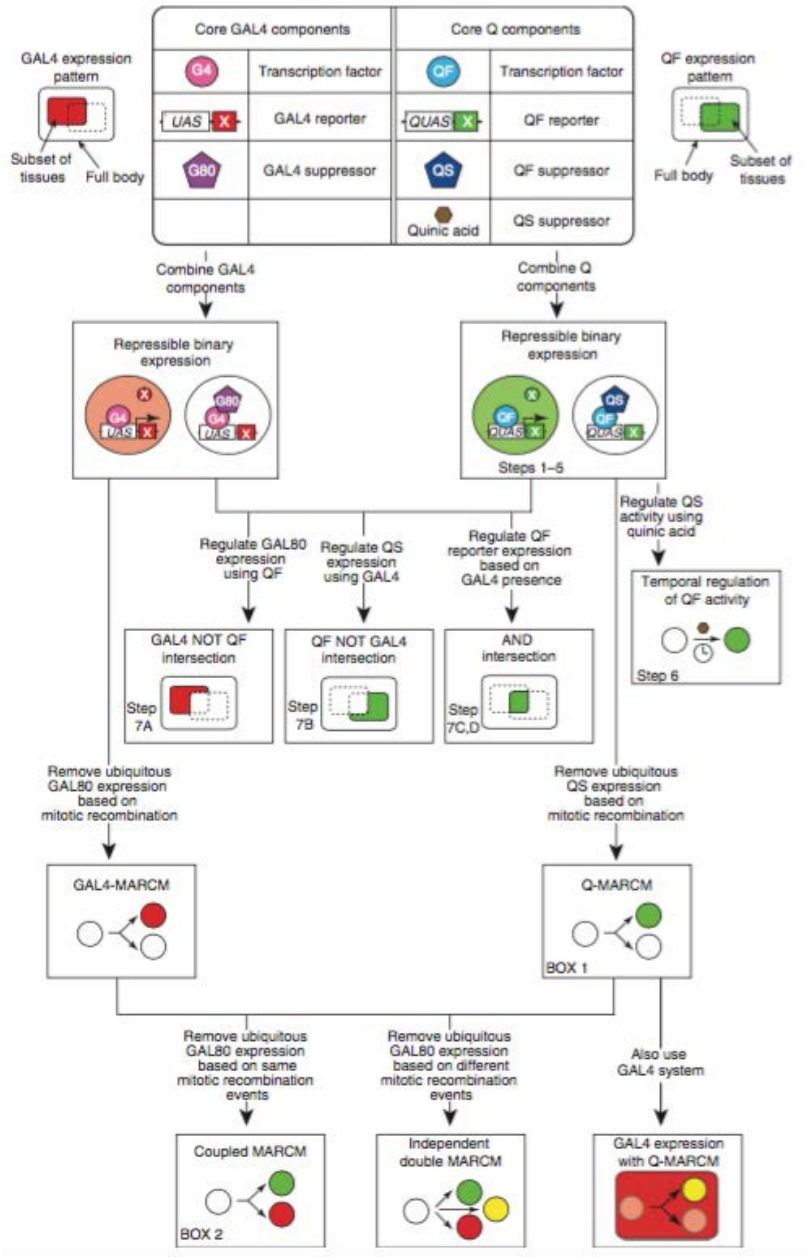


Figure 1. 2 Possible uses of Gal4 and Q binary expression systems.

Adapted from Potter, 2011 [49].

An important goal in neuroscience research is to understand how neurons process information and use relevant external sensory information to generate appropriate behaviors. In order to satisfy this goal, it is very useful to be able to study how neurons fire. This is mainly achieved in two ways in *Drosophila*:

Calcium Imaging: Using a binary expression system, a calcium indicator can be expressed in neurons. Calcium levels surge when neurons fire, causing the indicator to increase its fluorescence. The most common genetically driven calcium indicator is a molecule called GCaMP [50, 51]. GCaMP is a split GFP attached to a calcium-sensing molecule called calmodulin and also to the calmodulin binding protein M13. When calmodulin binds calcium, a conformational change brings the two halves of GCaMP together by the binding of M13 to calmodulin to make a whole, fluorescent molecule.

Single sensillum recording (SSR): SSR is a way to measure the electrophysiological responses of OSNs. An electrode is inserted into a single sensillum, and field potential recordings are recorded from the neurons that innervate that sensillum when an odorant is puffed on the sensillum [52]. Three variations on SSR have proven to be powerful tools in the study of olfaction:

The 'empty neuron' has been essential in matching Ors with their cognate odorants [24]. Dobritsa et al. [53] knocked out Or22a and Or22b, two odorant receptors expressed in the same neuron, creating a neuron that no longer expressed its natural olfactory receptors (hence 'empty'). This eliminated the neuron's natural response to odorants that would normally activate the now absent Or22a and Or22b. Or22a/b OSNs innervate a particular class of sensilla that are easy to identify and easy to record from. Without the native

olfactory receptor, the authors could now use the empty neuron to systematically match odorants to Ors by using an *Or22a-Gal4>UAS-OrX* crossing scheme.

A simple but extremely useful elaboration on SSR is the newly published technique of Fluorescence guided SSR [54]. Previous to the development of FgSSR, identifying specific sensilla types to record from was difficult and required testing each sensillar response with a panel of diagnostic odors. FgSSR aids in quickly and accurately identifying sensilla by detection of GFP in the sensillum of interest (*OrX-Gal4>15xUAS-GFP (III) or OrX-Gal4>10XUAS (II)*). The sensillum can then be easily located using a fluorescence microscope.

This work describes the development of a novel genetic tool that utilizes the Gal4/UAS binary expression system to activate OSNs (described in Chpater 2). This tool was validated then used to study how olfactory inputs influence oviposition decisions in female flies.

2. A Novel Chemogenetic Method for Studying Chemosensation

2.1 INTRODUCTION

2.1.1 Challenges in studying olfactory sensory neurons

Olfaction has been challenging to study. Unlike the visual and auditory systems, where stimuli can be rank ordered and easily classified, the olfactory system must detect a near-infinite number of odorants with highly variable chemical structures [35]. Perhaps as a result of the diversity of possible stimuli, the olfactory system has many more receptor

classes - *Drosophila melanogaster* having 62, humans 388, and mice having 1200 [35] - compared to four opsins in mammalian vision and two types of hair cells in mammalian audition. Further complicating the study of OSNs is that most odorants are capable of binding several different olfactory receptor classes, which precludes a precise odor-driven experimental study of OSNs [24].

2.1.2 Known connections between olfactory neurons and odor-guided behaviors

The inability to specifically activate OSN classes has resulted in gradual and slow characterization of individual OSNs. Functions for a select few OSNs have been identified through laborious SSR screening and calcium imaging using odorants of interest. These strategies only allow for the study of a few glomeruli at a time. Table 2.1 provides examples of specific OSNs and how their functions were discovered.

Olfactory Receptor	Chemical/Receptor Identification Method	Odorant	Function	Citation
Flight				
Gr21a/Gr63a	Established Literature	CO_2	Attraction during flight	Wasserman, et al 2003
Ir64a	Established Literature	Acids	Attraction during flight	Wasserman, et al 2003
General (Feeding?)				
Or42b	Ca^{2+} imaging/natural odorant screen	Apple Cider Vinegar	Increases attraction, especially when starved	Semmelhack and Wang, 2009
Or92a	Ca^{2+} imaging/natural odorant screen	Apple Cider Vinegar	Increases attraction, especially when starved	Semmelhack and Wang, 2009
Or83c	SSR/odorant screen	Farnesol	Attractive to starved flies	Ronderos et al, 2014
Or85a	Ca^{2+} imaging/natural odorant screen	High concentrations ACV	Decreases attraction to high concentrations ACV	Semmelhack and Wang, 2009
Ir92a	Ca^{2+} imaging with odor stimulation	Amines and Ammonia	Promotes attraction in walking assays	Min et al, 2013
Gr21a/Gr63a	Ca^{2+} imaging with odor stimulation	CO_2	Promotes aversion in walking assays	Suh et al, 2004
Ir64a	Ca^{2+} imaging with odor stimulation	Acids	Promotes aversion in walking assays	Ai et al, 2010
Courtship				
Or47b	GC-SSR screen/Ca^{2+} imaging	Methyl Laurage, Methyl Myristate, Methyl Palmitate	Mediates male courtship success and female receptivity	Dweck et al, 2014
Or67d	Established Literature	cVA	Inhibits male courtship, promotes female receptivity	Kurtovic et al, 2007
Or88a	GC-SSR screen/Ca^{2+} imaging	Methyl Laurate	Promotes attraction between two sexes	Dweck et al, 2014
Ir84a	SSR screen	Phenyl Acetic Acid	Promotes male courtship mediated by food odors	Grosjean et al, 2011
Oviposition				
Or7a	Behavior, GCMS and SSR	9-Tricosene	Aggregation pheromone that attracts both sexes; Positive oviposition cue for females	Lin et al, 2015
Or19a	GC-SSR screen/Ca^{2+} imaging	Citrus volatiles	Positive oviposition cue for anti-parasitic odors	Dweck et al, 2013
Or49a	GC-SSR screen/Ca^{2+} imaging	Parasitoid Wasp Pheromones	Causes oviposition avoidance to interspecific parasite	Ebrahim et al, 2015
Or56a	SSR screen	Geosmin	Aversion in walking flies as well as negative oviposition cue	Stensmyr et al, 2012
Or67d	Established Literature	cVA	Positive oviposition cue	Bartelt et al, 1985
Or71a	GC-SSR screen with natural odorant	Dietary Antioxidants	Attraction in walking flies and oviposition	Dweck et al, 2014

Table 2. 1 Summary of odorant receptors linked to a behavioral effect.

Blue indicates attractive, Red indicates aversive.

2.1.3 Existing strategies of experimental neuronal activation

In the field of neuroscience, being able to functionally interrogate individual neurons in a circuit through silencing and activation has proven to be extremely valuable for studying how the brain generates behavior [43]. For our experiments, we require a method to activate individual types of OSNs (genetically defined by the olfactory receptors expressed in that neuron type) in which the activation method alone does not alter fly behaviors as a byproduct. The following are several existing strategies we considered for manipulating the activity of olfactory neurons:

Temperature Sensitive Proteins – Using temperature sensitive proteins to conditionally affect neuronal firing rate is a common strategy for looking at a neuron's function in behavior [43]. *Drosophila* TRPA1 is a nonselective cation channel that opens in response to warming starting at 25^{o}C [55] causing TRPA1-expressing neurons to fire. In contrast, temperature sensitive *shibire* is a *dynamin* mutant that prevents vesicle scission, inhibiting neurotransmitter release [56] Using binary expression systems, these two proteins can be expressed in neurons of interest (*Promoter-Gal4>UAS-TrpA1 or Promoter-Gal4>UAS-shibire^{ts}*) [40] Typically, temperature is raised from room temperature to 29^{o}C in behavioral experiments to increase or inhibit a neuron's activity using TRPA1 or shibire respectively. Activation and inhibition happen relatively quickly, and the effects of these proteins are reversible – lowering back to ambient temperature restores the neurons to a normal state. These two valuable tools have been used to study a wide range of behaviors including sleep, feeding, and courtship [57-59], comparing behavior at room temperature when neurons fire normally to behavior at elevated temperature where firing is modified. However, maintaining a focal area at a set

temperature in an open space, and flies' preference for their optimal metabolic temperature of 25°C, precludes the use of temperature sensitive proteins in studying behaviors that involve choice [60].

To address the issue of temperature, we sought to use TRPM8, another cation channel of the TRP family of thermosensors. TRPM8 is the mammalian cold receptor, but it also responds to the odorant-ligand menthol [61]. Thus by using the Gal4/UAS system (*OrX-Gal4; UAS-TRPM8*), we planned to genetically drive the expression of TRPM8 in OSNs and stimulate them with menthol, eliminating the need for a temperature change that would affect behavior [62]. A *UAS-TRPM8* was generated (cloning described in Experimental Methods section). Unfortunately, in parallel control experiments in wild-type flies not expressing TRPM8, flies appeared to nevertheless be able to detect and behaviorally respond to menthol (Four-Field Olfactometer: O Riabinina, S. Chin, data not shown; also see [63] and [64]), which contrasted a previous report that flies were anosmic to menthol [65]. The chemosensors in the fly responding to menthol are currently unknown. They could be olfactory receptors, gustatory receptors, or both; nonetheless, it would be difficult to avoid these responses when using menthol as a TRPM8 agonist.

Optogenetics - Microbial opsins have allowed for the control of neuronal activity with high temporal precision [43]. In the presence of retinal, a chromophore whose conformation changes when it absorbs a photon, expressing channelrhodopsin or halorhodopsin in neurons renders their electrical signals sensitive to blue and yellow light, respectively [66, 67]. These popular tools have revolutionized the field of neuroscience, but they present some limitations when studying behavior in flies. Foremost, activating photoswitchable proteins requires intense light in order to penetrate the fly's cuticle. The

heat generated from such intense light, and also the fact that *Drosophila* are highly phototactic, can confound the interpretation of behavior [68]. Recent developments in optogenetics have yielded a red-shifted channelrhodopsin called Chrimson [69]. However, using a 750 nm infrared light elicits behavior in a four-field olfactory arena (data not shown), even though fly opsin parameters would suggest that *Drosophila* should not be able to see in that spectral range. Instead it appears likely that flies maybe able to detect steep gradients of 750 nm light. A diffuse light source may counteract this effect, but temperature changes to the arena would still persist.

Pharmacogenetics - DREADDs (Designer Receptors Exclusively Activated by Designer Drugs) are muscarinic acetylcholine G protein-coupled receptors from mammals that have been mutated to eliminate the receptor's affinity to acetylcholine, its natural ligand, and instead to be activated upon binding to the synthetic ligand clozapine-N-oxide [70]. DREADDS have been shown to function in insect cells [71]. However, the activating drug is usually administered through ingestion. Clozapine-N-oxide seems generally non-toxic to flies, but it is difficult to determine whether or not the drug has pharmacological effects that could affect behavior. Also, since ingestion is the route of administration, fast temporal resolution is compromised and acutely using DREADDs to activate neurons on a short time scale is not possible.

Odorants - SSR empty neuron experiments have been used to match olfactory receptors with odorants that activate them on a large scale [24, 53, 72]. Screening through large panels of odorants shows that most olfactory receptors can respond to multiple odorants, oftentimes of structurally different chemical classes [13, 24, 72], with higher concentrations of odorants recruiting more olfactory receptors. This broad tuning

supports the idea that combinatorial coding is the predominant way to organize olfactory information in the brain, but subsequent research, as described in Table 2.1, suggests that at least some olfactory receptors may be specifically tuned [73, 74]. If each olfactory receptor could be paired with a ligand that only activated that receptor, then those ligands could be used to activate OSNs specifically. This has been done for some of the 19 olfactory receptors in *Drosophila* larvae, but several ligands still activated multiple, albeit fewer, receptors [75]. Matching these odorants to receptors required screening through ~500 odorants [75]. Finding specific ligands for all olfactory receptors in the adult would potentially require screening through many more odorants with the risk of missing uncommon, commercially unavailable odorants such as pheromones. Furthermore, since specific activation oftentimes depends upon concentration, and concentrations of odorants across different experimental settings can be difficult to ascertain, the specificity of OSN activation would be circumspect.

2.1.4 Or56a is specifically tuned to the odorant geosmin

The olfactory receptor *Or56a* was identified by Stensmyr et al (2012) [28] as being specifically tuned for detecting the odorant geosmin. No other Ors appear to be activated by geosmin. This rare instance of specificity can be used to our advantage. We developed a novel chemogenetic approach in which the odorant geosmin, when matched to the experimental expression of the odorant receptor *Or56a*, could be used to investigate odor-driven and OSN-guided behaviors.

2.2 RESULTS

2.2.1 Or56a and geosmin can be used to activate olfactory sensory neurons

Our chemogenetic approach requires three components: *Or56a* mutant animals (to eliminate wild-type responses to geosmin), a *UAS-Or56a* transgene to drive *Or56a* in olfactory neurons, and *OrX-GAL4* lines to direct which olfactory neurons express *UAS-Or56a*. Thus by using the binary Gal4-UAS system (*OrX-Gal4>UAS-Or56a*) in an *Or56a* mutant background, Or56a can be ectopically expressed in OSNs that can then be specifically activated by geosmin (Figure 2.1). An extensive collection of *OrX-GAL4* lines is available so we focused on generating *Or56a* mutants and *UAS-Or56a* transgenic lines [76, 77].

2.2.2 Generation of an *Or56a* mutant knockout

The Or56a knockout was created using accelerated homologous recombination [78]. Procedural information about its generation can be found in the Experimental Methods section of this chapter.

We verified the *Or56a* knockout using PCR with the forward primer within the GAL4 sequence of the targeting pTV^{Cherry} construct and a genomic primer downstream 81 bps downstream of the end of the *Or56a* 3' homology arm. The expected band size was 3524 bps. The reverse primer was also used to sequence the junction between the 3' homology arm and genomic sequence. The sequencing read showed excellent alignment, as expected for a successful knockout (Figure 2.2).

Given that PCR and sequencing suggested an *Or56a* knockout, we next validated *Or56a* knockout animals using SSR. Knocking out *Or56a* results in silencing the smaller neuron of antennal basiconic sensillum 4 (ab4B) while the larger amplitude neuron (ab4A),

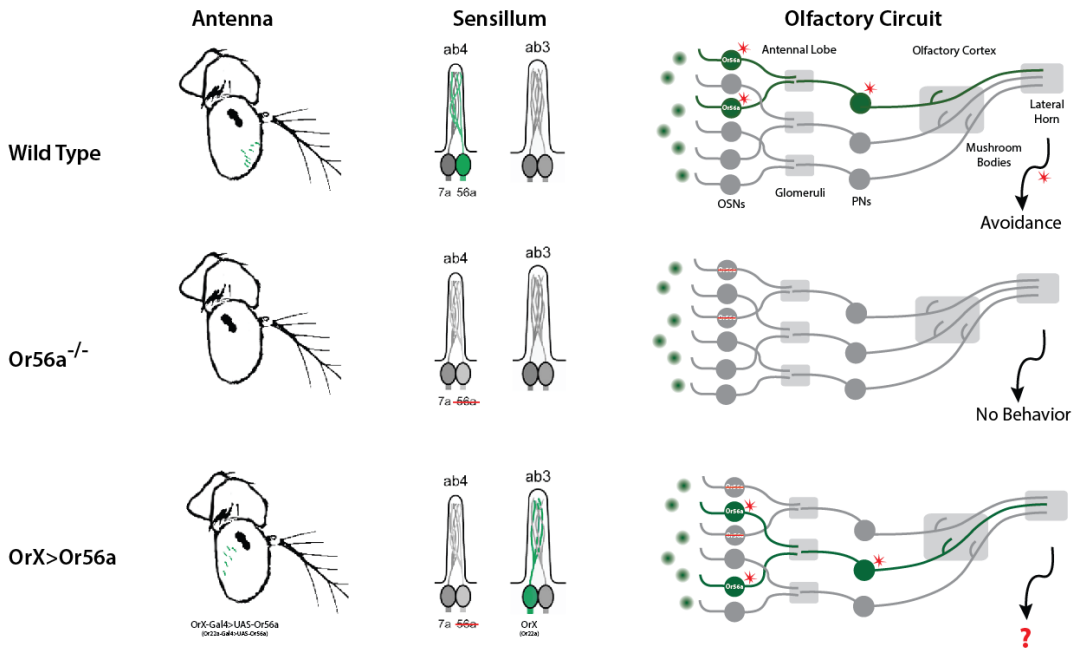


Figure 2. 1 Schematic of Or56a-Geosmin Chemogenetic Activation of OSNs.

Knocking out Or56a eliminates the fly's native response to geosmin. Ectopically expressing Or56a in an Orco+ olfactory neuron using a genetic driver (UAS-Or56a) allows that neuron to be activated by geosmin.

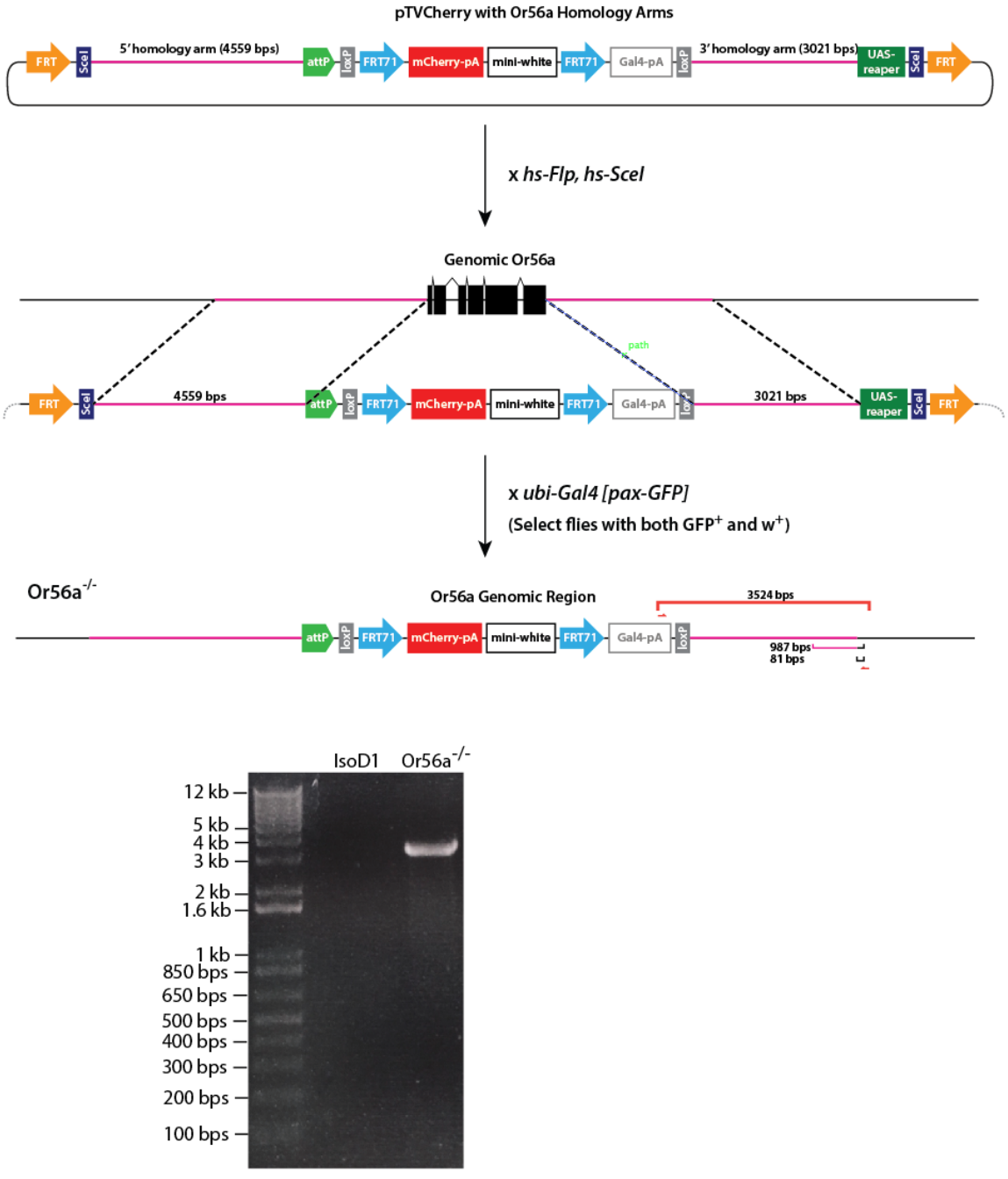


Figure 2. 2 Using accelerated homologous recombination to generate the Or56a knockout.

Cloning of Or56a homology arms into pTV^{Cherry} and primer positions for validating correct insertion of knockin construct into Or56a locus of genome.

which expresses Or7a, fires normally. Fluorescence-guided SSR was used to ensure identification of ab4 [54] and the pheromone 9-tricosene was used to activate Or7a (ab4A) as a positive control in ab4 SSR experiments (Figure 2.3).

2.2.3 Testing the ability of *UAS-Or56a* and geosmin to activate olfactory neurons

Four types of sensillum, classified according to morphology, cover the fly's antenna and maxillary palps. Based on electrophysiological odorant-Or screening, basiconics have classically been thought to detect food odors, trichoids detect pheromones, and intermediate sensillum may detect kairomones [24, 54]. Coeloconics express a relatively newly discovered class of olfactory receptors called ionotropic receptors that are highly conserved across different species of insects and have been associated with detecting acids, amines, and humidity [19, 22, 23, 79]. Studies of antennal trichoid 1 (at1), which is innervated by a single neuron expressing Or67d, suggest that the internal molecular environment of different sensillum types may differ. Or67d has been shown to specifically detect the pheromone 11-cis-vaccenyl-acetate (cVA). Successful signaling in at1 requires additional odorant binding proteins LUSH and SNMP1 [80, 81]. A recent study deorphanizing Or83c in antennal intermediate 2 (ai2) suggests that intermediate sensilla are more similar to trichoids than basiconics [82]. Thus, we used SSR to determine if ectopically expressed Or56a in non-ab4B neurons would be functional and activated by geosmin.

UAS-Or56a was expressed in ab3A (*Or22a-GAL4*), pb1B (*Or71a-GAL4*), ai2B (*Or23a-GAL4*), at1 (*Or67d-GAL4*) and at4A (*Or47b-GAL4*). This sampling of neurons covers both olfactory organs (antenna and palp) and three of the four major sensillum types.

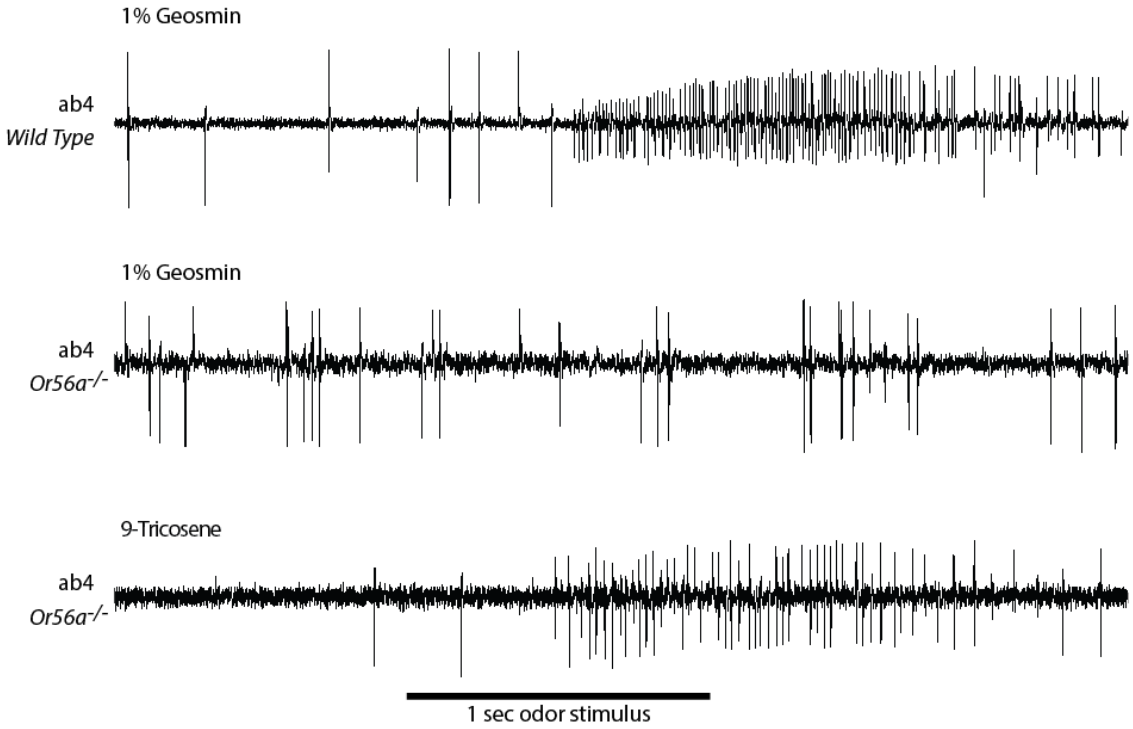


Figure 2. 3 Single sensillum validation of wild type and *Or56a* knockout controls.

Top recording is wild type ab4 response to geosmin. Or56a is expressed in neuron B so stimulating with 1% Geosmin activates the smaller amplitude neuron. Middle recording is from a successful *Or56a* mutant knockout. In the absence of *Or56a*, the B neuron no longer fires. Bottom trace depicts *Or56a* knockout stimulated with 9-tricosene. Since 9-tricosene activates Or7a expressed in ab4A neurons, 9-tricosene acts as a positive control to confirm that the recording is indeed from ab4.

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 2.4). While we were primarily testing the response of ab3A (*Or22a-GAL4>UAS-Or56a*), geosmin also appeared to cause a change in firing rate of its neighboring B neuron (Figure 2.4). The ab3B neuron responds to the pure mineral oil control. Since neurons housed in the same sensillum share hemolymph, the firing of one neuron has the ability to inhibit firing in its neighbors through the phenomenon of ephaptic coupling, possibly due to depletion of electrochemical gradients when a neuron fires strongly {Su:2012hr}. Ephaptic coupling along with ab3B's native response to the vehicle mineral oil is likely why presentation of geosmin to activate ab3A also silences ab3B.

Basiconic sensilla express canonical Ors along with the co-receptor Orco, with one exception. The ab1C neuron expresses two gustatory receptors - Gr21a and Gr63a - that, together, detect CO_2. ab1C neurons do not express any Ors or Orco. Given that the mechanism of activation and necessary intracellular signaling components of Grs are not well studied, it was unclear whether an Or could be used to activate a gustatory receptor neuron (GRN). Experimentally expressing both *Or56a* and *Orco* in ab1C (*Gr63a-Gal4>UAS-Or56a, UAS-Orco*) and stimulating with geosmin indicates that ab1C can, indeed, be activated using Or56a-geosmin chemogenetics (Figure 2.5). Thus, ectopic expression of Or56a allowed geosmin to activate antennal and palp basiconics in all examined cases. Notably, 1% Geosmin stimulation to ab1C elicited typical firing dynamics with a continuous burst of firing after odor presentation that gradually returned to baseline. However, this was not the case with a 10% Geosmin stimulation. In 5 of the 6

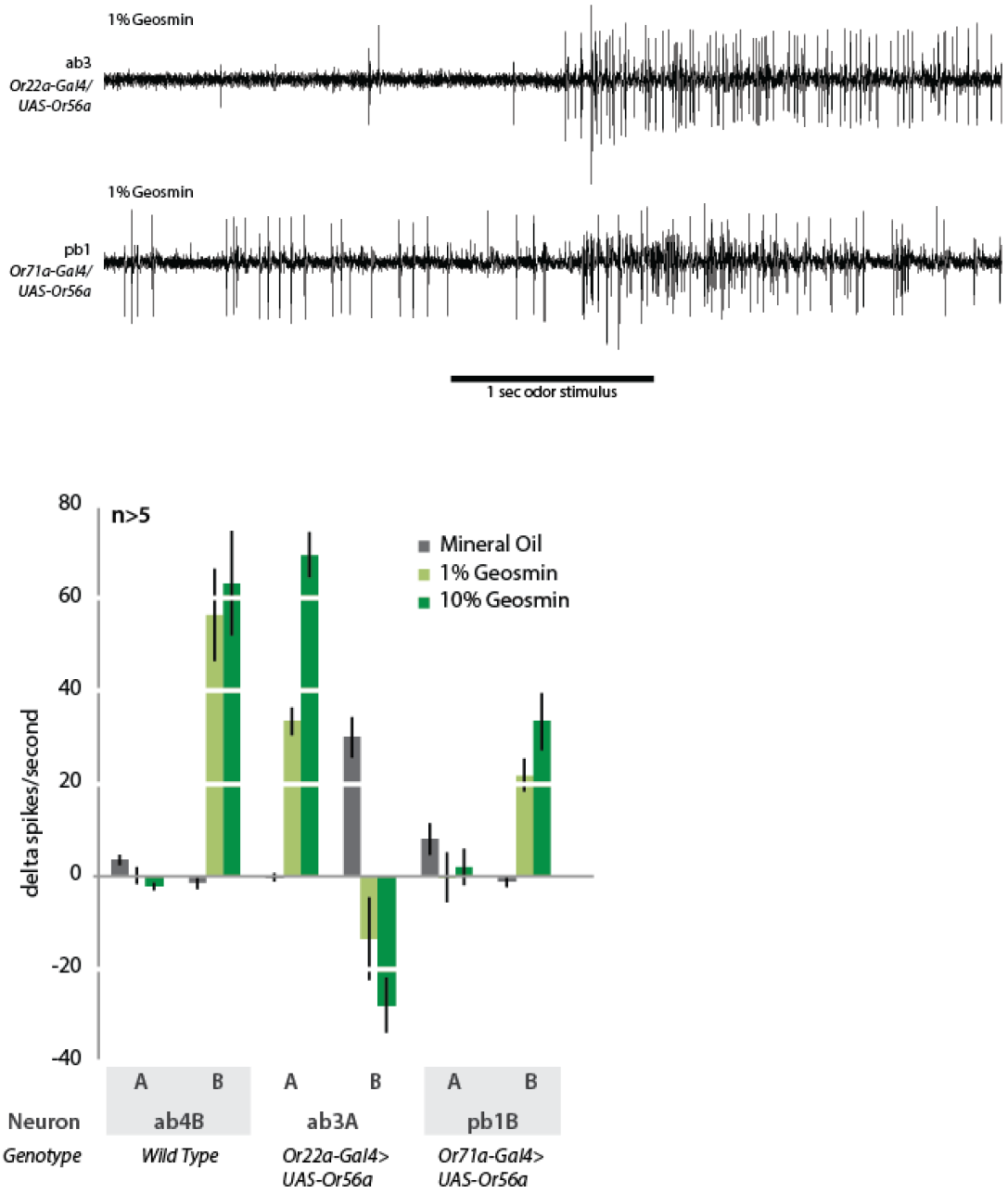


Figure 2.4 Activation of basiconic sensilla through ectopic expression of Or56a.

Ectopic expression of Or56a (*OrX-Gal4>UAS-Or56a*) enables basiconic OSNs to be activated by geosmin. Or22a is expressed in ab3A, and Or71 is expressed in pb1B. Bottom panel is quantification.

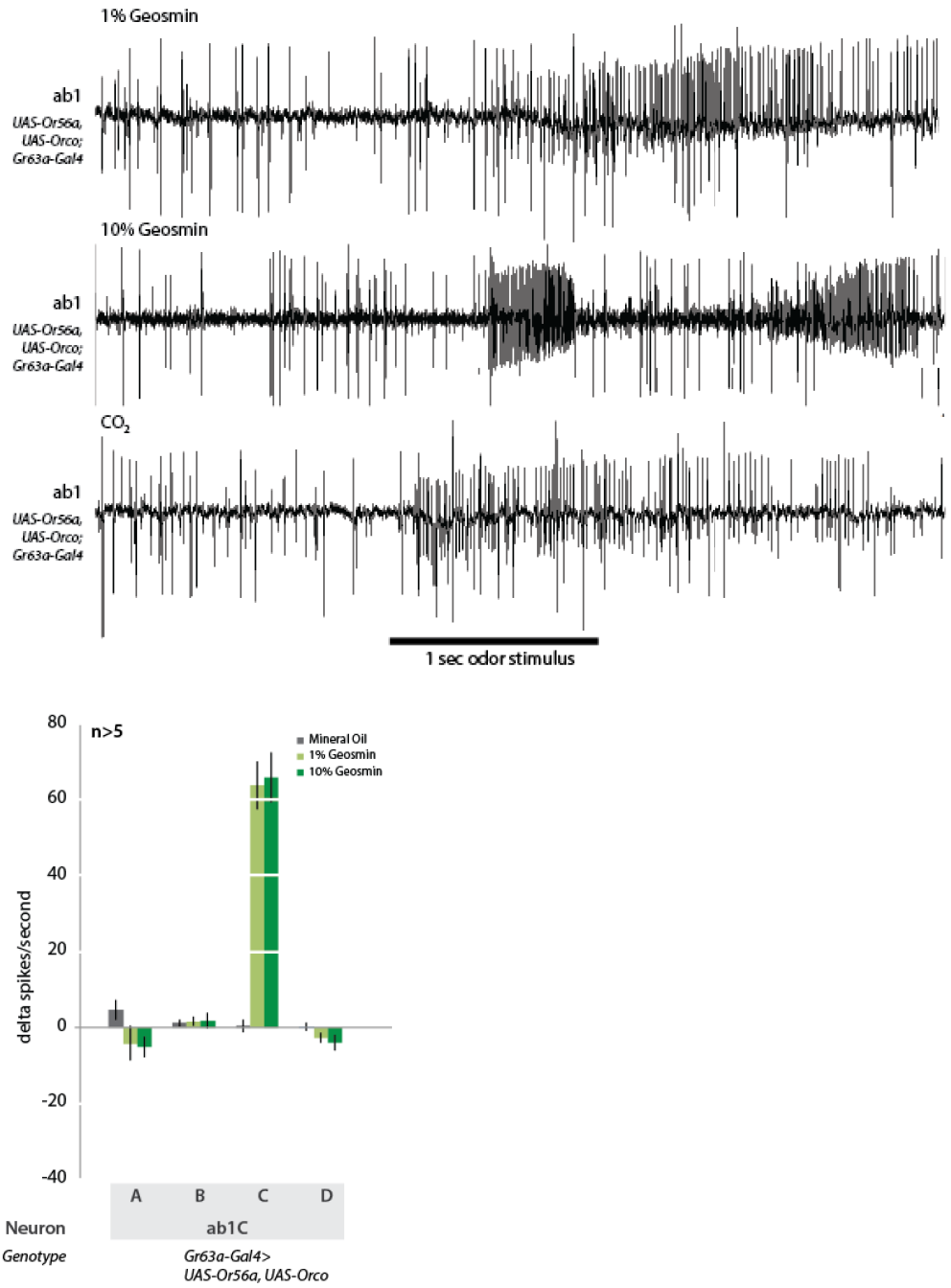


Figure 2.5 Activation of olfactory gustatory receptor neuron ab1C using chemogenetics.

When Orco is expressed with Or56a, the CO_2-sensing GRN ab1C can be activated with geosmin. Using a higher concentration of geosmin produces a biphasic response (middle trace). Bottom panel is quantification.

recordings using 10% geosmin, we observed a biphasic response where, upon initial presentation of the odor, a very high firing rate occurred followed by a rapid decrement to no firing, and then a second burst of firing occurred shortly thereafter (Figure 2.5, Top). 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 [87, 88]. The difference in firing dynamics in ab1C suggests that Grs may have different downstream second messengers that act at a different time scale than those of Ors, or perhaps they can act to temporarily repress Orco-Or56a receptor complex signaling.

The intermediate sensillum neuron ai2B also reliably increases firing, but only by <10 Δspikes/second (Figure 2.6). It is possible that non-basiconic intermediate sensilla contain different molecular components (e.g., odorant binding proteins, co-receptors, or odorant degrading enzymes), that might be responsible for weak activation [83-85]. Therefore, we also tested expressing *Or56a* in ai2A (*Or83c-GAL4*), the companion neuron to ai2B in the same sensillum (Figure 2.6). The ai2A neuron was robustly activated by geosmin (30-50 delta spikes/sec), suggesting that basiconic Ors can function in intermediate sensilla.

While Or56a can function in intermediate sensilla, surprisingly we were not able to elicit a notable response to geosmin when expressing Or56a in trichoids. We tested both of the trichoid sensilla at1 and at4 (at1: *Or67d-Gal4>UAS-Or56a; at4: Or47b-Gal4>UAS-Or56a*), and neither produced a response towards geosmin. Trichoids, as pheromone detectors, may have developed abilities to robustly detect only specific odorants as evidenced by the lack of ligands found to activate trichoid Ors in basiconic empty neuron

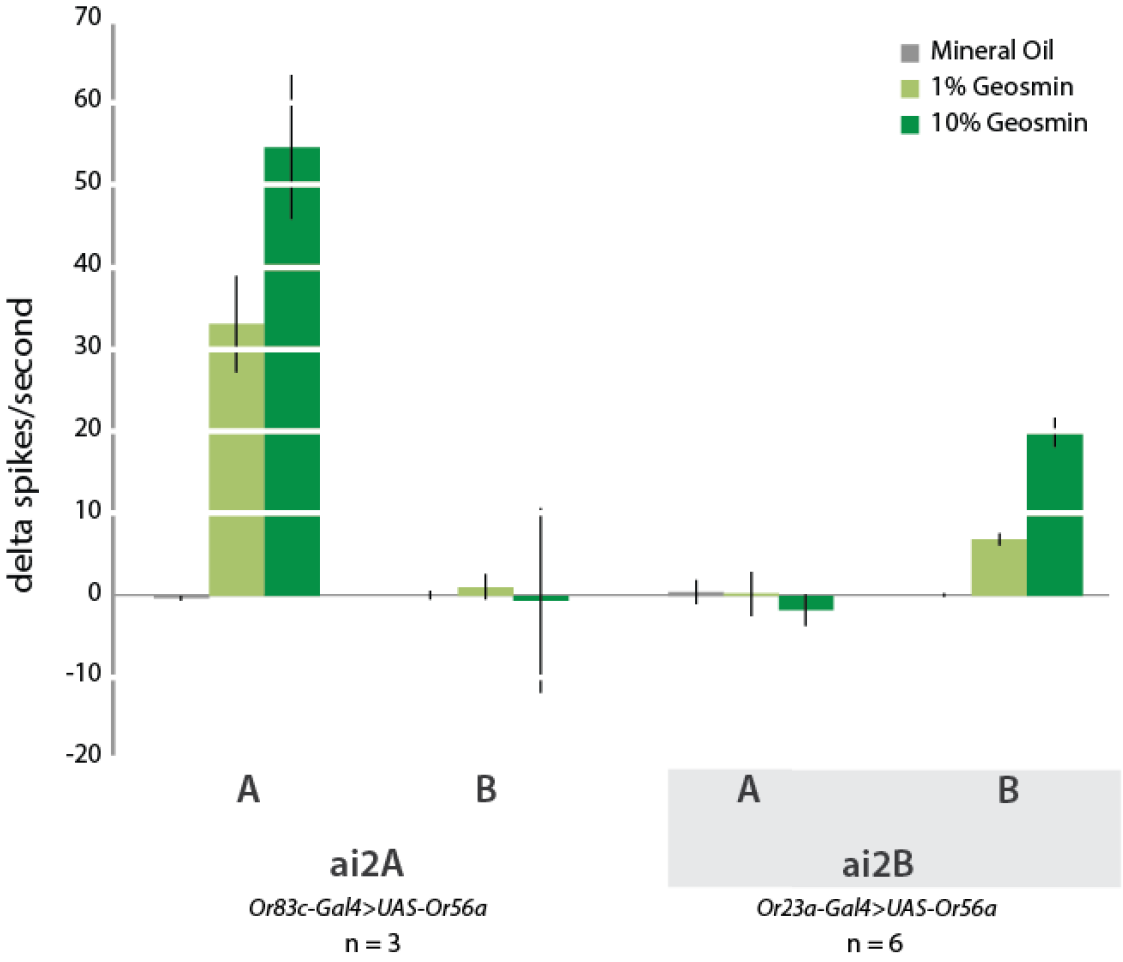


Figure 2.6 Single sensillum recordings comparing ectopic Or56a expression in ai2A and ai2B. Intermediate sensillum ectopically expressing Or56a can be activated by geosmin. Using *Or23a-Gal4* to drive Or56a expression confers low geosmin-induced activation. This is not likely due to Or23a being expressed in an intermediate sensillum neuron as *Or83c-Gal4* to drive Or56a in ai2A neurons confers comparable geosmin-induced activation levels to basiconics (See figures 2.4, 2.5).

studies [24, 86]. The example of at1 is instructive. The at1 sensillum exclusively contains Or67d-expressing OSNs and needs both the odorant binding protein LUSH, present in sensillar lymph, and also the sensory neuron membrane protein, SNMP1, at the OSN cell surface in order to detect its specific ligand cVA. This suggests that trichoid sensilla in general may utilize molecular components that are not only required for detection of a specific pheromone but may also limit the neuron's ability to functionally detect other odorants [80, 81]. This interesting observation regarding a potentially unique function of trichoid neurons will be a subject for future studies in the Potter laboratory.

2.3 DISCUSSION

Issues with existing methods of neuronal activation led us to develop a novel chemogenetic approach to study olfactory circuits. By using the odorant geosmin, we can selectively activate olfactory neurons at physiologically relevant ranges. Channelrhodopsin and TRPA1 typically produce very strong firing of neurons that may lead to unnatural behavioral responses. Tuning neuronal firing rate in both methods is very difficult, and the stimuli for activating these two channels also tend to be challenging to localize. For example, in a two choice assay such as a T-maze or oviposition assay using opto- or thermo- genetics, light or heat would need to be restricted only to one side or portion of the assay.

Our Or56a-geosmin strategy uses a physiologically appropriate receptor and stimulus for olfactory experiments. There has been some controversy as to the mechanisms of intracellular signaling in OSNs, and using an Or circumvents possible problems with unnatural firing patterns of activation. Geosmin is an odor that has the dynamic features of natural olfactory stimuli. This eliminates confounding factors for behavior like phototaxis and temperature preferences. 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.

Our Or56a-geosmin chemogenetic approach leads to reproducible, albeit low (20-60 spikes/sec), increases in olfactory neuron signaling. This likely reflects normal activation of an olfactory neuron to low odorant concentrations. 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 one of the first instances where OSNs were systematically screened to a panel of odorants [24], responses were categorized as 'hits' if responses were greater than 50 spikes/second. Responses of ~150-200 spikes/second were considered as reflecting 'real' odorant-to-Or matches. A recent study [68] used an elaborate and sophisticated optogenetic setup to tightly control stimulus intensity towards individual moving flies. The study showed that high levels of neuron activity may not be required for generating behavior. In some cases, lower induced activity of the olfactory neuron of around 40-50 spikes/second generated stronger behavior. The efficient coding hypothesis [4] postulates that the level of a stimulus should match the level of neuronal firing in natural environments where an animal has evolved to survive. This optimizes 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 lab 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. All together, this suggests that the chemogenetic approach we have developed is an appropriate method for testing the neural connections between olfactory neurons and odor-driven behaviors.

2.4 EXPERIMENTAL PROCEDURES

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'-

GAATAGGGAATTGGGAATTCATGTTTAAAGTTAAGGATCTGTTGC-3' and 5'-ATCTGTTAACGAATTCCTAATACAAGTGGGAGCTACG-3'). InFusion cloning (Clonetech Laboratories, Inc) was used to subclone *Or56a* into the EcoRI cut site in the multiple cloning region of *pUAST* [40]. 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 in (Baena-Lopez et al. 2013). Briefly, 4559 bps of genomic sequence immediately upstream and 3021 bps immediately downstream to *Or56a* were PCR amplified using primers designed for InFusion cloning to create a 5' and 3' homology arm, respectively **(Or56a_5'homarm_REV** 5'-AGTTGGGGCACTACG**GTTAAACTGTTTAGCGTTAACCATATTC**-3'**, Or56a_5'homarm_FOR2** 5'**-**CTAGCACATATGCAG**CTCACAGCGCTTGTCGTAAT**-3'; **Or56a_3'homarm_FOR** 5'-ACGAAGTTATCA**AGGGAAAGCCTTTTCTTCAGG**-3', **Or56a_3'homarm_REV2** 5'-GATCTTTACTAG**TTTTCCGCTTCTGCTCTACG**-3';

Bolded nucleotides represent genomic sequence, and unbolded sequence indicates vector nucleotides. Sequentially, the 3' homology arm was InFusion cloned into the SpeI restriction site of MCS B in *pTV*^{Cherry} (vector from lab of J.P .Vincent), and the 5' homology was cloned into the NheI site of 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 hours and 72 hours after egg-laying for 1 hour 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.

Chemicals: Geosmin in methanol was purchased from Sigma-Aldrich at highest available purity, ≥97% (Product #: G5908-1ML, Lot #: BCBP7178V). Chemical as received was dried out to remove methanol and then diluted to 4 mg/mL in mineral oil (Sigma-Aldrich, Product #: 330779-1L, Lot #: MKBF6530V). Methyl laurate (Product #: 234591-2.5G, Lot #: BCBQ6830V) 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 described in Lin and Potter 2015 [54].

UAS-TRPM8 Cloning: pUAST was digested using EcoRI and XbaI. *TRPM8* was PCR amplified from UAS-TRPM8 flies (provided by Ben White) and InFusion cloned (InFu_5XUAS_TRPM8_EcoRI_FOR: ; InFu_5XUAS_TRPM8_XbaI_REV:).

3. Behavior

3.1 INTRODUCTION

3.1.1 Studying olfactory behavior in *Drosophila*

The vinegar fly *Drosophila melanogaster* is an ideal organism to study olfactory sensory processing because the anatomical circuitry of the fly olfactory system has been well-characterized, and flies exhibit easily measured robust behaviors [6, 30, 31, 59]. Powerful genetic tools enable the manipulation of neuronal activity and ease of obtaining animals allows for repetition of experiments to draw statistically strong conclusions [40, 41, 43, 59].

Many assays are used to evaluate olfactory behavior in flies. Importantly, the salience of a stimulus is dependent on a fly's current internal and external conditions. Also, the variable nature of odorants and their aerodynamic features in different types of physical space makes it difficult to compare results across different behavioral assays since, to our knowledge, there are no reliable ways to consistently and accurately measure odorant concentrations and gradients without disturbing these characteristics. When interpreting olfactory experiments, it is important to consider the factors that can change behavioral output:

Population of Flies: The age and sex ratios that compose an experimental group as well as the total number of flies loaded into an assay can be very important. Flies exhibit many social behaviors, and the presence of pheromones can heavily influence behavioral outcome in many contexts [10, 89, 90]. Thus, a fly behaving in isolation will most likely behave very differently than in a group, and the presence of two sexes vs. one, or the

sexual state of females (naïve vs. mated), is important for behavioral responses to odorants [29].

Both extremes of age must also be considered in olfactory assays. The adult olfactory system is most sensitive 3 days post eclosion [20], and conversely, it has been shown that older flies have reduced responses to attractive odors because OSNs begin to degenerate with age [91].

Rearing Conditions: Larval density during development has long-term effects on adults. Overcrowding can increase competition for resources resulting in smaller body size and metabolic differences that later affect adult fecundity and mating behaviors [92]. Background odorants during larval as well as adult rearing can also have long-term effects on odor-driven behaviors [93].

Satiety State: Many locomotion-based assays require food-depriving flies prior to experiments to encourage exploratory activity. Foraging and nutrient intake are crucial for the fly's day-to-day health and survival, so satiety state is a powerful modulator of preference. Satiety state has been directly linked to behavioral sensitivity to odorants that results from changes in sensitivity of specific OSNs to odorants in starved versus fed states [8]. As with temperature and age, metabolic changes in the fly can drastically affect behavior.

Timescale of Experiments: Long assays introduce variables of thirst and starvation, and it is unclear how the occurrence and timing of phenomena such as sensory habituation affect behavior. Furthermore, by the principle of diffusion, every assay that uses an odor source produces odor gradients, and odorants inevitably equilibrate and possibly saturate

the assay. Therefore, long assays are not optimal for understanding a fly's sensitivity to concentrations of odorants.

Physical Parameters of Assay: The physical dimensions, spatial structure, temperature, and humidity of olfactory assays can all affect behavior. Assay size and structure determines odor distribution, which is important for being able to clearly interpret results. Odor dynamics within an assay can also be affected by the presence or absence of airflow. Along with changing gradient effects of odor presentation, airflow also provides mechanosensory input to the fly. Airflow helps mimic odor dynamics involved in flight in which plume tracking seems to be the dominant behavior [9].

Behavior is challenging to study because changes in any of these parameters have the potential to signify ethologically different behavioral contexts for the fly, dramatically changing responses to a stimulus. Therefore, it is important to consider what ethological behavior is being tested by an assay. This is best exemplified with the example of CO_2. Initial experiments characterizing CO_2 utilized a two-choice T-maze assay and categorized CO_2 as an aversive stimulus [94]. The T-maze is a standard olfactory behavioral assay that consists of two tubes with different odorants plugged into the T-maze apparatus [95]. The odor-filled tubes are small enough in diameter that flies cannot engage in flight, and airflow is not usually applied through the experimental space [28, 96]. Flies are lowered through an 'elevator' into a space at the center of the apparatus between the two tubes and allowed to choose an odor side. What ethological behavior is this assay measuring? Assays that involve starvation most likely measure foraging activity, but in this case, flies were not starved. When CO_2 was tested in an olfactory assay involving airflow in a setup simulating flight, attractive tracking behavior was

indistinguishable from vinegar [9]. Interestingly, the flight assay revealed that CO_2 plume tracking during flight is mediated by the acid-sensing Ir64a rather than Gr21a/Gr63a, which have been shown to directly respond to CO_2 and mediate walking CO_2 avoidance. These findings illustrate the sensory complexity involved in contextualization of olfactory stimuli, showing not only that attraction and repulsion can depend on the task at hand, but that these different responses arise from context-dependent recruitment of different parts of the olfactory system.

3.1.2 Semiochemicals

A class of chemical that has been of particular interest is the pheromone. *Drosophila* and other organisms have dedicated neural circuits for processing conspecific chemical cues [34, 97]. More broadly, semiochemicals are any chemicals involved in animal communication [98, 99]. This communication can be with conspecifics or interspecifics. Pheromones are monomolecular (as in the case of cVA) or mixtures of chemicals used to signal between individuals of the same species. They are frequently used for tasks such as conspecific recognition, mating cues, and aggregation. While communication between flies is highly important for species survival, so is the fly's ability to read olfactory cues from other organisms in its environment. Allelochemicals are another class of semiochemical that contain compounds or mixtures of compounds that are used to signal between different species. These can be classified based on which individual, sender or receiver, benefits from the signal: kairomones (receiver), allomones (emitter), and synonome (both) [100]. Since organisms that share a complex environment coexist, interactions between interspecifics can exert evolutionary pressure to develop a response to specific chemicals. Interestingly, many of the same chemicals are used by different

species for pheromonal and allochemical communication. For example, the pheromone of one species could be used as an allelochemical for another; this is the case for situations when predators locate prey organisms by 'eavesdropping' on the prey's pheromonal signals, or one animal species uses another's alarm pheromone to avoid a common predator [101]. This is likely because, evolutionarily speaking, organisms across taxa share common ancestry and there is limited variation in biochemical processes used for synthesizing molecules [98].

3.1.3 Oviposition

Egg laying is an important behavior for species survival. Throughout her lifespan, a female's egg laying capacity maximizes at approximately four to five days after eclosion and gradually decreases after about 15 days into senescence [102]. Flies have been shown to lay eggs in bouts, laying several eggs within a few minutes rather than continuously laying eggs at a uniform rate.

The female reproductive tract is not fully developed until after mating, and mating triggers broad changes in female behaviors that divert the female's priorities from courtship and copulation to increased feeding and oviposition [103, 104]. A combination of compounds in seminal fluid, the presence of sperm, and pheromonal inputs from males are thought to mediate this dramatic shift in behavioral prioritization [105-107].

Since larvae cannot fly, their ability to survive is highly dependent 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 - maximization of nutrient intake and protection from parasites and disease-causing microorganisms [108]. Females achieve this by continuously probing their environment with proboscis and ovipositor and using

many sensory features to aid in decision making such as substrate color, texture, temperature, and the presence of UV radiation [109, 110].

Drosophila lay eggs on their food substrate, rotting fruit, so chemosensation has been heavily implicated in oviposition choice since smell and taste provide essential information about the composition of a food source such as nutritional content and toxicity. Along with antennal and palp olfactory receptors and gustatory receptors in the labellum, female flies also have gustatory receptors on their wing margins, labellum, legs, and ovipositors. In the labellum, there are receptors for the standard taste modalities like sweet and bitter, but unlike olfactory receptors that have been odor-matched through large-scale screens using SSR, there are no broad studies of chemical panels for Grs [20]. This may be because single Gr neurons (GRNs) typically coexpress a large number of Grs (up to 29 in one bitter-sensing neuron) and the mechanistic biology of GRNs is still widely unknown. What is known is that expressing different sets of Grs seems to modulate the activity taste receptors as seen in the case of bitter-sensing GRNs inhibiting firing of sugar-sensing GRNs [20, 111-113].

The primary studies on chemosensation in oviposition have involved Grs. Generally, flies are attracted to calorie-rich sugar substrates and avoid substrates that contain bitter compounds. Counterintuitively, it was initially discovered that flies prefer to oviposit in sugarless substrates to sweetened substrates and sometimes even prefer bitter oviposition substrates to sweet [11]. This was later shown to be a function of substrate area size, which determines the ease with which a larva could move to another patch to forage [114]. Small patches close together minimize larval foraging costs since the larvae do not need to travel far in order to reach a calorie rich sugar patch. Therefore, 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, it is more advantageous for the female fly to directly lay eggs on sweet substrate.

It is difficult to distinguish between an odorant and a tastant – a bitter volatile chemical could potentially be tasted by the gustatory system as well as smelled by the olfactory system. This distinction may seem inconsequential, but 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 [115]. A similar phenomenon has been observed with olfactory vs. gustatory responses to acetic acid [116]. The integration of these two sensory modalities along with elements of environment such as patchiness of food resources illustrates that oviposition choice is a complex decision making task.

Five olfactory receptors have been specifically associated with oviposition. Or19a and Or49a are implicated in avoidance of larval parasitization by wasps. Or19a mediates positive oviposition and responds to citrus volatiles repellent to wasps, and Or49a detects parasitoid wasp semiochemicals which female flies want to avoid during oviposition. Or56a and Or71a are implicated in avoiding the negative effects of infection by microorganisms. Or56a detects geosmin, which is emitted by harmful microorganisms

[28, 117, 118], and Or71a promotes attractive oviposition because it is thought to detect antioxidants in food that can attenuate oxidative stress resulting from exposure to toxins [119, 120]. Or7a has been shown to detect the pheromone 9-tricosene and mediates geographical tagging of food sites by males used to attract females [29]. 9-tricosene has also been shown to positively stimulate oviposition through Or7a [29].

With this limited knowledge of olfactory contributions to oviposition, and given that oviposition is such an important ethologically relevant behavior, we utilized a chemogenetic approach to conduct a behavioral screen to identify olfactory receptor neurons involved in oviposition.

3.2 RESULTS

3.2.1 Validating the three-well two choice oviposition assay

We used the novel chemogenetic system described in Chapter 1 to evaluate olfactory neurons in the context of oviposition. Our three-well assay contains two control wells loaded with only agarose and vehicle (in most cases mineral oil), and one well loaded with agarose containing geosmin or another test odorant. Though the arena used in our oviposition assay has three wells, the assay is a two-choice assay with the odorant well typically placed in the center well. This mimics the standard oviposition assays used in the field that typically have three zones (odor, neutral, odor) [121, 122]. In the case of our assay, the odorant well is in the center section of the assay. This gives the flies more opportunity to choose or avoid the odor well since it is surrounded on both sides by non-odorant substrate rather than the boundary wall of the assay. We first sought to ensure that the three-well design of our assay did not have any undesired biases. Since flies tend to primarily explore the boundaries of an open field, we first aimed to determine that the

physical features of the arena would not skew behavior. In Figure 3.1, we tested *Or56a* knockout flies and moved the geosmin well into each of the three positions. Positional effects of the odorant well are not statistically significant.

The original Or56a-geosmin study [28] screened for olfactory responses using SSR on antennal sensilla. While the authors conducted behavioral experiments silencing Or56a OSNs using temperature sensitive *shibire*, they neither generated an *Or56a* knockout nor tested for behavior in *Orco* mutants. Since our chemogenetic tool relies on highly specific activation of Or56a and only Or56a, we deemed it necessary to conclusively exclude the possibility that non-olfactory receptor based chemosensation (Grs and non-olfactory Irs) might contribute to behavioral responses to geosmin (as was found for menthol). In addition, since temperature can affect volatility of chemical odorants as well as the metabolic functions of flies, we wanted to confirm that our behavioral settings also maintained geosmin specificity (which was not previously examined). This was achieved by testing three genotypes: 1) the *Or56a* mutant 2) the *Orco* mutant and 3) a quadruple anosmic mutant generated by Richard Benton's

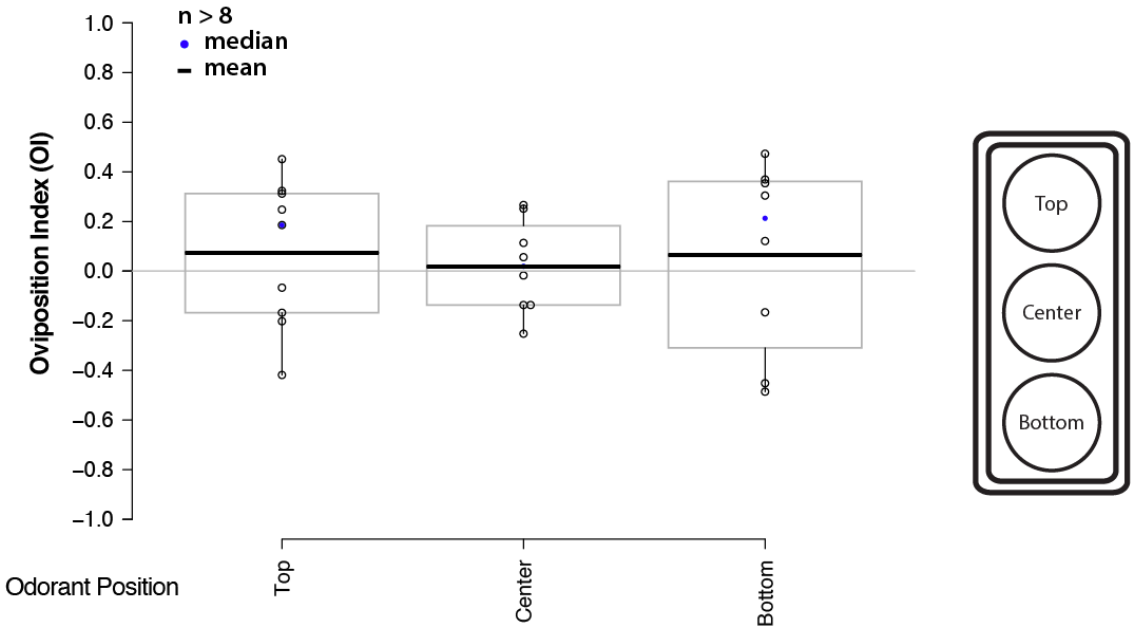


Figure 3. 1 Positional controls for three-well oviposition assay.

Odorant well containing 1% geosmin was moved to different positions. We used *Or56a* knockout flies to test. There appear to be no positional effects. Differences not statistically significant.

laboratory that is mutant for two ionotropic receptor coreceptors (*Ir8a* and *Ir25a*), *Orco*, and *Gr63a*. All three control genotypes exhibited neutral oviposition indices to geosmin, suggesting that knockout of *Or56a* eliminates behavioral response to geosmin in our oviposition assay, and that gustatory and ionotropic receptor inputs do not contribute to the behavioral response in this assay (Figure 3.2). There are fewer *Orco* mutant experiments because *Orco* mutants frequently did not lay sufficient numbers of eggs in the assay.

3.2.2 Screening *OrX-Gal4* lines in search of oviposition cues

We next tested twenty-three *OrX-Gal4* lines in the oviposition assay using the Chemogenetic tool described in Chapter 1 (Figure 3.3, Schematic 3.4). These lines were chosen to interrogate OSNs for which PN morphology is known. All *OrX-GAL4* lines were backcrossed into the same isogenic background, and combined into the *Or56a* mutant background. PN analysis appears in the subsequent Anatomy Chapter 4.

Our data show that activating OSNs using Or56a-Geosmin chemogenetics can successfully elicit behavior. In our oviposition assay, we observed a range of oviposition indices, suggesting that the results we see are not artifacts resulting from an uncontrolled parameter of the assay. All of the statistically significant hits from our screen appear to mediate negative oviposition in our three-well assay (Figure 3.5). Or92a looks like a positive oviposition olfactory channel but the behavioral results do not reach statistical significance ($p > 0.05$).

As mentioned in Chapter 2, we had difficulty obtaining any geosmin-directed activation in SSR recordings for trichoids expressing Or56a. However, the second strongest

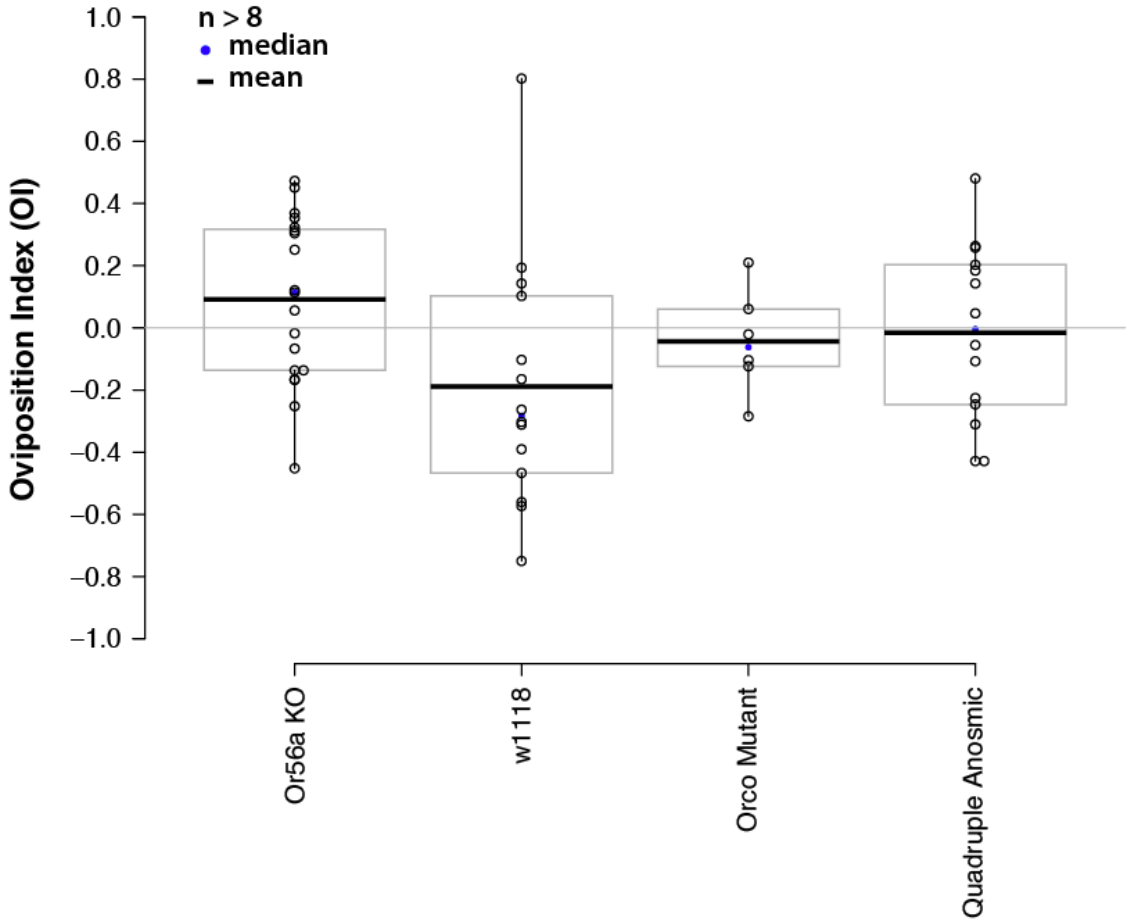


Figure 3. 2 1% Geosmin controls in mutants and wild type.

All experiments involved presenting 1% geosmin in the center well. Wild type w^{1118} flies appeared to find geosmin mildly repellant but narrowly missed statistical significance when compared to the *Or56a* knockout (p = 0.053). *Orco* mutant and quadruple anosmic fly experiments show that gustatory or other unidentified chemosensory receptors do not cause behavior in response to geosmin.

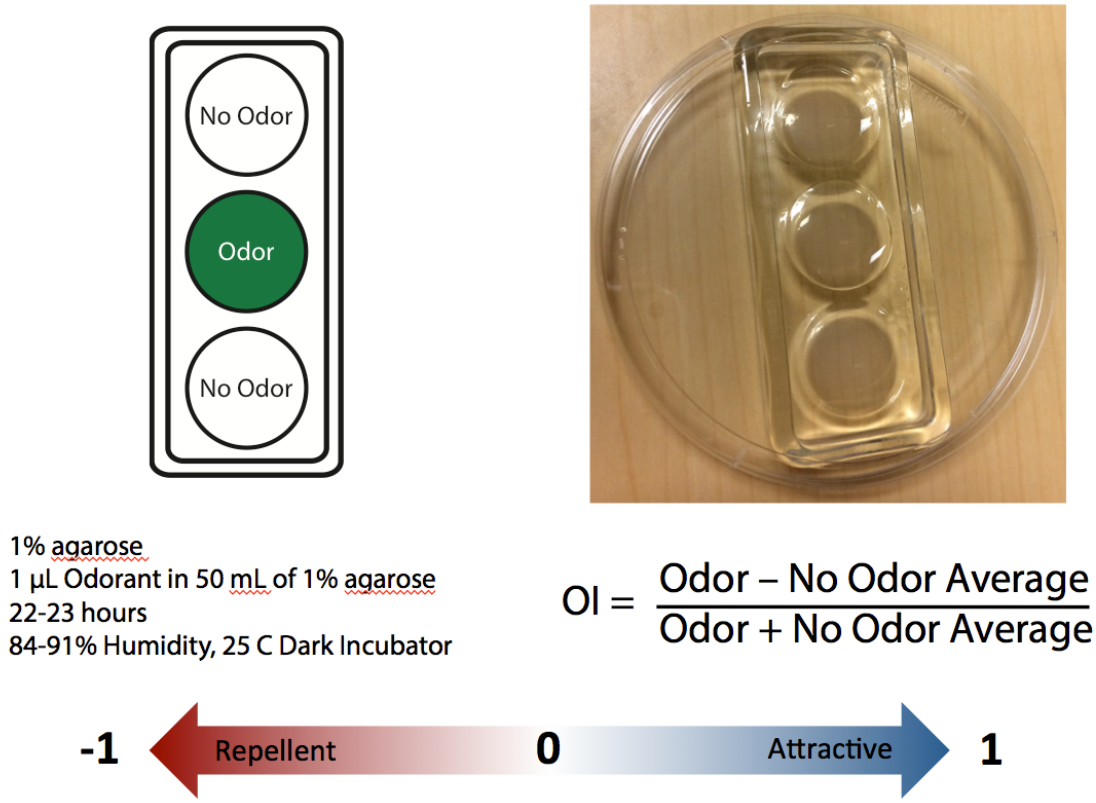


Figure 3. 3 Experimental setup in three-well assay.

Schematic and photo of the agarose-based oviposition assay. Environmental parameters and Oviposition Index (OI) calculation shown.

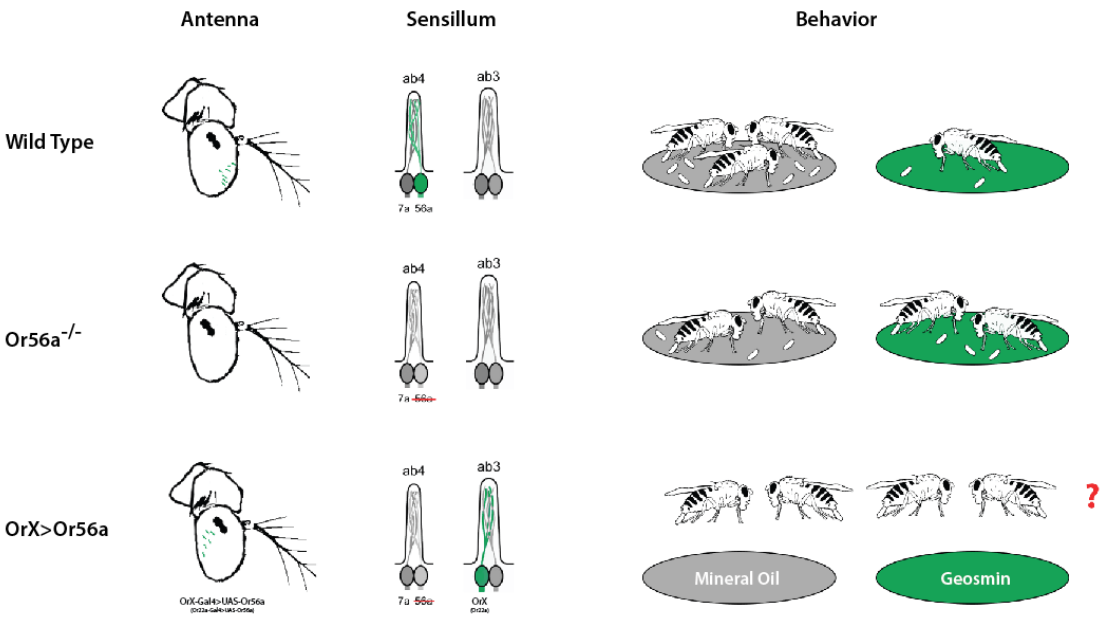


Figure 3. 4 Schematic of oviposition experiments.

The experiments for the body of this thesis use chemogenetics to test the effects of activating specific OSN types expressing the same olfactory receptors on egg laying behavior.

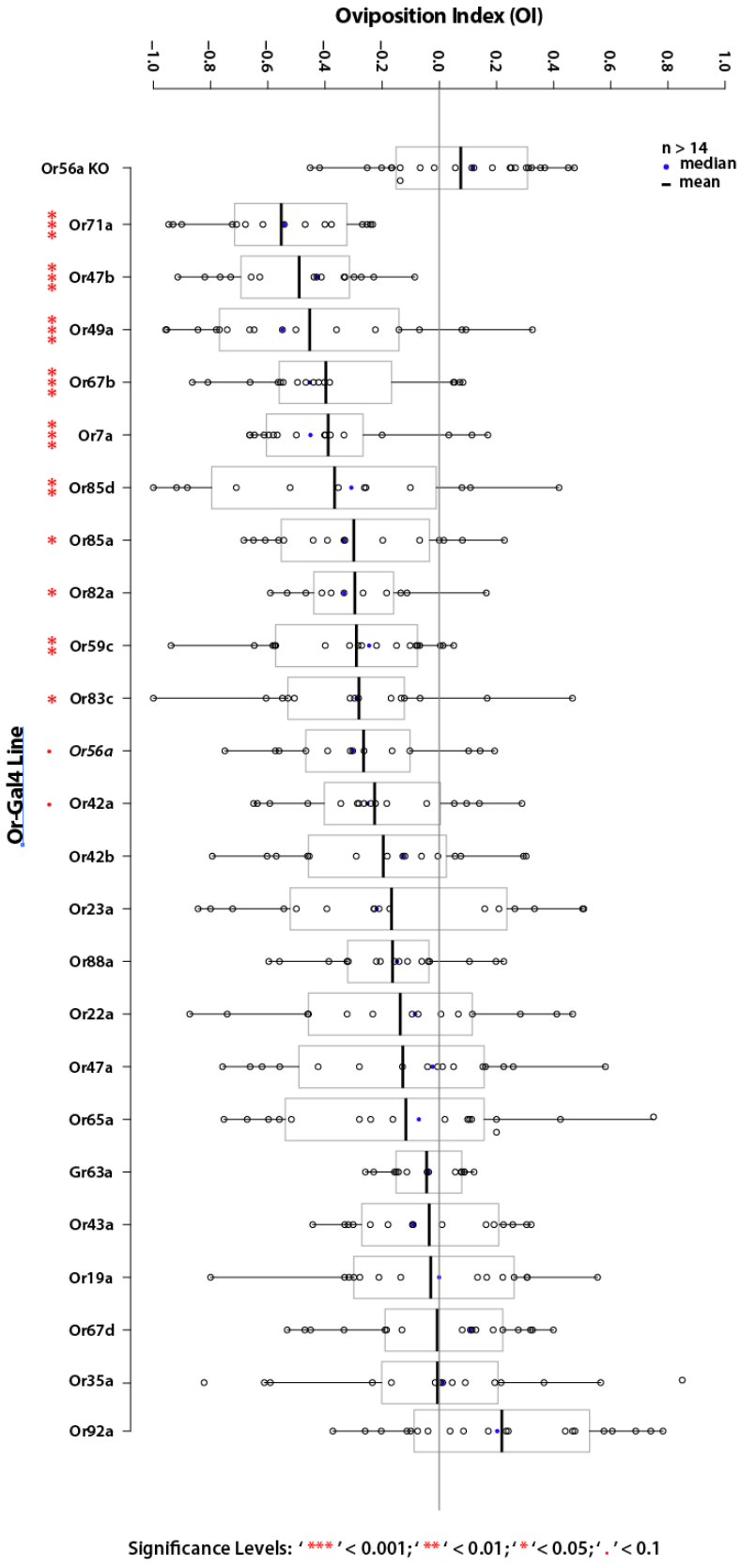


Figure 3. 5 Assaying the effects of activating individual olfactory sensory neuron types in egg laying decisions.

OrX-Gal4 lines were tested by crossing to UAS-Or56a in the *Or56a*$^{-/-}$ background. *Gr63a-GAL4* was crossed to *UAS-Or56a, UAS-Orco* in the *Or56a*$^{-/-}$ background. The Or56a experimental (italicized) results are wild type flies' response to 1% geosmin in mineral oil. Statistics presented are a Dunnett's Many-to-One Comparisons Test compared to *Or56a*$^{-/-}$.

mediator of oviposition in our chemogenetic behavioral screen is Or47b, whose OSN dendrites innervate antennal trichoid 4. In SSR, at4 responded normally to the pheromone methyl laurate but we were not able to elicit a response to even 100% geosmin in *Or47b-Gal4;UAS-Or56a* flies. It has been postulated that trichoids and intermediates may require additional molecular elements such as odorant binding proteins or membrane proteins that interact with receptor or ligand to truly activate. The ai2A neuron was only weakly activated, with a small but reliable <10 delta spikes/sec, by geosmin when Or56a was ectopically expressed (*Or23a-Gal4;UAS-Or56a*). These spikes could only clearly be observed because the baseline firing of ai2 is low. The at4 sensillum houses three neurons whose baseline firing is high. Therefore, small changes in firing rate may be missed and would require many more experimental replicates in order to detect such a small change with statistical significance. Nonetheless, the chemogenetic activation of Or47b resulted in robust oviposition avoidance. It is possible that geosmin does activate Or47b OSNs but that the activation is subtle. Perhaps, since it is not a fatty pheromone, the efficiency of geosmin entering the hemolymph of antennal trichoids is extremely low but existent. The behavioral effect on egg laying could be the result of slow accumulation of geosmin that eventually reaches threshold to activate Or47b OSNs. Alternatively, Or47b could be expressed in non-antennal organs. To this end, we crossed *Or47b-Gal4* to a strong reporter (*Or47b-Gal4, 10XUAS-IVS-mCD8-GFP*) and looked for possible *Or47b*>GFP expression on other parts of the body. Antennal expression was as expected, but we did not see fluorescence in legs, wing margins, or ovipositor. If *Or47b* is expressed somewhere other than antenna, it must be at a very low level.

Few olfactory receptors have relatively specific odorant ligands. Interestingly, four of our top five most statistically significant hits have been associated with relatively specific ligands in previously published literature. Or71a is activated by 4-ethyl guaiacol, Or47b is activated by methyl laurate, Or49a by two wasp semiochemicals, and Or7a is activated by the pheromone 9-tricosene. Given our SSR findings showing that chemogenetic activation results in low level firing of OSNs (Chemogenetics Chapter 2), we tested the chemicals listed above at low concentrations to determine if our assay results behaviorally recapitulate natural low-level stimulation from the identified OSNs' specific ligands. Or49a was not tested because wasp semiochemicals are not commercially available (they were synthesized in house for the published study ([123])). The behavioral responses of chemogenetic activation recapitulated low concentration chemical activation for Or71a and Or7a OSNs, but not for Or47b OSNs (Figure 3.6). This could be because methyl laurate, the odorant used to activate Or47b neurons, while relatively specific also activates one other class of OSNs that express Or88a. It is possible that the combinatorial activation of both Or47b and Or88a OSNs accounts for the difference between chemically and chemogenetically generated behaviors.

3.3 DISCUSSION

3.3.1 Assay design can influence behavioral results

Using our novel chemogenetic approach, we have systematically screened through twenty-three olfactory receptor Gal4 (*OrX-GAL4*) lines to identify olfactory inputs involved in female oviposition. Chemogenetic activation of neurons was sufficient to

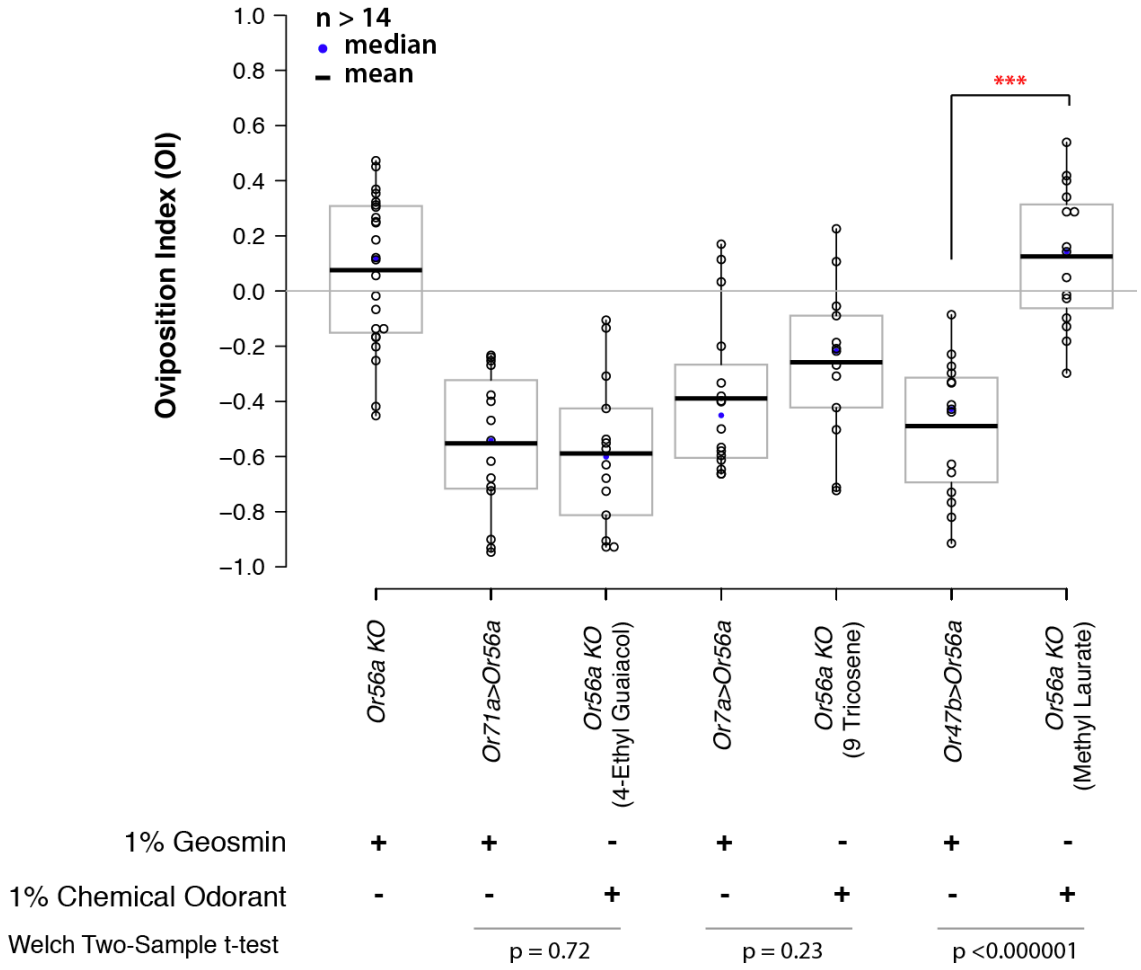


Figure 3. 6 Chemical vs. Chemogenetic activation of olfactory sensory neurons.

Comparison of chemical and chemogenetic activation of OSNs with identified specific odorants. Or71a and Or56a responses are statistically the same. However, Or47b responses are different ($p < 0.001$) possibly because methyl laurate also activates the Or88a receptor.

```
        Simultaneous Tests for General Linear Hypotheses

Multiple Comparisons of Means: Dunnett Contrasts


Fit: aov(formula = OI ~ Or.Order)

Linear Hypotheses:
                        Estimate Std. Error t value Pr(>|t|)
Or71a - Or56a KO == 0 -0.62825    0.10868  -5.781  < 0.001 ***
Or47b - Or56a KO == 0 -0.56530    0.10868  -5.201  < 0.001 ***
Or49a - Or56a KO == 0 -0.52862    0.10467  -5.050  < 0.001 ***
Or67b - Or56a KO == 0 -0.47257    0.10657  -4.434  < 0.001 ***
Or7a - Or56a KO == 0  -0.46461    0.10657  -4.360  < 0.001 ***
Or85d - Or56a KO == 0 -0.44213    0.11674  -3.787  0.00384 **
Or85a - Or56a KO == 0 -0.37462    0.10868  -3.447  0.01337 *
Or82a - Or56a KO == 0 -0.37068    0.12023  -3.083  0.04164 *
Or59c - Or56a KO == 0 -0.36517    0.10296  -3.547  0.00937 **
Or83c - Or56a KO == 0 -0.35698    0.11104  -3.215  0.02788 *
Or56a - Or56a KO == 0 -0.34074    0.11371  -2.997  0.05383 .
Or42a - Or56a KO == 0 -0.30225    0.10657  -2.836  0.08413 .
Or42b - Or56a KO == 0 -0.27154    0.10868  -2.499  0.19431
Or23a - Or56a KO == 0 -0.24254    0.10657  -2.276  0.31172
Or88a - Or56a KO == 0 -0.23941    0.10657  -2.247  0.32985
Or22a - Or56a KO == 0 -0.21179    0.11104  -1.907  0.58314
Or47a - Or56a KO == 0 -0.20284    0.10657  -1.903  0.58682
Or65a - Or56a KO == 0 -0.19250    0.10657  -1.806  0.66638
Gr63a - Or56a KO == 0 -0.12010    0.11104  -1.082  0.99545
Or43a - Or56a KO == 0 -0.11063    0.10868  -1.018  0.99794
Or19a - Or56a KO == 0 -0.10545    0.11104  -0.950  0.99922
Or67d - Or56a KO == 0 -0.08304    0.10467  -0.793  0.99995
Or35a - Or56a KO == 0 -0.08208    0.10868  -0.755  0.99998
Or92a - Or56a KO == 0  0.14343    0.09997   1.435  0.91786
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
(Adjusted p values reported -- single-step method)
```

Table 3. 1 Summary of comparison statistics for oviposition screen.

Post hoc Dunnett's many-to-one comparisons test compared to Or56a Knockout control.

produce statistically significant olfactory behaviors in oviposition (Table 3.1). Our five top statistically significant hits ($p < 0.001$) correspond to neurons expressing Or71a, Or47b, Or49a, Or67b, and Or7a. 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 [29, 123, 124]. Three of the five top hits also have been directly linked to oviposition. Or71a was reported to mediate attraction to dietary antioxidants, Or7a allows males to contribute to female egg laying decisions through the use of 9-tricosene, and Or49a is used to avoid laying eggs in substrate inhabited by parasitic wasps.

Interestingly, we only saw statistically significant negative oviposition behavior. Stimulating Or92a OSNs, neurons that contribute to attraction to apple cider vinegar [125], is the only behavioral result that yielded a positive average oviposition index, but this result was not statistically significant. Or71a, Or19a, and Or7a have been behaviorally shown to detect positive oviposition cues, however, our OSN activation screen produced no attractive oviposition when we chemogenetically stimulated these classes of OSNs. This can be explained in several ways.

As mentioned in the introduction of this chapter, parameters of behavior assays can drastically 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 individual chemical components of a naturalistic odor such as fly food can interact with each other in unpredictable and complicated ways. The results from human psychophysics studies suggest that odor

mixtures oftentimes form a gestalt perception from which humans have difficulty discriminating individual components of the mix [126], and insect studies show that background odor can change behavior and physiology of olfactory neurons [14, 127, 128]. This explains why the activation of Gr63a OSNs produced a neutral oviposition behavior. If the hypothesis that activating CO_2-sensing OSNs causes obligate, or at least strongly, repulsive behavior were true, this would be a surprising result. However, the neutral rather than negative response seen in our oviposition assay adds to the increasing evidence that context plays an important role in olfactory behavior [9, 94, 129, 130].

Or7a was discovered to be involved in oviposition in our lab. In Lin et al, 2015 [28], Or7a and 9-tricosene were shown to positively induce egg-laying. However, the equivalent experiments conducted for this study show the opposite. This most likely results from differences in how experiments were conducted. The experiments from the present study were carried out in the highly controlled environment of an incubator while the original experiments were conducted in a room. Humidity seems to have been an important factor for producing consistent chemogenetic data. There were also differences in technique. Liquid warm agarose was pipetted in this study while agarose was poured into wells for Lin et al 2015 [29].

Why did we only see negative oviposition? Foraging cost seems to be a major consideration of female flies when choosing an oviposition site. The initial ovipositional chemosensation study [11] that observed sugar avoidance and increased egg laying on lobelline infused substrate used assay chambers that were 19 mm in diameter. Since the preference for a bitterant seemed counterintuitive to the general observation that *Drosophila* exhibit attraction to sucrose and avoidance to bitter compounds, Schwartz et

al extended this avenue of study [114]. They found that using larger plates with a 35 mm diameter resulted in preference for sucrose instead of lobeline, reversing the result previously observed in 2008. The spatial dimensions of the region that flies can explore in our assay are 75 mm x 25 mm x 4 mm (Figure 3.7). This corresponds more closely to the larger assay from the 2012 study so it is unlikely that the repulsion seen in our data is derivative of our assay mimicking small patch size. Instead, we hypothesize that since the three-well oviposition assay is agarose-based rather than food-based, we are likely minimizing the olfactory background and getting low-level activation of single classes of OSNs that project to a single glomerulus. This could be significant because it has been shown that low levels of activation of single OSN types can lead to aversion [131]. It is possible that attraction requires the activation of several olfactory inputs and glomeruli rather than a single glomerulus. This is considered further in the main discussion.

3.3.2 Importance of inter- and intra- specific chemical cues in oviposition

Oviposition is a highly complex behavior where female flies need to integrate information from many senses [11, 114-116]. In particular, olfactory cues provide diverse information regarding many features of an egg laying substrate [26-28]. In the laboratory, flies' exposure to hazardous stimuli and diverse environments is limited, but evolutionarily, *Drosophila* exists in a complex ecological space with many food and oviposition site options, and in addition the presence of predators and non-predator interspecifics must be taken into account [132]. Chemosensory interactions between these different organisms along with the yeast and microbial milieu that exists on rotting food substrates necessitate the ability to process many types of chemical cues coming from many different types of organisms. When an organism needs to be able to detect another

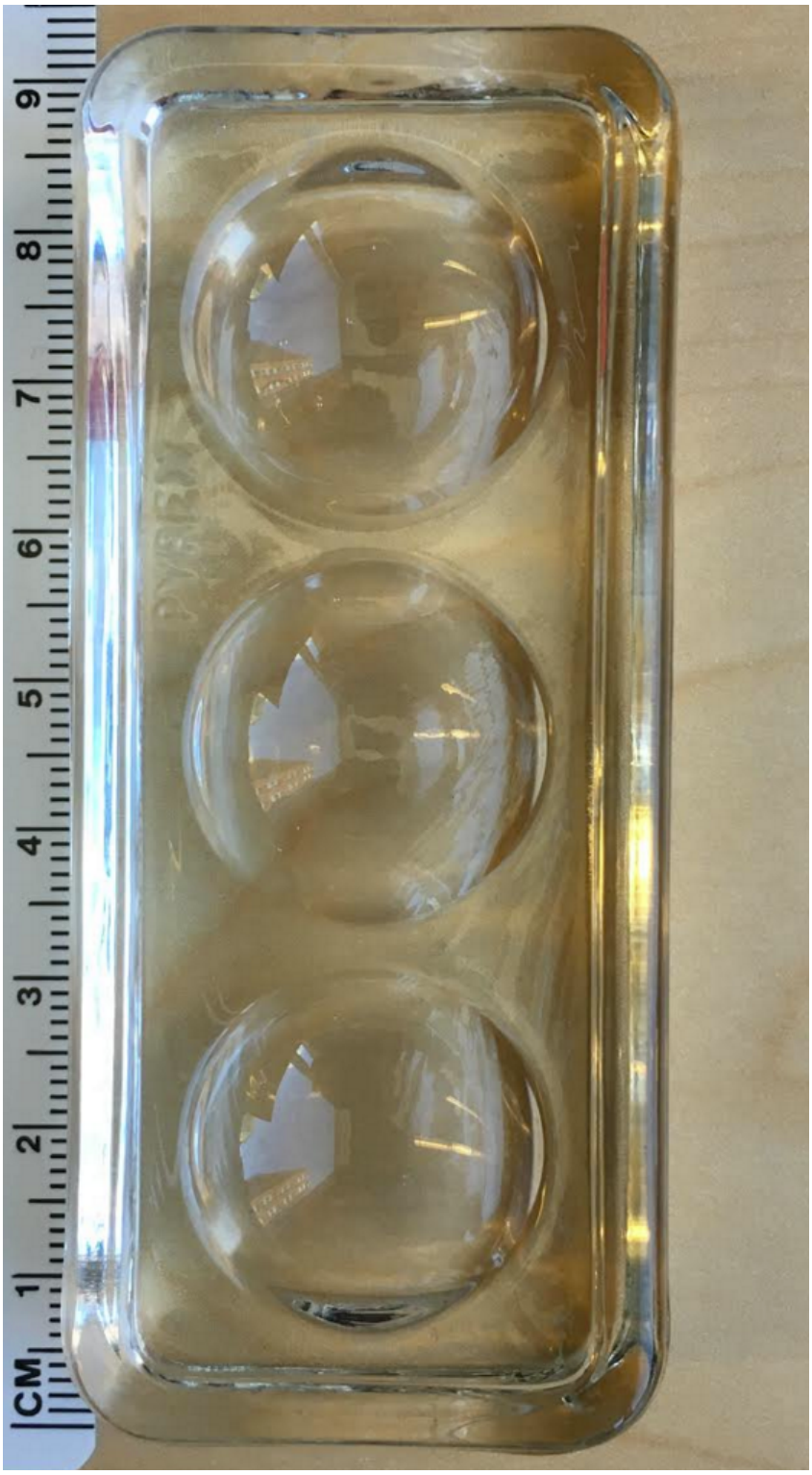


Figure 3. 7 Dimensions of three-well oviposition assay.

Space in which flies can freely move is approximately 75 x 25 mm.

species, it oftentimes co-opts a chemical emitted by the object of detection as an allelochemical [98, 100]. This is relevant to our study because expanding our analysis of OSNs involved in negative oviposition ($p < 0.05$) indicates that the olfactory receptors identified in our oviposition behavior screen detect compounds such as farnesol, 9-tricosene, and other chemicals that have been identified to be semiochemicals in other insect species (http://neuro.uni-konstanz.de/DoOR/content/). This suggests that the main concern of female flies, in our assay conditions, is to avoid laying eggs on substrate where other organisms may have previously occupied.

As argued in the paper that identified Or49a as a direct detector of wasps, wasp semiochemicals can be used to avoid predation and parasitism of larvae [123]. Studies have shown that hosts of plant-eating insects can release chemicals that attract protective parasites [133]. One such chemical is E2-hexenal, which is both a leafy green volatile (smells grassy) and is used as an aggregation pheromone, or allomone, by many insects including bed bugs and parasitic wasps [134-136]. Plants have been shown to release E2-hexenal in response to cellular damage as an attractant to parasitic wasps, presumably to reduce the population of the insects damaging the plant. Thus, chemicals like E2-hexenal could be used by *Drosophila* as an indicator, by proxy, of the presence of parasitic wasps.

Another benefit of avoiding semiochemicals is to reduce competition for survival of a female's offspring. A crowded food source could potentially be depleted before larvae become adults, resulting in malnutrition and decreased fitness for mating and survival. At the extreme, larvae raised in starvation states have even been shown to resort to conspecific predatory cannibalism whereby early instar larvae will attack and consume larger, less mobile, wandering third instar larva on the cusp of pupating [137].

It is worth noting that, given the complicated nature of semiochemicals as illustrated by the case of E2-hexenal, we should urge caution when exclusively attributing a chemical signal to a specific use. For example, Or71a is thought to mediate attraction to proxies of dietary antioxidants produced by *Brettanomyces* yeast, but the strongest activator identified in the study, 4-ethylguaiacol, has been identified as a component in the male sex pheromone mixture of cockroaches [138]. It is impossible to know what ecological context the fly uses to interpret the perception of this smell. We tend to assume a receptor's ethologically relevant ligand is its strongest activator, but recent studies, along with those discussed above, suggest that low levels of olfactory activation can be relevant drivers of behavior [68].

3.4 EXPERIMENTAL PROCEDURES

Fly Stocks: Wildtype 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 Lin & Potter 2015, Table 1. *Or56a* mutant and *UAS-Or56a* lines were generated as detailed in experimental procedures section of Chapter 2. Flies used for *Orco* mutant experiments contained two different alleles as reported in Larsson et al 2004.

Oviposition Assay: Equal numbers of female and male adult flies were collected within 24 hours of eclosion and group housed on fly food for three days. On Day Four, all flies were transferred to a vial with only yeast paste (powder baker's yeast + propionic acid) to prime females for egg laying. 50 mL of 1% agarose in double distilled water was allowed

to cool to precisely 65°C. 1 uL of odorant 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 No 7223-34 - discontinued (20 drops per well); Replica three-well spot plate printed in porcelain with matte black or polished white finish through Shapeways (14 drops per well)) using a pipet-aid with a 10 mL serological pipette (Danville Scientific, Part No: P7134). Flies were briefly anesthetized on ice for 3-5 minutes, and males were removed. ~10 female flies were tapped onto each spot plate and the lid of a 100x20 mm tissue culture dish (Corning Incorporated, Product No: 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 5 and 7 pm, and flies were incubated on the assay in a dark, humidified incubator at 25°C and 89-94% humidity for 22-23 hours, 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 (# of eggs in well/number of flies). We discarded experiments in which flies laid fewer than 8 eggs/fly/day.

Oviposition index was calculated as follows:

$$OI = (O - NO^{avg})/(O + NO^{avg})$$

OI = Oviposition Index

O = # of eggs in well containing odorant

NO^{avg} = Average # of flies between two vehicle control wells

We also analyzed the data according to percentage where no preference would mean that flies laid equal proportions of eggs in each of the three wells so that the odor well should

have 33% of the total eggs laid.

> *Nota bene:* We found that maintaining high humidity (RH = 84-91%) was very important for getting reliably large number in egg laying.

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 posthoc Dunnett's Many-to-One Multiple Comparisons test, with each experimental group compared to the Or56a knockout control, indicates that 9 out of the 22 tested OrX-Gal4 lines statistically significantly induce aversive behavior in our oviposition assay. These tests were all run in R.

Chemicals: Geosmin in methanol was purchased from Sigma-Aldrich at highest available purity, ≥97% (Product #: G5908-1ML, Lot #: BCBP7178V). Chemical as received was dried out to remove methanol and then diluted to 4 mg/mL in mineral oil (Sigma-Aldrich, Product #: 330779-1L, Lot #: MKBF6530V). Methyl laurate (Product #: 234591-2.5G, Lot #: BCBQ6830V) and farnesol (Product #: F203-25G, Lot #: MKBG0101V) were purchased from Sigma-Aldrich.

4. Anatomical Tracing in the Lateral Horn

4.1 INTRODUCTION

4.1.1 Higher order olfactory processing across taxa

How does the brain process olfactory information? Anecdotally, humans have difficulty verbally describing the estimated 4500 to a trillion odorants [139, 140] that we may be able to detect [126]. This attests to the idea that olfactory processing is a complex task, and human psychophysics does not provide clues as to what the brain does with raw information to generate a percept.

OSNs collect chemical information directly from the environment and send those signals to secondary neurons. One of the purposes of early neurons in the olfactory circuit seems to be sorting odors by function. Zebrafish accomplish this through the use of a 'chemotopic' map where the olfactory bulb appears to be segmented into regions based on chemical structure [141]. However, the neural maps of early olfactory processing in rodents and insects are weakly chemotopic at best [77, 142]. Instead, olfactory inputs may be sorted by biological function. In rodents, this begins at the level of OSNs in that there are at least four distinct neural structures that seem to detect odorants. The two largest and best studied are the main olfactory epithelium (MOE) and the vomeronasal organ (VNO). The MOE is the largest olfactory sensory region in rodents and seems to contain broadly tuned OSNs whose organization is largely unknown except for a class of OSNs that express trace amine-associated receptors (TAARs). These TAAR OSNs respond to compounds in cat urine, which are highly aversive to mice, and they innervate a distinctly defined region in the main olfactory bulb (MOB) where second order neurons receive information from MOE OSNs [143, 144]. The vomeronasal organ seems to detect

conspecific pheromones and also compounds that indicate physical health in social peers [35, 145]. Axons from the VNO innervate the accessory olfactory bulb (AOB), which further segments conspecific olfactory inputs based on sex and state of sexual maturity [146].

4.1.2 Functional information sorting in the lateral horn

Strikingly, the division between pheromonal and general odors seems to be paralleled in the insect olfactory system. Using mosaic analysis with repressible cell marker (MARCM), many of the projection neurons that are included in the expression pattern of the driver line *GH146-Gal4* have been traced and mapped [34, 47]. *GH146-Gal4* expresses in approximately 60% of all excitatory projection neurons [34, 147]. Warping the traces into a standard coordinate system allowed for the precise comparison of innervation patterns of these PNs in the lateral horn. Since the OSN to PN connection is well understood and the two are reliably connected to the activity of the olfactory receptors that activate them, PNs were thus matched with classes of odorants that stimulate them [34, 148]. These classifications taken in light of PN innervation patterns demonstrated that the lateral horn appears to be divided into two sections denoting food and pheromone odors. Several subsequent studies suggest that there may be domains for attraction and repulsion in the lateral horn [23, 149], but no definitive domains have been identified.

A few specific odorants have been identified (CO_2, acid, and geosmin) that lead to selective activation of olfactory neurons and aversive chemotaxis [22, 28, 94]. In addition, the chemogenetic experiments reported in Chapter 3 identified classes of olfactory neurons that can drive negative oviposition. In this chapter, we asked if the projection

neurons most likely activated by these OSNs exhibit shared innervation patterns, which might indicate shared olfactory processing centers in higher brain regions.

4.2 RESULTS

4.2.1 Mapping projection neurons in the lateral horn associated with repulsion

V PN – Carbon dioxide is an odorant that has been identified as robustly aversive to flies [94]. Flies release CO_2 as a stress odorant, and high concentrations of CO_2 can pose a survival hazard and render flies unconscious. The only OSN – ab1C – that expresses gustatory receptors (Gr63a/Gr21a) senses CO_2. These OSNs send axons to the V glomerulus. V PNs were not mapped in the original GH146 study since the V PN is not $GH146^+$ and has not been registered (see Experimental Procedures) onto a standard brain in any subsequent PN studies. The driver line *NP7273-Gal4* includes the V PN, so we used MARCM to sparsely label, trace, and map the 'aversive' CO_2 neuron (see Experimental Procedures, below). MARCM relies on the activation of a heat-shock inducible Flippase from yeast (FLPase) to properly recombine genetic components during development to sparsely label neurons in a larger expression pattern. The 'birthdate' of the V PN was previously unknown, and we determined that the V PN is born either at a late embryonic stage or very early during larval stages of development. Therefore, heat-shocking between 0-1 hours of larval hatching yielded MARCM-labeled V PNs. Alternatively, the V PN was expressed in an enhancer trap screen QF line (*12B-QF*) and could be sparsely labeled through intersectional genetics with *NP7273-Gal4*. The *12B-QF* line was unfortunately lost, but traces derived from this intersectional genetic combination were used in the statistical analysis described below (traces from Chun-

Chieh Lin, Potter Laboratory). Therefore, we have included the genotype in our Experimental Procedures.

The V projection neuron has a distinctive axon that does not follow either of the neuronal tracts projection neurons usually use to enter the lateral horn (Figure 4.1). The axonal innervation of the V PN in the lateral horn is also distinctive in that it seems to cup the outer, medial edge of the lateral horn.

DA2 PN – The geosmin-sensing Or56a neuron has also been shown to mediate aversion ([28] and Chapter 2). Or56a neurons contact DA2 PNs in the antennal lobe. Since this PN is GH146+, we used GH146 MARCM to label, trace, and map DA2 PNs (Figure 4.1). We heat-shocked at 86-88 hours after larval hatching, as reported in Lin et al 2012 [150].

The DA2 PN also has a distinctive cup-shaped final innervation pattern, and when the warped traces of the geosmin and CO_2 sensing PNs are examined together on a standardized coordinate system, they appear to innervate a similar area of the lateral horn.

DL4 PN – A third OSN type that expresses acid-sensing Ir64a has been described as mediating aversive behavior [22]. The DL4 PN associated with Ir64a OSNs was visualized using photoactivatable GFP, but the neuron trace has not been registered. Nonetheless, qualitatively, the neuron trace reported appears as though it might also

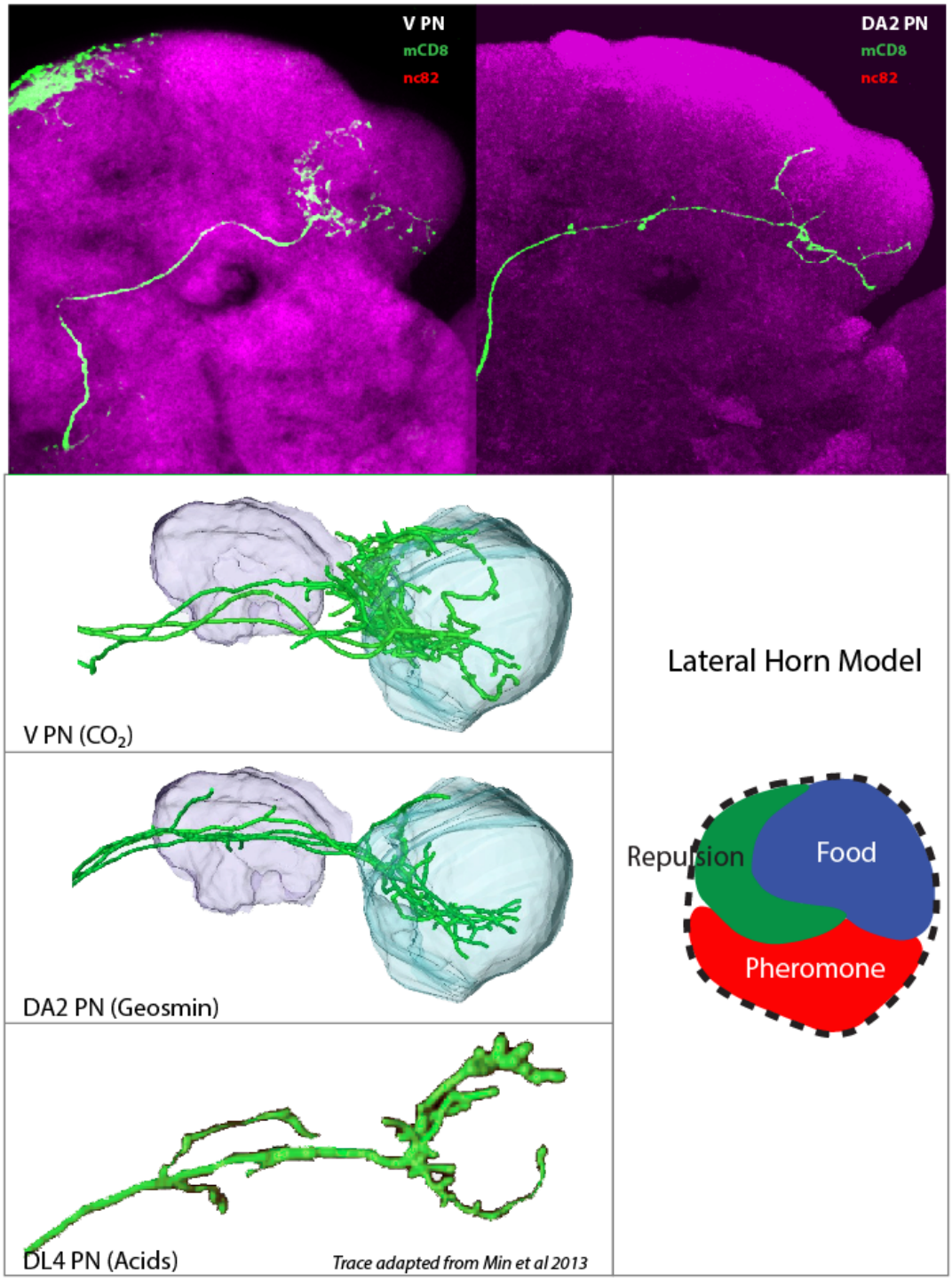


Figure 4. 1 Projection neurons associated with aversive behaviors.

Cup shaped morphology is associated with repulsion. Top: Confocal images of individual neurons labeled using MARCM. Left: Traced neurons with lateral horn and mushroom body calyx. Right: Hypothesized Model of the Lateral Horn.

innervate the cup shaped outer edge of the lateral horn. Based on this visually striking morphology and the behavioral data reported in the literature, we hypothesized that the outer medial edge of the lateral horn is a region that functionally encodes repulsive olfactory stimuli (Figure 4.1 Model).

4.2.2 Projection neurons in the lateral horn involved in negative oviposition

We next analyzed PNs that correspond to our chemogenetic oviposition behavior data set. Negative oviposition PNs seem to inhabit a central region of the lateral horn (Figure 4.2). While it appears that some of the PNs of interest may assume the 'cup' morphology in the LH that we hypothesize denotes repulsion, others do not. For clarity, we visualized candidates according to significance level when compared to the *Or56a* knockout (no trace for Or49a PN DL4). Close examination of these traces suggests that a negative oviposition domain may exist in the posterior region of the lateral horn coinciding with innervation of one branch of the bifurcating cup shaped neurons (Figure 4.2). The exception to this trend is the DC1 PN, which responds to Or19a OSNs (Figure 4.3). Its innervation pattern seems to be interspersed with the negative ovipositions OSNs, but no egg laying phenotype was seen in the chemogenetic oviposition assay.

4.2.3 Possible distinct region in lateral horn encoding negative oviposition

Statistical analysis was performed on the negative oviposition PNs to compare their projection patterns to each other as well as other PNs. The analysis calculates distance between the processes of two neurons so that a distance of '0' describes two identical neurons, and a distance of 1.8 is the maximum difference between two neurons in the lateral horn [151]. Negative oviposition neurons appear to be statistically similar to one

another when compared to other neurons, with an averaged calculated distance amongst the oviposition projection neuron of 0.69 (Figure 4.4).

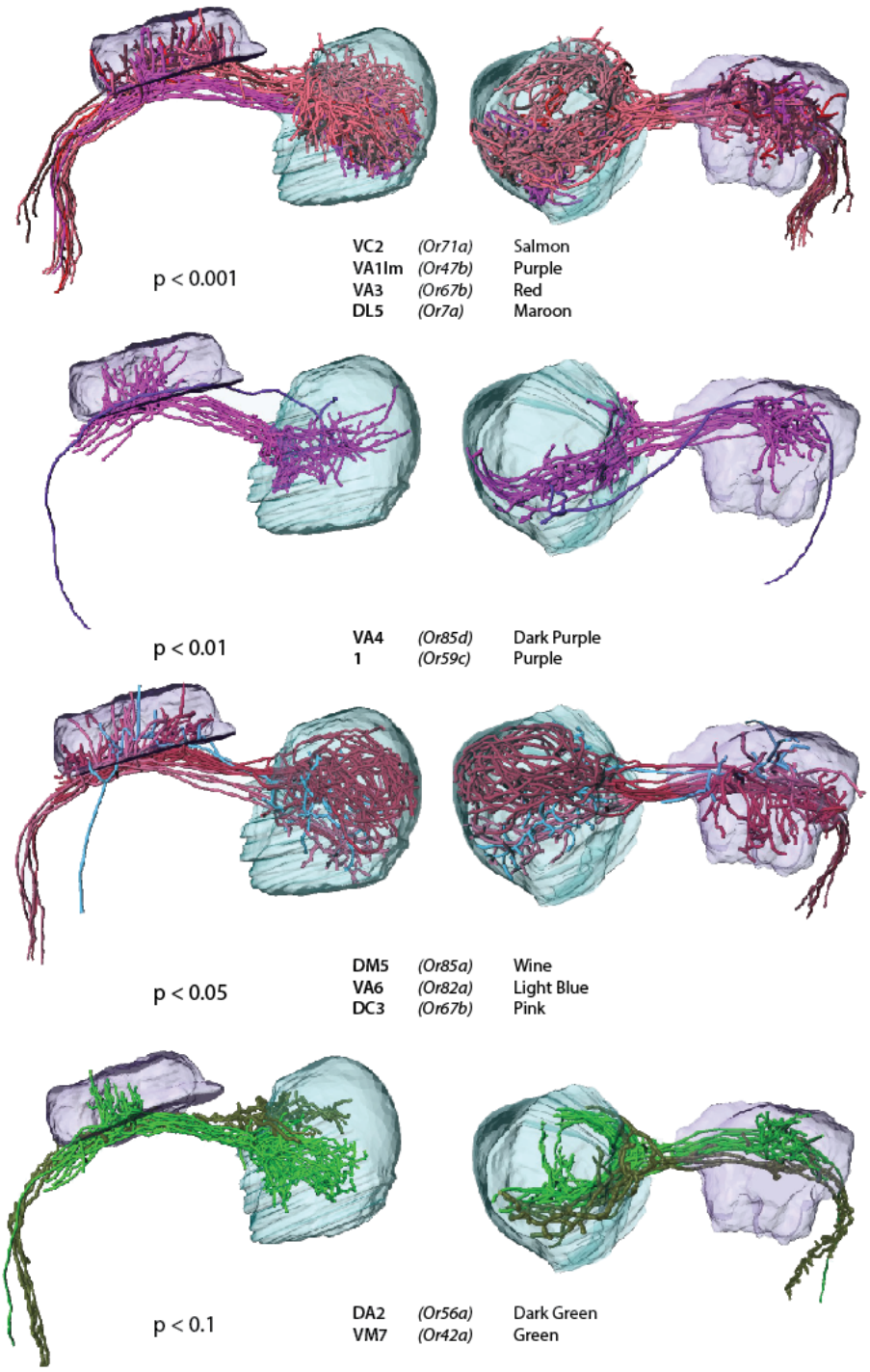


Figure 4. 2 Projection neurons associated with negative oviposition.

Traces are sorted according to statistical significance level. Left column: Anterior view of LH and MBC. Right column: Dorsal-posterior view of LH and MBC. Branch in this region of LH seems to be common feature in negative oviposition PNs.

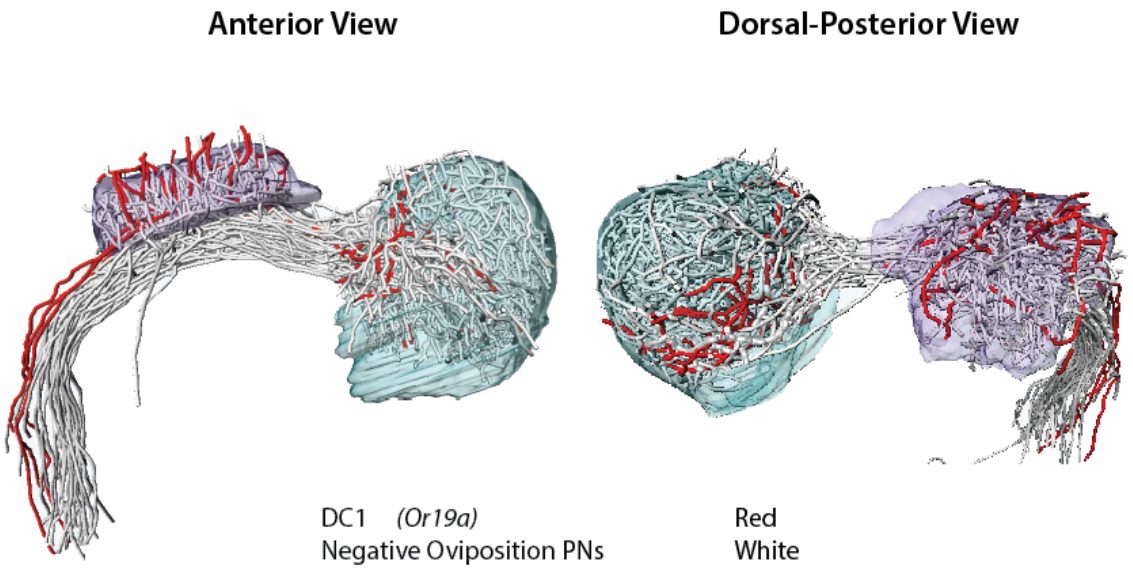


Figure 4. 3 DC1 does not follow negative oviposition trend.

Despite having the dorsal-posterior branch, Or19a OSNs mediated neutral oviposition in our assay.

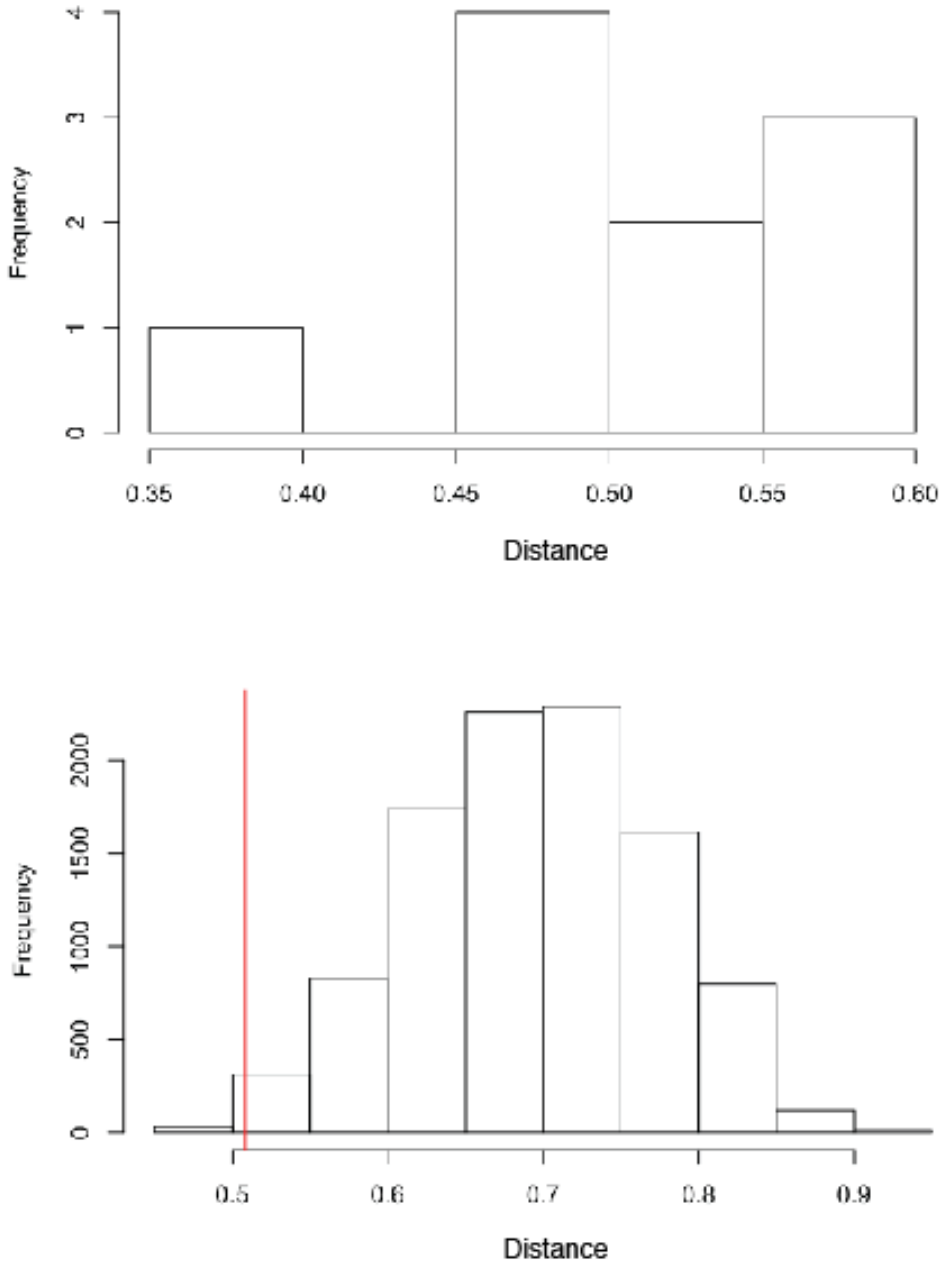


Figure 4. 4 Statistical analysis of negative oviposition PN data.

Top: Distance calculations between top negative oviposition hits. Bottom: Distance analysis from random sampling all PN traces 1000 times. Red line is average of negative oviposition PNs. Value of 0 indicates two identical neurons, farthest possible distance value ('most different') is 1.8.

4.3 DISCUSSION

4.3.1 Lateral horn domains may functionally segment projection neurons

The initial study looking for an olfactory neural map identified two 'domains' in the lateral horn: Food and Pheromone. While it has been speculated that the lateral horn may have a repulsion domain, and that this would be encoded in the brain as a labeled line, our data indicate that the actual PN organization may be more complicated than simple positive or negative valence. Activation of Or56a OSNs with geosmin in wild type flies has been reported to mediate aversive behaviors in the trap assay and an oviposition assay that included fly food. However, testing wild type response to geosmin in our agarose-based oviposition assay resulted in mild negative oviposition that narrowly missed the $p < 0.05$ significance level ($p = 0.053$). The chemogenetic activation of Gr63a-expressing OSNs, which normally detect 'repulsive' CO_2, yielded a neutral oviposition index. We typically think of repulsion and attraction categorically since these responses are thought to be the purest of 'innate' behaviors, but it is possible that absolute attraction and repulsion do not exist. That is, for example, that activation of 'repulsive' CO_2 or geosmin sensing neurons will only lead to repulsion under particular contexts [9, 94, 129]. While some PNs associated with negative oviposition have the cup-shaped morphology that we had previously associated with aversive olfactory behaviors, this is not true in all cases. However, it appears that those that do have a cup morphology may signify a bifurcation with the dorsal posterior branch signifying negative oviposition (Figure 4.2, 4.5). This implies that functional domains in the lateral horn may be defined by structurally similar segments of neurons with different gross morphologies, and that these domains may take into account context (in this case, oviposition). Since elements of behavioral reactions to

olfactory sensations such as downstream motor programs may have some similarities for repulsive stimuli in different contexts (i.e. turning away from an aversive stimulus), perhaps positioning segments with conditionally similar functions together may prove more efficient. This is supported by the similarity in shape of DA2 and V PNs to some of the negative oviposition PNs. The anterior branch of cup-shaped PNs may be involved in the neurons' roles in other contexts (Figure 4.6).

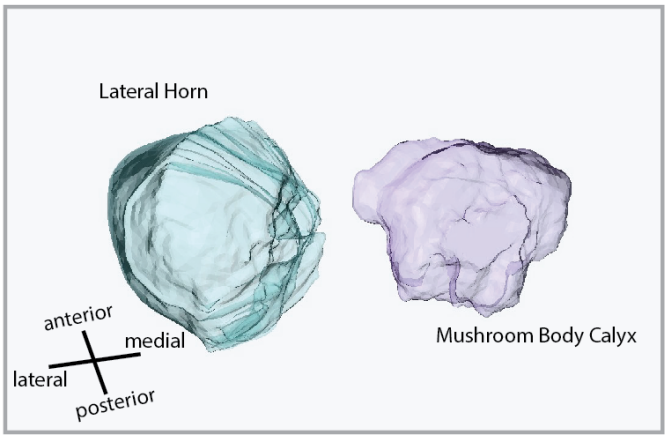
4.3.2 PN Involvement in different contexts of olfactory behaviors

While it does not clearly bifurcate (Figure 4.7), a notable case in which a PN has now been implicated in contributing to multiple behaviors is the VA1 PN, innervated by Or47b OSNs. In females, Or47b seems to facilitate copulation by mediating the female's receptivity to males [152]. Inhibiting Or47b OSNs decreased receptivity in females, suggesting that activation of Or47b OSNs denotes a positive mating cue. The authors of this study hypothesize that Or47b contributes to same-species recognition in both male and female flies, and that activation along with exposure to the pheromone 7-T enhances female receptivity [153]. This underscores the importance of behavioral context in studying olfactory behavior. Literature in the field of fly reproductive behavior demonstrates that copulation and oviposition are two behaviors that directly oppose one another since the reproductive canal is used in both acts [154]. Perhaps activating Or47b OSNs in females induces a behavioral switch that overwhelmingly puts the individual in a pro-copulatory state at the expense of oviposition.

Based on our analysis, it appears that negative oviposition is an important context for innate olfactory behaviors in female flies. Strikingly, as discussed in the Behavior chapter above, many of the odorants detected by negative oviposition OSN and PNs seem to be

Top Negative Oviposition PNs

Olfactory Receptor	Projection Neuron	Color
Or71a	VC2	Purple
Or47b	VA1lm	Green
Or49a	DL4	NA
Or67b	VA3	Red
Or7a	DL5	Blue



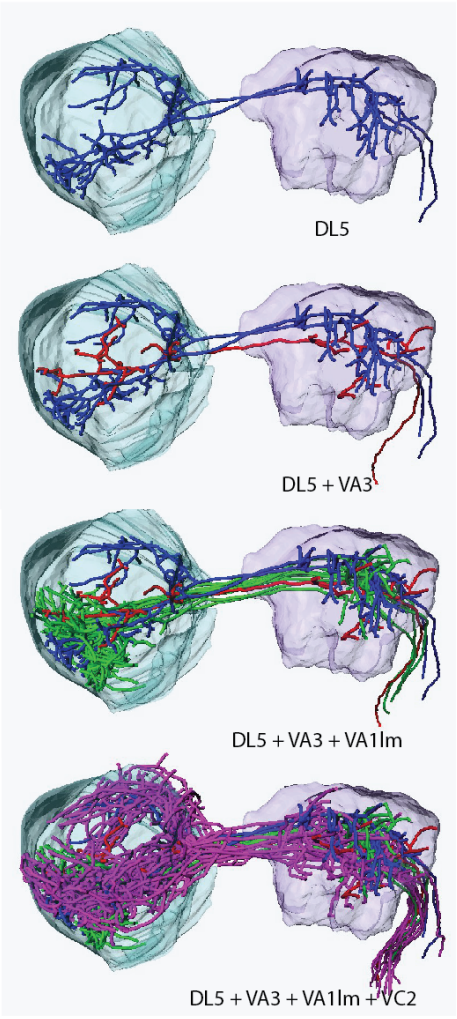


Figure 4. 5 Most aversive oviposition PNs.

Dorsal-Posterior view of four of top five most aversive oviposition PNs ($p < 0.001$), sequentially added according to magnitude of OI. VA3 (red) and VA1lm (green) are not cup shaped, but rather just the dorsal-posterior branch.

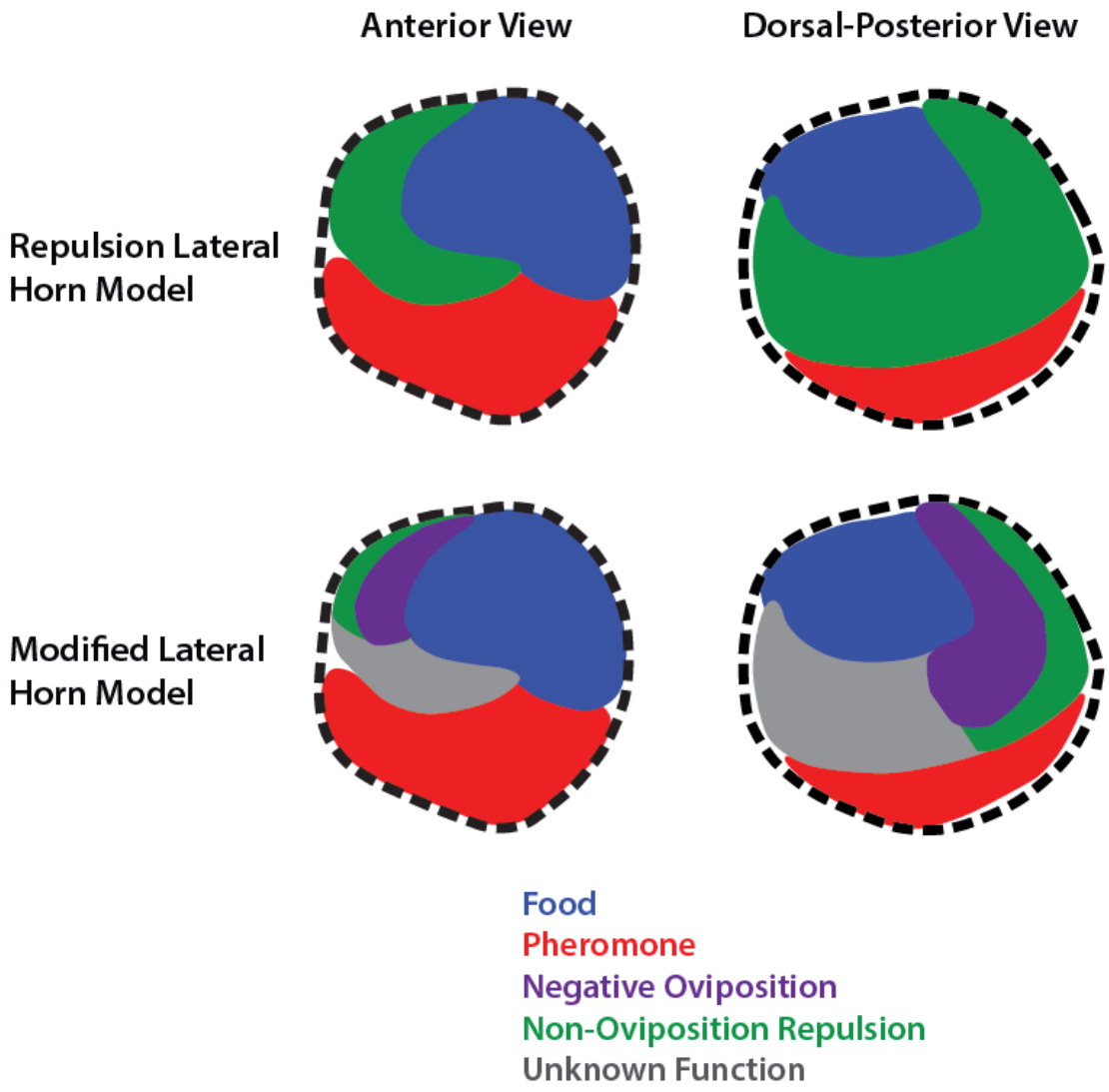


Figure 4. 6 Models of the Lateral Horn.

Lateral horn may be sectioned into functional domains. We propose a negative oviposition domain (purple) that is in the dorsal-posterior region of the lateral horn. It is unclear what the anterior branch of cup shaped neurons signifies.

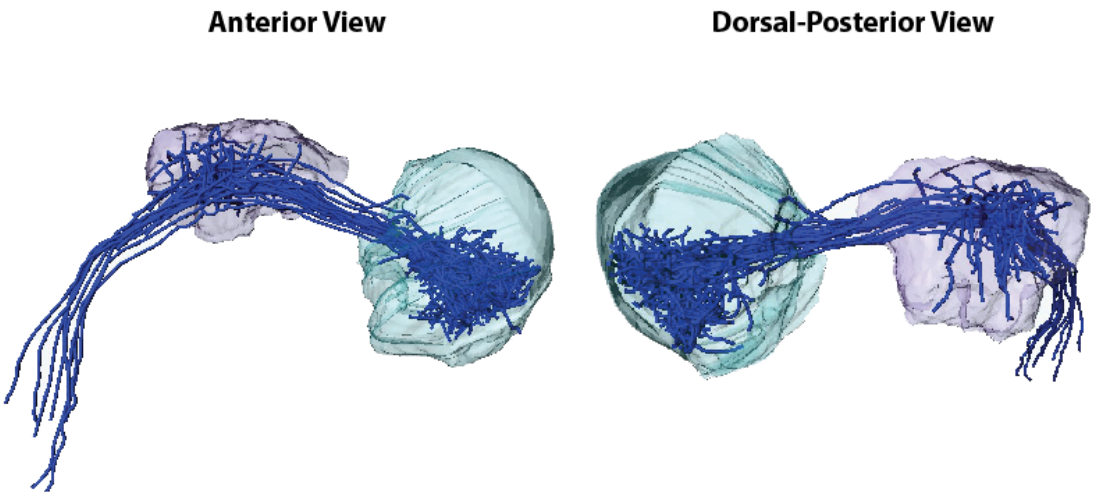


Figure 4. 7 VA1 Projection Neurons.

VA1lm PNs respond to Or47b OSNs. Or47b seems to mediate several different female-specific behaviors.

'animal' in nature. These odorants may be kairomones and allomones – signals that come from other insects that have variable relevance and value to flies. In the context of egg laying, this 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.

4.3.3 Sexual dimorphism in PNs

Oviposition is a female-specific behavior, but males express the same complement of receptors as females. Interestingly, the second strongest mediator of repulsive oviposition OSN identified was Or47b. Male Or47b neurons are positive for the male-specific splice form (fru^M) of *fruitless*, a transcriptional regulator that effects sexual dimorphic behaviors through its expression in neurons. Other fru^M OSNs are Ir84a OSNs which are thought to affect male courtship through food-derived odors, and Or67d OSNs which detect the pheromone cVA and have been shown to affect male courtship and female receptivity behaviors [155-157]. These OSNs connect to PNs that are in the 'pheromone' section of the lateral horn. Or47b is a recently deorphanized receptor that detects the pheromone methyl laurate. Methyl laurate and the activation of Or47b neurons are positive drivers for male mating success and are also involved in facilitating males identifying younger, more fertile mates [158, 159]. As mentioned above, Or47b can also mediate female receptivity as well as negative oviposition.

Both male and female flies express the same complement of receptors capable of detecting the same odor space at the primary level, and no OSN has been shown to have sexually dimorphic responses to monomolecular odorants [160]. As an extension, the antennal lobe exhibits minimal sexual dimorphism, and only three glomeruli are

innervated by fru^M positive neurons [10]. This suggests that most of the negative oviposition neurons identified by our behavioral assay do not grossly differ in anatomy and physiology. How, then, do similar structures between males and females elicit and regulate different behaviors?

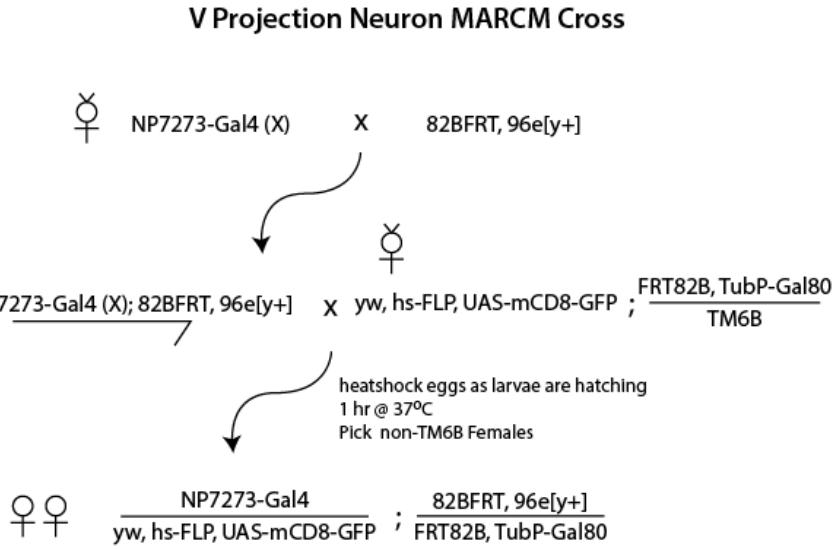
In flies, regions of sexual dimorphism were identified by comparing volumes of major areas throughout the brains of male and female flies. Brain regions identified as different between the sexes were then examined and compared to regions directly innervated by fru^M expressing neurons. It is presumed that differences in *fruitless* neuron connections between males and females indicate sexually dimorphic connections. This analysis indicates that there are neuronal connections that are made exclusively in males or in females, allowing the brain to route sex-relevant information to different central circuits [161, 162]. This same study looked at the neuroblast lineages of the identified sexually dimorphic brain regions throughout, and it was found that as many as one third of regions different between males and females were in regions involved with olfaction, and further that seven of the eight neuroblasts that generate lateral horn projections are sexually dimorphic. This suggests that though detection of odors in female and male flies may be highly similar, the fly brain uses downstream neurons in the central brain to enact sexually dimorphic behaviors, and that olfaction may be a large contributor to sexually dimorphic behaviors. As third order lateral horn and downstream neurons are mapped, it will be interesting to identify how the downstream effectors differ between males in females for our putative negative oviposition region of the lateral horn.

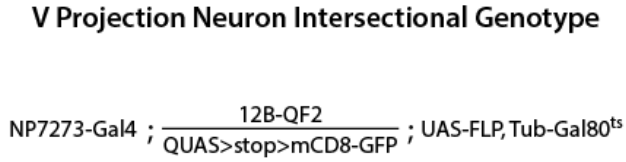
Or47b OSNs and their PN partner VA1 have been discussed above as mediating many sex-specific behaviors such as mating behaviors and oviposition, but these neurons are

clearly molecularly sexually dimorphic through different splice forms of *fruitless*. What function might *fruM* negative 'negative oviposition PNs' be serving in males? A study in *C. elegans* described how, mechanistically, the same olfactory neuron detecting a specific pheromone can elicit different behaviors in different sexes (hermaphrodite vs. male) and behavioral phenotypes (social vs. solitary) [163]. These different behaviors could be opposite between the two sexes (attractive vs. repulsive) and/or existent in one behavioral phenotype but nonexistent in another (repulsive vs. no behavior). This wide range of sexually dimorphic and behavioral possibilities in response to neuronal activation also exists in flies. Or7a OSNs have been shown to stimulate males to geographically tag food-rich locations through the pheromone 9-tricosene [29]. 9-tricosene acts an aggregation pheromone and attracts both males and females. Females presumably come for the food, while large groups of flies signify to the male fly both food and the presence of females to court. In females, 9-tricosene also induces egg laying, allowing the male to contribute to the decision of where a female oviposits. This diverse response to the same chemical further supports the idea that there may be few or no behavioral absolutes in terms of labeled lines in *Drosophila* olfactory circuits. It would be interesting to systematically interrogate these so far female-associated OSNs using chemogenetics to study courtship or foraging and elucidate the behaviors these neurons regulate in males.

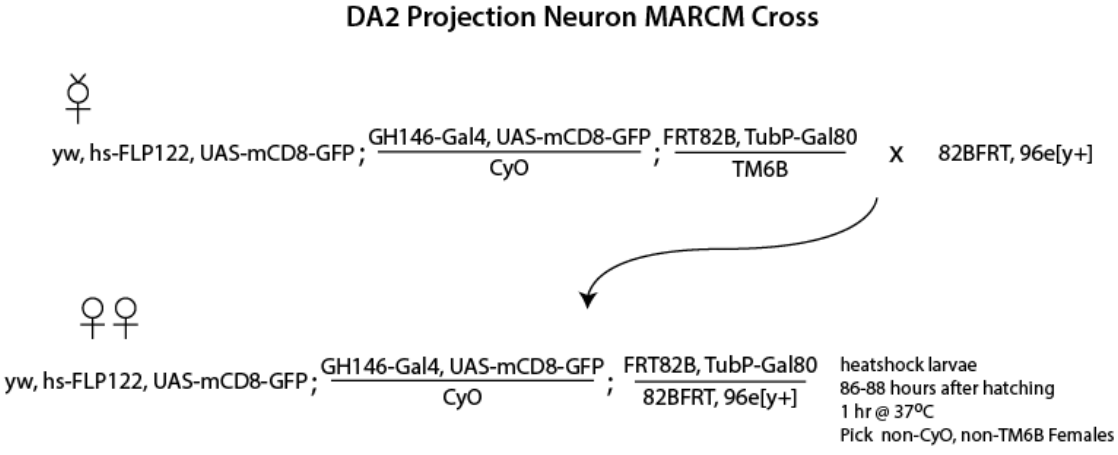
4.4 EXPERIMENTAL PROCEDURES

Crossing schemes for V and DA2 PN MARCM





Flies reared at 18°C. Upon eclosion, adults were transfered to 29°C for 4 days before brain dissections and staining.



Immunostaining and Imaging: Detailed procedures for immunostaining are described in Wu and Luo (2006). Briefly, brains were dissected in 1XPBS, fixed in 4% PFA for 20 minutes, washed over the course of several hours, and blocked in 5% Normal Goat Serum for 30 minutes at RT. Brains were then incubated at 4^{o}C in primary antibody for 3 days. Primary antibodies were mouse α nc-82 (DSHB, 1:25) and rat α CD8 (Caltag Laboratories, 1:250) diluted in 5% Normal Goat Serum. Primary was washed off in three washes of 0.3% PBT over the course of a day, and secondary antibodies were added. Secondary antibodies were incubated for 2-days. The secondary antibodies used were Goat α mouse Alexa568 (Invitrogen Cat# A11031, 1:500) and Goat α rat Alexa488 (Invitrogen Cat# A11006, 1:500). Finally, secondary was washed off in 0.3% PBT over the course of several hours, and brains were mounted whole in SlowFade Gold (ThermoFisher).

Confocal images were obtained using the Zeiss LSM 700 Confocal microscope.

Warping/Registration and Tracing: LSM confocal files were processed in FIJI by splitting the two channels (red and green) and converting each channel into the .nrrd file format. Brain registration was performed using CMTK Registration software and methods described in Jefferis et al 2007 with Cell07 reference brain but using new parameters detailed in Cachero et al 2010. Registration maps neuron traces onto a reference brain. 'Affine' parameters transform the x-y axes, and 'warp' transforms the z axis. Successfully warped images were converted to .am files using FIJI and imported into Amira 5.5.0. Traces were generated using Amira's automated Filament Editor combined with manual correction for inaccuracies.

Statistical Analysis: Negative Oviposition PNs were compared to each other using a 'distance' measurement generated by the R package Nblast. We first compared the top five negative ovipositions PN to each other and generated a mean distance (top Figure 4.4). The program then randomly sampled 10,000 times, groups of five projections neurons (bottom Figure 4.4). The red line on the r10000 is the original mean distance for the top five negative oviposition PN. The probability estimate of obtaining this result randomly is 0.0035. This analysis was programed and performed by Paavo Huoviala from Greg Jefferis's lab.

5. Discussion

Olfaction is considered one of the most primal senses because all animals use chemical information to interact with their environments. The olfactory system has the ability to generate a percept that drives integral survival behaviors such as finding nutritious food, identifying an attractive mate, and avoiding ingestion of disease-causing microbes and toxins. The vinegar fly *Drosophila melanogaster* is a highly smell-driven animal whose olfactory system has been studied dating back to the early twentieth century when William Barrows began comparing behavioral reactions between monomolecular and mixes of odorants [164]. In the past century, further studies of olfaction have shown that olfactory cues greatly influence a wide range of fly behaviors including those mentioned above and also oviposition, courtship, aggregation, flight, and aggression [3, 9, 26, 158]. The chemical world is one chockfull of information with an unimaginably large number of compounds. How does the brain make sense of it all?

5.2 Hypotheses for odor coding

There are several alternative hypotheses about how the brain processes incoming olfactory information. The 'labeled line' hypothesis perpetuated 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. Some postulate that most receptors have a 'most relevant' ligand yet to be identified [73]. This is probably not the case since the olfactory world of an animal like the vinegar fly likely contains more important biologically relevant stimuli than olfactory neuron types. As evidenced by contradicting behaviors seen in different assays (including ours; Table 2.1 from Chemogenetics chapter), even olfactory circuits previously thought to be labeled

lines for attraction or repulsion do not absolutely produce the same behavior in all contexts. All together, this indicates we should shift how we think about the rigidity of 'innateness'. Instead, every result should be considered in its behavioral context, and studies like ours that look at organization of an 'innate' center of the brain like the lateral horn should be studied in respect to ethological behaviors (egg laying, courtship, foraging) rather than searching for absolutes for all situations (attractive or repulsive).

While few dedicated channels have been identified, the much higher prevalence of broadly tuned odorant receptors and promiscuity with which odorants bind Ors suggests that it is more likely that odorant information is processed via a combinatorial code [73]. Within a combinatorial code, odorant identity and the generation of behavior from that information potentially happens in several different ways. The olfactory lobe could linearly summate all inputs from activated and inhibited glomeruli and/or use coincidence detection of simultaneously activated OSNs to determine odor identity [68, 100, 130].

5.3 Lateral inhibition in the antennal lobe

Generally, we treat each type of OSN independently with the underlying assumption that driving inputs into a specific glomerulus linearly causes activation in a labeled line fashion [26-28, 123, 124]. The Wilson lab has published a large body of work showing that neurons innervating one glomerulus tend to interact with neurons innervating another glomerulus, and that the activation of one glomerulus can inhibit or excite other glomeruli [36]. By recording from PNs in the antennal lobe [165] it was found that this modulation is weighted rather than general [166]. This is achieved through both excitatory and inhibitory local interneurons (eLNs and iLNs) [36]. A subset of excitatory local interneurons has been shown to form gap junctions with multiple PNs [167]. These

interneurons typically innervate many glomeruli and serve a gain control function to boost weak signals and reduce noise in stronger olfactory signals. eLNs also connect with iLNs, in the form of chemical synapses, and this connection is stronger than that of eLNs and PNs [168]. This interconnectedness of PNs, eLNs, and iLNs is thought to prevent saturation of the PN signal towards odors [36]. Interconnectedness could also be a functional property, as seen in moths. Some interneurons in the moth antennal lobe are blend-specific and need particular combinations of OSN activation in order to respond [169]. Based on our results using Or56a-Geosmin chemogenetics, we speculate that low level OSN activation minimally engages the typical levels of antennal lobe lateral inhibition in response to high concentration or generalist odors. If true, we are studying relatively linear OSN inputs that are unmodified by surrounding neurons. To our knowledge, there are no studies that perform a large-scale systematic screen to examine OSN inputs in this fashion.

5.4 Context dependence of valence representation in the antennal lobe

It appears that the background odor environment is quite important for olfactory processing [72] Badel et al [130] used *in vivo* calcium imaging to record antennal lobe activity in an awake behaving flies. This was valuable because the experimental setup generated directly matched neuronal activity and behavioral data. The authors modeled the matched datasets by mapping the calcium imaging data onto their olfactory valence behavioral data. This revealed that the computation occurring in the fly's antennal lobe involves normalizing all inputs according to previous experience (context) and then linearly summating the remaining 'weights' of the activated glomeruli in response to an odorant or odorant mixture. This model was highly predictive for odor mixtures and

silencing of specific OSN channels. This context dependent normalization may account for the differences in valence observed in our oviposition assay compared to results reported by others. Since we are only activating one glomerulus in our chemogenetic assay, and if most glomeruli linearly summate, then we should be measuring the valence of each glomerulus in the specific context of our experiments. If true, then conducting chemogenetic screens may lead to the identification of those olfactory neuron classes that are sufficient to drive behaviors on their own. If an olfactory class needed a specific combination of glomeruli or activation of another class of cues such as ionotropic or gustatory receptor neurons, then it would not be picked up in our screen. Interestingly, for oviposition in our experimental settings, single olfactory neuron classes only had 'negative' valences, suggesting that the decision influence for single olfactory neuron classes regarding oviposition may be avoidance.

5.5 Activation of single olfactory receptor neuron classes

If olfaction probably uses a combinatorial code, then what is the utility of testing single OSN classes as we have done in this body of work? Single OSNs still may impart important olfactory information that is modulated or influenced by the activity of other OSNs. By identifying what behavior, if any, can be driven by a single OSN, this might highlight how that OSN functions when utilized in combination with others. To our knowledge two studies have employed systematic single OSN type activation. The first used a strategy of silencing all OSNs using *Orco-Gal4* to drive *UAS-shibire*^{ts} and rescuing *Orco* function in one or few OSNs through the use of *OrX-Gal80* lines [131]. The authors concluded that aversion only needs sparse input while attraction is sensitive to several Or inputs. However, eliminating activity of all Orco neurons dramatically

reduces baseline activity within the antennal lobe. It is unclear what effect this has on olfactory processing, and the highly artificial nature of silencing most baseline activity makes these results difficult to interpret in light of normal olfactory circuit function.

A more informative study was conducted by Bell and Wilson [68]. These experiments involved using low-level optogenetic activation of OSNs in a two choice walking assay with airflow. This low level of activation (< 35 spikes/sec) was demonstrated to be sufficient to elicit behavior because the authors were able to obtain attractive and repulsive behavior upon stimulating eight OSN classes previously identified as attractive or repellent at this low spike rate. After characterizing the baseline valence of each OSN type, the authors sought to understand how glomeruli interact and generate a behavior so pairs of attractive OSN classes were activated simultaneously. These pairwise studies revealed that activation of certain glomerular pairs resulted in behavioral output that summed linearly, but others did not. The findings from pairs that did not linearly sum exhibited a level of behavior lower in magnitude than that expected from linear summation, and the magnitude of attraction was close to that of the more attractive glomerulus of the pair when tested alone. No changes in 'sign' were ever observed when pairing two attractive glomeruli together, but pairing attractive OSNs with repellent OSNs that activate V glomerulus did decrease attraction. Individually activating V glomerulus OSNs did not produce strong aversion, and the authors posit that firing rates matter in that the aversion observed in previous studies was due to stronger activation. This idea that firing rates can significantly change behavior is also evidenced by the case of pairing the V innervating OSNs with DL4 innervating OSNs in which only stimulating at their maximum light intensity (though still low activation) produces a dramatic

decrease in attraction.

As an aside: An intriguing observation we made in the course of our study was that while geosmin response was abolished in both *Orco* mutant and Quadruple Anosmic flies (Figure 3.2 from Behavior Chapter), *Orco* mutant experiments frequently (~ 40% of experiments) did not meet the egg laying rate threshold of 8 eggs/fly/day (data not shown). This could be due to genetic background since outcrossing the four transgenes in the Quadruple Anosmic was unpractical, but the low egg-laying rate may result from the dynamics of the antennal lobe in these olfactory mutants. In the *Orco* mutant, most but not all OSNs are silenced, leaving low levels of background firing from the Gr-driven ab1C neuron and Ir-expressing OSNs. The Quadruple Anosmic flies are mutant for all co-receptors of any olfactory neurons expressed in the antenna. This largely eliminates background firing. It is possible that low level baseline firing somehow inhibits oviposition behavior more than no firing at all.

5.6 Chemogenetics

Our study created a novel genetic olfactory tool, highlights olfactory neurons and brain regions involved in negative oviposition, and adds to the understanding provided by Bell & Wilson [68] that low neuronal activation of single OSN classes can generate robust behavior. This is novel because preceding studies used high odorant concentrations [170] which could lead to unnaturally high levels of neuronal activation, overdriving neuronal circuits. The chemogenetic approach enabled us to systematically identify olfactory receptors involved in oviposition choice, possibly without the confounding factor of lateral excitation and inhibition due to the low activation level driven by the ectopically expressed Or56a (as monitored by SSR). In light of the context of background odors

playing a significant role in modulating OSN activity, it is important to be able to study OSNs in 'cleaner' environments [130]. Complex odor mixtures such as fly food are non-standard between labs, so studying behavior in a low background environment will be crucial to obtaining robust, reproducible results.

Since glomerular activity eliciting behavior in a specific context has been shown to retain sign (negative or positive) [68], then the negative oviposition OSNs we identified in our study most likely add 'negative' weight to the overall antennal lobe activation when a female makes an oviposition choice [130]. Activation of any one of our hits on its own is sufficient to evoke negative oviposition, but it is unlikely any one channel is absolutely necessary to induce oviposition since behavioral output involves total summation that could be mediated by many different OSNs. Based on observations about summation, we predict that activating two negative oviposition OSN classes together could either generate a linearly summed, higher magnitude aversive behavior or may exhibit behavior consistent with the 'more negative' of the two OSN types. If this were not the case, then repulsion functions differently than attraction. It has been shown that certain glomeruli have greater influence over others [166]. 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. This would support the labeled line theory of olfactory coding. 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.'

5.7 Segmentation of individual PNs in the LH

It appears from our results that the lateral horn does indeed functions as a categorizer of salient olfactory information. Published studies have defined domains in the lateral horn based on the entire axonal morphology of PNs [34]. Our analysis of PNs involved in negative oviposition suggests that information may be sorted based on segments of axons sharing a section of the lateral horn as seen in the dorsal posterior branch of cup shaped negative oviposition PNs, and that section categories can be different between the two sexes (males do not oviposit). The non-oviposition branch of cup-shaped PN neuronal target regions may encode another as yet unknown domain in the lateral horn since they seem to localize together. One way to identify other domains may be to classify PNs by cell type. Sakurai et al (2013) identified a cluster of PNs that express a protein called *spin*. Two of these PNs (VA1 and VA3) were hits in our oviposition screen, and two (VA1 and VM5) were shown to mediate female receptivity. Both of these phenotypes involve female-specific behaviors, suggesting that neurons expressing Spin possibly share functional coding traits. An Or56a-Geosmin chemogenetics strategy could be used to activate these neurons in a complement of behavioral assays that measure female-specific behaviors.

5.8 Future directions

Mated *Drosophila* females actively probe the environment in search of oviposition sites. Several studies have shown that social conditioning and memory can play a role in oviposition preference [171, 172]. For example, females can remember cues associated with food sources teeming with larvae and will prefer laying eggs on future food sources with similar scents. It would be interesting to see if larval pheromones activate some of the OSNs identified in our adult screen. Conversely a recent RNA seq study conducted

by Darya Task, a graduate student in the Potter laboratory, suggests that Orco and Ors previously thought to be exclusively expressed in adults may also be expressed in sensory structures at the larval tail. Chemogenetics could be used to aid in identifying the functions of these tail receptors in larvae towards odorants. This likely would prove technically easier than using optogenetics or thermogenetics, especially in targeting the olfactory system of larvae. Since maternal oviposition choice and larval survival are closely linked, comparing the results from this study with a larval study might elucidate how larval needs influence a female fly's oviposition decisions.

Similarly, chemogenetics could be used to study any behavior amenable to use of binary expression system genetics and odor presentation. Ectopic expression of Or56a and activation by geosmin could be used to singly interrogate OSNs in many olfactory settings and would allow for the identification of putative receptors involved in a behavior of interest such as male courtship. More interestingly, the ability to include multiple *Gal4* lines in an experiment allows for testing behavioral effects of multiple OSN type activation, and if the system works in non-olfactory Grs, combinations of ORN and GRN activation could be used to study sensory integration.

5.9 Some observations about olfaction

With its large array of receptor types, olfaction may be the sensory modality most adaptable to incorporate nuances or improvement with other sensory inputs, and thereby increase the overall behavioral efficiency and fitness of the animal. This may be why olfactory responses are malleable and heavily influenced by context. A looming visual stimulus indicates a physical object about to strike or crush an animal. In contrast, olfactory cues seem to be subtler. As an example, this is often seen in human studies of

flavor. In one human psychophysics study, artificially formulated strawberry juice was presented to trained tasters. Omission of any one of six high concentration component odorants normally found in natural strawberry juice produced a detectable change in strawberry flavor [173]. This indicates that olfaction is influencing the perception of taste and increasing the complexity of flavor. For an animal in the wild, the increased sensory complexity could prove beneficial and significant. This is supported by recent literature examining adaptive radiation and speciation in flies on the Hawaiian islands. A recent study performed comparative genome sequencing on three recently diverged *Drosophila* species and found Ors and Grs to be 'Among the most abundant groups of overrepresented genes driven by positive selection' [21].

Consider the variations in perception that human subjects reported when presented with the odorant androstenone (unpleasant – 'sweaty, urinous'; pleasant – 'sweet, floral'; and odorless) [65]. The cause of this heterogeneous response was traced back to single nucleotide polymorphisms that caused one or two amino acid changes in the human odorant receptor Or7D4. Thus, it is interesting that speciation would favor 'using' olfaction. A minor mutation can cause drastic changes in olfactory perception, allowing the individual with the mutation to potentially be able to perceive new compounds or perceive common compounds in a way that is different than other conspecific individuals. This could lead to adaptations like gaining the ability to find alternative food sources or better avoidance of harmful microorganisms and parasites. Similarly, since olfaction is highly important in mating behavior, mutations in key Ors that respond to pheromones could lead to mating with a zygotically compatible interspecific.

Interestingly, a recent study in *Drosophila erecta*, a close relative of *D. melanogaster,* found that one glomerulus is enlarged compared to the equivalent structure in *D. melanogaster*. This increased volume was the result of having a greater number of OSNs that express Ors tuned to the chemicals released by the species' specific host plant [174]. An earlier study had a similar finding of an enlarged glomerulus in the species *Drosophila sechillia* that was also related to host plant volatiles [175]. Strikingly, while presenting the odorant associated with the host volatile-detecting Or generated increased attraction, more dramatic results were elicited toward blends of chemicals and this was posited to affect oviposition behavior. In light of our study and other recent studies previously mentioned in discussion, these findings support the idea that changes in the 'weights' of a single OrX OSN type can lead to major changes in an animal's olfactory percepts [68, 130]. Thus, determining the responses of individual OSN types in different behavioral contexts may be informative.

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CURRICULUM VITAE

Sonia Chin October 2016

Educational History

Ph.D.	2016	Neuroscience Program	Johns Hopkins School of Medicine Mentor: Christopher Potter
B.S.	2008	Neurobiology	University of Texas at Austin

Professional Experience

Thesis Research	2010-2016	Lab of Christopher Potter, Johns Hopkins University
Summer Instructor	2014-2016	Biotechnical Institute of Maryland, Baltimore, MD
Senior Technician	2008-2010	San Diego Zoo, San Diego, CA
Undergrad Research	2006-2008	Lab of David Crews, University of Texas at Austin

Publication

Dias BG, **Chin SG**, Crews D. Steroidogenic enzyme gene expression in the brain of the parthenogenetic whiptail lizard, Cnemidophorus uniparens. *Brain Res.* 2009 Feb 9;1253:129-38.

Posters, Abstracts, Presentations

Chin S, Potter CJ. Characterizing valences driven by single olfactory sensory neuron types. Poster at CSH Neurobiology of Drosophila Conference, 2015.

Chin S, Potter CJ. Olfactory Processing of Repulsive Odor Cues in *Drosophila melanogaster.* Poster at Department of Neuroscience Retreat, 2013.

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Zhao J, **Chin S**, Dias BG, Crews D. Gene expression of steroidogenic enzymes in the whiptail lizard Cnemidophorus uniparens. Poster at the Undergraduate Research Forum, University of Texas at Austin, 2007.