Subunit Contributions to the Structure and Function of Insect Olfactory Receptors

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UNIVERSITY OF MIAMI

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

SUBUNIT CONTRIBUTIONS TO THE STRUCTURE AND FUNCTION OF INSECT Olfactory RECEPTORS

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Insects detect specific chemicals in the environment with olfactory receptors (ORs), which represent a novel class of ligand-gated ion channel. Insect ORs are comprised of at least one common subunit (OR83b in *Drosophila*) and at least one odorant-binding subunit. However, the molecular details of insect OR architecture, such as how they bind odorants, are unknown. This lack of knowledge hinders the development of compounds that may modulate OR function and potentially control insects involved in disease propagation and agricultural damage. The intent of this project is to investigate the structure and function of insect ORs. To this end, the utility of the *Xenopus* oocyte heterologous expression system was explored. Assay optimization, accuracy, and investigations on functional requirements were first performed using the *Drosophila* OR (DmOR) 35a/83b. The utility of the assay system was also demonstrated by identification of the honey bee (*Apis mellifera*) OR 11/2 as a receptor for the queen pheromone, 9-oxo-2-decenoic acid. A series of DmORs was cloned and expressed in *Xenopus* oocytes and individual receptors were selected for further study. DmOR85a/83b was shown to possess an incredibly high degree of enantioselectivity for the odorant ethyl 3-hydroxybutyrate. The receptive range of DmOR67a/83b was explored and
observations were made on potential features of the odorant-binding site and a ligand odorophore. DmORs were also used to investigate the contributions of individual subunits toward the odorant-binding site and pore structure. Also, evidence for receptor antagonism by odorants was revealed. DmORs were screened with methanethiosulfonate reagents and the substituted cysteine accessibility method to identify residues 146-150 of DmOR85b as functionally important in receptor activation. This region, located at the predicted interface between transmembrane segment 3 (TMS3) and extracellular loop 2, was shown to be physically adjacent to the odorant-binding site itself. Finally, residues within the extracellular half of TMS3 in DmOR85b were implicated in odorant-induced activation by screening DmOR85b mutants for altered ligand preferences. Therefore, this project provides the first identification of insect OR subunit components involved in odorant recognition, and represents an important starting point for detailed analysis of the molecular basis for insect OR activation by odorants.
ACKNOWLEDGMENTS

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<table>
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<th>Description</th>
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<tbody>
<tr>
<td>AgOR</td>
<td><em>Anopheles gambiae</em> olfactory receptor</td>
</tr>
<tr>
<td>AmOR</td>
<td><em>Apis mellifera</em> olfactory receptor</td>
</tr>
<tr>
<td>BmOR</td>
<td><em>Bombyx mori</em> olfactory receptor</td>
</tr>
<tr>
<td>cAMP</td>
<td>3'-5' cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>cDNA</td>
<td>Copy deoxyribonucleic acid</td>
</tr>
<tr>
<td>cGMP</td>
<td>3'-5' cyclic guanosine monophosphate</td>
</tr>
<tr>
<td>cRNA</td>
<td>Copy ribonucleic acid</td>
</tr>
<tr>
<td>δ-</td>
<td>Partial negative charge</td>
</tr>
<tr>
<td>DEPC</td>
<td>Diethylpyrocarbonate</td>
</tr>
<tr>
<td>DmOR</td>
<td><em>Drosophila</em> olfactory receptor</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>EC₅₀</td>
<td>Half-maximal excitatory concentration</td>
</tr>
<tr>
<td>ECL</td>
<td>Extracellular loop</td>
</tr>
<tr>
<td>EtOH</td>
<td>Ethanol</td>
</tr>
<tr>
<td>Gα₁₅</td>
<td>G alpha 15 subunit</td>
</tr>
<tr>
<td>Gαolf</td>
<td>G alpha olfactory subunit</td>
</tr>
<tr>
<td>Gαq</td>
<td>G alpha q subunit</td>
</tr>
<tr>
<td>Gαs</td>
<td>G alpha stimulatory subunit</td>
</tr>
<tr>
<td>GPCR</td>
<td>G-protein coupled receptor</td>
</tr>
<tr>
<td>GR</td>
<td>Gustatory receptor</td>
</tr>
<tr>
<td>IC₅₀</td>
<td>Half-maximal inhibitory concentration</td>
</tr>
<tr>
<td>ICL</td>
<td>Intracellular loop</td>
</tr>
<tr>
<td>IR</td>
<td>Ionotropic chemosensory receptor</td>
</tr>
<tr>
<td>LN</td>
<td>Lateral interneuron</td>
</tr>
<tr>
<td>mAChR1</td>
<td>Muscarinic acetylcholine receptor subtype 1</td>
</tr>
<tr>
<td>MTS</td>
<td>Methanethiosulfonate</td>
</tr>
<tr>
<td>MTSEA</td>
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<tr>
<td>MTSES</td>
<td>Ethylsulfonate methanethiosulfonate</td>
</tr>
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<td>MTSET</td>
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</tr>
<tr>
<td>nA</td>
<td>Nanoamperes</td>
</tr>
<tr>
<td>ng</td>
<td>Nanograms</td>
</tr>
<tr>
<td>OBP</td>
<td>Odorant Binding Protein</td>
</tr>
<tr>
<td>OnOR</td>
<td><em>Ostrinia nubilalis</em> olfactory receptor</td>
</tr>
<tr>
<td>OR</td>
<td>Olfactory Receptor</td>
</tr>
<tr>
<td>OSN</td>
<td>Olfactory Sensory Neuron</td>
</tr>
<tr>
<td>PLC</td>
<td>Phospholipase C</td>
</tr>
<tr>
<td>PN</td>
<td>Projection neuron</td>
</tr>
<tr>
<td>QRP</td>
<td><em>Apis Mellifera</em> queen retinue pheromone blend</td>
</tr>
<tr>
<td>RR</td>
<td>Ruthenium red</td>
</tr>
<tr>
<td>SCAM</td>
<td>Substituted cysteine accessibility method</td>
</tr>
<tr>
<td>TMS</td>
<td>Transmembrane segment</td>
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CHAPTER I: INTRODUCTION

**Insect chemical detection**

Insect olfaction is integral for survival, enabling the survey and interpretation of a rich external environment of volatile compounds. Similar to other animal olfactory systems, insects have developed a general framework and strategy for chemical detection. Peripheral stimuli, or odorants, are first recognized by chemosensory receptors, which are exposed to the environment on the surface of sensory neuron plasma membranes. The recognition event is then converted into an electrical signal within the sensory neurons and this information is sent via neuronal networking to the central nervous system for interpretation. Accurate and powerful odorant discrimination is obtained by the use of a large repertoire of olfactory receptors (ORs), which can be activated by multiple odorants. In turn, individual odorants can activate multiple receptors and are represented by a combinatorial code of receptor activation patterns (5,6). Using this approach, a vast degree of chemical space can be covered. This discriminatory power of the olfactory system is used by an individual insect to detect the presence of predators, food sources, and conspecifics. Based on this information, important behavioral decisions can then be made. Indeed, olfaction drives a large degree of observable behaviors in insects.

**Insects and human society**

Insect behavior, and thus insect olfaction, has profound influences on human health and quality of life. Agricultural researchers estimate 35% of the world’s crops, a full 87 crop types, are significantly impacted by animal pollinators such as insects (7). Crop-destroying insects account for an estimated 30-40% of agricultural production loss
Furthermore, an additional 60-90% of plant species, many with central roles in human society other than food sources, are dependent on animal pollination. Of course, the success of plant reproduction has strong implications in ecosystem vitality. One way to increase yields in crop and plant production could involve manipulation of the insect olfactory system, driving beneficial behaviors such as pollination and predation of detrimental insects. Such strategies may become increasingly important as rising food demands have led to problems with proper land management and the use of high-density agricultural methods. The control of harmful insects also offers significant challenges. Insects as biological vectors for diseases in humans, animals and plants are endemic worldwide. Annually, insect-based disease transmission to humans exceeds 300 million cases, of which 1 million are fatal. Forty percent of the world’s population is at risk to malaria alone. At present, many effective insect control strategies involve the use of dangerous pesticides and toxins, which create additional concerns for human health.

Since the insect olfactory system is responsible for regulating many beneficial and harmful behaviors, new strategies on insect control may involve olfactory-based intervention and manipulation. This may allow the development of effective agents with less toxicity and higher specificity. Research on insect olfaction has yielded much recent progress, but we particularly lack a strong understanding of the molecular basis of olfaction. Of particular importance is elucidating how insect ORs specifically recognize odorants and transform this event into a neuronal signal. This information is critical if new insect control strategies, based on the modulation of olfactory function, are to be realized.
**Organization of the insect olfactory system**

For clarity, my discussion of insect olfactory system anatomy will focus on the adult *Drosophila melanogaster* fruit fly. It should be noted that the general structure and organization of the fruit fly olfactory system is common for all insects (10). The main peripheral structures of the *Drosophila* olfactory system are the third segment of the antenna and the maxillary palp. On the surface of these organs are hair-like hollow bristles, called sensilla, which house olfactory sensory neurons (OSNs). Sensilla provide physical protection of the OSNs and, by way of pores on the sensillum surface, help concentrate odorants from the external environment. These sensory structures also contain a mucosal layer of lymph, secreted by a collection of glial-like support cells in the antenna (11). While specific details are mostly unknown, odorant binding proteins (OBPs) present in the lymph may bind odorants for further odorant enrichment and delivery to the *Drosophila* olfactory receptors (DmORs) themselves (12). Alternatively, OBPs may play a role in efficient removal or binding of odorants for adaptation and the detection of concentration gradients. One well studied example is LUSH, an OBP molecule that directly associates with the *Drosophila* male-produced sex pheromone, *cis*-vaccenyl acetate, to form the actual pheromone receptor ligand (13). Between one and four OSN cell bodies are situated at the base of a sensillum, each projecting an OR-enriched ciliated dendrite into the sensillum cavity for odor detection, plus a single axon that extends into the antennal lobe (14). The basic structures of a sensillum are presented in Figure 1.1.
Sensilla can be morphologically categorized into five main classes (large basiconic, small basiconic, coeloconic, intermediate, and trichoid) and are arranged on the antennal and maxillary palp surfaces in a stereotyped manner (15). Furthermore, the sensilla distribution can be organized based on functional and physiological properties of odorant sensitivity, conferred by the repertoire of OSNs housed within (14,16,17). At present, it is unknown why particular OSNs and their ORs are associated together within a particular sensillum type, even though this association is also highly stereotyped (14,17). No obvious logic related to odorant specificity exists (14).

In most cases each OSN only expresses a single OR, plus the widely expressed OR83b (expanded upon below) (18-20). It should be noted that a small population of OSNs instead express other chemosensory receptors, including ionotropic receptors (IRs) and gustatory receptors (GRs), described briefly below. Axons from particular OR-expressing OSNs converge onto discrete locations in the antennal lobe called glomeruli (14,21). Each glomerulus also contains inputs from projection neurons (PNs) and lateral interneurons (LNs) (14,21). Odorant activation of a population of OSNs is thus imparted
onto the antennal lobe as a map of glomerular activation patterning. There are, at present, 43 identifiable glomeruli in the adult fly (22). The activation or inhibition of individual glomeruli can be controlled by various modes of neurotransmission. For instance, cholinergic PNs provide an excitatory link to higher brain regions, inhibitory GABAergic LN s play a role in lateral suppression between glomeruli (23,24), while still other populations of LNs may induce interglomerular excitation through cholinergic innervation (25). Recent work suggests this organization provides more complex coding of information by way of gain control circuits and the recruitment or inhibition of neighboring glomeruli (26,27). Therefore, glomeruli can be thought of simultaneously as individual functional units and features of an interconnected coding network, vastly increasing the discriminatory power of the animal. In summary, electrical signals originating from OR functional activity travel from the OSNs through the PNs of the antennal lobe and to higher order brain regions such as the mushroom body and lateral horn (21,23). This organization provides a mechanism for the brain to interpret the detection of an odorant in the periphery (Figure 1.2). Of course, this process begins with odorants binding to ORs.

**Insect chemosensory receptors**

The first candidate insect ORs were identified by the isolation of low abundance *Drosophila* antennal and maxillary palp-specific mRNA transcripts (28) and later by bioinformatic analysis (29). A highly divergent 62 member gene family sharing no obvious similarities with any other receptor class was later described in *Drosophila* (20,30).
ORs recognize various food odors, volatile chemicals and pheromones (10). The chemosensory GRs are mostly expressed in sensilla on the maxillary palp, legs, and wings, and are primarily used to detect non-volatile compounds such as sugars and bitter-tasting molecules (10). Similarities may exist between the GRs and ORs in structure and function, but little is known about GR complex formation and subunit composition. The low sequence conservation between GRs and ORs offers little insight (10). More recently, the chemosensory IRs were reported to be expressed in specific coeloconic sensilla and the arista (31). Highly related to glutamate ion channels, these proteins lack conserved glutamate-binding domains. This receptor class may play a role in detection of both internal and external cues, as evidence has been presented showing sensitivity to
water, ammonia, and some food odors (31). IRs may be strongly conserved through evolutionary time, and variants may serve a wide range of roles in plants, bacteria, and animals (31). Fully understanding insect chemosensation will undoubtedly rely on fundamental discoveries relating to all three classes of chemoreceptor. This project focuses on the ORs.

**Insect ORs are different than mammalian ORs**

Insect ORs are significantly different from the G-protein coupled receptor (GPCR) mammalian OR family. Mammalian ORs have seven transmembrane domains with an extracellular N-terminus and intracellular C-terminus, characteristics shared among all known GPCRs. However, extensive biochemical analysis including the use of engineered glycosylation sites as topology markers (32), inserted epitope tags on receptor sequence termini (33), and yellow fluorescent protein fusion experiments (19) conclude that insect ORs have an intracellular N-terminus and an extracellular C-terminus. Even the number of insect OR transmembrane segments is unknown, as algorithms do not entirely agree and usually predict between six and eight transmembrane segments. Furthermore, it cannot be ruled out that insect ORs contain loop sequences that enter the membrane but do not cross (i.e. pore loops).

Another difference between insect and mammalian ORs lies in the transduction mechanisms for the conversion of odorant binding events into a cellular signal. In mammals, OSN action potentials are achieved by a signal transduction system in which a functional OR triggers an increase in intracellular cAMP through $G_{olf}$-mediated adenylate cyclase activation (34,35). This increase in cAMP activates a cyclic
nucleotide-gated inward cation current which results in an increase in intracellular Ca\(^{2+}\) levels (36). This increase in Ca\(^{2+}\) activates a chloride channel, which further depolarizes the cell (36). As expected, mammalian OSNs are highly enriched in these 2\(^{nd}\) messenger signal transduction components and ion channels. Furthermore, genetic deletion of G\(_{\text{olf}}\)-coupled signal transduction components results in abolishment of olfactory responsiveness (37,38). Insect OSN dendrites do not express enriched levels of G protein subunits that would suggest the coupling of insect ORs to canonical GPCR primary signal transduction pathways (39-41). In addition, no direct association with G proteins has been firmly established. Nevertheless, studies have implicated various 2\(^{nd}\) messenger pathways, including G\(_{\text{as}}\), and G\(_{\text{aq}}\)-coupled signaling, for the modulation and extension of maximum responsiveness in insect OR function (42-46). These experiments have involved the genetic deletion or transient modulation of candidate pathway components resulting in reduced, but not abolished, olfactory responsiveness. Experiments reporting only behavioral changes may be interpreted as learning or coding defects of the fly (47), and not alteration of the OR transduction pathway. Unlike mammalian olfaction, these reports suggest a non-essential role for various 2\(^{nd}\) messengers in insect OR activation by odorants (4). But it should be noted that these results provide compelling evidence for a major role of insect OR functional modulation by various intracellular OSN components (4,33,45). The complete unrelatedness of insect ORs to other receptor classes provides a significant challenge to study the modulation of insect ORs by other proteins and molecules. For instance, insect ORs do not contain obvious regulatory regions or canonical protein-protein interaction sites, such
as the E/DRY motif, shared by many GPCRs (48). Therefore, if insect ORs indeed couple directly to G proteins, they associate in a completely novel way.

**The insect OR complex and signaling**

Insect ORs were recently reported to be ligand-gated ion channels, activating a non-selective cation current upon odorant binding (45,49). The discovery of insect ORs as functional ion channels was finally demonstrated by performing whole-cell patch recordings of heterologously expressed *Drosophila* and *Anopheles* receptors in *Xenopus* oocytes, HeLa cells, and HEK293 cell membranes and recording odorant-induced currents (45,49). The data directly opposed long-standing assumptions on the nature of insect OR signaling (see above). Also, this established insect ORs as a newly identified class of ligand-gated channel, which possibly utilize novel mechanisms for ligand binding and ion channel activation. The activation of ORs by odorants leads to depolarization of the OSN, however, it is unclear if OR activation is sufficient to reach threshold for the generation of an action potential.

Each functional OR is a complex of unknown stoichiometry, comprised of at least one copy of OR83b and at least one copy of a non-OR83b subunit (18,19,50,51). Non-OR83b subunits are thought to be involved in ligand binding and recognition, since changing this subunit can alter the ligand preference of the receptor complex (1). Mounting evidence suggests these specificity subunits cannot functionally activate a current without OR83b (1,6,49-53), however a small number of reports suggest some receptor subunits are weakly functional alone (33,54). Differences in assay design and recording methods may account for these discrepancies. The specificity subunits exhibit
a high degree of sequence variability (about 20-70%). This high degree of variation may enable a wide range of odorant structures to be recognized by an OR family.

Interestingly, the highly conserved OR83b subunits from various insect species can act interchangeably to form functional OR complexes, pointing to a conserved and common role of this subunit (55). OR83b directly associates with the specificity subunits through interactions with at least the 3rd intracellular loop (IC3) of each subunit (19). Additional interactions are certainly possible. While conserved residues are indeed more prominent toward the C-terminus of the OR83b protein, the exact residues responsible for subunit-subunit interactions are unknown. Also, the diverse specificity subunits do not exhibit any obvious regions of amino acid conservation that might indicate probable residues in IC3 responsible for OR83b association. This is surprising, since it is tempting to envision all specificity subunits similarly interacting with the common OR83b subunit. Hydrophobic associating regions of the subunits that contain a high degree of variability or a role for additional and unidentified chaperone proteins could account for this discrepancy. OR83b also acts as a chaperone, improving the targeting of the OR complex to the plasma membrane of the OSN dendrite (19). Proper localization of ectopically expressed OR83b suggests that this subunit can couple directly to common intracellular protein trafficking components found in a variety of ciliated cell types (19). No specific residues have been identified that confer this interaction, but the expanded intracellular loop 2 (IC2), as compared to specificity subunit IC2, has been implicated (19). Furthermore, heterologous expression of functional OR complexes suggests other cofactors that may be found only in OSN’s are not required for membrane targeting (45,49-51). The OR83b subunit does not respond to any odors by itself, but is required
for odorant-activated ion flux (1,45,49,51,53). This suggests OR83b might have a role in contributing to the channel itself, although details on channel structure are extremely limited. Recently, a group reported that deletion of specific residues within transmembrane segment 6 (TMS6) of OR83b altered the permeability of the activated OR complex (45), supporting the notion that OR83b is structurally involved in the channel pore. The same group also reported that the OR complex can be directly activated by cyclic nucleotides (cAMP and cGMP), generating a current through $G_{\alpha \text{s}}$-mediated signaling, and the receptor complex may associate directly with G-proteins. This “metabotropic” current was reported to build slowly, as opposed to the odorant-generated and fast “ionotropic” current, and is proposed to provide further sensitivity of the olfactory system. Such an interpretation is presently at the center of debate within the field; other labs have been unable to duplicate both the cyclic nucleotide sensitivity of insect ORs and the slower “metabotropic” current itself (49). Also contradictory is whether the insect OR complex associates directly with G protein subunits. To date, no definitive evidence has been presented to answer this question. As an alternative interpretation, the sensitivity of insect ORs to cyclic nucleotides may be a feature of their proposed ability to be modulated by additional, as yet unidentified, membrane or soluble proteins (4). A summary of the known signal transduction mechanisms of insect ORs, as well as molecules with possible modulatory functions in OR signaling, is presented in Figure 1.3. Clearly, more work is required before consensus is achieved (4). Evidence presented thus far does implicate insect ORs as a class of ligand-gated ion channel that functions through mechanisms not completely understood, and perhaps new to our understanding of sensory biology.
Studies on insect ORs

Research on odor coding and insect OR ligand specificities has relied mostly on the “empty neuron” in vivo expression system in Drosophila antenna (17,52). The system uses a transgenic approach where DmOR22a and b are deleted in ab3A neurons and replaced with a receptor of choice. Electrophysiological recordings from these transgenic neurons are measured by inserting a wire directly into the base of a sensillum as odors are puffed onto the antenna. Using this method, studies have successfully deorphaned a large number of adult and larval DmORs (1,56-58), and even identified Anopheles gambiae receptors (AgORs) responsible for the mosquito’s detection of human sweat components and CO2 (59,60). The main disadvantages of this in vivo approach include the generation of a new fly line for each receptor tested, and the
inability to study receptors in isolation from *Drosophila* OSN intracellular components that may affect receptor function. It has also been suggested that receptors from at least some insect species are incompatible with this expression system (K. Wanner, personal communication).

Heterologous expression systems offer distinct solutions to these problems by allowing the relatively quick investigation of protein function in isolated conditions. The *Xenopus* oocyte heterologous expression system as a method to study insect ORs was first reported using the *Drosophila* receptor DmOR43a. This receptor was coexpressed with the promiscuous G protein, G\(_{\alpha15}\), to presumably couple together and, upon odorant binding, activate internal Ca\(^{2+}\) release and the oocyte Ca\(^{2+}\)-activated Cl\(^{-}\) current (54). At the time, it was unknown if insect ORs require coupling to a signal transduction system for generation of odorant-induced currents. Unfortunately, this work omitted key control experiments (see Chapter III) which limit interpretations on the role, if any, of G\(_{\alpha15}\) in development of a recordable current signal. Following this study, another lab utilized the *Xenopus* expression system to characterize a pheromone receptor from the silk moth, *Bombyx mori* (50). This work first suggested that only the heterologous expression of a specificity subunit and an OR83b subunit is sufficient for odorant-induced current generation in heterologous expression systems. Later, the functional expression of AgORs in *Xenopus* oocytes was successfully demonstrated (60-62). These reports provided strong evidence for the successful functional expression of a variety of insect ORs in *Xenopus* oocytes. Therefore, this project sought to utilize this methodology to study insect ORs.
Statement of purpose

Insect ORs belong to a newly identified class of ligand-gated ion channel. These receptors adopt a multimeric structure of unknown stoichiometry, comprised of at least one OR83b subunit and one additional OR subunit presumed to confer ligand specificity. Odorant binding to a receptor complex results in opening of a non-selective cation channel, providing a mechanism for the detection of odorants in the environment. This receptor class shares no obvious structural similarities with other receptors or ligand-gated ion channels. Therefore, research efforts into the elucidation of insect OR structure and function are more difficult and of greater consequence.

Understanding OR function will provide new avenues toward effective insect control strategies. Currently, there are many outstanding questions on the molecular details of insect OR structure and function. How and where do insect ORs bind odorants? How is this event converted into the opening of the channel? What regions of the receptor are subjected to cellular regulation? What are the subunit contributions to both the odorant binding domains and channel structure? The intent of this project is to study the structure and function of insect ORs. Since this research represents the first foray into the structural details of insect ORs, a broad and unbiased methodology was selected to allow identification of any regions important for ligand binding, channel activation, or other functionally related roles. To this end, the development and validation of an insect OR functional assay was investigated using the *Xenopus* oocyte heterologous expression system. This work also reports the successful utilization of this assay system to characterize a newly cloned collection of *Drosophila* ORs (DmORs).
Investigations on the various contributions of OR subunits are provided, furthering our understanding of the requirements for a functional OR complex. Also, I report the identification and location of odorant recognition features on a particular DmOR. The discoveries reported herein provide the first structural details for the basis of ligand recognition in insect ORs and will initiate a new field of research on the activation and function of this novel class of ligand-gated ion channel.
CHAPTER II: MATERIALS AND METHODS

Materials

*Xenopus laevis* frogs were purchased from Nasco (Fort Atkinson, WI). The care and use of *Xenopus laevis* frogs in these studies were approved by the University of Miami Animal Research Committee and meet the guidelines of the National Institutes of Health. Odorants were purchased from Sigma-Aldrich (St. Louis, MO), unless otherwise noted.

Methanethiosulfonate (MTS) reagents were from Toronto Research Chemicals (Toronto, Canada). These reagents were stored long term in desiccant at -20°C. On the day of use, 1 M MTS reagent stock solutions were prepared in DMSO and stored at -20°C. Immediately before use (less than 2 min before application), MTS working solutions were prepared in ND96 (see below for ND96 constituents). This procedure limited the hydrolysis of MTS reagents before a given experiment. Table 2.1 lists the MTS reagents used and their relative half-lives in aqueous solution. Please refer to Figure 5.1 for MTS structures.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Abbreviation</th>
<th>Approximate Half-life (min) (at pH 7.5 and 25°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-Aminoethyl methanethiosulfonate hydrobromide</td>
<td>MTSEA</td>
<td>15</td>
</tr>
<tr>
<td>[2-(Trimethylammonium)ethyl] methanethiosulfonate bromide</td>
<td>MTSET</td>
<td>10</td>
</tr>
<tr>
<td>Sodium (2-sulfonatoethyl) methanethiosulfonate</td>
<td>MTSES</td>
<td>20</td>
</tr>
</tbody>
</table>

Table 2.1 - MTS reagents and their approximate half-lives expected in buffer solution (2).
**Oocyte preparation**

*Xenopus laevis* frogs were anesthetized in water containing 0.08% ethyl 3-aminobenzoate methanesulfonic acid and 0.08% sodium bicarbonate for 15 min. Oocyte pouches were removed by surgery and placed in Ca$^{2+}$-free Barth’s solution (in mM: 88 NaCl, 1 KCl, 2.4 NaHCO$_3$, 0.82 MgSO$_4$, 15 HEPES, pH 7.6, and 100 µg/mL amikacin). Frogs were then sutured, treated with gentamicin at the incision site, and returned to fresh water for recovery. Follicle cells and connective tissue was removed from the oocytes by treatment with Collagenase B (Roche, Indianapolis, IN) for 2 hours at room temperature. Washing was performed in Ca$^{2+}$-free Barth’s solution. Oocytes were maintained at 18°C in regular Barth’s solution (in mM: 88 NaCl, 1 KCl, 2.4 NaHCO$_3$, 0.3 CaNO$_3$, 0.41 CaCl$_2$, 0.82 MgSO$_4$, 15 HEPES, pH 7.6, and 100 µg/mL amikacin) for a maximum of 24 hours before injection.

**Preparation of total RNA from antenna**

Approximately 200 wild-type (Canton S) *Drosophila melanogaster* adult flies were frozen in liquid nitrogen and shaken through a three-stage sieve. The bottom of the sieve was verified to contain mostly antenna, heads and legs by microscopy. This tissue was collected and homogenized on ice in a Dounce homogenizer containing 2 ml of Trizol (Invitrogen, Carlsbad, CA). Total RNA was extracted with Trizol, following manufacturers’ directions, resuspended in DEPC-treated dH$_2$O and stored at -80°C. Using this procedure, approximately 50 µg of total RNA could be isolated.

An alternative method of tissue collection was later developed. Instead of antenna collection by sieve separation, flies were first anesthetized on an ice block. The
front half of each fly head, which included the antenna and mouth parts, was dissected and placed in a microfuge tube on dry ice. Total RNA was extracted using Trizol, as described above. Approximately 50 flies yielded 20 µg of total RNA.

**Receptor cloning**

_Drosophila_ antenna total RNA was used as template for 1st strand synthesis of various OR subunit transcripts using gene-specific primers. Approximately 5 µg of total RNA was used as source material for each reaction; however, reactions could be multiplexed (multiple gene-specific 1st strand primers in the same tube). 1st strand synthesis was performed using SuperScript III (Invitrogen). Reverse transcribed products were amplified by polymerase chain reaction (PCR) using Phuzion polymerase (NEB; Ipswich, MA) in a two step methodology. The first round of PCR utilized highly optimized primers ending exactly at the Start and Stop codons of each clone. Samples were separated and visualized on a 0.8% agarose gel, and bands at the approximate expected length for each clone (www.flybase.org) were excised. Gel pieces were frozen at -20°C for 30 min followed by high-speed centrifugation: an alternative “freeze and squeeze” approach. 1-2 µl of the resulting liquid was used as template for a second round of PCR utilizing primers with flanking restriction sites. After purification and digestion, products were subcloned into pGEMHE (63) and verified by sequencing (Genewiz; South Plainfield, NJ). DmORs 35a and 83b were generously provided by J. Carlson and L. Vosshall, respectively. Some DmOR clones contained differences in amino acid sequence, when compared to the annotated receptors published at www.flybase.org. All changes present in the newly cloned receptors were verified by sequencing additional
PCR products from duplicate synthesis reactions. These differences may be a result of annotation mistakes, or natural genetic variation among fly populations. A summary of the amino acid differences is presented in Table 2.2.

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Position and amino acid substitution in cloned receptors</th>
</tr>
</thead>
<tbody>
<tr>
<td>DmOR9a</td>
<td>0</td>
</tr>
<tr>
<td>DmOR22a</td>
<td>0</td>
</tr>
<tr>
<td>DmOR35a</td>
<td>0</td>
</tr>
<tr>
<td>DmOR43b</td>
<td>K328N</td>
</tr>
<tr>
<td>DmOR59b</td>
<td>0</td>
</tr>
<tr>
<td>DmOR67a</td>
<td>M100V, P120S, V124D</td>
</tr>
<tr>
<td>DmOR83b</td>
<td>0</td>
</tr>
<tr>
<td>DmOR85a</td>
<td>0</td>
</tr>
<tr>
<td>DmOR85b</td>
<td>M28L, N29K</td>
</tr>
<tr>
<td>DmOR98a</td>
<td>F171L, N250K</td>
</tr>
</tbody>
</table>

**Table 2.2 - Comparison of cloned *Drosophila* OR sequences with the annotated ORs at www.flybase.org. A value of zero indicates no difference between cloned and annotated ORs. Differences of cloned receptors are indicated as the second amino acid for each position reported.**

**Receptor mutagenesis**

Mutant receptors were constructed using QuickChange Lightning kits (Stratagene, La Jolla, CA). Mutagenic primers were designed using the QuickChange Primer Design program (www.stratagene.com). All mutant constructs were verified by sequencing.

**cRNA synthesis and injection**

Plasmid constructs containing inserts coding for OR subunits were linearized by digestion using a suitable restriction enzyme. Linearized DNA was purified by
phenol:chloroform extraction, followed by chloroform extraction and finally ethanol precipitation for use as template in cRNA synthesis reactions. Capped cRNA encoding various insect OR subunits was generated from these templates using mMMessage mMMachine kits (Ambion, Foster City, CA). All cRNAs were visualized in an agarose gel and concentrations were estimated by spectrophotometric reading (at A260) before use. Depending on the experiment, 5-35 ng of cRNA encoding each OR subunit was injected into Stage V-VI *Xenopus* oocytes. If additional cRNAs besides insect ORs were injected, adjustments were made to limit the total amount of cRNA presented to each cell to approximately 60 ng/oocyte. Oocytes were incubated at 18°C in Barth’s solution for 2-7 days prior to electrophysiological recording.

**Electrophysiology and data capture**

Odorant-induced currents were recorded under two-electrode voltage clamp from oocytes expressing various insect OR subunits, using an automated parallel electrophysiology system (OpusExpress 6000A; Molecular Devices, Sunnyvale, CA). Unless noted, oocytes were perfused with ND96 (in mM: 96 NaCl, 2 KCl, 1 CaCl2, 1 MgCl2, 5 HEPES, pH 7.5). On days of recording, odorants were freshly prepared by first making a 1 M stock solution in DMSO or EtOH, followed by dilution in ND96 at the testable concentration. Unless otherwise noted (described in detail in protocols below), odorants were applied for 20 sec at a flow rate of 1.65 ml/min. Subsequent odorant applications were preceded by extensive washing of the oocyte chamber in ND96 for 10 min at a flow rate of 4.6 ml/min. Oocytes were pierced by micropipettes filled with 3 M KCl and with resistances of 0.2-2.0 MΩ. Under voltage clamp, oocytes were held at a
potential of -70 mV. Current responses were filtered (4-pole, Bessel, low-pass) at 20 Hz (-3 db) and sampled at 100 Hz. Data was captured and stored using OpusXpress 1.1 software (Molecular Devices).

Data analysis

Initial analysis of electrophysiological data was done using Clampfit 9.1 software (Molecular Devices). Relevant current responses were measured by subtracting baseline current from the maximum recorded response during odorant application. Statistical analyses, curve fitting, EC50, and Hill slope calculations were done using Prism 4 (Graphpad, La Jolla, CA). Concentration-response data were fit to the equation: 

$$I = \frac{I_{\text{max}}}{1 + \left(\frac{\text{EC}_{50}}{X}\right)^n}$$

where I represents the current response at a given concentration of odorant, X; $I_{\text{max}}$ is the maximal response; EC50 is the concentration of odorant yielding a half maximal response; and n is the apparent Hill coefficient. For concentration-inhibition curves, data were fit to the equation: 

$$I = \frac{I_{\text{max}}}{1 + \left(\frac{X}{\text{IC}_{50}}\right)^n}$$

where IC50 is the concentration of inhibitor present that still allows a half maximal response from odorant. Statistical significance was assessed using a two-tailed unpaired t test, a one-tailed unpaired t test, a two-tailed paired t test, an F test, or a one-way analysis of variance (ANOVA) followed by the Bonferroni’s post-test or Dunnett’s multiple comparisons post-test, as appropriate.

Utilization of the OpusExpress two reservoir system

The OpusExpress machine is designed so that impaled oocytes are constantly bathed in a solution from one of two sources: a reservoir underneath the recording
chamber or a pipette tip robotically positioned above the recording chamber. Usually, simple protocols assaying receptor function during a single application of compound use the bottom reservoir as a source for the wash buffer (i.e. ND96) and the pipette (1 mL maximum volume) as a source for the compound diluted in the same wash buffer. The bottom reservoir is tapped during baseline recording and in between multiple compound applications as a wash step. The OpusExpress has two bottom reservoir pumps (A and B), allowing extended versatility in protocol design. I took advantage of this versatility for several experiments reported herein. For example, some experiments required the application of various reagents onto an activated receptor. To achieve this, the A reservoir was reserved for ND96 buffer and used traditionally as a wash solution. The B reservoir contained receptor agonist (odorant) diluted in ND96, and the pipette contained the same agonist, at the same concentration as the B reservoir, plus the testable reagent in ND96. An oocyte was challenged with odorant from the B reservoir, activating the receptor until the current response was at steady-state. Immediately following this, the pipette applied its contents. In this way, the receptor was maintained in the exact concentration of agonist, but subjected to the testable agent after a steady-state response is reached. Thus, changes in the current response as result of the testable reagent acting on the activated receptor could be measured in real time. This assay of receptor function was deemed the “new protocol” and proved useful to reveal functional inhibition and potentiation of activated insect ORs. Furthermore, this protocol avoided the issue of receptor desensitization (see below).
Receptor desensitization

Some insect ORs exhibited desensitization during experiments with high concentrations of multiple odorant applications, causing a potential problem in data interpretation. The desensitization was not dependant on the wash time, as extending wash times to >30 min failed to decrease the extent of desensitization. This effect was very consistent within and between oocyte batches. Therefore, when necessary, data could be normalized to account for this effect. Protocols requiring normalization of measurements were performed on the same day and batch of oocytes. Please refer to individual protocols (below) for various procedures on normalization.

Chapter III Experimental protocols

Dose-response analysis of DmOR35a/83b activation by hexanol presented in Figure 3.2 was performed by normalizing all responses to 30 μM odorant. Similarly, measurements of AmOR11/2 activation by 9-oxo-2-decenoic acid presented in Figure 3.13 were normalized to the response elicited by 10 μM odorant.

For the current amplitude comparisons in the presence of various external buffers presented in Figure 3.6, a control response to 30 μM hexanol was first measured in ND96. The ND96 buffer, containing 1 mM Ca$^{2+}$ and 1 mM Mg$^{2+}$, was washed out and replaced with ND96 containing altered concentrations of divalent cations for 10 min. Finally, a second response to 30 μM hexanol was measured. The common desensitization effect of 74.9% response remaining for DmOR35a/83b with 30 μM hexanol (see Figure 3.4) was then used as a normalization factor to calculate adjusted current amplitudes to the second hexanol response after buffer exchange.
For the Ca\textsuperscript{2+} chelation experiments presented in Figure 3.7, each oocyte was injected with DmOR35a (20 ng), DmOR83b (10 ng), muscarinic acetylcholine receptor 1 (20 ng), and G\textsubscript{a15} (10 ng). Pre-treatment responses to both 100 µM acetylcholine and 10 µM hexanol were recorded. Only positive responders to both agonists were removed from the OpusExpress and incubated in special wash buffer (ND96 without Ca\textsuperscript{2+}, but with 1 mM Ba\textsuperscript{2+} added) supplemented with 50 µM BAPTA-AM (in 0.5% DMSO) for 2 hours at room temperature. A control set of positive responders were incubated in special wash buffer supplemented with 0.5% DMSO. Oocytes were again clamped with the OpusExpress and post-treatment responses to 100 µM acetylcholine and 10 µM hexanol were recorded. All recordings were performed in special wash buffer.

The inhibition of DmOR35a/83b by transition metals, presented in Figure 3.9, was performed as follows: A pretreatment response was first determined by 10 µM hexanol application. After a 10 min wash period, oocytes were challenged with 10 µM hexanol plus 1 mM metal (nickel, cadmium, copper, or zinc). Effects of metal application on receptor function are presented as a percentage of the pretreatment response. To account for desensitization, a 10 µM hexanol application followed by 10 min wash was performed before the above procedure. This response was not included in calculations and served to simply remove the first and largest receptor response from consideration (see Figure 3.4).

The transition metal concentration-inhibition analysis, presented in Figure 3.10, also included a first response to 10 µM hexanol that was discarded from analysis. Each receptor response to 10 µM hexanol plus a specific concentration of metal was preceded by a response to 10 µM hexanol alone. This preceding response to hexanol alone was
used to normalize each metal-containing subsequent response to hexanol. All applications were followed by a 10 min wash.

**Chapter IV Experimental protocols**

Concentration-response analysis of DmOR85a/83b presented in Figure 4.9 was performed by normalizing all responses to 1 mM racemic ethyl 3-hydroxybutyrate. Curve fitting was not possible for the (R) ethyl 3-hydroxybutyrate, since a saturable effect was not reached at the highest concentration tested. Therefore, the line drawn for (R) ethyl 3-hydroxybutyrate is only for visualization and comparison.

Concentration-response analysis of DmOR67a/83b presented in Figures 4.14 was performed by normalization of current responses to that elicited by 40 µM methyl benzoate. This response was considered the approximate apparent EC$_{50}$ of methyl benzoate for DmOR67a/83b. In this way, odorants could be assayed for relative efficacy as compared to methyl benzoate.

Measurement of inhibition by ruthenium red presented in Figures 4.22 and 4.23 followed the “new protocol” method (described above). Briefly, application of odorant for 240 sec resulting in steady-state activation was followed by application of ruthenium red plus odorant for 60 sec. Finally, all reagents were washed out in ND96. The extent of inhibition by ruthenium red was considered the current amplitude immediately before washout (300 sec after initial application of odorant) divided by the current amplitude immediately before ruthenium red application (240 sec after initial application of odorant).
Chapter V Experimental protocols

For the MTS susceptibility screens presented in Figures 5.2, 5.3, and 5.4, a pre-MTS receptor response to odorant was measured by taking the average response from two odorant applications. To avoid desensitization, a preliminary application of odorant was performed but not included in the measurements. After a sufficient wash period, MTS reagent was applied at 1 mM for 2 min, followed by a 4 min wash period. Then, post-MTS receptor responses to the same odorant were recorded, with 10 min wash periods between each subsequent odorant application. For DmOR85a/83b screening, a 12 min wash period between post-MTS odorant applications was used. Post-MTS responses are presented as a percentage of the averaged pre-MTS odorant response for each post-treatment time point indicated.

For the partial reversal by dithiothreitol (DTT) of MTSES inhibition presented in Figure 5.5, a pre-treatment response was first determined by application of 100 µM 2-heptanone (HEP). Following a 10 min wash period, oocytes were presented with either ND96 (Sham or DTT alone treatments) or 10 mM MTSES (MTSES alone or MTSES+DTT treatments) for 2 min. After a 10 min wash period, oocytes were presented with either ND96 (Sham or MTSES alone treatments) or 20 mM DTT (DTT alone or MTSES+DTT) for 2 min, followed by a 10 min washout in ND96. Finally, a post-treatment response to 100 µM HEP was measured. Data was analyzed by calculating the response remaining (post-treatment HEP / pre-treatment HEP) and is presented by normalizing this value as a percent of the response remaining after sham treatment. Thus, any desensitization of the receptor was accounted for by normalizing the measurements to the sham treatment.
The MTS susceptibility screens presented in Figure 5.8 utilized the “new protocol”. Briefly, 300 µM 2-heptanone was applied for 240 sec, immediately followed by 2-heptanone plus 10 mM MTSES for 200 sec, followed by a washout in ND96. MTSES effects were calculated by measuring current amplitudes 440 sec after initiation of 2-heptanone application (response in the presence of 2-heptanone and MTSES) and dividing by the response amplitude 240 sec after the initiation of the 2-heptanone application (maximal response to 2-heptanone alone). Refer to Figure 2.1 for an example trace and the time points at which amplitudes were measured. The SCAM screen presented in Figure 5.16 utilized the same analysis, except 100 µM 2-heptanone was the activating concentration. Measurements of inhibition by 2-nonanone (Figure 5.21) were performed similarly, except 2-nonanone was applied for 60 sec in the presence of 2-heptanone, followed by an additional application of 2-heptanone alone for 60 sec before washout in ND96.

Figure 2.1 - Example of an MTS susceptibility protocol and the calculation of percent response remaining. Point A represents the maximal response to HEP alone (at 240 sec). Point B represents the receptor response to HEP and MTSES (at 440 sec). Notice the current responses are at a plateau for points A and B.
Control traces for the MTSES susceptibility screen were generated by measuring current amplitudes of receptors challenged with 300 µM 2-heptanone for 440 sec (no MTSES treatment). Presented in Figure 5.7, the degree of receptor desensitization was calculated by measuring current amplitudes 440 sec after initiation of the 2-heptanone application and dividing by the response amplitude 240 sec after initiation of the 2-heptanone application. These time points are equal to the time points used in the MTSES screens.

The concentration-response curves of DmOR85b cysteine mutant activation by 2-heptanone presented in Figure 5.9 were generated by normalizing current responses to that elicited by 100 µM 2-heptanone. Usually, multiple runs with different oocytes were required, but each experimental run included this 100 µM 2-heptanone application for normalization. Concentration-response analysis of DmOR85b activation by 2-heptanone in the presence of 1 mM 2-nonanone was also performed using a 100 µM 2-heptanone application as a normalizing factor (Figure 5.23). Concentration-response analysis of DmOR85b WT and F142C activation by 2-heptanol was also performed using a 100 µM 2-heptanone application as a normalizing factor (Figure 5.27).

Experiments on the long-term effectiveness of MTSES for the newly identified mutants (shown in Figure 5.17) were performed as follows. First, a pre-treatment response to 100 µM 2-heptanone was measured, followed by application of 10 mM MTSES or ND96 (sham) for 2 min. Finally, a 20 min washout preceded a post-treatment response to 100 µM 2-heptanone. Data was analyzed by first calculating the percent response remaining (post-treatment 2-heptanone / pre-treatment 2-heptanone) for both the sham-treated cells and the MTSES-treated cells. Data is presented by normalizing the
MTSES-treated percent remaining value as a percentage of the sham-treated percent remaining value. This effectively took into account any desensitization from multiple agonist applications.

For protection from MTSES action by 2-nonanone (NONA) presented in Figure 5.24, a pre-treatment response was first determined by application of 100 µM 2-heptanone (HEP). Following a 10 min wash period, oocytes were presented with either ND96 (Sham or MTSES alone treatments) or 6 mM NONA (MTSES+NONA or NONA alone treatments) for 50 sec, immediately followed by application of ND96 (Sham), 1 mM MTSES (MTSES alone), 1 mM MTSES and 6 mM NONA (MTSES+NONA), or 6 mM NONA (NONA alone) for 1 min, followed by a 10 min washout in ND96. Finally, a post-treatment response to 100 µM HEP was measured. Data was analyzed by calculating the response remaining (post-treatment HEP / pre-treatment HEP) and is presented by normalizing this value as a percent of the response remaining after sham treatment. All measurements were performed on the same day with the same batch of oocytes. Thus, any desensitization of the receptor was accounted for by normalizing the measurements to the sham treatment.

For the odorant preference ratio measurements in Figures 5.25 and 5.26, two sets of oocytes were utilized for each DmOR subunit tested. The first set of oocytes was challenged twice with 1 mM 2-heptanol and an average desensitization factor (D) was calculated by: 2-heptanol response #2 / 2-heptanol response #1. The second set of oocytes was challenged first with 1 mM 2-heptanol, then with 1 mM 2-heptanone and a preference ratio (P) was calculated by: 2-heptanone response / 2-heptanol response.
Finally, the normalized preference ratio corrected for desensitization was calculated as:

P/D. All odorant applications were followed by 10 min wash periods in ND96 before subsequent applications. Also, measurements were made on the same day and within the same batch of oocytes for fair comparison.
CHAPTER III:
USE OF THE XENOPUS OOCYTE HETEROLOGOUS EXPRESSION SYSTEM
FOR THE INVESTIGATION OF INSECT OLFACTORY RECEPTORS

Functional expression of a DmOR in Xenopus oocytes

Previous research reported that functional expression of *Bombyx mori* ORs (BmORs) in *Xenopus* oocytes required only the heterologous expression of a specificity subunit (BmOR1 or 3) with the DmOR83b ortholog, BmOR2 (50). Upon specific pheromone applications, these receptors (BmOR1/2, and BmOR3/2) generated an inward current in oocytes under voltage clamp at -80 mV. In addition, DmOR47a/83b expressed in oocytes selectively responded to the odorants 2-heptanone and pentyl acetate. Responses to any ligands required the coexpression of the common receptor, BmOR2 or DmOR83b (50). These encouraging results suggested that this methodology could be used as a general platform for insect OR functional studies. Therefore, I first asked if another DmOR could be functionally expressed in *Xenopus* oocytes.

35 ng of cRNA encoding a specificity subunit, DmOR35a, and 5 ng of the common subunit, DmOR83b, were injected into oocytes and allowed to express for 3 days. Oocytes were then held under two-electrode voltage clamp at -70 mV. Application of 30 µM hexanol, a known agonist based on *in vivo* work (1), resulted in robust inward currents which decayed immediately upon washout of the odorant (Figure 3.1, center). As expected, responses to hexanol required the presence of both DmOR subunits, as expression of only DmOR35a (Figure 3.1, left) or DmOR83b (Figure 3.1, right) failed to elicit a response. During a hexanol application of 20 sec the response approached a
plateau, so I used this application time for subsequent experiments. I conclude that co-injection of DmOR35a and DmOR83b in Xenopus oocytes results in expression of a functional odorant receptor.

Next, I asked if the expressed receptor (DmOR35a/83b) responds to odorant in a sufficiently sensitive, saturable, and concentration-dependent manner by performing concentration-response analysis. Indeed, DmOR35a/83b, when subjected to a range of hexanol concentrations, responded with concentration-dependence and could be maximally activated (Figure 3.2, left). Plotting the concentration-response relationship indicated that DmOR35a/83b is a sensitive receptor for hexanol, with an EC$_{50}$ of 674 ± 68 nM (n=3) (Figure 3.2, right). Therefore, heterologous expression in oocytes can be used to measure the sensitivity of this DmOR to odorants and may be extended to other insect ORs. Based on this preliminary data, I conclude that Xenopus oocytes are a suitable platform for insect OR studies in this project.
Optimization of DmOR35a/83b expression

Consistent and accurate recording of receptor responses in *Xenopus* oocytes usually requires the optimization of assay parameters. This is especially important when a new receptor class is being investigated. So, basic optimizations were carried out using DmOR35a/83b. Maximum current amplitudes measured in early experiments (such as the trace presented in Figure 3.1) only averaged several hundred nanoamperes (nA). These responses are fairly modest compared to those recorded in this laboratory from neuronal nicotinic receptors (64) and mammalian ORs (65). Therefore, I first attempted to optimize cRNA ratios and asked if current amplitudes could be improved. Regardless of receptor type, total cRNA injected per oocyte was generally limited to about 50-60 ng, as greater amounts result in quick oocyte death. Relative response amplitudes to a sub-maximal concentration of hexanol (1 μM) were measured in oocytes injected with 30 ng of DmOR35a and various amounts of DmOR83b (5, 15, 30 ng). The largest current amplitudes observed in oocytes (30 ng of DmOR35a and 15 ng of DmOR83b) were still limited to several hundred nA, but were significantly larger compared to oocytes injected
with 5 ng (p<0.001, one-way ANOVA) or 30 ng (p<0.05, one-way ANOVA) of DmOR83b (Figure 3.3). Based on this data, I chose to inject 30 ng of a specificity subunit and 15 ng of OR83b per oocyte. With these cRNA totals, I found receptor expression can be measured 2 days after injection and is maintained throughout the life of the oocyte. It should be noted that individual subunit cRNAs may have widely different translation efficiencies due to varying transcript character or secondary structures. Because of this, insights into receptor complex stoichiometry cannot be inferred from these optimized cRNA ratios.

Since experiments would require analysis of receptor function after multiple odorant applications, I next investigated DmOR35a/83b desensitization upon repeated odorant exposure. Oocytes were challenged with 4 applications of hexanol, with 10 min washouts in between applications. This wash time was sufficient to return current responses to a pre-application baseline and was subsequently used for all experiments.
Responses were then calculated as a percentage of the first response (Figure 3.4). Using a saturating concentration of hexanol (30 μM) (see Figure 3.2), significant desensitization was apparent between the 1st and 2nd applications (74.9 ± 2.1% response remaining, p<0.001, one-way ANOVA) (Figure 3.4, left). This effect was maintained through the recording timeframe for the 3rd and 4th odorant applications (67.0 ± 3.2 and 66.5 ± 3.7 % response remaining, respectively). But, comparing the 2nd through 4th responses, no further desensitization was observed (p>0.05, one-way ANOVA), indicating stable and persistent responses to repeated applications of odorant after the 1st response. Using a sub-saturating concentration of hexanol (1 μM) (see Figure 3.2), no significant desensitization was observed (p=0.13, one-way ANOVA) (Figure 3.4, right), suggesting desensitization effects can be minimized when odorant concentrations are sub-saturating. Therefore, protocols were adjusted when necessary to account for potential desensitization of current responses (see individual protocols).

Figure 3.4 - Desensitization of the DmOR35a/83b response to agonist. DmOR35a/83b expressing oocytes were challenged with 4 applications of 30 μM hexanol (Left) or 1 μM hexanol (Right). Odorant applications were followed by 10 minute washes in ND96 (4.6 ml/min). Data are presented as the percent of the 1st response to hexanol (bars are means ± sem, n=5 (left), n=10 (right)). Statistical significance was assessed by one-way ANOVA and Bonferroni’s post test, two asterisks: p<0.001.
Validation of accurate heterologous expression

Does heterologous expression of a receptor accurately recapitulate its function and behavior observed \textit{in vivo}? This is a question that must be repeatedly answered when utilizing any heterologous expression system. Fortunately, \textit{in vivo} DmOR activation patterns in response to a diverse odorant panel have been published, offering a source for comparison (1). This study presents receptor activation as an increase in neuronal spikes per second after odor puff application. The degree of response is binned into four categories of increasing spike rate (+ through +++++, please refer to Table 3.1 legend for further description). Admittedly, this generates limited information on receptor sensitivity, but can be used to broadly compare the activation patterns to oocyte data. All my previous work on DmOR35a/83b used hexanol, a strong activator according to the \textit{in vivo} study. Other alcohols were also reported to activate DmOR35a/83b with varied effectiveness (1). Therefore, I first asked if DmOR35a/83b, expressed in oocytes, could be differentially activated when challenged with a panel of alcohols ranging from four to nine carbons in chain length. Indeed, DmOR35a/83b was variably activated when subjected to the primary alcohols at 30 $\mu$M, a saturating concentration of hexanol (Figure 3.5, \textit{left}). I next compared response amplitudes by normalization to the hexanol response. DmOR35a/83b preferred hexanol over the other alcohols tested and preference diminished as carbon length increased or decreased beyond this “ideal” six carbon chain length (Figure 3.5, \textit{right}). This data backs up previous suggestions that insect ORs are tuned to various ligands, but can be activated to varying degrees by closely related structures (1,56).
I further expanded the known activation pattern of DmOR35a/83b in oocytes by screening a subset of the odorants used in the *in vivo* study by Hallem *et al.* (1). All odorants were screened at 30 μM and each experiment included a 30 μM hexanol application. Receptor activation was calculated as a percentage of this normalizing response to hexanol. This normalized activation profile was then compared to the Hallem *et al.* data (Table 3.1). As touched upon above, direct comparisons between the two methodologies is impossible. Potentially large differences in receptor sensitivity to specific odorants are lost using a binning strategy to report activation strength. Also, Hallem *et al.* used a 1/100 dilution of pure odorant as a source for puff application. But, the exact concentration of odorant observed by the receptor in a sensillum is affected by many complicating factors, including the presence of OBPs and differences in odorant volatility. Still, broad similarities are evident by this comparison, including the similar activation patterns elicited by the primary alcohols, hexanal, benzaldehyde, and amyl acetate (Table 3.1). Importantly, the heterologously expressed receptor was never appreciably activated when challenged with odorants reported to be inactive by Hallem *et al.*
Therefore, I suggest false positives in receptor screening are unlikely. This data provides evidence that insect ORs heterologously expressed in oocytes maintain proper ligand specificity.

<table>
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<tr>
<th>Odorant</th>
<th>in vivo expression</th>
<th>heterologous expression</th>
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<tr>
<td></td>
<td>(Hallem et al. 2006)</td>
<td>(Xenopus oocytes)</td>
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<tr>
<td></td>
<td>strength of response</td>
<td>% of response to hexanol</td>
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</tr>
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<tr>
<td>amyl acetate</td>
<td>+++</td>
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</tr>
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</table>

Table 3.1 - Accuracy of DmOR35a/83b heterologous expression. In vivo data is reproduced for comparison from Hallem et al. (1). In that study, neuron responses to individual odorants (10⁻² dilutions) were binned into categories based on degree of sensitivity, measured as spikes per second. Categories are: (•) less than 50 spikes/s, (+) 50-100 spikes/s, (++) 100-150 spikes/s, (+++) 150-200 spikes/s, (++++) over 200 spikes/s. Heterologous expression data was obtained by measuring current responses of DmOR35a/83b oocytes to 30 µM odorant applications. Data are presented as percent of the response elicited by 30 µM hexanol (means ± sem, n ≥ 6 for each odorant tested). Odorants of various chemical classes are color coded (blue, carboxylic acids; red, primary alcohols; green, aldehydes; black, other classes including a ketone, terpenes, an aldehyde, and esters).
Investigating the odorant-induced current in OR-expressing oocytes

Odorants activate a cation current in insect OR-expressing oocytes (49,50) and significant evidence exists that insect ORs are ligand-gated cation channels (45,49). Receptor responses of a variety of insect ORs show a slight outward rectification and are generally non-selective for cations (49,50). Therefore, I sought to investigate the nature of measurable OR activity in oocytes. I first asked what contributions divalent cations have on DmOR35a/83b function. Also, altering the divalent cation concentrations in the external wash buffer may have provided a way to increase current amplitudes in oocytes for future studies. I compared current amplitudes after application of 30 µM hexanol in DmOR35a/83b oocytes in the presence of various external concentrations of the divalent cations Ca\(^{2+}\), Mg\(^{2+}\), and Ba\(^{2+}\). After normalization that accounted for desensitization between subsequent 30 µM hexanol applications (see Methods), no difference in current amplitudes was observed (Figure 3.6).

![Figure 3.6 - Alteration of external divalent cations does not affect DmOR35a/83b current amplitudes](image)

DmOR35a/83b responses elicited by 30 µM hexanol were measured in the presence of various external concentrations of the divalent cations (barium\(^{2+}\), Ba; calcium\(^{2+}\), Ca; magnesium\(^{2+}\), Mg) (in mM: 1 Mg + 1 Ca, 1 Ba, 1 Mg + 10 Ca, 10 Mg + 1 Ca). Data are presented as a percent of the response elicited by 30 µM hexanol in ND96 buffer (bars are mean ± sem, n=4). Values were normalized to account for desensitization rates after subsequent 30 µM hexanol applications (see Figure 3.4 and Methods). Statistical significance was assessed by one-way ANOVA and Bonferroni’s post-test, ns: p > 0.05.
These results suggest none of these specific divalent cations are absolutely required for channel function and contributes to the idea that insect ORs flux a non-selective cation current. Furthermore, these divalents are not the major current carrier, since amplitudes were equal in the presence of 1 (1 Ba), 2 (ND96), or 11 (1 Mg/10 Ca, 10 Mg/1 Ca) mM total divalent cation concentration. Oocytes require the presence of divalents cations in the external bath for proper function, so I could not compare currents in a buffer lacking these ions.

I also wished to explore whether internal Ca\(^{2+}\) stores of the oocyte contributed toward odorant-activated currents. Ca\(^{2+}\) mobilization in insect OSNs may contribute to modulatory action of ORs (see Figure 1.3), which may require other intracellular components not present in *Xenopus* oocytes. However, internal Ca\(^{2+}\) release in oocytes can trigger activation of the endogenous Ca\(^{2+}\)-activated Cl\(^{-}\) channel, muddling interpretations of insect OR-specific function and the contributions of Ca\(^{2+}\). To investigate, I examined the effect of internal oocyte Ca\(^{2+}\) chelation with BAPTA-AM on DmOR35a/83b function. As an internal control for BAPTA-AM efficacy, the same oocytes were also injected with the muscarinic acetylcholine receptor subtype 1 (mAchR1), and G\(_{\alpha15}\). The mAchR1 couples to G\(_{\alpha15}\) and, upon activation with acetylcholine, triggers G protein dissociation and phospholipase C (PLC)-mediated Ca\(^{2+}\) release from internal stores. This Ca\(^{2+}\) increase leads to measurable activation of the endogenous Ca\(^{2+}\)-activated Cl\(^{-}\) channel, which can be inhibited by Ca\(^{2+}\) chelation. Treatment of oocytes with BAPTA-AM for 2 hours significantly diminished mAchR1 responses to 100 nM acetylcholine (14.2 ± 2.3% response remaining, n=6, p=0.001, unpaired t-test). However, this treatment failed to significantly diminish DmOR35a/83b
responses to hexanol (76.8 ± 7.6% response remaining, n=6, p=0.098, unpaired t-test).
The insignificant diminishment of the hexanol response may even be a result of receptor
desensitization. Testing the effects of Ca\textsuperscript{2+} chelation on two receptors in the same oocyte
provided definitive evidence that internal calcium levels had been chelated in each oocyte
measured. It should be noted that recorded currents from insect OR or mAchR
activation look identical. Importantly, each receptor ligand (acetylcholine and hexanol)
cannot activate the other’s cognate receptor. Therefore, currents observed after
acetylcholine or hexanol applications were due to the sole activation of DmOR35a/83b or
mAchR1, respectively. I conclude that large-scale release of Ca\textsuperscript{2+} from internal stores is
not required for DmOR35a/83b function, and the currents observed after application of
odorant are from the insect OR itself and not the Ca\textsuperscript{2+}-activated Cl\textsuperscript{-} channel of the oocyte.

![Figure 3.7 - Internal Ca\textsuperscript{2+} stores are not required for DmOR35a/83b function. Oocytes expressing DmOR35a, DmOR83b, muscarinic AchR1 (mAchR1) and G\textalpha_{15} were measured for responses to 100 nM acetylcholine and 3 \muM hexanol (corresponding to activation of mAchR1 and DmOR35a/83b, respectively) both before and after treatment with 50 \muM BAPTA-AM for 2 hrs. Data are presented as means ± sem (n=6). Statistical significance was assessed by unpaired t-test, ns: not significant; double asterisk: p = 0.001.](image-url)
Reports have provided conflicting evidence for a role of G_α_{15} in insect OR function expressed in *Xenopus* oocytes (50,54). While the results above suggest G_α_{15} activation and calcium release are not required for DmOR35a/83b functional responses to odorant, I wanted to explore the effect of G_α_{15} on DmOR35a/83b without the mAchR1 receptor present. Current responses elicited from a sub-saturating concentration of hexanol (1 µM) were measured in DmOR35a/83b oocytes with and without G_α_{15} (Figure 3.8). To minimize cell-to-cell variability, all measurements were performed on the same day and cRNA amounts of DmOR subunits were equal in both oocyte groups. No significant difference in averaged current amplitudes was observed between the two cell groups (p > 0.05, unpaired t-test).

![Figure 3.8 - Expression of G_α_{15} in oocytes does not affect DmOR35a/83b function.](image)

While not significant, the oocytes injected with G_α_{15} trended with lower current amplitudes. This may be a result of decreased DmOR35a and DmOR83b expression due to the presence of additional cRNAs and a higher load on oocyte transcriptional machinery. These results indicate G_α_{15} function does not contribute directly to odorant-induced DmOR35a/83b activation in oocytes. It should be noted that the G protein may...
be “silently” associating with the receptor complex and failing to induce functional effects. If so, *Xenopus* oocytes may simply lack the necessary effector components for $G_{\alpha15}$-mediated signal transduction.

Metal ions, and especially transition metals, can affect a variety of ligand-gated ion channels by blocking ion flux or binding to allosteric sites and modulating function (66,67). Since insect ORs are a newly identified family of ligand-gated ion channel, I looked at the modulation of DmOR35a/83b function by various transition metals.

DmOR35a/83b activation by 10 µM hexanol was measured with and without the addition of 1 mM nickel chloride, cadmium chloride, copper chloride, or zinc chloride (Figure 3.9). Each metal perturbed the receptor’s ability to be activated by hexanol, with copper as the strongest inhibitor of receptor function (58.4 ± 1.6 % response remaining, n=6). Concentration-inhibition analysis was used to calculate the apparent IC$_{50}$ values for each metal (Figure 3.10). The transition metals capable of DmOR35a/83b inhibition may be used as tools in the identification of residues involved in channel structure and gating.

![Figure 3.9 - Inhibition of DmOR35a/83b by transition metals. Current responses to 10 µM hexanol and 10 µM hexanol + 1 mM nickel chloride, cadmium chloride, copper chloride or zinc chloride were measured in oocytes expressing DmOR35a/83b. Data are presented as a percentage of the response to hexanol alone (bars are means ± sem, n = 6 for each).](image)
Deorphanization of ORs using the oocyte system

All OR functional recordings presented thus far are primarily responses to known odorants, based on the Hallem in vivo data (1). Can the Xenopus expression system be used to identify ORs for particular ligands? A collaboration was formed with Kevin Wanner and Hugh Robertson (University of Illinois, Urbana-Champaign), aimed to identify honeybee (Apis mellifera) ORs that respond to pheromones.

Queen bees release a blend of pheromones that help maintain dominance in a colony and trigger various honeybee behaviors (68). One such behavior is the long distance recruitment of male drones during mating flights, presumed to be mediated by pheromone release (69). We sought to identify specific receptors responsible for this pheromone detection by heterologous expression and screening of receptor candidates in our assay system. The Urbana-Champaign group utilized custom microarrays and, by screening for sex-biased OR expression in honeybee antenna, identified candidate sex-
specific pheromone receptors. We reasoned that four male-biased ORs may detect particular components of the queen retinue pheromone blend (QRP), which includes the pheromone responsible for controlling drone mating behaviors (70). Therefore, I expressed the four candidate ORs (AmOR10, 11, 18, and 170) with the OR83b honeybee ortholog, AmOR2, in Xenopus oocytes and screened for responsiveness to the four major components of QRP (Figure 3.11). AmOR11/2 responded strongly to the major QRP component, 9-oxo-2-decenoic acid (9-ODA). While this receptor was also weakly activated by 9-hydroxy-2-decenoic acid (9-HDA), contamination from 9-ODA is possible, since it is used to synthesize 9-HDA (51).

Figure 3.11 - 9-ODA activates AmOR11 + AmOR2. Oocytes injected with cRNA encoding AmOR10 + AmOR2, AmOR11 + AmOR2, AmOR18 + AmOR2, or AmOR170 + AmOR2 are challenged with 100 μM each of: methyl p-hydroxybenzoate (HOB), 9-oxo-2-decenoic acid (9-ODA), 4-hydroxy-3-methyoxyphenylethanol (HVA), 9-hydroxy-2-decenoic acid (9-HDA), and a blend of the four components (queen mandibular pheromone, or QMP) prepared such that the concentration of 9-ODA is ≈100 μM. Pheromone applications (20 sec, indicated by arrowheads) are followed by 10 min washing in ND96.
AmOR11 required the coexpression of AmOR2 for function, and AmOR2 expressed alone did not respond to any pheromones (Figure 3.12). This result extends the hypothesis that the role of OR83b subunits in insect olfaction is highly conserved, serving as a functional partner in all OR complexes. Interestingly, the ability of these OR complexes to detect only the honeybee pheromone differs from Drosophila pheromone detection system, in which the OBP LUSH is required for detection of the pheromone, cis-vaccenyl acetate (13).

I next characterized the activation of AmOR11/2 by 9-ODA. Concentration-response analysis revealed a highly sensitive receptor, with an apparent EC$_{50}$ of 280 ± 31 nM (Figure 3.13). This sensitivity to pheromone is similar to the reported responsiveness of the Bombyx mori pheromone receptors, BmOR1/2 and BmOR3/2, to bombykol and bombykal (50).
Insect ORs that respond to pheromones are hypothesized to be extremely narrowly tuned, which is in contrast to the other ORs responsible for general odor detection. This receptor characteristic would allow pheromones to trigger specific behaviors even in the presence of a blend of molecules with similar structures. Furthermore, highly specific pheromone receptors would allow tight control of behaviors linked to pheromone receptor activation. I further tested the specificity of AmOR11/2 by screening for responsiveness to four additional minor QRP components (methyl oleate, linolenic acid, coniferyl alcohol, and 1-hexadecanol), floral odorants (linalool and hexanol), and social pheromones (geraniol and citral) (Figure 3.14). The floral odorants and social pheromones have previously been shown to activate specific glomeruli in the honeybee antennal lobe, but not suspected pheromone receptor-innervated macroglomeruli (71). As expected for a highly specific pheromone receptor, AmOR11/2 did not respond to any of these additional compounds.
This investigation successfully identified AmOR11/2 as a receptor for 9-ODA, the major component to QRP, and a pheromone capable of attracting worker bees to the queen, physiologically inhibiting worker ovary development, and attracting male drones during mating (68). Furthermore, the broad utility of the Xenopus oocyte expression system to screen new insect ORs for ligand responsiveness is clearly demonstrated.
CHAPTER IV:  
THE CHARACTERIZATION AND ANALYSIS OF SUBUNIT CONTRIBUTIONS  
TO THE FUNCTION OF DROSOPHILA ODORANT RECEPTORS

Functional expression of a panel of DmORs

This project required the cloning, functional expression, and survey of a panel of DmORs in addition to DmOR35a/83b. Using the empty neuron approach, Hallem et al. screened the 32 receptors shown to be expressed in fly antenna by in situ hybridizations (20) against a panel of 100 diverse odorants. 24 receptors responded to at least one odorant (1) and I decided to clone eight of these 24 receptors (DmOR9a, 22a, 43b, 67a, 85a, 85b, and 98a). The in vivo results indicated the eight receptors respond to a reasonably diverse panel of odorant molecules. Also, many of the receptors clustered together in a phylogenetic relationship tree based on amino acid sequence (Figure 4.1). I reasoned that receptors with closely related sequence, but diverse ligand activity profiles, may be useful candidates for studies on the structural basis of OR ligand specificity.

Figure 4.1 - Phylogenetic tree (MacVector) depicting the relative similarities based on amino acid sequence of all DmORs with known odorant ligands (1). Red boxes highlight the receptors selected for cloning. Node values indicate the frequency of bootstrap proportion. DmOR83b (Dm83b) roots the tree.
Each of the eight newly cloned receptors were expressed in *Xenopus* oocytes, along with DmOR83b, and screened for responsiveness to known odorants. Six receptors were functionally activated by various odors (Figure 4.2). This represents the first reported heterologous expression of five receptors (DmOR59b, 67a, 85a, 9a, 85b). DmOR43b was expressed in oocytes previously (54). However, DmOR43b was coexpressed with $G_{\alpha 15}$ and not DmOR83b. Strangely, currents declined in the continued presence of odorant (54). This is a direct contradiction to my observations of DmOR43b activation, which persists for the duration of odorant application (see Figure 4.23). To this end, the data presented here is the first report of DmOR43b heterologously and functionally expressed with only DmOR83b, and not $G_{\alpha 15}$. I was unable to record functional responses from DmOR22a or DmOR98a in oocytes. These receptors may be unable to properly fold in tertiary structure, associate with OR83b, efficiently translate, or traffic to the oocyte plasma membrane.

I also further investigated the role of DmOR83b on the function of two newly cloned receptors (DmOR85a and DmOR85b). Only coexpression of both the specificity subunit and DmOR83b allowed functional responses to odorant, further confirming the OR83b subunit’s central role in receptor activation (Figure 4.3).

**Validation of accurate heterologous expression**

Three newly cloned receptors (DmOR67a/83b, 85a/83b, and 85b/83b) were screened with a panel of odorants to generate ligand activity profiles and for comparison to reported receptor specificities (1). Each receptor was challenged with both activating odorants and those expected to yield no responses. For comparisons, receptor activities
were normalized to a particular odorant response (highlighted in each summary table below). The specificity of DmOR67a/83b, DmOR85b/83b, and DmOR85a/83b is presented in Figures 4.4, 4.5, and 4.6, respectively. For all three cases, heterologously expressed receptors did not respond to any odorants listed as inactive \textit{in vivo} and most odorants expected to activate the receptors did so (1).

Figure 4.2 - Newly cloned DmORs are functional in the oocyte assay system. Each DmOR was coexpressed with DmOR83b and screened for activity against known odorants based on available \textit{in vivo} data (Hallem et al. 2006). Oocytes were challenged with 20 sec applications (arrowheads) of various odorants (at 1 mM): methyl acetate (MA), methyl benzoate (MB), ethyl benzoate (EB), ethyl 3-hydroxybutyrate (E3HB), (S)-ethyl 3-hydroxybutyrate ((S) E3HB), 2-heptanone (HEP).
Figure 4.3 - DmOR function requires coexpression of DmOR83b. (Top) Oocytes injected with DmOR85b and DmOR83b subunits are challenged with 20 sec applications (arrowheads) of 1 mM 2-heptanone (HEP). (Bottom) Oocytes injected with DmOR85a and DmOR83b subunits are challenged with 20 sec applications (arrowheads) of ethyl 3-hydroxybutyrate (E3HB).
**Figure 4.4** - Accuracy of DmOR67a/83b heterologous expression. *(Top trace)* An oocyte injected with DmOR67a/83b is challenged with 20 sec applications (arrowheads) of various odorants (at 1 mM): methyl benzoate (MB), hexanal (HEXA), ethyl benzoate (EB), heptanoic acid (HA), benzaldehyde (BENZ). *(Bottom table)* Comparison of receptor responses in oocytes (data are means ± sem, n=4) and *in vivo* data from Hallem et al. (2006). *In vivo* data analysis is the same as described in Table 3.1.

<table>
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<tr>
<th>Odorant</th>
<th><em>in vivo</em> expression (Hallem et al. 2006) strength of response</th>
<th>heterologous expression <em>(Xenopus oocytes)</em> % of response to methyl benzoate</th>
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<td>benzaldehyde</td>
<td>++++</td>
<td>1.2 ± 0.9</td>
</tr>
</tbody>
</table>
Odorant

<table>
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<tr>
<th></th>
<th>in vivo expression (Hallem et al. 2006) strength of response</th>
<th>heterologous expression (Xenopus oocytes) % of response to 2-heptanone</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-heptanone</td>
<td>+++</td>
<td>100</td>
</tr>
<tr>
<td>ethyl octanoate</td>
<td>•</td>
<td>2.2 ± 1.5</td>
</tr>
<tr>
<td>amyl acetate</td>
<td>+++</td>
<td>17.4 ± 2.8</td>
</tr>
<tr>
<td>methyl acetate</td>
<td>•</td>
<td>0.3 ± 0.2</td>
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<tr>
<td>butyl acetate</td>
<td>+++</td>
<td>38.9 ± 10.4</td>
</tr>
</tbody>
</table>

Figure 4.5 - Accuracy of DmOR85b/83b heterologous expression. (Top trace) An oocyte injected with DmOR85b/83b is challenged with 20 sec applications (arrowheads) of various odorants: 1 mM 2-heptanone (HEP), 3 mM ethyl octanoate (EO), 1 mM amyl acetate (AA), 3 mM methyl acetate (MA), 1 mM butyl acetate (BA). (Bottom table) Comparison of receptor responses in oocytes (data are means ± sem, n=9) and in vivo data from Hallem et al. (2006). In vivo data analysis is the same as described in Table 3.1.
Figure 4.6 - Accuracy of DmOR85a/83b heterologous expression. (Top trace) An oocyte injected with DmOR85a/83b is challenged with 20 sec applications (arrowheads) of various odorants (at 3 mM): hexanol (HEX), amyl acetate (AA), ethyl butyrate (EB), benzaldehyde (BENZ), ethyl 3-hydroxybutyrate (E3HB). (Bottom table) Comparison of receptor responses in oocytes (data are means ± sem, n=7) and in vivo data from Hallem et al. (2006). In vivo data analysis is the same as described in Table 3.1.

<table>
<thead>
<tr>
<th>Odorant</th>
<th>in vivo expression (Hallem et al. 2006) strength of response</th>
<th>heterologous expression (Xenopus oocytes) % of response to ethyl 3-hydroxybutyrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>ethyl 3-hydroxybutyrate</td>
<td>++++</td>
<td>100</td>
</tr>
<tr>
<td>hexanol</td>
<td>++</td>
<td>2.6 ± 2.4</td>
</tr>
<tr>
<td>amyl acetate</td>
<td>•</td>
<td>-0.6 ± 0.4</td>
</tr>
<tr>
<td>ethyl butyrate</td>
<td>++</td>
<td>20.5 ± 1.7</td>
</tr>
<tr>
<td>benzaldehyde</td>
<td>•</td>
<td>0 ± 0.1</td>
</tr>
</tbody>
</table>

DmOR85a/83b

HEX  AA  EB  BENZ  E3HB

50 nA  5 min
One exception to the overall accurate recapitulation of receptor specificity is the failure of DmOR67a/83b to respond to benzaldehyde (Figure 4.4). The sensitivity to benzaldehyde may require OBPs present in sensilla, or activation may be masked by the strong response and slow recovery of the receptor after ethyl benzoate (EB) treatment. While lack of activation from an expected agonist is problematic, greater complications in data interpretation would occur if heterologously expressed receptors gained sensitivity to odorants expected to be inactive. In general, this data suggests these heterologously expressed receptors maintain proper ligand specificity, and complements the same assertion made for DmOR35a/83b (Table 3.1). It should be pointed out, again, that direct comparisons between the in vivo method and the oocyte expression system are unreasonable and only comparisons of trending can be reported.

**Characterization of newly cloned DmORs**

The successful cloning and heterologous expression of a new set of DmORs allowed closer investigation of OR activation and specificity. Here, I report unique features of three individual DmORs, providing new advances in our understanding of DmOR specificity and function.

**DmOR85a distinguishes ligand enantiomers with high selectivity**

Combinatorial coding, in which odorants activate multiple receptors and receptors can be activated by multiple ligands, provides a mechanism by which a relatively small number of insect ORs can discriminate among odors within a vast molecular space. However, individual ORs capable of distinguishing among closely related structures,
such as enantiomers, may also be required for odor discrimination in complex mixtures. At present, the degree of selectivity for enantiomers is largely unknown for DmORs. Ethyl 3-hydroxybutyrate (E 3-HB) is an odorant with a chiral center and, thus, exists as two enantiomers (Figure 4.7). The racemic blend of ethyl 3-hydroxybutyrate strongly activates DmOR85a/83b (Figure 4.6), but the extent of enantioselectivity of this receptor has never been examined. I sought to investigate possible enantioselectivity.

![Figure 4.7 - The enantiomers of ethyl 3-hydroxybutyrate (E 3-HB). The green box indicates the chiral center of E 3-HB.](image)

Surprisingly, DmOR85a/83b is activated by 1 mM of the (S) enantiomer, but not 1 mM of the (R) enantiomer (Figure 4.8). Importantly, receptor responsiveness is maintained after application of (R) E 3-HB, indicating the receptor is still capable of being activated and does not undergo strong desensitization or inhibition that may skew results. Furthermore, enantiomers have the same physiochemical properties, such as solubility and volatility, ensuring the oocytes are being subjected to equal concentrations of the test odorants. I conclude that, even at high concentrations of odorant, DmOR85a/83b is quite enantioselective for E 3-HB.
I next performed concentration-response analysis on the activation of DmOR85a/83b by each enantiomer (Figure 4.9). The analysis shows DmOR85a/83b is a strikingly selective receptor for (S) E 3-HB, exhibiting an apparent EC$_{50}$ of 58 ± 10 µM. Furthermore, this EC$_{50}$ value is half of that elicited by the racemic mixture (racemic E 3-HB EC$_{50}$ = 104 ± 18 µM). Since the (R) E 3-HB is essentially inactive, this suggests that the racemic blend is approximately a 1:1 ratio. It should be noted that slight activation of the receptor by 10 mM (R) E 3-HB may be due to contamination with the (S) enantiomer.

Odorants activate multiple receptors in insect olfaction, and E 3-HB is no exception (1). So, are other receptors for E 3-HB enantioselective as well? Is the (R) enantiomer simply unable to activate other DmORs? I investigated the relative responsiveness of DmOR35a/83b, DmOR85b/83b, and DmOR9a/83b toward each of the E 3-HB enantiomers. These receptors are slightly responsive to E 3-HB, so a high concentration (3 mM) of odorant was required.
All three receptors were activated by both enantiomers of E 3-HB (Figure 4.10). DmOR35a/83b slightly preferred (R) E 3-HB approximately 3-fold over (S) E 3-HB (p=0.008, paired t-test, n=5). DmOR85b/83b preferred the (S) enantiomer approximately 1.5-fold over the (R) enantiomer (p=0.02, paired t-test, n=5) and DmOR9a/83b exhibited no statistically significant preference between the two odors (Figure 4.10). Admittedly, response amplitudes from DmOR9a/83b were quite small, making data analysis and accurate interpretation difficult. DmOR9a may be a poor receptor for E 3-HB, or might not robustly express in oocytes. In summary, while there is only slight enantioselectivity by DmOR35a/83b and DmOR85b/83b, DmOR85a/83b exhibits >3000 fold selectivity for the isomers of E 3-HB (Figure 4.9). This extraordinary stereoselectivity of DmOR85a/83b for (S) E-HB suggests the receptor has undergone positive selection to differentiate between the two enantiomers.
Why might *Drosophila* require such specific capabilities to discern the enantiomers of E 3-HB? Many volatile odorants detected by *Drosophila* are naturally occurring in various fruits and serve as attractants (72), driving flies to their natural food.
source: the yeast colonies growing on ripening and rotting fruit. Interestingly, a
*Saccharomyces cerevisiae* metabolic pathway can convert the passion fruit odorant ethyl
acetoacetate to (S) E 3-HB with high specificity (73). If ethyl acetoacetate, released by
ripening fruit, is converted by yeast to (S) E 3-HB, detection of the (S) enantiomer by
DmOR85a may serve as a marker for yeast-rich fruit sources. Since DmOR85a is only
activated by (S) E 3-HB, the firing of the DmOR85a-expressing neuron may serve as an
indicator for heavy yeast growth. E 3-HB naturally occurs in grapes, mango, and passion
fruit (www.thegoodscentscompany.com). Therefore, activation of other DmORs
responsive to both enantiomers, such as DmORs 35a, 85b, and 9a, might serve as general
detectors for these fruits. I cannot rule out the possibility that other DmORs not
examined here may also detect (R) E 3-HB. This hypothesis requires experimental
investigation on the effects of DmOR85a ablation on *Drosophila* behavior and food
acquisition.

**Rational identification of new ligands for DmOR67a**

Unlike other DmORs cloned for this project, DmOR67a responds to odorants with
cyclic structures (1). Therefore, I wanted to further investigate the specificity of
DmOR67a for aromatic odorants. Since insect ORs tend to be activated by odorants
sharing common features, I reasoned that new ligands may be identified by screening a
collection of compounds structurally related to known activators, such as methyl
benzoate and ethyl benzoate. A representative panel of such structurally related
compounds is presented in Figure 4.11.
I screened oocytes expressing DmOR67a/83b for responsiveness to a panel of 24 odorants (Figure 4.12). Odorants were tested at 1 mM, and activation of the receptor is presented as a percentage of the maximum response elicited by methyl benzoate. The odorant screen identified several new ligands for DmOR67a, including phenyl acetic acid, methyl phenyl acetate, 2-coumaranone, and amyl acetate. Many additional compounds were able to marginally activate DmOR67a. This finding provides evidence that DmOR67a/83b is indeed activated by many different ligands and these ligands probably share structural characteristics. Several odorants, including heliotropyl acetone, inhibited currents through a receptor-specific mechanism (Figure 4.12 and 4.13). This suggests some odorants may be receptor antagonists. It should be noted that Hallem et al.
reported widespread inhibition of OSN basal firing activity in response to various odorants (1). This suggests specific odorants might act as inverse agonists, or compounds capable of driving a receptor’s spontaneous activity to a truly inactive state. The potential antagonism witnessed for DmOR67a/83b may be an example of such inverse agonism. However, I cannot rule out the possibility that these odorants are antagonists blocking residual activity of the receptor after application of a strong agonist (see Figure 4.13). In the oocyte system, basal activity might be even be dependent on a previous event of receptor activation. Unfortunately, this leads to complications in interpreting the difference between inverse agonism of basal activity and antagonism of residual receptor activity.

![Chart showing the effect of various aromatic compounds on DmOR67a/83b activation. Each odorant was tested at 1 mM and data is presented as a percent of the maximum response elicited by methyl benzoate. Bars represent means ± sem (n ≥ 3 for each odorant tested). Green bars represent compounds exerting potential antagonism or inverse agonism.](image-url)
Dose-response analysis on select DmOR67a activators allowed calculation of apparent EC$_{50}$s and relative efficacies, using methyl benzoate as a reference for normalization (Figure 4.14). Comparing values indicates a range of both potency and efficacy. For instance, while methyl phenyl acetate and phenyl acetic acid are similarly efficacious compared to methyl benzoate (at 1 mM for each), phenyl acetic acid is much less potent as exhibited by a right-shifted dose-response curve (EC$_{50}$s in µM: methyl benzoate, 44.1 ± 7.0; methyl phenyl acetate, 20.6 ± 3.4; phenyl acetic acid, 1070 ± 182.9). Ethyl benzoate is the most efficacious and potent odorant tested. Interestingly, while amyl acetate exhibits strong potency similar to ethyl benzoate (EC$_{50}$s in µM: ethyl benzoate, 4.1 ± 0.35; amyl acetate, 8.9 ± 1.1), it is a relatively low efficacy odorant (57 ± 2.7 % response of methyl benzoate at 1 mM). With this information, one can consider the structural features of these odorants that may impart specificity.

Figure 4.13 - Heliotropyl acetate may block residual receptor activity. Current responses from DmOR67a/83b-expressing (left trace) and uninjected (right trace) oocytes challenged with 1 mM methyl benzoate (MB) and 1 mM heliotropyl acetate (HA). Odorant applications were for 20 sec and are denoted by arrowheads.
Predicting the odorophore of DmOR67a

Similar to a strategy employed by mammalian ORs, the binding pockets of insect ORs may be buried within the transmembrane segments and comprised of highly variable residue components capable of detecting a diverse range of odorant structures. Further complicating matters, insect OR binding sites may be comprised of residues from non-adjacent transmembrane segments. Currently, there is a lack of understanding on how insect ORs bind odorants and discriminate among similar ligands. But, how do we begin to study this ligand recognition problem? Insect ORs detect volatile compounds that are...
usually low molecular weight and thus fairly simple structures. But, many odorants are also straight chain aliphatics with many rotatable bonds. Such linear compounds are highly flexible and can adopt thousands of different conformations, only some of which may be suitable for activation of a particular receptor. To this end, flexible odorants may be able to activate more ORs than rigid compounds because of their inherent adaptability to the many binding pocket structures of a population of ORs (74). This feature of flexible ligands, however, makes the analysis of a ligand’s structural and conformational requirements for receptor activation extremely difficult to resolve. Instead, rigid ligands that adopt fewer conformations could more easily enable studies on receptor-ligand interactions. As an example, a recent report utilized a series of increasingly rigid molecules to probe the binding site of a mammalian OR (75).

The odorant panel screen for DmOR67a (Figure 4.12) provides information on a series of aromatic structures with varying capabilities at receptor activation. By comparing these structures, preliminary observations can be made about the common features, or “odorophore”, of DmOR67a ligands and the general shape of the binding pocket of the receptor. I propose a DmOR67a ligand must consist of two distinct structural features: an aromatic ring or hydrocarbon chain in a ring shape, and a group of atoms (most likely oxygens) carrying a partial negative charge. The DmOR67a binding site would have residues positioned to coordinate the ring structure, and residues carrying a partial positive charge for ionic interaction with the electronegative oxygens or residues capable of hydrogen bonding with the electronegative atoms. Proper coordination and orientation of both of these functional groups by the binding pocket residues would be required for receptor activation. Specific features will impart a ligand’s affinity for
various receptor states and thus its apparent EC$_{50}$ and efficacy. As such, the positioning of the oxygens from the ring structure and the overall strength of the electronegative charge may be key features in determining responsiveness of the receptor. In keeping with this hypothesis, guiacol may be a poor activator due to the positioning of its oxygen atoms too close to its aromatic ring (Figure 4.11). Another poor activator, hydrocinnamaldehyde, may have too weak of a negative charge from its single oxygen atom (Figure 4.11). Strong DmOR67a ligands contain an additional methyl or ethyl group adjacent to the region of oxygen atoms and opposite the ring structure (refer to methyl benzoate, ethyl benzoate, methyl phenyl acetate and amyl acetate structures in Figure 4.15 and EC$_{50}$s in Figure 4.14). This could be explained by the presence of a steric buttress in the active state conformation of the DmOR67a binding site. Acting as an anchor, a hydrophobic pocket could act as a buttress and bind the ligand’s terminal methyl or ethyl group, improving coordination of the partial negative charge with binding site residues. Consequently, a hydrophobic buttress with a higher affinity for an ethyl group over a methyl group may explain why ethyl benzoate is the most potent and efficacious ligand tested. Lack of this functional group in phenyl acetic acid may explain its relatively low affinity as an absence of steric buttressing would cause destabilizing flexibility of the ligand and its electronegative oxygens.

Interestingly, the straight chain compound amyl acetate can also activate the receptor, suggesting its hydrocarbon chain may adopt a ring-like conformation in the binding pocket and its terminal methyl group can associate with the steric buttress of the active state receptor to coordinate the region of negative charge (drawn in a particular ring-like conformation in Figure 4.15). Even with these features, amyl acetate has a
much lower efficacy compared to odorants such as ethyl benzoate. How might I model this low efficacy? It may be an effect of high flexibility of this straight chain molecule, which might overcome the steric buttressing and lead to destabilization of the ligand in the binding pocket. 2-coumaranone, a highly rigid molecule, is also a marginal activator of DmOR67a (Figure 4.15). While rigidity limits the number of conformations this ligand may adopt, such a feature may also impose on binding site fit or access. Additionally, 2-coumaranone lacks the extension for steric buttressing, which may limit potency or efficacy.

Several odorants were identified as potential antagonists. Presumably, these compounds still occupy the agonist binding site and either stabilize a particular inactive conformation of the receptor or act as steric inhibitors. While additional data is required to prove competitive antagonist action, I can begin to make predictions. All four of the
potential antagonists contain oxygens in a similar position as the DmOR67a agonists (Figure 4.16). However, there are differences in the positioning of additional carbons that may account for an altered fit in the binding site. For instance, heliotropyl acetate and helional both contain a secondary methyl group (instead of a primary methyl group), which could orient in a space below the region of electronegativity (Figure 4.16). Heptanoic and octanoic acid may also be capable of bending their hydrocarbon chains and positioning their terminal methyl groups in this area (depicted in Figure 4.16).

Functionally, an inactive receptor conformation could provide a second hydrophobic pocket, serving as an alternate steric buttress for the functional group orientations of the potential antagonists, pulling the ligands into a different orientation than the full agonists. This alternate buttress would be available for binding only in the inactive receptor and thus occupation by antagonists would stabilize the inactive conformation and inhibit receptor activation. Additionally, heliotropyl acetone and helional contain both an extra ring structure and only one oxygen in the predicted region of electronegativity. These features could further contribute to an improper fit in the active receptor’s binding site.

Figure 4.16 - Structures of DmOR67a/83b potential antagonists. Similar to the agonists, all potential antagonists contain a ring or ring-like structure and a region of electronegative oxygens. But, these compounds also can orient a secondary methyl group below this region of oxygens, as depicted by the red boxes.
In summary, the binding site of DmOR67a could consist of a region for occupation by a ring or ring-like structure and a region capable of coordinating with an electronegative functional group of the ligand. I suggest that strength of the electronegative charge region (i.e. number of electronegative oxygens in this region) and orientation of the oxygen-containing functional group contribute to a specific ligand’s apparent EC$_{50}$ and efficacy. As a mechanism for odorant stabilization in the binding space, active and inactive conformations of the receptor would provide mutually exclusive hydrophobic binding pockets for methyl and ethyl groups on a ligand. These hydrophobic pockets may act as steric buttresses which provide support for a ligand to adopt certain conformations. The active receptor conformation provides a space best occupied by methyl and ethyl groups adjacent to the region of electronegativity, which helps an agonist to stabilize the active state of the receptor. The inactive receptor conformation provides a different space, below the region of electronegativity, which can bind secondary methyl groups (i.e. heliotropyl acetate) or the terminal methyl group of a flexible hydrocarbon chain (i.e. octanoic acid). Occupation of this conformation by a ligand results in stabilization of the inactive receptor, inhibiting function. Furthermore, molecules with weak areas of electronegativity or additional ring structures (i.e. heliotropyl acetate and helional) might position correctly in the binding space, but fail to interact strongly enough with coordinating residues of the receptor. It should be noted that amyl acetate contains features capable of occupying both of these steric buttress orientations. The capability of amyl acetate to adopt either conformation might explain this ligand as a partial agonist. Indeed, partial agonists can be conceptualized as both antagonists and agonists, binding a receptor in various intermediate conformational states.
resulting in less than maximal activation. A summary of this hypothesis is provided in Figures 4.17 and 4.18. It should be emphasized that these details are only assumptions, but do provide a platform for future testing. For example, the region responsible for coordination of a partial negative charge might include positively charged residues. If the odorant-binding site does lie buried within the transmembrane segments of the receptor, charged residues would be in relatively low abundance (especially compared to the loop regions). A targeted mutagenesis approach aimed at the positively charged residues of DmOR67a predicted to lie within the plasma membrane might be feasible. Substitution of these charged residues with stronger, weaker, or opposite charges might alter odorant recognition substantially. This type of approach would rely on the isolation of mutant receptors with altered, but not abolished, responses to odorants. Therefore, subtle changes in residue side chain character may be most suitable for this type of hypothetical screen.

**Active Conformation:**

![Active Conformation](image1)

**Inactive Conformation:**

![Inactive Conformation](image2)

Figure 4.17 - Predicted conformational states of the DmOR67a binding site. Both active and inactive conformations contain a region to bind a ring structure (ring) and a region for coordination of a partial negative charge ($\delta^-$). Hydrophobic binding pockets (buttress) act as steric buttresses for additional hydrocarbon groups, leading to ligand orientation in either state.
Figure 4.18 - Orientation of DmOR67a ligands within the predicted binding site. Odorants from Figures 4.15 and 4.16 are redrawn to fit within the modules of the DmOR67a binding pocket depicted in Figure 4.17. Regions: ring (red), electronegative (blue), hydrophobic steric buttress (green).
Does DmOR83b influence ligand selectivity?

As described previously, DmOR83b acts as a functional partner with another DmOR subunit to form odorant receptor complexes. Established in both *in vivo* (1) and heterologous studies here, changing the non-OR83b subunit alters odorant responsiveness. This strongly suggests that the non-OR83b, or specificity subunit, is a major contributor to the odorant-binding site. It is unknown if the odorant-binding site is comprised of residues from one or more OR subunits. Since the mature receptor is probably formed by OR83b and specificity subunits, one cannot rule out the possibility that the binding site may partially consist of residues from OR83b. Admittedly, this possibility seems unlikely. The high diversity among specificity subunits provides a suitable explanation for the ability of DmORs to detect a wide range of chemicals (1). It is also difficult to envision OR83b directly contributing to a binding site on all DmORs, yet allowing such diverse odorant responsiveness. However, indirect influence of OR83b through allosteric mechanisms may contribute to binding site structure. Therefore, I sought to investigate the contribution, if any, of OR83b toward binding site character.

I first asked if the specificity subunit DmOR35a can functionally couple to OR83b subunits from other species. AmOR2 and OnOR2, the respective OR83b orthologs of the honeybee (*Apis mellifera*) and the European corn borer (*Ostrinia nubilalis*) were coexpressed with DmOR35a in oocytes and screened for functionality. Both receptor complexes properly responded to the odorant hexanol (Figure 4.19). It should be noted that while DmOR35a/AmOR2 and DmOR35a/DmOR83b response amplitudes were generally similar. DmOR35a/OnOR2 responded with much lower current amplitudes (not quantified), suggesting this receptor is not able to express
robustly or is otherwise functionally impaired. Interestingly, sequence alignments of OR83b orthologs show comparatively equal percent amino acid identities (DmOR83b and AmOR2, 63% identical; DmOR83b and OnOR2, 64% identical). Since much of the variation between orthologs occurs in the N-terminal 2/3 of sequence, perhaps this region contributes to subunit association, trafficking, or receptor activation. The relative poor function of DmOR35a/OnOR2 may be caused by specific residue interactions, or lack thereof, in this region.

If DmOR83b contributes to binding site structure, then receptor complexes comprising OR83b orthologs may have altered relative ligand responsiveness. Since the receptors with OR83b orthologs are indeed functional, I reasoned differences in ligand specificity may be quite subtle. Therefore, I investigated the activation of each DmOR35a receptor complex with a panel of closely related primary alcohols (Figure 4.20). Responses to various ligands were normalized to the response elicited by hexanol for each receptor. Data for the DmOR35a/DmOR83b receptor is reproduced from Figure
3.5. Comparing the ligand response profiles to a panel of primary alcohols, I conclude that no differences exist between DmOR35a complexes with various OR83b orthologs.

![Figure 4.20 - Profiles of alcohol activation are similar among the three DmOR35a receptor complexes. A panel of primary alcohols (30 μM each) was screened against each DmOR35a receptor complex indicated (z-axis). Data are expressed as percent of the response elicited by hexanol (y-axis). Compounds are presented as carbon chain length (x-axis) and bars are mean values (n ≥ 6 for each odorant tested).](image)

To expand upon this result, I screened a larger panel of odorants and again compared DmOR35a receptor responsiveness (Figure 4.21). Odorants were selected based on a rational expansion of the chemical space around hexanol. These included aliphatic hydrocarbon odorants ranging from 2-12 carbons and containing various functional groups such as aldehydes, monocarboxylic acids, dicarboxylic acids, bromocarboxylic acids, and ketones. Again, for each experiment, all responses were normalized to the response generated by hexanol. DmOR35a/OnOr2 was excluded since
this screen required robust current amplitudes for the comparison of odorants with relatively weak effects. The OnOR2 complex simply does not express well enough to be included in this study. Remarkably, no large differences in the activation profiles between DmOR35a/DmOR83b and DmOR35a/AmOR2 were apparent (Figure 4.21). I conclude that OR83b subunits do not influence the activation of DmOR35a and do not contribute to receptor structure that imparts odorant selectivity. Furthermore, the residue differences between OR83b orthologs probably do not influence odorant selectivity.

Since hexanol is the strongest identified agonist for DmOR35a/83b, odorants most closely related to hexanol are also able to activate the receptor. Expansion of carbon length and functional group character that is increasingly more distant from the structure of hexanol results in odorants with lower activity. This is apparent in the bell
shaped bar graphs of alcohol and aldehyde odorants (Figure 4.21). Interestingly, a new “pocket” of ligand activity was uncovered in which ligands containing 9-12 carbons, but not alcohols, were able to elicit receptor activity (Figure 4.21). Of notable activity in both receptor complexes (containing DmOR83b or AmOR2) are 2-decanone and dodecanoic acid. How might a receptor recognize these compounds, while the 6-7 carbon 2-ketones and carboxylic acids fail to appreciably activate the receptor? It may be significant that the longer odorants are roughly double in carbon length compared to the shorter odorants. Perhaps half of these longer odorants is actually involved in occupation of the binding pocket, while the other half is stabilizing the receptor by hydrophobic interactions. This may be another case of steric buttressing as a mechanism for ligands to adopt specific conformations (see explanations above on DmOR67a). Future detailed studies on the binding site of DmOR35a may enable a clearer understanding of possible mechanisms.

**Do specificity subunits contribute to the structure of the pore?**

Strong evidence suggests insect olfactory receptors are heteromeric complexes of subunits that bind odorants and flux ions through a channel pore (33,45,49). As I have shown, heterologous expression of only specificity subunits or OR83b fails to produce functional channels. While specificity subunits impart odorant specificity, OR83b has been implicated in channel formation. Evidence for this assertion includes the report of OR83b generating current in response to cyclic nucleotides and the mutation of specific OR83b residues resulting in altered channel properties (45). Therefore, it is reasonable to speculate that OR83b subunit residues contribute directly to channel structure. Many
ligand-gated ion channels are comprised of multiple protein subunits which are arranged around a central pore. Each subunit can help determine channel properties by contributing residues toward the structure of the channel, vestibule, and selectivity filter. Even though OR83b most probably contributes to the channel structure, it is presently unknown if specificity subunits do the same. The specificity subunits are highly variable, but could still contribute to overall channel structure. Even variable subunits can combine to form selectivity filters, based on orientation of carbonyl oxygens in the amino acid backbones and not various residue functional groups. However, if insect olfactory receptor channel pores are comprised of both OR83b subunits and the highly variable specificity subunits, slight variations in channel properties may be expected. For these experiments, I first sought a molecular tool capable of probing DmOR channel structure.

Ruthenium red, a non-selective cation channel blocker, has previously been shown to inhibit the *Bombyx mori* receptor, BmOR1/2, but not the *Anopheles gambiae* receptor, AgOR7/2 (49,50). This suggests the ability of ruthenium red as a blocker is dependent on residue variation near the receptor channel and may be a useful tool for investigation of receptor composition. I first asked if ruthenium red can block DmOR function. DmOR35a/83b currents, in response to hexanol, were indeed inhibited by 50 µM ruthenium red (Figure 4.22, *left*). This inhibition was partial, in contrast to the complete inhibition of BmOR1/2 and the lack of effect on AgOR7/2 (also by 50 µM ruthenium red) (49,50). If ruthenium red is a ligand-gated ion channel blocker, it should do so in a non-competitive fashion and, therefore, would inhibit OR function to the same extent at any ligand concentration. I examined this notion by measuring the ability of 50 µM ruthenium red to block DmOR35a/83b function in the presence of 3, 30, and 300 µM
hexanol (Figure 4.22, right). The extent of channel inhibition was the same for each hexanol concentration, indicating that ruthenium red acts as a non-competitive channel blocker.

I next investigated the ability of 50 μM ruthenium red to block current generated by several other DmORs (DmOR43b/83b, DmOR67a/83b, DmOR85a/83b, and DmOR85b/83b). Similar to the experiments described above, each receptor was activated by an odorant until current response plateau, followed by co-application of the odorant with 50 μM ruthenium red. All DmORs examined were blocked by ruthenium red treatment (Figure 4.23). Interestingly, the extent of ruthenium red block of DmOR function was variable (see below).

Figure 4.22 - Ruthenium red (RR) inhibits DmOR35a/83b through non-competitive channel block. (Left) An oocyte expressing DmOR35a/83b is challenged with 3 μM hexanol (HEX) for 210 sec, followed by co-application of 3 μM HEX and 50 μM RR for 30 sec. (Right) Block of DmOR35a/83b currents by 50 μM RR at different hexanol concentrations (bars are mean ± sem, 3 μM: n=11; 30 μM: n=9; 300 μM: n=6, the difference between means is not significant by one-way ANOVA).
Quantifying the percent block after ruthenium red treatment shows a statistically significant broad range of effectiveness, ranging from $40.6 \pm 3.1\%$ for DmOR35a/83b (n=11) to $105.4 \pm 7.1\%$ for DmOR67a/83b (n=11) (Figure 4.24). Since all receptors contained the common subunit DmOR83b, this suggests the variable effectiveness of ruthenium red inhibition is conferred by the specificity subunits. I have shown evidence that ruthenium red acts by non-competitive means, at a separate location from the odorant binding site (Figure 4.22). It is certainly possible that ruthenium red may bind to some allosteric site on the receptor. But, based on the general properties as a cation channel blocker, a more likely explanation is that ruthenium red blocks ion flux through physical pore obstruction. Therefore, this data suggests that the specificity subunits contribute to the shape or character of the channel pore and thus influence the ability of ruthenium red to inhibit channel function. The differential effects of ruthenium red on the Bombyx and
*Anopheles* receptors complement this study nicely (49,50). It should be noted that this interpretation is not conclusive, but may help in the elucidation of individual insect OR subunit roles in receptor function. These results also strengthen the argument that an insect OR complex contains the channel itself, and does not functionally link to another channel protein.

![Comparison of OR complex inhibition](image)

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Figure 4.24 - (*Graph*) Quantification of the percent block achieved by 50 µM ruthenium red (RR) for each odorant-activated DmOR, as described in Figure 4.23. Percent inhibition was calculated as the difference in the maximum response compared to the response in the presence of RR (bars are mean ± sem, 35a/83b: n=11; 85a/83b: n=5; 85b/83b: n=5; 43b/83b: n=3; p < 0.0001 by one-way ANOVA). (*Table*) Individual mean values were compared to assess statistical significance by Bonferroni’s multiple comparison test.

As an aside, I note that treatment of DmOR67a/83b by ruthenium red sometimes resulted in a measured inhibition above 100% (% inhibition: 105.4 ± 7.1, n=11). While not statistically significant from 100% inhibition, this trend could highlight the existence
of DmOR67a/83b basal activity. Since ruthenium red is a non-competitive channel blocker, this potential effect of basal block would be considered an additional component of the measurable current and separate from odorant-generated activity. Such basal activity may be highly dependent on unknown intracellular conditions, resulting in highly variable conditions between individual oocytes and batches. Unfortunately, this high variability adds a margin of difficulty in studying receptor basal activity and precludes any conclusions at this time.

**Conclusions and models from the OR subunit contribution studies**

The above studies offer evidence from which I can make preliminary hypotheses on the organization of heteromeric insect odorant receptors. The actual stoichiometry or number of subunits forming the channel and pore is unknown, but, for sake of visualization here, I will depict OR channels as tetrameric. Please note this is only for sake of discussion. One possible arrangement of insect OR subunits may consist of OR83b subunits solely contributing to a central channel, with specificity subunits associated on the outside for odorant recognition (Figure 4.25, left). However, the above data suggest that this type of arrangement is incorrect. Based on the conclusions garnered from the ruthenium red experiments, I propose that the insect OR channel properties are influenced by at least one specificity subunit (Figure 4.25, right). Future experiments may be designed to test this hypothesis. For example, after further elucidation and identification of regions near the pore, chimeric receptors may be
generated that could change ruthenium red sensitivity depending on the parent receptor used for chimera construction. The work here offers an excellent starting point for these types of studies.

Comparing the ligand specificities of DmOR35a paired with various OR83b ortholog subunits (DmOR83b, AmOR2, and OnOR2) showed no significant differences in ligand preference (Figures 4.20 and 4.21). This suggests that the odorant selectivity is shaped entirely by DmOR35a residues, and without any contributions from OR83b residues. Therefore, this data does not support an arrangement in which the odorant-binding site is at the interface between a specificity subunit and an OR83b subunit (Figure 4.26, top). Since specificity subunits impart ligand responsiveness, a model could be supported in which the binding site resides entirely within a single specificity subunit (Figure 4.26, bottom left and center). Also, these studies cannot rule out a model in which an odorant binding site is comprised of residues from more than one specificity.
subunit (Figure 4.26, bottom right). This type of arrangement is reminiscent of other ligand-gated ion channels, such as neuronal nicotinic acetylcholine receptors. These receptors contain multiple acetylcholine-binding sites formed at the interface between pairs of subunits (76).

These studies on subunit contributions provide data which favor specific arrangements of receptor complexes over others. However, I should emphasize that little to no detail is known about insect OR subunit stoichiometry. Future work should focus on testing the more probable receptor arrangements predicted by this data.

Figure 4.26 - Possible subunit arrangements for insect OR binding site localization. (Top) In this unsupported model, an odorant binding site is comprised of OR83b and specificity subunit residues. (Bottom) Equally supported by the data, one (Left) or more (Center) odorant binding sites reside within a single specificity subunit, or multiple specificity subunits contribute to a single binding site (Right). The arrows indicate cation flow through a central pore.
CHAPTER V: IDENTIFICATION OF FUNCTIONALLY RELEVANT FEATURES IN AN INSECT OLFACTORY RECEPTOR

**Scanning DmORs for susceptibility to MTS reagents**

I sought to identify specific regions of functional importance in insect ORs. Since these receptors belong to a novel class of ligand-gated ion channel with no known regions of conservation, few structural or functional features have been uncovered. One tool for the identification of functional regions of receptors and ion channels is the methanethiosulfonate (MTS) reagents (77). These molecules form disulfide bonds with the sulphhydryls of cysteine residues, potentially altering protein structure and function (Figure 5.1, top).

\[
\text{Protein} \text{SH} + \text{CH}_3\text{S-SR} \rightarrow \text{Protein} \text{S-SR} + \text{CH}_3\text{S-H}
\]

(MTS Reagent)

![MTS Reagents](image)

Figure 5.1 - (Top) MTS reagents react with sulphydryl groups on cysteines, forming a covalent disulfide bond. Thus, cysteines become derivatized by molecular “tags” (SR in blue) that may affect protein function. (Bottom) Examples of commonly used MTS reagents. Cysteine “tags” are highlighted in blue.

Conventionally, MTS reagents are used in the substituted cysteine accessibility method (SCAM), in which cysteine residues are sequentially inserted into regions of interest and mutants are tested for susceptibility to MTS treatment (77-79). However, our almost complete lack of structural knowledge about insect ORs reduces the feasibility of
this approach; a series of approximately 400 cysteine substitution mutants would have to be generated to scan an entire receptor sequence. At times, wild-type receptors or proteins may be naturally susceptible to MTS treatment due to positioning of specific cysteines. In these cases, use of SCAM requires construction of a “pseudo-wild-type” (pseudo-WT) receptor in which one or more cysteines are removed to create a receptor that is insensitive to MTS. This pseudo-WT receptor is then used as a platform (or backbone) for the generation of cysteine-insertion mutants. I reasoned that, among the highly variable DmORs, a WT receptor may contain a cysteine fortuitously located in a region of functional importance and thus be naturally susceptible to MTS treatment.

MTS reagents are of various sizes and charges. Thus, reagents are differentially effective at altering receptor function. I chose to screen several DmORs (DmOR35a/83b, DmOR85a/83b, and DmOR85b/83b) for susceptibility to covalent modification and functional perturbation by three MTS reagents (ethylsulfonate-MTS, MTSES; ethylammonium-MTS, MTSEA; and trimethylammonium-MTS, MTSET) (Table 2.1 and Figure 5.1, bottom). These reagents are charged and membrane impermeant, therefore only able to react with residues accessible to the extracellular space where odorants would presumably interact with the receptor.

Selection of a suitable MTS reagent and DmOR pair for use in functional studies should be based on strict criteria in order to avoid complications in data interpretation. I searched for an MTS reagent with a mechanism of action that, most likely, tags a receptor and changes functional properties by forming a disulfide bond with a cysteine residue. Specifically, functional modulation by MTS should be long-term, which suggests covalent disulfide bonds are formed with cysteine residues and are irreversible under
oxidizing conditions. Furthermore, the MTS reagent should not activate the receptor itself, which may suggest the reagent reversibly binds to the odorant binding site or an allosteric site by non-covalent means. Additionally, transient effects may indicate interference with the channel pore and not labeling of specific cysteine residues. While MTS reagents may have interesting effects with multiple components, the scope of this study was to identify an MTS reagent that most likely covalently tags a DmOR and “cleanly” alters function.

The MTS susceptibility screen was performed by recording pre-treatment responses to known odorants and comparing these amplitudes to multiple post-treatment responses after application and wash-out of a specific MTS reagent (applied at 1 mM for 2 min) (Figures 5.2, 5.3, 5.4). Odorant responses of DmOR35a/83b were unaffected by treatment with any of the three MTS reagents, and was discarded from further study (Figure 5.2). DmOR85a/83b was also unaffected by MTSES treatment, but was transiently potentiated by MTSEA and MTSET (Figure 5.3). This transient effect may be caused by electrostatic interactions of the positively charged MTS reagents and negatively charged residues close to the channel pore, rather than covalent modification of cysteine residues. Furthermore, MTSEA and MTSET slightly activated DmOR85a/83b in a reversible manner, suggesting additional components might be present in the recorded response after MTS treatment (Figure 5.3). Due to these complicating factors, DmOR85a/83b was discounted as a suitable receptor for study with these MTS reagents. Only DmOR85b/83b was inhibited by MTS treatment (Figure 5.4). DmOR85b/83b inhibition by MTSEA and MTSET appeared to be slowly reversible, as receptor responsiveness recovered after multiple post-treatment odorant applications. In
contrast, inhibition by MTSES was stably maintained for at least 54 min after washout of the reagent. While the effect of MTSES on OR85b/83b was partial (50 ± 2% response remaining, mean ± sem, n=4), this effect was saturating; increasing the concentration of MTSES to 10 mM had no further inhibitory effect (see Figure 5.8). These details suggest that MTSES is covalently modifying one or more cysteine residues of DmOR85b/83b and blocking receptor activation or function. Therefore, I chose to use MTSES to probe DmOR85b/83b structure.

Figure 5.2 - MTS reagent susceptibility screen of DmOR35a/83b. (Traces) Odorant induced current responses of DmOR35a/83b expressing oocytes before and after treatment with MTSES, MTSEA, and MTSET (1 mM, 2 min). DmOR35a/83b was activated by 3 µM hexanol (HEX). Odorant applications were for 20 sec and are denoted by arrowheads. (Graph) Quantification of odorant-induced current responses after MTS treatment (1 mM, 2 min). Remaining response values were assayed after a wash period of 4 min (4') and 14 min (14') and are presented as means ± sem (n values from left to right: 4,3,5,5,7,7).
Figure 5.3 - MTS reagent susceptibility screen of DmOR85a/83b. *(Traces)* Odorant induced current responses of DmOR85a/83b expressing oocytes before and after treatment with MTSES, MTSEA, and MTSET (1 mM, 2 min). DmOR85a/83b was activated by 100 µM ethyl 3-hydroxybutyrate (E3HB). Odorant applications were for 20 sec and are denoted by arrowheads. *(Graph)* Quantification of odorant-induced current responses after MTS treatment (1 mM, 2 min). Remaining response values were assayed after a wash period of 4 min (14') and 16 min (16') and are presented as means ± sem (n values from left to right: 5, 4, 5, 5, 3, 3).
Is MTSES inhibiting DmOR85b by covalent modification?

If MTSES is indeed forming a disulfide bond with the sulphydryl group of a cysteine residue on DmOR85b, the reaction should be reversible under reducing conditions. Therefore, I sought to recover DmOR85b function after MTSES action by application of the reducing agent dithiothreitol (DTT). As expected, WT DmOR85b/83b receptor responsiveness to 100 µM 2-heptanone was significantly inhibited after treatment with MTSES (10 mM, 2 min) compared to sham treatment (% response

Figure 5.4 - MTS reagent susceptibility screen of DmOR85b/83b. (Traces) Odorant induced current responses of DmOR85b/83b expressing oocytes before and after treatment with MTSES, MTSEA, and MTSET (1 mM, 2 min). DmOR85b/83b was activated by 100 µM 2-heptanone (HEP). Odorant applications were for 20 sec and are denoted by arrowheads. (Graphs) Quantification of odorant-induced current responses after MTS treatment (1 mM, 2 min). Remaining response values were assayed after a wash period of 4 min (4'), 14 min (14') and 54 min (54') and are presented as means ± sem (n values from left to right: 4,4,4,4,4,4,4,4,5).
remaining, 51.3 ± 6.1%, n=5, p<0.01, Dunnett’s multiple comparison test). However, application of MTSES (10 mM, 2 min) followed by application of DTT (20 mM, 2 minutes) resulted in significantly reduced receptor inhibition compared to MTSES treatment alone (% response remaining, 76.8 ± 5.1%, n=12, p<0.05, Dunnett’s multiple comparison test). Importantly, treatment with DTT alone (20 mM, 2 min) does not significantly affect the receptor response to 2-heptanone compared to sham treatment (Figure 5.5). Thus, application and washout with a high concentration of DTT does not alter the ability of the receptor to be activated by agonist. These results suggest treatment of DmOR85b/83b with DTT can partially reverse the functional inhibition caused by MTSES action. Furthermore, these results imply that MTSES acts to inhibit DmOR85b/83b function by covalent modification of cysteine residues through disulfide bond formation. I next sought the DmOR85b/83b cysteine target of MTSES.

Figure 5.5 - DTT partially reverses the inhibition of DmOR85b/83b by MTSES. Quantification of current responses from DmOR85b/83b expressing oocytes to 100 µM 2-heptanone after indicated treatment. MTSES was applied at 10 mM for 2 min, DTT was applied at 20 mM for 2 min. Sham is an ND96 application for 2 min. Data are presented as means ± sem (n values from left to right: 5,4,12,5). Statistical significance was assessed by one-way ANOVA and Dunnett’s multiple comparison post-test, one asterisk: p<0.05, two asterisks: p<0.01.
Identification of the DmOR85b cysteine target of MTSES

MTSES affected only one of the three DmORs tested, suggesting that the site of action is located on the specificity subunit DmOR85b and not the common DmOR83b subunit. While the charged MTSES is considered membrane impermeant and only cysteines accessible to the extracellular space are possible targets for modulation, transmembrane topologies for insect ORs are fairly weak in predictive strength. Thus, I constructed a series of mutant receptors in which each cysteine in OR85b (Figure 5.6) was replaced with a serine and screened for susceptibility to block by MTSES (Figure 5.8). Serine was chosen as the replacement residue since it is the most conservative amino acid substitution. However, serine does not contain a sulfhydryl group and cannot form disulfide bonds with MTS reagents.

Figure 5.6 - Predicted secondary structure of DmOR85b. Figure rendering was performed using the TMRPres2D program and transmembrane locations are from Swiss-Prot (which provides a consensus transmembrane predication based on three algorithms). Wild-type cysteine positions (approximate) are highlighted in yellow.
I wished to use a simpler protocol to screen DmOR85b mutants for MTS susceptibility (see Chapter II for a detailed description). However, this protocol required an extended odorant application time of 440 sec. Therefore, I first asked if DmOR85b subunits could undergo significant desensitization after a 440 sec odorant application that would skew my data interpretations. Current amplitudes were measured at 240 sec and 440 sec after initiation of a 300 µM 2-heptanone application (time points used for the pre and post MTSES current measurements). The 440 sec current amplitudes after odorant application were calculated as a percentage of the current amplitude at 240 sec after odorant application. No significant desensitization was apparent for WT or any mutant DmOR85b tested (Figure 5.7). I concluded that any changes in current after MTSES application would be a result of receptor modification and not desensitization.

Figure 5.7 - Lack of receptor desensitization after long-term odorant application. Oocytes expressing DmOR83b and either the WT DmOR85b subunit or one of several DmOR85b mutants (C124S, C146S, C208S, C278S, or C311S) were challenged with 300 µM 2-hepanone for 440 sec. (Graph) Data is presented as a ratio of the current amplitude at 440 sec over the current amplitude at 240 sec after initiation of odorant application. Bars are means ± sem (n values, WT: 9; C124S: 9; C146S: 12; C208S: 10; C278S: 8; C311S: 5). Statistical significance was assessed by one-way ANOVA followed by Dunnett’s multiple comparison post-test (not significant, p=0.18; p>0.05 for all values). (Trace) An oocyte expressing WT DmOR85b/83b is challenged with 300 µM 2-hepanone for 440 sec. Points at which amplitudes were measured are indicated by arrows.
I next screened the various DmOR85b subunits for MTS susceptibility using this protocol. Three mutant receptors (C124S, C208S, and C311S) were similarly susceptible to MTSES compared to wild-type (WT) DmOR85b (Figure 5.8). Receptors formed by two mutant DmOR85b subunits (C146S and C278S) showed reduced susceptibility to MTSES. However, block by MTSES was slightly more evident for the C278S receptor. The C278S receptor also exhibited extremely slow recovery after initiation of washout (Figure 5.8), suggesting this receptor might be more sensitive to agonist. To investigate, I performed concentration-response analysis on receptor activation by 2-heptanone for all the mutant receptors (Figure 5.9).

Figure 5.8 - Targeting the site of MTSES-directed inhibition of DmOR85b/83b function. Current responses are presented from DmOR expressing oocytes when challenged with 300 μM 2-heptanone (HEP) for 440 sec. MTSES (10 mM) was co-applied during the last 200 sec of the HEP application. This is the “new protocol” described in the Methods section. Oocytes expressed DmOR83b and either the wild-type DmOR85b subunit or one of several DmOR85b mutants: C124S, C146S, C208S, C278S, or C311S. Note the slow recovery after washout for the C278S receptor.
Mutant receptors C124S, C146S, C311S, are similarly sensitive to 2-heptanone compared to WT (Figure 5.9, *table*). The C208S receptor was approximately 5-fold and the C278S receptor was approximately 28-fold more sensitive compared to WT (p<0.0001, F-test for each). If the effect of MTSES is to interfere with odorant sensitivity, such an effect could be masked by the increased sensitivity of particular
receptor mutants; the screening concentration of 2-heptanone (300 µM) used in Figure 5.8 would be super-saturating, especially for the C278S mutant. I investigated this notion by testing the C278S mutant for MTSES susceptibility in the presence of a lower concentration of 2-heptanone. Indeed, decreasing the concentration of 2-heptanone to 30 µM resulted in a significant improvement of receptor block by 10 mM MTSES (average % current response remaining: 300 µM 2-heptanone, 99.3 ± 2.4, n = 9; 30 µM 2-heptanone, 77.1 ± 0.9, n = 5; p<0.0001, unpaired t-test) (Figure 5.10).

Figure 5.10 - The susceptibility of DmOR85b C278S to modulation by MTSES is dependent on the agonist concentration. An oocyte expressing DmOR85a C278S and DmOR83b activated by 300 µM 2-heptanone (HEP) (Top trace) is less susceptible to 10 mM MTSES treatment (avg. % current response remaining = 99.3 ± 2.4, n = 9) compared to an oocyte activated by 30 µM HEP (Bottom trace) (avg. % current response remaining = 77.1 ± 0.9, n = 5). Traces are representative examples. 300 µM HEP is super-saturating for DmOR85b C278S (Top Graph), while 30 µM is not (Bottom graph).
Since the effectiveness of MTSES to inhibit C278S is dependent on the concentration of agonist, I investigated this notion for the other DmOR85b mutants and the WT receptor. The effect of MTSES on WT receptor was much more pronounced at 30 µM 2-heptanone compared to the effect at 300 µM 2-heptanone (Figure 5.11). This 2-heptanone dose-dependence for MTSES susceptibility occurred with the C124S, C208S, C311S, and C278S mutants (Figure 5.11). Only the C146S mutant receptor remained insensitive to MTSES treatment at all 2-heptanone concentrations (Figure 5.11), indicating that C146 of the DmOR85b subunit is the sole site of action for MTSES.

Figure 5.11 - All DmOR85b receptors, except C146S, are differentially susceptible to MTSES depending on agonist concentration. The effect of MTSES treatment on the response of wild-type and mutant receptors to several 2-heptanone concentrations is presented. Bars are means ± sem (n values from left to right, WT: 4,5,4; C124S: 4,7,5; C146S: 5,9,4; C208S: 5,9,4; C278S: 4,5,9,4; C311S: 4,7,4).
Mechanism of MTSES inhibition of DmOR85b

The dependence on agonist concentration for MTSES effectiveness suggests the reagent interferes with receptor activation or responsiveness to agonist. Therefore, treatment with MTSES should shift the dose-response relationship to the right, rendering a receptor less responsive to 2-heptanone. I investigated this possibility by comparing dose-response curves before and after MTSES treatment. For these experiments, a pre-treatment (before MTSES or sham) application of 100 µM 2-heptanone was used as a normalization factor. Indeed, 10 mM MTSES (2 min application) significantly reduced the 2-heptanone sensitivity of WT DmOR85b/83b (apparent EC$_{50}$ before MTSES treatment: 71 ± 28 µM, n=20; apparent EC$_{50}$ after MTSES treatment: 472 ± 213 µM, n=10, p<0.02, F-test) (Figure 5.12). Thus, it seems derivatization of C146 by MTSES results in a receptor less sensitive to agonist-induced activation.

Figure 5.12 - MTSES treatment renders DmOR85b less sensitive to agonist. Oocytes expressing DmOR85b/83b were treated with 10 mM MTSES (2 min) prior to being challenged with increasing concentrations of 2-heptanone. Data for the MTSES curve is expressed as a percentage of a 100 µM 2-heptanone normalizing dose applied before MTSES treatment. The MTSES-treated curve data was then re-normalized to the untreated curve and fitted. EC$_{50}$ values (µM ± sem), untreated: 71 ± 28, n=20; MTSES treated: 472 ± 213, n=10. p < 0.02 by F-test.
There are essentially two reasonable explanations on how MTSES could be blocking DmOR85b function: an effect on agonist-induced activation or interference with the channel pore. While evidence presented above strongly implicates an effect on agonist-induced activation, I wished to perform current-voltage analysis to determine whether MTSES treatment also affects the general properties of the pore (Figure 5.13). As expected, MTSES treatment of WT receptor resulted in a reduced odorant-induced macroscopic current of a single recorded cell (54.7% at -70 mV), indicating that MTSES is indeed inhibiting the functional output of the receptor (Figure 5.13, left). Normalizing data to a particular voltage (+20 mV) results in current-voltage curves with unchanged reversal potentials and similar curve shapes (Figure 5.13, right). This indicates that MTSES treatment does not alter the general properties of the channel pore. Also, this is suggestive that C146 is not situated close to the channel pore itself.

Figure 5.13 - Current/voltage relationship of DmOR85b/83b, before and after treatment with MTSES. (Left) I/V measurements from an oocyte expressing DmOR85b/83b and activated by 100 µM 2-heptanone (HEP), before and after 2 min treatment with 10 mM MTSES. (Right) I/V curves obtained from oocytes expressing DmOR85b/83b and activated by 100 µM HEP, before and after 2 min treatment with 10 mM MTSES. Current values were normalized to +20 mV (data are means ± sem, n=5). Currents for both graphs were recorded during a voltage ramp from -100 mV to +60 mV over a period of 5 sec. Passive current values, obtained in the absence of HEP, have been subtracted.
SCAM of the region surrounding position 146 of DmOR85b

Residue C146 is in a suitable region of DmOR85b for further investigation, as witnessed by the functional perturbation of the receptor when this position is tagged by MTSES. Therefore, I targeted the region surrounding position 146 using the traditional SCAM approach (79). According to a consensus of transmembrane predictions (Swiss-Prot protein database), C146 lies at the interface between transmembrane segment 3 (TMS3) and extracellular loop 2 (ECL2). So, I chose to use SCAM on positions 134 to 154 in order to include residues predicted to reside in both of these regions (Figure 5.14).

A series of mutant DmOR85b subunits was generated in which the residues from positions 134 to 154 were sequentially replaced with cysteine. Since WT DmOR85b is susceptible to MTSES but the C146S mutant is not, all mutants generated for SCAM
were constructed within a C146S background (considered a “pseudo-WT”). It should be noted that the pseudo-WT C146S receptor is similarly responsive to agonist, compared to WT (Figure 5.9). Cysteine substitution mutants were then screened for the ability to be functionally affected by 10 mM MTSES in the presence of 100 µM 2-heptanone. This sub-saturating concentration of agonist was chosen to ensure any effects of MTSES treatment were not masked, as seen for the C278S receptor. All 20 DmOR85b mutant receptors responded to 2-heptanone when coexpressed with DmOR83b. Treatment with MTSES resulted in one of three results: no effect, inhibition, or potentiation of the response to 2-heptanone (example traces in Figure 5.15). A summary of the SCAM screen results is presented in Figure 5.16.

Figure 5.15 - Current responses from DmOR expressing oocytes challenged with 100 µM 2-heptanone (HEP) for 440 sec. MTSES (10 mM) was co-applied during the last 200 sec of the HEP application. Oocytes expressed DmOR83b and one of several DmOr85b mutants (each within the C146S pseudo-WT background): S136C (left), M148C (middle), E149C (right). MTSES application resulted in no effect (left), inhibition (middle), or potentiation (right) of the response to HEP.
Figure 5.16 - SCAM analysis identifies a functionally important region in DmOR85b. Quantification of the effect of MTSES treatment on the response of WT OR85b/83b (C146 (WT)) and mutant receptors to 100 µM 2-heptanone (data are means ± sem). Bars extending to the left indicate inhibition, while bars extending to the right indicate potentiation. Each mutant was constructed in the C146S pseudo-WT background. Statistical significance was assessed by one-way ANOVA and Bonferroni’s post-test comparing each mutant response to the C146S pseudo-WT response: two asterisks, p<0.001 (n values from top to bottom: 6,6,5,6,7,5,6,3,7,5,4,6,4,6,5,6,7,7,7,4,7).
Most of the mutant receptors were not affected by MTSES treatment (Figure 5.15, left and Figure 5.16). However, I found that MTSES could alter receptor function when a cysteine was placed at positions 147, 148, 149, and 150, further confirming the interpretation of position 146 and its surrounding region as functionally relevant. Similar to WT receptor, MTSES treatment of the M148C mutant resulted in inhibition of the response to 2-heptanone (% response remaining: WT, 49.8 ± 2.4, n = 3; M148C, 38.5 ± 4.6, n = 5) (Figure 5.15, center and Figure 5.16). Surprisingly, MTSES treatment resulted in potentiation of function for the V147C, E149C, and Y150C mutants (% response remaining: V147C, 292.3 ± 18.5, n=6; E149C, 265 ± 29.7, n=5; Y150C, 497.7 ± 50.1, n=6) (Figure 5.15, right and Figure 5.16). Both inhibition and potentiation of receptor response by MTSES reached a saturation of effect by the end of MTS application (examples in Figure 5.15). Furthermore, the responses properly decayed to baseline current levels when the washout step was initiated. These results provide evidence for residues 146-150 on DmOR85b comprising the first discrete and functionally relevant region identified in an insect OR.

**Validation of newly identified positions as true MTSES targets**

I next investigated if the newly identified positions (147, 148, 149, and 150) were affected by MTSES in a similar fashion as the WT receptor. Temporary and non-specific functional alteration of DmOR85a/83b and DmOR85b/83b with various MTS reagents was not maintained 20 min after the MTS application (Figure 5.3 and 5.4). But, derivatization of WT DmOR85b at position 146 by MTSES was maintained at least 20 min after MTS application (Figure 5.4). So, I asked if the effect of MTSES on a subset
of the mutant receptors was maintained for at least 20 min. Indeed, mutant receptor responses continued to be affected 20 min after MTSES application (Figure 5.17). This result suggests that MTSES is acting on the V147C, M148C, and Y150C mutant receptors by covalent modification, similar to proposed mechanism for the WT receptor.

The ability of MTSES to inhibit the WT receptor is dependent on agonist concentration. So, I investigated if increasing the agonist concentration would similarly reduce MTSES susceptibility for the newly identified receptor mutants. Receptor function, after MTSES treatment, was measured in the presence of 100 µM and 3000 µM 2-heptanone (Figure 5.18), as these concentrations elicited very different degrees of MTSES susceptibility for the WT receptor (Figure 5.11). For receptors that were inhibited by MTSES (WT and M148C), increasing the concentration of 2-heptanone significantly decreased the susceptibility to block by MTSES (Figure 5.18, left).
Concomitantly, receptors that were potentiated by MTSES (V147C, E149C, and Y150C) exhibited reduced potentiation at higher 2-heptanone concentration (Figure 5.18, right). Therefore, the effects of MTSES treatment, potentiation or inhibition, can be mitigated by increasing the agonist concentration. This suggests that MTSES also acts by altering the 2-heptanone agonist dose-response relationship of the newly identified SCAM mutant receptors.

Possible functional role of positions 146-150 in DmOR85b

The residues 146-150 clearly define a functionally important region of DmOR85b. C146 was identified during the original screen of WT DmORs for susceptibility to MTS reagents. Positions 147-150 were identifying in the SCAM screen,
in which a cysteine substitution at each position conferred MTSES susceptibility.

However, treatment with MTSES does not completely abolish receptor responsiveness to odorant for any mutant tested. Instead, labeling these positions results in altered agonist activation of the receptor. I suggest that positions 146-150 cannot be directly involved in odorant binding, but rather play a role in agonist access to the binding site or receptor conformational changes during activation.

**Potentiation of function by MTSES**

How might I explain the receptor mutants that result in response potentiation after MTSES treatment? Conclusions from Figure 5.18 suggest that the concentration-response curve of these mutants is shifted leftward after MTSES application; the receptors are more sensitive to 2-heptanone. However, it should be noted that V147C, E149C, and Y150C appear to be functionally impaired compared to WT and M148C and concentration-response analysis is highly difficult. Therefore, these mutations may have caused structural disruption of the receptor. The mutants may have a collapsed structure that normally contributes to odorant access or receptor conformational shifts upon activation. MTSES attachment at these positions could partially restore the structure in this region, resulting in improved receptor function. This would be measured as an increase in agonist responsiveness (potentiation) after MTSES treatment of a mutant receptor, which has an initially impaired ability to be activated by odorant.

An outstanding question remains: by what mechanism does derivatization of the cysteine with a sulfonate group result in partial restoration of function at positions 147, 149, and 150? Perhaps the bulk of the derivatized cysteine is all that is required for
structural restoration, pulling the loop away from its collapsed form. Alternatively, replacement of the glutamic acid negative charge (at position 149) by the negative sulfonate group may only need to occupy the same general area, thus positions 147 and 149 are sufficient. Furthermore, I note that mutation of position 148 to cysteine results in inhibition of the receptor after MTSES treatment, but changing the immediately flanking positions 147 and 149 to cysteine results in potentiation. This may indicate a defined structure in this sub-region of the receptor, and not an amorphous loop occupying extracellular space. Thus, positions 147, 149, and 150 may be oriented similarly while positions 146 and 148 are oriented in another direction, and attachment of MTSES in either of these orientations dictates the functional consequences of inhibition or potentiation.

**Identification of 2-nonanone as a receptor antagonist**

Evidence presented above suggests that positions 146-150 are physically close but are not directly associated with the odorant-binding site of DmOR85b. In order to investigate this notion, I sought to protect the receptor from MTSES action by occupation of the odorant-binding site. In other words, can I prevent MTSES action by co-application with a receptor ligand? Use of an agonist, such as 2-heptanone, to occupy the odorant-binding site for this experiment is problematic due to the high concentration and long application needed to achieve protection from MTSES derivatization. Therefore, I sought an antagonist-like compound that can occupy the odorant-binding site but does not activate the receptor. Based on my previous investigations on the specificity of DmORs, I reasoned that closely related compounds to known activators may also
interact with the DmOR85b odorant-binding site. 2-heptanone is the strongest known activator of this receptor (Figure 4.5), so I screened DmOR85b/83b against a panel of secondary ketones spanning from 5 to 9 carbons in length (Figure 5.19).

2-hexanone and 2-octanone were weak activators and 2-pentanone failed to activate the receptor. Surprisingly, application of 2-nonanone resulted in an upward (opposite) deflection of baseline current (Figure 5.20). Importantly, 2-nonanone did not elicit this effect in uninjected oocytes, indicating a specific interaction with the receptor. While the positive current inflection was interesting, these results also suggested 2-nonanone may be a receptor antagonist and warranted further study.

![Figure 5.19 - The odorant 2-nonanone exhibits negative efficacy on DmOR85b/83b. Quantification of current responses of DmOR85b/83b expressing oocytes challenged with a panel of ketones (applied at 1 mM, 5: 2-pentanone; 6: 2-hexanone; 7: 2-heptanone; 8: 2-octanone; 9: 2-nonanone). Zero indicates initial baseline current. Responses from each oocyte were normalized to the 2-heptanone response (data are means ± sem, n=9).](image-url)
I wished to confirm the notion that 2-nonanone is an antagonist of receptor activation. I first investigated if 2-nonanone is a direct competitor of 2-heptanone. Co-application of 1 mM 2-nonanone and 100 µM 2-heptanone results in inhibition of receptor activity elicited by 100 µM 2-heptanone alone (percent response remaining, 44.2 ± 1.7%, n=5) (Figure 5.21). This effect can be significantly diminished by increasing the concentration of 2-heptanone to 3 mM (percent response remaining, 92.8 ± 5.0, n=6, p<0.0001, unpaired t-test), suggesting the two odorants compete for the same binding space (Figure 5.21).

The results suggested that inhibition of 2-heptanone by 2-nonanone is concentration-dependent. I expanded upon this notion by performing a more complete concentration-inhibition analysis of 100 µM 2-heptanone block by 2-nonanone (Figure 5.22). This analysis revealed an apparent IC$_{50}$ of 550 ± 270 µM (n=4). Furthermore, a Hill coefficient ($n^H$) of -0.79 ± -0.2 (n=4) suggested 2-nonanone interacts with the receptor via a single binding site, similar to 2-heptanone.
Figure 5.21 - 2-nonanone competes with 2-heptanone on DmOR85b. (Left, traces) Current responses from DmOR85b/83b expressing oocytes challenged with HEP (100 or 3000 µM for 420 sec). NONA (1000 µM for 60 sec) was applied starting 240 sec after initiation of the HEP application. (Right, graph) Quantification of the remaining HEP response after NONA application as described above (bars are mean ± sem, 100 µM: n=5; 3000 µM: n=6). Statistical significance was assessed by unpaired t-test: three asterisks, p<0.0001.

Figure 5.22 - Concentration-inhibition relationship of 2-nonanone block of DmOR85b/83b activation by 100 µM 2-heptanone (IC_{50} = 550 ± 270 µM, means ± sem, n=4).
Finally, if 2-nonanone indeed competes with 2-heptanone for the odorant-binding space, then 2-nonanone should cause a rightward shift in the concentration-response curve of DmOR85b/83b activation by 2-heptanone. A 2-heptanone concentration-response curve was generated in the presence of 1 mM 2-nonanone (Figure 5.23), since this concentration elicited a reasonable degree of receptor inhibition (% response remaining of a 100 µM 2-heptanone treatment, 43 ± 2%, n=5, Figure 5.22). 2-nonanone significantly reduced the 2-heptanone sensitivity of DmOR85b/83b (apparent EC₅₀ untreated: 70 ± 20 µM, n=10; apparent EC₅₀ with 1 mM 2-nonanone treatment: 1700 ± 1020 µM, n=5; p=0.001, F-test). Importantly, block by 2-nonanone can be completely relieved by increasing the concentration of 2-heptanone to 10 mM (Figure 5.23). Taken together, these results suggest that 2-nonanone competes directly with 2-heptanone for binding to DmOR85b.

![Figure 5.23 - Dose-response relationship comparison for the activation of DmOR85b/83b by 2-heptanone untreated or in the presence of 1 mM 2-nonanone. Responses were normalized to the response elicited by 100 µM 2-heptanone for all data points (EC₅₀s, untreated: 71 ± 28 µM, n=20; 1 mM 2-nonanone treated: 1700 ± 1020 µM, n=5, means ± sem). Statistical significance was assessed by F-test of the EC₅₀ values (p=0.001).]
2-nonanone protects DmOR85b against MTSES action

The above results suggest 2-nonanone is an agonist competitor and antagonist of DmOR85b/83b activity. In this capacity, it may specifically associate with the odorant-binding space, but stabilize an inactive receptor conformation resulting in reduced receptor responsiveness. Since 2-nonanone does not elicit receptor activation, this compound should not desensitize the receptor at high concentrations. Also, 2-nonanone is larger than 2-heptanone and might be more effective at sterically inhibiting the ability of other molecules to associate with the receptor. Based on these conclusions, this odorant is ideal for use in a protection assay. I asked: can 2-nonanone inhibit the covalent association of MTSES with a residue position in the newly identified functional region of DmOR85b? Using a modified protocol (see Chapter II), I performed this experiment using the M148C C146S mutant of DmOR85b (Figure 5.24), since this receptor is robustly inhibited by MTSES (Figure 5.16) and expresses well in oocytes. As expected, M148C C146S receptor responsiveness to 100 µM 2-heptanone is significantly inhibited after pre-treatment with 1 mM MTSES for 1 minute compared to sham pre-treatment (% response remaining, 64.5 ± 3.3%, n=7, p<0.05, Bonferroni’s post-test). A pre-treatment co-application of 1 mM MTSES and 6 mM 2-nonanone, however, results in significantly reduced inhibition compared to MTSES alone (% response remaining, 102.7 ± 10.9%, n=6, p<0.05, Bonferroni’s post-test). Importantly, a pre-treatment application of 6 mM 2-nonanone does not significantly affect the receptor response to 2-heptanone compared to sham treatment (Figure 5.24). Thus, application and washout with such a high concentration of 2-nonanone does not alter the ability of the receptor to be activated by agonist. These results suggest pre-treatment of the receptor with 2-nonanone can
protect position 148 from MTSES labeling and functional inhibition. In order to confer this protection, 2-nonanone must associate with the receptor at a location near position 148. Figures 5.22 and 5.23 indicate that 2-nonanone competes directly with 2-heptanone for binding space. Taken together, the data suggest position 148 must lie close or adjacent to the odorant-binding site, which is able to be occupied by 2-nonanone.

**Figure 5.24 - 2-nonanone protects DmOR85b from MTSES.** Quantification of current responses from DmOR85b C146S M148C / DmOR83b expressing oocytes to 100 µM 2-heptanone after indicated treatment. MTSES was applied at 1 mM for 60 sec, 2-nonanone was applied at 6 mM. Sham is an ND96 application. Data are presented as means ± sem (n values from left to right: 12,7,6,8). Statistical significance was assessed by one-way ANOVA and Bonferroni’s post-test, asterisk: p<0.05.

**Identification of additional residues involved in DmOR85b activation**

The above results strongly implicate position 148 of DmOR85b, and by extension, the functionally relevant region of positions 146-150, as lying close to the odorant-binding site. However, identification of a portion of the odorant-binding site would be best demonstrated if changing the character of a binding site residue alters the
ligand specificity of the receptor. While all receptor mutants generated for the SCAM screen responded to 2-heptanone, obvious differences in functional robustness and current amplitudes were apparent. This provided circumstantial evidence that the MTS reagent may be disrupting residues involved in binding site structure. To this end, lack of an effect after MTSES treatment for some mutants may be due to restricted MTS access to target cysteines buried deep within TMS3. Consequently, residue positions involved in ligand recognition may be missed by this method. As an alternative screening method, I reasoned that comparing the relative ligand sensitivities of mutant receptors may uncover differences imparted by specific residue substitutions. Since differences in ligand preference may be subtle, I chose to screen a subset of mutant receptors for relative sensitivities to two structurally similar odorants (2-heptanol and 2-heptanone). Most likely, odorant binding occurs within the transmembrane segments, so I screened DmOR85b mutant receptors that contain residue substitutions within the extracellular half of TMS3 (positions 139-146). Receptors formed by WT DmOR85b, pseudo-WT (C146S) and most of the mutant receptors showed no preference between 2-heptanol and 2-heptanone, each applied at a concentration of 1 mM (Figure 5.25). However, two mutants (F142C and N143C) exhibited significantly altered odorant preferences, in which responses to 2-heptanone were larger than responses to 2-heptanol (indicated by normalized preference ratios <1, p<0.01 for N143C and p<0.001 F142C, Bonferroni’s post-test) (Figure 5.25). Therefore, positions 142 and 143 are implicated in odorant recognition and receptor activation.
I next investigated the mechanism for the odorant preference shift in the F142C mutant receptor. Increasing the odorant screening concentration to 10 mM resulted in a significantly diminished preference for 2-heptanone over 2-heptanol (preference ratio at 1 mM, 0.50 ± 0.04, n=18; preference ratio at 10 mM, 0.81 ± 0.06, n=5, p=0.0025, unpaired t-test) (Figure 5.26). This indicated that F142C might have a right-shifted concentration-response relationship to the odorants, compared to WT receptor. Increasing the concentration of 2-heptanol effectively compensated for this reduced sensitivity of the receptor. 2-heptanone, however, elicits the same response at 1 mM or 10 mM, indicating that 1 mM is at the top of its concentration-response curve.

Figure 5.25 - Residues 142 and 143 contribute to receptor activation of DmOR85b. Oocytes expressing WT and mutant forms of DmOR85b and DmOR83b were challenged with 2-heptanol and 2-heptanone (at 1 mM each). Data are presented as normalized odorant preference ratios between the odorants for each receptor. A ratio of <1 indicates a preference for 2-heptanone over 2-heptanol. Bars are means ± sem (n values from top to bottom: 13,6,6,5,5,18,7,7,7). Statistical significance was assessed by one-way ANOVA and Bonferroni’s post-test comparing each mutant response to WT: two asterisks, p<0.01; three asterisks, p<0.001.
I expanded upon this idea by generating concentration-response curves for 2-heptanone and 2-heptanol for both the WT OR85b receptor and the F142C OR85b receptor. In these experiments, the F142C OR85b receptor contained C146, as in WT. This allowed direct investigation of the 142 position and its effect on odorant sensitivity. The WT receptor was significantly less sensitive to 2-heptanol compared to 2-heptanone (Figure 5.27). Observing the tops of the curves, however, shows that the 1 mM test concentration used in Figure 5.25 is near saturation for both odorants, resulting in similar response amplitudes for that particular screen. Similarly, the F142C mutant was also less sensitive to 2-heptanol than to 2-heptanone, but both concentration-response curves were shifted to the right (Figure 5.27); F142C is less sensitive to both odorants compared to the WT receptor. Thus, the 1 mM test concentration used in Figure 5.25 is no longer near saturation for 2-heptanol and the response to 2-heptanol is less than the response to 2-heptanone. I conclude that odorant activation was altered upon mutation of the 142 position.

**Figure 5.26** - Increasing the ligand concentration diminishes the preference ratio for the F142C C146S DmOR85b mutant. Data are presented as normalized odorant preference ratios between the odorants (at 1 mM and 10 mM). Bars are means ± sem (n values: 1 mM = 18; 10 mM = 5). Statistical significance was assessed by unpaired t-test: two asterisks, p=0.0025.
Taken together, these results implicate position 142, and most likely 143, of DmOR85b in receptor activation after odorant exposure. Changing F142 and N143 to cysteines altered the ability of DmOR85b/83b to be activated and shifted the concentration-response relationships of the receptors for various odorants. While ligand recognition was altered in these mutants compared to WT receptor, the effect was fairly subtle. This suggests positions 142 and 143 probably do not specifically coordinate an
odorant within the binding pocket. Indeed, the fact that F142C and N143C DmOR85b subunits form functional receptors at all contributes to this argument; if these positions were critically involved in direct ligand coordination, mutations might be expected to completely abolish receptor function. Instead, positions 142 and 143 may contribute to receptor structural changes and activation. Also, mutation of these residues may alter the effectiveness of odorant to access the site or potentially de-optimizes the structure of the odorant-binding site to fit these specific odorants.

**Interpretations on odorant binding within DmOR85b**

Analysis of DmOR85b has identified residues strongly implicated in odorant recognition. These residues lie at the predicted transition from TMS3 to ECL2 (Figures 5.6 and 5.14). Treatment of WT receptor with MTSES results in partial inhibition of function through covalent modification of C146. SCAM analysis extended this study by uncovering an MTSES-susceptible region comprising residues 146-150. Finally, mutating positions 142 and 143 altered receptor activation. A common feature of these studies, however, is the partial effect of MTSES treatment or residue mutation on receptor function. I argue that alteration of residues involved in direct coordination of odorants in the binding site would result in more drastic and total functional perturbation. Rather, this region may be involved in receptor activation and conformational changes or by formation of the access pathway to the binding site.

According to transmembrane predictions, residues 142, 143,146, and perhaps 147 are part of the extracellular half of TMS3 (Figure 5.14, left). Therefore, these residues may be part of an α-helical structure. If these positions all play a role in odorant access to
the binding site, they might be positioned on the same face of the α-helix of TMS3. I attempted to strengthen this hypothesis by generating a helical wheel diagram for positions 130-147 of DmOR85b (Figure 5.28). Indeed, positions 142, 143, 146, and 147 orient to the same face of the helix. Additionally, this side of the helix carries a higher hydrophilic character compared to the opposite face (refer to color codes in the Figure 5.28 legend), suggesting this face is oriented toward a region with access to both the extracellular space and odorants.

![Helical wheel representation for residues 130-147 of TMS3 on DmOR85b.](image)

Figure 5.28 - Helical wheel representation for residues 130-147 of TMS3 on DmOR85b. The diagram is oriented with the extracellular end of TMS3 at the front of the page. Asterisks indicate residues of interest. Hydrophilic residues are circles and hydrophobic residues are diamonds. Colors indicate the degree of hydrophobicity (highest hydrophobicity is green and highest hydrophilicity is red). The helical wheel script was created by Don Armstrong and Raphael Zidovetzki, U. of California Riverside.

This interpretation reinforces the hypothesis that residues in the extracellular half of TMS3 contribute to receptor recognition of odorants, either through odor access or binding-site structure. Why then are positions 146-150 affected by MTSES treatment,
while positions 142 and 143 are not? A suitable explanation may be that the odorant access pathway is fairly constrained and narrow, limiting entrance of the sulfonate reagent but allowing access of straight chain molecules such as 2-heptanone, 2-heptanol and 2-nonanone. This structural space limitation may be caused by additional transmembrane segments which comprise the binding site structure. It should also be noted that the comparative ligand sensitivity screen does carry a trend of periodicity (Figure 5.25), as expected for a helical structure within a transmembrane segment. Mutations T141C and W140C do not change comparative ligand sensitivity, possibly due to the orientation of these residues on the opposite side of the helix (Figure 5.28). Interestingly, altered ligand specificity is also not apparent for the I139C, even though this residue is on the same face as the functionally relevant positions 142 and 143 (Figure 5.28). While the I139C mutation may not impart a noticeable change in receptor function, this position could be close to the bottom limit of the binding site or access path cavity.

If TMS3 is structurally rigid, the functionally relevant ECL2 region (positions 147-150) may be more flexible, as disordered loops are common in extracellular regions of proteins. This interpretation may help to explain observed differences in the functional effects caused by tagging specific residues with MTSES. For example, tagging position 148 or 146 with MTSES causes inhibition of receptor function, most likely by interfering with agonist access to the binding site. Orientation of these two positions may be similar with respect to the odorant access pathway. Alternatively, tagging positions 147, 149, and 150 with MTSES results in potentiation of receptor function. As described above, instead of interfering with odorant access directly, the MTS reagent may relieve a
collapsed disordered structure that inhibits odorant access in the mutant receptor. The negative charge of MTSES may also play a critical role in this effect.

### A model of DmOR85b

Examining the effect of various OR83b subunits on receptor specificity suggests that the “common” subunit is not involved in odorant recognition or binding (Figures 4.20 and 4.21). However, a discrete functional region of a specificity subunit, DmOR85b, has been identified as important in odorant binding and receptor activation. Three separate transmembrane algorithms (consensus from Swiss-Prot) strongly agree that this region lies between the extracellular half of TMS3 and ECL2. Since altering residues of TMS3 and ECL2 does not completely abolish receptor function, this infers that other regions of the receptor also contribute to the odorant-binding site. Furthermore, these identified positions in TMS3 probably do not directly coordinate the ligand, but rather contribute to overall structure of the receptor. What other regions may contribute to receptor activation? Since the C208S and C278S DmOR85b mutant receptors are more sensitive to agonist than WT (Figure 5.9), these regions of the receptor might play important functional roles. The 208 position lies within TMS4 and the 278 position lies at the predicted interface between TMS5 and ECL3 (Figure 5.6). Both of these locations may be integral in odorant recognition. However, I cannot rule out an alternative role in contributing to conformational changes that would occur upon activation. Nevertheless, this finding illustrates possible associations of residues from multiple transmembrane segments on DmOR85b in conferring odorant sensitivity. I suggest the odorant-binding site lies entirely within the specificity subunit, and contains
structural contributions from various transmembrane segments (Figure 5.29). Bioinformatic analysis of a collection of DmORs identified 12 specific residues predicted to determine odorant specificity (80). Some positions reside within the extracellular half of TMS3 (80). This data independently strengthens my findings, which implicate positions 142 and 143 of TMS3 within DmOR85b for conferring odorant sensitivity. In the same study, most of the additional residues predicted to be involved in odorant recognition lie within the extracellular portions of TMS2-6. These positions and the immediately surrounding residues represent new targets to study using site-directed mutagenesis and chimeric receptor functional comparison experiments. While odorant binding may occur within the transmembrane segments, ECL2 positions 147-150 were also functionally relevant, as indicated in my SCAM screen. This loop portion may lie directly above the binding site entrance or entry pathway, allowing for functional modulation of the receptor after modification by MTSES. The representative figure below (Figure 5.29) summarizes these ideas. The model shows an odorant-binding site as being influenced by multiple transmembrane segments and loops. This work has successfully provided the first localization of a component of an insect OR subunit that confers odorant sensitivity. Based on the information gained here, rapid experimental expansion can occur to begin the formidable task of describing the molecular basis of odorant recognition by receptors in insect olfaction.
Figure 5.29 - Schematic representation of the predicted location of the odorant-binding site of DmOR85b. TMS3 is shown as a blue cylinder. MTSES sensitive positions (red for inhibitory and green for potentiating positions) are indicated by small circles along ECL2 as it emerges from TMS3. The odorant binding space is indicated with a gray dashed-line oval.
Utility of the *Xenopus* oocyte expression system to study insect ORs

This project reports the successful heterologous expression of a variety of insect ORs in *Xenopus* oocytes. Heterologous expression is useful to study receptors in isolation, but always raises the issue of proper preservation of a normally behaving protein. Therefore, significant strides were made here to compare the specificities of heterologously expressed receptors to those expressed in insect OSNs. The extensive *in vivo* work provided by Hallem et al. (1) was used as a basis for comparison of receptor specificities for DmOR35a/83b, DmOR67a/83b, DmOR85a/83b, and DmOR85b/83b. Keeping in mind that the functional assays are quite different, I concluded that receptor specificity is accurately maintained in *Xenopus* oocytes. Importantly, no receptor expressed in oocytes gained responsiveness to odorants expected to be inactive. While the possibility of false negatives is a constant fear in screening for new receptor-ligand associations, the lack of false positives is encouraging and indicates the ability to use the *Xenopus* oocyte expression system for deorphanization of insect ORs.

To this end, the *Xenopus* oocyte system could be used to identify ligands for the 19 remaining orphan DmORs. Using a panel of 100 odors, Hallem et al. were able to deorphan 24 of the 31 *Drosophila* receptors previously shown to be expressed in antenna (1,20). Similar studies were also carried out for DmORs expressed in the larva (57). As expected from the combinatorial theory of olfactory receptor activation, most of these receptors responded to multiple odorants. Therefore, it is feasible to deorphan the remaining receptors with a similarly sized odorant panel. Hallem et al. show the broadest
activation of the DmORs with esters, alcohols, and aromatics (1). Expansion of these odor classes to create a new panel of testable compounds would provide a good strategy for future screening.

It should be noted that screening heterologously expressed receptors in oocytes may nicely complement the “empty neuron” system. Some receptors may only recognize a ligand complex of odorant and OBP, as in the pheromone detection of LUSH (13). Since the “empty neuron” approach involves the ectopic expression of DmORs in ab3a neurons, the necessary OBPs may not be expressed in these specific neurons and sensilla. Certainly a demanding experiment, it may be possible to express OBPs in oocytes and allow enrichment of OBP levels between the plasma membrane and vitelline membrane. Could these OBPs successfully interact with applied odors and be detected by DmORs? If so, the oocyte expression system would be useful to investigate OBP/odorant interactions.

**Odor-evoked currents from insect ORs**

The kinetics of odor-evoked currents from insect ORs raise intriguing questions on the nature of OR function. I observe persistent and stable currents during long-term odorant applications with little desensitization (Figure 5.6). However, the approach to a current maximum is achieved over a fairly long time-scale (seconds), especially when compared to other ligand-gated ion channels such as neuronal nicotinic acetylcholine receptors (<1 sec). Additionally, upon washout of odor, insect OR responses require several minutes to return to baseline. This effect is certainly more pronounced when high concentrations of odorant are used, probably due to inefficient washout of compounds.
that may concentrate in lipid-rich oocytes. Nevertheless, the recovery phase can be slow for the many different insect ORs described in this dissertation and tested across a range of odorant concentrations. The slow inactivation is even apparent in current recordings of DmORs expressed in HeLa cells (49). Why is the current generation and decay of insect ORs so slow? Perhaps the answer is central to the ongoing debate on the nature of insect OR channel function. As reviewed in Chapter I, a consensus proposal states that these proteins are ligand-gated ion channels capable of being heavily modulated by intracellular components (4). Upon odorant binding, the OR opens a channel pore and moves ions. But, this event could also trigger the modification of the OR complex by various intracellular molecules such as cyclic nucleotides, G-proteins, phospholipids, phosphorylation events, or ions. This modification could then allow additional structural changes in the receptor leading to increased ion flux. Similarly, the slow return to baseline after odorant is removed could be dependent on the reversal of these intracellular modifications. I note that oocytes would be required to contain similar intracellular transduction components as those found in OSNs. In experiments not included in this dissertation, I investigated the effects of specific agents such as phorbol myristate acteate and cyclic nucleotide analogs on insect OR function. Results did not yield significant effects, but these experiments were preliminary and focused only on DmOR35a/83b function. It cannot be ruled out that individual insect ORs are differentially modulated. Alternatively, the dependence on various receptor modifications on function could be experimentally tested by measuring the activation and decay slopes of odor-evoked insect OR activity after treatment with various agents. This approach might provide more sensitivity to uncover otherwise cryptic modulatory effects.
Interestingly, I have occasionally observed an oocyte-expressed OR that settles upon a new baseline after high concentration odorant application and washout. Specific batches of oocytes may exhibit this trend more prominently. This phenomenon could be a result of oocytes being unable to adequately handle the potential reversible modifications needed to deactivate the OR. Experimentally, this could be tested by showing ruthenium red treatment can block this “new baseline” and return the receptor baseline toward a pre-odorant level.

Subunit stoichiometry and OR complex organization

The insect OR is comprised of a complex of at least one specificity subunit and one “common” OR83b subunit. Stoichiometry of this complex, however, is completely unknown. This work has provided the first evidence for favoring specific complex arrangements over others, including the contribution of odorant-binding residues from only specificity subunits and the contribution of specificity subunit residues toward pore structure (Figures 4.25 and 4.26). Construction and functional testing of concatemers would help elucidate this question, but positioning of the N and C termini on opposite sides of the plasma membrane requires transmembrane linker segments. Proper folding of this type of concatemer would be difficult to achieve. Estimates of stoichiometry could be made by western blotting of complexes under native conditions. Since OR83b is slightly larger than many specificity subunits, inferences can be made on subunit contributions based on the molecular weight of the complex as a sum of individual subunit molecular weights. Perhaps most technically challenging, a series of functional mutant subunits with different channel properties can be generated and co-expressed with
WT subunits. Identification of various receptor complexes that have channel properties with a combination of WT or mutant receptor characteristics can then allow subunit contributions to be deduced. This method has been used to report the pentameric structure of nicotinic acetylcholine receptors (81). While the insect OR channel pore location is currently unknown, mutation of some residues of TMS6 in OR83b did result in altered conductance and may be useful as a starting point for mutagenesis and generation of the necessary receptors with varying conductances (45).

**Future mutagenesis studies**

I have presented evidence that positions 142 and 143 of DmOR85b contribute to receptor activation. As described above, I fail to see altered ligand sensitivity when residues below position 142 are altered to cysteine, suggesting a lower limit to a ligand-accessible space. It would be of interest to investigate this hypothesis by further mutational analysis of these positions. An example experiment would be to generate a double mutant receptor at positions 142 and 143, to see if ligand specificity can be shifted to a greater extent. Also, the lower limit of an observed shift in ligand specificity from the screen (Figure 5.25) might be changed by substituting smaller amino acids, such as glycine or alanine, at position 141. Conversely, increasing the bulk of side chains in this relevant region may result in the opposite trend. If odorant indeed passes near positions 142 and 143, placing charged residues in this region may affect ligand specificity more severely. Of course, since this is within a transmembrane segment, charged amino acids may result in substantial disruption of packed helical structure and render the receptor non-functional.
The functionally relevant residues between positions 146-150 lie at the interface of TMS3 and extend into ECL2. Treatment of specific cysteine substitution mutants with MTSES resulted in either inhibition or potentiation of receptor function. Future studies should focus on additional mutagenesis of this location as well, including double mutants. For example, D150 could be changed to a positive charge to investigate the role of electrostatic interactions in this area. Further testing with additional MTS reagents, especially the large MTS-biotin, may give clues as to what mutations should be pursued.

It should be noted that changing positions 208 and 278 to a serine results in substantial shifts in the sensitivity to odorant (approximately 5 and 28-fold, respectively). This is a fairly drastic change, indicating both positions as functionally relevant. Positions 208, 278 and the respective surrounding regions should also be targeted for future mutagenesis studies and screened for changes in sensitivity to odorant.

The odorant-binding site of other insect ORs

This work represents the first identification of residues contributing to formation of an insect OR odorant-binding site. Is this position conserved across all insect ORs? It would be useful to select a series of additional insect ORs to screen. Homologous residues to DmOR85b positions 142, 143, 278 and 146-150 could be identified by analysis of predicted topologies. Mutation of these positions in other insect ORs may then yield functional differences when compared to WT receptors. Perhaps receptors which recognize more structurally complex odors may be affected to a greater extent. If altering homologous positions in several receptors yield functional changes, it would suggest insect OR specificity subunits form ligand-binding structures in similar locations.
and it is the diversity of residues in certain positions that dictates responsiveness to individual odors.

**Inverse agonism of insect ORs**

The results presented in Chapter V provide strong evidence that 2-nonanone is an antagonist of DmOR85b/83b and competes for the same binding space as 2-heptanone. While future experiments must still rule out a general effect of odorant antagonists simply inhibiting residual currents, Figures 4.12 and 5.20 suggest odorants might be capable of inhibiting basal activity of a DmOR and decrease baseline current. This effect could be described as inverse agonism, which requires basal activity of a receptor at “rest”. Inverse agonism has been described for other receptors, such as GPCRs, which dynamically associate with signal transduction components to produce significant basal activity (82). While basal activity of ion channels is usually low, outside-out patch-clamp recordings of a DmOR do show spontaneous openings (49), suggesting these channels exhibit some degree of spontaneous activity. This may be a different type of activity compared to the basal activity potentially modulated by intracellular components (4). While 2-nonanone may be an inverse agonist of DmOR85b, the basal activity of a particular OR may be inhibited by multiple odorants, similar to receptor activation by multiple odorants. The large activation profile of 24 DmORs by Hallem *et al.* does report various odorants able to inhibit the basal firing rate of OSNs (1). Based on the data generated here, I contend the inhibitory compounds from Hallem *et al.* might be inverse agonists as well. Adding to this argument, I note that octanoic acid was reported to inhibit spontaneous activity of OSNs expressing DmOR67a/83b (1). In this work, I
provide data that octanoic acid might inhibit basal activity of DmOR67a/83b in oocytes (Figure 4.12), providing a potential link between the \textit{in vivo} data and inhibition of isolated receptor activity shown here. More work must be performed to confirm this hypothesis. Interestingly, if inverse agonism exists in heterologously expressed receptors, this raises the possibility that insect ORs are basally active without the presumed endogenous 2\textsuperscript{nd} messenger modulating systems, or that oocytes may contain the necessary components to allow basal activity. Either explanation would allow the use of oocytes for additional studies on the structural basis of inverse agonism in insect ORs.

Widespread inverse agonism in insect olfaction would strongly implicate this effect as an additional dimension for olfactory coding and processing (1). Discriminatory power in olfaction is enriched based on combinatorial processing whereby an odorant is capable of activating multiple receptors and receptors are activated by multiple ligands. This combinatorial patterning may also extend to inverse agonists. Furthermore, inverse agonists may span a range of efficacies similar to full agonists (i.e. partial inverse agonism). Blends of odorants encountered in the environment could be interpreted in widely varying ways depending on very subtle differences in odorant concentrations, or the presence of specific inverse agonists. For example, the olfactory percept of a specific blend of odorant agonists is the combined effect of these compounds on a population of receptors, and thus glomerular activation patterns. However, this percept would be very different depending on the presence of odorant receptor antagonists or inverse agonists. Thus, inverse agonism on receptors may provide another mechanism for olfactory information processing and could be an important component of the extreme sensitivity and discriminatory power of the insect olfactory system.
New insect control strategies

The control of insect behavior through interference of the insect olfactory system is currently underutilized in insect management strategies. Since insect ORs are of novel structure and humans and other mammals lack homologous receptors, these proteins could be targeted by chemical agents with higher selectivity and lower environmental toxicity than commercial insecticides. However, a significant understanding of insect OR structure is required to allow the development of new agents. The discoveries presented in this dissertation provide key advances to this end. These include the first identification of residues that contribute to formation of the odorant-binding site, and advances in elucidating the subunit contributions toward insect OR function. A firm understanding of general receptor features would allow the rational design of agents capable of interacting with specific receptors. Furthermore, understanding the behavioral circuits underlying each receptor would be of great utility.

Inhibition of receptors and attractant circuits used for searching out human targets is an obvious area of interest. This strategy may help limit the transmission of harmful diseases carried by insect vectors. For example, the *Anopheles gambiae* mosquito may use a combination of gustatory receptors AgGR22, AgGR23, and AgGR24 to detect CO2 plumes emanating from humans (60). It should be noted that information obtained from OR structure/function studies may be applicable to the GR class of chemosensory receptors, which also recognize odors. To this end, a group recently identified odorants capable of inhibiting *Drosophila* and *Culex quinquefasciatus* mosquito CO2-responsive receptors (83). Functional interference of the receptors resulted in block of the aversive behaviors mediated by the CO2 receptor behavioral circuit in *Drosophila*. Presumably,
block of the attractant CO$_2$-responsive behavioral circuit in mosquitoes would also alter
behavior. Instead of inhibition of receptor function, compounds could be designed for
specific receptor activation. These agents would lead to activation of attractive
behavioral circuits and could be employed to lure insects into traps.

As more insect genomes are sequenced and chemosensory receptor families are
uncovered, it is becoming increasingly clear that OR families have diverged greatly. For
example, while the *Drosophila*, *Anopheles* and pea aphid *Acyrthosiphon pisum* OR
families are composed of a similar number of receptors (62, 79, and 79, respectively), the
honeybee *Apis mellifera* genome has expanded to 170 OR members (84-86).
Interestingly, the pea aphid OR family is suggested to have arisen by rapid expansion
and, thus, carries few orthologs to other species’ ORs (85). This suggests that
evolutionary pressure imposed upon insect OR gene families is strong, providing a
mechanism to generate very different chemosensation strategies for the specialized needs
of insect species. I note that the relative diversity of insect OR families among species
might allow an extreme level of precision for insect control strategies. Such highly
specific agents could be quite useful in the protection of agricultural crops in which many
insect species, both detrimental and beneficial, share the same environment. Elucidation
of insect OR binding site features is a critical requirement to realize these endeavors.
REFERENCES


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