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The Role of Drosophila Odorant Receptors in Odor Coding

Elane Fishilevich

THE ROLE OF *DROSOPHILA* ODORANT RECEPTORS IN ODOR CODING

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Elane Fishilevich

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Elane Fishilevich, Ph.D.

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Drosophila melanogaster is a powerful genetic model organism, and a promising model system in olfaction. At the onset of my thesis research, the expression patterns of fly's 62 odorant receptors (ORs) were largely unknown. I set out to understand the rules of connectivity of olfactory sensory neurons and the resulting properties of olfactory circuit. Consequently, we assembled maps of the olfactory neuron projections in the fly brain and characterized the contribution of several ORs to olfactory-guided behavior.

We compiled near-complete maps of OR-specific neuronal projections to the antennal lobe glomeruli of adult and larval fly brains. We analyzed expression profiles of 42 ORs, 31 of which are expressed in the adult and 21 in the larva, with an overlap of 10 ORs between the two developmental stages. Our results show surprising complexity in organization of the fly's olfactory circuit. Four adult olfactory neuron populations co-express two ORs each and another olfactory neuron population expresses one odorant and one gustatory receptor. One glomerulus receives co-convergent input from two separate populations of olfactory neurons. Three ORs label sexually dimorphic glomeruli implicated in sexual courtship, and are thus candidate *Drosophila* pheromone receptors. The organization of larval antennal lobe is remarkably similar to that of adult flies and

mammals; each glomerulus occupies a unique stereotyped position in the antennal lobe. Unlike in adults, each OR is expressed in only one neuron, forming glomeruli with single afferents. The olfactory sensory maps provide experimental framework for relating ORs to olfactory neuroanatomy, and ultimately, to output of the olfactory system.

The Drosophila larval olfactory system shows great promise as a behavioral model. Larvae exhibit robust chemotaxis to odors and have a simple olfactory system. We utilized larvae to study response properties of three olfactory neurons to a large panel of odors. Behavioral assays of larvae with single olfactory neurons ablated, showed minimal effects on chemotaxis response, and thus great redundancy in function of olfactory neuron populations. Larvae with only *Or42a* olfactory neurons functional are able to chemotax robustly, demonstrating that chemotaxis is possible in the absence of the remaining elements of the olfactory circuit.

Dedicated to my family, friends, and colleagues who

supported me throughout my studies

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Abbreviations

AL - antennal lobe, site of first olfactory synapse in insects

AOB - accessory olfactory bulb, site of first synapse for vomeronasal neurons

ARM - anesthesia resistant memory

DO - dorsal organ, larval olfactory organ

Dscam - *Drosophila* homolog of human Down syndrome cell adhesion molecule

EM – electron microscopy

fru **-** *fruitless*, transcription factor gene that determines courtship behavior

G protein – guanine-nucleotide binding protein

GPCR - G protein-coupled receptor

GR - gustatory receptor

IP_3 - inositol 1,4,5 triphosphate, signaling molecule, downstream of G protein

LAL - larval antennal lobe, site of first olfactory synapse in insect larva

LH - lateral horn of protocerebrum, olfactory processing center in insects

LTM - long-term memory

MB - mushroom body, learning, memory and olfactory processing center in insects

MGC - macroglomerular complex, sexually dimorphic glomeruli

MOE - main olfactory epithelium

OB - olfactory bulb, site of first olfactory synapse in mammals

OBP- odorant-binding protein

OR - odorant receptor

OSN - olfactory sensory neuron

PN - projection neuron, second-order olfactory neurons in insects

STM - short-term memory

TO - terminal organ, larval taste organ

tra **-** *transformer*, sex-determination transcription factor gene in *Drosophila*

VNO - vomeronasal organ, pheromone-sensing organ in mammals

UAS - upstream activating sequence

yw - yellow body, white eyes

1 Introduction to Olfaction in Flies

1.1 Introduction

Sensory systems open a window onto how our brains receive and interpret external stimuli. Olfaction is one of key sensory systems that enable animals to respond to their environment. The olfactory system of insects is similar to, yet consists of far fewer neurons than ours. Powerful genetic and molecular techniques in fruit fly, *Drosophila melanogaster*, will enable us to decipher how the olfactory information is encoded in the brain. Despite its perceived simplicity, much is to be learnt about the *Drosophila* olfactory system. This chapter focuses on some of the findings and challenges in olfaction in *Drosophila* and other organisms.

1.1.1 Sensory Systems

We sense our environment through five modalities: touch, vision, hearing, taste, and smell. The sensory modalities differ in the range of signals that they perceive, in organization of the afferent input, and processing of sensory information. The inputs into sensory systems can have single or multiple attributes. The sensory systems may distinguish not only the presence but also the position, duration and other properties of the stimulus.

1.1.2 Olfactory Systems in Mammals

We recognize familiar odors, even if they represent very complex blends, such as coffee or bread. Every time we inhale, compounds from our rich chemical environment interact with odorant receptors (ORs) in our olfactory sensory neurons (OSNs), triggering olfactory circuits to generate a sensation of

smell. The OSNs that receive olfactory inputs protrude their ciliated dendrites into a layer of mucus on the main olfactory epithelium (MOE) that is located on the roof of the nasal cavity. The MOE of humans contains an astounding 10 million OSNs (Dennis, 2004). Other sensory neurons that are present in the nasal cavities of mammals other than humans are vomeronasal sensory neurons; these reside in vomeronasal organ (VNO), a structure separate from the MOE. While the MOE is thought to mediate olfactory information of food and other conventional volatiles, the VNO is thought to respond to odors that mediate conspecific communication (detect pheromones). The neurons within VNO can also respond to conventional odors, hence the functions of these two systems may overlap (Sam et al., 2001).

OSNs direct their axons to the olfactory bulb (OB), which relays the information via mitral and tufted neurons to the olfactory cortex. The processing that leads to conscious perception of odors is thought to take place in orbitofrontal and frontal cortices of the brain. Some odors may lead to strong emotional responses. Olfactory-induced emotions are thought to result from olfactory information targeting the amygdala and the hypothalamus, structures that regulate non-voluntary activities that include emotions (Winston et al., 2005; Zald et al., 2002; Zald and Pardo, 1997). While the OB neurons project mostly to the olfactory cortex, the vomeronasal information is relayed primarily to the amygdala (Scalia and Winans, 1975).

With about 1000 ORs in mammals (Buck and Axel, 1991; Zhang and Firestein, 2002), the olfactory system is designed to respond to small and large

molecules, single compounds and complex mixtures. Size, shape, and functional groups of odor molecules are thought to determine their interactions with the ORs. It is believed that the ORs recognize only certain features of the odorants, leading to a combinatorial code, where a single odorant may activate many ORs and a single OR may be activated by many odorants (Malnic et al., 1999). The combinatorial codes in the olfactory system allow us to perceive far more odors than the number of ORs.

Mammals are believed to express single OR in each OSN. In the OB, the axons of OSNs organize into glomeruli according to the ORs that they express. The positions of glomeruli are conserved from animal to animal and are mirror-symmetric within the OB. The targeting of millions of OSNs to thousands of glomeruli is a remarkable feat and hallmark of the mammalian olfactory system.

1.1.3 Olfaction in Nematodes

The logic of olfactory connectivity in nematode *C. elegans* is different from that of mammals. *C. elegans* have only four bilaterally symmetric neurons that are responsible for sensing volatile chemicals (AWA, AWB, AWC, and ADL) (Bargmann et al., 1993). These cells harbor as many as 1000 different ORs (Troemel et al., 1995). Some of the *C. elegans* OSNs mediate attraction while others mediate repulsion, the expression of the ORs in *C. elegans* (Bargmann et al., 1993; Troemel et al., 1997). Interestingly, chemical preferences of *C. elegans* can be reversed by misexpressing an OR that normally senses attractive odors in an OSN that mediates repulsion (Troemel et al., 1997). These studies are

important models for “simple” olfactory systems, where activation of a single OR or a single OSN can lead to reliable behavioral consequences.

1.1.4 Olfaction in Insects

The connectivity of the olfactory system in most insects is similar to that of mammals; each insect OSN expresses one or few ORs and the axons of insect OSNs organize into glomeruli in the brain. However, insects have only about 100 ORs.

The insect olfactory organs are antennae and in some cases maxillary palpi. Honeybee (*Apis mellifera)* maxillary palp is not olfactory, mosquito (*Anopheles gambiae)* maxillary palp is key to sensing CO_2 (Grant et al., 1995), an important food attractant, while fruit fly (*Drosophila melanogaster)* maxillary palp seems to functionally overlap with the antenna (Ayer and Carlson, 1992; Charro and Alcorta, 1994). The olfactory organs of insects are covered with porous hair-like olfactory sensilla. The sensilla house the dendrites of one or several OSNs. Electorophysiological responses of OSNs can be measured by single sensillum electrophysiological recordings, or on a more global level by electroantennograms.

In insects, the OSNs synapse in a structure analogous to the OB, called the antennal lobe (AL). The logic of organization of the antennal lobe in most insects is the same as in mammals, OSNs expressing the same OR converge upon one or two glomeruli in the AL (Vosshall et al., 2000). The axons of OSNs synapse with projection neurons (PNs) that relay olfactory information to mushroom bodies (MB) and the lateral horn of protocerebrum (LH), the main olfactory

processing centers (Stocker et al., 1990; Strausfeld et al., 1998). The glomeruli of the AL are also innervated by local interneurons that non-uniformly innervate most of the glomeruli of the AL (Stocker et al., 1990; Wilson and Laurent, 2005) and are thought to sharpen the input into AL via inhibitory interactions.

In most insects, the AL glomeruli can be identified by their position, size, and shape. The ease with which the glomeruli are recognized facilitates studies that use calcium-sensitive dyes to measure neural activity in the antennal lobe to reveal specific responses of glomeruli to odors.

Based on their size, brain structure, and genetic tools available, specific insects are preferred for studies of different olfactory problems. Locusts are valuable in electrophysiological recordings from various cell types in the olfactory circuit, however their AL structure does not follow “one OR – one glomerulus” logic. Honeybees were used to pioneer the activity-sensitive calcium imaging techniques in olfaction (Galizia et al., 1997). Honeybees are also excellent model systems for associative olfactory learning. Moths have large sexually dimorphic glomeruli that are tuned to known pheromones. In bees and flies sexual dimorphism is not readily noticeable (Laissue et al., 1999; Stocker et al., 1990). The following sections describe the structure and function of the *Drosophila* olfactory circuit and integrate information learnt from other insects and mammals.

1.2 Olfactory Circuit of *Drosophila melanogaster*

Over 100 years of genetics research in *Drosophila melanogaster* have yielded tools that allow us to mark and follow alleles and introduce or delete genes from the *Drosophila* genome. With only 62 ORs in its genome (Clyne et al.,

1999; Vosshall et al., 1999) and approximately 45 glomeruli in the AL (Laissue et al., 1999), the logic of connectivity of the olfactory system in fruit fly presents a tangible problem. Fly larval olfactory system is an even simpler olfactory circuit that expresses a subset of the total ORs.

1.2.1 Olfactory "Epithelium" in *Drosophila*

In adult fruit flies, an equivalent of mammalian olfactory epithelium consists of about 1200 sensilla on the third segment of the antenna, plus 120 sensilla on the maxillary palp (**Figure 1.1A**). Based on shape and electron microscopic ultrastructure, fly sensilla can be classified into three major categories: trichoid, basiconic, and coeloconic (**Figure 1.1C**). One to four OSNs insert their sensory dendrites into each sensillum. The dendrites of OSNs are bathed in the sensillum lymph that is secreted by the support cells (**Figure 1.1B**). The sensillum lymph contains high quantities of odorant binding proteins (OBPs) that are thought to function as chaperones in bringing odorant to the OR or in clearance of the odorants (Tsuchihara et al., 2005; Vogt and Riddiford, 1981). Positions of the sensilla seem to be relatively invariant; the pairing of OSNs in the sensilla is also invariant (de Bruyne et al., 1999; de Bruyne et al., 2001). The findings above have prompted studies that identified ORs that confer specific odor responses upon numerous OSNs (Goldman et al., 2005; Hallem et al., 2004a).

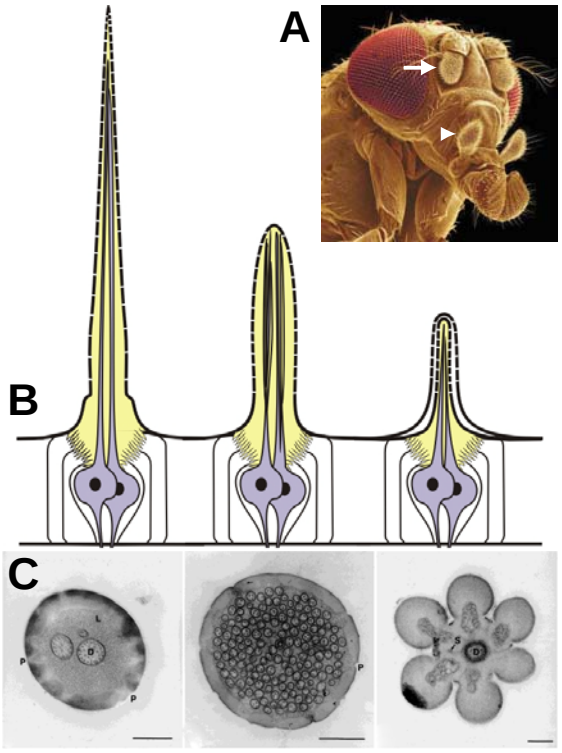

Figure 1.1: Olfactory sensilla of *Drosophila melanogaster*. A. Olfactory organs of the fly are antenna (arrow) and maxillary palp (arrowhead). B. Schematic representation of trichoid (left), large basiconic (middle), and coeloconic (right) sensilla with two OSNs each. OSNs are in blue, sensillum lymph is yellow, support cells are vertical black lines. C. Cross-sections through the shafts of antennal sensilla visualized by transmission electron microscopy. Trichoid sensillum (left), large basiconic sensillum (middle), and coeloconic sensillum (right). L, sensillum lymph; P, wall pore; S, spoke channel (equivalent to wall pore). Scale bars 0.5 mm (left, middle), 0.1 mm (right). Reproduced from (Stocker, 2001) and (Shanbhag et al., 1999).

Trichoid sensilla. The 150 trichoid sensilla are distributed in a distal lateral region of the third antennal segment on both anterior and posterior surfaces. There are three subtypes of trichoid sensilla that are innervated by one to three OSNs. The trichoid sensilla and their associated glomeruli that were studied thus far are sensitive to very few odors (Hallem et al., 2004a; Wang et al., 2003a) and may participate in pheromone reception (Xu et al., 2005).

Basiconic sensilla. The basiconic sensilla are present throughout the surface of the third antennal segment, but are concentrated in the medial proximal surfaces of the antenna. While the dendrites of trichoid sensilla are unbranched the dendrites of basiconic sensilla are branched extensively (**Figure 1.1B** and **C**). All of the maxillary palp sensilla are basiconic. The basiconic sensilla (n = 200) are further subdivided into at least six morphological subtypes that include large, thin, and small. Eighteen types of OSNs have been identified in basiconic sensilla, one sensillum type containing four OSNs and seven sensillum types containing two OSNs each (de Bruyne et al., 1999; de Bruyne et al., 2001; Elmore et al., 2003; Hallem et al., 2004a). The aforementioned studies show that basiconic sensilla respond to various aliphatic and aromatic odors.

Coeloconic sensilla. The trichoid and basiconic sensilla have single walls, while coeloconic sensilla are double walled. The outer wall has deep longitudinal grooves, whereas the inner wall envelopes the dendrites (**Figure 1.1B** and **C**). The coeloconic sensilla are innervated by two or three OSNs. They are distributed throughout the antenna and the sacculus, an organ thought to mediate hygroreception (water), thermoreception (heat/cold), and olfaction (Shanbhag et al., 1995). Although morphologically homogenous, four types of coeloconic sensilla have been identified electrophysiologically (Yao et al., 2005). Only one OR has been identified in coeloconic sensilla (Couto et al., 2005; Yao et al., 2005). Thus, it is possible that the majority of coeloconic sensilla are not olfactory.

Proper formation of sensilla and their positions on the antennal surface are determined by expression of transcription factors that include Lozenge, Atonal, and Amos. Atonal specifies the fate for coeloconic sensilla (Gupta and Rodrigues, 1997), while Amos specifies the basiconic and trichoid sensilla (Goulding et al., 2000). The choice between basiconic and trichoid identities is determined by Lozzenge, which regulates Amos (Gupta et al., 1998).

1.2.2 Odorant Receptors – Expression

It is well established that each mammalian OSN expresses only one OR (Chess et al., 1994; Malnic et al., 1999; Serizawa et al., 2003). Furthermore, the OR gene product is necessary for successful inhibition of expression of other ORs (Serizawa et al., 2003). The “one OR - one OSN” system is prudent since the ORs play a role in axon guidance and precise coalescence of a glomerulus (Feinstein et al., 2004; Feinstein and Mombaerts, 2004). It is understood that *Drosophila* ORs are not necessary for the proper targeting of the OSNs to glomeruli (Larsson et al., 2004). There are also several examples of two *Drosophila* ORs being expressed within same OSN (Dobritsa et al., 2003; Goldman et al., 2005). In addition to 62 ORs in *Drosophila*, several of about 70 gustatory receptors (GRs) are expressed in the antenna and may function in olfaction (Dunipace et al., 2001; Robertson et al., 2003; Scott et al., 2001).

The ORs in *Drosophila* are expressed non-homogeneously in overlapping domains throughout the third antennal segment and in the maxillary palp (Vosshall et al., 2000). The basiconic *Or22a* is expressed in a proximal medial region in about 20 OSNs (Dobritsa et al., 2003; Vosshall et al., 2000), while a

trichoid *Or47b* is expressed in about 60 OSNs in a distal lateral region (Vosshall et al., 2000). As mentioned earlier, the OSNs that express specific ORs are restricted to their corresponding sensillum types and observe invariant pairings within the sensillum. Although, the expression of ORs on the antennal surface is invariable, it is not predictable and has to be determined experimentally. The determinants of antennal versus maxillary OR expression are partially identified. A regulatory promoter motif named MP dyad, $CTA(N)_9TAA$, is deemed necessary for the OR expression in the maxillary palp, and MP oligo motif, CTTATAA, seems to be necessary for restriction of the OR expression to the antenna (Ray et al., 2003). The factors that determine which OR will be expressed within a particular sensillum are largely unknown.

1.2.3 Formation of Olfactory Glomeruli

Since OSNs that express a particular OR are spread throughout olfactory epithelium and intermingle with other OSNs and other sensillum types, the guidance of OSN axons to correct glomeruli in the AL is a challenging problem. Multiple factors seem to be involved in OSN axon path-finding and glomerular coalescence. The cues that ensure proper targeting of OSN populations in the AL include Down syndrome cell adhesion molecule (Dscam) which is differentially required by OSN populations and is necessary for the maxillary palp OSNs to enter the antennal lobe (Hummel et al., 2003). N-cadherin seems to be ubiquitously required by OSNs to form protoglomeruli and ensure proper synapses with the PN targets (Hummel and Zipursky, 2004). Null mutants for POU domain transcription factor Acj6 exhibit lack of expression for some of the

ORs, mistargeting of some OSNs, or have no defect (Komiyama et al., 2004). Further, the requirement for Acj6 can be either cell autonomous or cell non-autonomous. Although all studied populations of OSNs that require Acj6 cell autonomously express antennal ORs, no clear patterns for Acj6 requirement are known. Dreadlocks (Dock) an SH2/SH3 adapter and serine/threonine kinase Pak have also been found to influence OSN targeting in the brain (Ang et al., 2003). Perturbation of Roundabout (Robo) family members also causes mistargeting effects of OSNs (Jhaveri et al., 2004).

The PNs reach their targets within the AL, independently of OSNs (Jefferis et al., 2004; Jefferis et al., 2001; Marin et al., 2002; Wong et al., 2002). This observation leads to a conclusion that the neuroconnectivity of OSNs and PNs is independently programmed. Differentially expressed in the PNs, POU domain transcription factors Acj6 and Drifter are required for proper dendritic targeting (Komiyama et al., 2003).

Larval olfactory structures may also be important for axon guidance within the adult olfactory system. Interestingly, the activity of larval OSNs in not necessary for proper formation of adult olfactory glomeruli (Larsson et al., 2004). Larval OSNs degenerate during metamorphosis, but the larval antennal nerve may serve as a guiding track for the adult antennal nerve during development (Tissot et al., 1997). On the other hand, some larval PNs survive metamorphosis (Marin et al., 2005).

1.2.4 Olfactory System of *Drosophila* Larva

Larval chemosensory organs are the dorsal organ (DO) and terminal organ (TO) at the anterior tip of the animal (**Figure 1.2**). The functions of the DO and the TO were initially subdivided into olfactory and gustatory, respectively, based on the structure of pores through which chemical stimuli can access the dendrite (Singh and Singh, 1984; Stocker, 1994). The dome of the DO is perforated at the base and could be accessed only by volatiles, while the single-walled pores of the TO and additional pores at the base of the DO can allow for the direct contact with the chemicals. Twenty one neurons insert their dendrites into the dome of the DO, their cell bodies are part of the DO ganglion (Python and Stocker, 2002). These neurons are arranged into seven bundles of three neurons each (**Figure 1.2B**). Ablation of the 21 OSNs renders larvae anosmic, further proving their necessity for larval olfaction (Larsson et al., 2004). The axons of larval OSNs project to the larval antennal lobe (LAL) in the brain (Tissot et al., 1997) (**Figure 1.2A**). Each of larval OSNs terminates in a restricted area similar to an adult glomerulus (Ramaekers et al., 2005).

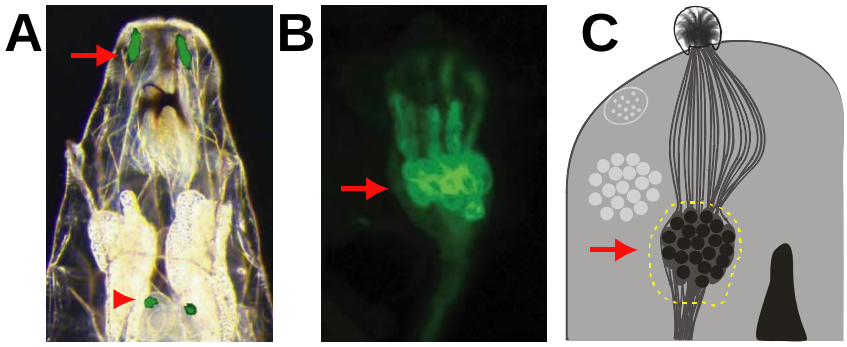

Figure 1.2: Olfactory organ of *Drosophila* larva. A. Whole-mount anterior tip of the larva visualized with light microscopy. The OR83b-positive olfactory neurons are stained with GFP, fluorescent and light images are superimposed. Arrow points to the

ganglion of the dorsal organ in A, B, and C. Arrowhead points to the axon termini in the brain. B. An immunofluorescence image of *Or83b→Or83b::Myc* stained with anti-Myc antibody. Seven bundles of dendrites are apparent. C. Schematic representation of the larval dorsal (dark grey) and terminal (light grey) organ positions. Dome of the dorsal organ is at the top; mouth hook is in lower right.

1.3 Olfactory Signal Transduction

The ORs in most organisms are thought to be seven-transmembrane G protein-coupled receptors (GPCRs). GPCRs represent the largest receptor gene family in mammals; they are also important pharmacological targets. Further, the OR family is the largest family of GPCRs. Thus, understanding the events associated with the function of ORs may prove useful beyond the scope of olfaction.

1.3.1 Odorant Receptors – Identity

The ORs in mammals, nematodes, and fish are GPCRs. Mammalian ORs are believed to be structurally similar rhodopsin, a well-studied light-sensitive GPCR in the retina. Sequence similarity to rhodopsin has allowed for computational modeling of the ORs and predictions for ligand binding (Katada et al., 2005). Although *Drosophila* ORs are predicted to have seven transmembrane topology, these ORs bear no sequence similarity to mammalian ORs (Clyne et al., 1999; Vosshall et al., 1999). Furthermore, they bear no sequence similarity to any of GPCRs. Hence, no predictions about ligand binding can be made.

Although, insect ORs are predicted to have seven transmembrane domains, in many cases the definition of the domains is vague (Benton et al., 2006). Furthermore, computational and experimental evidence suggests that in contrast

to mammalian ORs, at least some of *Drosophila* ORs have an intracellular N-terminus (Benton et al., 2006; Nakagawa et al., 2005).

1.3.2 GPCR Signaling

GPCRs interact with guanine nucleotide-binding proteins (G proteins). G proteins play a central role in signal transduction and are subdivided into several categories based on the pathways they tend to stimulate. Upon activation, G proteins disassociate into α and βγ subunits; with some debate (Robishaw and Berlot, 2004), the α subunit is thought to confer receptor and effector specificities of the G proteins. There are at least five β, twelve γ, and sixteen α mammalian subunit genes that are divided into four functional classes (Hurowitz et al., 2000). $G\alpha_s$ is likely to activate adenylate cyclase, $G\alpha_i$ inhibits it, $G\alpha_q$ stimulates the inositol triphosphate (IP_3) pathway through its interaction with phospholipase C, and $G\alpha_{12}$ directly regulates Na+/K+ channels (Neer, 1995).

In mammals, ORs couple to $G\alpha_{olf}$ (Belluscio et al., 1998; Jones and Reed, 1987). The G_{olf} is of the G_s family, and although IP_3 levels rise in OSNs when they are stimulated with odor (Ronnett et al., 1993; Schandar et al., 1998), cAMP is thought to be the predominant olfactory signal transduction pathway in mammals. Activation of the insect olfactory epithelium with an odor leads to an increase in IP_3 concentrations (Breer et al., 1990). Furthermore, $G\alpha_q$ and $G\alpha_s$ proteins were isolated from insect olfactory epithelium (Jacquin-Joly et al., 2002; Laue et al., 1997; Miura et al., 2005; Talluri et al., 1995). Thus, G protein-mediated signal transduction cascade is postulated for insect olfaction.

1.3.3 Heterodimerization of ORs and GPCRs

While rhodopsin and the mammalian ORs are thought to function as monomers, dimerization of GABA subunits is required for proper transport to the cell surface (Margeta-Mitrovic et al., 2000; White et al., 1998). It is now evident that heterodimerization of insect ORs is necessary for their activity (Larsson et al., 2004; Nakagawa et al., 2005; Neuhaus et al., 2005). OR83b of *Drosophila* and its homologs in other insects interact with conventional ORs and are required for localization of the ORs to the dendrite (Benton et al., ; Larsson et al., 2004). Presence of OR83b is also sufficient for OR function in the cell; it is possible to reconstitute OR activity by expressing both a conventional OR and OR83b in *Xenopus* oocytes (Nakagawa et al., 2005). Although in presence of G proteins the ORs initiate low-level potentials (Nakagawa et al., 2005), it is not clear whether G proteins are the natural components of the insect olfactory signal transduction cascade.

1.4 Receptive Fields of Odorant Receptors

The olfactory circuit functions in combinatorial manner; such that many receptors are activated by a single odorant and each receptor is activated by many ligands. The first cues that the olfactory system abides by a combinatorial code came from mammals, but the studies in insects have yielded comprehensive information about the receptive ranges of ORs in the olfactory circuit. Information in ligand specificity of the ORs and olfactory activity maps in the brain improve our understanding of how the olfactory information is coded in

the brain. Yet, much needs to be discovered, since many ORs still remain "orphan".

1.4.1 Function of Homologously and Heterologously Expressed ORs

First insights into OR odor binding properties came from successful transvection of the olfactory epithelium with I7, a rat OR, coupled to electrophysiological recordings (Zhao et al., 1998). Similarly, to identify ligands for the *Drosophila* OR43*a*, it was genetically overexpressed in the antenna (Störtkuhl and Kettler, 2001). Electrophysiological studies of the ORs expressed in an otherwise empty OSN are also homologous expression studies (**Section 1.4.5**).

Expressing ORs in a heterologous expression system potentially allows for a large scale ligand identification screen. A heterologous expression system in human embryonic kidney cells (HEK293) has deorphanized several mouse and human receptors (Fukuda et al., 2004; Krautwurst et al., 1998; Spehr et al., 2003; Wetzel et al., 1999). Recently identified receptor-transporting transmembrane proteins RTP1 and RTP2 that promote functional cell surface expression of the mammalian ORs greatly increase the potential of the HEK293 system (Saito et al., 2004).

Expression of insect ORs in heterologous systems has proved difficult. This situation is largely due to lack of knowledge about the insect olfactory signal transduction mechanism. Successful attempts to express a *Drosophila* OR along with G-proteins $G\alpha_{15}$ (Wetzel et al., 2001) or $G\alpha_{q}$ (Nakagawa et al., 2005) in *Xenopus* oocytes have yielded weak but consistent responses to odors. Recent

studies indicate that a member of OR family OR83b or its homologs are necessary for successful trafficking of the conventional ORs and their function (Larsson et al., 2004; Nakagawa et al., 2005; Neuhaus et al., 2005). In Xenopus oocytes, in presence of the OR83b homolog, insect ORs exhibit significant changes in membrane potential (Nakagawa et al., 2005). An observation that $G\alpha_q$, when expressed in *Xenopus* oocytes in addition to OR83b homolog and an OR does not induce higher currents further questions the relevance of G proteins for insect olfactory signal transduction.

1.4.2 Lessons from Rodents

Metabolic activities in mouse olfactory bulbs have been monitored with radioactively labeled 2-deoxyglucose (Imamura et al., 1992). Many glomeruli are tuned to particular functional group within one or two chain lengths, further, similarly tuned glomeruli are located next to each other, in domains or clusters (odotopic organization) (Meister and Bonhoeffer, 2001; Takahashi et al., 2004; Uchida et al., 2000). ORs do participate guiding OSNs to specific glomeruli in the olfactory bulb (Feinstein et al., 2004; Feinstein and Mombaerts, 2004). If we assume that similar ORs respond to similar odors, is not surprising to find co-activated neighboring glomeruli. An OR with a single mutation in a trans-membrane domain presumably retains a very similar, if not identical odor tuning, but leads to segregation of axons in the OB (Feinstein and Mombaerts, 2004). Whether the odotopy observed in mammalian OBs holds true in ALs of insects such as *Drosophila* awaits further proof.

1.4.3 Optical Calcium-Sensitive Imaging of Honeybee Brain

Changes in intracellular calcium levels in neurons are related to activities such as neurotransmitter-release and ion channel gating. Changing calcium levels in the AL can reveal the patterns of glomerular activation (odor maps) that are due to a particular chemical or a mixture.

Measurements of intracellular calcium changes in the AL were pioneered in honey bees (Galizia et al., 1997). Honeybee has about 160 glomeruli (Gascuel and Masson, 1991) and a window of 40 glomeruli can be imaged (Sachse et al., 1999). The types of fluorescent dyes that have been used in these studies are single excitation dyes, such as calcium-green (Galizia et al., 1997), or dual excitation ratiometric dyes, such as Fura (Sachse and Galizia, 2002). When the dye is bath-applied, the readout consists of responses by OSNs, PNs and interneurons in the AL, although the majority of the signal is thought to stem from OSNs.

Calcium imaging is a reliable and reproducible technique to monitor odor-invoked activity in glomeruli. The activation patterns are conserved between the brain hemispheres and from animal to animal (Galizia et al., 1998). There is a range in the time of signal onset and the level of activation and inhibition of different glomeruli in response to odors (Sachse and Galizia, 2002). Detailed studies in moths and honeybees reveal that a glomerulus may respond to compounds with different functional groups but with similar carbon chain-lengths (Meijerink et al., 2003; Sachse et al., 1999). There also seems to be a directional progression of the glomerular activation pattern within functional groups with

change in the carbon chain length (Sachse et al., 1999). These studies suggest that there is some chain length/functional group subdivision within insect AL.

1.4.4 Optical Calcium-Sensitive Imaging of *Drosophila* Brain

The smaller size of fruit flies in comparison to honey bees, greatly restricts the resolution between glomeruli in calcium imaging studies. However, the advantage of *Drosophila* studies is that instead of dyes one can genetically express calcium-sensitive fluorescent proteins within neurons of interest. Additionally, the glomerular identities of many fly ORs are already known, allowing for direct link between the sequences of the ORs, their electrophysiological responses within OSNs, and the resulting activities in various neurons in the AL.

Studies that measured odor-evoked calcium activities in the *Drosophila* OSNs and PNs revealed specific glomerular responses but no logical odotopy (Fiala et al., 2002; Ng et al., 2002; Wang et al., 2003a). Misexpression of *Or43a* in *Or47a* OSNs revealed that an OR defines the calcium response profile of a glomerulus (Wang et al., 2003a). Future studies that will genetically express calcium-sensitive proteins in OSNs or PNs that are associated with single OR, promise to yield high-throughput comprehensive descriptions of odor representations in the AL.

1.4.5 Electrophysiological Recordings from Olfactory Neurons in Flies

Physiological responses of *Drosophila* OSNs to odors have been studied extensively in basiconic sensilla. The OSNs in basiconic sensilla were initially classified electrophysiologically by their response profiles to a small panel of

odors (de Bruyne et al., 1999; de Bruyne et al., 2001). Subsequent studies have unveiled the OR identities of many of the OSNs (Goldman et al., 2005; Hallem et al., 2004a). Responses of individual OR that vary from none (OR49a) to nine (OR42a) out of 27 odors tested, reveal a broad range of OR tuning (Kreher et al., 2005). The ORs that correspond to basiconic sensillum type respond from one to six of twelve tested odors (Hallem et al., 2004a). Since flies may not encounter many of the odors at such high concentrations as presented under experimental conditions, not all electrophysiological responses can be deemed natural stimuli. When the concentration of the stimulant odor is decreased, some ORs decrease their responses more abruptly than others (Hallem et al., 2004a). Although low odorant concentrations are thought to be more physiologically relevant, these studies reveal the capabilities and possible adaptability of the olfactory system. We need not forget that many other factors such as synergy of odor mixtures may influence responses of OSNs and their postsynaptic neurons.

The ORs of trichoid sensilla are not characterized well electrophysiologically. The only trichoid OR, OR47b, either has no response or is inhibited by common odors that were tested (Hallem et al., 2004a). The only coeloconic OR, OR35a responds to as many as 13 of 31 test-odors (Yao et al., 2005). The response ranges of other coeloconic OSNs are very limited. Two of the eight (or nine) coeloconic OSNs respond to nitrogen containing compounds, one specifically to ammonia and another to 1,4-diaminobutane (Yao et al., 2005). Two coeloconic sensillum types respond to changes in humidity (Yao et al., 2005). Thus, it is

possible that the majority of coeloconic sensilla are occupied by receptors other than ORs.

It is plausible that the ORs and the GRs of antenna can respond to nonconventional odorants or other stimuli. For example, while most of the basiconic sensilla respond to conventional odorants, the antennal basiconic 1 (ab1) sensillum type contains a neuron expressing Gr21a; this neuron is specific in its responses to changes in CO_2 concentration (Couto et al., 2005; Scott et al., 2001; Suh et al., 2004) .

1.5 Olfactory Information Processing

Relatively little is known about processing of the olfactory information once it passes the first synapse. In mammals, large numbers of OSNs and mitral and tufted cells make it difficult to correlate their activities. In insects, electrophysiological and calcium-imaging studies that evaluated the processing of olfactory signal in the AL lead to inconsistent results. The conflicting hypotheses of olfactory signals being sharpened, being diffused, or being passed on faithfully from OSNs to PNs are all supported by evidence.

1.5.1 Optical Imaging in Insect Antennal Lobe

Comparisons of calcium-related activity of the honeybee PNs and overall activity conclude that there is refinement of signal at the PN level (Sachse and Galizia, 2002). Sharpening of the signal as information is passed from OSNs to PNs could be due to local GABAergic local interneurons; blocking GABA receptors with picrotoxin causes a decrease in signal sharpening (Sachse and Galizia, 2002). Residual modulation that is unaffected by picrotoxin, suggests an

additional interaction network within the AL. Contrast-enhancement of odor representations in the AL, implicates interneural network in preserving the odor identity at various odor concentrations (Sachse and Galizia, 2003).

In contrast to honeybees, studies that used calcium-sensitive proteins in *Drosophila* to image odor-evoked activities of OSNs and PNs observed that these readouts were nearly identical (Ng et al., 2002; Wang et al., 2003a).

1.5.2 Recordings from Projection Neurons in Moths

Moths are large insects, they are also extremely sensitive to species-specific pheromone blends. Moth's pheromone sensitive OSNs terminate in large sexually dimorphic glomeruli, termed the macroglomerular complex (MGC). Most PNs that project to MGC glomeruli are selectively activated by major and minor pheromone blend components that are particular to the species (Vickers and Christensen, 1998; Vickers and Christensen, 2003). Electrophysiological recordings from the PNs that terminate in an isomorphic glomerulus, concluded that isomorphic glomeruli can also be narrowly tuned to plant volatiles, reproducibly across individuals (Reisenman et al., 2005).

1.5.3 Temporal Component of Odor Coding in Locusts

It has been proposed that the temporal firing patterns of assemblies of PNs are just as important for the odor code as the spatial representation of an odor in the AL. It is known that PNs synchronize their activities upon odorant stimulation (Wehr and Laurent, 1996). The debate on whether this synchronization provides olfactory information was addressed by electrophysiological recordings from locust PNs.

Locust, *Schistocerca Americana,* is yet another insect model system in olfaction. The locust olfactory circuit is amenable to electrophysiological recordings from OSNs, PNs, local interneurons, and Kenyon cells that receive their input from PNs. Alas, the recognition of each glomerulus from animal to animal impractical, since locust AL contains a large number (~1000) of glomeruli. Also unlike in flies, locust PNs terminate in multiple glomeruli.

Synchronized activation of PNs is deemed necessary to activate Kenyon cells in the mushroom body that require strong coincident input for their activity (Perez-Orive et al., 2004). For example, application of picrotoxin desynchronizes PN activity leading to lower activation of Kenyon cells and impairs fine odor discrimination by higher level neurons (Stopfer et al., 1997). Additional studies argue that the temporal firing patterns of the PN assemblies carry critical information as to odor identity and intensity (Stopfer et al., 2003).

1.5.4 Recordings from Projection Neurons in Flies

True comparisons between the receptor and projection neurons activities from the same glomerulus have been accomplished. It is observed that the PNs that terminate in *Or22a* glomerulus respond to more odors than the OSNs. The broader response of the PNs could be attributed to the activities of interneural network in the AL. A recent study of GABAergic local interneurons in AL has confirmed a GABA-mediated, non-uniform, and odor dependant inhibition of the PNs in *Drosophila* (Wilson and Laurent, 2005). The unexpected observations in *Or22a* glomerulus may also be due to another population of OSNs converging upon the same glomerulus.

1.5.5 Olfactory Representations and Processing in Higher Brain

From the AL the olfactory information is sent to the MB and the LH (Stocker et al., 1990; Strausfeld et al., 1998). The olfactory information in the MB and the LH is not as well spatially segregated as in AL. Although the axons of PNs have very stereotypic branching patterns in the LH, they greatly overlap with axons of other PNs (Marin et al., 2002; Wong et al., 2002). A later study that considered groups of PNs revealed spatial segregation of several PN subgroups both in the LH and in the MB (Tanaka et al., 2004). Thus, while the information at the second olfactory synapse is not represented in the same spatial arrangement as in AL, some of its features persist.

The functions of MB and LH can be separated behaviorally. Reportedly, the MB is involved in associative aspects of olfaction, while the LH is thought to mediate more reflexive behaviors. To this end, if the activity of the MB is blocked, only the attractive but not the avoidance olfactory behavior is abolished (Wang et al., 2003b). It is also known that the function of MB neurons is important for olfactory and other types of associative learning and memory (Besson and Martin, 2005; Liu et al., 1999). The olfactory memory can be separated into long term memory (LTM), anesthesia resistant memory (ARM), and short term memory (STM) (Isabel et al., 2004; Tully et al., 1994; Zars et al., 2000). Unlike the other types of memory, the LTM requires spaced learning and protein synthesis.

1.6 Olfactory-Guided Behavior

If the sensation of smell is the chief output of the olfactory system, behavior is the ultimate output and the definitive readout that we can observe. The logic by

which the activation of an ensemble of neurons translates into animal's ability to detect and appropriately interpret large numbers of odors is the ultimate task of olfactologists.

1.6.1 Odor Plumes

Contrary to what one may first imagine, odors are not homogenous nor are gradients, but travel in plumes. Since molecular diffusion of volatiles is usually slower than the air turbulence, odor molecules are carried in packets by the "wind" (Murlis and Jones, 1981). Plumes can be continuous or discontinues, wide or thin, with varying odor concentrations based on the air turbulence and the distance they traveled. Variable plume structures inevitably add variables that influence odor perception and subsequently behavior. Although we cannot identify the source of the odor in a room, insects can track sources of odors by traveling upwind in zigzag patterns. The ability to travel upwind in response to a plume of odor is particularly important for successful mate location in moths.

1.6.2 Responses to Pheromones: Olfactory-Guided Behavior in Moths

Pheromones are means of conspecific communication. Pheromone perception can be defined as chemical spying by members of the same species. In fact, many pheromones have evolved from products or byproducts of normal metabolism (Wyatt, 2003).

The simplicity of the female-produced sex pheromone blends and the robustness of male behavioral responses make moths classic model systems for conspecific communication. The first identified pheromone compound was a double unsaturated 16-carbon alcohol, bombykol, which is released by female

silkmoth *Bombyx mori* (Butenandt, 1959). Female moths of various species produce straight unsaturated hydrocarbon chains of 5 to 22 carbons long. Most blends of two or more chemicals are emitted at exact proportions. In most moths, the pheromone blend is produced and emitted by the female. Males typically have large highly branched antennae; most of the OSNs in male antenna are sensitive to pheromone compounds. The pheromone induces males to fly upwind (Vickers and Baker, 1994) from miles away.

1.6.3 Olfactory-Guided Behavior in Rodents

Rodent urine volatiles are important cues for mice to determine the age, sex, reproductive, and social status of the scent donor. Some of these volatiles are pheromones and are detected by the VNO (Keverne, 1999) and some by main olfactory bulb (Lin da et al., 2005). The removal of VNO leads to defects in inter-male aggression, male sexual behaviors, and maternal aggression (Wysocki and Lepri, 1991), while deletion of a large subgroup of pheromone receptors leads to a subset of these defects (Del Punta et al., 2002).

Even monomolecular compounds can prompt robust behavioral responses in mammals. Rabbit mammary pheromone 2-methylbut-2-enal, allows pups to rapidly locate mother's nipples and obtain milk (Schaal et al., 2003). A mouse urine compound (methylthio)methanethiol specifically activates a set of mitral cells and increases the attraction by female mice (Lin da et al., 2005).

Rodents readily use their sense of smell to find food. They can also distinguish chemicals that are very similar in structure and have almost identical representations in the brain (Linster et al., 2001; Rubin and Katz, 2001). Rats, for

example need less than 200 ms to distinguish odors that invoke similar glomerular activities in the brain (Uchida and Mainen, 2003).

1.6.4 Olfactory-Guided Behavior in *Drosophila*

Fly aversive olfactory-guided behavior has been studied in the "T-maze" in learning and memory paradigms (Tully et al., 1994). Experiments that used high and low concentration of fruit volatiles in the T-maze observed that flies tend to be repelled at high concentrations and attracted at low concentrations by these chemicals (Stensmyr et al., 2003).

Attractive behaviors are associated with important tasks such as finding food, mates, and sites for laying eggs. Traps that capture flies from a relatively large area (1 liter – greenhouse) simulate these search behaviors more closely than the T-maze. However, attractive behaviors may be harder to observe and quantify because they are subject to fly's motivation. To obtain meaningful information about levels of adult fly attraction to various chemicals in 2 liter traps, each experiment spans 20 hours (Park et al., 2002). Furthermore, the results obtained in laboratory conditions may not be readily translated into the field (Zhu et al., 2003).

1.6.4.1 Pheromone-Guided Behavior in Adult Flies

It was commonly believed that sexual dimorphism in *Drosophila* AL does not exist (Laissue et al., 1999; Stocker, 1994). A study that challenged this belief identified several species of Hawaiian *Drosophila* that exhibit sexual dimorphism in select glomeruli (Kondoh et al., 2003). Closer measurements of glomerular volumes in *D. melanogaster* revealed that glomeruli VA1lm and DA1 are

significantly larger in males than females (Kondoh et al., 2003). These observations leave room for sex pheromone-induced responses in fly's olfactory system.

Although volatile *Drosophila* pheromones have not been identified, it is believed that fly courtship has an olfactory component (Ferveur et al., 1995; Heimbeck et al., 2001). Hydrocarbons C20 to C30 in length are present on fly's cuticle to prevent the fly from desiccation (Gibbs et al., 2003). These cuticular hydrocarbons also function as pheromones that may signal fly's species, age, sex, and possibly other characteristics. The shorter hydrocarbons could be short-range volatiles, while the longer ones may be sensed through direct contact. Changing the male cuticular hydrocarbon profile to female by selectively misexpressing a feminine form of the *transformer (tra)* gene, does not change their behavior but induces courtship by other males (Ferveur et al., 1997). The receptors for cuticular hydrocarbons could be GRs. For example, silencing male-specific neurons in the foreleg that express *Gr68a* significantly decreases male courtship (Bray and Amrein, 2003; Thorne et al., 2005).

Fruitless (fru) gene encodes a sex-specifically spliced putative transcription factor that acts as a genetic switch for male courtship behavior (Demir and Dickson, 2005; Manoli et al., 2005; Stockinger et al., 2005) and controls the development of a single muscle in male's abdomen (Usui-Aoki et al., 2000). Expressing a masculine *fru* product in females transforms their courtship behavior to that of males (Demir and Dickson, 2005; Manoli et al., 2005). *Fru* is widely expressed in the central nervous system. *Fru* is also expressed in several

sexually dimorphic glomeruli in the AL, further implying an olfactory component to male courtship behavior (Stockinger et al., 2005).

Male-produced pheromone 11-cis vaccenyl acetate can act as an aggregation pheromone or inhibit courtship behavior (Ferveur, 2005). A subtype of trichoid olfactory sensilla on the antenna is sensitive to 11-cis vaccenyl acetate (Clyne et al., 1997). OBP LUSH seems to be expressed in all trichoid sensilla; a mutation in OBP LUSH abolishes both electrophysiological and behavioral response of flies to this pheromone (Xu et al., 2005). This study strongly implies trichoid sensilla, if not trichoid ORs in pheromone sensing.

1.6.4.2 Olfactory-Guided Behavior in Larvae

While in laboratory conditions, adult flies can survive for up to a month, the generation time of fruit flies at 25^{o}C is only 12 days. The life stages proceed from embryo to larva to pupa and then to the adult fly. Flies lay their eggs directly on the food source. The first 24 hours of its life, *Drosophila* is an embryo. Embryo hatches to become first instar larva, whereupon it undergoes two molts. The larval stage lasts about four days, but may be longer depending on food availability and other factors that influence the release of a molting hormone, ecdysone (Caldwell et al., 2005; Mirth et al., 2005). The metamorphosis of the fly takes another 100 hours, it starts at the end of the third instar stage, when larvae crawl out of the food and pupate.

The short developmental period that leads to the larval stage, presents larvae as convenient experimental animals. Furthermore, larvae are not distracted by complex visual cues or activities such as mating. The “job” of larva

is to gain weight to ensure proper development of the imaginal discs that later become adult organs. Since larvae spend most of their time in or on food, they need an olfactory system that differs in tuning and sensitivity from that of adults. Auspiciously, larvae exhibit robust chemotactic attraction to many odors (Boyle and Cobb, 2005; Cobb and Dannet, 1994; Cobb and Domain, 2000).

1.6.5 Fruit Volatiles and *Drosophila*

Fruit volatiles play central role in *Drosophila melanogaster's* life cycle. Flies are likely to meet at the fruit to mate and to lay their eggs. Thus, adult flies need to sense fruit from long distances. On the other hand, larvae live directly in the fruit and experience much higher concentrations of chemical stimuli. *Drosophilae* accomplish this formidable task of short and long range chemo-attraction with only 62 ORs in their arsenal.

The volatiles produced by various fruits are incredibly complex. With detailed analysis, one can extract more than 300 volatile components from a given type of fruit (Pino et al., 2005). The ratios of volatiles emitted tend to change as the fruit ripens and can span several orders of magnitude (Beaulieu and Grimm, 2001). When considering fruit volatiles, we cannot assume that ones at the highest concentrations are the ones that are most attractive. Neither can we assume that the volatiles that best represent this fruit to us are relevant to flies. Most abundant fruit volatiles, many of which smell fruity to us are esters. Esters and alcohols are also commonly used as odorants in *Drosophila* behavioral essays. In addition to those used in behavioral essays, fruit produce more complex volatiles that warrant testing. Several studies have coupled

extraction of fruit volatiles to direct behavioral (Oppliger et al., 2000; Zhu et al., 2003) or electrophysiological (Stensmyr et al., 2003) testing in adult flies or in larvae. These approaches uncover reliable and strong natural attractants for flies, but since the methods of volatile extraction differ, they may miss less abundant or less attractive volatiles.

1.7 Significance of Studying Olfaction in Flies

Why do we care about fly olfactory system? As discussed in this chapter, fly's olfactory system is much simpler, yet operates under principles that are similar to mammal's. Any differences that we observe between these systems will also be valuable to our understanding of how sensory systems work.

Although *Drosophila melanogaster* is not an agricultural pest, many other flies, moths, and beetles are. Setting pheromone traps as a method for mating disruption effectively controls populations of several moth species and provides a safer alternative to pesticides (Kovanci et al., 2005).

Mosquito, *Anopheles gambiae* is the prime vector for malaria, a deadly disease that affects 300 to 500 million people every year and kills over one million. There is a dire need for more effective and safe malaria prevention and treatment. Mosquitoes and other insects are harder to rear in laboratory than *Drosophila*. Additionally, genetic and transgenic techniques are not well developed in mosquitoes. Encouragingly, *Anopheles gambiae* ORs that respond to 2-ethylphenol and 4-ethylphenol, key components of sweat, were identified by functional expression in *Drosophila* olfactory sensilla (Hallem et al., 2004b). Hence, *Drosophila* is also an attractive model system for malaria research.

2 Odorant Receptor Map in the Fly Antennal Lobe

2.1 Introduction

Drosophila melanogaster is a powerful genetic model, and a promising model system in olfaction. The neuronal connectivity of the *Drosophila* olfactory system is similar to that of mammals; a set of OSNs that express the same OR project to one or two glomeruli, and a homogenous population of OSNs is thought to occupy each glomerulus. Easy recognition of fly's glomeruli allows for precise olfactory-related activity imaging studies in the AL in both presynaptic and postsynaptic neurons. Electrophysiological responses of OSNs to odors are also reliably recorded from the surface of antenna and maxillary palp. Recently, most of the ORs expressed in basiconic sensilla were identified in a homologous gene expression model (Hallem et al., 2004a). Alas, it is not possible to correlate electrophysiological recordings at cell body and the calcium-associated activity in the brain.

The factors that determine OR expression within each OSN are not known. It is currently not possible to predict the positions of glomeruli from either the OSN positions on the antenna or the OR sequence. Thus, the OR–OSN–glomerulus correlations need to be determined experimentally. Lack of knowledge of the OR identities of glomeruli in the fly impedes our understanding of information processing in the insect AL. Filling in the OR identities of all the glomeruli within the antennal lobe may also uncover additional unexpected characteristics of the olfactory system in insects.

Genetic marking of several OSN populations using the OR promoters tethered to *Gal4* (Brand and Perrimon, 1993) have allowed for direct visualization of OSNs and glomeruli that express specific ORs (Vosshall et al., 2000). Some features of the OSN connectivity to the AL are known. It is known that OSNs expressing a single OR can project to two glomeruli as is the case of *Or23a* (Vosshall et al., 2000). Two ORs can also be co-expressed in the same cells in *Or22a/Or22b* OSNs in the antenna (Dobritsa et al., 2003) and in the *Or33c/Or85e* OSNs in the maxillary palp (Goldman et al., 2005). The *Gal4/UAS* system also allows for genetic manipulation such as neuron inactivation, misexpression of other ORs, or expression of markers such as calcium sensitive dyes. The availability of this toolkit for all of the 43 glomeruli (Laissue et al., 1999) within the AL will greatly increase the power of *Drosophila* as a model system for olfaction.

Among the 62 known ORs in the *Drosophila* genome, 39 are expressed in antenna or maxillary palpi, as determined by RNA *in situ* hybridization (Vosshall et al., 2000). These ORs are the prime candidates for *OR→Gal4* construction. The remaining ORs are undetectable in the antenna and their expression patterns are not known. It is possible that some of the ORs are expressed at low levels in fly olfactory tissues and their RNA is not detectable *in situ*. The Gal4/UAS amplification process may reveal the expression of such low-abundant transcripts. It is also possible that the undetected 13 ORs are expressed in other tissues. Since the *Drosophila* larvae robustly chemotax to odors, it is likely that the remaining ORs are larval.

To contextualize the data from the imaging studies and to compare it with electrophysiological findings, we set out to determine the OR identities of fly's glomeruli. We were also interested in the rules for expression of the ORs and connectivity of OSNs. To understand the rules of connectivity in the antennal lobe, we compiled a near-complete map of OR-specific OSNs projections to the AL. In total, we analyzed the expression profile of 25 antennal and five maxillary palp ORs. The results show surprising complexity in organization of the fly's olfactory circuit. Four populations of OSNs co-express two ORs, and a fifth expresses one OR and one GR. One glomerulus receives co-convergent input from two separate populations of OSNs, leading to mixed sensory input. Three ORs label sexually dimorphic glomeruli implicated in sexual courtship, and are thus candidate *Drosophila* pheromone receptors. This olfactory sensory map provides an experimental framework for relating ORs to glomeruli and ultimately to output of the olfactory system.

2.2 Materials and Methods

2.2.1 *Drosophila* Stocks

All fly stocks were maintained on conventional cornmeal-agar-molasses medium under a 12 hour light:12 hour dark cycle at 18^{o}C or 25^{o}C. Transgenic constructs were injected into yw embryos using standard procedures and single transformants outcrossed to autosomal balancers for chromosomal mapping. Fly stocks were kindly provided by: *UAS→GFP and UAS→IMPTNT* (Bloomington Stock Center); *UAS→CD8::GFP* (Liqun Luo, HHMI); *UAS→n-Syb::GFP* (Mani

Ramaswami, The University of Arizona); *Or46a→n-Syb::GFP* (Lawrence Zipursky, HHMI).

2.2.2 Odorant Receptor Promoter Element Transgene Construction

To mark the OSNs that express each of the ORs, we employed *Gal4/UAS* system (Brand and Perrimon, 1993). We used putative OR promoters to express Gal4 and genetically label the OSNs. We assumed that bulk of the promoter lies within 10 Kb upstream of the transcription start. Since no information is available about 5' untranslated regions (UTRs) of the ORs, we used 5' sequence up to the predicted translation start. The sequences were selected to exclude verified or predicted genes on the same strand, and where possible on the opposite strand of DNA. Following sequence lengths directly upstream of the first Methionine were used: *Or10a*, 6.868 Kb; *Or13a*, 8.199 Kb; *Or19a*, 9.934 Kb; *Or22a*, 7.717 Kb (Vosshall et al., 2000); *Or23a*, 7.818 Kb (Vosshall et al., 2000); *Or33a*, 5.155; *Or33b*, 8.086 Kb; *Or33c*, 7.156 Kb; *Or35a*, 3.880 Kb; *Or42a*, 4.184 Kb; *Or42b*, 8.039 Kb; *Or43a*, 3.424 Kb (Wang et al., 2003a); *Or46a*, 1.875 Kb (Vosshall et al., 2000), *Or47a*, 8.239 Kb (Vosshall et al., 2000); *Or47b*, 7.467 Kb (Vosshall et al., 2000); *Or49b*, 8.834 Kb; *Or56a*, 5.385 Kb; *Or65a*, 7.764 Kb; *Or67b*, 2.740 Kb; *Or67d*, 7.272 Kb; *Or69a*, 1.923 Kb; *Or71a*, 2.282 Kb (Hummel et al., 2003); *Or82a*, 1.865 Kb; *Or83c*, 7.843 Kb; *Or85a*, 2.791 Kb; *Or85e*, 7.5 Kb (Vosshall et al., 1999); *Or85f*, 8.961 Kb; *Or88a*, 1.656 Kb; *Or92a*, 9.247 Kb; *Or98a*, 9.538 Kb.

The above regions were amplified using Expand High Fidelity PCR system (Roche) from *Drosophila melanogaster* Oregon R genomic DNA. The PCR

products were cloned into pGEM-T easy (Promega), and then subcloned into pCaSpeR-AUG-*Gal4* (Vosshall et al., 2000), upstream of *Gal4.*

The following *OR→Gal4* lines did not express or were expressed too weakly to be analyzed: *Or2a*, 0.900 Kb; *Or7a*, 4.203 Kb; *Or22b*, 0.900 Kb; *Or65b*, 3.405 Kb, and *Or98b*, 5.120 Kb. *Or59c* (5.280 kb) and *Or85c* (7.588 Kb) showed ectopic expression that did not faithfully reproduce the expression of the endogenous OR. No PCR product could be obtained from the regions upstream of the following ORs, due to either annotation or technical issues: *Or9a*, *Or19b*, *Or43b*, *Or67a*, *Or67c*, *Or69b*, and *Or94a*. The following ORs were too closely linked to other genes to attempt an *OR→Gal4* fusion construct: *Or46b*, *Or59b*, *Or65c*, *Or85b*, *Or85d*, and *Or94b*. Eleven additional *OR→Gal4* transgenes *Or1a, Or22c, Or24a, Or30a, Or45a, Or45b, Or49a, Or59a, Or63a, Or74a,* and *Or83a* are expressed only in larval OSNs.

2.2.3 Labeling Olfactory Sensory Neurons

2.2.3.1 β-galactosidase Activity Staining

To visualize the expression of ORs in the antenna, flies homozygous for *OR→Gal4* were crossed to flies carrying cytoplasmic *LacZ* under control of upstream activating sequence *(UAS→LacZ*). Heads of the heterozygous progeny were stained for *β-galactosidase* activity (Wang et al., 1998). Antennae were removed from the flies' heads and photographed under light microscope with 40X lens.

2.2.3.2 Immunohistochemistry

For immunohistochemical analysis, a synaptic marker n-Synaptobrevin was expressed under control of *UAS* regulatory element. Flies homozygous for *OR→Gal4* were crossed to flies that carry UAS*→n-Synaptobrevin::Green Fluorescent Protein* (*UAS→n-Syb::GFP*). Brains were dissected and immunostained using a previously described protocol (Laissue et al., 1999) with nc82 antibody (Reinhard Stocker, University of Fribourg, Switzerland) to reveal features of neuropil at 1:10 dilution, which was visualized with goat Cy3-coupled antibody at 1:200 dilution (Jackson ImmunoResearch). Expression of n-Syb::GFP was detected with rabbit anti-GFP antibody (Molecular Probes) at 1:1000 dilution and visualized with goat AlexaFlour488-coupled antibody at 1:100 dilution (Molecular Probes).

To differentially label *Or46a* versus other maxillary palp glomeruli, we used a synaptic epitope *Or46a→n-Syb::GFP* coupled to *OR→Gal4* and *UAS→IMPTNT* (Sweeney et al., 1995), another synaptically targeted marker. Brains of *Or46a→*n-Syb::*GFP/OR→Gal4;UAS→IMPTNT* animals were visualized with anti-GFP, as described above, and anti-tetanus toxin at 1:10000 dilution (Thomas Binz, Medizinische Hochschule Hannover, Germany) antibodies.

Brains were mounted in Vectashield® (Vector Laboratories) with 11 x 22 mm No.1 cover slips as spacers. Z-sections 0.45 µm thick that spanned all of AlexaFlour488 signal were recorded under LSM510 Zeiss confocal microscope with 40X lens. Images of ALs represent collapsed Z-series that provide relative spatial context the stained glomerulus. The segmentation software Amira™

(Mercury Computer Systems, Berlin) was used to examine and rotate confocal Z-stacks of *Or46a→n-Syb::GFP/OR→Gal4;UAS→IMPTNT* brains.

2.2.4 Verification of Transgene Expression

Fluorescent two-color RNA *in situ* hybridization was used to verify expression of *OR→Gal4* transgenes. RNA *in situ* hybridization was performed as previously described (Vosshall et al., 1999). We used digoxigenin- and fluorescein-labeled riboprobes. Fluorescein riboprobes were detected with TSA-Plus Fluorescein System (PerkinElmer). After one-hour quenching period with 3% hydrogen peroxide, digoxigenin riboprobes were detected with TSA-Plus Cyanine5 System (PerkinElmer). Anti-digoxigenin-POD and anti-fluorescein-POD antibodies were diluted to a ratio of 1:500, by volume (Roche). At least two *OR→Gal4* lines were examined for each OR.

2.3 Results

2.3.1 Peripheral Organization of the *Drosophila* Olfactory System

This work represents a genetic effort to label OSNs that express most of the *Drosophila* OR genes and map their projections to morphologically defined glomeruli in the adult AL. Putative regulatory regions upstream of 49 ORs were cloned in front of the *Gal4* transcription factor. Expression of the *OR→Gal4* transgenes was visualized in the antenna and the maxillary palp with *UAS→LacZ*.

Thirty of the 49 *OR→Gal4* transgenes, 25 antennal and five maxillary palp ORs, exhibit restricted gene expression in subpopulations of adult OSNs (**Figure 2.1**). Although cytoplasmic LacZ, which primarily stains OSN cell body, seems to be a less sensitive than GFP in axons, *LacZ* is expressed in stereotyped

overlapping patterns in the antenna. *LacZ* expression ranges from few cells (*Or22a*) to many (*Or47b, Or67d,* and *Or88a*) that broadly correspond to sensillum zones of large basiconics and trichoids, respectively (de Bruyne et al., 2001; Shanbhag et al., 1999). Of the remaining 19 *OR→Gal4* constructs, 11 are selectively expressed in the larval olfactory system, *Or83b* is broadly expressed in most OSNs where it plays an essential role in olfaction (Larsson et al., 2004), and seven other ORs either show no expression or are ectopically expressed (**Section 2.2.2**).

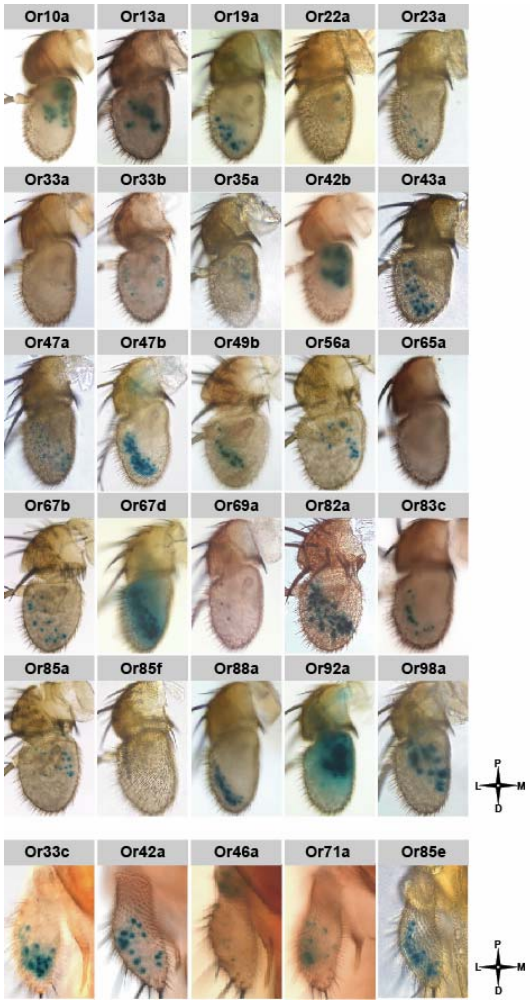

Figure 2.1: Odorant receptor expression in *Drosophila* chemosensory organs. Expression of *OR→Gal4* transgenes, visualized with LacZ activity staining

(blue) reveals stereotyped yet overlapping patterns of gene expression in antenna (top) and maxillary palp (bottom). *Or85f→Gal4* is weak and *Or65a→Gal4* staining is not detectable under these conditions. Orientation as specified at lower right: P=proximal; D=distal; L=lateral; M=medial.

Patterns of *OR→Gal4:UAS→lacZ* gene expression of 30 transgenes in the antenna and maxillary palp (**Figure 2.1**) are similar to those obtained by RNA *in situ* hybridization (Vosshall et al., 2000) and follow the same strict segregation of ORs expressed in the antenna and maxillary palp, with no *OR→Gal4* lines expressed in both organs. No β-galactosidase was detected in other sensory organs of the head (data not shown). For about half of the *OR→Gal4* constructs, β-galactosidase staining was performed in the whole fly and no labeling was seen in body parts other than antenna of maxillary palp (data not shown), leading us to believe that expression of ORs is restricted to fly's olfactory organs.

To verify that *OR→Gal4* lines reflect faithful expression of OR mRNA, we performed RNA *in situ* hybridization against *Gal4* and the endogenous OR. Transgene expression reflects that of an endogenous OR, except in few cases where the levels of fluorescence differed or had incomplete overlap (data not shown). For presented subset of ORs, though the levels of staining vary, all cells appear to have both endogenous OR and *Gal4* transcripts.

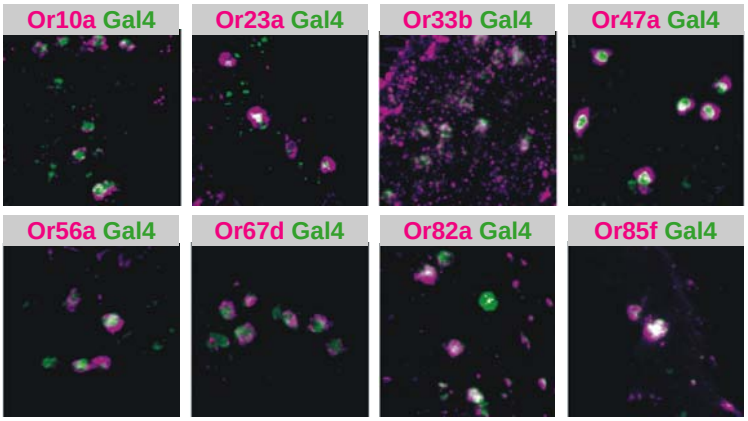

Figure 2.2: Two-color RNA *in situ* hybridization supports faithful expression of *OR→Gal4* transgenes. Frozen sections of *OR→Gal4* fly heads were hybridized with anti-OR digoxigenin (magenta) probes and anti-*Gal4* fluorescein (green) riboprobes.

2.3.2 Odorant Receptor-Map in the Adult Antennal Lobe

2.3.2.1 Glomerular Map of the Antenna

To determine how OSNs expressing different ORs connect to the brain, we labeled the termini of their axons. Analysis of glomerular projections of the 25 antennal *OR→Gal4;UAS→n-Syb::GFP* constructs reveals that 23 populations of OSNs that express different ORs target single glomeruli, while two (*Or33b* and *Or67d*) project to two glomeruli (**Figure 2.3A**). Both *Or33b→Gal4* and *Or67d→Gal4* seem to be co-expressed with their respective endogenous ORs (**Figure 2.2**).

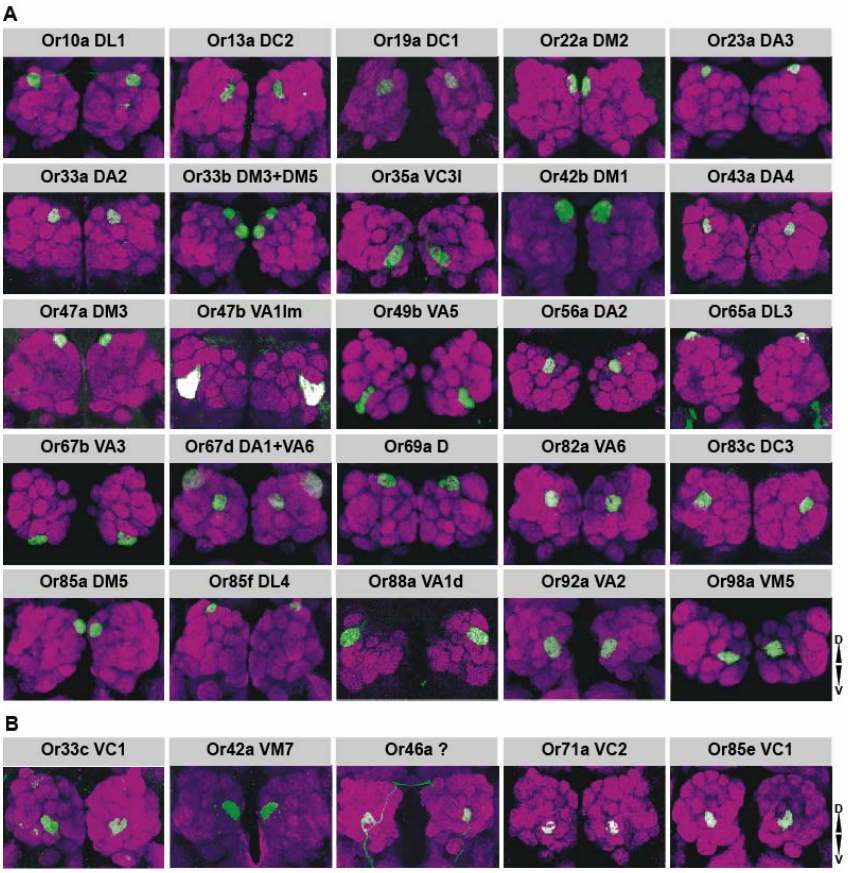

Figure 2.3: Axonal projections of fly's olfactory sensory neurons converge upon precise glomerular targets. (A) Whole mount immunofluorescence of adult ALs from antennal *OR→Gal4;UAS→n-Syb::GFP* animals stained with anti-GFP (green) and nc82 (magenta). Each data panel is labeled with a given *OR→Gal4* line and the corresponding glomerulus. (B) Analysis of maxillary palp *OR→Gal4* lines performed as in (A) above, except that *Or46a→Gal4* was crossed to *UAS→CD8::GFP*. Multiple independent samples were examined to make glomerular assignments. Orientation as specified at lower right, D = dorsal, V = ventral. All images are projections of confocal Z-series, available as raw confocal stacks at http://www.rockefeller.edu/labheads/vosshall/reprints/fishilevich_vosshall_fig2.php.

In some cases, there is weak or variable labeling in secondary glomeruli. As noted by others (Bhalerao et al., 2003), *Gal4* expression in secondary glomeruli reveals either real variability in the expression levels of ORs in different subpopulations of OSNs, or reflects transgene variability. Distinguishing between

these possibilities is constrained by detection thresholds of *in situ* hybridization technique, which may not detect OR transcripts in cells weakly positive for the *OR→Gal4* transgene. As previously reported, some *Or23a→Gal4* lines mark a second, weaker glomerulus at a more ventral location, possibly DP1m (Gao et al., 2000; Vosshall et al., 2000). Other cases of weak or variable secondary innervations include *Or65a→Gal4*, in vicinity of D; *Or85a→Gal4*, DM3; *Or56a→Gal4*, possibly DL4; *Or10a→Gal4*, in vicinity of VA7m; *Or33b→Gal4*, variable ectopic expression in multiple glomeruli. We base our general conclusions below on those glomeruli that show strong and reproducible labeling, while recognizing that the weakly labeled glomeruli may also contribute to the odor code.

2.3.2.2 Glomerular Map of the Maxillary Palp

Axonal projections of the five maxillary palp *OR→Gal4* lines were examined in brain whole mounts (**Figure 2.3B**). All palp neurons target a tight cluster of glomeruli in the ventomedial region in the AL that does not receive projections from antennal OSNs (**Figure 2.3B**). The segregation of antennal and maxillary palp projections in *Drosophila* was previously noted in anatomical tracing studies that preceded the advent of OR markers (Stocker, 1994). The functional significance of antennal/maxillary palp segregation remains obscure because no exclusive function was found for the maxillary palp.

In contrast to previous reports which mapped *Or46a* to VA5 (Komiyama et al., 2004), we could not assign *Or46a*-expressing OSNs to a known glomerulus, due to diffuse boundaries and somewhat variable position of this glomerulus. To

clarify the position of *Or46a* relative to other maxillary palp glomeruli, we simultaneously examined the axonal projections of *Or46a*-expressing and other palp OSNs. The *Or71a* glomerulus is located in the same anterior-posterior plane but medial to *Or46a*, while the *Or33c*/*Or85e* glomerulus is located posterior and medial to *Or46a* (**Figure 2.4**).

Figure 2.4: Three-dimensional reconstructions of close-lying maxillary palp glomeruli. To clarify the positions of known palp glomeruli relative to *Or46a*, brains of *Or46a→n-Syb::GFP/OR→Gal4;UAS→IMPTNT* animals are visualized with anti-GFP (cyan) and anti-tetanus toxin (magenta) antibodies. Z-series of glomeruli are reconstructed and rotated using segmentation software Amira™. Orientation as specified at lower right: A=anterior; P=posterior; D=dorsal; V=ventral.

2.3.3 Complexity of Olfactory Circuit: Co-expression and Co-convergence

From the glomerular assignments, it is apparent that several OSN types map to the same glomeruli (**Figure 2.3**). A complete list of ORs examined here and the glomeruli they target is presented in **Figure 2.5**, sorted by the name of glomerulus. There are five cases in which two *OR→Gal4* lines label the same glomerulus and one case where a single glomerulus is marked by an *OR→Gal4* and a *GR→Gal4* transgene. The above observations can be explained by co-expression of two ORs in the same ONS or co-convergence of two OSN populations onto the same glomerular target.

To test these hypotheses, we performed RNA *in situ* hybridization against pairs of candidate ORs (**Figure 2.5**). Maxillary *Or33c* and *Or85e* are co-expressed (Goldman et al., 2005) and these OSNs target the VC1 glomerulus (**Figure 2.5**). *Or33a* and *Or56a* are also expressed in the same OSNs that target DA2; same is true for the *Or10a/Gr10a* pair that project to DL1. Additionally, the expression of *Or33b* partly overlaps with both *Or47a* and *Or85a*. However, there seems to be no overlap between *Or47a* and *Or85a* expression (data not shown). *Or67d* and *Or82a* are not co-expressed, suggesting that these OSN populations co-converge.

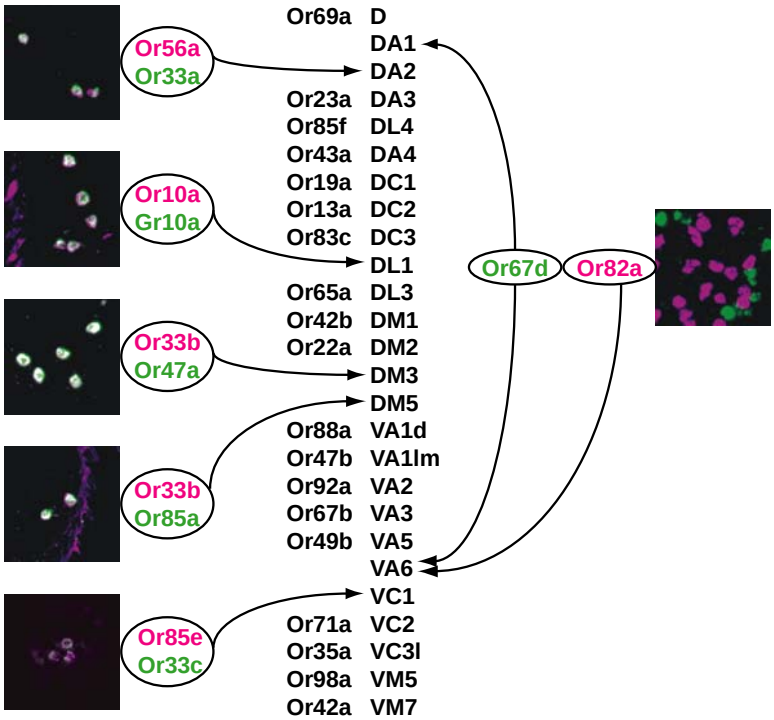

Figure 2.5: The glomerular identities of ORs reveal unexpected complexity of OR co-expression and OSN co-convergence. Summary of OR/glomerulus assignments, sorted by glomerulus, with cases of co-expression at the left and co-convergence at the right. RNA *in situ* results are shown for cases of possible co-expression and co-convergence, with digoxigenin- (magenta) and fluorescein-labeled (green) anti-OR riboprobes.

2.3.4 Topological Organization of the *Drosophila* Antennal Lobe

The availability of a near-complete map of AL projections allowed us to examine the organizational logic at the first olfactory synapse. There seems to be a relation between OSN location on the surface of sensory organs and their targets in the brain. Medial region of the antennal lobe is occupied by maxillary palp glomeruli. The ORs that are expressed at the distal/lateral periphery of the antenna correspond to glomeruli in lateral region of the antennal lobe. The OSNs in the proximal/medial region of the antenna map primarily to the medial region of AL.

Although we did not identify sensillum types in which each of the ORs are expressed, the segregation is most likely related to the topographic segregation of trichoid and basiconic classes of sensilla on the surface of the antenna (de Bruyne et al., 2001; Shanbhag et al., 1999; Stocker, 1994). *Or47b*, *Or88a*, and *Or67d* are all likely trichoid and *Or42b*, *Or33b*, and *Or22a* are basiconic (Hallem et al., 2004a). There are exceptions, notably *Or19a* targets a dorsal/medial glomerulus although *Or19a* OSNs are located in the lateral/distal domain in the antenna.

Of the 43 glomeruli and 47 distinct glomerular compartments (Laissue et al., 1999), this study assigns a genetic OR identity to 26. We were unable to assign a name to *Or46a*, which may be a previously unnamed glomerulus. Other studies mapped *Gr21a* OSNs to V (Scott et al., 2001) and *Or59c* OSNs to 1 (Komiyama et al., 2004), bringing the total known number of OR assignments to AL glomeruli to 29. Only few glomeruli mapped to the most posterior and ventral regions of the

antennal lobe. The missing assignments may correspond to unassigned ORs*, GRs,* or other receptors.

2.3.5 Functional Organization of the *Drosophila* Antennal Lobe

With receptotopic map of the AL at hand, we sought to determine the logic of organization in the *Drosophila* AL. It is unknown whether the organization of glomeruli within AL follows sequence of the ORs, is guided by the chemistry of ligands, or is determined by other factors. We integrated known ligands from previous studies for the ORs (Goldman et al., 2005; Hallem et al., 2004a; Kreher et al., 2005; Störtkuhl and Kettler, 2001; Wetzel et al., 2001) or glomeruli (Wang et al., 2003a) onto receptotopic map to generate an odortopic map (**Figure 2.6**). The limited and non-overlapping collections of odorants used by different groups as well as differences in experimental techniques made it difficult to compare across studies. In summation, each OR/glomerulus was screened with a small subset of the 76 odors used across these six studies and thus no comprehensive survey of the ligand specificity of a given OR/glomerulus exists. Several glomeruli respond selectively to aromatic odors. We also find a greater tendency for OSNs expressing broadly responsive ORs to project to dorsal/medial glomeruli while the more selective glomeruli are located at ventral/lateral positions (**Table 2.1** and **Figure 2.6**). However, there are many exceptions to this rule and the ordered chemotopy described in the mouse olfactory bulb (Uchida et al., 2000) is not obvious in the fly AL.

OR	glomerulus	strong ligands
Or10a	DL1	3/28: isoamyl acetate [1, 2], acetophenone [2], methyl salicylate [2]
Or13a	DC2	2/16: 1-octen-3-ol [1], 3-octanol [1]
Or19a	DC1	11/30: cineole [1], hexane [1], isoamyl acetate [1, 2], pentyl acetate [2], ethyl butyrate [2], 1-hexanol [2], 1-octen-3-ol [2], 2-heptanone [2], 2-octanone [2], 1-octanol [2], butyl acetate [2]
Or22a	DM2	11/29: r-carvone [1], octanal [1], 3-octanone [1], caproic acid [1], hexane [1], isoamyl acetate [1, 2], pentyl acetate [2], 1-octen-3-ol [2], ethyl butyrate [2], ethyl proprionate [2], butyl acetate [2]
Or23a	DA3	0/16 [2]
Or33a	DA2	0/16 [1]
Or33b	DM3+DM5	0/28 [1, 2]
Or33c	VC1	1/11: E2-hexenal [3]
Or35a	VC3l	11/17: pentyl acetate [2], ethyl butyrate [2], 1-hexanol [2], 1-octen-3-ol [2], E2-hexenal [2], cyclohexanone [2], 2-heptanone [2], 1-butanol [2], 1-octanal [2], ethyl 3-hydroxy butyrate [2], 1-nonanal [2], 2-pentanone [2]
Or42a	VM7	9/30: ethyl acetate [3, 4], isoamyl acetate [3, 4], E2-hexenal [3, 4], 2-heptanone [3, 4], ethyl butyrate [4], 1-hexanol [4], 2,3-butanedione [4], propyl acetate [4], 1-butanol [4]
Or42b	DM1	11/16: cyclohexanone [1], r-carvone [1], s-carvone [1], linalool [1], 3-octanone [1], 3-octanol [1], 1-octen-3-ol [1], benzaldehyde [1], caproic acid [1], hexane [1], isoamyl acetate [1]
Or43a	DA4	9/39: cineole [1], hexane [1], isoamyl acetate [1], hexanol [2], 1-octen-3-ol [2], cyclohexanol [2, 5, 6], cyclohexanone [2, 5, 6], benzaldehyde [5, 6], benzyl alcohol [5, 6]
Or46a	?	1/11: 4-methylphenol [3]
Or47a	DM3	6/28: hexane [1], isoamyl acetate [1], ethyl acetate [2], pentyl acetate [2], 2-heptanone [2], 3-(methylthio)-1-propanol [2]
Or47b	VA1l/m	0/15: [2]
Or49b	VA5	2/17: 3-methyl phenol [2], 2-methyl phenol [2]
Or56a	DA2	0/16 [1]
Or65a	DL3	0/17: [2]
Or67b	VA3	13/39: cyclohexanone [1], 3-octanone [1], 1-octen-3-ol [1], hexane [1], pyridine [1], pentyl acetate [4], 1-hexanol [4], E2-hexenal [34], 2-heptanone [4], 1-butanol [4], 1-heptanol [4], benzaldehyde [4], acetophenone [4]
Or67d	DA1+VA6	N.D.
Or69a	D	1/16: isoamyl acetate [1]
Or71a	VC2	1/23: 4-methylphenol [3]
Or82a	VA6	1/17: geranyl acetate [2]
Or83c	DC3	N.D.
Or85a	DM5	4/30: ethyl butyrate [2], 1-hexanol [2], ethyl 3-hydroxybutyrate [2], E2-hexen-1-ol [2]
Or85e	VC1	3/11: ethyl acetate [3], cyclohexanone [3], (-) fenchone [3]
Or85f	DL4	0/16 [2]
Or88a	VA1d	2/29: hexane [1], isoamyl acetate [1]
Or92a	VA2	3/16: r-carvone [1], s-carvone [1], octanal [1]
Or98a	VM5	7/15: pentyl acetate [2], ethyl butyrate [2], 1-hexanol [2], 1-octen-3-ol [2], 2-heptanone [2], E2-hexen-1-ol [2], linalool [2]

Table 2.1: Summary of odorant receptor ligand specificity. Maxillary palp genes are highlighted in light gray. Assignments for *Or46a* and *Or83c* differ from a previous study (Komiyama et al., 2004). Odors that activate each OR or its corresponding glomerulus are listed in the right column and are drawn from previous studies. Listed are the number of strong ligands/total ligands tested in all studies that examined the OR/glomerulus, the references, and a list of strong ligands. “Strong ligands” include odorants that: yielded activity at 20% saturated vapor in imaging experiments [1] (Wang et al., 2003a), produced more than >100 spikes/sec in electrophysiological experiments [2] (Hallem et al., 2004a), [3] (Goldman et al., 2005), and [4] (Kreher et al., 2005), or stimulated activity in heterologous expression systems [5] (Störtkuhl and Kettler, 2001) and [6] (Wetzel et al., 2001). N.D. = not done.

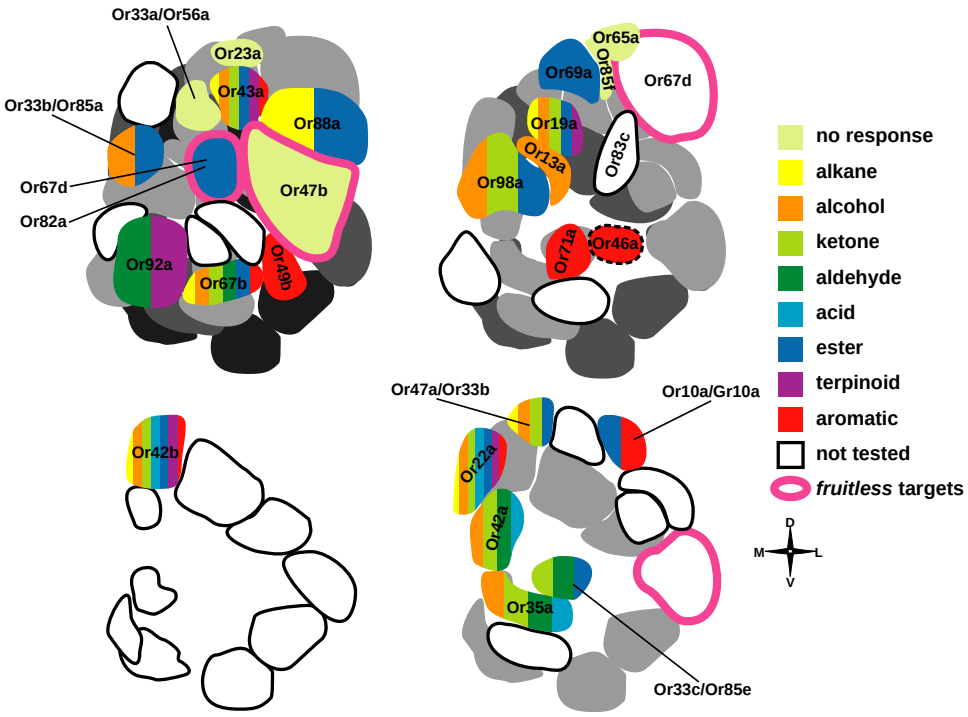

Figure 2.6: Odotopic map of olfactory projections reveals functional subdomains in the *Drosophila* antennal lobe. Schematic map coded for functional properties, the glomeruli are colored according to the functional groups of odorants that activate ORs/glomeruli. The odorants are classified by the functional group with the highest priority (IUPAC nomenclature), aromatic ring compounds and terpene derivatives are placed in separate categories (see **Table 2.1**); *fru* target glomeruli outlined in pink (Manoli et al., 2005; Stockinger et al., 2005); Estimated position of *Or46a* is mapped posterior of VA7l (dashed line). Antennal lobe model adapted from Figure 2 of Laissue *et al.* with permission; antennal lobe sections appear from anterior to posterior, clockwise from top left, using grey-scale depth-coding. Map represents 47 individual glomerulus compartments (Laissue et al., 1999), along with *Or46a*.

2.3.6 Implications of Co-expression for Odor Coding

Five subpopulations of OSNs that express multiple receptors along with the universal co-receptor OR83b (Larsson et al., 2004). What might be the function of such OR co-expression? In case of OR22a/OR22b co-expression, only OR22a is thought to contribute to the OSN response profile (Dobritsa et al., 2003). OR33c that is co-expressed with OR85e, exhibits relatively weak responses to

odors (Goldman et al., 2005). Even if it produces weak responses on its own, the second OR potentially modulates responses of the OSN. For example, OR33b does not respond to any known odors (Hallem et al., 2004a). However, when present together with OR47a or OR85a, OR33b could change the response profiles of these OSNs. In support, both OR47a and OR85a respond to more odors when ectopically expressed without OR33b in the ab3A "empty" neuron than the native neuron (Hallem et al., 2004a). Confirmation of modulatory role of co-expressed ORs awaits further genetic analysis *in vivo*.

Another possible function of co-expressed ORs is to act as mixture-specific detectors. It is believed that adding an additional OR to an OSN produces a linear increase in the activity of that OSN (Hallem et al., 2004a). However, it is not known how a linear change in the firing rate of the OSN affects the activity of PNs or Kenyon cells. It is possible that activation of two ORs within OSN sets the OSN activity over certain threshold that is meaningful to a fly. Co-expression may be a particularly useful strategy that minimizes the number of OSNs necessary to respond to an ecologically relevant mixture. Since the responses of OSNs saturate at about 250 spikes/second (de Bruyne et al., 2001), having two ORs within OSN also puts a cap on the maximal output of this OSN when a relevant mixture is presented.

Another intriguing population of OSNs co-expresses members of the OR and GR families, along with *Or83b*. The open reading frames of *Or10a* and *Gr10a* are only 350 nucleotides, suggesting that they are co-expressed (Robertson et al., 2003). *Or10a* and *Gr10a* do not have the sequence identity to

be considered recent gene duplication (Robertson et al., 2003), however, it is possible that one have jumped and is a “hitchhiker” on the promoter of another. In larvae the *Or10a/Gr10a* promoter is expressed in the gustatory organ (data not shown), thus it is unclear which gene is the hitchhiker. The role of GRs in the antenna is poorly understood, although *Gr21a* is expressed in neurons that respond to carbon dioxide (Scott et al., 2001; Suh et al., 2004). It will be interesting to determine whether GR10a contributes to the detection of odors in the antenna along with OR10a and serves an olfactory instead of gustatory function.

2.3.7 Candidate *Drosophila* Pheromone Receptors

Recent work examining the expression of the male-specific isoform of the *fruitless* (*fru*) transcription factor implicates two large, sexually dimorphic glomeruli (VA1lm and DA1) in male courtship behavior (Demir and Dickson, 2005; Manoli et al., 2005; Stockinger et al., 2005). This study has revealed the molecular identity of OSNs projecting to VA1lm and DA1 as *Or47b* and *Or67d* expressing OSNs, respectively. Other glomeruli that receive input from *fru*-expressing OSNs are VL2a (Manoli et al., 2005; Stockinger et al., 2005) and occasionally VA6 (Stockinger et al., 2005), which we identify as receiving input from both *Or82a*- and *Or67d*-expressing OSNs. The identity of the VL2a-projecting OSNs remains obscure.

The *fru*-expressing OSNs and the glomeruli to which they project show little or no activation in response to general odors (**Table 2.1** and Figure 2.6) and (Hallem et al., 2004a; Wang et al., 2003a). The exception is OR82a, which

responds very selectively to geranyl acetate, a green leaf volatile that is also a major component of medfly male sex pheromone (Hallem et al., 2004a; Light et al., 1999). Based on genetic anatomical marking, *Or82a*- and *Or67d*-expressing OSNs target the VA6 glomerulus. Whether they synapse uniformly upon the same population of postsynaptic PNs or whether the glomerulus has sub-compartments is not known. In either scenario, the glomerulus might act as a coincidence detector that would require both the OR82a ligand and the unknown OR67d ligand for activation.

Courtship behavior in *Drosophila* involves multi-modal input of visual, gustatory, auditory, and olfactory cues (Yamamoto et al., 1997). The involvement of volatile pheromones in *Drosophila* sexual behavior has long been inferred, but neither the volatile sex pheromones nor the receptors that detect them are known. Our observations suggest that the ORs in *fru*-expressing OSNs respond to volatile pheromones. In support of this, silencing or reprogramming these OSNs leads to selective disruption in male sexual behavior (Manoli et al., 2005; Stockinger et al., 2005).

2.3.8 Independent Validation of Receptotopic Map of the Antennal Lobe

A paper that was published concurrently with our report of this work has mapped the OR identities of 44 OSN populations that project to 37 glomeruli (Couto et al., 2005). The glomerulus assignments agree for all assigned glomeruli that overlap between the reports. The conclusions from the glomerular map generally agree, with some differences in interpretations. Couto *et. al.* mapped electrophysiological responses of the ORs (de Bruyne et al., 1999; de

Bruyne et al., 2001; Hallem et al., 2004a) onto the AL and observed a segregated cluster of glomeruli that respond to aromatic compounds. When we add results from a study that imaged odor-evoked activity in the AL (Wang et al., 2003a), this trend is not as strong (**Figure 2.6**). They also report a progression of responses for short- to long-chained esters from medial to lateral regions of the AL. Another analysis by Couto *et. al.* revealed a moderate correlation of sequence similarity and separation of glomeruli in basiconic sensilla. Our analysis using phylogenetic trees of the OR family sequences did not discover a trend that could be used as a predictor of glomerulus positions. Despite efforts of multiple research groups, there are more than a dozen glomeruli with their receptor identities unknown (**Figure 2.7**). Future work will reveal the receptors and possible novel functions for these unassigned glomeruli.

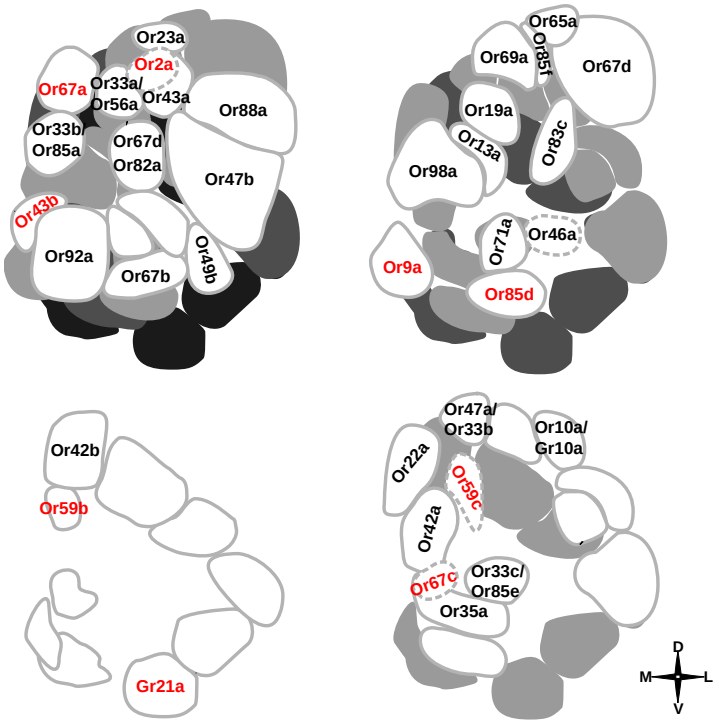

Figure 2.7: Known glomeruli of the *Drosophila* antennal lobe. Receptor assignments in black are same as in Figure 2.6. Receptors in red are *Gr21a* (Scott et al.,

2001) and *Or59c* (Komiyama et al., 2004), rest (Couto et al., 2005). *Or59c* glomerulus 1 is shown by dashed line. *Or2a* and *Or67c* OSNs map to previously unidentified glomeruli shown by dashed line (Couto et al., 2005). The receptor identities of blank glomeruli are unknown.

2.4 Conclusions

Here we present a nearly complete map of olfactory projections to the fly AL. From this map, we identify five populations of OSNs that express multiple receptors and two populations of OSNs expressing different ORs that co-converge upon a common glomerulus. An analysis of published odor response profiles for these ORs and their glomeruli suggests that OSNs that are tuned to a broad range of odors map to the dorsal/medial domain of the AL, while more restricted OSNs map to ventral/lateral glomeruli. We also identify candidate *Drosophila* pheromone receptors by virtue of their innervations of sexually dimorphic *fru*-positive glomeruli. A number of intriguing questions follow from our study. First, what is the genetic identity of the projections that target the posterior face of the antennal lobe? These glomeruli may receive input from OSNs expressing OR or GR genes that we did not examine in this study. Second, it will be of interest to understand in greater detail what effects receptor co-expression and OSN co-convergence have on the capacity of the fly to detect and discriminate odors. Finally, the availability of candidate pheromone receptors in *Drosophila* will make it possible to study sex pheromones in a genetically tractable organism from the circuits they activate to the stereotyped behaviors they elicit.

3 Expression of Odorant Receptors in *Drosophila* Larvae

3.1 Introduction

A fly passes through three developmental stages: embryo, larva, and pupa before emerging as an adult. *Drosophila* larvae can smell and chemotax to both synthetic and natural olfactory stimuli (Cobb and Dannet, 1994; Larsson et al., 2004; Monte et al., 1989; Oppliger et al., 2000). Larvae have only 21 OSNs in two bilaterally symmetric dorsal organs (**Figure 1.2**) and (Python and Stocker, 2002; Singh and Singh, 1984; Tissot et al., 1997). All of larval OSNs express *Or83b* (**Figure 1.2B**), an OR essential for olfaction (Larsson et al., 2004; Neuhaus et al., 2005).

For almost 250 years, the dorsal organ (DO) and the terminal organ (TO) have been presumed to modulate chemoreception in larva (Cobb, 1999). Only recently, the 21 *Or83b*-expressing OSNs of the DO were established to solely mediate olfaction (Larsson et al., 2004). However, the contributions of each of the 21 larval OSNs toward olfactory perception are unknown.

Like in adults, the first olfactory synapse in larval brain is organized into glomeruli (Python and Stocker, 2002), with structure analogous to that of vertebrate olfactory bulb. Studies that were published while the manuscript associated with this work was under review examined the glomerular structure of larval antennal lobe (Ramaekers et al., 2005) and inferred expression of about 23 members of the OR gene family in larvae (Couto et al., 2005; Kreher et al., 2005). The parsimonious design of the olfactory system of *Drosophila* larva makes it an

excellent genetic model to test the contribution of single ORs toward neural processing and olfactory perception.

We provide a comprehensive molecular and neuroanatomical survey of the olfactory system of the *Drosophila melanogaster* larva. Genetic markers that we generate for most of larval OSNs are key for future physiological and behavioral studies of larval olfaction.

3.2 Materials and Methods

3.2.1 *Drosophila* Stocks

The fly stocks were generated and maintained as noted in **Section 2.2.1**. Fly stocks were kindly provided: *UAS→GFP* (Bloomington Stock Center); *UAS→CD8::GFP* (Liqun Luo, HHMI); *UAS→DTI14* (L. Stevens, Albert Einstein College of Medicine); *Gr→Gal4* lines (Kristen Scott, UCSF and Hubert Amrein, Duke University); *Or83b→Or83b::Myc* (Leslie Vosshall, The Rockefeller University).

3.2.2 Generation of Odorant Receptor Promoter Element Transgenes

Putative promoter sequences for the ORs obtained by PCR were cloned into pCaSpeR-*AUG*-*Gal4* as described in **Section 2.2.2**. Reverse primers were placed immediately upstream of the predicted ATG initiation codon and forward primers were at the following distances upstream: *Or1a*, 6.285 Kb; *Or22c*, 7.156 Kb; *Or24a*, 8.72 Kb; *Or30a*, 9.148 Kb, *Or45a*, 9.556 Kb; *Or45b*, 4.764 Kb; *Or49a*, 3.799; *Or59a*, 7.8 Kb; *Or63a*, 4.205 Kb; *Or74a*, 7.226 Kb; *Or83a*, 1.628 Kb; *Gr63a*, 2.635Kb.

3.2.3 Verification of *OR→Gal4* Transgene Expression

Multiple independent transgenic lines were generated for each construct, all were analyzed for expression in the larva. To detect Gal4 expression in dorsal organs, *OR→Gal4* flies were crossed to *Or83b→Or83b::Myc;UAS→GFP* flies. To detect Gal4 expression in larval brains, *OR→Gal4* flies were crossed to *UAS→GFP* or *UAS→CD8::GFP* flies (these reporters produced indistinguishable results). There was some inter-line variability in the number of cells labeled in the DO and the occasional labeling of TO or other non-sensory cells in the larva. Where the ectopic expression was not supported by *in situ* results, such lines were not used for behavioral analysis, with the exception of *Or49a→Gal4*, whose expression was not detected by RNA *in situ* hybridization. *Or98b→Gal4* (5.12 Kb) did not express and *Or85c→Gal4* (7.588 Kb) showed ectopic expression. For *Or30a→Gal4,* there were reliably two additional cells of unknown function at the midline, posterior to the mouth hooks. *Or24a→Gal4* is expressed in the adult maxillary palp, although *Or24a* mRNA is not detected in this tissue (data not shown). The following 21 adult *OR→Gal4* lines do not express in larval OSNs: *Or19a, Or22a, Or23a, Or33c, Or43a, Or46a, Or47b, Or49b, Or56a, Or59c, Or65a, Or67d, Or69a, Or71a, Or83c, Or85a, Or85e, Or85f, Or88a, Or92a,* or *Or98a*.

Or83b→Or83b::Myc contained 7.76kb of genomic DNA upstream of the *Or83b* initiation codon and full-length Or83b cDNA (Genbank accession AY567998), fused to five Myc epitopes (MEQKLISEEDLNE), and followed by the endogenous 3' UTR of *Or83b* and the SV40 polyadenylation sequence in

pCasper4PLX. The OR83b::Myc protein does not localize to dendrites of OSNs, but rather serves as a pan-OSN marker.

3.2.3.1 Immunocytochemistry

Antibody staining of late third instar larvae was performed similarly to methods described previously (Python and Stocker, 2002). Larval brains and cuticle that contained DOs was carefully dissected. Larvae were fixed for one hour in 4% paraformaldehyde/1X PBS/0.2% Triton-X100. The incubation periods were overnight at 4^{o}C and for four hours at room temperature, for primary and secondary antibodies, respectively. Primary rabbit anti-GFP at 1:1000 dilution (Molecular Probes), mouse anti-Myc 9E10 at 1:10 dilution (Thomas Jessell, HHMI), mouse anti-Elav 9F8A9 at 1:10 (DSHB, University of Iowa), and mouse nc82 at 1:10 dilution (Reinhard Stocker, University Fribourg, Switzerland) antibodies were used. Secondary goat anti-rabbit Alexa488-coupled (Molecular Probes) and goat anti-mouse Cyanine3-coupled antibodies were used at 1:100 and 1:200 dilutions, respectively. In some cases, nuclei were counterstained with a 1:1000 dilution of TOTO-3 (Molecular Probes). Larval brains were mounted with 11 x 22 mm No.1 cover slips as spacers. Confocal Z-series using 0.45 μm thick sections that spanned the GFP signal were collected with a Zeiss LSM510 confocal microscope.

3.2.3.2 RNA In Situ *Hybridization*

Wild type (Oregon-R) third instar larvae were decapitated in 1x PBS, and carefully dissected to remove the digestive tube posterior to the esophagus, the salivary glands, and fat body. The larval heads were then transferred to plastic

embedding molds containing Tissue-Tek O.C.T., and were aligned so that the dorsal side faced the bottom surface. The samples were frozen and 12 μm frozen sections processed for *in situ* hybridization and visualized with alkaline phosphatase reaction, as previously described (Vosshall et al., 1999). Anti-sense digoxigenin-labeled riboprobes were transcribed from OR templates derived from genomic DNA (Vosshall et al., 1999). Alkaline phosphatase RNA *in situ* hybridization was performed by Kenta Asahina (RU).

Two-color *in situ* hybridization was performed as above, using digoxigenin- and fluorescein-labeled riboprobes. The riboprobes corresponding to two OR genes were mixed in the same hybridization buffer and detected first with TSA-Plus Fluorescein System (PerkinElmer) and then after quenching the fluorescein reaction for a one hour with 3% hydrogen peroxide, with TSA-Plus Cyanine5 System (PerkinElmer). Anti-digoxigenin-POD and anti-fluorescein-POD were diluted 1:500 (Roche). Sections were examined under confocal microscope and with Nomarski optics that permitted identification of the dorsal organ ganglion by its characteristic position and morphology.

3.3 Results

3.3.1 Larval Odorant Receptor Gene Expression

To identify larval ORs, we examined 42 of 62 possible transgenes that drive the expression of Gal4 protein (Brand and Perrimon, 1993) under the control of OR promoter elements (Vosshall et al., 2000). To visualize transgene expression in the dorsal organ, individual *OR→Gal4* lines were crossed to *UAS→GFP* and the OSN marker *Or83b::Myc*. Transgene *Or83b→Gal4* is broadly expressed

throughout the DO ganglion (Larsson et al., 2004) and (**Figure 3.1A**, green); twenty other *OR→Gal4* transgenes label single larval OSNs in DO (**Figure 3.1A**, green) that also express *Or83b::Myc* (**Figure 3.1**). *Or49a→Gal4* labels one DO OSN along with a single TO gustatory neuron (**Figure 3.1A**, **Figure 1.2C**). The positions of OSN cell bodies seem to be relatively static from animal to animal; however, the nature of immunocytochemical preparation makes it difficult to generate a map.

Gustatory receptor (GR) genes are expressed in both olfactory and gustatory organs of the adult fly (Dunipace et al., 2001; Scott et al., 2001). In larva, *GR→Gal4* transgenes are expressed only in the gustatory TO or in non-olfactory DO neurons that do not express *Or83b::Myc* (**Figure 3.1B**). The *Or10a/Gr10a* transgene is expressed in a single TO neuron (**Figure 3.1B**). Lack of TO markers makes it unfeasible to confirm expression of the endogenous receptor in these neurons.

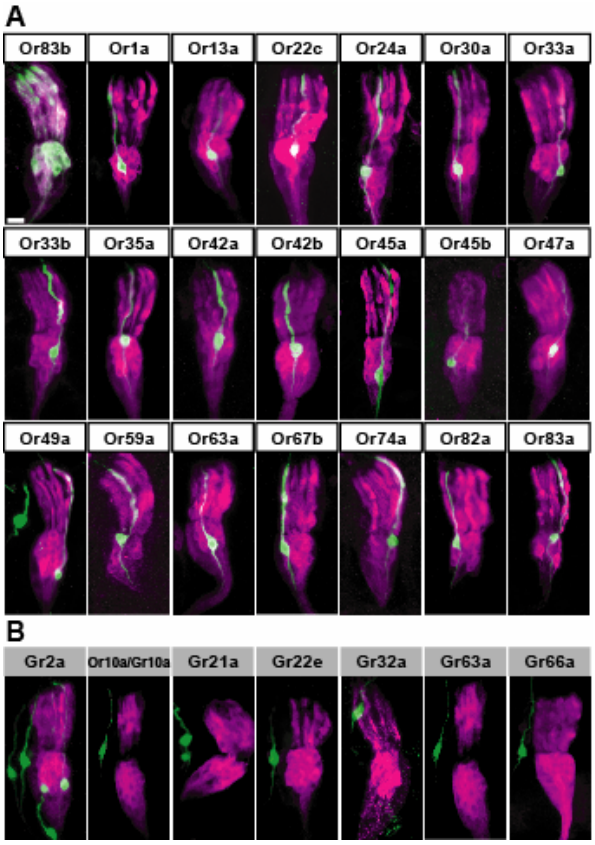

Figure 3.1: Twenty-one *OR→Gal4* transgenes are expressed in *Drosophila* larva. (A) Whole mount immunofluorescence staining of left larval dorsal organ with *OR→Gal4;UAS→GFP* transgenes in green and *Or83b::Myc* in magenta. *Or49a→Gal4;UAS→GFP* labels a single TO gustatory neuron in addition to a single DO OSN. (B) *GR→Gal4;UAS→GFP* positive neurons (green) are distinct from OSNs labeled for *Or83b::Myc* (magenta). (A) and (B) scale bar=10 μm.

Expression of all (except *Or49a→Gal4*) transgenes was verified by RNA *in situ* hybridization (data not shown). RNA *in situ* analysis identified expression of five additional ORs in the DO. Thus, we identify a total of 25 *Drosophila* ORs expressed in the larval DO. Of these, 14 are only expressed at the larval stage, while 11 are utilized by both larval and adult olfactory systems (**Table 3.1**).

OR	validation
Or1a	●
Or2a	○
Or7a	○
Or13a	●
Or22c	●
Or24a	●
Or30a	●
Or33a	●
Or33b	●
Or35a	●
Or42a	●
Or42b	●
Or45a	●
Or45b	●
Or47a	●
Or49a	•
Or59a	●
Or63a	●
Or67b	●
Or74a	●
Or82a	●
Or83a	●
Or83b	●
Or85c	○
Or94a	○
Or94b	○

KEY	larva	Larva/adult
In situ	○	○
Gal4	•	•
In situ/Gal4	●	●

Table 3.1: Summary of *Drosophila* ORs expressed in larvae and/or adults. Twenty-five ORs that are expressed in larva are color coded according to KEY for larval/adult expression. The symbols indicate OR expression validation in larva by RNA in situ hybridization and/or transgene expression.

3.3.2 Organizational Logic of the Larval Dorsal Organ

We postulated that the 25 ORs we identified in larva are distributed among the 21 *Or83b*-positive OSNs of the DO. Since the number of ORs exceeds the number of OSNs, there may be up to four OSNs that express two ORs; at the other extreme, 20 OSNs express one OR each, and the 21st OSN expresses five ORs. To ask how OR genes are distributed among the DO neurons, animals with two independently labeled OSNs were examined (**Figure 3.2**). In total, we

examined 21 different strains of transgenic larvae carrying two different *OR→Gal4* transgenes along with the *UAS→GFP* (cytoplasmic) or *UAS→CD8::GFP* (membrane-associated) reporters. There are always two GFP-labeled neurons in such animals, demonstrating that in all cases examined OR genes are not co-expressed (**Figure 3.2**). In some cases, the neurons are directly adjacent, but the presence of two distinct dendrites innervating the dorsal organ dome confirms that these neurons are distinct (e.g. *Or22c*/*Or49a* and *Or45a*/*Or74a*).

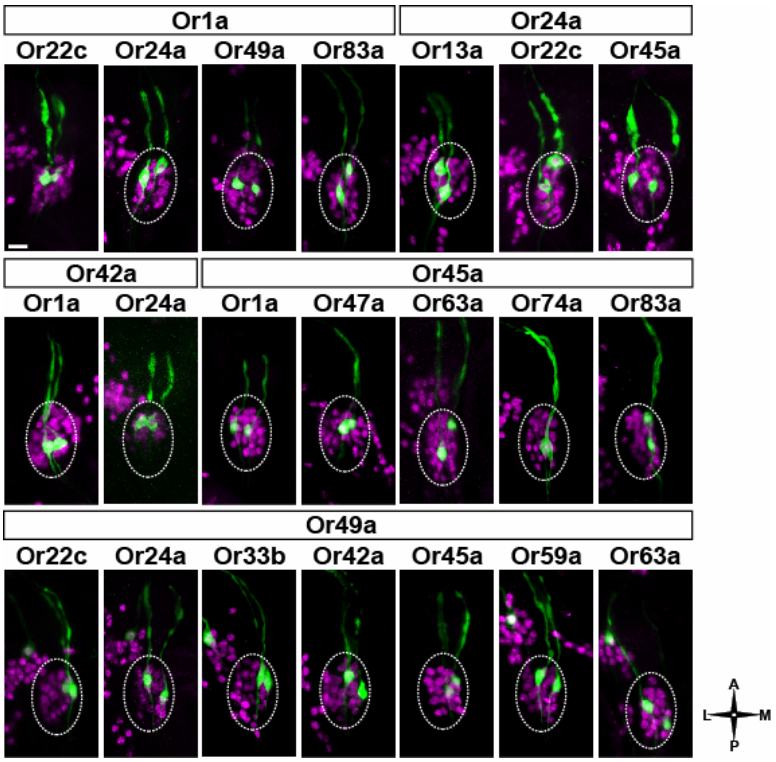

Figure 3.2: Most larval ORs are expressed in distinct olfactory sensory neurons. Whole mount immunofluorescence preparations of *OrX→Gal4/OrY→Gal4; UAS→GFP* or *UAS→CD8::GFP* animals, where *OrX* and *OrY* are two different ORs, show that each *OR→Gal4* is expressed in a different OSN (green). Neuronal cell bodies in the DO ganglion (white dashed circle) and the TO are counterstained with the neuron-specific Elav-9F8A9 antibody (magenta). Images are collapsed confocal Z-stacks that encompass the entire larval dorsal organ. Scale bar = 10 μm.

We used results from immunocytochemical analysis of one and two ORs to deduce candidates for OR co-expression. Henceforth, we performed RNA *in situ* hybridization to differentially label the ORs. While some OSN cell bodies are adjacent, two OSNs co-express pairs of ORs: *Or33b*/*Or47a* and *Or94a*/*Or94b* (**Figure 3.3**). Note that *Or33b* and *Or47a* are also co-expressed in the adult OSNs (**Figure 2.5**) Other instances of OR co-expression are also documented for the adult olfactory system (Dobritsa et al., 2003; Goldman et al., 2005).

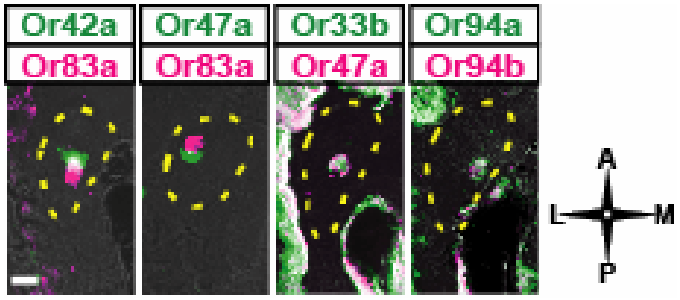

Figure 3.3: RNA *in situ* hybridization reveals two cases of OR co-expression. RNA of two ORs detected with digoxigenin- (magenta) and fluorescein-labeled (green) riboprobes. The border of the dorsal organ cell body ganglion is indicated by the yellow dotted line in each sample. Orientation of samples is indicated at the right: A = anterior, P = posterior, L = lateral, M = medial.

3.3.3 The Glomerular Map of Larval Olfactory Projections in the Brain

Larval OSNs project long axons to the larval antennal lobe (LAL) of the brain (**Figure 3.4A**) and (Python and Stocker, 2002; Ramaekers et al., 2005). Patterns of axonal projections to the larval antennal lobe were examined in larvae carrying *Or83b→Gal4* and 20 other larval *OR→Gal4* transgenes, visualized with *UAS→GFP* or *UAS→CD8::GFP* reporters (**Figure 3.4B** and **C**). *Or83b→Gal4* fills the entire LAL, while other *OR→Gal4* transgenes label single

axonal arbors (larval glomeruli) whose characteristic position is conserved across animals.

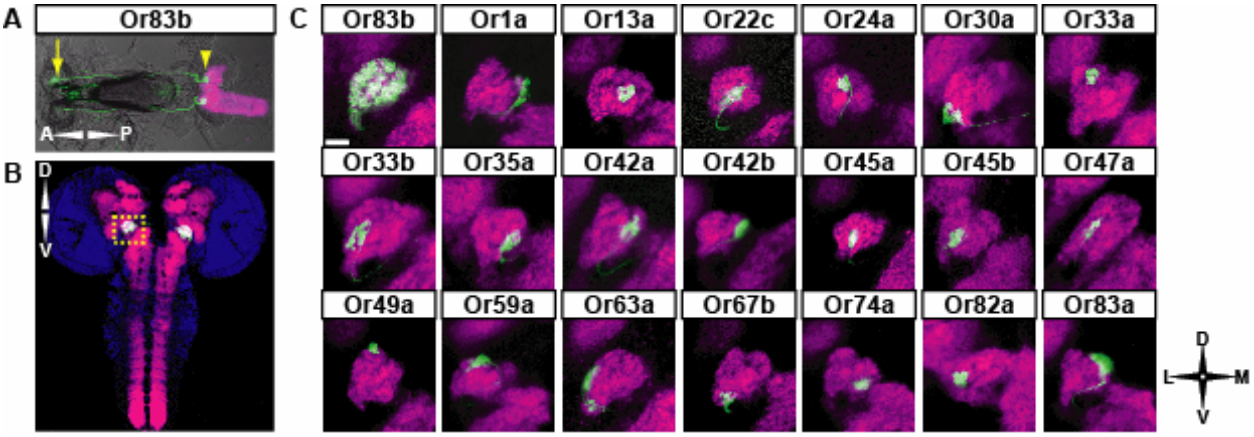

Figure 3.4: Larval olfactory sensory neurons project to single glomeruli in the antennal lobe. (A) Dorsal view of the anterior tip of an *Or83b→Gal4;UAS→GFP* larva reveals the OSNs (green) of the DO (yellow arrow) and the olfactory nerve extending axons to the LAL (yellow arrowhead). Sample orientation: A = anterior, P = posterior. Larval brain is counterstained with the neuropil-specific antibody nc82, magenta (A)-(C). Animal is oriented anterior left, posterior right. (B) Whole mount immunofluorescence staining of an *Or83b→Gal4;UAS→GFP* (green) reveals boundaries of the AL [yellow dashed square is magnified in (C)]. Nuclei are stained with TOTO-3, blue. Sample orientation: D = dorsal, V = ventral. (C) Left antennal lobes of *OR→Gal4;UAS→GFP* or *UAS→CD8::GFP* animals stained with anti-GFP (green) and nc82 (magenta). The left larval AL is centered in the box and the subesophageal ganglion is located at the lower right. Sample orientation: D = dorsal, V = ventral, L = lateral, M = medial. Scale bar = 10 μm.

Although the glomeruli in the LAL have poor morphological definition (Python and Stocker, 2002; Ramaekers et al., 2005), our analysis of 20 uniglomerular larval *OR→Gal4* transgenes reveals discrete axonal targets for each OSN. In some cases, neurons expressing different ORs converge upon the same sub-region of the antennal lobe (**Figure 3.4C**). Convergence of multiple OSNs onto the same rather than merely adjacent glomeruli, may lead to

convergent input to second-order olfactory neurons (larval PNs). *OR→Gal4* tools make it possible to for us to characterize the glomerular organization in more detail.

We generated 21 fly strains, each with two different *OR→Gal4* transgenes and a *UAS→GFP* or *UAS→CD8::GFP* reporters (**Figure 3.5**). In some cases, the glomeruli are adjacent (*Or22c/Or24a, Or45a/Or47a, Or45a/Or63a, Or24a/Or49a,* and *Or33b/Or49a*). Nonetheless, each strain clearly contains two labeled glomeruli, suggesting that for the cases examined each larval OSN targets a discrete sub-region of the antennal lobe.

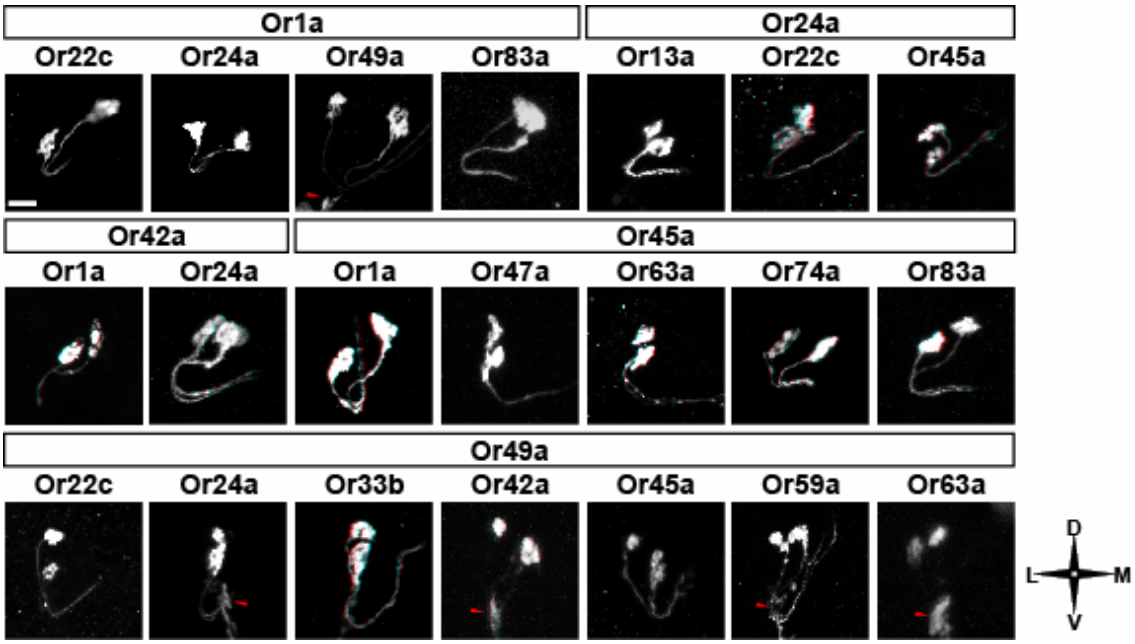

Figure 3.5: Larval olfactory sensory neurons target discrete glomeruli. Whole mount immunofluorescence preparations of *OrX→Gal4/OrY→Gal4;UAS→GFP* or *UAS→CD8::GFP* larval brains, where X and Y are two different ORs. Images are collapsed confocal Z-stacks that encompass the entire larval antennal lobe. TO projections of *Or49a→Gal4*-positive gustatory neurons are marked with a red arrowhead. Scale bar = 10 μm.

Analysis the LAL glomeruli using one or two *OR→Gal4* transgenes reveal a stereotyped glomerular pattern. The shapes of the glomeruli vary from globular to oblong, from animal to animal for the same *OR→Gal4*. The distances between two labeled OSNs also vary slightly (up to one glomerulus diameter). The relatively large numbers of OSNs that we can label, make it impractical to examine all two-OR combinations, thus restricting our resolution of the LAL glomeruli positions. Nonetheless, we observe reproducible characteristic positions of glomeruli within LAL. Approximate positions of larval olfactory glomeruli are schematized in **Figure 3.6**.

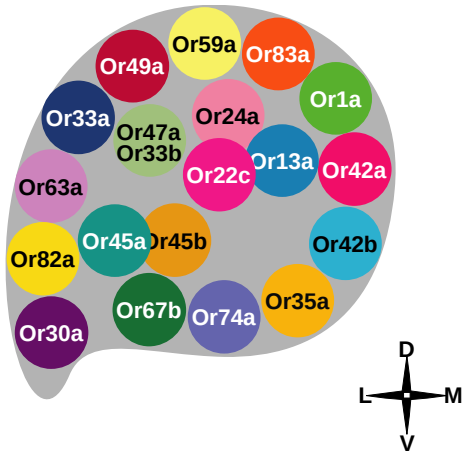

Figure 3.6: Map of the glomerular targets in larval antennal lobe. Flattened representation shows approximate positions of glomeruli receiving input from OR-expressing OSNs. Partially overlapping circles represent glomeruli whose relative position cannot be unambiguously resolved. Orientation: D = dorsal, V = ventral, L = lateral, and M = medial.

3.4 Discussion and Conclusions

3.4.1 Larval Odorant Receptor Repertoire

The olfactory circuit of the *Drosophila* larva remains a relatively simple system. Each of the 21 larval OSNs expresses one or two of the 25 ORs that we

identified in larva, along with *Or83b* (Larsson et al., 2004; Neuhaus et al., 2004). We do not expect the number of ORs in larva to exceed greatly the 25 that we identified. Independent studies stipulated 23 by RT-PCR (Kreher et al., 2005) and 18 by *OR→Gal4* transgene expression (Couto et al., 2005), mostly overlapping ORs to be expressed in larva. In contrast to RT-PCR results for *Or19a* and *Or88a* (Kreher et al., 2005) and transgene expression of *Or22a*, we did not detect expression for these *OR→Gal4* transgenes in larva. We also lack *Or43b→Gal4* (Kreher et al., 2005) and *Or85d→Gal4* (Couto et al., 2005) to confirm larval expression. Overall, the three studies support expression of 30 or less ORs in *Drosophila* larva.

3.4.2 Distinct, yet Overlapping Olfactory Systems

We hypothesized correctly that the ORs that are not detected by RNA *in situ* hybridization in the adult olfactory organs are larval. Unexpectedly, we find that among the 25 larval ORs, 11 ORs are shared between the adult and larval *Drosophila*. The high overlap in the OR expression in the two life stages may be due to a shared niche.

Rotting fruit is a key odor for both adults and larva. Adults seek it from long distances to mate and lay eggs and larva live in the fruit as they mature. The absolute concentrations of volatiles that the two life stages experience are different due to different proximities to the odor, hence the need for different ORs. Yet, adults and larvae are able to share many ORs. Further studies of OR response dynamics and olfactory information processing promise to uncover how animals recognize odors at various concentrations.

3.4.3 A Simple Mammal-Like Olfactory Circuit

Each of the *Drosophila* 21 larval OSNs extends a single axon that arborizes in a unique olfactory glomerulus in the LAL. In our effort to understand the organization of the *Drosophila* larva olfactory circuit, we developed markers for most larval glomeruli. Since larval glomeruli are not readily apparent (Ramaekers et al., 2005), the genetic markers will serve as useful identifiers of the synaptic olfactory targets in larvae.

The olfactory system in larva is almost as simple as in nematode, *C*. elegans. *C. elegans* have only four bilaterally symmetric neurons that are responsible for sensing volatile chemicals (Bargmann, 1993). Unlike *Drosophila*, the OSNs of *C. elegans'* OSNs harbor as many as 1000 different ORs (Bargmann, 1993; Troemel et al., 1995). The first olfactory synapse of *C. elegans* also does not exhibit glomerular structure. Although the systems are differently organized, experiments that identified behavioral functions of single olfactory neurons in *C. elegans* (Troemel et al., 1997) are commended in larvae.

The glomerular organization of the LAL is reminiscent of the mammalian OB. However, instead of a thousand glomeruli with two plains of symmetry that are present in mice, *Drosophila* larvae have one glomerulus for each of its 21 OSNs. Thus, input into each larval OSN or each glomerulus represents a large fraction of the total input into the olfactory system.

4 Chemotaxis Behavior Mediated by Larval Olfactory Neurons

4.1 Introduction

The olfactory system permits animals to detect, discriminate, and produce appropriate behavioral responses to a vast number of different odors. The first step in odor coding occurs at the periphery, where odor molecules interact with the OR proteins on the surface of OSNs. Each OR is thought to interact with a number of odor ligands and each ligand interacts with multiple ORs, leading to a combinatorial model in which odor identity is encoded by the activation of distinct ORs (Araneda et al., 2000; Hallem et al., 2004a; Malnic et al., 1999; Touhara et al., 1999). OSNs are known to express only one OR in mammals, or up to two ORs in insects (Dobritsa et al., 2003; Goldman et al., 2005). Receptive properties of the ORs are critical for OSN activation. Once an OSN is activated, neuron connectivity determines the output of the olfactory circuit. Hence, activation of an OSN will not confer olfactory sensation, if its information is not processed to be relevant or salient. Little is known about fate of olfactory signals once they leave OSNs. The parsimonious *Drosophila* larval olfactory circuit is an excellent model system to parse out the contributions of single ORs/OSNs to olfactory perception.

Manipulations to the olfactory sensory input would affect the output of the olfactory circuit and ultimately larval behavior. Genetic markers for larval OSNs allow us to manipulate the functionality of the peripheral system of the larva by either ablating single OSNs or constructing larvae with one or two functional OSNs. This approach allows us to deconvolute the sensory input to the olfactory

system and to examine the individual contribution of OSNs to chemotaxis behavior.

An observation that larvae chemotax robustly toward single compounds allows for well-controlled experiments, where the quantity and the quality of the stimulus are known. While the natural stimuli for larvae contain numerous volatiles at low concentrations, a single compound may require higher concentrations to elicit larval behavior. Therefore, to study the function of individual OSNs, we chose higher concentrations of odorants that produce robust chemotaxis in wild-type larvae.

Ablation of single OSNs reveals extensive functional redundancy in the larval olfactory system: a given OR/OSN is only necessary for chemotaxis to a small subset of odors tested. Animals with only a single functional OSN can chemotax robustly toward a number of odor stimuli. Combinatorial coding afforded by the entire ensemble of ORs is not strictly necessary for an animal to perceive and chemotax toward an odor. However, adding to a single-functional-OSN animal a second functional OSN, which by itself is not sufficient to mediate chemotaxis, produces enhanced behavioral responses to a subset of odors. These results demonstrate at a behavioral level that a single OSN is sufficient to detect the presence of an olfactory stimulus and that the combinatorial activation of different ORs participates in the formation of olfactory percepts.

4.2 Materials and Methods

4.2.1 *Drosophila* Stocks

The fly stocks were maintained as described in **Section 2.2.1**. Flies used as wild-type controls for behavioral experiments were of same genotype as for generation of transgenic flies, yw;+/+;+/+ (yellow body, white eyes). A cell-autonomous version of Diphtheria toxin (DTI) was used to silence larval OSNs; *UAS→DTI14* stock was generously provided by LM Stevens, Albert Einstein College of Medicine. Flies mutant for *Or83b*, (*Or83b*$^{-/-}$, strain *Or83b*^{1}/*Or83b*^{1} or *Or83b*^{2}/*Or83b*^{2}) were previously described (Larsson et al., 2004).

4.2.2 Measurement of Larval Glomerular Volumes

Immunostaining of larval brains and dorsal organs was performed as in **Section 3.2.3.1**. For volume measurements, three-dimensional Z-series of the GFP-labeled *Or1a* glomeruli in wild-type (*Or1a→Gal4/UAS→CD8::GFP;TM2/+*), *Or1a*-functional (*Or1a→Gal4, UAS→Or83b/UAS→CD8::GFP;Or83b*^{1}*/Or83b*^{2}), and *Or83b* mutant animals (*Or1a→Gal4/UAS→CD8→GFP;Or83b*^{1}*/Or83b*^{2}) were obtained by confocal microscopy. Images for all three genotypes were acquired under identical confocal settings. The volumes of *Or1a* glomeruli were measured with segmentation software, Amira^{TM} (TGS), with manual trimming of afferent axons. Left and right glomeruli were averaged for each animal before the mean was calculated.

4.2.3 Larval Chemotaxis Assay

Larval behavioral assays were carried out as described (Larsson et al., 2004). Odor stimuli (2 µl, 4 µl, or 20 µl neat or smaller quantities diluted in paraffin oil) were pipetted onto a filter placed inside a plastic cap located at one side of the 85 mm Petri dish. Most experiments used stimulus strength of 2 µl of neat odor because this elicits strong chemotaxis responses across a broad range of structurally different odors (Cobb et al., 1992; Cobb and Dannet, 1994; Monte et al., 1989). All odorants were supplied by Sigma-Aldrich and were of the highest purity available. Natural odors were commercial balsamic vinegar used at full strength or diluted in water, a liquid paste of 50% w/v ripe banana mashed in water, and 20% w/v paste of activated baker's yeast in water. Single third-instar larvae were transferred to the plate, and their locomotor activity was recorded for five minutes as X-Y coordinates at a sampling rate of 6 Hz with EthoVision® (Noldus Information Technology) tracking software. The assay was multiplexed, with up to 12 individual larvae assayed simultaneously in separate 85 mm circular arenas. Each animal was assayed only once. We minimized the presence of airflow in these experiments by conducting the assay in Petri dishes with closed lids. Animals were tested within a few seconds of odor application.

4.2.4 Statistical Analyses

Data were exported from EthoVision® and analyzed by Matthiew Louis using MATLAB® (The MathWorks). To filter out experiments with technical noise due to light scattering or tracking failures, any tracks shorter than 270 s or with a mean velocity = 0 cm/s or > 0.2 cm/s were discarded; all tracks that passed

these criteria were included in the analysis. Larval chemotaxis behavior was quantified as the distance of the animal from the odor. For each larva, distances to odor, calculated at each time-point, were averaged over the 5 minute trial (mean distance to odor).

Unless indicated otherwise, all statistical analyses were performed with one-tailed nonparametric Wilcoxon rank-sum tests that evaluate differences between control and experimental data sets. All ablated OSN or one- or two-functional OSN(s) genotypes were compared to appropriate parental genotypes. We denote the ablated genotypes as follows: (α_1) *Or83b→Gal4/+;UAS→DTI/+*; (α_2) *Or1a→Gal4/+;UAS→DTI/+*; (α_3) *Or42a→Gal4/+;UAS→DTI/+*; and (α_4) *Or49a→Gal4/+:UAS→DTI/+*. The one-functional OSN genotypes are denoted as (ρ_1) *Or1a→Gal4/UAS→Or83b;Or83b^1/Or83b^1*, (ρ_2) *Or42a→Gal4/UAS→Or83b; Or83b^1/Or83b^1*, (ρ_3) *Or49a→Gal4/UAS→Or83b;Or83b^1/Or83b^1* and two-functional OSNs as (ρ_4) *Or42a→Gal4,Or1a→Gal4/UAS→Or83b;Or83b^1/Or83b^1*, and (ρ_5) *Or49a→Gal4,Or1a→Gal4/UAS→Or83b;Or83b^1/Or83b^1*. The genotypes used for controls are as follows: (η_1) *UAS→DTI*, (η_2) *Or83b→Gal4*, (η_3) *Or1a→Gal4*, (η_4) *Or42a→Gal4*, (η_5) *Or49a→Gal4*, (η_6) *Or83b^1/Or83b^1*, (η_7) *UAS→Or83b/UAS→Or83b;Or83b^1/Or83b^1*, (η_8) *Or1a→Gal4/Or1a→Gal4; Or83b^1/Or83b^1*, (η_9) *Or42a→Gal4/Or42a→Gal4*;*Or83b^1/Or83b^1*, and (η_{10}) *Or49a→Gal4/Or49a→Gal4;Or83b^1/Or83b^1*. For OSN ablation experiments, the following comparisons were performed according to pairs of genotypes↔controls: $\alpha_1 \leftrightarrow \{\eta_1,\eta_2\}$, $\alpha_2 \leftrightarrow \{\eta_1,\eta_3\}$, $\alpha_3 \leftrightarrow \{\eta_1,\eta_4\}$, and $\alpha_4 \leftrightarrow \{\eta_1,\eta_5\}$. In one- and two-functional

OSN(s) experiments, comparisons were made as follows: *yw*↔{η_6}, (ρ_1)↔{η_7,η_8}, (ρ_2)↔{η_7,η_9}, (ρ_3)↔{η_7,η_{10}}, (ρ_4)↔{η_7,η_8,η_9}, and (ρ_5)↔ {η_7,η_8,η_{10}}.

To address the problem of multiple testing, we used a permutation-resampling-based technique to adjust the nominal p values and to keep the family-wise error rate to 0.05 everywhere (Korn et al., 2004). The approach we implemented accounts for possible non-independence of the tests and allows us to control the False-Discovery Rate (FDR) among the set of null hypotheses rejected by the Wilcoxon tests. When the FDR is chosen as zero, our analysis approximates the stringent Bonferroni correction, given the low level of dependency between tests for different odors (Korn et al., 2004; Shaffer, 1995).

To study the influence of individual OSNs on the olfactory responses of larvae, we proposed a linear model for dominant function of OSNs. We considered whether animals with two functional OSNs chemotax significantly better than those with one OSN, in the following model (Equation 1).

Equation 1: Linear model for dominant function of olfactory neuron

$$d_i=\beta_0+\beta_1.\Gamma_{\text{one OR}}+\beta_2.\Gamma_{\text{two ORs}}+\varepsilon_i$$

where d_i denotes the distance of the i^{th} larva to a given odor, ε_i is the i^{th} residual, and $\Gamma_{\text{one OR}}$ and $\Gamma_{\text{two ORs}}$ are binary indicators (0 or 1), 1 indicates the presence of functional neuron(s). “One OR” refers to the OR/OSN that mediates the stronger chemotaxis, “two ORs” refers to genotype with two functional OSNs.

We estimated coefficients β_0, β_1, and β_2 by multiple linear regression (MATLAB®) method. Potential cases of synergism are related to significant increases in attraction, and are associated with values of $\beta_2 < 0$. Conversely,

potential cases of OSN inhibition are related to significant decreases in attraction, and are associated with values of $\beta_2 > 0$. To test whether the estimated β_2 values are significantly different from zero, we used student two-tailed t tests with a significance level of 0.05.

To analyze the nature of OSN interaction identified by **Equation 1**, we considered an additive model (**Equation 2**).

Equation 2: Linear model for additive function of olfactory neurons

$$d_i=\beta_0+\beta_{Or1a}.\Gamma_{Or1a}+\beta_{Or42a}.\Gamma_{Or42a}+\beta_{Or1a/Or42a}.\Gamma_{Or1a}.\Gamma_{Or42a}+\varepsilon_i$$

where the binary indicator Γ_{OR} is equal to 1 when the corresponding OSN is functional, and 0 otherwise. The regression coefficients β are estimated by the multiple linear regression (MATLAB®) method. Cases where two functional neurons lead to chemotaxis that is greater than the sum of the chemotaxis characterizing single functional neurons could be considered true synergy, these cases are associated with values of $\beta_{Or1a/Or42a}$ significantly smaller than 0. Conversely, cases where the relationship between the behavioral enhancement is less than additive will be reflected by values of $\beta_{Or1a/Or42a}$ significantly larger than zero. Values of $\beta_{Or1a/Or42a}$ that fall between the aforementioned extremes cannot be distinguished from additive enhancement of chemotaxis.

4.3 Results

The genetic tools that uniquely label larval OSNs allow us to manipulate the odor code by deconstructing the peripheral olfactory input and examining effects on behavioral output. Toward this end, we established a chemotaxis assay of sufficient sensitivity to quantify differences in odor-evoked behavior. The assay

involves single-animal analysis in which the coordinates of individual larvae are tracked on Petri dishes over the course of a 5 minute experiment (Larsson et al., 2004). We tested larval chemotaxis for a large panel of synthetic monomolecular odorants, most of which are also emitted by ripening fruit.

For each larva, we obtained mean distance to the odor source. For each experimental condition (genotype/odor), more than 20 usable recordings were obtained. Since some of the data were not normally distributed, we chose to use medians rather than the means when compiling data for multiple larvae; thus, we plot population medians of individual mean distances represented by pseudocolor scale from zero (maximal attraction) to 8.5 cm (maximal repulsion) (**Figure 4.1**A). Distance to odor is used as an indicator of chemotaxis intensity with an assumption that the degree of attraction correlates inversely with distance to the odor.

4.3.1 Wild-type Larvae Chemotax Strongly Toward Many Odorants

Chemotaxis of wild-type (*Or83b*$^{+/+}$) and *Or83b* mutant (*Or83b*$^{-/-}$) larvae were measured in response to 53 synthetic odorants, median distances to odor are plotted in pseudo-color scale (**Figure 4.1B**). Forty of these 53 odors are naturally present in fruit (Argenta et al., 2004; Idstein et al., 1984; Jordan et al., 2001; Pino et al., 2005) (**Figure 4.1B**); and of these, 13 are known to elicit behavioral (Oppliger et al., 2000; Zhu et al., 2003) and electrophysiological (Stensmyr et al., 2003) responses in *Drosophila* (**Figure 4.1B**). *Or83b*$^{-/-}$ larvae are not attracted by any of the odors tested (**Figure 4.1B**) and (Larsson et al., 2004), but wild-type (*yw*) larvae respond to many odors with strong chemotaxis (**Figure 4.1B**).

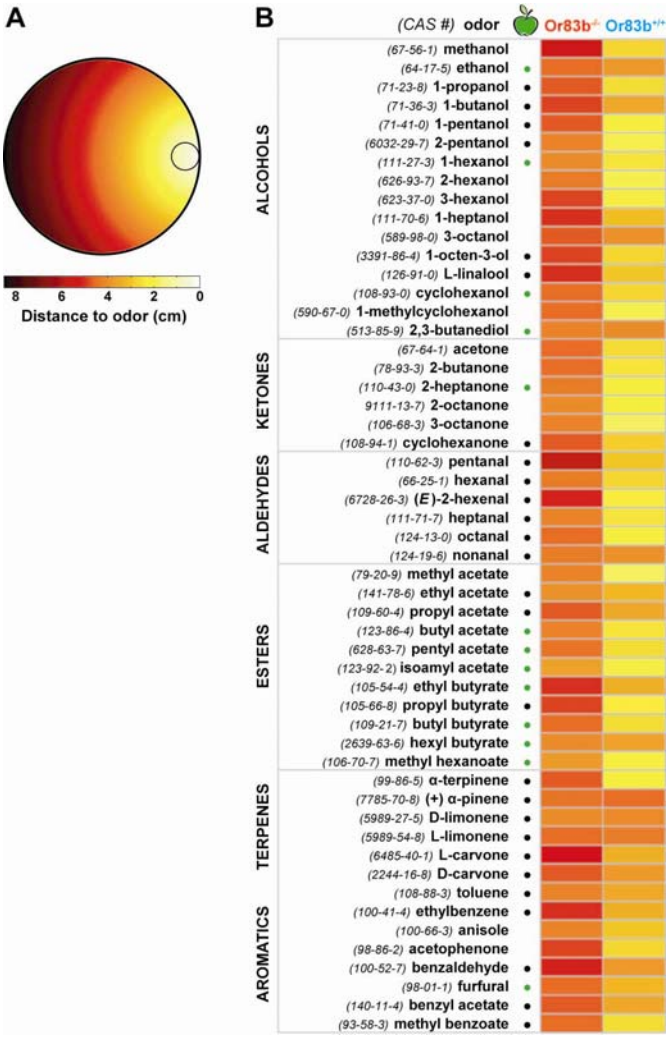

Figure 4.1: Larvae respond to odors with chemotaxis. (A) Schematic of larval plate assay, pseudo-color coded for distance to odor, with the scale at bottom. Odor stimulus is placed on round filter, at the right edge of the plate. (B) Responses of *Or83b* mutant (red) and wild-type (cyan) larvae to a panel of 53 synthetic odors presented at a dose of 2 µl. Median distance to odor is expressed with the pseudo-color scale in (A). Dots under the apple graphic indicate odors that are found in apple (Argenta et al., 2004), cherimoya fruit (Idstein et al., 1984), banana (Jordan et al., 2001), or in at least three of 20 assayed varieties of mango (Pino et al., 2005). Green dots indicate odors that were found to be relevant for flies via single-sensillum electrophysiological recordings in adult flies (Stensmyr et al., 2003) or behavioral assays in adult flies (Zhu et al., 2003) or larvae (Oppliger et al., 2000). In total, 4780 larvae were tested; mean n = 45 (range 20–85) per odor and genotype. Chemical Abstracts Service (CAS) numbers are to the left of each odor.

Or83b$^{-/-}$ larvae in presence of odor behave similarly to *Or83b*$^{+/+}$ larvae in absence of odor; they do not stay at the center of dish where they start but explore the plate. The distribution of anosmic *Or83b*$^{-/-}$ (red) and wild-type *Or83b*$^{+/+}$ (cyan) larvae in space over the course of a five minute experiment is represented by sector plots and box plots in **Figure 4.2**. The 8.5 cm plate is divided into 21 sectors, and the average percent time an animal spends in each sector is plotted in grayscale (**Figure 4.2**, left). Note that only *Or83b*$^{+/+}$ larvae are strongly attracted to the sector containing the odor. Box-plot distributions accompany sector plots (**Figure 4.2**, bottom). The distribution of *Or83b*$^{+/+}$ larvae in response to no odor is used as an empirical reference throughout this study.

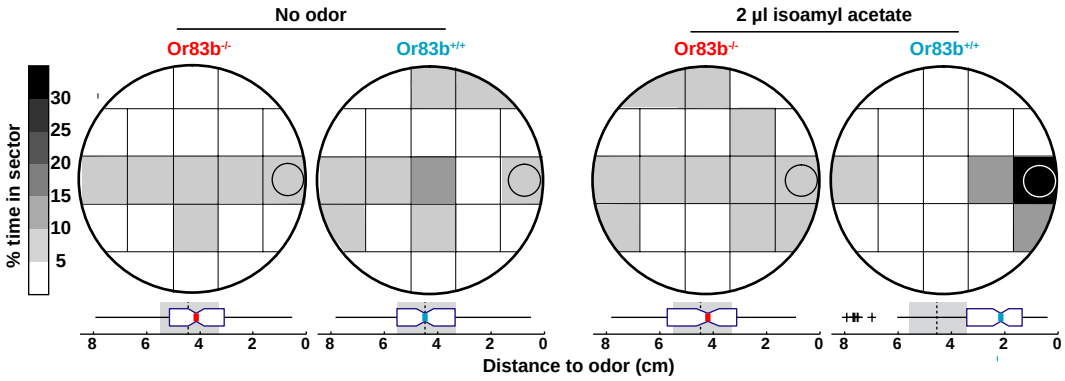

Figure 4.2: Sector plots illustrate spatial distribution of larvae in response to odor. Odor source (2 µl of isoamyl acetate) is represented by circle (right side of plate). Sectors are shaded according to the percentage time in sector (scale at left). Box plot representations of each experiment are under sector plots. The median is indicated by colored vertical line inside box plot, box boundaries represent first and third quartiles, whiskers are 1.5 interquartile range, and outliers are indicated by hatch marks. Genotypes, left to right: *Or83b*^{1}/*Or83b*^{1}, n = 111; *yw*, n = 111; *Or83b*^{1}/*Or83b*^{1}, n = 91; *yw*, n = 112. Median (black dashed line at 4.45 cm) and first to third quartiles (grey shaded area from 3.30 cm to 5.50 cm) of yw response distribution to “no odor” are present in all box-plots.

4.3.2 Larval Odor-Response Thresholds Vary Greatly

At 2 µl doses of different odors, larvae exhibit a range from strong to no observable chemotaxis. Next, we examined how larval sensitivity changes when we decrease the dose for three potent odors (**Figure 4.3**). The responses to 1-hexanol drop sharply with decrease in concentration, while dilutions of isoamyl acetate induce sustained chemotaxis (**Figure 4.3A**) In fact, wild-type larvae exhibit chemotaxis that is different from anosmic controls at all tested concentrations of isoamyl acetate (**Figure 4.3B**). At low concentration(s) of 1-hexanol and heptanal chemotaxes are weak and not statistically different from anosmic controls (**Figure 4.3B**). In conclusion, response thresholds to heptanal and isoamyl acetate are one and two log orders, respectively, below that of 1-hexanol (**Figure 4.3**).

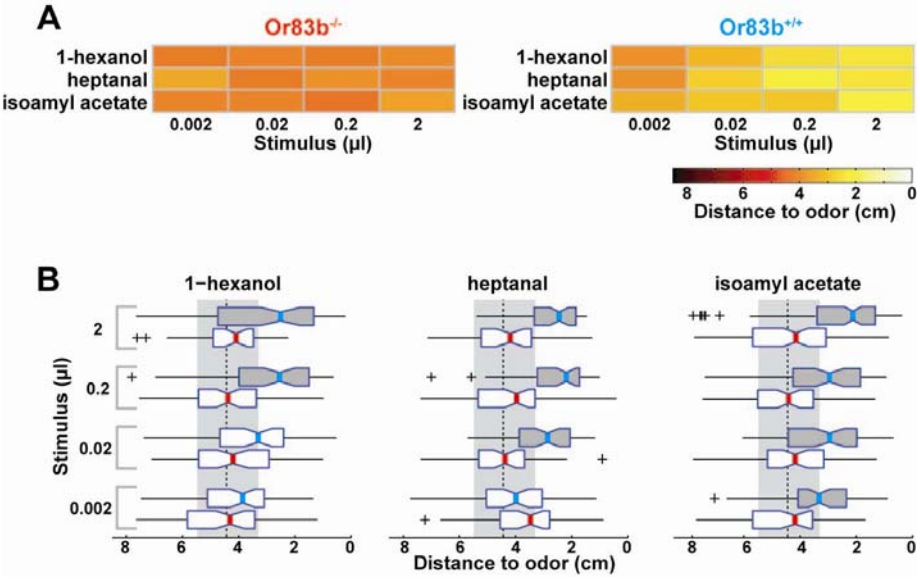

Figure 4.3: Concentration dependence of larval odor responses. (A) Median distances to 10X dilutions of odor are coded in pseudo-color scale (bottom), for anosmic *Or83b*$^{-/-}$ (red; left) and wild-type *Or83b*+/+ (cyan; right) larvae. (B) Box plots of results in (A). Significance was established by Wilcoxon rank-sum tests comparing data from

Or83b$^{+/+}$ (cyan) to *Or83b*$^{-/-}$ controls (red) with Bonferroni correction for multiple comparisons. Box plots shaded dark gray indicate significant chemotaxis ($p < 0.05/4 = 0.0125$). In total, 1183 larvae were tested; mean n = 49 (range 30–64) per odor and genotype.

4.3.2.1 Increased Doses of Some but not All Odors Attract Larvae

At 2 µl dose, some odors elicit weak or no chemotaxis. To test whether some odors have high detection thresholds, we tested seven odors at 20 µl dose (**Figure 4.4**). Under these conditions, only 1-butanol and 2,3-butanediol elicit significant chemotaxis, and the remaining five odors do not. Thus increasing odor dose from 2 µl to 20 µl will not render all stimuli attractive.

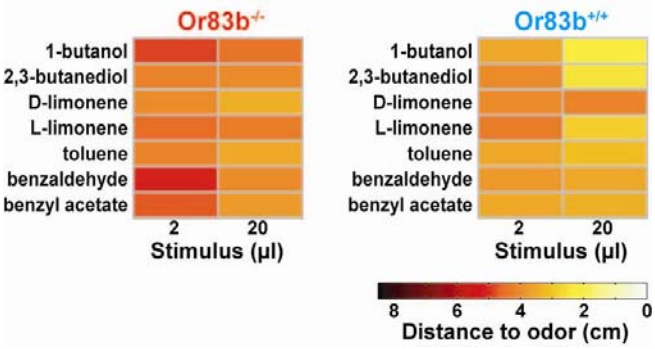

Figure 4.4: Summary of concentration-dependent changes in larval behavior. Median distances to odor are indicated in pseudo-color scale (bottom), for anosmic *Or83b*$^{-/-}$ (red; left) and wild-type *Or83b*$^{+/+}$ larvae (cyan; right) at 2 µl and 20 µl concentrations for seven odors that induce no chemo-taxes at 2 µl.

For all conditions tested, odor responses seem to plateau for higher concentrations; we find no evidence that higher concentrations elicit repulsion (**Figure 4.1B**, **Figure 4.4**, and data not shown). The 2 µl stimulus dose elicits robust chemotaxis across a large group of different odors (**Figure 4.1B**), in accordance with previous behavioral studies (Cobb et al., 1992; Cobb and Dannet, 1994). Detection thresholds and concentration curves for each odor

need to be empirically determined; without this information, the 2 µl stimulus is a reasonable global standard.

4.3.2.2 Larvae are Attracted by Complex Natural Stimuli

We examined whether chemotaxis elicited by single odors is comparable to that obtained with natural stimuli. Chemotaxis was measured in the same assay to mashed banana, balsamic vinegar, and yeast paste at different concentrations. We find that attraction elicited by single synthetic odors is qualitatively similar to that obtained with natural odor blends and that the same steep threshold and stable plateau properties are seen for both stimulus types (**Figure 4.5**).

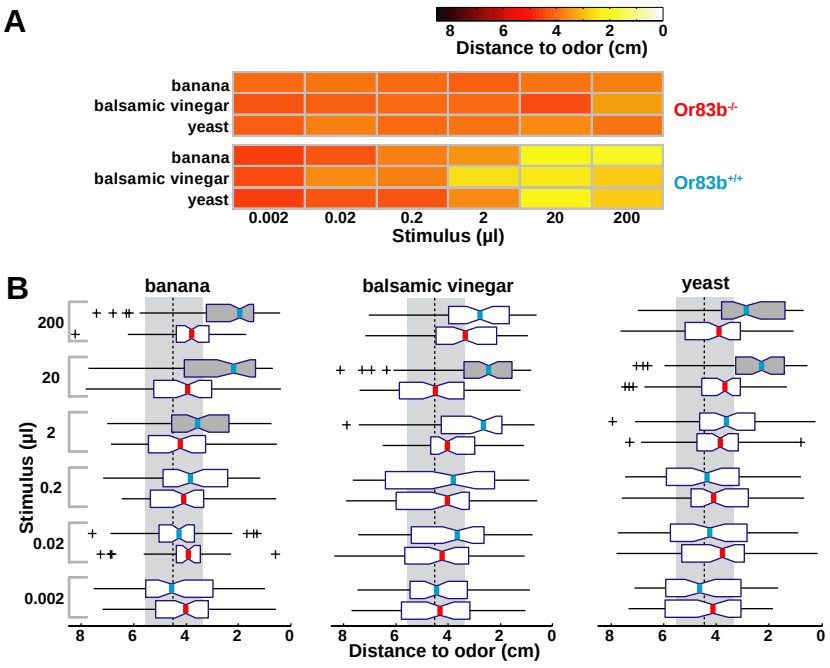

Figure 4.5: Larval responses to complex natural stimuli. (A) Summary of responses of anosmic *Or83b*$^{-/-}$ (red, top) and wild-type *Or83b*$^{+/+}$ (cyan, bottom) larvae to natural stimuli. Median distances are plotted in pseudo-color scale. (B) Box plots data for *Or83b*$^{-/-}$ (red) and *Or83b*$^{+/+}$ (cyan) larvae in (A). Box plots shaded dark gray indicate significant chemotaxis with Bonferroni correction ($p < 0.05/6 = 0.0083$). The highest dose of balsamic vinegar acidifies the agarose in the plate, attracting *Or83b*$^{-/-}$ larvae via their

intact gustatory system (data not shown). In total, 2177 larvae were tested, mean n = 60 (range 36–67) per odor and genotype.

The quantities of natural stimuli that induce chemotaxis are 2 µl or greater. Although the total output of volatiles from the natural stimuli is not known, it is likely to be less than from 2 µl of the synthetic odors that we used. Use of natural stimuli may permit us to use lower concentrations of volatiles, however, these stimuli are not well defined and are difficult to control.

Another factor to consider when choosing odor quantities for assay is vapor pressure. Upon loading 2 µl of an odorant stimulus in the closed-dish assay, the spatial distribution and average airborne concentration of odor in the dish will be greatly determined by the odor's vapor pressure. An alternate way to standardize odor concentration, would be to adjust quantities for vapor pressure. Observations that odorants with higher vapor pressures are likely to have higher detection thresholds (Keller and Vosshall, unpublished), discourages us from standardizing to vapor pressure, as the olfactory systems already compensate for the differences in volatility of odors. In the initial phases of this study, we found no clear correlation between the vapor pressure of a given odor and its corresponding behavioral efficacy (data not shown). We therefore decided to avoid any normalization of stimulus concentration and used the same quantity of odor (2 µl) for all 53 stimuli tested.

4.3.3 Genetic Ablation of Single Larval Olfactory Neurons

We next asked, what is the relative contribution of any given OSN to the formation of an odor percept? Diphtheria toxin (DTI), an attenuated version of protein-translation inhibitor diphtheria toxin (Bellen et al., 1992; Han et al., 2000),

was used to selectively ablate identified OSNs. When both DTI and GFP are expressed in all larval OSNs under control of *Or83b→Gal4,* these OSNs are atrophied to the degree that they do not express GFP (**Figure 4.6A**, left). When the dendrites of DTI-expressing larval OSNs are visualized with electron microscopy (EM), the few remaining sensory dendrites are severely atrophied (**Figure 4.6A**, right). In *Or49a*-ablated animals, the GFP marker is not visible and the overall structures of the DO and TO ganglia are not perturbed, suggesting cell autonomy (**Figure 4.6B**).

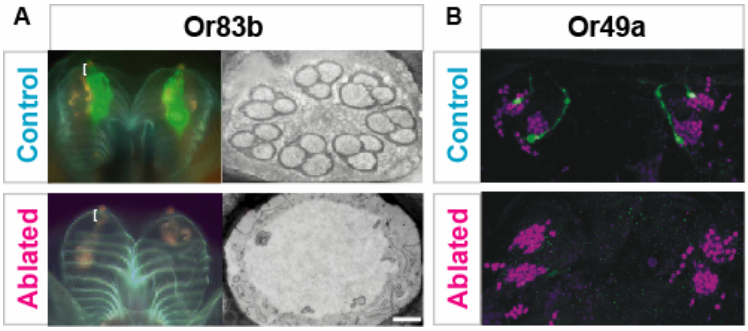

Figure 4.6: Diphtheria toxin atrophies olfactory sensory neurons. (A) Whole-mount GFP fluorescence (left; green) and thin-section electron micrographs (right) of *Or83b→Gal4;UAS→GFP/TM6B* larvae (top) and *Or83b→Gal4;UAS→GFP/UAS→DTI* larvae (bottom). Horizontal EM sections (right) were obtained from the region indicated by the bracket in the left panels. Scale bar = 1 μm. (B) Whole-mount GFP fluorescence (right, green) of *Or49a→Gal4;UAS→GFP/TM6B* larvae (top) and *Or49a→Gal4; UAS→GFP/UAS-DTI* larvae (bottom) is shown. Neuronal nuclei (DO and TO) are labeled with ELAV (magenta).

Chemo-taxes of animals with single OSNs ablated (*Or1a*, *Or42a*, or *Or49a*) or all OSNs ablated (*Or83b)* were measured with a panel of 20 odors. To control for effects of genetic background, we compared the behavior obtained with each ablated animal to the corresponding parental controls. Nonparametric Wilcoxon rank-sum tests were performed to establish significantly impaired chemotaxis in

ablated animals, correcting for multiple tests with the False Discovery Rate (FDR) method (**Section 4.2.4**). The summary plot in **Figure 4.7** is masked to show only those values with impaired chemo-taxes that are statistically significant. With the conservative statistical approach of zero false discoveries, *Or83b*-ablated larvae fail to respond to 17/20 odors (**Figure 4.7**). If we allow for a single false discovery (FD ≤ 1), *Or83b*-ablated animals fail to respond to 19/20 odors. At FD = 0, *Or1a*-ablated and *Or49a*-ablated animals each show reduced chemotaxis to a single odors, (*E*)-2-hexenal and 1-hexanol, respectively, but show normal chemotaxis to the other 19 odors (**Figure 4.7**). In contrast, ablation of the *Or42a* OSN causes decrease in chemotaxis to four of 20 odors (**Figure 4.7**).

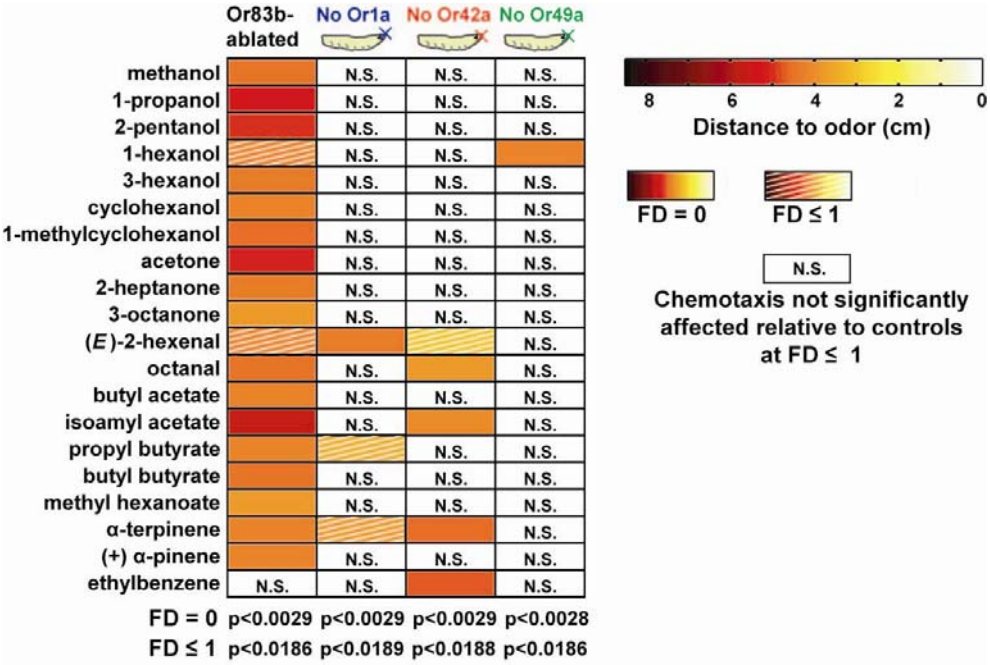

Figure 4.7: Genetic ablation reveals redundancy in the larval odor code. Summary of behavioral data to 20 odor stimuli presented at a dose of 2 µl. The difference in chemotaxis observed between the ablated genotypes and their parental controls was assessed upon adjustment of the nominal significance levels of Wilcoxon tests to maintain the family-wise type I error rate smaller than 0.05, while allowing zero or one False Discoveries (FD). Where chemo-taxes are significantly impaired relative to

controls, median distance-to-odor values are filled in pseudo-color scale. All cases that do not meet these statistical thresholds are masked in white, labeled N.S. (not significant, level 0.05 at FD ≤ 1). Corrected nominal significance levels at FD = 0 and FD ≤ 1 for all genotypes are listed below the table.

4.3.4 Larvae with One Functional Olfactory Neuron Can Smell

We next asked which OSNs are sufficient to produce chemotaxis to a given odor by constructing animals with only one or combinations of two functional OSNs. This was achieved by exploiting the *Or83b* mutation, which prevents OR trafficking to the sensory dendrite (Benton et al., 2006; Larsson et al., 2004; Neuhaus et al., 2005). *Or83b* function is restored in individual OSNs by crossing animals with specific *OR→Gal4* drivers to *UAS→Or83b* animals, allowing us to assess the contribution of single neurons to odor-evoked behavior in the OR-functional progeny.

We find no evidence that the glomerular map is distorted by the activation of a single OSN in a background of nonfunctional neurons as evidenced by the normal position and volume of the *Or1a* glomerulus (**Figure 4.8A**). When all larval OSNs are nonfunctional, the *Or1a* glomerulus appears normal in approximately two-thirds of animals (data not shown) but shows stray fibers leaving the glomerulus in about one-third of animals (**Figure 4.8A**, right panel). Only a single OR83b-positive neuron is seen in *Or1a*-, *Or42a*-, and *Or49a*-functional OSN animals, whereas two OR83b-positive neurons are visible in *Or1a-/Or42a*- and *Or1a-/Or49a*-functional OSNs animals (**Figure 4.8B**, top). The remaining OSNs are present but unlabeled in these animals because the *Or83b* mutation eliminates OR83b protein expression.

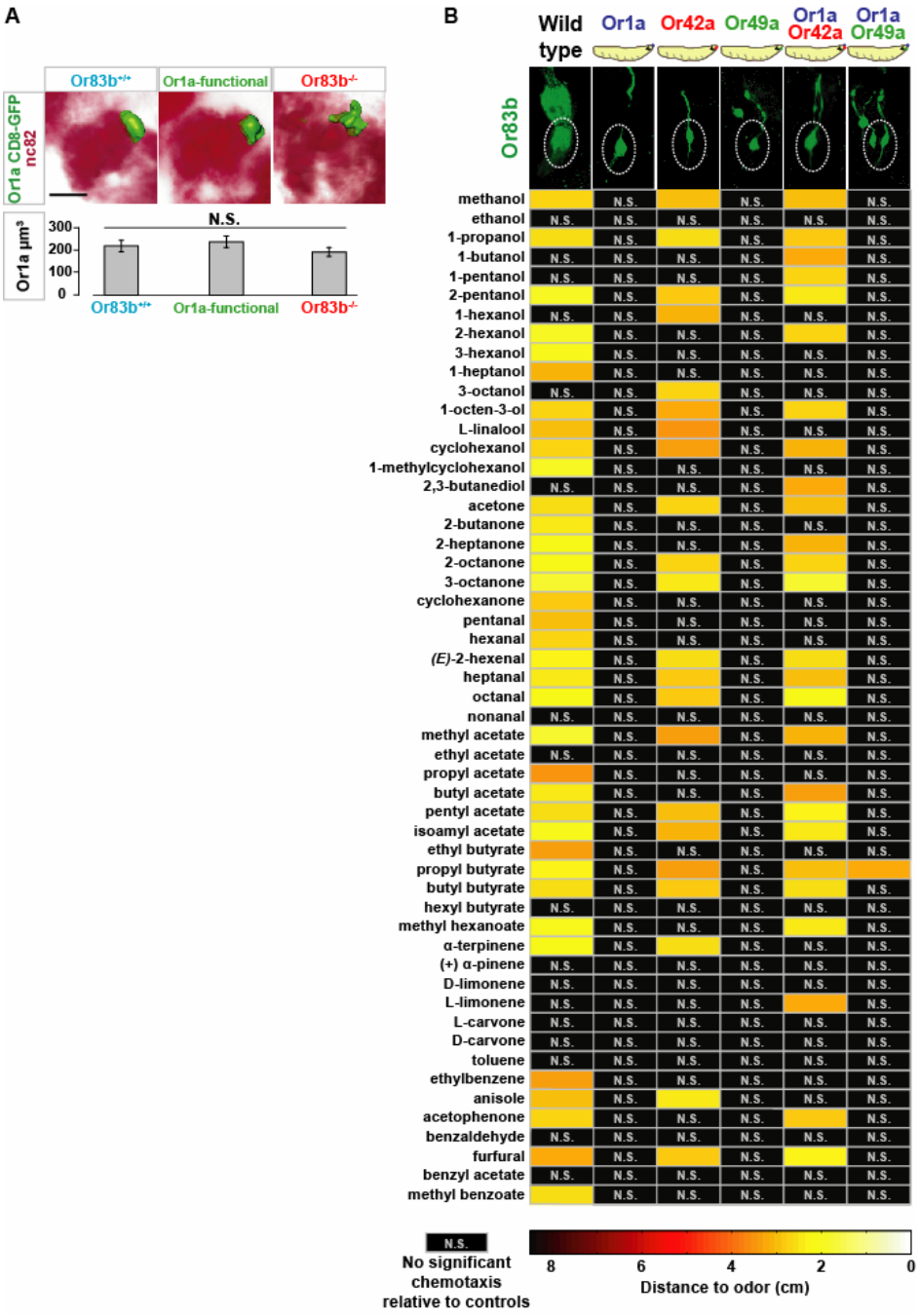

Figure 4.8: Chemotaxis produced by single functional olfactory neurons. (A) Surface view of the *Or1a* glomerulus in wild-type, *Or1a*-functional, and *Or83b* mutant backgrounds (green), along with a direct volume rendering of the larval antennal lobe stained with nc82 (dark red). Scale bar = 10 mm. Mean volume +/- standard error of the mean (SEM), n = 9 animals, n = 18 glomeruli. Glomerular volume differences are not significant among these genotypes ($p > 0.2$; two-tailed t test). (B, top) schematic of larvae having single or pairs of functional OSNs. Immunofluorescence of OSNs stained

with anti-OR83b antibody (green) of *yw*, (ρ1), (ρ2), (ρ3), (ρ5), and (ρ6) larvae (left to right), see **Section 4.2.4** for genotypes. White dotted line indicates boundary of dorsal-organ cell-body ganglion, as visible in the *yw* genotype. The terminal-organ neuron expressing *Or49a*-Gal4 is visible outside of the boundary of the dorsal organ. (Bottom) Summary of behavioral data to 53 different odor stimuli, presented at a dose of 2 µl. Cases with significant chemotaxis (level 0.05; FD = 0) relative to anosmic controls show the distance to odor with the scale at bottom. All cases that do not meet this statistical threshold are masked with a black box labeled N.S. (not significant at level 0.05 and FD = 0). In total, 29,235 larvae were tested, mean n = 50 (range 20–146) per odor and genotype. Corrected nominal significance levels for each genotype are $p < 0.0012$ for *Or83b* and $p < 0.0011$ for the remaining five genotypes.

Larvae with one or two functional OSNs, along with genetically matched control larvae were screened for chemotaxis to 53 odors by using the same behavioral assay and nonparametric statistical analysis as for the ablation experiments. The resulting median distances to odors are summarized in **Figure 4.8B** (bottom). The summary plot is masked to show only those values with statistically significant chemotaxis allowing FD = 0. At FD = 0, *Or42a*-functional animals respond to 22 odors compared to 36 odors in $Or83b^{+/+}$ controls that have 21 functional OSNs. Consistent with the *Or42a*-ablated phenotypes (**Figure 4.7**), *Or42a*-functional animals respond to three of four odors to which *Or42a*-ablated animals are anosmic (**Figure 4.8B**). The broad behavioral response profile we observe for *Or42a*-functional larvae is in agreement with the broad ligand specificity of this OR as defined by electrophysiological experiments (de Bruyne et al., 1999; Goldman et al., 2005; Kreher et al., 2005).

In contrast to the broad odor response profile of *Or42a*-functional larvae, *Or1a*- and *Or49a*-functional OSN animals do not show significant chemotaxis to

any of the 53 odors tested, consistent with the weak phenotype of ablating either the *Or49a*-expressing or *Or1a*-expressing neuron (**Figure 4.7**). These behavioral results are also in accord with the ligand profiling of *Or49a*, which does not show strong electrophysiological responses to any of 27 odors tested (Kreher et al., 2005).

4.3.5 A Second Functional Olfactory Neuron Enhances Chemotaxis

Although *Or1a*- and *Or49*-functional larvae do not chemotax to any odors tested, we analyzed whether these neurons contribute to chemotaxis in concert with the *Or42a* neuron. Chemotaxis performance of larvae with two functional neurons was compared to data from animals with only a single functional neuron. Larvae with two functional neurons respond to a somewhat different subset of odors than animals having either single functional neuron (**Figure 4.8B**).

To examine the existence of interactions between these neurons and identify cases of combinatorial enhancement, we developed an exploratory linear model to compare chemotaxis data across genetically matched controls for larvae with one or two functional OSNs (**Equation 1**). The model was designed to identify potential cases where single-neuron chemotaxis behavior differs from two-neuron behavior. In six cases, larvae with *Or1a- and Or42a*-functional OSNs chemotax significantly differently from larvae with single functional OSNs (**Figure 4.9** left, highlighted in red). In each case, the value of β_2 is smaller than zero, reflecting an enhancement in chemotaxis. For the *Or1a/Or49a* OSNs, three cases of significant difference are also highlighted in red (**Figure 4.9** right); all

three odors are associated with values of β_2 larger than zero, which reflect decreases in chemotaxis.

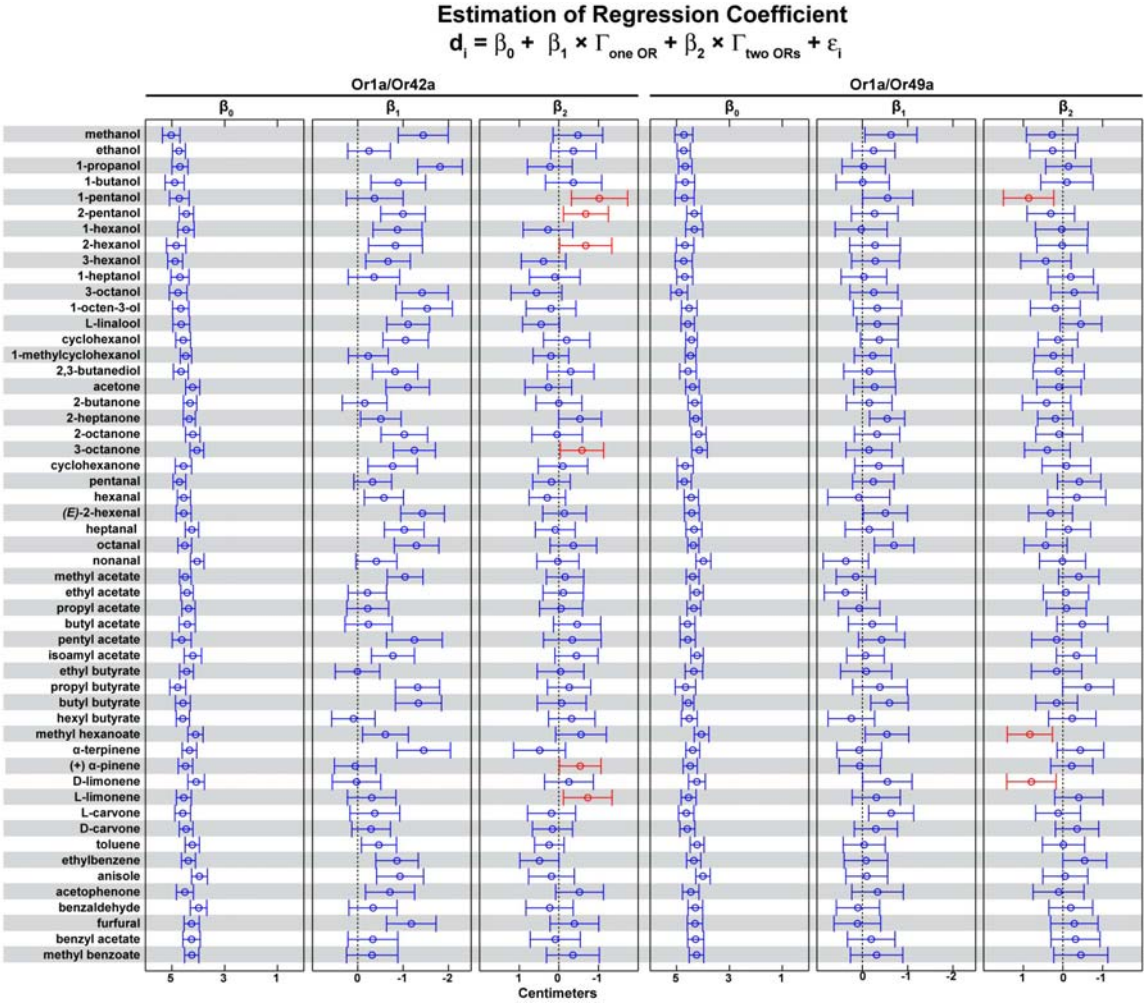

Figure 4.9: Linear model highlights potential cases of olfactory neuron interaction. Exploratory linear model applied to 53 odors to identify cases of chemotaxis enhancement (or inhibition) between the double and the single functional genotypes (**Figure 4.8**). Estimated coefficients β_0, β_1, and β_2 are represented by circles. The confidence interval of each estimate is represented by a horizontal bar calculated at a significance level 0.05.

We sought to investigate experimentally the potential cases of positive cooperativity between *Or1a* and *Or42a* OSNs. Additional chemotaxis experiments were carried out with four odors (1-pentanol, 2-pentanol, 2-hexanol,

and 3-octanone) at three concentrations. 1-pentanol shows significantly stronger chemotaxis in *Or1a*/*Or42a*-functional animals than *Or42a*-functional or *Or1a*-functional animals at all three concentrations (**Figure 4.10A**). A qualitative view of this behavioral enhancement is presented as sector-plot distributions; a progressive increase in chemotaxis to 1-pentanolincrease from *Or83b*$^{-/-}$ anosmic condition is seen for *Or1a*-functional, *Or42a*-functional, and *Or1a*/*Or42*-functional OSNs (**Figure 4.10B**). The *Or1a*/*Or42*-functional animals spend comparatively more time in the sector containing the odor than animals having either single functional neuron alone. For the other three odors, most odor concentrations show a trend towards cooperativity of *Or1a* and *Or42a* OSNs, however the cooperative effect is significant only at a single odor concentration (**Figure 4.10A**).

The cooperative function of *Or1a*- and *Or42a*-expressing OSNs for the four odors tested in **Figure 4.10** could be additive, sub-additive, or super-additive (true synergy). To test for synergy, we applied a second model, which assumes that addition of a functional neuron leads to an additive change in distance to odor (**Equation 2**), to data for 1-pentanol, 2-pentanol, 2-hexanol, and 3-octanone from **Figure 4.8B** and **Figure 4.9**. Because no estimates of $\beta_{Or1a/Or42a}$ are significantly different from zero ($p < 0.0125$), the combined effects of the *Or1a* and *Or42a* OSNs are not significantly greater than the sum of the individual contributions (**Figure 4.11**). Thus, we can only conclude that cooperative interactions between *Or1a*- and *Or42a*-expressing OSNs for 1-pentanol, 2-pentanol, 2-hexanol, and 3-octanone are neither synergistic nor sub-additive.

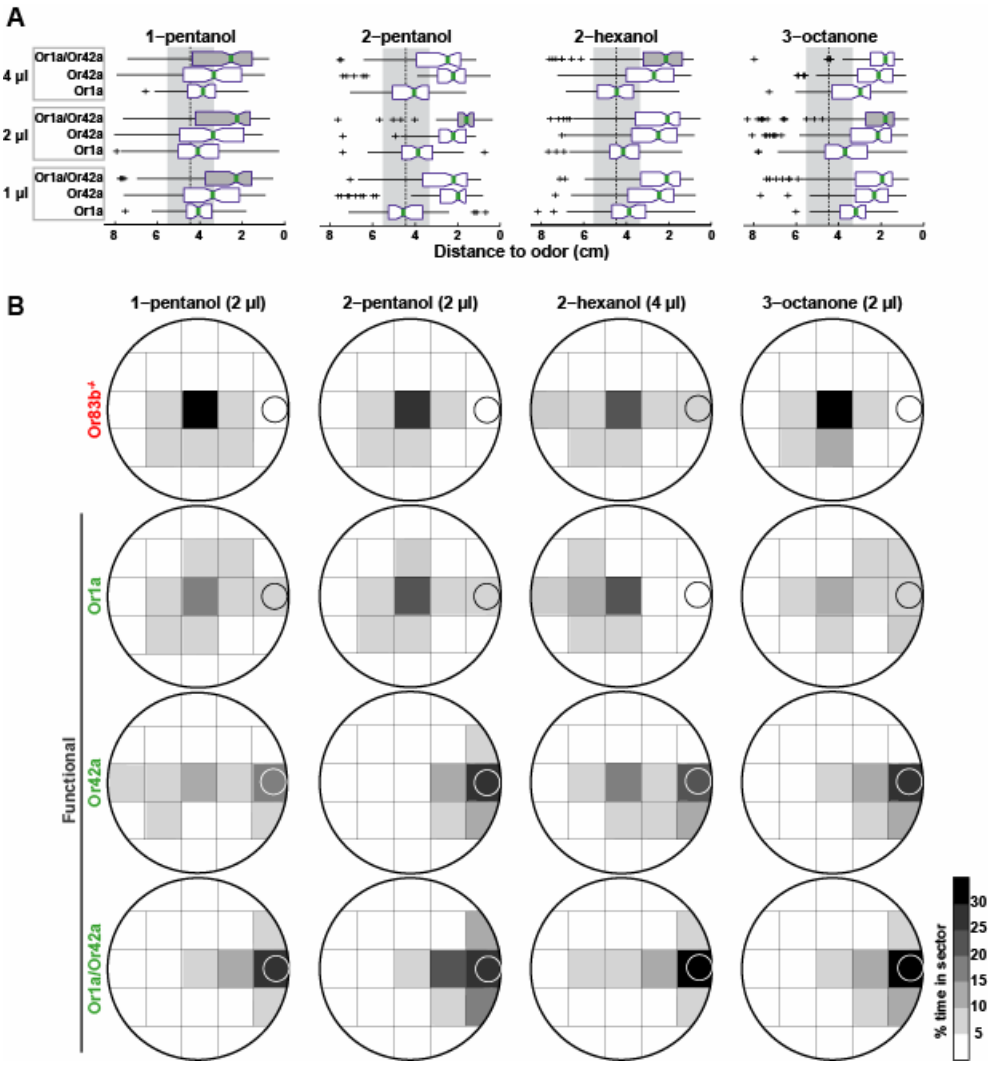

Figure 4.10: Larvae with two functional olfactory neurons (as compared to one) exhibit enhanced chemotaxis at a range of odor concentrations. (A) Box plots of responses of larvae with *Or1a*-, *Or42a*-, and *Or1a/Or42a*-functional OSNs to 1-pentanol, 2-pentanol, 2-hexanol, and 3-octanone at 1 µl, 2 µl, and 4 µl doses. Box plots shaded dark gray indicate significantly different chemotaxis of *Or1a/Or42a*-functional from *Or1a*- and *Or42a*-functional genotypes, determined with Wilcoxon rank-sum tests and Bonferroni correction ($p < 0.05/3 = 0.017$). In total, 3088 larvae were tested, mean n = 87 (range 47–165) per odor and genotype. (B) Sector plots showing the averaged spatial distribution of animals from (A) for a single concentration of each odor.

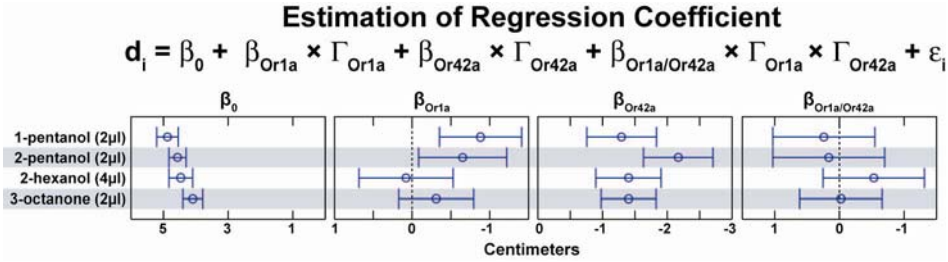

Figure 4.11: Behavioral contributions *Or1a* and *Or42*a olfactory neurons are not different from additive. Linear model applied to test for synergistic interactions of the single OSNs. Coefficients (*β*) were approximated by multiple linear regression (MATLAB®) method, represented by circles, at a significance level adjusted with the Bonferroni correction (p = 0.05/4 = 0.0125) represented by horizontal bar.

4.4 Discussion

In this study, we use behavioral analysis to measure the contribution of individual neurons to the odor code and provide a missing link between our understanding of the molecular biology of ORs, the neurophysiological properties of the olfactory network, and complex odor-evoked behaviors. We investigated how the combinatorial activation of ORs encodes odor stimuli and elicits olfactory behavior.

Our results suggest that there is a high level of redundancy in the larval olfactory system, such that ablating a single OSN has minimal effects on odor detection. The *Or42a* OSN plays a more important role in odor detection than the *Or1a* or *Or49a* OSNs. *Or42a* OSN is both necessary and sufficient for chemotaxis to several odors, as schematized in **Figure 4.12**. *Or1a* and *Or49a* OSNs are necessary for wild-type chemotaxis to a single odor each, and are sufficient only in concert with another functional OSN (**Figure 4.12**). At various concentrations, chemotaxis of larvae with *Or1a*- and *Or42a*-functional OSNs are

enhanced to four odors, relative to *Or42a*-functional larvae. Thus, while olfactory input contributed by some OSNs is not sufficient alone to elicit robust chemotaxis, it enhances the perception of odors in conjunction with the information transmitted by other OSNs.

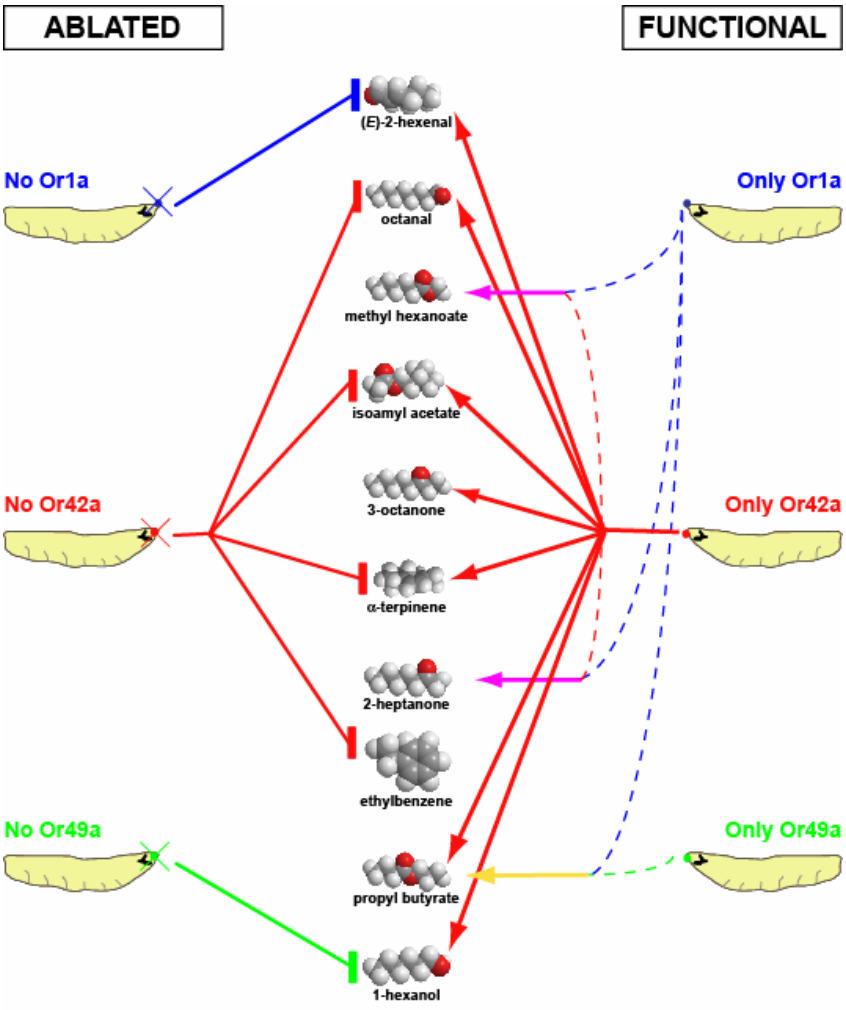

Figure 4.12: Larval chemotaxis behavior is integrated across multiple olfactory neurons. This schematic diagram outlines the general conclusions from our behavioral observations. At left, ablated OSNs are necessary for chemotaxis to a subset of odors (lines ending in vertical bars). At right, single OSNs (solid lines) or combinations of two functional OSNs (dashed lines) are sufficient to mediate chemotaxis (lines ending in arrows). This schematic includes results from **Figure 4.7** and a subset of **Figure 4.8B**. Although an OSN may be necessary for wild-type chemotaxis to an odor, other OSNs

may be sufficient for chemotaxis to the same odor that is significantly stronger than anosmic condition.

A number of conclusions about odor coding in the *Drosophila* larva can be drawn from our work. Larvae can chemotax robustly to many odors even when they have only one functional OSN. There appears to be no clear structural relationship between the odors that elicit chemotaxis mediated by a given OSN, as previously observed in studies of ligand response properties of ORs in the adult fly (Hallem et al., 2004a; Kreher et al., 2005). Some OSNs may play a more dominant role in the olfactory circuit than others in both their odor receptive ranges and the behavioral consequences of their activation.

It remains to be seen what percentage of larval OSNs fall within the high influence on chemotaxis (*Or42a*) category or the less influential (*Or1a/Or49a*) category. Interestingly, the behavioral response profile of the *Or42a*-functional genotype indicates that an OR may not need to be strongly activated by a given odor to allow for chemotaxis toward the odor source. This point is best illustrated by 3-octanol and anisole, which both elicit strong chemotaxis in *Or42a*-functional animals whereas they seem to induce relatively weak electrophysiological activity (Kreher et al., 2005).

The behavioral receptive field of animals having combinations of functional OSNs cannot be explained by the simple linear models explored in this study. Although the examples of cooperative interactions between OSNs that we explored do not differ from additivity, the additive model may not hold true for all neurons since activation of single neuron can already lead to wild-type-like chemotaxis. We explored behavioral properties that stem from activities of only

three of the 21 larval OSNs. There may exist combinations of OSNs that produce strong synergistic or inhibitory effects; these OSNs are yet to be studied.

The chemotaxis results we report highlight the existence of strong nonlinearities in the processing of olfactory information; consequentially, in the arithmetic of sensory coding, the whole is greater than what the parts can produce independently. Such a scheme would be consistent with the extraordinary needs of the olfactory system to detect numbers of odors that greatly exceed the number of OR genes in any given animal. The functional redundancy we observe here would allow larvae to chemotax to single odors and complex stimuli; it could also buffer the olfactory system against mutations and allow animals to adapt to changing or new odor environments.

Behavior is the ultimate output of a sensory system that integrates all aspects of external-information processing. Our experiments demonstrate the feasibility and value of integrating behavioral analysis into the study of odor coding. We propose that the simple olfactory system of *Drosophila* larvae will be an invaluable model in any attempt to correlate the cellular basis of the odor code with its behaviorally relevant output.

4.5 Contributions

I was fortunate to be a part of the team that carried out the larval chemotaxis study. I established expression of *OR→Gal4* transgenes that led to DTI ablation and single functional neuron experiments. I also participated in experimental design and analysis of the data. Several members of the Vosshall laboratory and The Rockefeller University contributed to this work. Kenta Asahina

(graduate fellow, RU) carried out RNA *in situ* hybridization to confirm OR expression in larvae. A chemotaxis assay that uses Ethovision® was developed by Andreas Keller (postdoctoral fellow, Vosshall lab, RU) for tracking adult flies; this assay was adapted for larvae by Ana Domingos (graduate student, Vosshall lab and Instituto Gulbenkian de Ciencia, Portugal). Laboratory helpers Silvia Vasquez and Lylyan Salas collected most of the larval tracks under the supervision of Ana Domingos and Matthiew Louis (postdoctoral fellow, Vosshall lab, RU). Félix Naef (RU and ISREC, Switzerland) and Joel Cohen (professor, RU) advised on data analysis. Matthiew Louis performed statistical analysis of data and evaluated exploratory linear models. Leslie Vosshall advised in the design and interpretation of the experiments.

5 Implications of Current Study and Future Prospects

Since discovery of the *Drosophila* ORs (Vosshall et al., 1999), significant progress was made in our understanding of insect olfaction. At the onset of my thesis research, the ORs in *Drosophila* larvae were not known. Larvae exhibit robust chemo-attractive behaviors and have only 21 OSNs (Larsson et al., 2004). Therefore, we hypothesized that a subset of *Drosophila* ORs are expressed in larval OSNs. We identified 25 and genetically marked 20 ORs that are expressed in single OSNs in larvae. With the *OR→Gal4* drivers, we ablated individual larval OSNs and generated larvae with only one or two functional OSNs. Subsequently, we analyzed whether particular larval OSNs were necessary or sufficient to confer chemotaxis to odors. In total, we genetically marked two thirds of *Drosophila* ORs. With *OR→Gal4* markers, we visualized patterns of OR

expression in the adult and larval fly olfactory organs and traced projections of their respective OSNs to the AL of the brain. We assigned OR identities to olfactory glomeruli and thus generated a “receptortopic” map of the AL.

In the passages below, I discuss how my thesis work may impact future work in the general field of sensory neuroscience. Our receptortopic map has highlighted the OR identities of glomeruli that express *fruitless*, gene that acts as a genetic switch for male courtship behavior (Demir and Dickson, 2005; Manoli et al., 2005; Stockinger et al., 2005). If the implicated ORs contribute to *Drosophila* courtship, targeted mutation of these ORs (Rong and Golic, 2000; Rong and Golic, 2001), will lead to defects in courtship behavior and mating.

To understand how odors are converted to olfactory perceptions, we need to know how chemical stimuli are encoded at the level of input to olfactory system. Further, we need to understand how the olfactory information is transformed in the AL and at higher levels. Our neuroanatomical description of olfactory sensory input into the adult and larval ALs promotes further studies of global properties of OSN activation in response to odors. Receptortopic maps provide tools to relate odor maps collected with calcium-sensitive imaging techniques to the ORs. Calcium imaging of the entire AL has an advantage of capturing global and temporal properties of activation of the AL glomeruli. Expression of calcium-sensitive proteins under control of the OR promoters will yield precise maps of OSN activation within AL. These experiments are already underway in the laboratories of Dr. C Giovanni Galizia (University of Konstanz, Germany) and Dr. Richard Axel (Columbia University). The resulting odor maps

of the AL will set the foundation to decode the olfactory code. These maps will provide information as to which ORs are most strongly activated by a given odor. Odor maps could be compared to electrophysiological response properties of the ORs to evaluate possible presynaptic modulation of olfactory input. OSN responses could also be compared to PN responses to reveal the transformation of olfactory information in the AL. Comparisons of the OSN and PN responses were already possible for one OR population in *Drosophila* (Ng et al., 2002; Wilson et al., 2004). These experiments revealed that the PNs responded to a broader range of odors than the OSNs. Our genetic tools will allow to extend these analyses and to determine whether broader responses by PNs are general properties of this neuron type.

While the glomeruli of the larval AL are not distinguishable morphologically, we can mark them genetically. Calcium-based activity imaging in LAL will be invaluable in studies of olfactory information processing in glomeruli. Activation of one OR may lead to activities of several glomeruli via the interneural network within AL. In *Drosophila* larvae, it is now possible to monitor global activities of the AL with calcium-sensitive proteins, while only one OSN is functional. These experiments in progress in the laboratory of Leslie Vosshall will unveil the excitatory and inhibitory neuronal interactions within larval olfactory circuit.

Our behavioral studies of larval olfactory system are initial steps in understanding how activations of individual OSNs lead to olfactory percepts. While we studied behavioral contributions of only three of the 21 OSNs in larvae, we generated tools to study most. It is not clear yet whether individual OSNs

mediate different behaviors in larvae, as they do in *C. elegans* (Bargmann et al., 1993; Troemel et al., 1997). Genetic experiments that will express heterologous receptors in *Drosophila* larval OSNs will uncouple the properties of OSN activation from the properties of ORs. For example, in *Drosophila,* behavioral experiments were possible with photo-activation of neurons (Lima and Miesenbock, 2005). In *C. elegans,* expression of mammalian TRP receptor (VR1) that responds to capsaicin, the active ingredient of chili peppers, induces worm repulsion to this molecule (Tobin et al., 2002). In a recent study, Marella et al. successfully expressed VR1E600K, a variant of VR1, in *Drosophila* taste neurons; they observed capsaicin-induced neuron activation and attractive and avoidance behaviors (Marella et al., 2006). Insect pheromone receptors, such as silk moth *Bombyx mori BMOr1* (Nakagawa et al., 2005), are other candidate heterologous receptors to be expressed in *Drosophila*. These or similar "remote control" experiments in *Drosophila* larval olfactory neurons will reveal the behaviors mediated by each OSN.

The imminent studies of how the olfactory information is represented and processed in the AL of *Drosophila* and then translated into chemotaxis behavior may help us understand human olfactory systems. We expect that some of the odor coding mechanisms of *Drosophila* will hold true in mammals because of the underlying neuroanatomical similarities between these animals.

Publications

The findings from my Ph.D. training are included in the following publications:

Fishilevich, E., Domingos, A. I., Asahina, K., Naef, F., Vosshall, L. B., and Louis, M. (2005). Chemotaxis behavior mediated by single larval olfactory neurons in *Drosophila*. Curr Biol *15*, 2086-2096.

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