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STRUCTURE-FUNCTION ANALYSIS OF

INSECT OLFACTORY RECEPTORS

A Thesis Presented to the Faculty of

The Rockefeller University

in Partial Fulfillment of the Requirements for

the degree of Doctor of Philosophy

by

Maurizio Pellegrino

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STRUCTURE-FUNCTION ANALYSIS OF

INSECT OLFACTORY RECEPTORS

Maurizio Pellegrino, Ph.D.

The Rockefeller University 2011

Organisms use their senses to transform external stimuli into an internal representation of the world. Insects employ their keen sense of smell for a variety of tasks including location of food sources, which can vary from yeast growing on ripe fruits for the vinegar fly *Drosophila melanogaster* to mammals for blood-feeding insects such as the mosquito *Anopheles gambiae*. The first informational relay between the external environment and the organism is the olfactory sensory neuron (OSN), whose activation translates the intensity, quality, and temporal features of volatile chemicals into spike trains. This dissertation focuses on understanding how the insect olfactory system functions at the periphery, shedding light on the molecular players involved and the interactions between environmental chemicals and OSNs.

In *Drosophila*, most of the ~1,200 OSNs express members of the olfactory receptor (OR) protein family (Stocker, 1994; Vosshall et al., 1999). The functional OR complex comprises at least one variable odorant-binding subunit and one constant subunit named OR83b (Benton et al., 2006). Insect ORs have historically been grouped with mammalian and nematode ORs, both of which are G protein coupled receptors (GPCRs), whose activation leads to increased concentrations of intracellular second messengers and opening of cyclic nucleotide-gated channels (CNG; Buck and Axel, 1991; Colbert et al., 1997; Firestein et al., 1991; Nakamura and Gold, 1987; Troemel et

al., 1995). Insect ORs lack similarity to GPCRs (Benton et al., 2006; Vosshall et al., 1999), and we hypothesized that they function as odorant-gated ion channels. We showed that expression of insect ORs in heterologous cells generates odorant-evoked currents that are resistant to G protein inhibitors, independent of cyclic nucleotides, and whose properties change based on OR subunit composition (Sato et al., 2008). This surprising discovery supports our hypothesis that insect ORs are indeed odorant-gated ion channels.

Concurrently with these findings, we investigated the mode of action of DEET, the most widely used topical insect repellent, and showed that ORs are among its molecular targets. We demonstrated that DEET suppresses *Drosophila* food-seeking behavior, modulates OSN activity, and decreases OR-mediated currents in heterologous cells (Ditzen et al., 2008). Moreover, we showed that a missense polymorphism in a ligand-binding OR subunit leads to pharmacological resistance to the repellent *in vivo*. This is the first finding that identifies a molecular target of DEET.

Within the OR complex, OR83b plays an essential role. Ligand-binding subunits fail to localize properly at the OSN dendrite in the absence of OR83b, resulting in almost complete loss of sense of smell (Benton et al., 2006; Larsson et al., 2004). We identified a putative localization motif in the OR83b protein, and showed that mutations in conserved residues abolish proper OR trafficking and impair odorant-evoked responses. This discovery defines critical amino acids that might be used as possible targets of future repellents to modulate the activity of insect OSNs.

The discoveries described in this thesis will have an impact on the design of better and safer insect repellents and the control of insect-borne diseases.

Acknowledgments

Table of Contents

v

List of Figures

List of Tables

Abbreviations

2-MP – 2-methyl phenol
ab – antennal basiconic
ACIII – adenylyl cyclase III
AR – adrenergic receptor
cAMP – 3'-5'-cyclic adenosine monophosphate
CDC – Center for Disease Control and Prevention
CFTR – cystic fibrosis transmembrane conductance regulator
cGMP – 3'-5'-cyclic guanosine monophosphate
CNBD – cyclic nucleotide binding domain
CNG – cyclic nucleotide gated channel
cp – capitate peg
DAG – diacylglycerol
DEET – N,N-diethyl-3-methylbenzamide
EAG – ether-a-gogo
EPA – US Environmental Protection Agency
ER – endoplasmic reticulum
FPRL – formyl peptide receptor-like protein
FSK – forskolin
GDP-β-S – guanosine 5'-(β-thio)diphosphate
GG – Grüneberg ganglion
GPCR – G protein-coupled receptor
GR – gustatory receptor
IC - intracellular
iGluR – ionotropic glutamate receptor
IP3 – inositol-1,4,5,-trisphosphate
IR – ionotropic receptor
NA – niflumic acid
NMDG$^+$ – N-methyl-D-glucamine$^+$
mOR-EG – mouse eugenol olfactory receptor
MOS – main olfactory system
OB – olfactory bulb
OE – olfactory epithelium
OR – olfactory receptor
OSN – olfactory sensory neuron
PA – pentyl acetate
PLC – phospholipase C
PO – paraffin oil
SO – septal organ
TAAR – trace amine-associated receptor
TEVC – two-electrode voltage clamp
TM - transmembrane
TRP – transient receptor potential
VNO – vomeronasal organ
VR – vomeronasal receptor

1 Introduction to olfaction

1.1 Why do we need chemosensation?

1.1.1 Use of the chemosensory system in living organisms

Sensory systems allow organisms to perceive the surrounding world to interact with the environment and survive. The central nervous system collects stimuli and translates them into an inner representation, which is used by the organism to respond with the most appropriate behavior.

Chemosensation, the ability to sense external chemicals, is one of the oldest of our senses. Even unicellular organisms like bacteria can respond to changes in environmental chemicals through chemotaxis (Engelmann, 1883; Pfeffer, 1884), migrating up gradients of nutrients (Adler, 1966), and away from harmful stimuli like hydrogen peroxide (Benov and Fridovich, 1996). This behavior is also necessary for bacteria like *Helicobacter pylori* and *Campylobacter jejuni* to properly colonize the site of infection and become pathogenic (Foynes et al., 2000; Takata et al., 1992).

With the evolution of higher organisms, the ability to detect chemicals has developed into two systems that differentiate between water-soluble and volatile cues: the sense of taste (gustation) and smell (olfaction). This dissertation is focused particularly on the latter.

Similar to unicellular organisms, the nematode *Caenorhabditis elegans* interprets environmental cues to chemotax (Bargmann, 2006; Bargmann et al., 1993; Bargmann and Horvitz, 1991; Grewal and Wright, 1992), to avoid detrimental conditions (Pradel et

al., 2007; Troemel et al., 1997), and to alter its developmental stage depending on external circumstances (Golden and Riddle, 1982; Golden and Riddle, 1984a; Golden and Riddle, 1984b).

Although plants do not move from place to place and cannot rapidly follow gradients, they can use chemical cues to communicate over short and long distances. Infected plants, for example, can emit volatile organic compounds that are detected by nearby conspecifics, which in turn increase the expression of resistance genes and mount defense responses (Baldwin and Schultz, 1983; Engelberth et al., 2004; Karban et al., 2006; Shulaev et al., 1997; Yi et al., 2009). Moreover, it has recently been shown that the parasitic dodder plant *Cuscuta pentagona* uses volatile cues to locate and specifically recognize its tomato plant host *Lycopersicon esculentum* (Runyon et al., 2006).

In vertebrates and invertebrates, volatile signals are fundamental for survival and social interactions. They mediate choices among possible mates (Andersson et al., 2007; Bateman and Toms, 1998; Baum and Kelliher, 2009; Blows and Allan, 1998; Charpentier et al., 2008; Cross et al., 2009; Dickson, 2008; Fabre, 1911), discrimination between self and non-self (Bloss et al., 2002; Bonadonna, 2009; Carr et al., 1979; Carr et al., 1976; Thunken et al., 2009), and location of suitable oviposition sites (Elnaiem and Ward, 1992; Joseph et al., 2009; Pickett and Woodcock, 1996). Moreover, olfactory cues can elicit very strong behavioral responses. Substantial evidence has linked the innate fear responses in the rat *Rattus norvegicus* to the detection of traces of a single compound, 2,3,5-trimethyl-3-thiazoline, a component of fox predator urine (Fendt and Endres, 2008; Morrow et al., 2000). Studies in the mouse *Mus musculus* and rat have

shown how volatile chemicals are used for kin recognition (Brown et al., 1987; Todrank et al., 2005), social dominance (Drickamer, 2001; Lacey and Hurst, 2005), health status (Liberles et al., 2009; Riviere et al., 2009), and as a way for pups to recognize their own mother and initiate suckling behavior (Brake, 1981; Bruno et al., 1980; Teicher and Blass, 1976; Teicher and Blass, 1977).

Nearly every species on Earth detects and responds to volatile chemicals present in its surrounding environment, and has developed a unique chemical language that mediates a wide variety of behaviors, tuning its olfactory system to discriminate, among all possible odorants, those that are essential for its survival. Understanding how chemical cues are sensed will serve as a new Rosetta Stone that will help us decipher the complex communication networks existing in nature.

1.2 The peripheral olfactory system in commonly used model organisms

Although different species respond to chemosensory cues depending on their needs, many adopted similar solutions to sense volatile chemicals.

The mouse *Mus musculus*, the rat *Rattus norvegicus*, the nematode *Caenorhabditis elegans*, and the vinegar fly *Drosophila melanogaster* have been extensively used as model organisms to unravel the mechanisms underlying olfaction. In these species, volatile chemicals known as odorants are sensed by specialized sensory neurons that extend ciliated dendrites into an odor-rich environment. These

cells are characterized by expression of receptor proteins on their surface that directly bind odorants. Upon docking, the odorant changes the spontaneous activity of the neuron, either by increasing or inhibiting it. This represents the first step in odorant recognition, and sensory neurons are the first relay that translates the chemical signal into an electrical one by means of trains of action potentials ("spikes"). The neuronal change in activity is then interpreted by higher centers in the brain, which lead to a behavioral output of the organism.

The ligand specificity of the receptor proteins varies considerably: some are specifically tuned to recognize only one or few chemicals, while others can vary broadly in specificity, likely reflecting the biological relevance of some scents compared to the large number of potential odorants that an organism can encounter in a lifetime. Surprisingly, a single receptor can detect chemicals with fundamentally different structures. Moreover, a single compound can often be recognized by multiple receptors with different affinity (Araneda et al., 2000; Araneda et al., 2004; Hallem and Carlson, 2006; Katada et al., 2005; Malnic et al., 1999; Peterlin et al., 2005; Sengupta et al., 1996).

1.2.1 Cellular and molecular components of the peripheral olfactory system in rodents

In rodents and most mammals, the detection of olfactory cues is divided among functionally and anatomically distinct organs: the main olfactory system (MOS), the accessory olfactory system or vomeronasal organ (VNO), the septal organ (SO), the Grüneberg ganglion (GG), and the guanylyl cyclase-D-expressing cells (Figure 1.1A).

The MOS and SO are activated by a vast variety of volatile chemicals (Breer et al., 2006; Buck, 1996; Kaluza et al., 2004; Sicard and Holley, 1984; Tian and Ma, 2004). Many substances conveying social and sexual signals, called pheromones, are sensed mainly by the VNO (Doving and Trotier, 1998; Halpern, 1987; Holy et al., 2000; Leinders-Zufall et al., 2000; Stowers et al., 2002; Wysocki and Lepri, 1991), as are cues from other species called kairomones (Sam et al., 2001; Spehr et al., 2006; Trinh and Storm, 2003; Wang et al., 2006; Xu et al., 2005). The functional relevance of the GG is still under investigation, although it has recently been shown that it can detect an alarm pheromone (Brechbuhl et al., 2008) and low ambient temperatures (Mamasuew et al., 2008), potentially functioning as a way for the pups to remain close to their mothers.

In the MOS, odorants inhaled through the nose are detected by the olfactory epithelium (OE). Located on the roof of the nasal cavity, it contains the olfactory sensory neurons (OSN), whose dendrites lie in the OE mucus. Each of these primary sensory neurons expresses only one type of odorant receptor gene (OR) on its surface [a discovery formalized as the "one neuron—one receptor" rule (Axel, 2005; Buck and Axel, 1991; Chess et al., 1994; Malnic et al., 1999)]. The axons of neurons expressing the same OR converge into spherical structures known as glomeruli in the olfactory bulb (OB), where the information from the periphery is related to higher brain centers (Figure 1.1B).
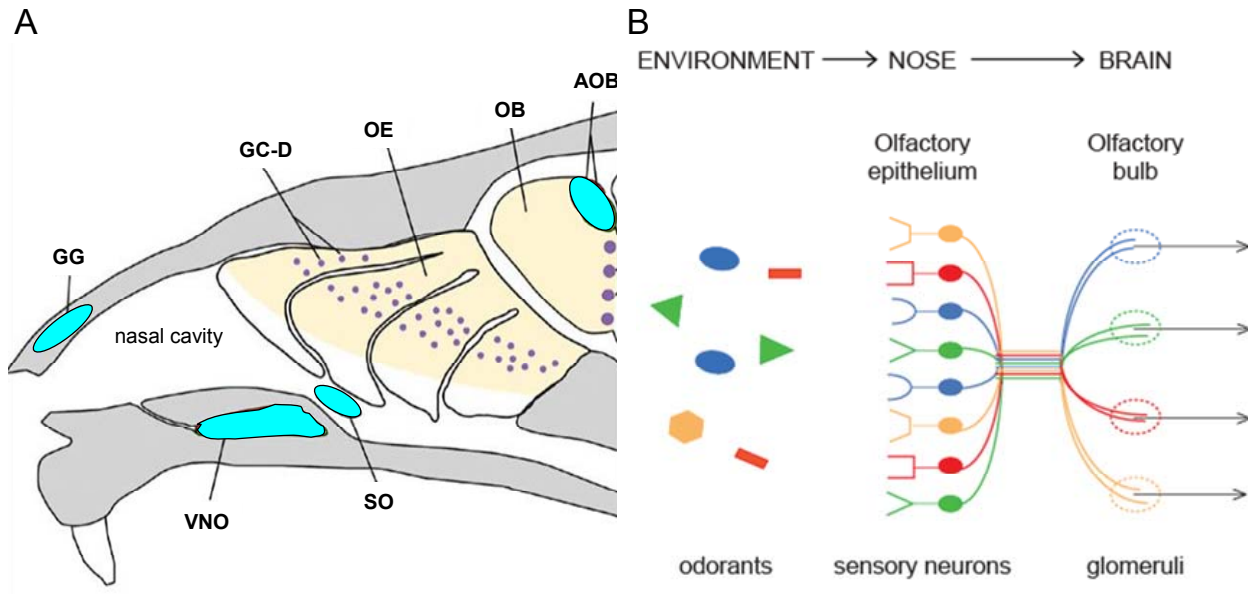
Figure 1.1 Anatomy of the mouse olfactory subsystems.
(**A**) Sagittal view of a rodent head, representing the different organs involved in odorant detection. AOB, accessory olfactory bulb; GC-D, guanylyl cyclase-D; GG, Grüneberg ganglion; MOS, main olfactory system; OB, olfactory bulb; OE, olfactory epithelium; SO, septal organ; VNO, vomeronasal organ. Adapted from Brennan and Zufall (2006). (**B**) Schematics of the connections between sensory neurons in the olfactory epithelium and the glomeruli in the olfactory bulb. Adapted from Pellegrino and Nakagawa (2009).

The molecular identity of the receptors underlying the sense of smell was elucidated in 1991, with the groundbreaking discovery of the olfactory receptor protein family by Buck and Axel (Buck and Axel, 1991). In the mouse and rat, this protein family comprises ~1,000 different members (Zhang and Firestein, 2002; Zhang et al., 2004; Zhang et al., 2007) expressed in peripheral neurons of the OE (Buck and Axel, 1991), the SO (Kaluza et al., 2004; Tian and Ma, 2004), and in specific areas of the GG (Fleischer et al., 2006). These proteins contain seven transmembrane domains and

belong to the rhodopsin superfamily of G protein-coupled receptors (GPCRs; Buck and Axel, 1991).

Upon ligand binding, GPCRs activate a heterotrimeric G protein, a complex composed of α, β, and γ subunits with GTPase activity. Depending on the type of α subunit, G proteins can be divided into four major subfamilies, the $G\alpha_{i/o}$, $G\alpha_q$, $G\alpha_{12/13}$, and $G\alpha_s$ groups, each one coupled to a different downstream pathway (Alberts et al., 2002).

In the main olfactory system, odorant binding activates the OR, which stimulates G_{olf}, an OSN-specific $G\alpha_s$ protein (Jones and Reed, 1989). This triggers rapid synthesis of 3'-5'-cyclic adenosine monophosphate (cAMP) by adenylyl cyclase III (ACIII; Pace et al., 1985; Sklar et al., 1986), and opening of the cAMP-sensitive Na^+/Ca^{2+}-permeable cyclic nucleotide gated channel (CNG) CNGA2/A4/B1 (Firestein et al., 1991; Nakamura and Gold, 1987). The resulting influx of Ca^{2+} into the neuron (Frings et al., 1995; Leinders-Zufall et al., 1997) opens Ca^{2+}-dependent chloride conductances, likely through the ANO2 protein (Figure 1.2; Kurahashi and Yau, 1993; Lowe and Gold, 1993; Stephan et al., 2009). Both entry of Ca^{2+} and outflow of Cl^- are responsible for the depolarization of the neuron and generation of action potentials.
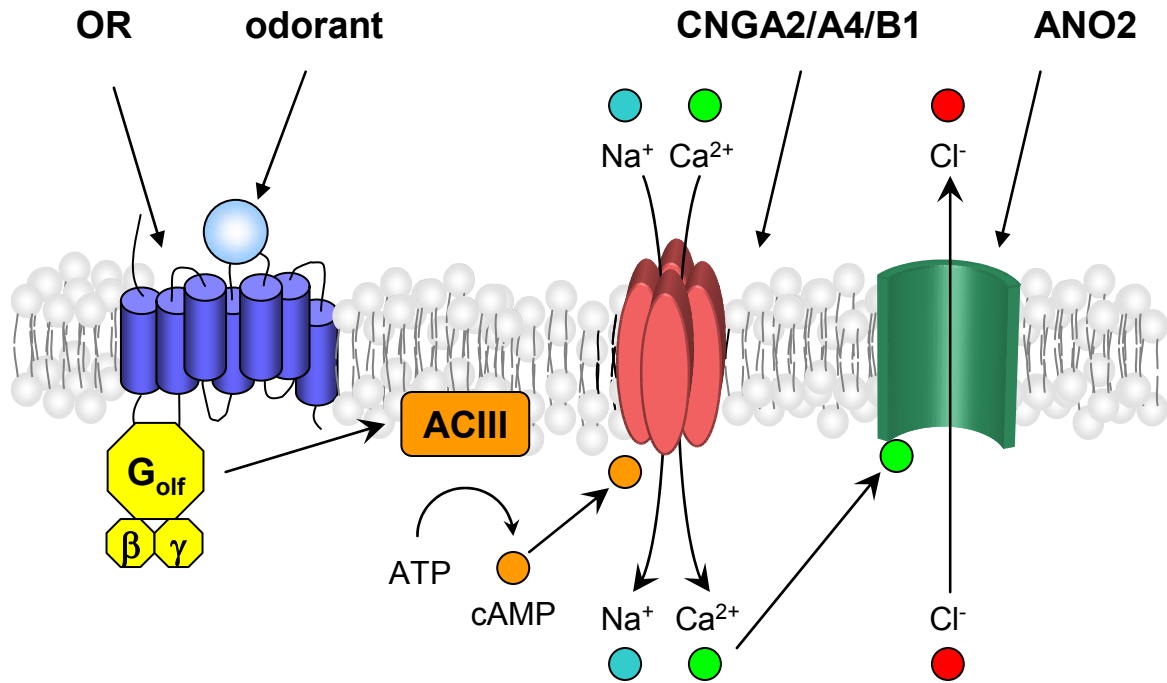
Figure 1.2 Signal transduction cascade in mammalian OR-expressing neurons.
Binding of an odorant to the olfactory receptor (OR) activates the G protein G_{olf} which in turn stimulates production of cAMP through adenylyl cyclase III (ACIII). This leads to the opening of a cAMP-gated CNG channel (CNGA2/A4/B1). The influx of Ca^{2+} through the CNG channel opens the chloride channel ANO2.

ORs are not the only receptors expressed in the OE. The trace amine-associated receptors (TAARs) represent a second class of chemosensory molecules that, similar to ORs, conform to the "one-neuron one-receptor" rule (Borowsky et al., 2001; Fleischer et al., 2007; Liberles and Buck, 2006). There are 15 TAAR genes in the mouse (Borowsky et al., 2001). Also found in some GG neurons (Fleischer et al., 2007), their transduction mechanisms are likely to be G_{olf} and cAMP-mediated (Liberles and Buck, 2006). This protein family recognizes volatile amines present in mouse urine, such as β-phenylethylamine, isoamylamine, and trimethylamine, which have been previously associated with the communication of social signals like stress levels (Dourish et al.,

1982; Paulos and Tessel, 1982) and sexual maturity (Liberles and Buck, 2006; Price and Vandenbergh, 1992).

One population of olfactory neurons in the MOS does not express the elements of the cAMP-mediated pathway, but is identified by the presence of the guanylyl cyclase GC-D (Fulle et al., 1995), the 3'-5'-cyclic guanosine monophosphate (cGMP)-dependent phosphodiesterase PDE-2 (Juilfs et al., 1997), and a cGMP-sensitive CNG channel (Meyer et al., 2000). These neurons are thought to respond to mouse urinary peptide hormones (Leinders-Zufall et al., 2007) and CO_2 (Hu et al., 2007), but the molecular identity of receptors expressed in these cells, if they are not the GC-D itself, is still unknown.

The VNO, located within the nose septum, contains sensory neurons whose axons extend to glomeruli in the accessory olfactory bulb (Figure 1.1A). With the exception of a few sporadic neurons, the VNO does not express members of the OR and TAAR families. Instead, the sensitivity of the VNO to chemosensory signals is provided by three different classes of GPCRs, the vomeronasal receptor superfamilies V1Rs (Dulac and Axel, 1995; Pantages and Dulac, 2000; Rodriguez et al., 2002; Zhang et al., 2007) and V2Rs (Herrada and Dulac, 1997; Matsunami and Buck, 1997; Ryba and Tirindelli, 1997), and the formyl peptide receptor-like proteins (FPRL; Liberles et al., 2009; Riviere et al., 2009). The mouse genome has about 200 V1R genes (Zhang et al., 2004; Zhang et al., 2007), 100 V2R genes (Shi and Zhang, 2007; Yang et al., 2005), and seven FPRL genes (Liberles et al., 2009; Riviere et al., 2009). As is seen with receptors expression in OSNs of the MOS, the three VNO receptor families are

expressed in non-overlapping zones and each neuron expresses only one receptor type (Dulac and Axel, 1995; Dulac and Axel, 1998; Liberles et al., 2009; Riviere et al., 2009). The types of ligands detected by these three gene families also appear to be distinct: V1Rs detect volatile chemicals (Del Punta et al., 2002; Leinders-Zufall et al., 2000), while V2Rs respond to small peptides (Chamero et al., 2007; Kimoto et al., 2005; Kimoto et al., 2007; Leinders-Zufall et al., 2004), and both can be activated by sulphated steroids (Nodari et al., 2008). The ligands activating FPRLs are less well understood, but one group has provided evidence that FPRLs detect ligands related to disease and inflammation status of the individual (Riviere et al., 2009). All three VNO receptor families are implicated in social communication in mice (Chamero et al., 2007; Hurst et al., 2001; Kimoto et al., 2005).

The V1R family is co-expressed with the $G\alpha_{i2}$ protein subunit and V2R-expressing cells co-express the $G\alpha_o$ subunit (Berghard and Buck, 1996; Berghard et al., 1996; Herrada and Dulac, 1997; Jia and Halpern, 1996). In both cases, ligand binding is thought to activate the corresponding $G\alpha$ subunit, which then detaches from the $G\beta\gamma$ components allowing the $G\beta$ protein to activate phospholipase $C\beta2$ ($PLC\beta2$; Runnenburger et al., 2002). This event triggers the generation of downstream products such as inositol-1,4,5,-trisphosphate (IP3) and diacylglycerol (DAG; Krieger et al., 1999; Kroner et al., 1996; Sasaki et al., 1999; Wekesa and Anholt, 1997). DAG mediates the opening of the transient receptor potential channel C2 (TRPC2; Lucas et al., 2003). The influx of Na^+ and Ca^{2+} through this channel is responsible for the depolarization of the neuron (Figure 1.3A and B). Recently, a TRPC2-independent pathway involving

arachidonic acid has been proposed as an alternative transduction mechanism (Spehr et al., 2002; Zhang et al., 2010).

FPRL-positive neurons show co-expression of either $G\alpha_{i2}$ or $G\alpha_o$ subunits. Therefore, PLC is thought to be involved in the signaling cascade following receptor activation in these cells as well (Liberles et al., 2009).

Although unrelated, the five GPCR families employed by the mammalian olfactory system rely on G protein-dependent transduction mechanisms to trigger neuronal activity.
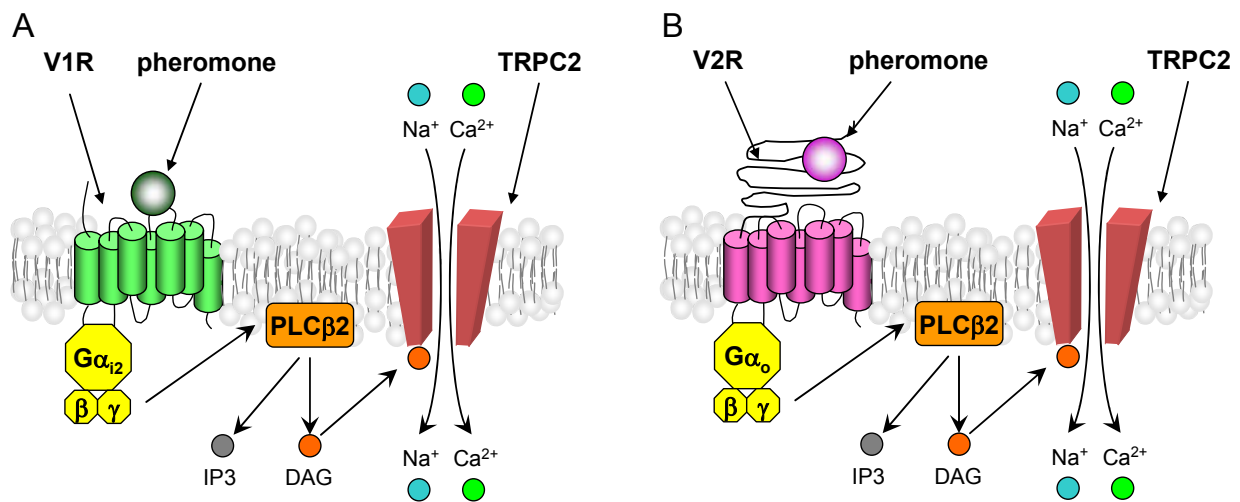
Figure 1.3 Signal transduction cascade in mammalian vomeronasal neurons.
(**A**) Signal transduction in V1R-expressing neurons. Upon pheromone activation, the $G\alpha_{i2}$ protein induces production of inositol-1,4,5,-trisphosphate (IP3) and diacylglycerol (DAG) through the phospholipase Cβ2 (PLCβ2). Subsequent opening of transient receptor potential C2 (TRPC2) causes influx of cations and neuronal depolarization. (**B**) Signal transduction in V2R-expressing neurons. The activated receptor stimulates a $G\alpha_o$ protein. This leads to production of DAG through PLCβ2 and opening of the TRPC2 channel.

1.2.2 Cellular and molecular components of the peripheral olfactory system in the nematode *Caenorhabditis elegans*

Like that of mammals, chemoreception in *Caenorhabditis elegans* is confined within distinct groups of cells: the amphid, inner labial, and phasmid neurons (Ward et al., 1975; Ware et al., 1975) which together total 32 neurons involved in chemosensory detection. Sensory cilia of these neurons are either enclosed within a sheathing cell or are exposed directly to the environment (Bargmann, 2006; Perkins et al., 1986; Ward et al., 1975), and axons are connected to the nerve ring, the largest neuropil in the head of the worm (Figure 1.4; Ward et al., 1975; Ware et al., 1975).

The nematode genome contains over 1,000 rhodopsin-related GPCRs that may function as chemoreceptors (Chen et al., 2005; Colosimo et al., 2004; Sengupta et al., 1996; Troemel et al., 1995; Zhang et al., 1997), representing about 7% of all worm genes (Robertson and Thomas, 2006). Worm chemosensory receptor genes can be grouped into eight different families, most distantly related to vertebrate GPCRs (Robertson and Thomas, 2006). Given the large number of putative chemoreceptors and the limited number of sensory neurons, it is not surprising that, unlike mammalian OSNs, *Caenorhabditis elegans* olfactory neurons express multiple chemoreceptors with different ligand specificities (Battu et al., 2003; Chen et al., 2005; Robertson and Thomas, 2006; Sengupta et al., 1996; Troemel et al., 1995).
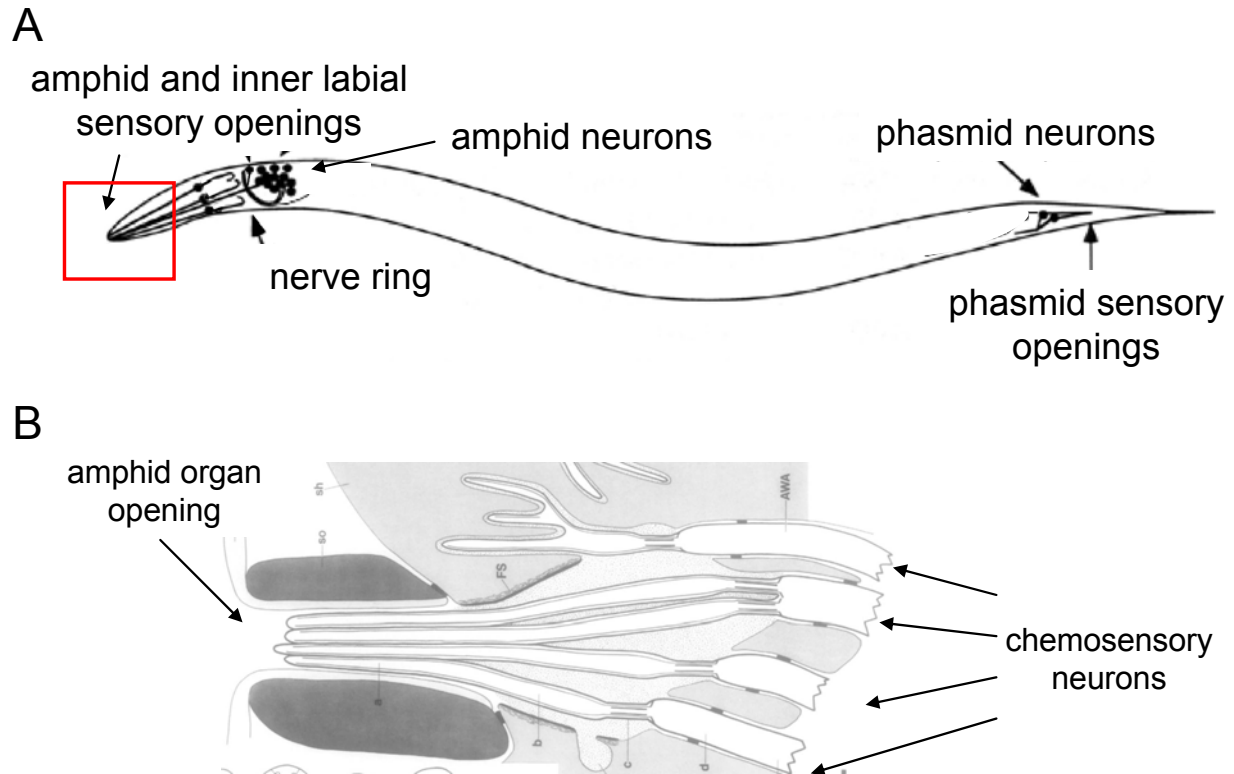
Figure 1.4 Anatomy of the nematode olfactory subsystems.

(**A**) Location of chemosensory neurons in *Caenorhabditis elegans*. Circled in red is the amphid sensory organ. Adapted from Bargmann (2006). (**B**) Details of one of the two amphid organs circled in red in **A** showing the chemosensory neurons projecting towards the amphid organ opening. Adapted from Perkins et al. (1986).

Substantial lines of evidence suggest that there are two transduction mechanisms following ligand binding to nematode chemosensory receptors. Genetic and gene expression analysis indicate that the $G\alpha_i$-like protein ODR-3 plays a major role downstream of odorant receptors for proper odorant-evoked responses (Lans et al., 2004; Roayaie et al., 1998). Subsequent inhibition of a cGMP phosphodiesterase, or possible activation of the guanylyl cyclases ODR-1 and DAF-11 (Birnby et al., 2000; L'Etoile and Bargmann, 2000; Torayama et al., 2007; Vowels and Thomas, 1994), leads to increased intracellular levels of cGMP and opening of the cGMP-gated CNG channel

TAX-2/TAX-4 (Coburn and Bargmann, 1996; Coburn et al., 1998; Komatsu et al., 1999; Komatsu et al., 1996), followed by cation influx and neuronal depolarization (Figure 1.5A). A second G protein-dependent mechanism in a different pool of sensory neurons involves the $G\alpha_i$–like ODR-3 and GPA-3 proteins. Following G protein activation, production of polyunsaturated fatty acids through yet unknown mechanisms opens the TRPV cation channel OSM-9/OCR-2 (Colbert et al., 1997; Kahn-Kirby et al., 2004; Tobin et al., 2002), and causes neuronal depolarization (Figure 1.5B). Analogous to mammals, *Caenorhabditis elegans* relies on its olfactory system for survival and employs a structurally unrelated family of GPCRs to detect volatile chemicals.
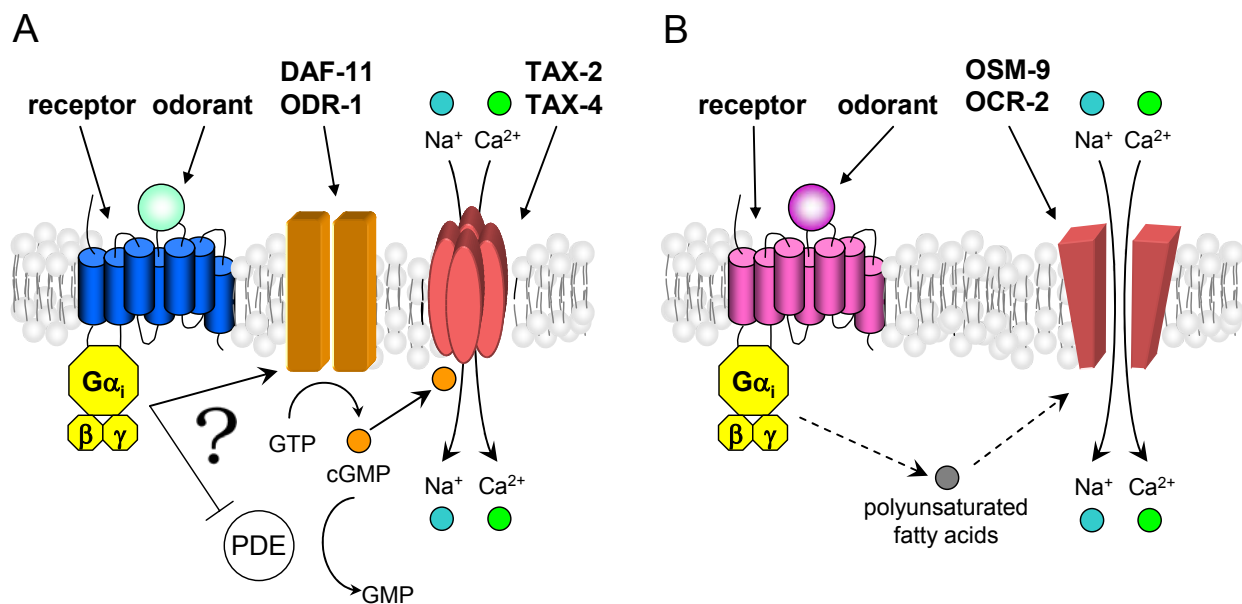
Figure 1.5 Signal transduction cascade in nematode chemoreceptor neurons.
(**A**) Some chemoreceptors activate the $G\alpha_i$ protein ODR-3 which regulates the production of cGMP through activation of the guanylyl cyclases DAF-11 and ODR-1 or inhibition of a phosphodiesterase. In either case, increase in cGMP concentrations opens the CNG channel TAX2/TAX-4. (**B**) An alternative signaling pathway involves the activation of the $G\alpha_i$ protein ODR-3 or GPA-3 and production of polyunsaturated fatty acids, which eventually leads to opening of the TRPV channel OSM-9/OCR-2.

1.2.3 Cellular and molecular components of the peripheral olfactory system in insects

In adult insects, olfactory sensory neurons are located in the antennae and maxillary palps (Keil, 1999), two pairs of appendages protruding from the head (Figure 1.6A). Sensory neurons extend their dendrites into hair-like structures, called sensilla. Each sensillum houses between one and 50 neurons, depending on the species (Esslen and Kaissling, 1976; Ochieng et al., 1998). The sensillar cuticle is perforated by pores (Riesgo-Escovar et al., 1997a; Riesgo-Escovar et al., 1997b; Stocker, 2001) through which odorants dissolve into the fluid lymph surrounding the OSNs. In the vinegar fly *Drosophila melanogaster*, the shape and location of each sensillum is stereotyped across individuals. All *Drosophila* sensilla have been characterized electrophysiologically for responses to odorants and subsequently classified into distinct groups based on their response profiles (de Bruyne et al., 2001; van der Goes van Naters and Carlson, 2007; Yao et al., 2005). Antennal sensilla are morphologically and functionally divided in 10 distinct types of club-shaped basiconic (antennal basiconic or ab sensilla, numbered ab1 to ab10), four types of sharp-tipped trichoid sensilla (at1-at4), and four dome-shaped coeloconic sensilla (ac1-ac4; Benton et al., 2009; Couto et al., 2005; de Bruyne et al., 2001; Shanbhag et al., 1999; Shanbhag et al., 2000; Stocker, 2001; Yao et al., 2005). Maxillary palps are anatomically simpler and contain only three types of basiconic sensilla (palp basiconic or pb1-pb3; Figure 1.6B; Couto et al., 2005; Shanbhag et al., 1999).
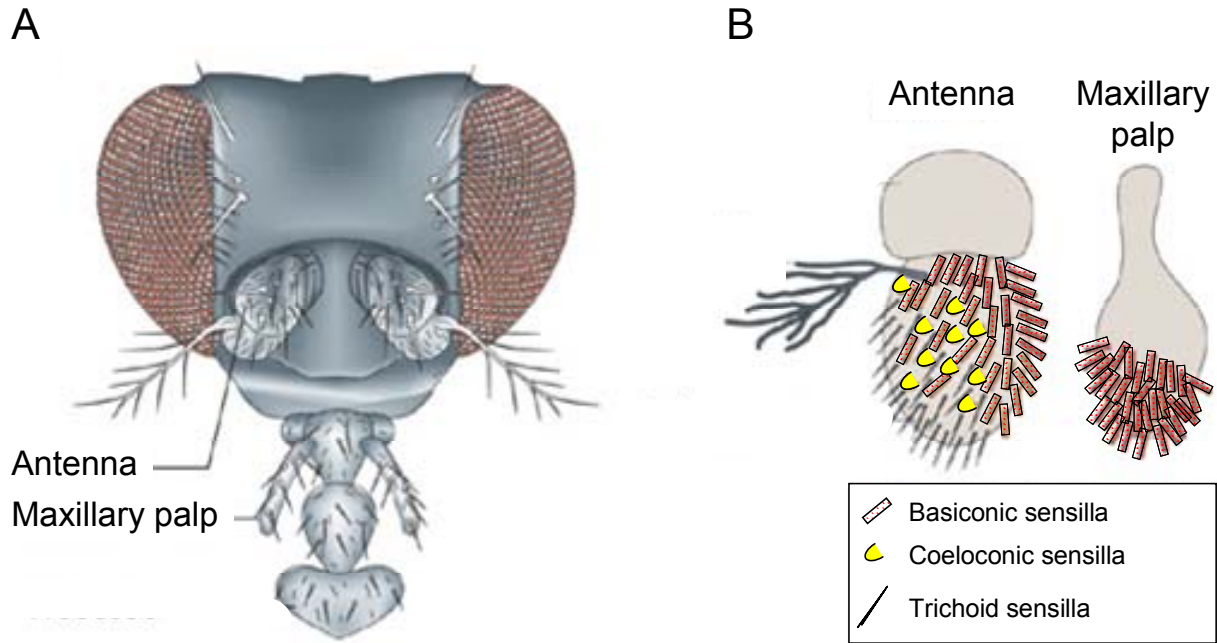
Figure 1.6 Anatomy of the insect peripheral olfactory system.
(**A**) Head of an adult vinegar fly *Drosophila melanogaster*. The two olfactory organs, the antenna and maxillary palp, are indicated. (**B**) Schematic representation of the types of sensilla covering the olfactory organs in the vinegar fly. Adapted from Kaupp (2010).

For the past 50 years, neurophysiological and behavioral research in insect olfaction focused on large insects such as moths (Boeckh et al., 1960; Boeckh et al., 1965; Fabre, 1911; Schneider et al., 1964), honeybees (Boeckh et al., 1965; Esslen and Kaissling, 1976; Kaissling and Renner, 1968), locusts (Blaney, 1977; Ochieng et al., 1998), and beetles (Inouchia et al., 1987; Merivee et al., 2001; Merivee et al., 2002). However, the molecular basis of insect olfaction has begun to be elucidated only in the past 10 years, thanks to the discovery of two unrelated protein families expressed in OSNs of the vinegar fly *Drosophila melanogaster*: the olfactory receptors (ORs; Clyne et al., 1999; Gao and Chess, 1999; Vosshall et al., 1999) and the ionotropic receptors (IRs; Benton et al., 2009).

1.3 The molecular constituents of the insect olfactory sensory system

1.3.1 Insect IR and OR families: speculation on their origin, evolution, and function

In *Drosophila melanogaster*, IRs include 61 genes with similarity to ionotropic glutamate receptors (iGluR; Benton et al., 2009). Classic iGluRs function in the central nervous system to bind to the neurotransmitter glutamate, and are characterized by two glutamate-binding modules separated by an ion channel pore (Mayer, 2006). Despite the conserved organization with iGluR structural domains, IRs lack the critical residues coordinating the glutamate, and have been shown to detect volatile chemicals instead (Benton et al., 2009). IRs are expressed in sensory dendrites of coeloconic sensilla, as well as gustatory neurons in the proboscis and mechanosensory neurons (Benton et al., 2009). In addition, two IRs, IR8 and IR25a, are widely expressed in overlapping neuronal populations (Benton et al., 2009). The connection of IR-expressing OSNs to higher brain areas has not been fully described, but neurons expressing a single IR converge their axons to a single antennal lobe glomerulus (Benton et al., 2009). It will be fascinating to discover how the presence of multiple IRs in a single cell affects the wiring to higher brain centers and how this impacts the fly's ability to discriminate odorants.

The sequence identity across the IR family ranges between 10% to 70% in *Drosophila melanogaster*, with the highest conservation in the channel pore region (Benton et al., 2009). This strongly suggests that IRs maintain the ability to function as

ion channels upon ligand binding, similar to the canonical iGluR family members in the NMDA, AMPA, and kainate receptor subtype families. It is unknown if the influx of cations depolarizes the neuron directly, or indirectly by activating other ion channels through still undefined mechanisms.

The presence of IRs in organisms lacking a nervous system, like the plant *Arabidopsis thaliana* (Chiu et al., 1999; Lam et al., 1998), can now be revisited. It is tempting to speculate that IRs could have odorant-sensing functions in plants and might mediate, for example, the attraction of the parasitic dodder plant *Cuscuta pentagona* to its tomato plant host (Runyon et al., 2006).

In summary, while vertebrates use iGluRs as a means of neural communication system between neurons, insects have evolved additional iGluR-like genes to function as chemosensory receptor family enabling communication between neurons and the external environment.

ORs are structurally different from IRs and represent an insect-specific seven transmembrane domain protein family, with 50-250 members in each insect species that has been examined so far (Nei et al., 2008). The genome of *Drosophila melanogaster* contains 62 OR genes (Clyne et al., 1999; Gao and Chess, 1999; Vosshall et al., 1999). OR expression does not overlap with IR-containing neurons [with one exception (Benton et al., 2009)] and ORs are expressed in all trichoid OSNs, one coeloconic OSN, and all basiconic OSNs except the ab1C neuron that is sensitive to carbon dioxide (Couto et al., 2005; Hallem et al., 2004a). OR family members are extremely variable in sequence within and across species (Clyne et al., 1999; Hill et al., 2002; Vosshall et al.,

1999) and, in *Drosophila melanogaster*, their amino acid identity ranges between 15% and 30% between each pair (Vosshall, 2003).

The molecular and anatomical organization of OR-expressing cells shows some similarities with their mammalian counterparts: although most peripheral neurons express on the surface only one type of ligand-specific receptor gene, respecting the "one neuron—one receptor" rule (Clyne et al., 1999; Gao and Chess, 1999; Vosshall et al., 1999), there are 13 known cases of multiple receptors co-expressed in a single neuron (Couto et al., 2005; Fishilevich and Vosshall, 2005; Vosshall and Stocker, 2007). Neurons expressing the same odorant receptor target the same glomerular structures in the antennal lobe (Couto et al., 2005; Fishilevich and Vosshall, 2005), the insect olfactory bulb, in an anatomical parallel to the glomeruli found in the mammalian olfactory system. The anatomical similarities between the mammalian and insect olfactory systems, the presence of seven transmembrane domains, and the fact that previous work in mammals and nematodes identified G protein coupled receptors as olfactory sensors led to the erroneous classification of insect ORs as GPCRs.

1.3.2 The unusual receptor OR83b

OR83b, a member of the OR family, is different from other olfactory receptors in that it is expressed in OSN along with a neuron-specific conventional OR that interacts with odorant ligands. In addition, it is the only OR whose protein sequence is extremely conserved across species. In fact, OR83b amino acid sequences from 15 insect species, separated by 350 million years of evolution (Grimaldi and Engel, 2005; Hennig, 1981), share on average 75% identity (Figure 1.7). As a consequence, OR83b orthologues

from other species can functionally substitute for *Drosophila* OR83b (Jones et al., 2005),

highly suggestive of an exceptional and conserved role for this protein in insect olfaction.
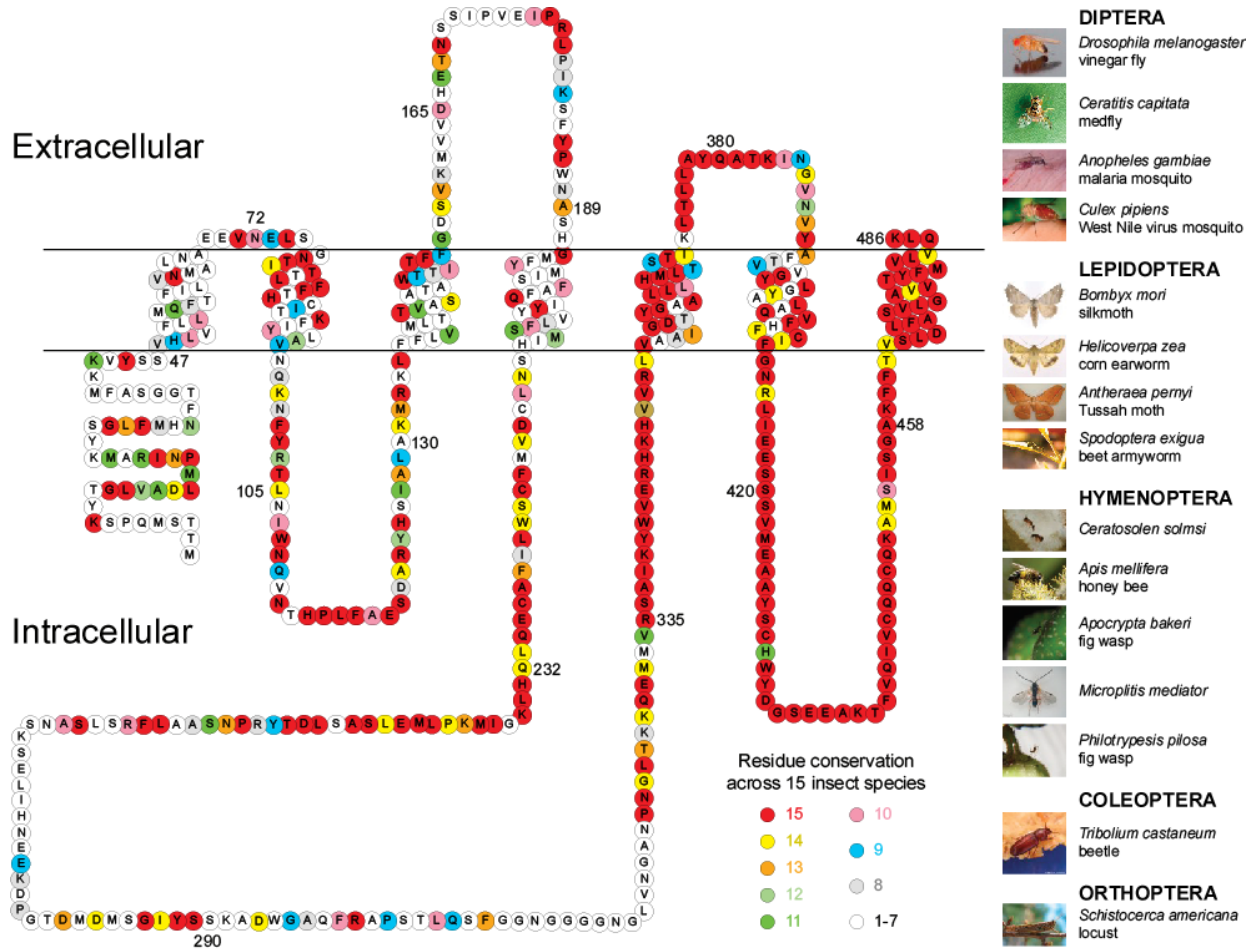
Figure 1.7 Snake plot of *Drosophila melanogaster* OR83b.
Each amino acid residue is color coded according to the degree of identity across the
15 insect species pictured in the right panel.

Although co-expressed with other ORs, this atypical receptor does not seem to

be involved in specific odorant recognition (Dobritsa et al., 2003; Hallem et al., 2004a).

Instead, experimental evidence has shown that OR83b is necessary and sufficient to

mediate oligomerization with ligand-specific ORs, which is fundamental for proper

targeting to cilia of olfactory neurons (Figure 1.8; Benton et al., 2006). *Or83b* null

mutants lack functional ORs on the ciliated dendrite and are therefore seriously

impaired in olfactory behavior and physiology (Larsson et al., 2004).
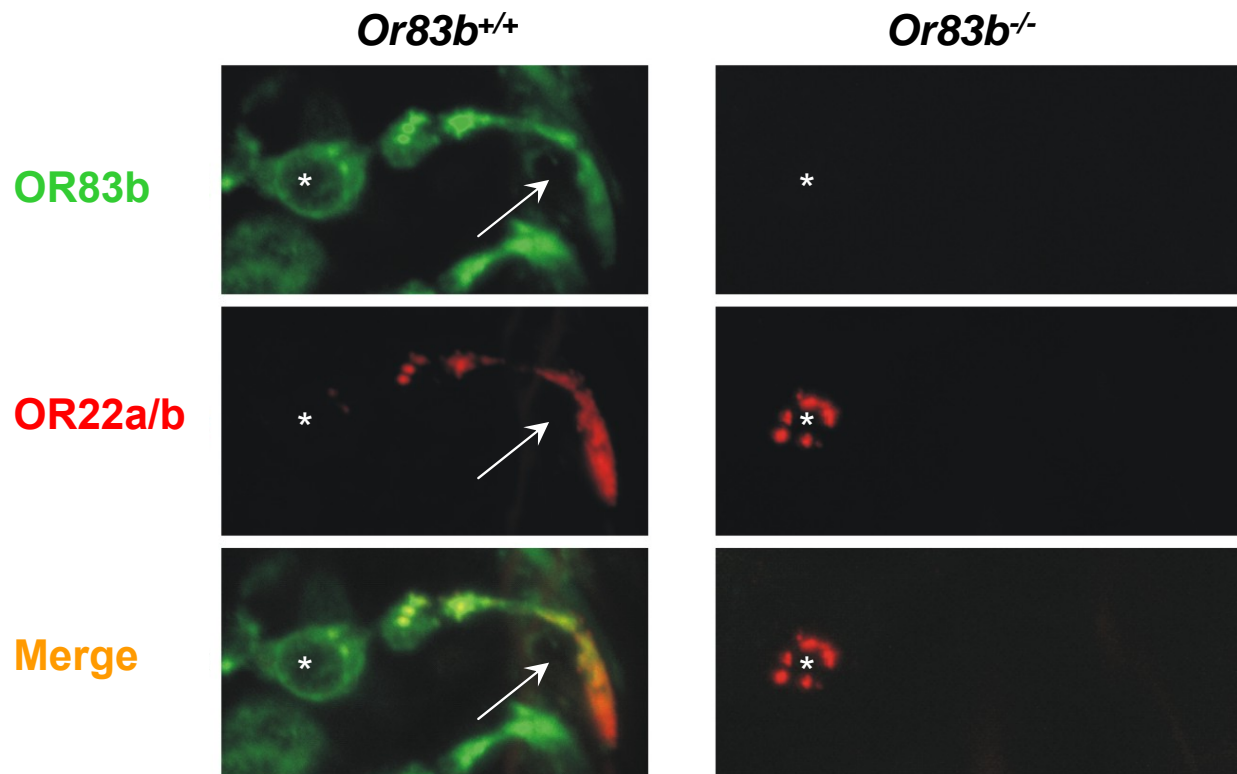
Figure 1.8 OR83b is necessary for proper localization of OR22a/b.

Immunostaining for OR83b (green) and OR22a/b (red) in sections of adult antennae of

wild type (*left panel*) and *Or83b* knock-out flies (*right panel*). The asterisk indicates the

olfactory neuron cell body, while the arrow points at its ciliated dendrite. Adapted with

permission from Benton et al. (2006).

1.3.3 The topology and subunit association of insect ORs

Mammalian and nematode olfactory receptor proteins belong to the seven

transmembrane domain superfamily of rhodopsin-like GPCRs, their N-terminals

exposed to the extracellular environment and the C-terminal lying intracellularly (Palczewski et al., 2000).

In contrast, bioinformatics, cellular, and glycosylation studies (Benton et al., 2006; Lundin et al., 2007; Wistrand et al., 2006) have shown that OR83b and other ORs (Jordan et al., 2009; Smart et al., 2008) adopt an inverted topology, questioning the general assumption that insect ORs are GPCRs (Figure 1.7). Although the precise stoichiometry of the complex has not been explicitly determined, interactions that promote the assembly of OR/OR83b complexes are likely to occur in the C-terminal half of the protein, as shown by yeast two-hybrid and chimeric receptor analysis (Benton et al., 2006). Furthermore, protein complementation assays *in vivo* have shown that at least two OR83b and two ligand-specific subunits lie in close proximity within the membrane, suggesting that the functional complex is a heteromultimer (Benton et al., 2006).

OR83b is thus likely to play a major role as an intermediary between a diverse family of receptors and a common transduction mechanism, and a structure-function analysis of this protein is needed to unveil its function in the receptor complex.

1.3.4 Evidence for and against involvement of G proteins in insect olfactory sensory neurons *in vivo*

There is extensive and compelling evidence for the role of G proteins in the signal transduction of both the mammalian and nematode olfactory systems. In contrast, studies on the signaling pathways of insect olfactory receptors have come to contradictory conclusions.

Localization studies in moth and mosquito species of different $G\alpha$ subunits showed a generalized expression in olfactory organs, suggesting that G proteins may be involved in the transduction mechanisms. $G\alpha_q$ and $G\alpha_s$ are present in dendrites of olfactory sensory neurons, where the signaling cascade following ligand activation begins, while $G\alpha_o$ is localized to the nerve bundle (Jacquin-Joly et al., 2002; Laue et al., 1997; Miura et al., 2005; Rutzler et al., 2006), suggesting a minor role in the initiation of signal transduction. Similar experiments in *Drosophila melanogaster* have revealed generalized expression of all $G\alpha$ subunits types in the olfactory organs, including glia and support cells surrounding the sensory neurons (Boto et al., 2010; Talluri et al., 1995).

Although important in mammalian olfaction, the role of $G\alpha_s$/cAMP in insect olfactory transduction seems to be minor. Independent studies failed to observe production of cAMP following pheromone stimulation of cockroach and moth antennae (Boekhoff et al., 1993; Breer et al., 1990; Ziegelberger et al., 1990). In addition, reduction of cAMP levels by genetic manipulations in *Drosophila* olfactory neurons impairs behavioral responses to some, but not all, odorants (Gomez-Diaz et al., 2004; Martin et al., 2001). But even these effects are very mild when compared to the striking reduction of odorant-evoked responses in the absence of G_{olf} in mice (Belluscio et al., 1998). Similarly, inhibition of $G\alpha_o$ signaling by pertussis toxin in OR83b-expressing neurons does not abolish olfactory responses, but decreases sensitivity to odorants and

possibly extends the response duration (Chatterjee et al., 2009), possibly suggesting a modulatory role of the odorant responses.

On the other hand, there is some evidence for the involvement of $G\alpha_q$/PLC/IP3– dependent pathways in the insect olfactory signaling cascade. Several studies reported production of IP3 and cGMP and the presence of a cGMP-activated ion channel upon pheromone stimulation of moth or cockroach antennae (Boekhoff et al., 1993; Boekhoff et al., 1990; Breer et al., 1990; Stengl, 1994; Ziegelberger et al., 1990; Zufall and Hatt, 1991). Furthermore, activation of $G\alpha$ subunits or DAG in pheromone-responsive neurons induces currents similar to those evoked by the cognate ligand (Pophof and Van der Goes van Naters, 2002). However, the role of cGMP remains unclear, and cGMP seems to underlie the adaptation of pheromone responses, without participating directly in the response itself (Boekhoff et al., 1993; Dolzer et al., 2008; Ziegelberger et al., 1990).

In *Drosophila melanogaster*, genetic tools allowed the manipulation of several components of the $G\alpha_q$ pathway. Reduction of either $G\alpha_q$ (Kain et al., 2008; Kalidas and Smith, 2002), PLCβ (Kain et al., 2008), DAG (Kain et al., 2008), or IP3 (Gomez-Diaz et al., 2006) levels in adult olfactory neurons decreases, but does not abolish, physiological and behavioral responses to odorants. However, more recent work by Yao and Carlson (Yao and Carlson, 2010) calls these prior results into question. These authors find no role whatsoever for G protein signaling in the *in vivo* function of *Drosophila* ORs.

In summary, the olfactory phenotypes described in these studies suggest that G proteins and/or downstream effectors may play a role in the function of the adult insect olfactory system. However, it is difficult to discriminate between a direct role in olfactory transduction mechanisms and a more general function of the G proteins in the maintenance or biological functions associated with the sensory neuron.

In Chapter 2, we investigate the signaling cascade mechanisms necessary to trigger odorant-evoked responses of insect olfactory receptors. We show that insect ORs do not rely on cyclic nucleotides as second messengers and that G proteins do not play a significant role in the initiation of the odorant response. In addition, we provide initial evidence that insect ORs constitute a new family of ligand-gated cation channels. In Chapter 3 we demonstrate that ORs are molecular targets of the insect repellent DEET and identify a single amino acid polymorphism that renders an insect OR DEET-insensitive. Finally, in Chapter 4 we present structure-function analysis of residues in the OR83b co-receptor that are important for receptor function.

2 Signaling cascade mechanisms of insect olfactory receptors

In this chapter, we will analyze the components of the signaling cascade underlying insect OR activation. Both mammalian and nematode ORs rely on second messengers generated by G proteins to induce neuronal responses. Based on heterologous expression of insect ORs, we provide evidence that G proteins and classical second messengers are not necessary to induce activation of insect OR-dependent currents, therefore suggesting fundamentally different mechanisms of signal transduction.

2.1 Heterologous expression of insect ORs in *Xenopus* oocytes

To directly investigate the signal transduction mechanisms of olfactory receptor complexes, we established a heterologous expression system where we could study receptor complexes. We isolated and expressed in *Xenopus laevis* oocytes ligand-specific odorant receptors from the vinegar fly *Drosophila melanogaster* and the malaria mosquito *Anopheles gambiae*, along with the corresponding co-receptor, OR83b and GPROR7, respectively. We tested the functional expression of OR complexes by performing two-electrode voltage clamp (TEVC) recordings. With this technique, we were able to fix the voltage across the oocyte cell membrane and record the currents necessary to maintain it. In agreement with previous *in vitro* (Nakagawa et al., 2005; Neuhaus et al., 2005) and *in vivo* (Benton et al., 2006) experiments, only the presence of both OR83b-like and ligand-specific subunits could confer odorant sensitivity to *Xenopus* oocytes (Figure 2.1A-C). The odorant specificity observed in this heterologous

system resembles the tuning previously described *in vivo* (Hallem and Carlson, 2004; Hallem and Carlson, 2006; Hallem et al., 2004b): OR47a/OR83b, GPROR1/GPROR7, and GPROR2/GPROR7 complexes specifically responded to their cognate ligands pentyl acetate, 4-methyl phenol, and 2-methyl phenol, respectively (Figure 2.1A-C). This allowed us to perform dose-response curves to establish a suitable odorant concentration range for further experiments (Figure 2.1D-F).
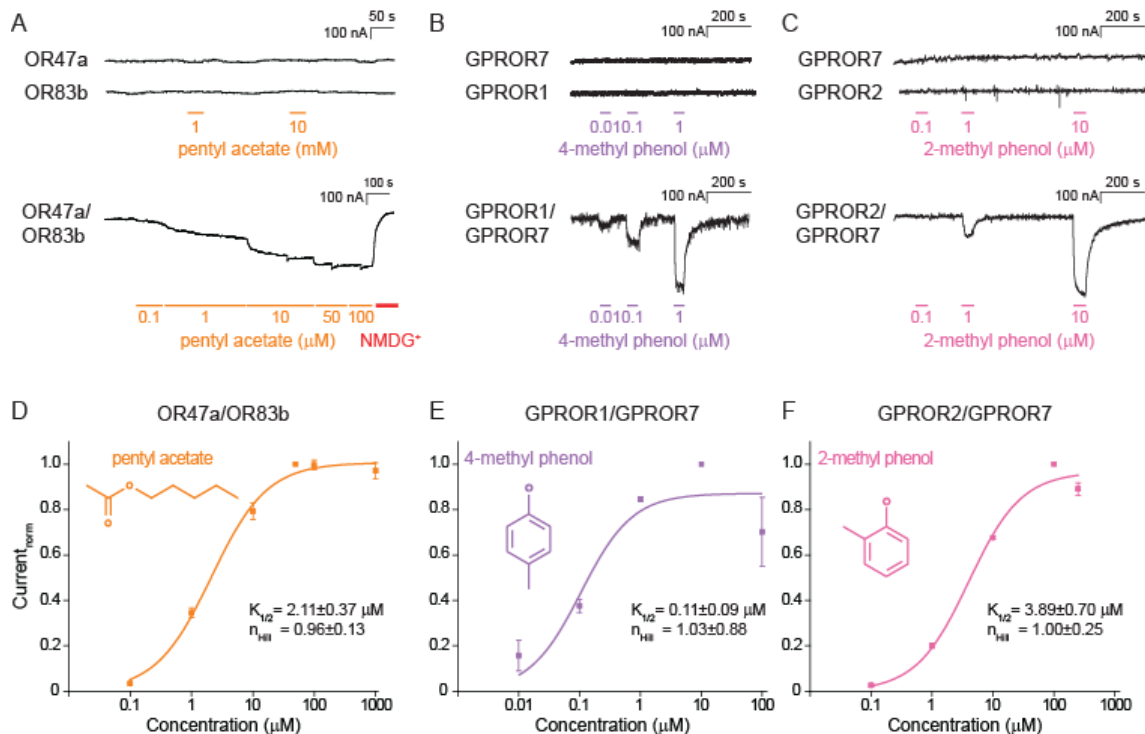
Figure 2.1 Odorant stimulation of *Xenopus* oocytes expressing insect ORs generates inward currents.

(**A-C**) Response profiles of OR47a, OR83b, and the complex OR47a/OR83b (**A**), GPROR1, GPROR7, and GPROR1/GPROR7 (**B**), GPROR2, GPROR7, and GPROR2/GPROR7 (**C**) to pentyl acetate, 4-methyl phenol, and 2-methyl phenol, respectively. The bars below each trace represent ligand application. (**D-F**) Dose-response curves of OR47a/OR83b (**D**), GPROR1/GPROR7 (**E**), and GPROR2/GPROR7 (**F**) to cognate ligands shown in **A-C**. Curves were fitted by a Hill equation (n=6, 3, 8). Hill coefficient n and apparent association constant $K_{1/2}$ values are indicated. Adapted from Sato et al. (2008).

Upon stimulation with the appropriate odorant, we could observe an inward current in cells at a holding potential of -60 mV. By changing the holding potential of the cell membrane from -80 mV to +40 mV and plotting the corresponding current (current-voltage relationship or I-V curve), we observed that these currents were symmetric at positive and negative potentials, but exhibited a slight outward rectification similar to what had been previously reported for the moth receptor BmOR1 expressed with BmOR2, the OR83b orthologue from this moth species (Figure 2.2A-C; Nakagawa et al., 2005). In Ringer's solution, composed mainly of Na^+ and Cl^-, the potential at which no net flux of current is observed (reversal potential) was not statistically different for all the OR complexes tested (Table 2.1).

Inward currents are carried by either an influx of positive ions or by an outflow of negative ions. To identify which ions carry the observed currents, we performed ion substitution experiments in which Na^+ or Cl^- ions present in the aqueous solution bathing the oocytes were substituted with equimolar concentrations of N-methyl-D-glucamine ($NMDG^+$) and sulfamate salts, respectively. These ions do not easily permeate through ion channels due to their size, but maintain a unitary charge and therefore do not change the osmolarity of the solution. I-V relationship analysis of the odorant-evoked currents in the different solutions revealed that, for all the OR- pairs tested, the elimination of Na^+ shifted the reversal potential to more negative values (Figure 2.2A-C and Table 2.1), while removal of Cl^- had no effect. Although removal of Ca^{2+} (Figure 2.2A-C and Table 2.1) or Mg^{2+} (data not shown) did not change the reversal potential, we cannot exclude that these ions also carry the currents observed

because of their low abundance in the solution. We then tested whether the apparent affinity of OR47a/OR83b or GPROR2/GPROR7 to their cognate ligands was voltage dependent, but found no effect of voltage on ligand affinity within the voltage range examined (Figure 2.2D and E). Taken together, these data suggest that odorants activate an OR-dependent cation conductance.
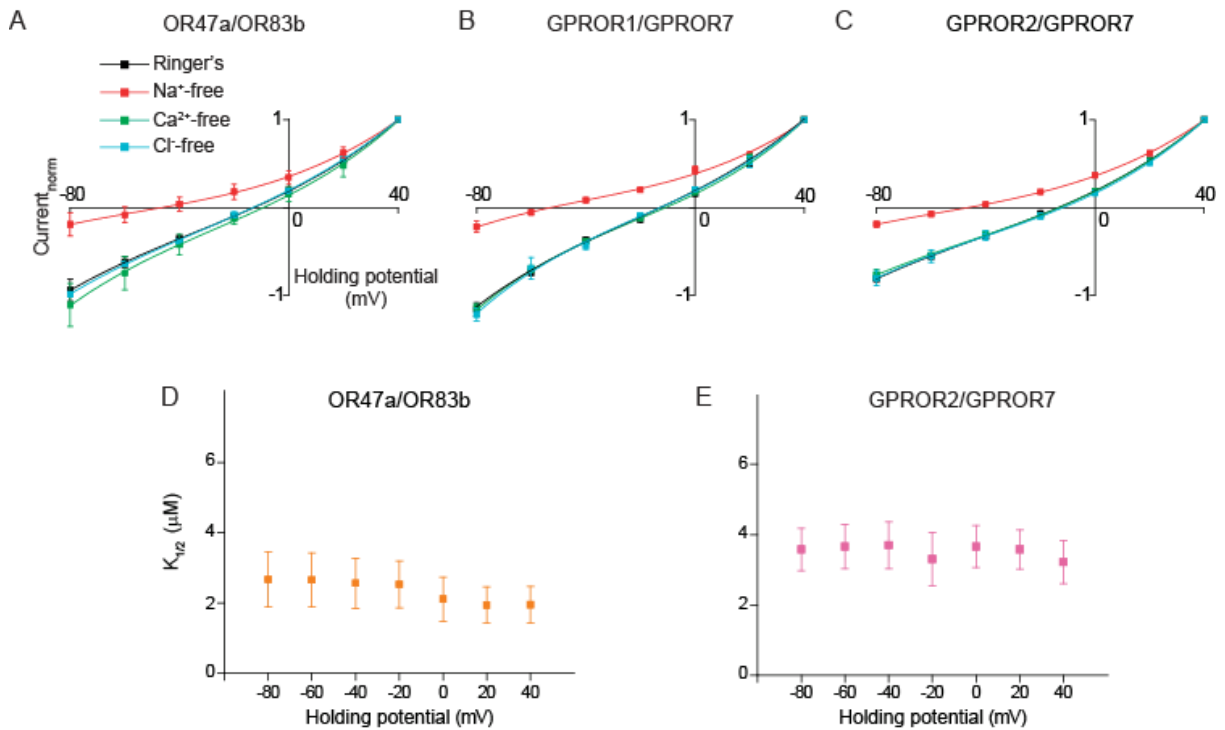
Figure 2.2 Effects of ion removal and voltage change on odorant-evoked currents. (**A-C**) I-V relationship of OR47a/OR83b- (**A**), GPROR1/GPROR7- (**B**), and GPROR2/GPROR7-dependent (**C**) currents. I-V curves were obtained by changing voltage from -80 mV to +40 mV (20 mV steps). The current magnitude was normalized at a holding potential of +40 mV (n=5, 3, 3). Different curves represent I-V relationships under different ionic conditions: oocyte Ringer's solution (black); Na^+-free solution (red); Ca^{2+}-free solution (green); and Cl^--free solution (blue cross). Adapted from Sato et al. (2008). (**D-E**) $K_{1/2}$ values for OR47a/OR83b (**D**) and GPROR2/GPROR7 (**E**) stimulated with pentyl acetate and 2-methyl phenol, respectively, at holding potentials ranging from -80 mV to +40 mV (20 mV steps, n=6, 5). Data are shown as mean±SEM.

Table 2.1 Reversal potential of OR complexes under different ionic conditions

	Reversal potential (mV)		
	OR47a/OR83b	GPROR1/GPROR7	GPROR2/GPROR7
Ringer's	-13.68±5.21	-11.15±1.53	-13.46±0.22
Na⁺-free	-54.19±4.40	-54.06±3.03	-45.33±2.40
Ca²⁺-free	-9.39±2.20	-12.65±0.50	-13.15±0.15
Cl⁻-free	-12.90±1.80	-13.07±0.37	-12.00±1.50

Table 2.1 Summary of the reversal potential values in the different ionic conditions analyzed in Figure 2.2A-C (mean±SEM). The shift in Na$^+$-free conditions is highlighted in grey.

2.2 Role of cyclic nucleotides and intracellular soluble components in the initiation of olfactory transduction mechanisms

If stimulation of the odorant receptor complex activates Gα_i or Gα_s pathways in ways similar to mammal or nematodes chemoreceptors, we would expect an increase in cAMP or cGMP levels that would open downstream channels. Therefore, we would expect that artificial increase of the intracellular concentrations of these cyclic nucleotides would lead to the opening of the ion channels independent of the activation of the olfactory receptor complex. To test this hypothesis, we applied 8-bromo-cGMP, a permeable cGMP analogue, to oocytes expressing the GPROR2/GPROR7 complex, but failed to detect current activation (Figure 2.3A). Similarly, application of forskolin (FSK), a direct activator of adenylyl cyclase, failed to generate currents in GPROR2/GPROR7-expressing cells (Figure 2.3B). In both cases, the functional expression of the complex was tested by successful stimulation with the cognate ligand

2-methyl phenol. Our cyclic nucleotide manipulations were effective, as the cGMP-sensitive rat olfactory cyclic nucleotide gated (CNG) channel (CNGA1/A2/B4) expressed in oocytes was effectively activated by perfusion with 8-bromo-cGMP (Figure 2.3C). The observed currents were blocked by addition of external Mg^{2+}, as shown in previous reports (Frings et al., 1991; Frings et al., 1995), confirming the stimulation of the CNG channel. Likewise, we could detect currents when forskolin was applied to cells expressing the chloride channel cystic fibrosis transmembrane conductance regulator (CFTR), whose opening is indirectly regulated by increases in intracellular cAMP. As expected, the observed inward currents were also sensitive to niflumic acid (NA), a general chloride channel blocker (Figure 2.3D).

To test whether stimulation of GPROR2/GPROR7 leads to production of intracellular cAMP, we applied 2-methyl phenol (2-MP) to cells that simultaneously expressed GPROR2/GPROR7 and CFTR. If the activated receptor complex increases cAMP levels, these would indirectly open the cAMP-sensitive CFTR channel, and we would observe NA-sensitive currents. Although we could successfully stimulate the GPROR2/GPROR7 complex with 2-methyl phenol, we failed to detect CFTR-evoked currents, unless we stimulated the cells with FSK (Figure 2.3E). This suggests that either GPROR2/GPROR7 does not produce cAMP, or that it does not produce it in sufficient quantities to open CFTR. To investigate whether other soluble components are necessary to produce OR-dependent currents, we performed outside-out patch-clamp recordings on oocyte membranes expressing OR47a/OR83b or GPROR2/GPROR7. This technique enabled us to isolate patches of membrane, and to control the composition of both the intracellular and extracellular environment.
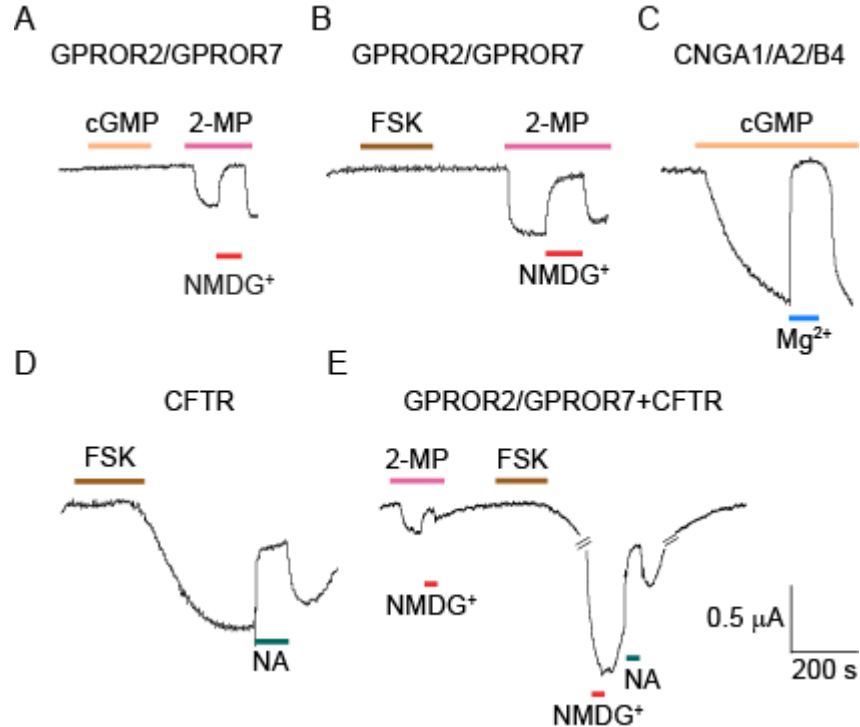
Figure 2.3 Increase of cyclic nucleotide levels does not induce inward currents in oocytes expressing insect ORs.

(**A-E**) Current recording of oocytes expressing GPROR2/GPROR7 (**A-B, E**), the rat olfactory cyclic nucleotide-gated channels (CNGA1/A2/B4; **C**), and cystic fibrosis transmembrane conductance regulator (CFTR; **D-E**), as indicated at the top of each trace. 8-bromo-cAMP (cAMP, 100 µM), 8-bromo-cGMP (cGMP, 100 µM), Mg^{2+} (10 mM), and forskolin (FSK, 40 µM), 2-methyl phenol (2-MP, 10 µM), $NMDG^+$ (84.5 mM), and niflumic acid (NA, 1 mM) were applied during the time indicated by the bars above or below each trace. Adapted from Sato et al. (2008).

Patches from uninjected oocytes did not show current responses to 2-methyl phenol (2-MP) and pentyl acetate (PA), indicating that odorants do not induce non-specific currents by interfering with the membrane or its components (Figure 2.4A). In the absence of intracellular components, outside-out patches from oocytes injected with either *Or47a*/*Or83b* or *GPROr2*/*GPROr7* mRNA showed transient currents that

resemble *Drosophila* and *Anopheles* receptor-dependent spontaneous activity of OSNs in the absence of ligands (Figure 2.4B and C). A larger number of events were observed with the application of the cognate ligands pentyl acetate (Figure 2.4D) and 2-methyl phenol (Figure 2.4E), respectively. The increase in events was dose-dependent (Figure 2.4F) and the magnitude of the odorant-evoked currents increased when the holding potential was progressively shifted from +40 mV to -120 mV (Figure 2.4G), as expected from the I-V relationship (Figure 2.2C), in patches expressing OR47a/OR83b and GPROR2/GPROR7, respectively. These data show that insect ORs expressed in a heterologous system generate cation currents that are independent of cyclic nucleotides. To derive the channel conductance, we analyzed the recordings from OR47a/OR83b-injected oocytes stimulated by 300 μM pentyl acetate (Figure 2.5A-C), and plotted the distribution of events and their relative current. At a holding potential of -80 mV, we calculated the mean channel current for single events at 1.2±0.03 pA (Figure 2.5D). To test whether ATP- or GTP-dependent signal transduction components were involved in odorant-evoked currents, we supplemented the intracellular patch solution with ATP (1 mM) and GTP (0.1 mM), but we could not detect any difference in the activity (Figure 2.5E-G). Although we observed OR-dependent odorant-evoked currents in outside-out patches, we failed to observe macro-currents, suggesting either that the expression levels on the membranes are not sufficient to generate them or that other elements not present on the membrane patch are necessary for the channels to remain in an open position for a longer period of time.

Figure 2.4 Properties of odorant-evoked currents in Xenopus outside-out membrane patches.

(**A**) Outside-out patch clamp recording of an uninjected oocyte clamped at -90 mV. Arrows indicate valve openings that delivered pentyl acetate (PA, 300 μM), 2-methyl phenol (2-MP, 300 μM), or oocyte Ringer's solution. (**B-C**) Outside-out patch clamp recording of an oocyte injected with OR47a/OR83b (**B**) or GPROR2/GPROR7 (**C**) in the absence of odorant ligand. (**D-E**) Outside-out patch clamp recording of an OR47a/OR83b- (**D**) or GPROR2/GPROR7-injected oocyte (**E**) stimulated for 14 sec with 300 µM of the non-agonist odorant 2-MP or PA, respectively, followed by a 14 sec application of 300 µM of the cognate agonist. Delay in current response to the ligand is due to ~2 sec bath perfusion time lag. Oocyte Ringer's solution was perfused after the 14 sec ligand stimulation. The voltage was clamped at -90 mV. Bottom trace shows a time expansion during the ligand stimulation phase. (**F**) Dose-response and ligand selectivity of OR47a/OR83b-expressing patches in an outside-out configuration clamped at -90 mV. (**G**) Currents elicited in the same patch of a GPROR2/GPROR7-injected oocyte by 300 µM 2-MP at various holding potentials from +40 mV to -120 mV. Adapted from Sato et al. (2008).
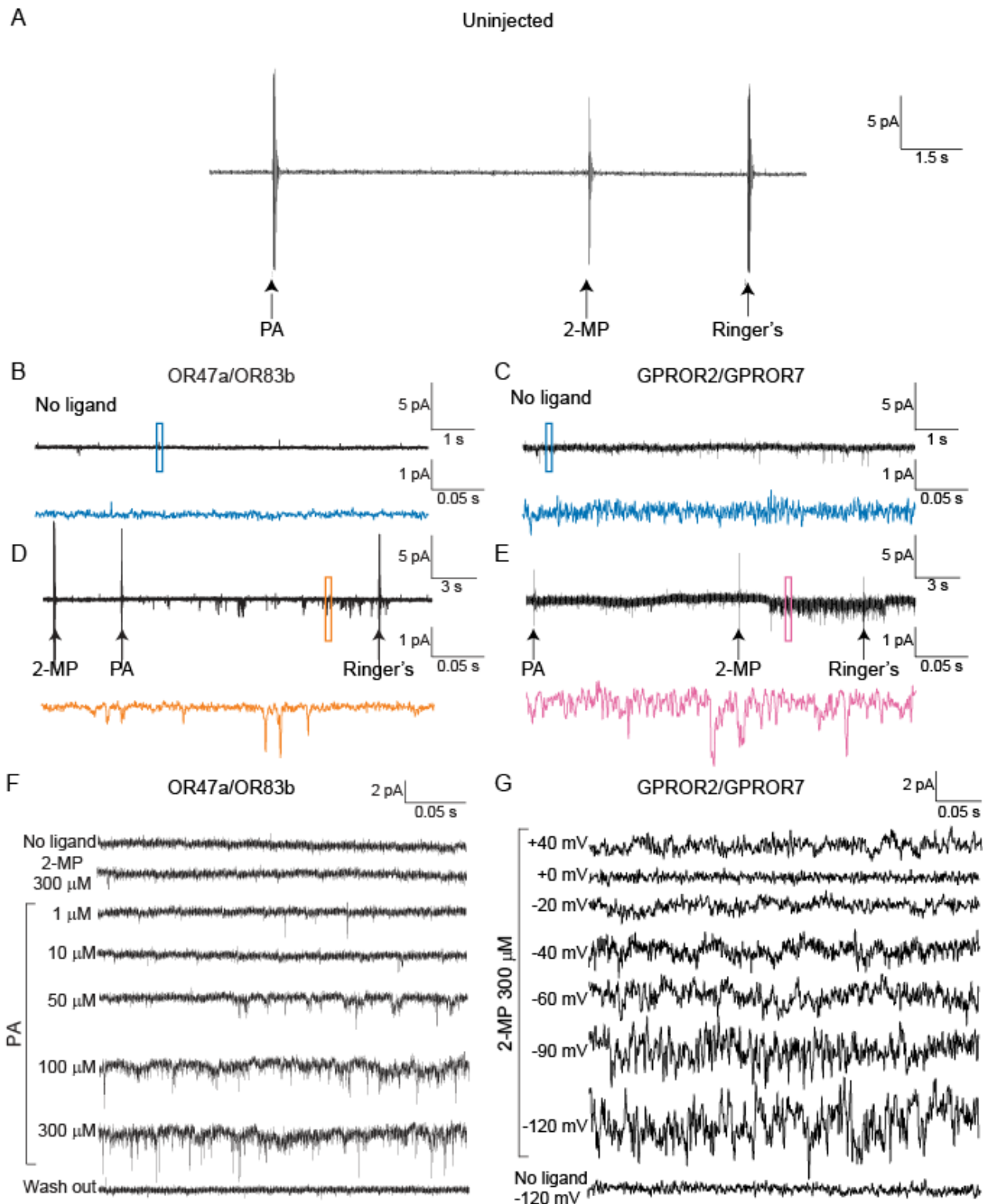
Figure 2.4 Properties of odorant-evoked currents in *Xenopus* outside-out membrane patches.
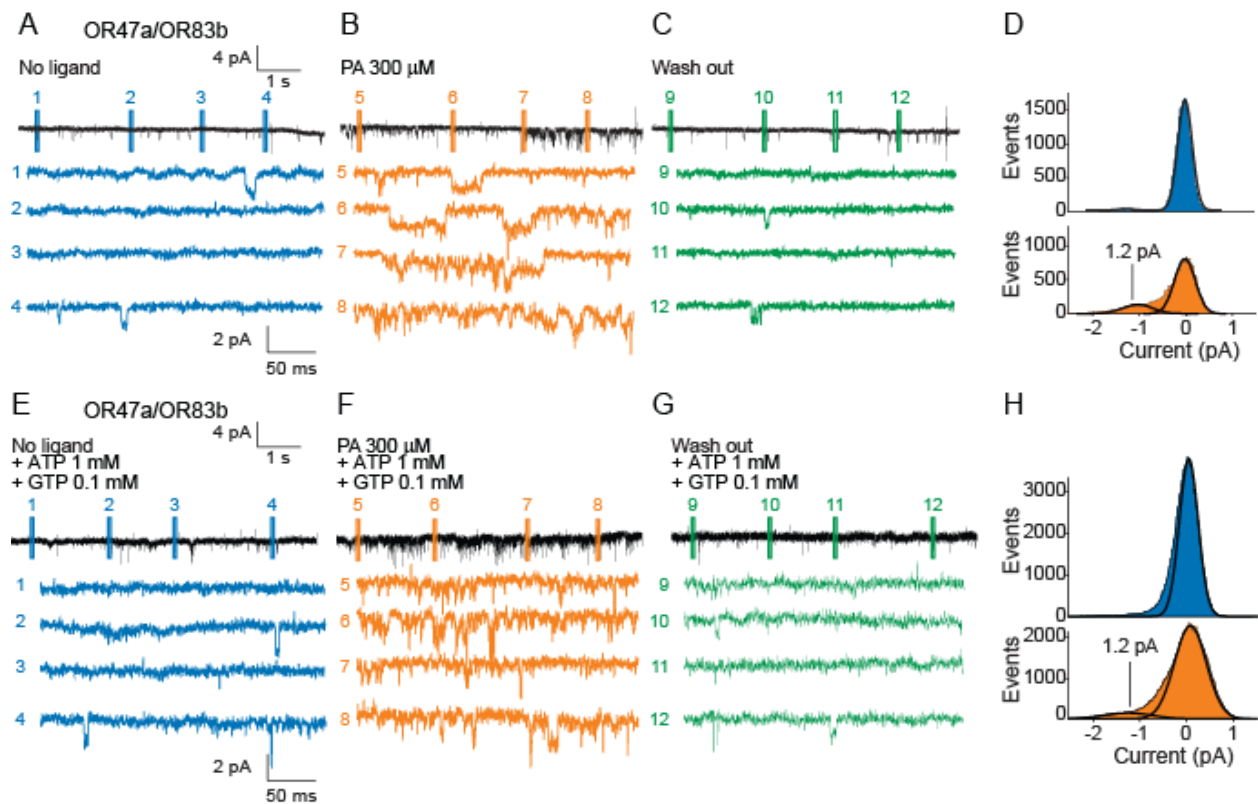
Figure 2.5 Odorant-evoked currents in excised outside-out patches of membranes expressing OR47a/OR83b.

(**A-C**) Outside-out patch-clamp recording of a *Xenopus* oocyte membrane expressing OR47a/OR83b before stimulation (**A**), during stimulation with 300 µM pentyl acetate (PA; **B**), and after wash out (**C**). (**E-G**) Same as in **A-C**, but ATP (1 mM) and GTP (0.1 mM) were added to the pseudo-intracellular solution. The bottom traces of each panel indicate expansions of 300 ms current traces of single-channel recording at the positions indicated by the numbers. The data for **A-C** and **E-G** were obtained from two cells with voltage clamped at -80 mV. Scales for **A-C** and **E-G** are indicated at the top in **A** and **E**, and scales for the expansions are at the bottom in **A** and **E**. (**D, E**) All-point current histograms of unitary events before (blue) and during (orange) application of the ligand PA in **B** and **F**, respectively. Amplitude distributions were fitted with two Gaussian components (black lines). Adapted from Sato et al. (2008).

2.3 G protein pathways are not involved in the initiation of odorant-evoked responses of insect ORs

To examine the possibility that G proteins mediate the activation of OR-dependent currents, we introduced GDP-β-S, a competitive inhibitor of G proteins, in the pseudo-intracellular solution, and performed outside-out patches on OR47a/OR83b-expressing membranes. Even in the presence of GDP-β-S, we still detected pentyl acetate-evoked currents (Figure 2.6), suggesting that G protein signaling cascades are unlikely to underlie the activation of OR-dependent currents.
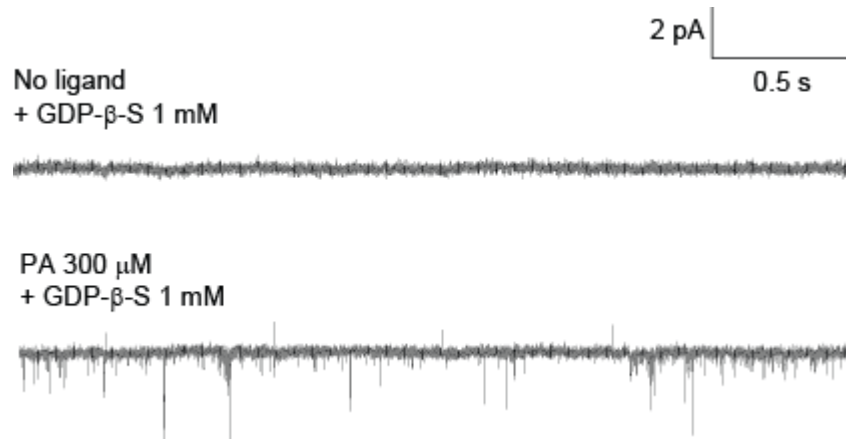
Figure 2.6 Outside-out patches of membranes expressing OR47a/OR83b exhibit odorant-evoked currents in the presence of the G protein inhibitor GDP-β-S. Outside-out patch clamp recording of an oocytes expressing OR47a/OR83b in the absence (*upper trace*) and presence (*lower trace*) of 300 μM pentyl acetate (PA). The intracellular solution contained 1 mM GDP-β-S. Voltage was clamped at -80 mV. Adapted from Sato et al. (2008).

Similar results were obtained in a separate heterologous system by Dr. Koji Sato expressing OR complexes that were tested in *Xenopus* oocytes (Sato et al., 2008). To test whether $G\alpha_s$ is recruited after odorant stimulation, levels of cAMP were measured in HEK293T cells expressing *Drosophila* OR47a/OR83b, *Anopheles* GPROR2/GPROR7, or *Bombyx* BmOR1/BmOR2, the moth receptor complex responsible for the detection of bombykol (Nakagawa et al., 2005). Stimulation with the cognate ligands failed to increase intracellular cAMP levels. However, a rise in cAMP was observed after stimulation of the mouse olfactory receptor mOR-EG, as expected (Figure 2.7A).

To investigate a possible role of $G\alpha_q$/PLC, the phospholipase inhibitor U73122 was applied before and throughout ligand stimulation in HeLa cells expressing OR47a/OR83b, but failed to affect the odorant-evoked response. In control experiments, the response of the GPCR α1-adrenergic receptor was completely abolished (Figure 2.7B).

To test whether other G protein pathways were recruited after OR activation, *Xenopus* oocytes expressing BmOR1/BmOR2 were injected with GDP-β-S. Similar to the outside-out patches in Figure 2.6, this treatment did not affect odorant-evoked currents, but drastically decreased G protein-mediated responses of the GPCRs α1- and β2-adrenergic receptors (Figure 2.8). These results support the hypothesis that OR activation is largely independent of G protein signaling.
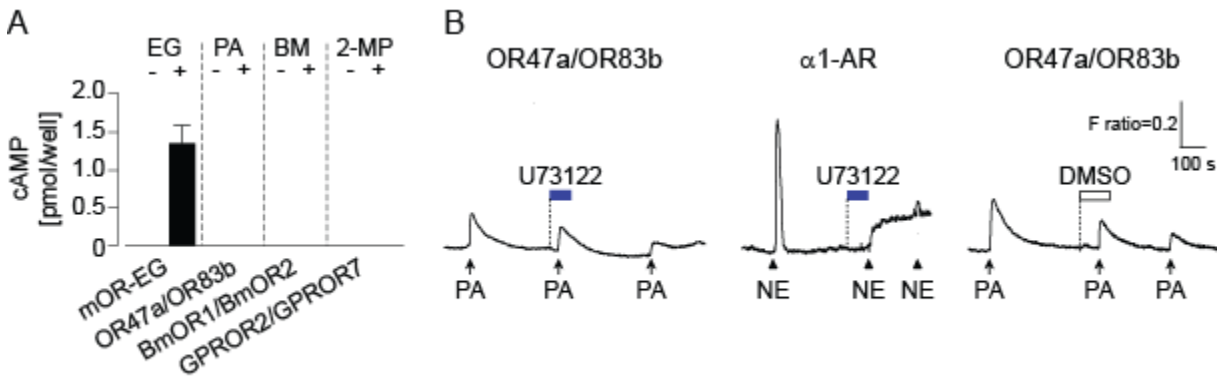
The experiments in this figure were performed by Dr. Koji Sato

Figure 2.7 Insect OR activity is independent of cAMP and PLC signaling pathways.
(**A**) cAMP production in HEK293T cells expressing mOR-EG, OR47a/OR83b, BmOR1/BmOR2, or GPROR2/GPROR7 stimulated with eugenol (EG, 1 mM), pentyl acetate (PA, 100 μM), bombykol (BM, 10 μM) or 2-methyl phenol (2-MP, 100 μM), respectively. (**B**) Ca^{2+} responses of HeLa cells expressing OR47a/OR83b or α1-adrenergic receptor (α1-AR) to a 10 s stimulation with 100 μM PA or 100 nM noradrenaline (NA) with application of 10 μM U73122 (filled bar) or 0.1% DMSO (open bar). Data are shown as mean±SEM. Adapted from Sato et al. (2008).
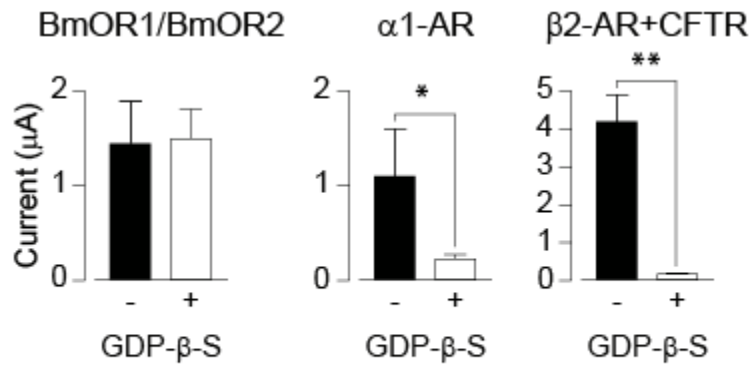
Figure 2.8 Insect OR activity is independent of G protein signaling.

Effect of GDP-β-S on ligand-induced inward currents in *Xenopus* oocytes expressing BmOR1/BmOR2 (30 μM bombykol), α1-AR (1 μM noradrenaline) or β2-AR+CFTR (10 μM isoprenaline). Significance assessed by t-test: *, $p < 0.05$; **, $p < 0.01$; n=5 each. Data are shown as mean±SEM. Adapted from Sato et al. (2008).

2.4 Insect ORs are ligand-gated ion channels

Taken together, these data provide compelling evidence that insect ORs function independently of G protein signaling cascades unlike their mammalian and nematode counterparts. Since insect ORs can function independently of intracellular components, it raises the possibility that either the OR complexes couple to an ion channel present on frog, mammalian, and insect cells, or that ORs themselves are the ion channels responsible for the odorant-evoked currents. If the latter is true, it is likely that the properties of ligand-evoked currents would change depending on the subunit composition of the complex. Indeed, odorant-evoked currents of HeLa cells expressing OR47a/OR83b and OR47a/BmOR2 show different reversal potential and outward rectification (Figure 2.9A-B). Despite a lack of homology with any previously described

ion channel, these data suggest that insect ORs are the ion channels responsible for the odorant-evoked currents.
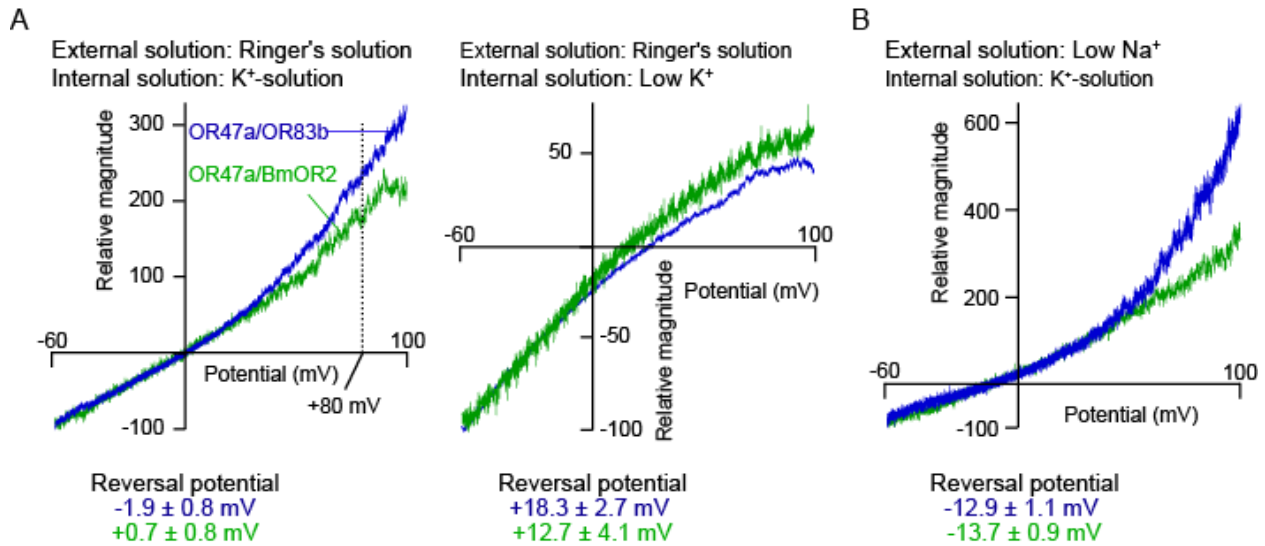
The experiments in this figure were performed by Dr. Koji Sato

Figure 2.9 The functional properties of odorant-evoked currents are dependent on OR subunit composition.

(**A**; *left panel*) I-V curves of OR47a/OR83b (blue) and OR47a/BmOR2 (green) expressing membranes when normal Ringer's solution and K^+-internal solution were perfused externally and internally, respectively (n=14, 13). The dotted line indicates a holding potential at +80mV. (*right panel*) Same as in **A**, but normal Ringer's solution and $NMDG^+$-internal solution were perfused externally and internally, respectively (n=11, n=13). (**B**) Same as in **A** with $NMDG^+$-external solution and K^+-internal solution perfused extracellularly and intracellularly, respectively. The I-V curve was obtained by ramp voltage from -60 mV to +100 mV. The magnitudes of currents were standardized at a holding potential of -60 mV. The reversal potentials are indicated as mean±SEM (n=9, 5). Adapted from Sato et al. (2008).

Subsequent studies from other laboratories also failed to observe G protein involvement in odorant-evoked currents of the *Drosophila* OR43a subunit expressed in Sf9 and HEK293T cells (Smart et al., 2008).

Given the number of OR protein members in different insect species (Nei et al., 2008), insect olfactory receptors may be the largest family of ligand-gated ion-channel proteins found in any organism (Figure 2.10A).

2.5 Controversial ideas in insect olfaction: a comparison of the ion channel versus channel-GPCR models

An alternative hypothesis regarding the nature of OR-dependent currents was proposed by Wicher and colleagues (Wicher et al., 2008), who suggested that ligand-binding subunits activated $G\alpha_s$ at low odorant concentrations, and that subsequent production of cAMP activated the CNG-like channel OR83b directly (Figure 2.10A-B). The authors claimed that OR83b has CNG-like activity in the absence of ligand-binding ORs. In their heterologous expression system, this led to metabotropic currents that developed over the course of ~60 seconds. At high odorant concentrations, the ligand-binding subunits coupled directly to OR83b, resulting in a much faster current activation, peaking at ~1 second. According to their model, this would allow for a larger working range of the insect olfactory system (Figure 2.10A-B).

Unlike what was shown in our study, Wicher and co-workers found that the ligand-binding subunits OR22a or OR47a are necessary and sufficient to raise the

intracellular concentration of cAMP through a $G\alpha_s$ pathway. However, it is peculiar that this group found that application of the G protein inhibitor GDP-β-S did not abolish odorant responses, but only decreased the apparent affinity of the OR for its ligand. It is also intriguing that Wicher et al. found that both ligand-binding subunits and the co-receptor OR83b showed functional expression independently of each other in HEK293T cells, although co-expression of a ligand-binding subunit and the co-receptor is required *in vivo* (Benton et al., 2006; Jones et al., 2005; Larsson et al., 2004), and in some heterologous cell studies (Nakagawa et al., 2005; Nichols and Luetje, ; Wang et al., 2010).

While there is agreement that insect ORs are a new family of odorant-gated cation channels, the exact role of G proteins and cyclic nucleotides in signal transduction and the subunit composition of the ion channel remain controversial. The discrepancies observed in the experimental data between our paper (Sato et al., 2008) and that of Wicher *et al.* (Wicher et al., 2008) could be due to different heterologous systems used to express the OR complexes and/or to the endogenous proteins expressed along with insect olfactory receptors. To reconcile the models proposed by our group along with Dr. Touhara and the competing group, it would be useful to test the OR22a/OR83b receptor complex in either *Xenopus laevis* oocytes or HeLa cells. However, neither I nor members of the Touhara laboratory have been able to obtain functional expression of this receptor complex in any cell type tested (data not shown). Thus, the nature and origin of currents described by Wicher *et al.* remain unknown.
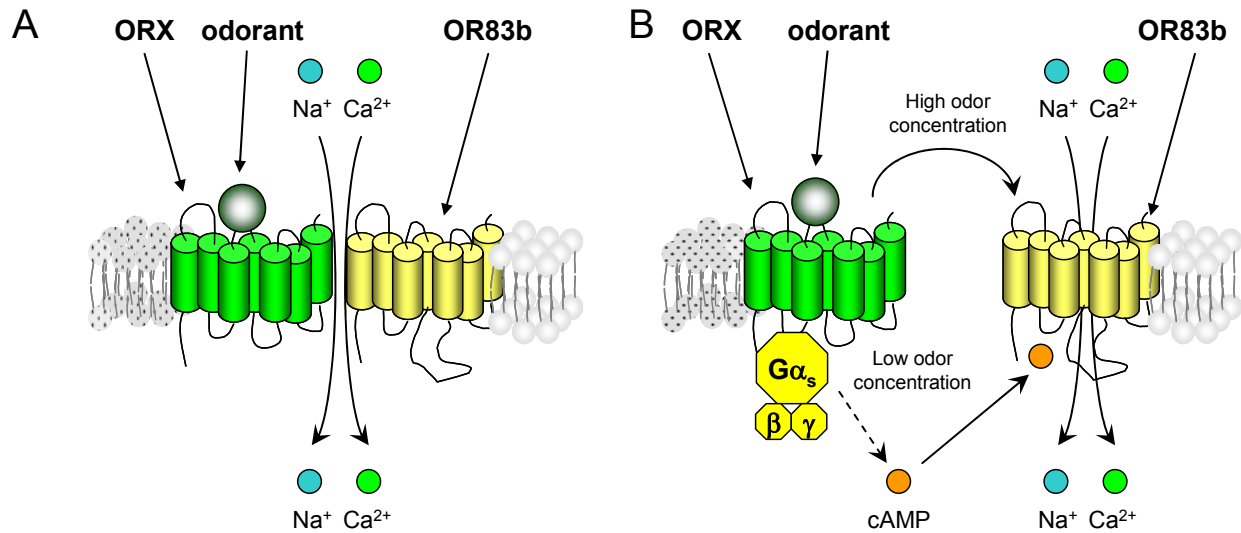
Figure 2.10 Models of insect OR transduction mechanisms.
(**A**) Ion channel model proposed by our group with Dr. Touhara and co-workers: insect ORs form a ligand-gated ion channel directly gated by odorants. (**B**) Channel-GPCR model proposed by Dr. Wicher and co-workers: at low odorant concentration, the ligand-specific subunit ORX activates $G\alpha_s$ that increases cAMP concentrations. Cyclic nucleotide binds and opens the CNG-like ion channel OR83b. At high odorant concentrations, the binding of the ligand to the OR opens OR83b directly.

2.6 OR83b does not contain a predicted cyclic nucleotide binding domain

For OR83b to be directly modulated by cyclic nucleotides, as proposed by Wicher *et al.* (Wicher et al., 2008), a cyclic nucleotide binding domain (CNBD) is required.

CNBDs are conserved from prokaryotes to eukaryotes, and contain six invariant amino acids located within antiparallel β-barrel structures (Shabb and Corbin, 1992). These residues are the basis for two signature patterns (CNBD_BINDING_1 and

CNBD_BINDING_2, Table 2.2) used to identify them in novel proteins (Hulo et al., 2008). The first pattern spans the first two invariant residues, and the second pattern includes the remaining four. Both signatures can recognize CNBD motifs with specificity bigger than 0.95 (probability of identifying true negative hits) and sensitivity of about 0.5 (probability of identifying true positive hits; Hulo et al., 2008).

Neither of the two motifs is present within the OR83b protein (data not shown). However, given the low sensitivity of the patterns, there is a possibility that a degenerate CNBD exists in OR83b or other OR family members, or that they contain a different kind of CNBD.

To partially address this issue, we looked for a degenerate consensus in two protein sets based on the information provided by the database of protein domains PROSITE (Hulo et al., 2008). The first group includes proteins in which the algorithm could not detect a CNBD, but that have similarity to other proteins with a known CNBD (false negatives); the second group consists of proteins that are not known to bind to cyclic nucleotides (true negatives). Using the Network Protein Sequence Analysis PROSCAN algorithm (Combet et al., 2000), we identified putative degenerate CNBD domains and compared the average identity to the signature patterns within the two groups. Among false negatives, the average percentage identity to the CNBD_BINDING_1 was 82.00±11.01%, compared to 61.63±7.75% for true negatives (p=0.001, Student's t-test, Table 2.2). Similarly, the average percentage identity to CNBD_BINDING_2 was 79.00±4.23% for false negatives, and 59.38±1.24% for true negatives (p=0.005, Student's t-test, Table 2.3). OR83b falls within the range of the true

45

negatives for both patterns, with a percentage identity of 59% and 56%, respectively. Given these results, we think it is unlikely that OR83b contains a degenerate canonical cyclic nucleotide-binding domain.

All proteins in the false negative group had domains with at least 60% similarity to the first signature pattern. However, the cGMP-dependent kinase KGP1_RABIT and the transcriptional regulator ARCR_STAA1 did not show any similarity to the second signature pattern, suggesting that they also lack a CNBD, although they are classified as false negatives. It is important to highlight that the function of these proteins is based only on sequence similarity to known proteins in other model organisms. Therefore, it is possible that the CNBD in these two proteins is non functional or, alternatively, they contain a non-canonical CNBD. Therefore, direct experimental evidence is necessary to show the existence of a functional cyclic nucleotide binding domain both in these proteins and in OR83b.

In conclusion, the exact role of G proteins and second messenger-mediated mechanisms in insect olfactory signal transduction remain controversial, and regulation of insect OR channels by G proteins still needs to be fully explored.

Table 2.2 Percent identity to the CNBD_BINDING_1 pattern in false and true negative protein groups.

CNBD_BINDING_1

False negatives (Best similarity, %)		True negatives (Best similarity, %)	
HCN3_MOUSE K$^+$/Na$^+$ channel *Mus musculus*	93%	76%	ARI2_DROME RING finger protein *Drosophila melanogaster*
KGP1_RABIT cGMP-dependent kinase *Oryctolagus cuniculus*	92%	68%	CP1A1_RAT Cytochrome P450 *Rattus norvegicus*
CNG11_ARATH CNG channel *Arabidopsis thaliana*	89%	65%	1B78_HUMAN HLA-I antigen *Homo sapiens*
Y2565_MYCTU NTE family protein *Mycobacterium tuberculosis*	87%	62%	ENO_DROME Enolase *Drosophila melanogaster*
CNBD1_HUMAN CNBD containing protein *Homo sapiens*	81%	57%	Q26433_DROME Myosin heavy chain *Drosophila melanogaster*
RPGF3_RAT Rap GEF 3 *Rattus norvegicus*	80%	57%	DPOLA_MOUSE PolymeraseA1 *Mus musculus*
SKOR_ARATH K$^+$ channel *Arabidopsis thaliana*	74%	55%	SAPA_ECOLI ABC binding protein *Escherichia coli*
ARCR_STAA1 Transcriptional regulator *Staphilococcus aureus*	60%	53%	Q9UE34_HUMAN Fibrinogen *Homo sapiens*
mean±SEM	**82.00±11.01%**	**61.63±7.75%**	**mean±SEM**
		59%	OR83B_DROME Or83b *Drosophila melanogaster*

Table 2.2 Proteins are identified by their UniProtKB identifier. The common name and the organism of origin are indicated.

Table 2.3 Percent identity to the CNBD_BINDING_2 pattern in false and true negative protein groups.

CNBD_BINDING_2

False negatives (Best similarity, %)			True negatives (Best similarity, %)
HCN3_MOUSE K$^+$/Na$^+$ channel *Mus musculus*	91%	64%	1B78_HUMAN HLA-I antigen *Homo sapiens*
SKOR_ARATH K$^+$ channel *Arabidopsis thaliana*	86%	63%	ENO_DROME Enolase *Drosophila melanogaster*
Y2565_MYCTU NTE family protein *Mycobacterium tuberculosis*	85%	63%	Q9UE34_HUMAN Fibrinogen *Homo sapiens*
RPGF3_RAT Rap GEF 3 *Rattus norvegicus*	78%	59%	Q26433_DROME Myosin heavy chain *Drosophila melanogaster*
CNBD1_HUMAN CNBD containing protein *Homo sapiens*	70%	58%	ARI2_DROME RING finger protein *Drosophila melanogaster*
CNG11_ARATH CNG channel *Arabidopsis thaliana*	64%	57%	CP1A1_RAT Cytochrome P450 *Rattus norvegicus*
KGP1_RABIT cGMP-dependent kinase *Oryctolagus cuniculus*	Not found*	56%	DPOLA_MOUSE PolymeraseA1 *Mus musculus*
ARCR_STAA1 Transcriptional regulator *Staphilococcus aureus*	Not found*	55%	SAPA_ECOLI ABC binding protein *Escherichia coli*
mean±SEM	**79.00±4.23%**	**59.38±1.24%**	**mean±SEM**
		56%	OR83B_DROME Or83b *Drosophila melanogaster*

* Not considered in the average

Table 2.3 Proteins are identified by their UniProtKB identifier. The common name and the organism of origin are indicated.

3 Insect repellents

3.1 Introduction to insect repellents: a way to reduce insect-borne diseases

3.1.1 The socio-economic impact of arthropods

Arthropods shaped human civilization in ways that are often overlooked. The relationship between human societies and arthropods, and insects in particular, heavily depends on the species under consideration. Beetles, ants, termites, and caterpillars, to name a few, have been used as food source, and bees are exploited to produce honey, wax and to pollinate flowers. Pigments, silk, and resins are derived from the cochineal *Dactylopius coccus*, the moth *Bombyx mori*, and the scale insect *Kerria lacca*, respectively. Furthermore, arthropods have been used in agriculture as biological agents for pest control (Bale et al., 2008; Neuenschwander et al., 2003; Smith, 1996), in the biomedical field in maggot therapy (Jones, 2009; Sherman et al., 2000; Whitaker et al., 2007), as source of the anti-wart agent cantharidin (Moed et al., 2001), and in forensics to date and interpret crime scenes (Wells and Stevens, 2008).

However, the economic, biomedical, and social impact of arthropods has a dark side: these organisms can harm animals, damage crops, and, most importantly, be vectors of human diseases. For example, arthropods can carry the agents responsible for Lyme disease and Rocky Mountain spotted fever (deer ticks of the genus *Ixodes*), yellow and Dengue fever (the mosquito *Aedes aegypti*), plague (rat flea *Xenopsylla cheopis*), Chagas disease (assassin bugs of the *Triatoma*, *Rhodnius*, and

Panstrongylus genera), and sleeping sickness (tsetse flies of the genus *Glossina*). Malaria, carried by mosquitoes of the genus *Anopheles*, claims more than 1 million lives per year (Breman et al., 2001; WHO, 2009), and about half of the world population is at risk of contracting the disease (WHO, 2009). Besides the impact on human lives, it is estimated that malaria is responsible for a reduction in economic growth of 1.3% and a loss of $12 billion per year in Africa alone (Gallup and Sachs, 2001).

In all these cases, the hematophagous arthropod vector acquires the disease agent while blood feeding on an infected host, and transmits it during a successive meal.

3.1.2 DEET is the most widely used insect repellent

Prevention of insect-bone disease can be achieved in several ways: vaccines and antibiotic treatments against the disease agent, when available, can be used in the developed world, while physical barriers and chemical means are being implemented in developing countries (Genton, 2008; Qazi and Shaikh, 2007; Sharma and Singh, 2008). In particular, insecticides and insect repellents play a crucial role in preventing blood-feeding insects from biting humans. Insecticides have the general advantage of killing the potential disease vector, but are often toxic at high doses, can be concentrated in the food chain, and are not species specific (Stuetz, 2006; Tanabe, 2002; van den Berg, 2009). Moreover, insects tend to develop resistance through natural selection (Oyarzun et al., 2008; Rosario-Cruz et al., 2009; Soderlund, 2008; Soderlund and Knipple, 2003). On the other hand, insect repellents currently used are less toxic and reduce exposure to a blood-feeding vector without killing it. Among insect repellents, DEET (N,N-diethyl-3-methylbenzamide), picaridin (2-(2-hydroxyethyl)-1-piperidinecarboxylic acid 1-

methylpropyl ester   or KBR 3023), oil of lemon eucalyptus (p-menthane 3,8-diol or PMD), and IR3535 (3-[N-Butyl-N-acetyl]-aminopropionic acid, ethyl ester) are repellents recommended by the Center for Disease Control and Prevention (CDC) that are also registered with the US Environmental Protection Agency (EPA; http://www.cdc.gov/ncidod/dvbid/westnile/RepellentUpdates.htm), because of their long lasting effects.

DEET is the most widely used ingredient in topical formulations of insect repellents. Developed in 1946 at the US Department of Agriculture with Department of Defense funding (McCabe et al., 1954), this compound was registered for civil use in 1957 (http://www.epa.gov/pesticides/factsheets/chemicals/deet.htm) and has proven effective against ticks (Couch and Johnson, 1992), sand flies (Naucke et al., 2006), mites (Ho and Fauziah, 1993; Tilak et al., 2001), fleas (Mehr et al., 1984; Rutledge et al., 1982), bedbugs (Kumar et al., 1995), cockroaches (Rao and Rao, 1991), and mosquitoes (Coleman et al., 1993).

The precise mode of action of DEET is largely unknown, although published data show that close contact to the insect repellent affects behaviors such as probing time and blood feeding rates, and reduces overall survival, revealing some insecticidal properties in the mosquito *Anopheles quadrimaculatus* (Xue et al., 2007). However, DEET can also affect mosquitoes through gas phase exposure up to a distance of approximately 40 cm (Schreck et al., 1970), can block both the behavioral attraction to lactic acid (Dogan et al., 1999) and the evoked activity of lactic acid-responsive OSNs in *Aedes aegypti* (Davis and PG., 1976). Therefore, it is likely that the olfactory system is

involved in mediating these effects. Three main hypotheses have been recently suggested to account for this: olfactory inhibition (Plettner and Gries, 2010), olfactory repulsion (Stanczyk et al., 2010; Syed and Leal, 2008), and direct interaction with odorants (Syed and Leal, 2008).

We performed behavioral, electrophysiological, and molecular analysis to investigate the mode of action of DEET, and provide evidence for a direct effect of the insect repellent on insect olfactory receptors.

3.1.3 Vinegar flies as a model to study the molecular action of DEET

The attraction of arthropods towards human hosts has been extensively studied for the vector mosquitoes of the genera *Anopheles* (Annis, 1990; Pridgeon et al., 2009; Robert et al., 1991; Schreck and Kline, 1989; Yap, 1986) and *Aedes* (Frances et al., 1993; Licciardi et al., 2006; Schreck et al., 1984). The attraction of mosquitoes is largely mediated by volatile compounds emitted from the human body, such as lactic acid, CO_2, and 1-octen-3-ol (Takken and Knols, 1999). These molecules are sufficient to mediate host-seeking behavior and are effectively used as baits in mosquito traps (Hoel et al., 2007; Kline et al., 1990).

Unlike blood feeding arthropods, vinegar flies do not show attraction towards humans. However, it is possible to establish an *in vivo* assay to study food-seeking behavior, similarly to what has been done in mosquitoes. As a result, it has been shown that vinegar flies avoid DEET-treated food traps probably due to airborne vapors of the insect repellent (Reeder et al., 2001).

To study this avoidance behavior, Dr. Mathias Ditzen established a two-choice assay (Figure 3.1A). In the absence of any food bait or DEET, flies distributed equally among the trap vials (Figure 3.1B, *right two bars*). When the entrance of one trap was treated with 100% DEET, flies avoided the vial (Figure 3.1B), but this behavior was reduced when they were shielded from direct contact via a wire mesh or a perforated polypropylene barrier, and when the amount of DEET was decreased to 10% (Figure 3.1B). With these physical barriers, no contact effects of DEET could be observed, allowing us to isolate its airborne mode of action.

In the presence of food baits (Figure 3.1C), flies distributed roughly equally between the two vials. However, when one of the two entrances was treated with 10% DEET, more flies chose the untreated side (Figure 3.1D), despite the fact that at this concentration DEET did not show any repellent effect *per se* (Figure 3.1B). When DEET was applied to both entrances, the distribution of flies in the two vials was again similar (Figure 3.1D).

These data show that *Drosophila melanogaster* can be used as a model to study the effects of DEET in the gas-phase on food-seeking behavior.
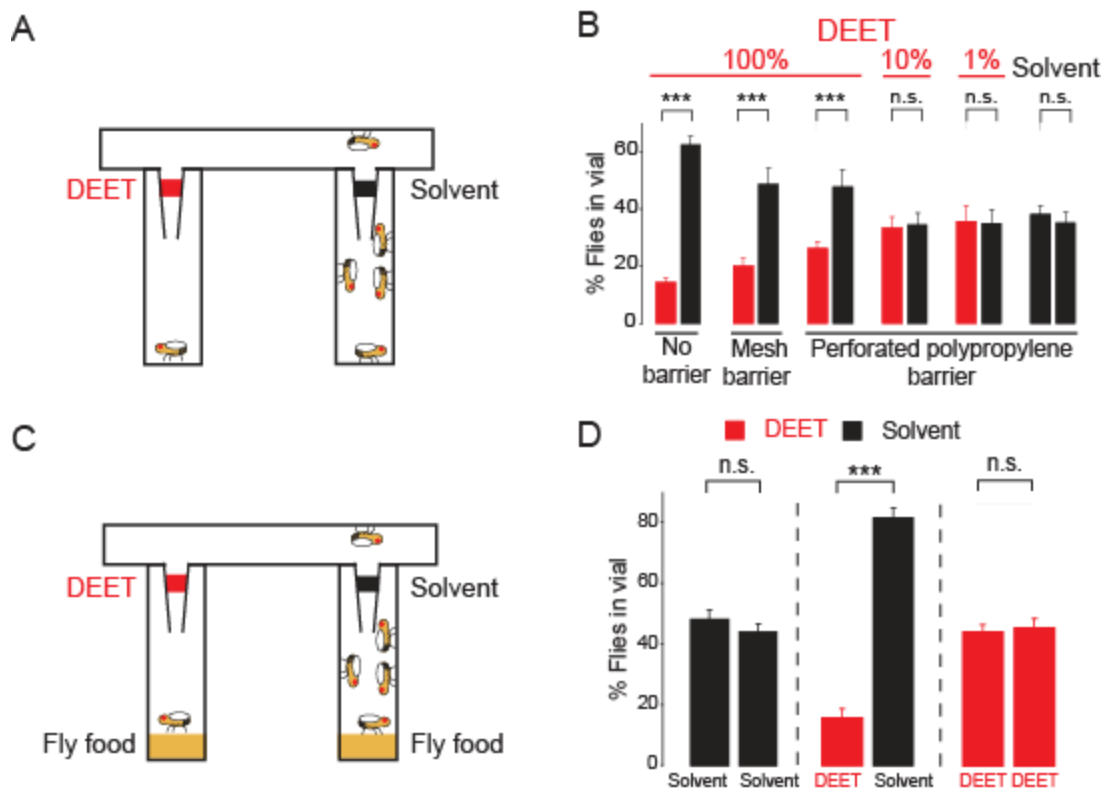
The experiments in this figure were performed by Dr. Mathias Ditzen

Figure 3.1 DEET reduces attraction of *Drosophila melanogaster* to food odor.

(**A, C**) Schematic of trap assay without (**A**) or with (**C**) food bait (yellow). Entrance to trap is coated with DEET (red) or solvent (black). (**B**) Repellency of varying concentrations of DEET in the trap assay without food bait, with different barriers to impede direct contact with DEET (***, p<0.001; n.s., not significant, Mann-Whitney test; mean±SEM, n=11 to 12). (**D**) Repellency of 10% DEET with perforated polypropylene barrier in the trap assay with food bait (***, p<0.001; n.s., not significant, Mann-Whitney test; mean±SEM, n=12, 22, 12). Adapted from Ditzen et al. (2008).

3.1.4 The effects of DEET on the *Drosophila melanogaster* olfactory system are OR83b-dependent

To investigate whether DEET acted at the peripheral level of the olfactory system, Dr. Ditzen tested flies with an impaired sense of smell in the two-choice assay. While intact flies and flies with only one antenna still preferred the non-treated vial, antenna-less flies entered both food vials, with a slight preference for the DEET-treated trap (Figure 3.2A). Flies lacking *Or83b*, in which ~80% of the OSNs are genetically silenced, also do not show avoidance to DEET-treated traps (Figure 3.2B). These data show that the olfactory system plays a crucial role in mediating the effects of the insect repellent in *Drosophila melanogaster*.
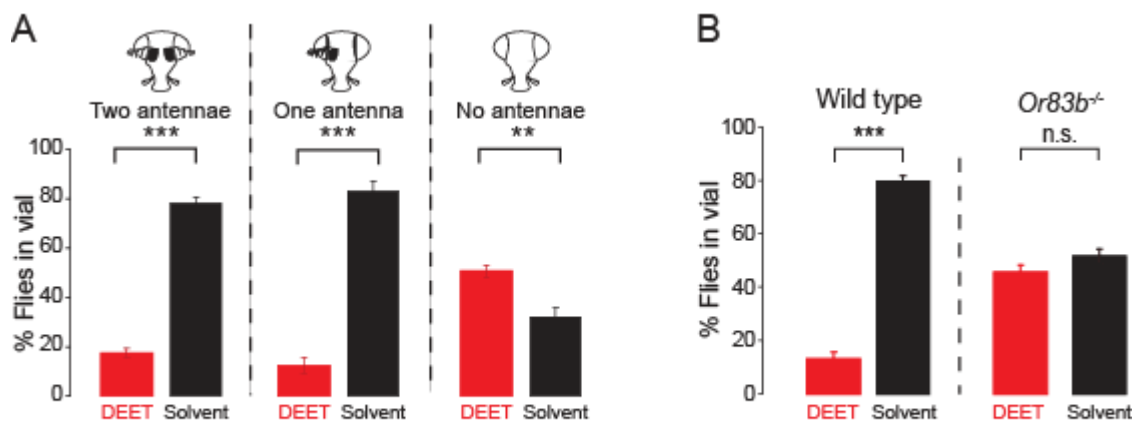
The experiments in this figure were performed by Dr. Mathias Ditzen

Figure 3.2 DEET-mediated behavioral inhibition is OR83b-dependent.
(**A**) Repellency of 10% DEET with perforated polypropylene barrier in the trap assay with food bait with surgically de-antennated flies (**, $p<0.01$; ***, $p<0.001$; Mann-Whitney test; mean±SEM, n=12). (**B**) Same assay as (**A**) with wild-type and *Or83b*$^{-/-}$ flies (***, $p<0.001$; n.s., not significant, Mann-Whitney test; mean±SEM, n = 13, 46). Adapted from Ditzen et al. (2008).

To determine whether DEET affected responses to food odorants in all or a subset of the OSNs, Dr. Ditzen recorded the extracellular electrical activity of the antennal olfactory neurons stimulated with the odor of fly food in the absence or presence of DEET (Figure 3.3A). These experiments showed complex effects of the insect repellent. In most OSNs, food-evoked responses were not affected by DEET, while a few showed potentiation (ab1A, ab3B, ab7, ab8) or inhibition (ab1B, ab5, atδ). The strongest inhibition was seen in the ab5 sensillum, which houses two olfactory sensory neurons, the ab5A and ab5B cells, expressing the OR82a/OR83b and OR47a/OR83b complexes, respectively. Responses elicited by a cognate ligand for OR47a, 3-methylthio-1-propanol (Figure 3.3B), and behavioral attraction mediated by the same odorant (Figure 3.3C) were decreased by the presence of DEET, correlating the electrophysiological phenotype to a behavioral outcome (Ditzen et al., 2008).

Similar results were obtained when DEET was applied to the *Anopheles gambiae* maxillary palp capitate peg (cp) sensilla, which house two cells: the CO_2-sensitive cpA cell, expressing the gustatory receptors GPRGR22/GPRGR23/GPRGR24, and the 1-octen-3-ol-sensitive cpB cell, expressing the olfactory receptors GPROR8/GPROR7 (Lu et al., 2007). We found that the presence of DEET inhibited cpB odorant-evoked responses (Figure 3.3D) but not the cpA CO_2-evoked activation (Figure 3.3E).

3.2 Insect ORs are molecular targets of DEET

Although the previous experiments are suggestive of an interaction of DEET with the olfactory system, they do not prove that olfactory receptors are directly affected by it. To test this, we carried out experiments in which responses of different insect ORs evoked by odorants in the presence of DEET were examined in heterologous cells.

We performed TEVC recordings in *Xenopus* oocytes expressing OR47a/OR83b. This receptor complex is particularly interesting because the sensory neurons in which it is expressed, the ab5B cell, showed a strong inhibition to food odor-evoked responses in the presence of DEET (Figure 3.3A). Treatment of OR47a/OR83b-expressing oocytes with high DEET concentrations did not generate currents nor did it prevent pentyl acetate-evoked currents in the same oocyte (Figure 3.4A). This suggests that DEET does not have non-specific effects on the cell membrane or endogenous membrane proteins. Oocytes stimulated with pentyl acetate for a sustained period of time showed slow inactivation of the current, which was not affected by intermittent stimulations with the same ligand concentration (Figure 3.4B). However, when DEET was applied along with the ligand, the odorant-evoked inward currents showed a dose-dependent and reversible decrease (Figure 3.4C). DEET similarly affected two *Anopheles gambiae* OR complexes, GPROR1/GPROR7 and GPROR2/GPROR7 (Figure 3.4E and G), tuned to the human sweat odorants 4- and 2-methyl phenol, respectively (Hallem et al., 2004b), and an additional OR complex, GPROR8/GPROR7, tuned to 1-octen-3-ol, a highly potent mosquito attractant (Figure 3.4I). This is in agreement with our *in vivo* results showing DEET inhibition of the OSN housing the GPROR8 receptor (Figure 3.3D).

Figure 3.3 DEET affects odorant-evoked responses in sensory neurons.

(**A**) Single-sensillum electrophysiology. Responses of OR83b-dependent antennal basiconic (ab) and trichoid (at) sensilla stimulated with food odorants along with solvent (black bars) or DEET (red bars). Data are plotted as mean corrected spikes/s±SEM (n=5-17 sensilla). Circles above bar graph indicate the fold change in response in the presence of DEET (filled circles, decrease; open circles, increase). (**B**) Dose-response curves of ab5B stimulated with 3-methylthio-1-propanol with solvent (black) or DEET (red; mean±SEM, n=4). (**C**; *left panel*) Trap assay in which one vial is baited with pure 3-methylthio-1-propanol (*p<0.05, Mann-Whitney test; mean±SEM, n=4). (*right panel*) Repellency of 10% DEET with perforated polypropylene barrier in the trap assay with pure 3-methylthio-1-propanol as bait (***p<0.001, Mann-Whitney test; mean±SEM, n=12). (**D-E**) Dose-response curves of mosquito cpA and cpB cells to CO_2 and 1-octen-3-ol with (red) or without (black) DEET (mean±SEM, n=7-13). Adapted from Ditzen et al. (2008).

The experiments in this figure were performed by Dr. Mathias Ditzen
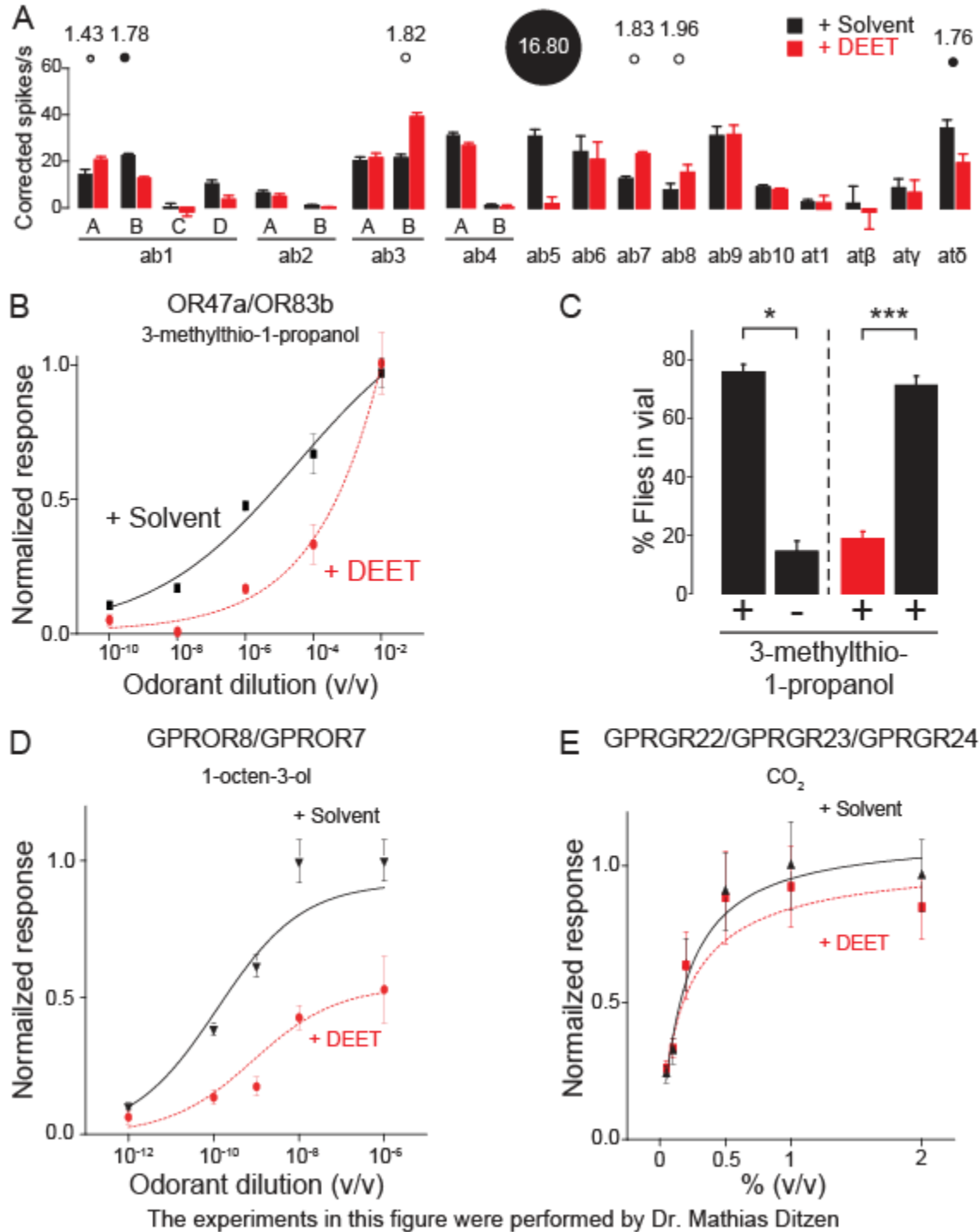
Figure 3.3 DEET affects odorant-evoked responses in sensory neurons.

To investigate whether DEET could affect the properties of OR-dependent currents, we analyzed the I-V curve relationships when the complexes where stimulated in the presence and absence of the insect repellent. In all cases, the effects on the odorant-evoked currents were symmetric at positive and negative potentials, and no change in reversal potential was observed (Figure 3.3D, F, H, and J). This could be explained by a reduction in ion permeability that does not affect ion selectivity through the OR channel.

Although DEET decreased the evoked currents for all the ORs tested, the extent of the effects was dose-dependent and differed according to the specific OR pair (Figure 3.3M). The insect repellent was not able to influence chloride currents elicited by the CFTR channel stimulated with forskolin (Figure 3.3K and L), nor impair the activation of the mouse eugenol olfactory receptor (mOR-EG) and subsequent cAMP production, which was observed by activation of CFTR (Figure 3.3N). However, DEET did inhibit other cation channels not related to insect ORs: the mouse transient receptor potential M8 (mTRPM8), the olfactory heteromeric CNG channel (CNGA2/A4/B1), and the *Drosophila* ether-a-gogo potassium channel (Figure 3.3N).

Taken together, these data suggest that insect olfactory receptors are direct targets of the insect repellent DEET. Since OR83b-dependent OSNs are affected differently in *Drosophila*, and some are not affected at all, it is unlikely that OR83b itself represents the main target of this insect repellent.

3.3 Controversial ideas on the mode of action of DEET

A recent study challenged our published results and suggested that DEET does not act by inhibiting olfactory responses, but acts to decreases the volatility of 1-octen-3-ol, and possibly other odorants, within the odorant delivery system through a not well described "fixative mechanism" (Syed and Leal, 2008). We were interested in evaluating Syed and Leal's claims but needed to find a compelling experimental paradigm to do so.

In vivo analysis both in our laboratory and Leal's laboratory relies on odorant-delivery devices in which the absolute concentration of odorant and DEET that reaches a given insect olfactory sensillum are essentially impossible to control or to measure between laboratories or even within the same laboratory. On the other hand, heterologous expression systems have not been successful for the expression of the majority of insect ORs. Moreover, they intrinsically lack additional components present in insect olfactory organs that might play a role for the proper action of insect repellents, such as odorant degrading enzymes, odorant binding proteins, and intracellular neuronal constituents. To overcome these limitations, we reasoned that we could distinguish between the various models proposed for DEET function by analyzing the activity of OSNs housed within the sensillum of *Drosophila* antennae. In each sensillum, every neuron is exposed to the same concentration of odorant and DEET, and shares the same lymph components. This system allows us to directly compare responses of multiple receptor complexes to single odorants and highlight the differential effects of DEET on separate ORs. By *in vivo* extracellular recordings of OSNs, we provide evidence that the insect repellent DEET directly modulates the OR ligand-binding subunit.

Figure 3.4 DEET decreases odorant-evoked currents in *Xenopus* oocytes.

(**A-B**) Pre-exposure to increasing concentrations of DEET (**A**) and inactivation of pentyl acetate-evoked currents (**B**) in oocytes expressing OR47a/OR83b. (**C-J**) Ligand-evoked currents in the presence of DEET in oocytes expressing OR47a/OR83b (**C**), GPROR1/GPROR7 (**E**), GPROR2/GPROR7 (**G**), and GPROR8/GPROR7 (**I**). (**D**, **F**, **H**, and **J**) show current-voltage (I-V) relationships during ligand stimulation in the absence (black squares) or presence (red circles) of 1000 μM DEET. Current was normalized to the value of +40 mV in the absence of DEET (mean±SEM, n=3 to 6). (**K**, **L**) Forskolin-evoked currents in the absence (**K**) or presence (**L**) of 1000 μM DEET. (**M**) DEET effects on ligand-dependent currents of insect ORs (mean±SEM, n=3-5). Current was normalized to the value of the current in the absence of DEET. (**N**) Normalized stimulus-evoked currents in oocytes expressing various receptors or ion channels in the presence of 1000 μM DEET (CFTR: 40 μM forskolin; mOR-EG+CFTR: 50 μM eugenol; GPROR2/GPROR7: 10 μM 2-methylphenol; GPROR1/GPROR7: 0.5 μM 4-methyl phenol; OR47a/OR83b: 100 μM pentyl acetate; GPROR8/GPROR7: 5 μM 1-octen-3-ol; CNGA2/A4/B1: 100 μM cAMP; ether-a-go-go: voltage steps from -60 mV to +20 mV; mTRPM8: 50 μM menthol). Bars labeled with different letters are significantly different (p<0.05, Kruskal Wallis test with posthoc multiple comparison correction against the CFTR control; mean±SEM, n=4-7). Adapted from Ditzen et al. (2008).

Figure 3.4 DEET decreases odorant-evoked currents in *Xenopus* oocytes.

63
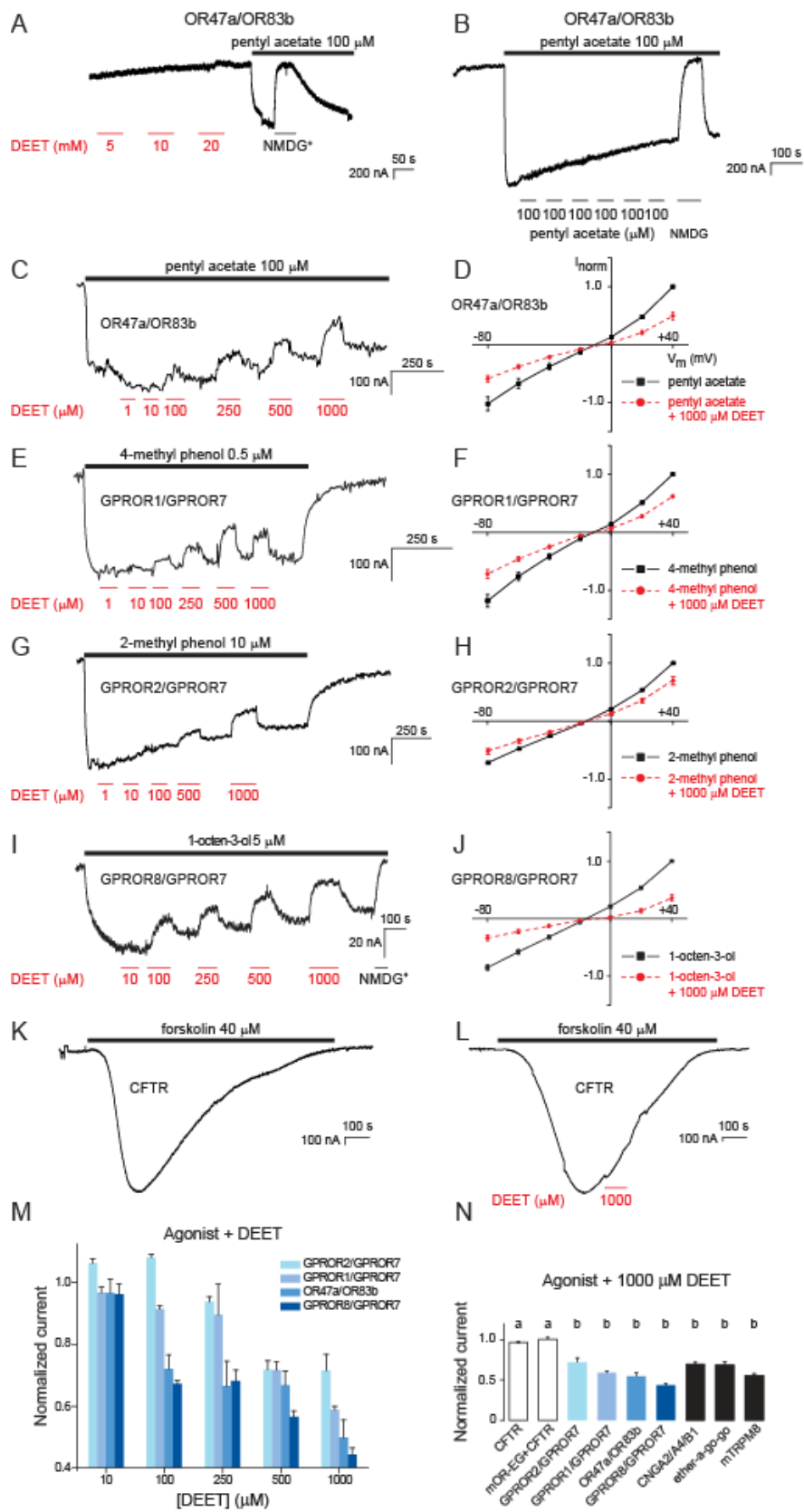
3.4 DEET modulation of responses to single compounds is OSN-, odorant-, and concentration-dependent

To assess the pharmacological action of DEET on ORs, we focused our analysis on the ab3 sensillum, which houses an A and a B cell that express the ligand-specific OR22a/22b, and OR85b subunits, respectively, along with OR83b. Both OR22a and OR85b have been shown to respond to a variety of alcohols and esters (Hallem and Carlson, 2006). OR22b is expressed along with OR22a but does not appear to be a functional ligand-binding receptor in *Drosophila melanogaster* (Dobritsa et al., 2003). The food odor we used in previous experiments to examine the effect of DEET on fly OSNs is a complex mixture of chemicals in variable ratios, and is therefore unsuitable for more detailed analysis. Instead, we selected a subset of single odorants that can stimulate both OSNs in a given sensillum, and analyzed the effects of DEET on the responses to increasing concentrations of ligands (Figure 3.5A-B).

The effects on responses elicited by single ligands were OSN-, odorant-, and concentration-dependent. Both ab3A and B cells stimulated with 1-octen-3-ol (Figure 3.5C-D) and 2-heptanone (Figure 3.5E-F) showed an apparent decrease in affinity when DEET is applied along with the odorant. Co-stimulation with pentanal and the insect repellent caused a minor effect on the A cell (Figure 3.5G), but abolished responses of the B cell (Figure 3.5H). DEET had no effect on the pentanoic acid-induced inhibition of ab3B while still decreasing odorant-evoked responses of ab3A neurons (Figure 3.5I-J).

Conversely, the inhibitory effects on the ab3A cell were dependent on the dose of DEET applied (Figure 3.6A). Although DEET seems to act over a large spectrum of the chemical space, ab3A responses to butyraldehyde were not significantly reduced in its presence (Figure 3.6B).

Taken together, these results are in agreement with the multitude of effects observed with food odor (Figure 3.3A), and with the idea that DEET might function by differentially altering the ability of the OSNs to respond to odorants.

Similarly, DEET affected odorant-evoked responses of OSNs housed in the ab2 sensillum expressing the OR59b/OR83b and OR85a/OR83b complexes (Figure 3.7 and Figure 3.8). It is known that a given OR complex can be inhibited and activated by different odorants (de Bruyne et al., 2001; Hallem and Carlson, 2006). Interestingly, 1-octen-3-ol can function on the OR59b/OR83b complex both as an inhibitor and an activator in a concentration-dependent manner. The activity of the ab2A cell was decreased by a 10^{-3} dilution of the odorant to levels below the spontaneous firing rate and the small solvent-induced activity. When the odorant was presented at a 10^{-2} dilution, the cell was effectively silenced (Figure 3.7D). Increasing the odorant dilution to 10^{-1} led to activation similar to paraffin oil, and at 10^{-0} the neuron was significantly activated.

Figure 3.5 Odorant-dependent effects of DEET on OSNs in the ab3 sensillum.

(**A**) Schematic of the odorant delivery protocol. Increasing concentrations of the indicated odorants were delivered to the ab3 sensillum in the absence or presence of DEET. Responses from ab3A neurons expressing OR22a/b/OR83b and ab3B neurons expressing OR85b/OR83b were recorded simultaneously and subsequently separated by spike-sorting algorithms. (**B**) Representative spike traces of ab3 sensillum recordings showing responses of the OR22a/b/OR83b OSN (*left*) and the OR85b/OR83b OSN (*right*) to 10^{-2} 1-octen-3-ol, in the absence (*top*) or presence (*bottom*) of DEET. Spikes corresponding to the relevant cell are highlighted in red, while those of the other cell are in black. Bars above traces represent the 1 s odorant stimulus. The delay in odorant response onset is a function of the odorant delivery system. (**C-J**) Dose-response curves of OR22a/b/OR83b (**C**, **E**, **G**, **I**) and OR85b/OR83b (**D**, **F**, **H**, **J**) stimulated with 1-octen-3-ol (**C**, **D**), 2-heptanone (**E**, **F**), pentanal (**G**, **H**), and pentanoic acid (**I**, **J**), with (dark color) or without (light color) DEET. Bar plots next to each dose-response curve represent responses to the solvent (PO, paraffin oil) in absence (grey) or presence (black) of DEET (significance assessed with F-test using Bonferroni correction. **, $p < 0.01$; ***, $p < 0.001$; n.s.=not significant; mean±SEM, n=9–16).
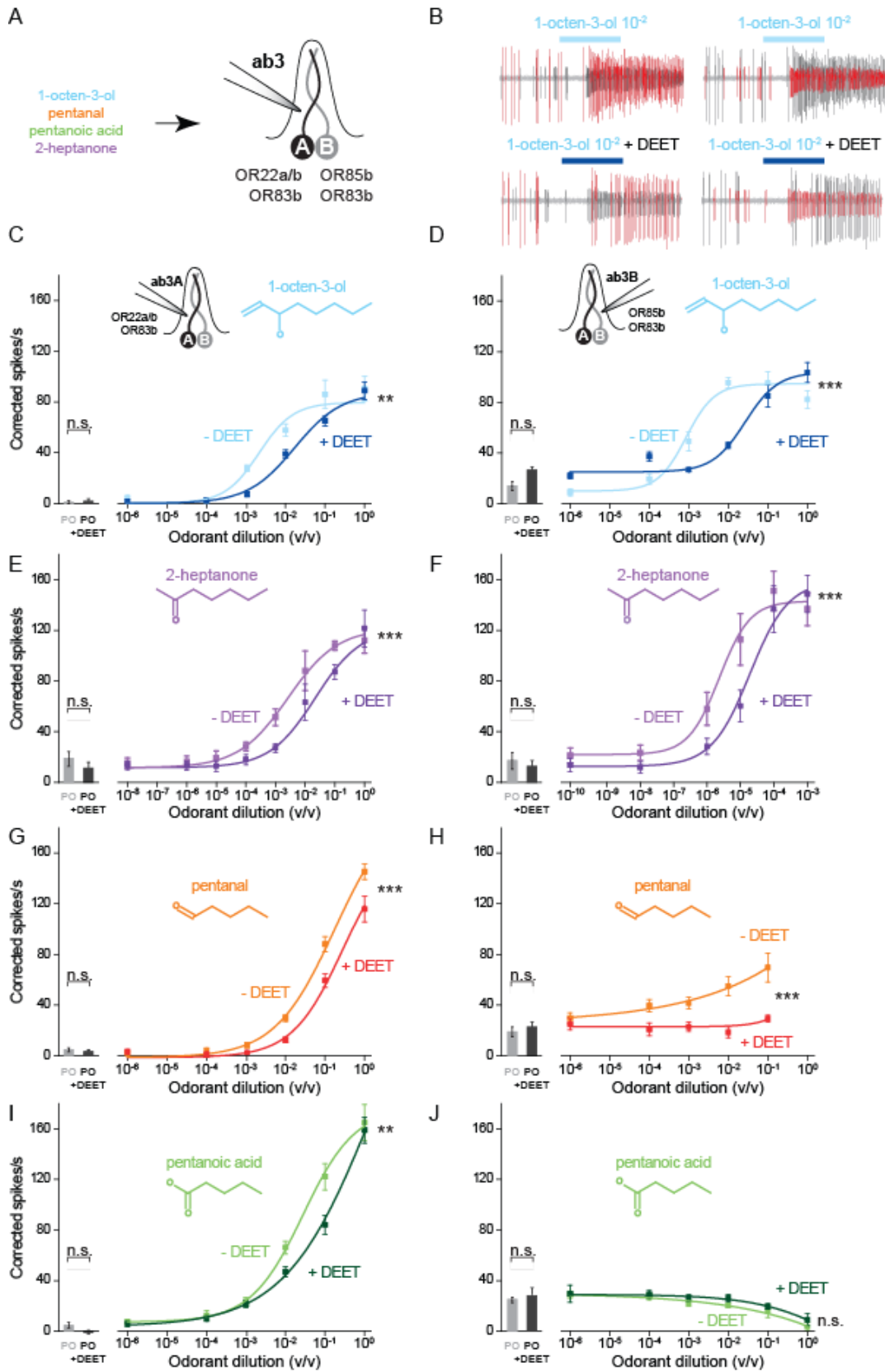
Figure 3.5 Odorant-dependent effects of DEET on OSNs in the ab3 sensillum.
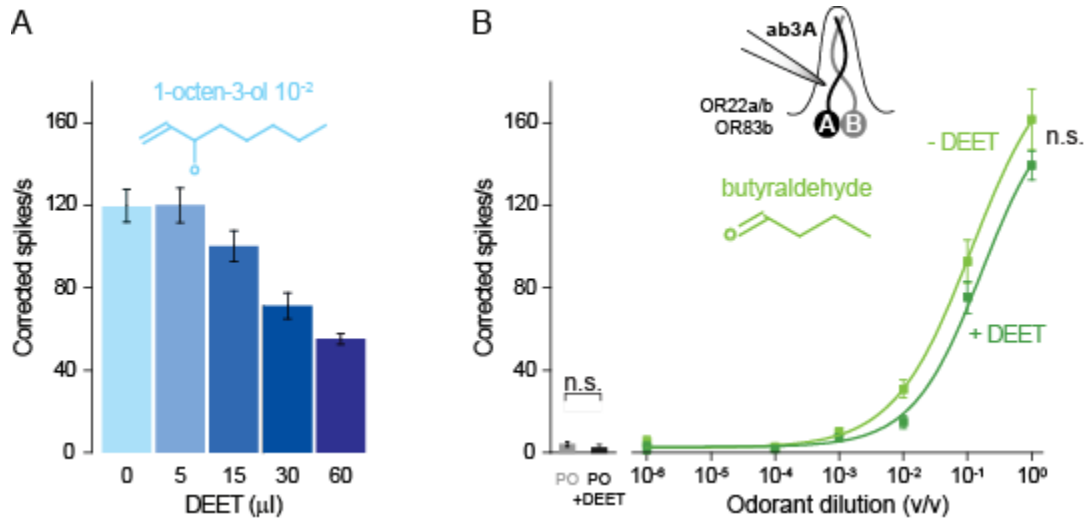
Figure 3.6 DEET effects on the OR22a/b/OR83b complex are odorant- and concentration-dependent.

(**A**) Dose-inhibition curve of DEET on OR22a/b/OR83b OSNs activated by 10^{-2} 1-octen-3-ol. (**B**) Dose-response curve of OR22a/b/OR83b OSNs stimulated with increasing concentrations of butyraldehyde in the absence (light green) or presence (dark green) of DEET. Bar plots (*left*) represent responses to the solvent (PO, paraffin oil) in absence (grey) or presence (black) of DEET (significance assessed with F-test using Bonferroni correction: n.s.=not significant; mean±SEM, n=6-11).

Although the corrected spike counts in these experiments fall below the arbitrary 50 spikes/sec threshold imposed by Hallem and Carlson (Hallem and Carlson, 2004; Hallem and Carlson, 2006), the responses are highly significantly different from responses evoked by solvent alone. Moreover, the actual number of spikes needed to elicit a behavioral output in any insect is unknown, but likely occurs below the arbitrary 50 spikes/sec threshold. Application of DEET along with the odorant suppressed the inhibition of the neuron, but did not alter its activation (Figure 3.7D). In contrast, the ab2B cell in the same sensillum showed activation to 10^{-2} 1-octen-3-ol, which was effectively suppressed when DEET was co-applied (Figure 3.7E).
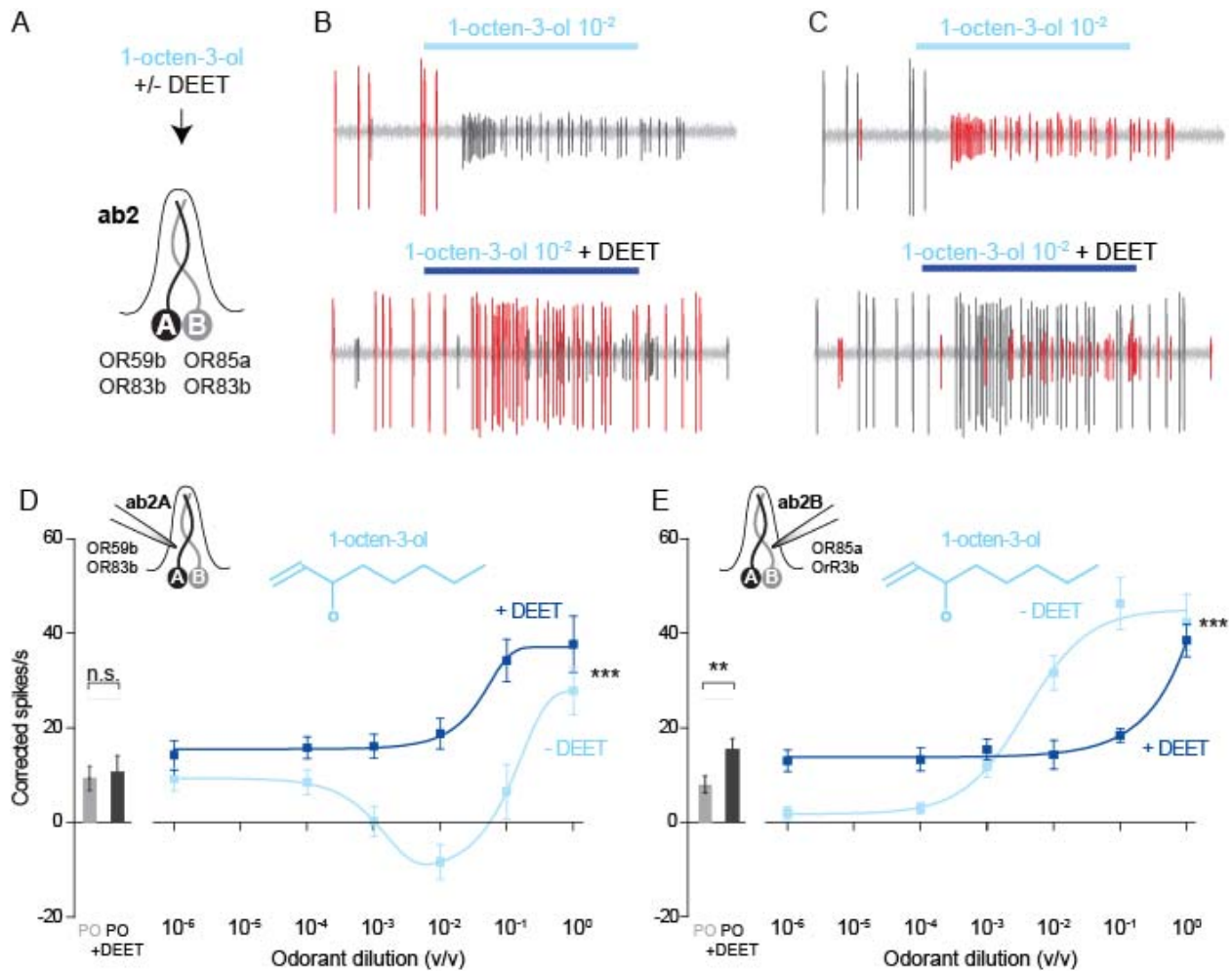
Figure 3.7 DEET affects odorant-evoked inhibition of the OR59b/OR83b complex.
(**A**), Schematic of the delivery protocol. Increasing concentrations of 1-octen-3-ol were delivered to the ab2 sensillum in absence or presence of DEET, and responses from OR59b/OR83b and OR85a/OR83b expressing neurons were recorded simultaneously. (**B-C**). Representative traces of single sensillum recordings. Bars represent 1 s odorant stimulation. Spikes corresponding to ab2A are in red in panel **B** and black in panel **C**. Spikes corresponding to ab2B are in red in panel **C** and black in panel **B**. The delayed response onset is a function of the odorant delivery system. (**D-E**) Dose-response curves of OR59b/OR83b (**D**) and OR85a/OR83b expressing cells (**E**) stimulated with 1-octen-3-ol in the absence (light color) or presence (dark color) of DEET. Bar plots next to each dose-response curve represent responses to the solvent (PO, paraffin oil) in the absence (grey bar) or presence (black bar) of DEET (***, p<0.001, F-test with Bonferroni correction; mean±SEM, n=8–22).

Therefore, the simultaneous application of odorant and DEET to two neurons in the same sensillum resulted in opposite effects: suppression of odorant activation in the ab2B cell and suppression of odorant inhibition in the ab2A cell. These results are not consistent with the contention that DEET acts by inhibiting 1-octen-3-ol volatility prior to reaching the olfactory sensillum (Syed and Leal, 2008). To test whether DEET can generally prevent odorant-evoked inhibition in the ab2A cell, we performed dose-response curves with two additional inhibitory odorants, linalool (Figure 3.8A) and 1-octanol (Figure 3.8B). In both cases, the presence of DEET caused a partial suppression of inhibition, with similar maximal inhibition reached at higher ligand concentrations. However, activation of the cell by methyl acetate and 2,3-butanedione was not affected by DEET (Figure 3.8C and D). Conversely, the activation of the ab2B neuron in the same sensillum, expressing the OR85a/OR83b complex, could still be affected when DEET was presented with 1-octanol and ethyl-3-hydroxybutyrate (Figure 3.8E and Figure 3.8F).
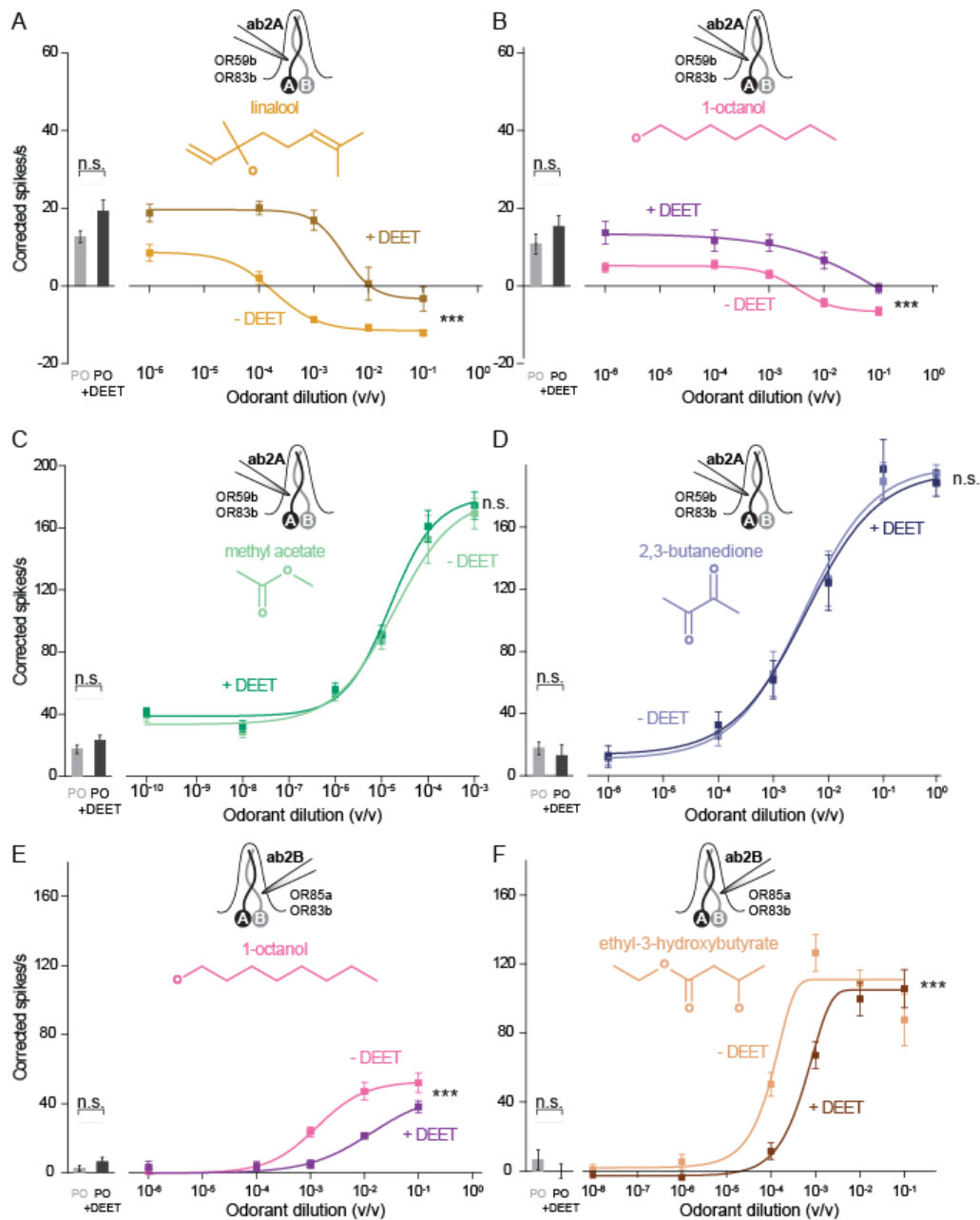
Figure 3.8 DEET affects odorant-dependent excitation in ab2B but not ab2A cells.
(**A-F**) Dose-response curves of the OR59b/OR83b (**A**, **B**, **C**, **D**) and OR85a/OR83b (**E**, **F**) complex stimulated with increasing concentrations of linalool (**A**), 1-octanol (**B**, **E**), methyl acetate (**C**), 2,3-butanedione (**D**), and ethyl-3-hydroxybutyrate (**F**) in the absence (light color) or presence (dark color) of DEET. Bar plots next to each dose-response curve represent responses to the solvent (paraffin oil, PO) in the absence (grey bar) or presence (black bar) of DEET (***, p<0.001; n.s.=not significant, F-test with Bonferroni correction; mean±SEM, n=6–22).

71

3.5 *Or59b* is polymorphic in 18 wild type populations of *Drosophila melanogaster*

To test whether DEET has a direct effect on ORs *in vivo*, we focused our attention on the responses of OR59b/OR83b to 1-octen-3-ol. We used *Or59b* allelic variants in populations of *Drosophila melanogaster* to analyze the effects that naturally occurring polymorphisms have on OR-odorant interaction sites and their effects on sensitivity to DEET.

Naturally occurring polymorphisms can result in amino acid changes in a protein, leading to changes in its function. Polymorphisms have been previously connected to differential odorant sensitivity in humans (Keller et al., 2007; Menashe et al., 2007), and behavioral responses to oxygen and carbon dioxide in the nematode *Caenorhabditis elegans* (McGrath et al., 2009).

Using 18 strains of *Drosophila melanogaster* collected in different locations around the world (Figure 3.9), we assessed responses of the OR59b/OR83b receptor complex to 10^{-2} 1-octen-3-ol in the absence or presence of DEET (Figure 3.10). ab2 sensilla were identified in each strain by the characteristic size and location of the sensilla and responses of the A cell to a 10^{-5} dilution of its cognate ligand methyl acetate (data not shown). Since all strains belong to the *Drosophila melanogaster* species, OR expression patterns in identified sensilla are likely to be conserved.

Figure 3.9 World map indicating the origin of the 18 wild type *Drosophila melanogaster* strains analyzed.

World map indicating the location where the founders of 18 *Drosophila melanogaster* strains were collected.

When stimulated with 1-octen-3-ol, 17 strains showed w^{1118}-like inhibition that could be suppressed by co-application of DEET. However, the Brazilian strain Boa Esperança lacked 1-octen-3-ol inhibition, and the presence of DEET did not affect its response to the odorant (Figure 3.10B and D).

Aside from a lack of inhibition by 1-octen-3-ol, ab2 sensilla of the Brazilian strain had response profiles otherwise similar to our w^{1118} control both for the A (Figure 3.11A-C) and B cell (Figure 3.11D). This suggests that the sensillum expresses the characteristic receptors found in ab2 sensilla, OR85a/OR83b and OR59b/OR83b, and that there is a potential OR59b^{Boa} variant in this strain.

Figure 3.10 Responses of OR59b/OR83b to 1-octen-3-ol and sensitivity to DEET vary across wild type *Drosophila melanogaster* strains.

(**A**, **C**; u*pper panel*) Schematic of the screening protocol: 10^{-2} 1-octen-3-ol (**A**) or solvent (**C**; PO, paraffin oil) was delivered in the absence (light color) or presence (dark color) of DEET. (*Lower panel*) DEET suppresses 1-octen-3-ol-evoked activity of w^{1118} ab2A neuron (***, p<0.001; t-test with Bonferroni correction; mean±SEM, n=10). (**B**, **D**) Bar plots of odorant-evoked responses of 18 wild type strains to 10^{-2} 1-octen-3-ol (**B**) or PO (**D**) in the absence or presence of DEET (***, p<0.001; n.s.=not significant, t-test with Bonferroni correction; mean±SEM, n=10–17).
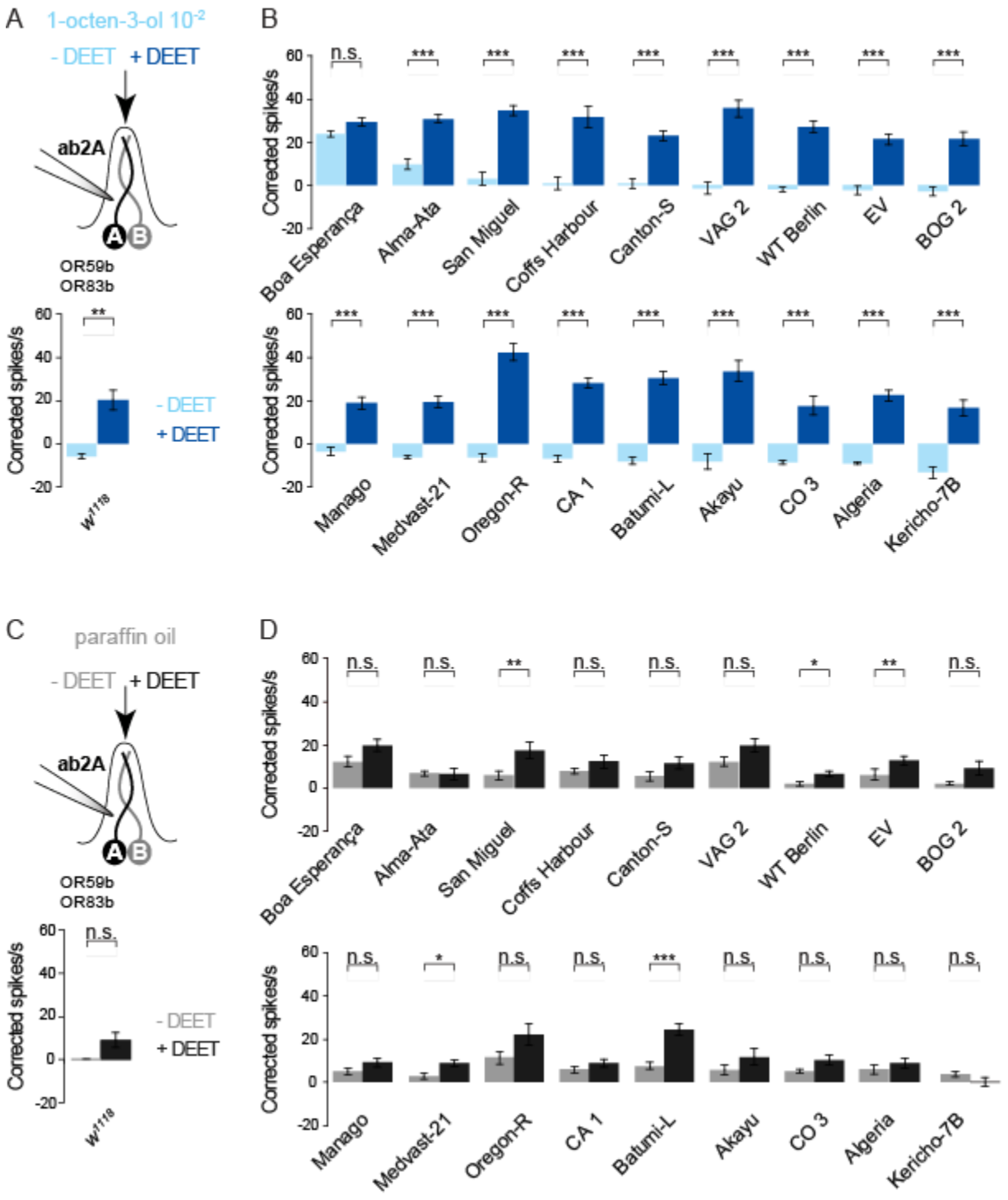
Figure 3.10 Responses of OR59b/OR83b to 1-octen-3-ol and sensitivity to DEET vary across wild type *Drosophila melanogaster* strains.

Figure 3.11 Comparison of responses of OSNs housed in the ab2 sensillum of *w¹¹¹⁸* **and Boa Esperança.**

(**A**) Schematic of the odorant delivery protocol. The indicated odorants were delivered to the ab2 sensillum of w^{1118} and Boa Esperança, and responses from the ab2A and ab2B OSNs were recorded simultaneously. (**B**) Representative traces of single sensillum recordings. The red traces show responses of the w^{1118} (*upper panel*) and the Boa Esperança (*lower panel*) ab2A cells to 10^{-5} methyl acetate. The delay in odorant response onset is a function of the delivery system. (**C**, **D**) The responses of the w^{1118} ab2A (**C**) and ab2B (**D**) cells are plotted against the corresponding Boa Esperança ab2A and ab2B cell, respectively. The dotted lines show the linear regression fit of the values (mean±SEM, n= 9).
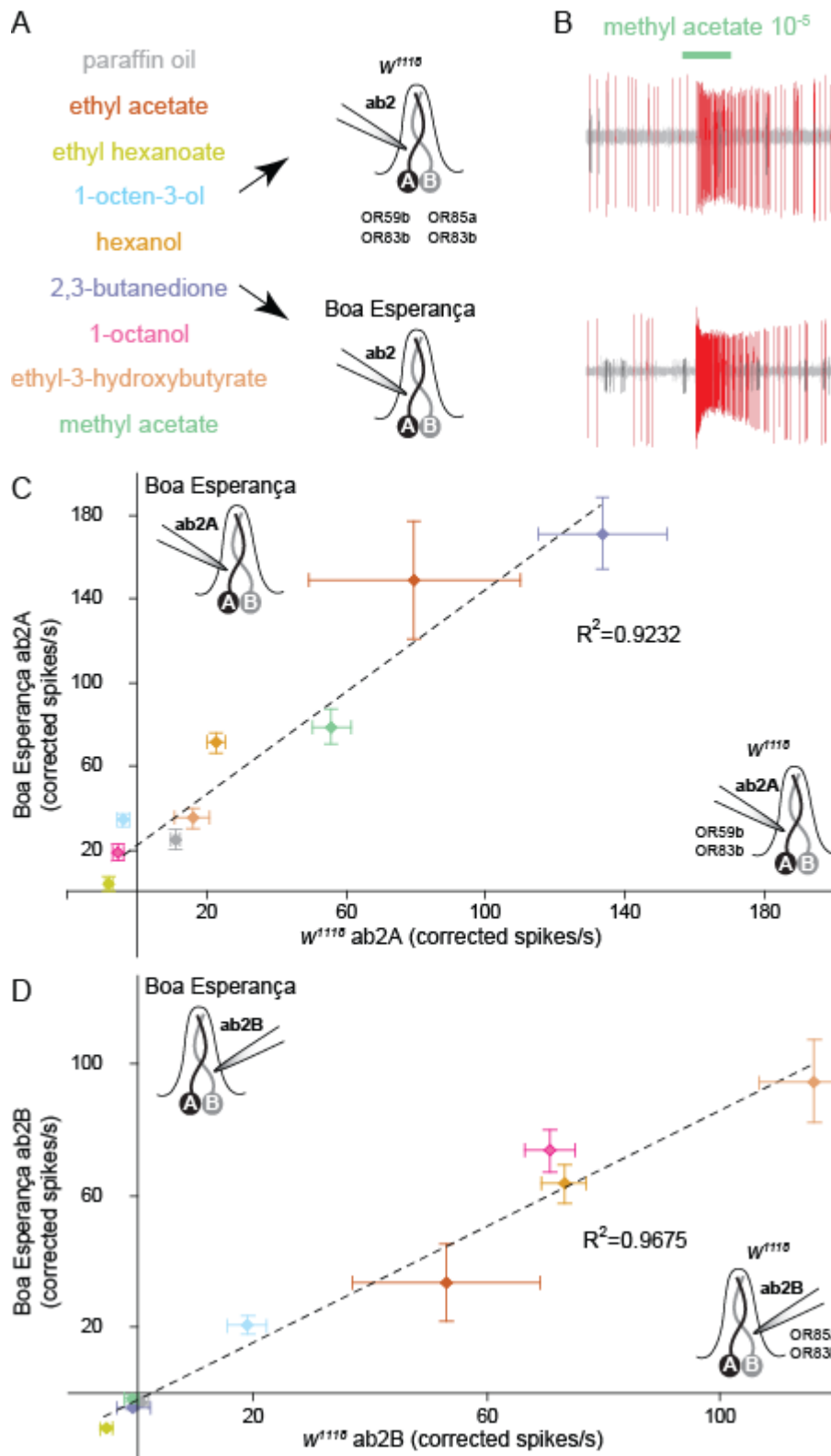
Figure 3.11 Comparison of responses of OSNs housed in the ab2 sensillum of w^{1118} and Boa Esperança.

3.6 Boa Esperança ab2A neurons exhibit decreased levels of odorant-evoked inhibition compared to w^{1118}

We then compared the full dose-response to 1-octen-3-ol with responses of OR59b from w^{1118} (Figure 3.12A). Unlike our control strain, OR59b^{Boa} expressing neurons were not inhibited by 1-octen-3-ol for all dilutions tested, but still preserved odorant-evoked excitation at higher ligand concentrations. Furthermore, DEET did not modulate 1-octen-3-ol-evoked activity of OR59b/OR83b in the Boa Esperança strain (Figure 3.12B). Excitatory responses were also not affected, as the response profile to methyl acetate, both in the absence and presence of DEET, did not differ when compared with the corresponding w^{1118} neuron (Figure 3.12C and D).

We then asked whether the loss of inhibition affected only 1-octen-3-ol, or if it similarly modulated the effect of other inhibitory ligands. As shown in Figure 3.12E, the ab2A cell in Boa Esperança also showed impaired inhibitory responses to 1-octanol and ethyl hexanoate. However, linalool was still able to inhibit the spontaneous activity of the neuron to the same extent of the w^{1118} OR59b-expressing OSN. Neither excitatory nor inhibitory responses of the B cell to the same odorants were altered (Figure 3.12F). These results eliminate the possibility that the observed differences in the Boa Esperança strain can be attributed to changes in either the sensillum lymph or other pan-neuronal cellular components, and strongly argue in favor of mutations present on the OR59b/OR83b complex expressed on the ab2A neuron of Boa Esperança.

Figure 3.12 ab2A neurons in w^{1118} and Boa Esperança differ in odorant-evoked inhibition and sensitivity to DEET.

(**A-C**) Dose-response curves of the OR59b/OR83b complex in the wild type w^{1118} (solid line) and Boa Esperança (dotted line) strains stimulated with of 1-octen-3-ol (**A**) or methyl acetate (**C**; ***, p<0.001; n.s.=not significant, F-test with Bonferroni correction; mean±SEM, n=5–14). The dose-response curve of w^{1118} to 1-octen-3-ol is reproduced from Figure 3.7D for comparison. (**B**, **D**) Dose-response curves of the OR59b/OR83b complex in the wild type w^{1118} (solid line) and Boa Esperança (dotted line) strains stimulated with increasing concentrations of 1-octen-3-ol (**B**) or methyl acetate (**D**) in the presence of DEET. Bar plots next to dose-response curves represent responses to the solvent paraffin oil (PO) in the absence (grey bar) or presence (black bar) of DEET (n.s.=not significant, F-test with Bonferroni correction; mean±SEM, n=5–11). (**E-F**) Bar plots comparing responses of the OR59b/OR83b (**E**) and OR85a/OR83b complexes (**F**) in the w^{1118} (solid bar) and Boa Esperança (dotted bar) strains to 10^{-2} 1-octen-3-ol, 10^{-1} 1-octanol, 10^{-1} ethyl hexanoate, and 10^{-1} linalool (**, p<0.01; ***, p<0.001; n.s.=not significant, t-test with Bonferroni correction; mean±SEM, n=9–11).
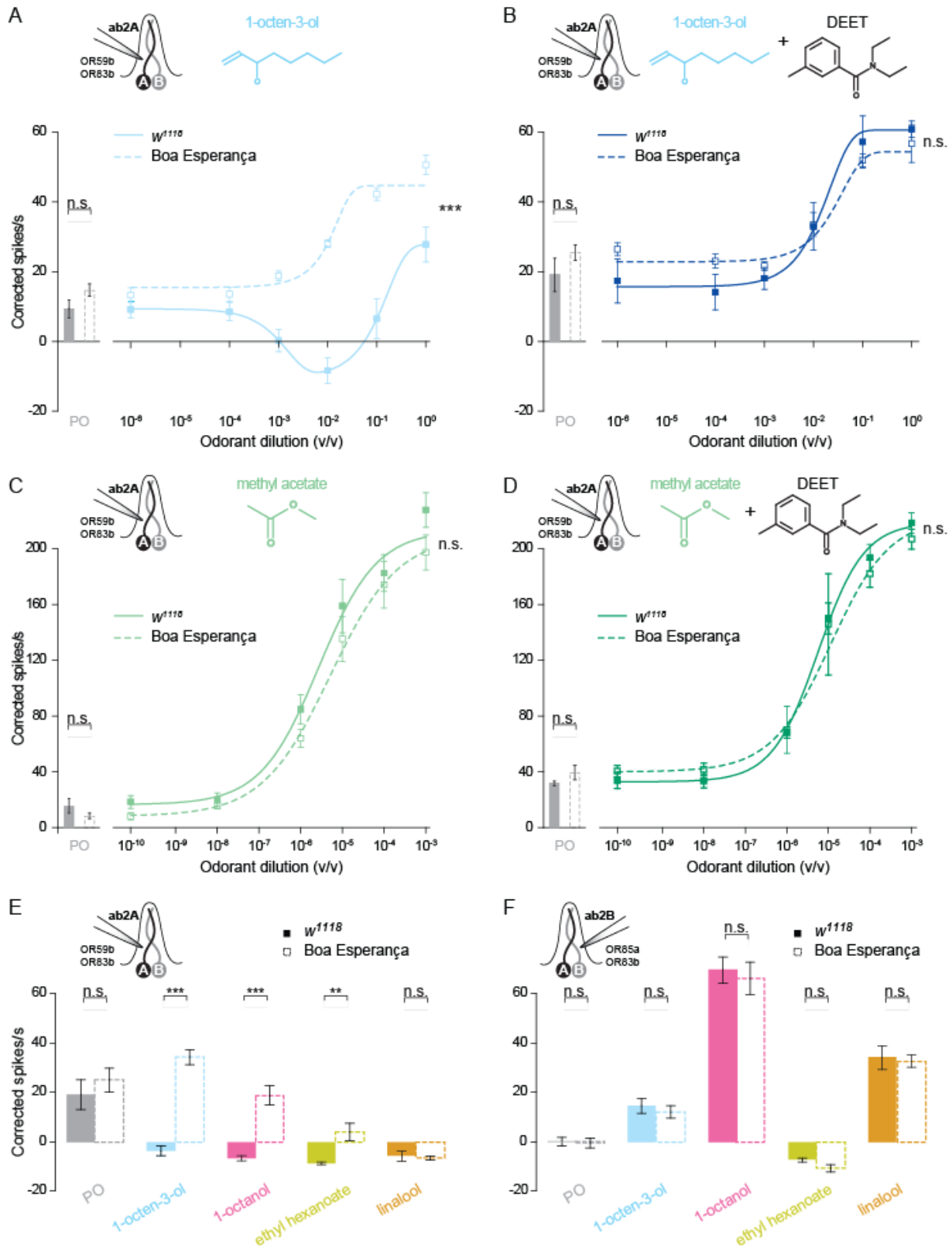
Figure 3.12 ab2A neurons in *w¹¹¹⁸* and Boa Esperança differ in odorant-evoked inhibition and sensitivity to DEET.

3.7 A single natural missense polymorphism in *Or59b* confers pharmacological resistance to DEET

To verify the hypothesis that DEET acts directly on OR59b to modulate the receptor complex, we sequenced the coding region of *Or59b* in the 19 wild type strains and compared them to the published *Or59b* sequence (NCBI reference number NP_5238822.1). *Or59b* is one of the most highly conserved odorant receptor genes among closely related *Drosophila* species separated by 12 million years of evolution (McBride et al., 2007). Within our *Drosophila melanogaster* strains, we identified seven missense polymorphisms that allowed us to group OR59b into distinct protein haplotypes (Figure 3.13A). Based on limited within-strain sampling, we detected only one protein haplotype per strain, with the exception of the w^{1118} control for which we identified two separate sequences (Figure 3.13A and Table 3.1), one identical to the published OR59b sequence (OR59b$^{NCBI\ REF}$), and one containing two missense polymorphisms (OR59b$^{M352I\ T376S}$). Since we did not observe two different phenotypes in our electrophysiological recordings for this strain, both proteins are likely to have similar functional properties for the odorants tested. Interestingly, Boa Esperança was the only strain containing four missense polymorphisms (V41F, V91A, T376S, and V388A). The V41F and V91A polymorphisms, located in the N-terminus intracellularly near TM1 and within TM2, respectively, are unique to this strain (Figure 3.13B).
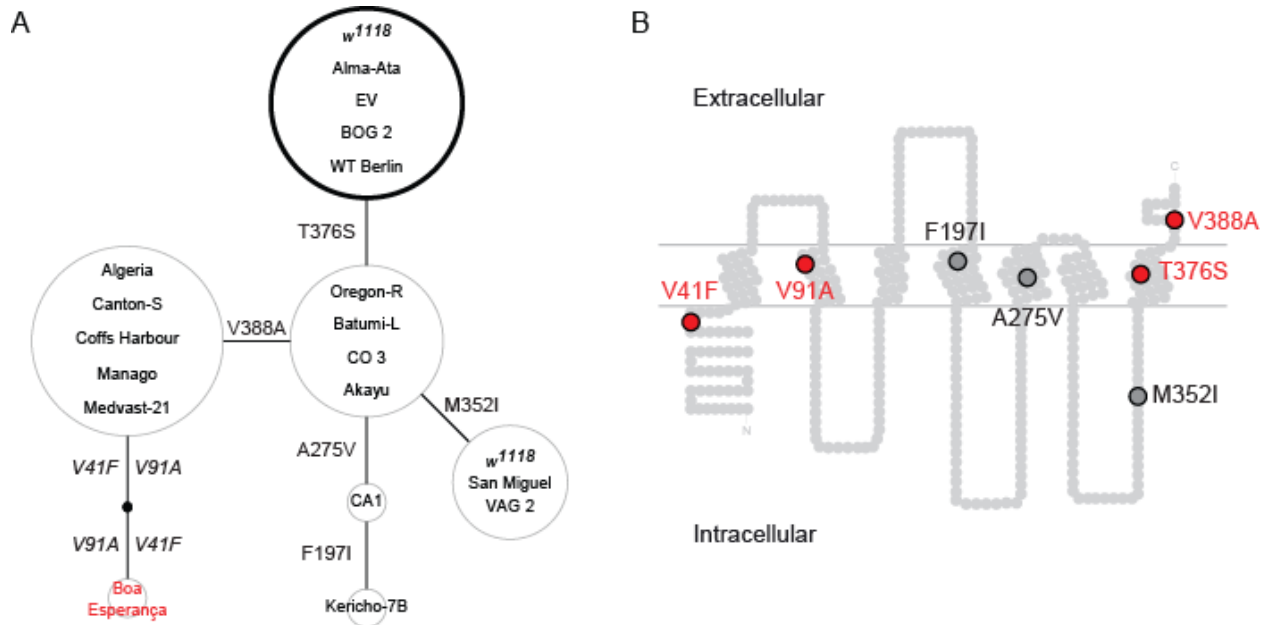
Figure 3.13 Summary of OR59b missense polymorphisms in the 19 wild type strains of *Drosophila melanogaster* analyzed.

(**A**) Haplotype network for OR59b protein sequences. Each circle represents a unique OR59b protein haplotype, its size proportional to the number of strains containing each variant. Connecting lines show the type of amino acid substitutions that separate each haplotype. The bold circle represents the reference NCBI haplotype NP_5238822.1. The Boa Esperança strain is shown in red. (**B**) Snake plot of OR59b showing the location of missense polymorphisms. Substitutions in Boa Esperança are shown in red.

In addition to the seven missense polymorphisms that induced amino acid substitutions, we found 36 silent polymorphisms (see Table 3.1 and Figure 3.14). Since the co-receptor OR83b is an essential component of the OR complex, we sequenced the *Or83b* gene in both *w^{1118}* and Boa Esperança strains but did not detect any missense polymorphisms relative to the NCBI reference sequence NP_524235.2 (data not shown).

To test whether missense polymorphisms in *Or59b* are responsible for the sensitivity to 1-octen-3-ol and DEET, we generated receptor variants containing each one of the four polymorphisms (V41F, V91A, T376S, and V388A), and a combination of the two polymorphisms unique to the Brazilian strain (V41F V91A), or the polymorphisms shared with other strains (T376S V388A), based on the OR59b$^{NCBI\ REF}$ backbone. To test the function of each OR59b variant, we expressed cDNA in the *Drosophila* "empty neuron" (Hallem et al., 2004a). This system uses *Δhalo* flies (Gross et al., 2003) containing a synthetic deletion encompassing the *Or22a* and *Or22b* genes normally present in ab3A neurons. This allows the expression of *Or59b* using the GAL4-UAS system under the control of the *Or22a* promoter, therefore functionally replacing the endogenous ligand-binding OR protein with a given OR59b mutant.

Table 3.1 Silent and missense polymorphisms of *Or59b* in 19 *Drosophila melanogaster* strains

Strain	Origin	Silent polymorphisms	Missense polymorphisms
BOG2	Bogota	P78, A94, N133	NCBI REF*
Alma-Ata	Kazakhstan	P78, A94, N133	NCBI REF*
WT Berlin	Berlin, Germany	R27, C51, A55, P78, A94, N133, D206	NCBI REF*
Boa Esperança	Minas Gerais, Brazil	S95, D206, L348	V41F V91A T376S V388A
Manago	Hawaii, USA	R27, V355, I359	T376S V388A
Algeria	Algeria	R27, L103, V355, I359	T376S V388A
Canton-S	Canton, Ohio, USA	R27, C51, A55, P78, A94, N133, D206	T376S V388A
w^{1118}†	Oregon, USA	L25, R27, R43, C51, A55, P78, A94, N133, I186, D206	NCBI REF*
			M352I T376S
Oregon R	Oregon, USA	R27, L103, G282, V355, I359, K379	T376S
EV	Ellenville, New York, USA	R27, C51, A55, P78, A94, D206	NCBI REF*
Coffs Harbour	New South Wales, Australia	R27, C51, A55, P78, A94, D206	T376S V388A
San Miguel	Buenos Aires, Argentina	P78, A94, N133, I186, D206, V227	M352I T376S
Medvast-21	Finland	R27, C51, A55, P78, A94, D206, I322, V355, I359	T376S V388A
VAG2	Athens, Greece	R27, C51, A55, P78, A94, I186, D206, G297	M352I T376S
CO3	Commack, New York, USA	R27, C51, A55, P78, A94, N133, K379, F380, I385	T376S
Kericho-7B	Kericho, Kenya	R27, P78, Y92, E112, L276, L302, P313, R343, I359, K379, F380	F197I A275V T376S
Batumi-L	Batumi, Georgia	I186, D206, F274, G282, T306, P313, I322, V355, I359, S370, K379, F380, I385	T376S
CA1	Cape Town, South Africa	T13, R27, A55, F56, T77, P78, Y92, S117, L118, D206, L276, L302, P313, R343, I359, K379, F380	A275V T376S
Akayu	Akayu, Japan	R27, C51, A55, P78, A94, N133, F274, G282, T306, P313, I322, V355, I359, S370, K379, F380, I385	T376S

*NCBI REF corresponds to the OR59b NCBI reference sequence NP_5238822.1

†We found two different protein haplotypes that co-segregate in w^{1118}
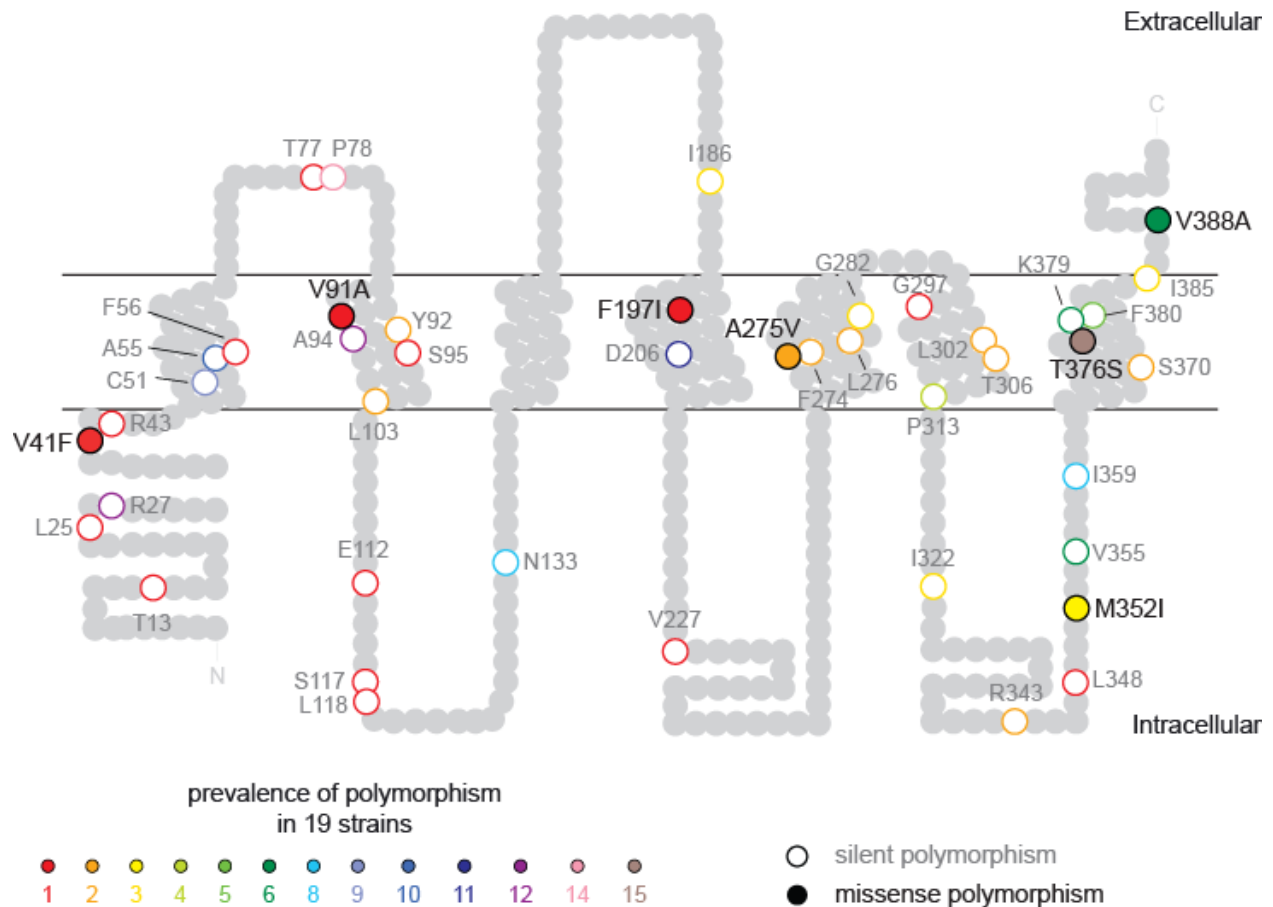
Figure 3.14 Silent and missense polymorphisms of OR59b.

Snake plot of OR59b showing the prevalence and location of missense (filled circles) and silent (open circles) polymorphisms in the 19 strains analyzed. The position of each polymorphism is reflected on the corresponding amino acid and color coded according to its occurrence.

OR59b[NCBI REF] expressed in ab3A neurons showed a decrease in spontaneous activity comparable to the endogenous receptor in w^{1118} animals after application of 10^{-2} 1-octen-3-ol (See Figure 3.15 and Figure 3.10 for comparison). In contrast, OR59b[Boa] expressed in ab3A neurons showed activation after application of 10^{-2} 1-octen-3-ol comparable to the response of the ab2A neuron in the Boa Esperança strain (Figure 3.15 and Figure 3.1). OR59b[T376A], OR59b[V388A], and OR59b[T376A V388A] were also inhibited by the odorant (Figure 3.15A).

The V91A polymorphism was the only one necessary and sufficient to phenocopy the electrophysiological effects of the endogenous Boa Esperança OR59b (Figure 3.15A). Both the single amino acid substitution (V91A) and any combination tested (V41F V91A and V41F V91A T376S V388A) showed the same DEET insensitivity and loss of odorant inhibition (Figure 3.15A).

For each experiment, we verified that responses of endogenous OR59b in the native ab2A neuron on the same antennal preparation showed normal inhibition by the odorant (data not shown).

A recent paper documented an effect of "silent" synonymous SNPs on the function of the ABC transporter MDR1, presumably because rare codons affected timing of co-translational folding and membrane insertion (Kimchi-Sarfaty et al., 2007). Although we did not explicitly test the role for the many synonymous *Or59b* SNPs identified in the course of our analysis, it is unlikely that silent changes in the *Or59b* coding region affect its inhibition to 1-octen-3-ol since its effects are reproducible across different strains that contain multiple and diverse SNP patterns.
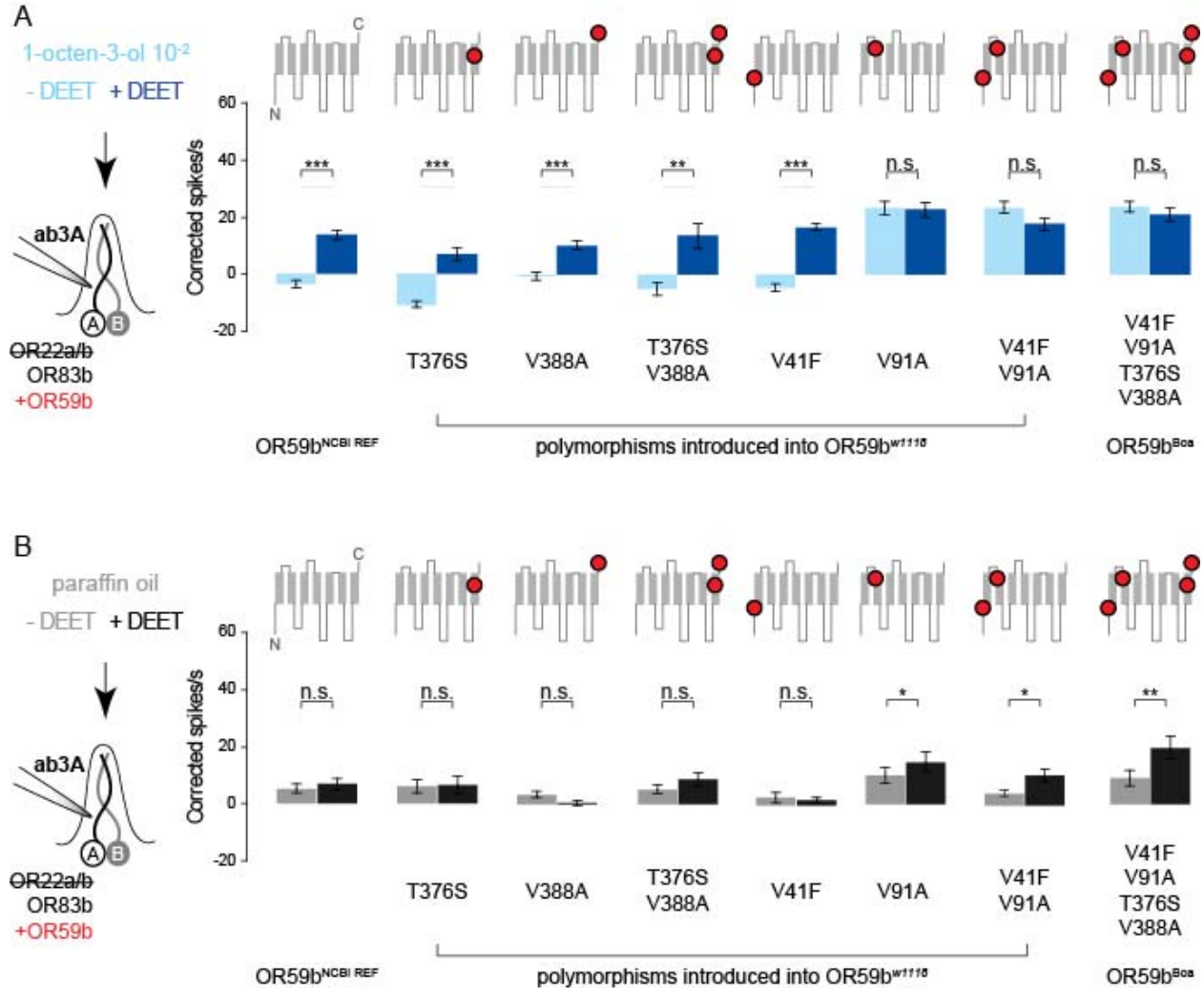
Figure 3.15 A single natural polymorphism in *Or59b* confers pharmacological resistance to DEET.

(**A-B**) Bar plots show the responses of OR59b variants expressed in *Δhalo* ab3A neurons, which lack endogenous *Or22a* and *Or22b* genes, to 10^{-2} 1-octen-3-ol (**A**) or the solvent (**B**; PO, paraffin oil) in the absence (light color) or presence (dark color) of DEET. The location of variant amino acids in OR59b is depicted in the cartoon snake plot on top of each bar plot (*, $p<0.05$; **, $p<0.01$; ***, $p< 0.001$, n.s.=not significant, t-test with Bonferroni correction; mean±SEM, n=7–11).

3.8 Model of the odorant receptor complex OR59b/OR83b

Based on these results, we can speculate about a possible model of the odorant receptor complex OR59b/OR83b. Although the stoichiometry of any OR complex is still unknown, experimental evidence suggests that the functional OR is composed of at least two OR83b subunits and two ligand binding subunits (Benton et al., 2006).

The dose-response curve of the OR59b/OR83b complex to 1-octen-3-ol (Figure 3.7D) might be explained by the presence of two different binding sites that lead to different conformational changes in the OR channel (Figure 3.16A-B). At lower concentrations of 1-octen-3-ol, the odorant occupies the inhibitory site, leading to a closed state of the OR complex and inhibition of the neuron (Figure 3.16C). At higher odorant concentrations, occupation of the excitatory site leads to an open channel conformation and activation of the neuron (Figure 3.16D). The effects of DEET on the dose-response curve in Figure 3.7D can be explained by postulating interactions between the repellent and binding of the odorant to the inhibitory site, either directly (Figure 3.16E) or through some allosteric modulation. At low odorant concentrations, the insect repellent would then effectively block the inhibition of the OR complex (Figure 3.16E), without interfering with the excitation observed at higher odorant concentrations (Figure 3.16F). This model is supported by the fact that DEET does not interfere with ligands that act as pure agonists, as shown in Figure 3.8C and D, suggesting that DEET does not have access to the excitatory binding site. Moreover, odor-evoked inhibition and sensitivity to DEET are specifically abolished by the polymorphism V91A (Figure 3.12 C and D, Figure 3.15A). This shows that a mutation in the ligand-binding subunit can uncouple inhibitory and excitatory responses.
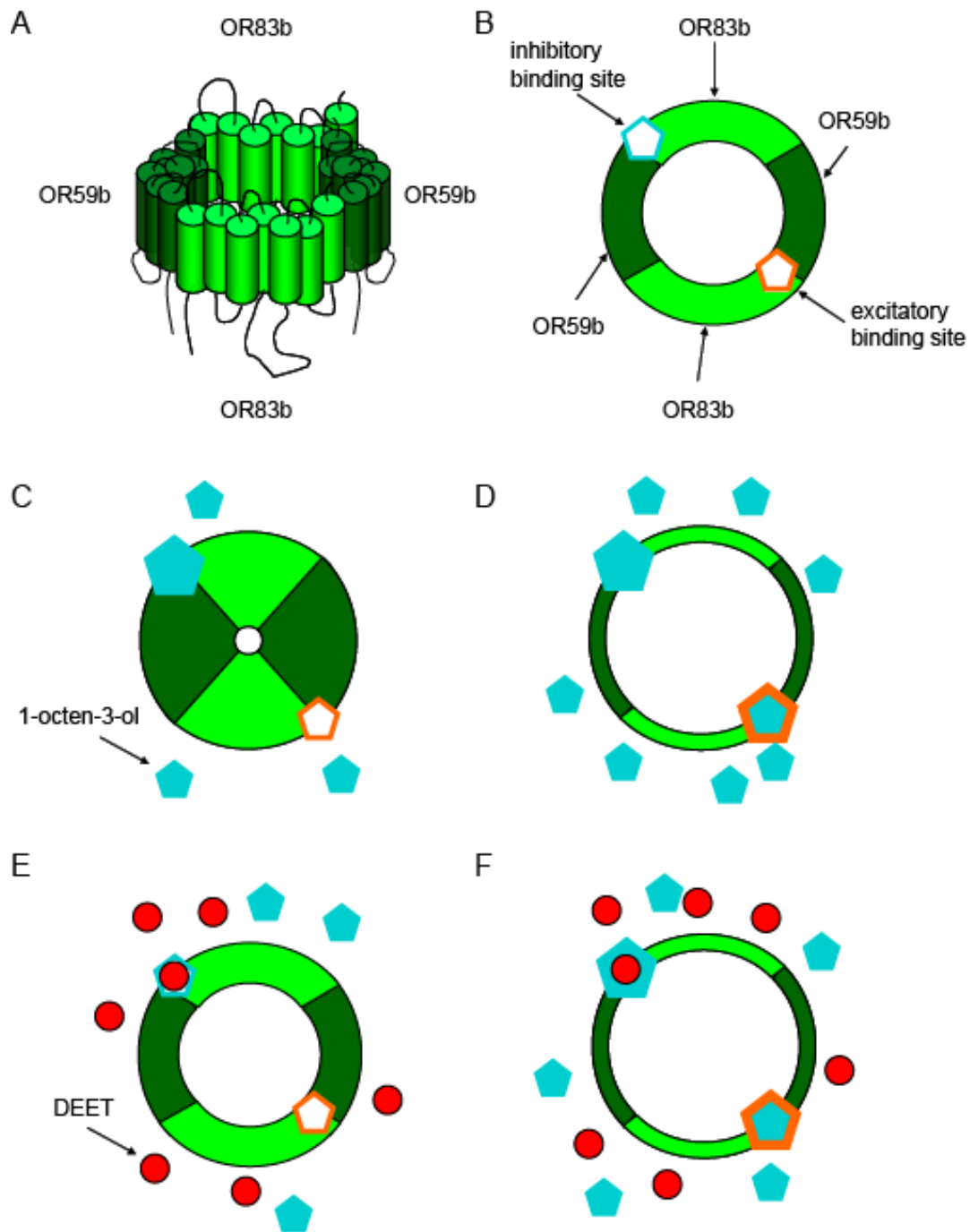
Figure 3.16 Model of the OR complex OR59b/OR83b.

(**A-B**) 3D (**A**) and 2D (**B**) model of the OR59b/OR83b receptor complex, based on Benton et al. (2006). The location of the binding sites is arbitrary (**C-F**) Putative model for the interactions of 1-octen-3-ol and DEET on the receptor complex. Refer to the text for a complete explanation of the model.

3.9 DEET as a molecular confusant of insect olfactory receptors

In this study, we investigated the mechanisms of action of the insect repellent DEET acting in the gas phase. Previous studies have described insecticidal properties (Pridgeon et al., 2009; Xue et al., 2007), active repellency (Plettner and Gries, 2010; Syed and Leal, 2008), and a direct effect on odorants (Syed and Leal, 2008), but its precise mode of action is still controversial.

Our *in vivo* results using food-seeking behavior in *Drosophila melanogaster* demonstrated that the insect olfactory system is necessary for DEET to exert its effects (Figure 3.1-Figure 3.3). Moreover, OR expression studies in *Xenopus* oocytes showed that OR-generated currents are decreased by DEET, suggesting a direct effect of the insect repellent on the insect olfactory receptors (Figure 3.4).

To investigate whether DEET has "fixative" effects on odorants, therefore preventing them from being released from the delivery system (Syed and Leal, 2008), we studied the electrophysiological responses to odorants that can stimulate OSNs housed in the same sensillum. We found that DEET affects responses to single odorants in an OSN-, odorant-, and concentration-dependent way (Figure 3.5-Figure 3.8). Because DEET has opposing effects on 1-octen-3-ol responses on two neurons housed in the same sensillum (Figure 3.7D-E), we can exclude the proposed artifacts of reduced odorant delivery as the mechanism by which DEET reduces OSN activation (Syed and Leal, 2008).

In addition we reasoned that, if DEET were acting directly on olfactory receptors, mutations in specific OR residues could lead to DEET-resistant OR complexes. The

OR59b/OR83b complex is expressed in the ab2 sensilla and is inhibited and activated by increasing concentrations of 1-octen-3-ol, and DEET can specifically suppress its odorant-evoked inhibition (Figure 3.7). We searched through 18 populations of *Drosophila melanogaster* for ab2 sensilla with altered responses to 1-octen-3-ol (Figure 3.10) and found that the Brazilian strain Boa Esperança displays impaired inhibitory responses of OR59b/OR83b but retains normal odorant-evoked excitation (Figure 3.12). The *Or59b* gene in this strain contains two unique missense polymorphisms, among which V91A is sufficient and necessary to confer the same loss of inhibition observed in Boa Esperança when the OR59b receptor was misexpressed in the 'empty neuron' system, therefore rendering the receptor resistant to DEET (Figure 3.15). Unfortunately, we were unable to obtain functional heterologous expression of OR59b in HEK293T cells, and so were unable to probe the interactions of DEET with the various OR59b receptors variants in greater mechanistic detail (data not shown).

The missense changes occurring in the OR59b receptor within *Drosophila melanogaster* populations are also shared with other non *melanogaster* species. By comparing OR59b sequences in 12 *Drosophila* species (Ware et al., 1975) we found that an alanine at position 91 is also present in *Drosophila yakuba* (*melanogaster* subgroup) and *Drosophila grimshawi* (*Hawaiian Drosophila* group), while the valine is found in the other 10 species (data not shown).

It has recently been proposed that activation of a sensory neuron in a short antennal trichoid sensillum is responsible for the repellent effect of DEET and other compounds in the Southern house mosquito *Culex quinquefasciatus* (Syed and Leal,

2008). Furthermore, a recent paper (Stanczyk et al., 2010) documented behavioral insensitivity to DEET in *Aedes aegypti* mosquitoes. The authors suggested that genetic alterations in olfactory sensilla explained the observed behavioral effects but the responsible gene(s) were not identified. Although appealing, it is improbable that a single OR could mediate the repellency effects of DEET across such a large number of highly evolutionarily divergent insect species, given the very low level of similarity of chemoreceptors genes even within the same species (Abdel-Latief, 2007; Benton et al., 2009; Engsontia et al., 2008; Hill et al., 2002; Robertson and Wanner, 2006; Robertson et al., 2003). Furthermore, DEET alone has been shown to be a mild attractant for *Aedes* and *Anopheles* mosquitoes (Dogan et al., 1999; Mehr et al., 1990), while still inhibiting behavioral attraction when in combination with other odorants (Dogan et al., 1999). Therefore, DEET is unlikely to function as an active repellent.

Our data show that a single missense polymorphism can affect the sensitivity of odorant receptors to DEET by modifying the interactions of specific odorants while leaving other odorant-evoked responses intact. Furthermore, we showed that these mutations can occur spontaneously and are present in natural populations of insects, both within and across species. We propose that DEET acts as a molecular "confusant" to modulate OR responses to some, but not all, odorants.

Although we showed that olfactory receptors are direct targets of DEET, there are likely to be other protein targets of this insect repellent. We showed that DEET inhibits TRP and K^+-channels expressed in *Xenopus* oocytes (Figure 3.4) and it seems likely that DEET exerts its effects on other still unknown targets. For example, while ticks are sensitive to DEET, there is no evidence of *Or83b* homologues or any ligand-

92

binding ORs in the deer tick (*Ixodes scapularis*) genome (H.M. Robertson, personal communication), suggesting that in this animal DEET is acting on non-OR protein targets. Unlike mosquitoes, ticks do not fly, and usually wait in tall grass to attach to passing hosts. It is therefore possible that DEET exerts its protective effects not in the gas phase, as for flying insects, but through direct contact. Gustatory receptor (*Gr*) genes, which normally respond to non-volatile compounds and are distantly related in sequence to *Or* genes (Robertson et al., 2003), are present in the tick genome and could therefore mediate the protective effects of DEET in this species.

In addition, a recent study (Corbel et al., 2009) provided evidence for DEET-mediated inhibition of both insect and mammalian cholinesterase activity *in vitro*, suggesting that the modes of action of this insect repellent might extend to non-chemosensory systems.

4 Structural domains of insect olfactory receptors

Insect ORs are a highly divergent family of proteins that share no similarity with other proteins in non-insect species (Clyne et al., 1999; Hill et al., 2002; Vosshall et al., 1999). Even across insect species, it is often difficult to find highly homologous sequences, and the rapid sequence divergence is also observed within the same species. In *Drosophila*, for example, the overall protein identity ranges between 15% and 30% (Vosshall, 2003). The only exception to this rule is OR83b, which retains on average 75% of sequence identity in species separated by up to 350 million years of evolution (Figure 1.7). Given its key role in the insect olfactory system and its conservation across species, OR83b represents an ideal candidate to investigate functional regions that allow the dimerization, targeting, and regulation of function of the OR complex. Thanks to the powerful genetic tools available in *Drosophila*, potentially interesting domains can be mutated and their function directly assessed *in vivo* in native olfactory sensory neurons.

4.1 Bioinformatics analysis of putative functional domains of insect ORs

We carried out a bioinformatics analysis on OR83b and its orthologues. Given the lack of homology with other protein families, we searched for motifs that have been shown to mediate dimerization, trafficking, and turnover of other membrane proteins (summarized in Figure 4.1 and Table 4.1), or were putative phosphorylation sites (summarized in Figure 4.2 and Table 4.2).
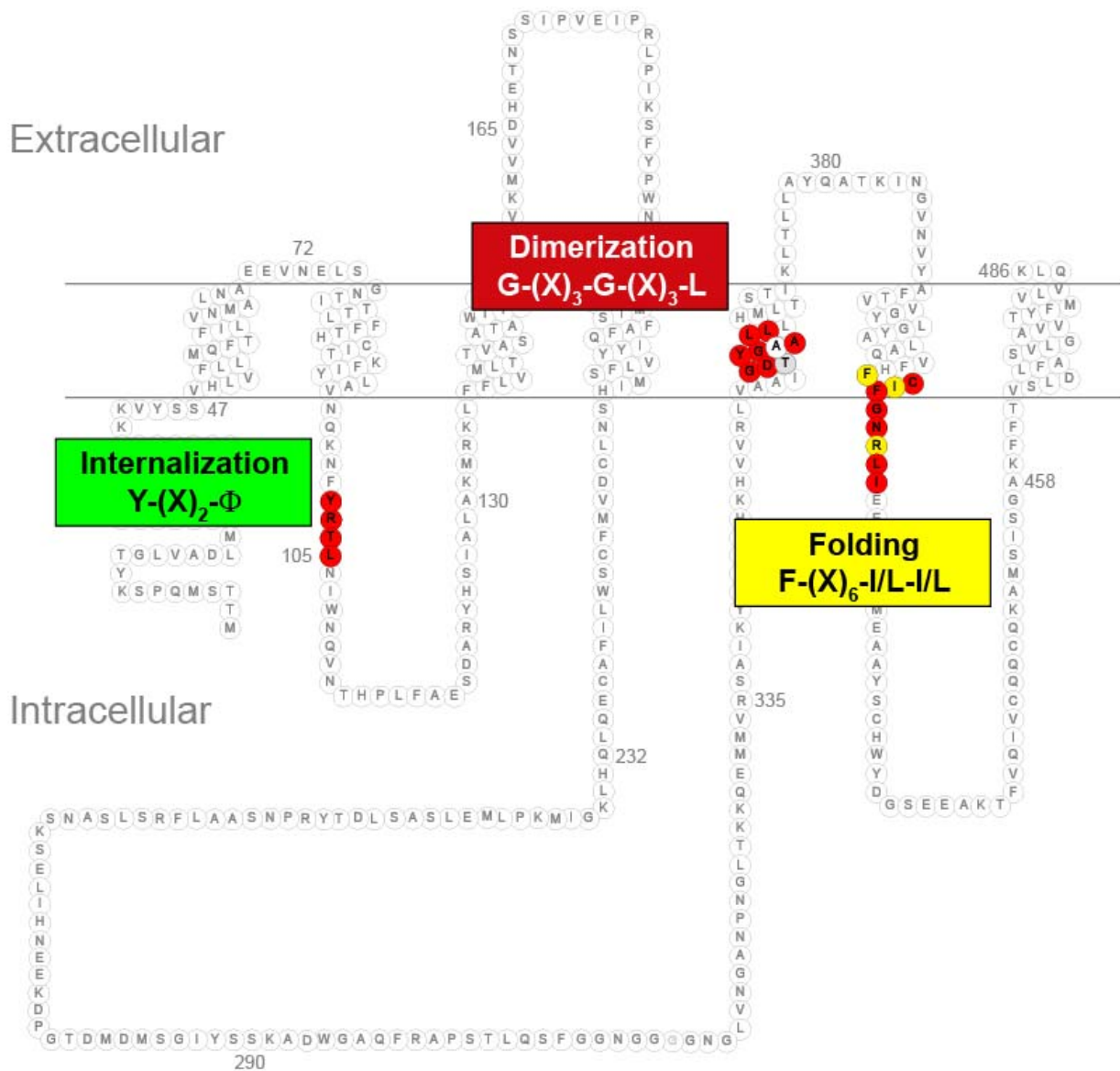
Figure 4.1 Conserved motifs in OR83b orthologues with homology to dimerization and trafficking motifs in other membrane proteins.

The position of an internalization (Y-(X)$_2$-Φ), dimerization (G-(X)$_3$-G-(X)$_3$-L), and folding (F-(X)$_6$-I/L-I/L) motif is shown on the snake plot adapted from Figure 1.7. Only the residues that are part of the motifs are color coded according to their degree of conservation.

Table 4.1 Summary of conserved motifs found in OR83b orthologues.

Motif	Function	Position	Reference
$Y\text{-}(X)_2\text{-}\Phi$	clathrin-mediated internalization	IC1 (102)	(Rapoport et al., 1997)
$G\text{-}(X)_3\text{-}G\text{-}(X)_3\text{-}L$	$\beta2$-adrenergic receptor dimerization	TM5 (356)	(Hebert et al., 1996; Salahpour et al., 2004)
$F\text{-}(X)_6\text{-}I/L\text{-}I/L$	receptor folding and ER export	IC3 (408)	(Duvernay et al., 2004)

Table 4.1 Φ = bulky hydrophobic residue [ILVMFYW]; X = any residue; IC = intracellular loop; TM = transmembrane domain. The numbers in parenthesis indicate the amino acid position for the beginning of each motif.
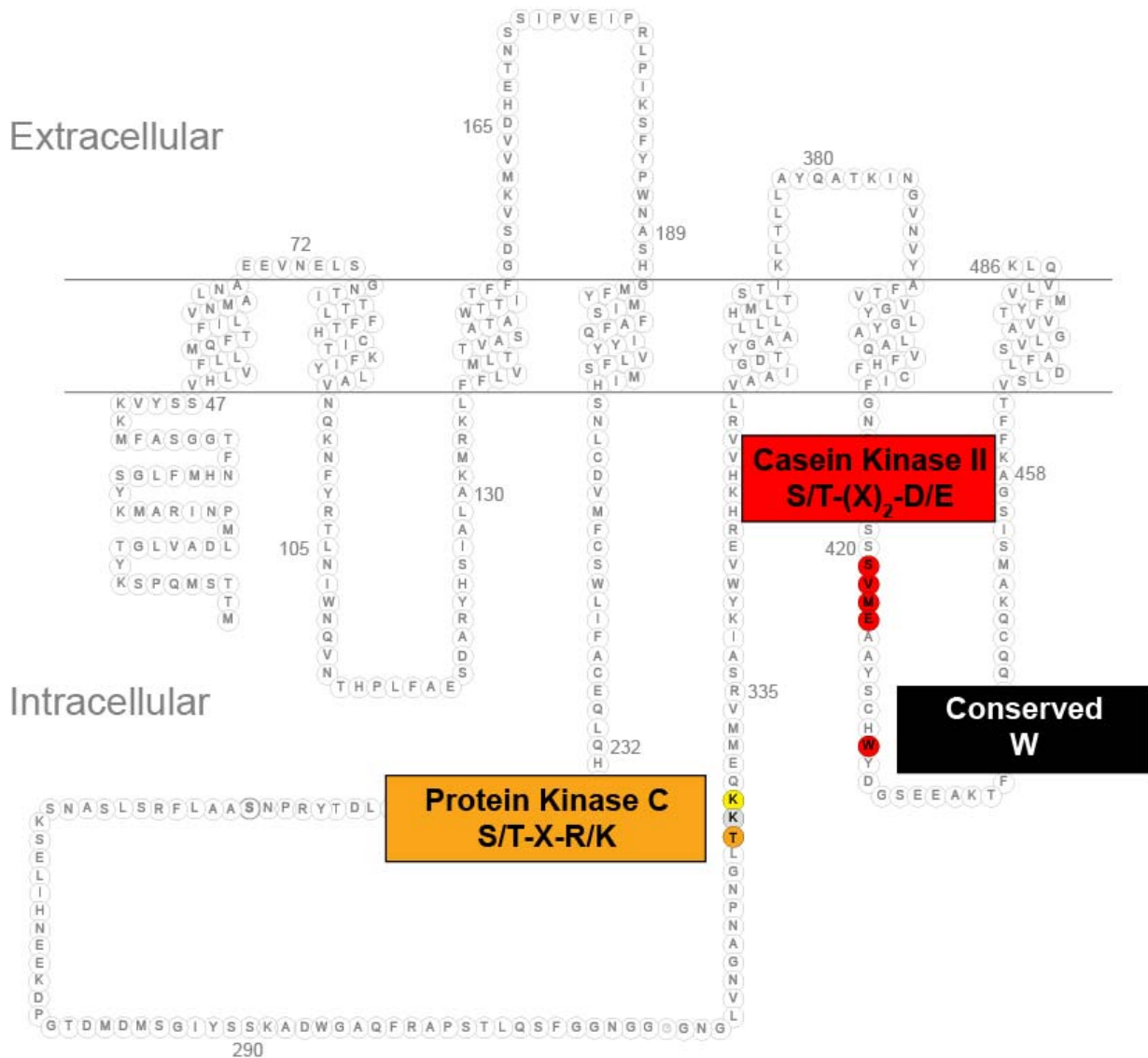
Figure 4.2 Putative phosphorylation consensus sequences in OR83b orthologues and conserved residues.

The location of a putative protein kinase C and casein kinase II phosphorylation sites, and the conserved tryptophan are shown on the snake plot adapted from Figure 1.7. Only the residues that are part of the motifs are color coded according to their degree of conservation.

Table 4.2 Summary of putative phosphorylation sites found in OR83b orthologues.

Consensus	Function	Position	Reference
S/T-X-R/K	protein kinase C phosphorylation	IC2 (327)	(Dai et al., 2009; Hecquet et al.)
S/T-(X)$_2$-D/E	casein kinase 2 phosphorylation	IC3 (421)	(Faber, 2009)

Table 4.2 X = any residue; IC = intracellular loop. The numbers in parenthesis indicate the amino acid position for the beginning of each consensus site.

In addition, we searched for extremely conserved amino acids by aligning the entire OR repertoire of *Drosophila melanogaster* and *Anopheles gambiae*. Across 138 sequences, we could identify one tryptophan in the third intracellular loop that is present in 57/61 ORs from *Drosophila melanogaster* and 70/75 ORs from *Anopheles gambiae*, and is located at position 431 in *Drosophila* OR83b (W431). Interestingly, this residue is part of one of three motifs that have been recently described through an independent bioinformatics analysis of *Drosophila melanogaster*, *Apis mellifera*, and *Anopheles gambiae* ORs (Miller and Tu, 2008). Although very little is known about the functional significance of these domains, it has been previously shown that this region is necessary for dimerization between the ligand-binding subunit and OR83b in a yeast two-hybrid assay (Benton et al., 2006). In addition, a recent study has shown that a tryptophan to cysteine mutation in the vertebrate endo-alpha-1,2-mannosidase, a single span transmembrane protein, causes impaired trafficking of the protein from the endoplasmic reticulum (ER) to the Golgi, where it normally resides (Torossi et al., 2007). Therefore, it is tempting to speculate that the W431 residue may play a role at the

interface between OR subunits acting within a retention, localization, or dimerization motif. Given the striking conservation of the tryptophan across ORs, and its position in a region mediating OR-OR interaction, I will discuss the functional role of this residue within OR83b.

4.2 The W431 residue in OR83b is part of a potential localization motif

To test whether OR83b and the vertebrate endomannosidase might use similar localization signals, we compared the region surrounding the tryptophan in OR83b and the endomannosidase form *Rattus norvegicus, Homo sapiens*, and *Canis familiaris* (Figure 4.3).

```
endomann.    169                         216
Rattus          IETHMKQMRSASIGVLALSWYPPDASDENGEATDYLVPTILDKAHKYN

endomann.    169                         216
Homo            IETHMRQMRSASIGVLALSWYPPDVNDENGEPTDNLVPTILDKAHKYN

endomann.    185                         232
Canis           IESHMKQMYSASIGVLALSWYPPGTNDENGENTDDLVPTILDKAHKYN

OR83b        412                         456
Drosophila      GNRLIEESSSVMEAAYSCHWYD---GSEEAKTFVQIVCQQCQKAMSIS

                 :   :.:   *.  .. :   **    ..*:.:   :*    :** . .

Identity                    S       WY     E        V      KA
```

Figure 4.3 Partial sequence alignment of endomannosidases and *Drosophila melanogaster* OR83b.

Residues surrounding the tryptophan (red) in endomannosidases from human (*Homo sapiens*), dog (*Canis familiaris*), and rat (*Rattus norvegicus*) compared to the putative homologous region in *Drosophila* OR83b (***** = identical residue; **.** = conserved substitutions; **:** = semi-conserved substitution). Identical residues are reported on the bottom row. The red box surrounds residues mutated within OR83b.

99

In the 45 amino acid region surrounding the conserved tryptophan, OR83b shows ~30% conserved substitutions with the endomannosidase protein family, with seven identical residues. We carried out site-directed mutagenesis of the *Or83b* gene to investigate the functional importance of the conserved residues W431 and Y432 by generating a series of point mutations summarized in Table 4.3. We hypothesized that mutations in any of the identical residues in this motif may cause a disruption in OR83b localization, therefore leading to accumulation in the ER compartment (Benton et al., 2006). In control experiments, we induced a conservative mutation of W431 to phenylalanine (W431F) and mutated the non-conserved residue D433 to alanine (D433A).

Table 4.3 Summary of mutations in the OR83b intracellular loop 3 and their expected phenotypes.

Residue	Mutations	Expected phenotype on evoked responses	Expected phenotype on localization
W^{341}	W431A W431F	No activity Wild type	Mislocalization Wild type
Y^{432}	Y432A	No activity	Mislocalization
D^{433}	D433A	Wild type	Wild type

Transgenic *Drosophila melanogaster* flies were generated for each mutant (Or83b^{MUT}). To assess the functional consequences of our mutations, we expressed each Or83b^{MUT} in OSNs lacking the endogenous *Or83b* gene, and tested the ability of the mutant to rescue the null phenotypes of OR trafficking and responsiveness to odorants.

4.3 OR83b W431 and Y432 mutants show impaired spontaneous activity and odorant-evoked responses in a subset of OSNs

We performed extracellular recordings from the big basiconic ab2 and ab3 sensilla stimulated with their cognate ligands. Each one of these sensilla houses two olfactory sensory neurons: the ab2A and ab2B (expressing OR59b/OR83b and OR85a/OR83b, respectively), and the ab3A and ab3B neurons (expressing OR22a/b/OR83b and OR85b/OR83b, respectively). To control for a general deleterious effect of our transgenic constructs on the antenna, we also recorded the activity of the ab1 sensillum, which houses one OR83-independent CO_2-responsive neuron (ab1C) in addition to three OR83b-dependent cells (ab1A, ab1B, and ab1D; see Table 4.4 for a summary of cells and their cognate ligands used to stimulate them). We did not consider the activity of the ab1D cell because of its small spike amplitude.

Table 4.4 Summary of the sensilla analyzed, the OR complex expressed, and the preferred cognate ligand.

Cell	OR83b-dependent	OR complex	Stimulating compound
ab1A	YES	OR92a/OR83b	ethyl acetate ethyl butyrate
ab1B	YES	OR42b/OR83b	ethyl butyrate
ab1C	NO	NA	CO_2
ab2A	YES	OR59b/OR83b	methyl acetate
ab2B	YES	OR85a/OR83b	ethyl-3-hydroxybutyrate
ab3A	YES	OR22a/b/OR83b	ethyl butyrate
ab3B	YES	OR85b/OR83b	2-heptanone

As expected, sensilla of *Or83b*^{-/-} flies did not exhibit spontaneous or evoked activity, except for the ab1C sensillum that expresses the CO_2 receptor subunits *Gr21a* and *Gr63a* (Figure 4.4A; Jones et al., 2007; Scott et al., 2001), while a wild type OR83b transgene could restore neuronal activity (Figure 4.4).

Mutations in both W431 and Y432 OR83b residues altered spontaneous and odorant-evoked activity in a cell-dependent way. In flies carrying the OR83b[W431A] mutant, most of the sensilla analyzed lacked odorant-evoked responses. The ab1 sensillum could be identified due to its sensitivity to CO_2, but both spontaneous and odorant-evoked activity of all the OR83b-dependent cells was abolished (data not shown; see summary in Table 4.5).

In flies expressing the OR83b[W431A] mutant, we failed to identify any sensillum with ab2-like responses. Instead, sensilla with no spontaneous or evoked activity were present in the same location where ab2 sensilla are usually found (Figure 4.4C). Moreover, three out of 13 sensilla (~25%) in the same area showed very sparse spontaneous activity but no odorant-evoked responses. This suggests that both the A and B cells in ab2 sensilla are mostly non-functional. In the ab3 sensillum of the same flies, only the B cell showed spontaneous and ligand-evoked activity. In six out of nine sensilla (~60%), the A cell was activated only when the B cell responded to its cognate ligands, but otherwise showed sparse spontaneous activity and lacked odorant-evoked responses to ethyl butyrate (Figure 4.4).

102

Figure 4.4 Phenotypes of OSNs expressing OR83b mutants.

(**A-B**) Representative traces of OSNs in ab2 (two *left columns*) and ab3 (two *right columns*) sensilla of *Or83b$^{-/-}$* flies (**A**, *CyO/Bl; Or83b^2*) rescued with an *Or83b* wild type transgene (**B**, *Or83b-Gal4/UAS-Or83b; Or83b^1/Or83b^2*). (**C-F**) Peristimulus plots (upper panels) and representative traces (lower panels) of OSNs in ab2 (two *left columns*) and ab3 (two *right columns*) sensilla of *Or83b$^{-/-}$* flies rescued with OR83b^{W431A} (**C**, *Or83b-Gal4/UAS-Or83b^{W431A}; Or83b^1/Or83b^2*), OR83b^{W431F} (**D**, *Or83b-Gal4/UAS-Or83b^{W431F}; Or83b^1/Or83b^2*), OR83b^{Y432A} (**E**, *Or83b-Gal4/UAS-Or83b^{Y432A}; Or83b^1/Or83b^2*), and OR83b^{D433A} (**F**, *Or83b-Gal4/UAS-Or83b^{D433A}; Or83b^1/Or83b^2*) transgenes. Responses from the OR83b wild type (WT=wild type, black circles) and mutant (red) rescue are superimposed in the peristimulus plots (n=3-6). The plots represent the number of spikes grouped in 200 ms bins of the cell highlighted in red in the traces. Spikes from the other neuron sharing the same sensillum are shaded in grey. The black bars above traces and plots represent 1 s stimulation of the sensillum with a 10^{-5} dilution of the specified ligand. In *Or83b$^{-/-}$* flies, each sensillum was classified based on its size and location on the antenna.
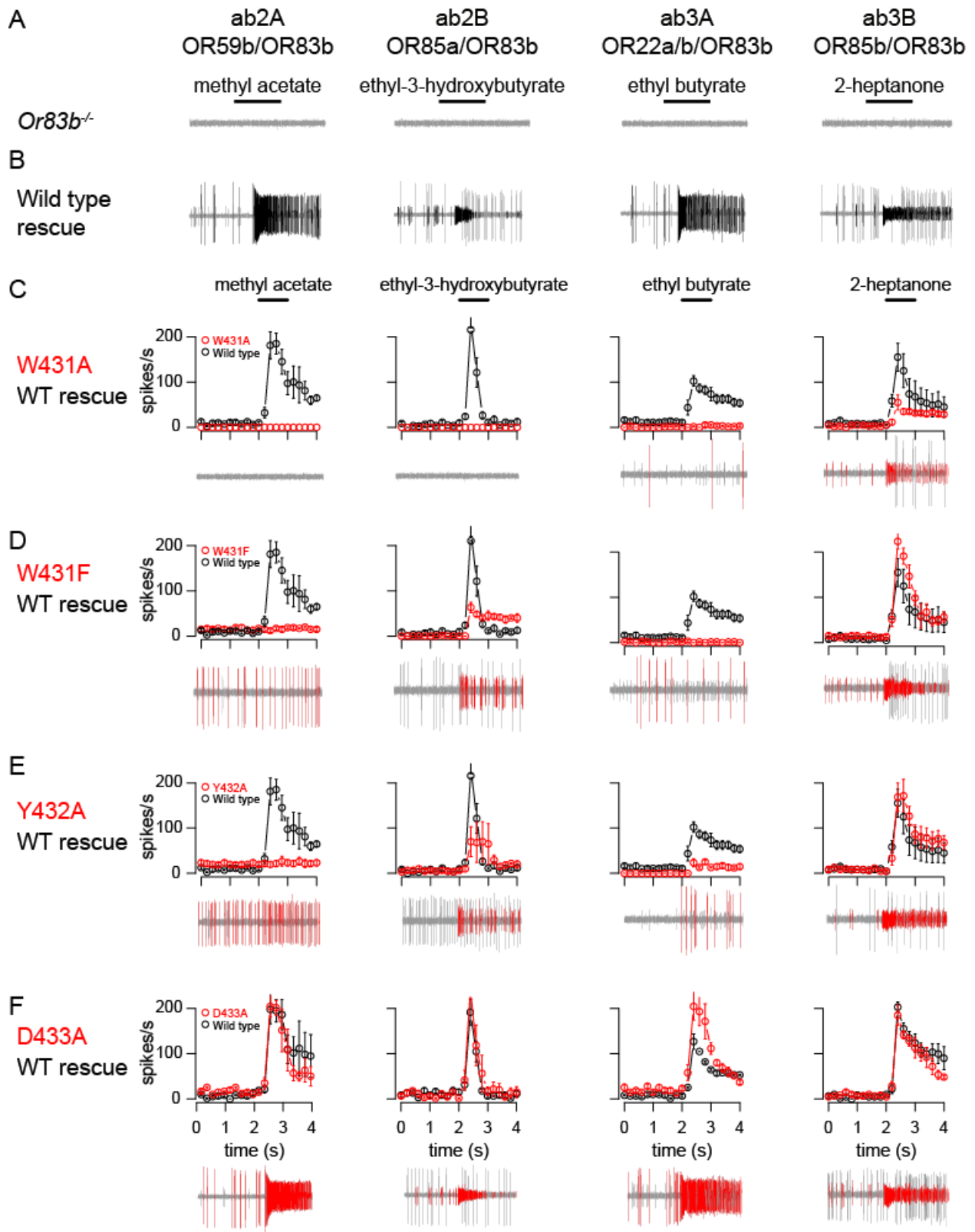
Figure 4.4 Phenotypes of OSNs expressing OR83b mutants.

Less severe phenotypes were observed in OSNs expressing the OR83b^{W431F} or OR83b^{Y432A} mutations, where the spontaneous activity of one and two cells, respectively, was restored in the ab1 sensillum (see Table 4.5). Although OR83b^{W431F} and OR83b^{Y432A} showed spontaneous activity, we could not elicit odor-evoked responses with either mutant, and therefore failed to identify the molecular identity of the neurons showing spontaneous activity.

In ab2 sensilla of animals expressing either OR83b^{W431F} or OR83b^{Y432A} transgenes, the spontaneous activity of both the A and B cell was restored, but only the B cell could be stimulated with its cognate ligand ethyl-3-hydroxybutyrate (Figure 4.4D and E). In the ab3 sensillum of the same animals, the A neuron showed sparse spontaneous activity in ~50% of the cells analyzed and weak odorant-evoked responses could be stimulated when expressing OR83b^{Y432A}, but not OR83b^{W431F}. Instead, the ab3B neuron exhibited normal odorant activation when either mutant was expressed (Figure 4.4D and E).

Expression of OR83b^{D433A} showed no difference from the wild type OR83b rescue (Figure 4.4F), as expected from the lack of conservation of the D433 residue between the endomannosidase family and OR83b (Figure 4.3).

Table 4.5 Rescue of the *Or83b⁻/⁻* phenotype by different OR83b mutants in single sensillum recordings

Mutant	Cell	OR83b-dependent	Spontaneous activity	Odor-evoked activity
W431A	ab1A/B	Yes	No	No
	ab1C	No	Yes	Yes
	ab2A	Yes	No	No
	ab2B	Yes	very sparse in 25% of cells analyzed	No
	ab3A	Yes	sparse in 60% of cells analyzed	No
	ab3B	Yes	Yes	Yes
W431F	ab1A/B	Yes	1 cell	No
	ab1C	No	Yes	Yes
	ab2A	Yes	Yes, faster than WT	No
	ab2B	Yes	Yes, slower than WT	Yes
	ab3A	Yes	sparse in 50% of cells analyzed	No
	ab3B	Yes	Yes	Yes
Y432A	ab1A/B	Yes	2 cells	No
	ab1C	No	Yes	Yes
	ab2A	Yes	Yes, faster than WT	No
	ab2B	Yes	Yes, slower than WT	Yes
	ab3A	Yes	sparse in 50% of cells analyzed	Yes, weak
	ab3B	Yes	Yes	Yes
D433A	ab1A/B	Yes	Yes	Yes
	ab1C	No	Yes	Yes
	ab2A	Yes	Yes	Yes
	ab2B	Yes	Yes	Yes
	ab3A	Yes	Yes	Yes
	ab3B	Yes	Yes	Yes

Table 4.5 Spontaneous activity and odorant-evoked responses were color-coded according to the degree of rescue achieved compared to the wild type transgene. Green=full rescue; yellow=partial rescue; red=no rescue. ab1C neurons are not color coded because they are OR83b-independent

4.4 OR83b W431 and Y432 mutants show abnormal localization *in vivo*

The absence of odorant-evoked responses in the OR83b mutants analyzed could be due to a primary effect on the function of OR83b as an odorant-gated channel or due to a trafficking defect that either causes OR83b to be mislocalized and/or to fail to interact with its OR cargo.

To test whether OR83b mutants could localize to the dendrite of the OSN, we performed immunostaining on adult antennal sections. As previously reported (Benton et al., 2006; Larsson et al., 2004), $Or83b^{-/-}$ flies did not contain any OR83b protein in OSN dendrites (Figure 4.5B). We could rescue this phenotype by expressing wild type OR83b (Figure 4.5C) or our control OR83b^{D433A} mutant (Figure 4.5F).

OR83b^{W431A}, OR83b^{W431F}, and OR83b^{Y432A} mutants (Figure 4.5D-F) failed to traffic properly and remained trapped within the cell body, with some faint dendrite staining in OR83b^{Y432A}-expressing cells. In these animals, the ligand-binding subunit OR22a/b also failed to localize to dendrites (data not shown), consistent with the hypothesis that these mutant OR83b proteins fail to traffic ligand-binding ORs to the dendrite. Unfortunately, attempts to raise antibodies to detect the OR85b subunit expressed in ab3B neurons failed, so we were unable to examine the localization of this receptor in neurons that express impaired OR83b mutants yet continue to function normally.

It has been previously shown that the OR22a/b subunit co-localizes with an ER marker in the absence of OR83b (Benton et al., 2006). In $Or83b^{-/-}$ flies (Figure 4.5B) and

in animals expressing the wild type OR83b transgene (Figure 4.5C) or the OR83b[D433A] mutant (Figure 4.5G), visualization of the ER resident protein BOCA (Culi and Mann, 2003) produced a faint cellular staining. Consistent with the published results, the same staining in antennae expressing OR83b[W431A] (Figure 4.5D), OR83b[W431F] (Figure 4.5E), or OR83b[Y432A] mutants (Figure 4.5F) displayed brighter accumulation of BOCA in cell bodies and co-localization with OR83b in a fraction of cells. The cellular accumulations of BOCA are thought to be secondary to the trafficking defects, therefore explaining the low percentage of cells showing co-localization with OR83b.

Given the strong electrophysiology and localization phenotypes obtained with OR83b[W431A] or OR83b[W431F] mutants, we asked whether these proteins could have dominant negative effects, and expressed each mutant along with one copy of the endogenous *Or83b* gene. Extracellular recordings of ab1, ab2, and ab3 sensilla (data not shown), and immunostaining of antennal sections did not differ from the wild type (Figure 4.6), suggesting that mutations of the W431 residue did not affect the function of endogenous OR83b. However, our antibody staining cannot distinguish between mutant and endogenous proteins. Therefore, we cannot discern whether the functional complexes localized at the dendrites contain only the endogenous wild type protein or if the co-expression of endogenous OR83b serves to rescue the localization of OR83b mutants.

Taken together, these experiments suggest that the conserved W431 and Y432 residues are necessary for the proper localization of some OR83b-complexes to the ciliated dendrites.

Figure 4.5 OR83b trafficking defects lead to accumulation in the ER.

(**A**; *left*) Schematic of the third antennal segment. The field of view is represented here and in subsequent figures by the black square. (*Right*) Schematic of an olfactory sensory neuron, adapted from Benton *et al.*, 2006 (Benton et al., 2006). (**B-G**) Immunostaining for BOCA (green) and OR83b (red) in antennal sections of flies lacking OR83b (**B**, *CyO/Bl; Or83b^2*), or rescued with the wild type OR83b (**C**, *Or83b-Gal4/UAS-Or83b; Or83b^1/Or83b^2*), OR83b^{W431A} (**D**, *Or83b-Gal4/UAS-Or83b^{W431A}; Or83b^1/Or83b^2*), OR83b^{W431F} (**E**, *Or83b-Gal4/UAS-Or83b^{W431F}; Or83b^1/Or83b^2*), OR83b^{Y432A} (**F**, *Or83b-Gal4/UAS-Or83b^{Y432A}; Or83b^1/Or83b^2*), or OR83b^{D433A} (**G**, *Or83b-Gal4/UAS-Or83b^{D433A}; Or83b^1/Or83b^2*) transgenes. The dotted lines represent the boundary between the main antennal body where the cell bodies reside (*left*) and the sensilla where the outer segment of the dendrite is located (*right*). Arrows indicate cells where co-localization of OR83b and BOCA occurs. The images were taken with the same confocal settings to allow comparisons of signal intensities.
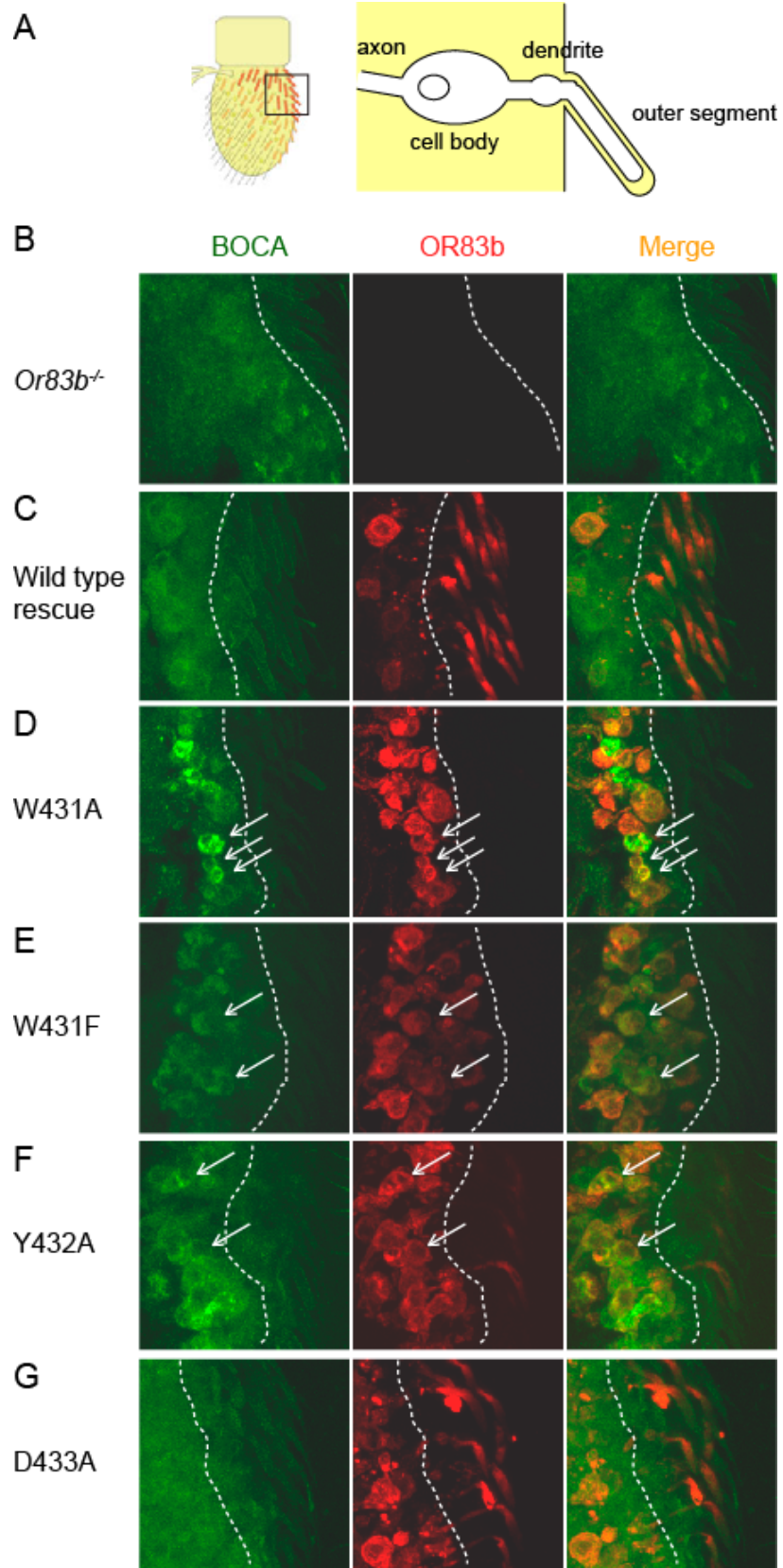
Figure 4.5 OR83b trafficking defects lead to accumulation in the ER.
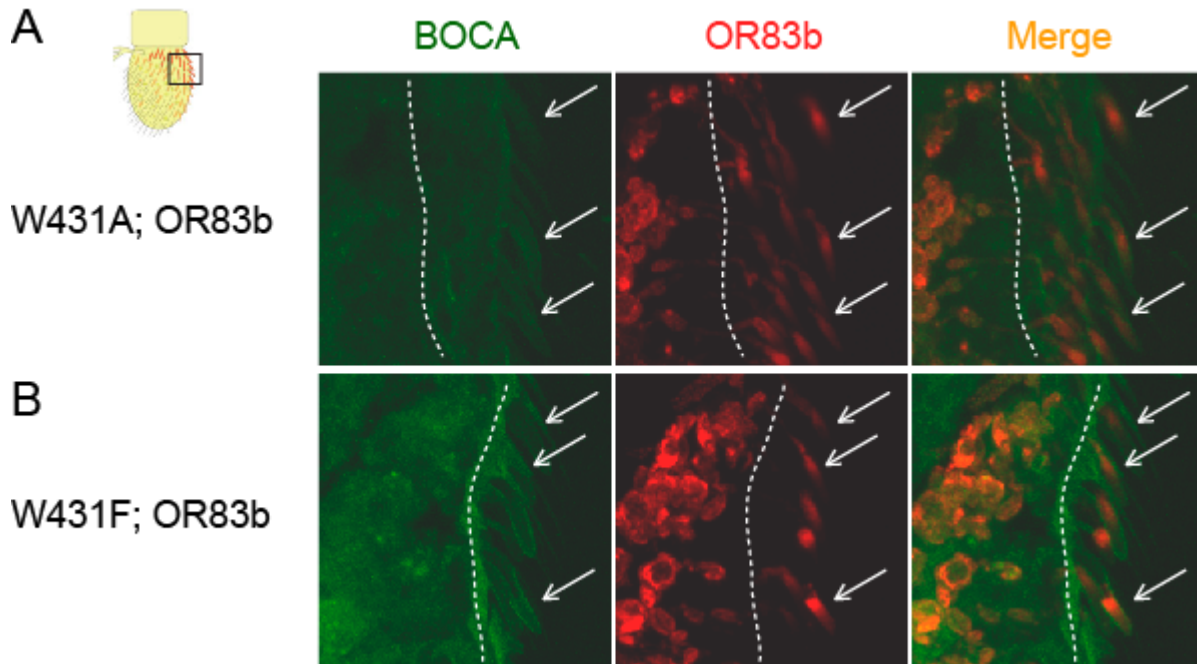
Figure 4.6 OR83b W431A and W431F do not act as dominant negative proteins.
Immunostaining for BOCA (green) and OR83b (red) in antennal sections of flies expressing OR83b^{W431A} (**A**, *Or83b-Gal4/UAS-Or83b^{W431A}; +/Or83b^2*) or OR83b^{W431F} transgenes (**B**, *Or83b-Gal4/UAS-Or83b^{W431F}; +/Or83b^2*), in the presence of one copy of the endogenous *Or83b* gene. The dotted lines represent the boundary between the main antennal body where the cell bodies reside (*left*) and the sensilla where the dendrites are located (*right*). Arrows indicate OR83b localization to the dendrite tip. The images were taken with the same confocal settings to allow comparisons of signal intensities.

4.5 C-terminal domains of OR83b mutants can interact in a yeast two-hybrid assay

It has been proposed that OR83b interacts with other OR subunits via its C-terminal third intracellular loop (IC3; Benton et al., 2006), which contains the conserved residues W431 and Y432. Given their phenotypes, we asked whether the mutations disrupted association within OR83b proteins and between OR83b and other ORs, therefore preventing the formation of most OR complexes.

As previously demonstrated (Benton et al., 2006), a yeast two-hybrid assay among OR IC3 domains can be used as a proxy for protein-protein association. Applying the same approach, we observed interactions of the wild type IC3 domain with all mutant IC3s (Figure 4.7A). The IC3 domain from each protein also associated with itself (Figure 4.7B).

Using the same assay, we failed to detect interaction between the OR83b wild type IC3 and the IC3 from OR43a or OR47a (data not shown), therefore preventing us from investigating the association of the mutated domains with the ligand-binding subunits. Interaction between the IC3 domains of OR83b and OR43a has been previously reported (Benton et al., 2006). The discrepancy between our results and the published interaction could be explained by the weak association of the OR83b and OR43a IC3 domains (R. Benton, personal communication) and the qualitative nature of the scoring system. A more quantitative approach, such as detection of β–galactosidase activity induced by the interacting moieties, could lead to improved sensitivity of the assay and the ability to test whether mutated OR83b IC3s can associate with the corresponding regions of the ligand-binding subunits.
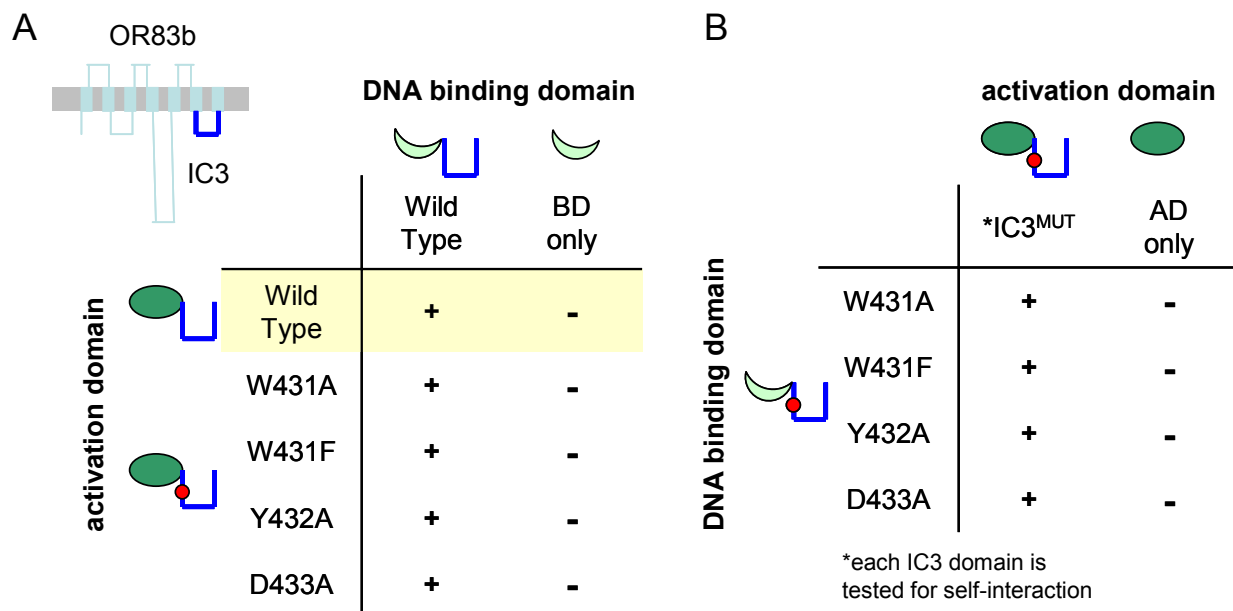
Figure 4.7 IC3 domains of wild type and mutant OR83b interact in a yeast two-hybrid assay.

(**A-B**) Interaction of IC3 domains tested by yeast two-hybrid assay scored for growth (+). (**A**) The cartoon depicts the location of the IC3 domain used (dark blue) and its location within the OR83b protein (light blue). Yeast growth was observed when either wild type or mutant IC3 domains were linked to the GAL4 activation domain and the wild type IC3 domain was linked to the GAL4 DNA binding domain. No growth was observed when the IC3 domains were co-expressed with the binding domain alone (BD, binding domain). (**B**) Each mutant IC3 domain was tested for self-interaction by co-expressing the same domain linked to the GAL4 activation and DNA binding domains. No growth was observed when the IC3s linked to the GAL4 activation domain were co-expressed with the GAL4 activation domain alone (AD, activation domain).

4.6 [431]WY[432] – part of a new ER export/localization motif?

While ORs rely on OR83b for proper localization, the OR83b protein alone is sufficient for trafficking to OSN dendrites (Benton et al., 2006). We identified a tryptophan and tyrosine residues conserved among members of the OR and a vertebrate endomannosidase family (Figure 4.3) that are necessary for proper localization of the OR complex. Disruptive mutations of these residues in the OR83b protein, W431A and Y432A, abolish or decrease spontaneous activity and odorant-evoked responses in some OSNs *in vivo* (Figure 4.4), and lead to lack of dendrite localization and retention of the protein in the cell bodies (Figure 4.5). A conservative mutation of the same tryptophan to phenylalanine (W431F) results in less severe electrophysiological phenotypes, but similar localization defects, while a disruptive mutation in the non-conserved amino acid D433 to alanine (D433A) does not affect OR83b function.

Cells expressing OR85b/OR83b and OR47a/OR83b still retain sensitivity to odorants, suggesting that OR83b mutations did not drastically alter these OR complexes. In *Or83b*$^{-/-}$ animals, these sensilla are electrically silent (Figure 4.4A and data not shown), confirming that their odorant-sensitivity is OR83b-dependent. The discrepancy between the extracellular recordings and the antennal immunostainings could be explained by the higher sensitivity of electrophysiological recordings in detecting smaller amounts of functional complexes on the cell surface. Our data therefore suggests that a small amount of functional OR complex is sufficient to restore odorant sensitivity in *Or83b*$^{-/-}$ OSNs.

OSNs lacking conventional ORs lack odorant-responses but still show low levels of spontaneous activity (Dobritsa et al., 2003; Elmore et al., 2003), which is absent in $Or83b^{-/-}$ (Figure 4.4A; Larsson et al., 2004). Sparse spontaneous activity is also present in ab2B cells expressing OR83b^{W431A}, and ab3A cells expressing the OR83b^{W431A,} OR83b^{W431F}, and OR83b^{D433A} mutants (Figure 4.4C-E and Table 4.5). This could be explained by the presence of OR83b on the cell surface and its ability to form homomers with channel activity.

The association of ORs with OR83b within the cell body forms an OR complex that is trafficked to dendrites. Mutations in OR83b W431 and Y432 residues did not disrupt the functional association with OR85b or OR47a, unlike what was observed for other OR complexes, suggesting that interactions between OR83b and each OR may be mediated by different subsets of residues within the OR83b protein (Figure 4.4). During the steps leading to the cell membrane, ligand-binding subunits are thought to assume a passive role and be simply guided to the dendrite thanks to the presence of OR83b. Our results suggest that conventional ORs might play a more important role within the OR complex than previously thought, in that they are not equivalent in the ability to interact with OR83b. The association of OR83b with OR85a and OR47a might be more stable than with other ORs, explaining the presence of evoked activity in our extracellular recordings with OR83b mutants in neurons expressing these two but not other ORs.

Alternatively, cellular components present only in subset of OSNs could help the formation of functional complexes with OR83b mutants. This could be tested by co-

expression of OR85b/OR83b^{W431A} into an ab3A neuron in a $\Delta halo$, $Or83b^{-/-}$ double mutant background. $\Delta halo$ contains a deletion of the *Or22a* and *Or22b* genes, creating an "empty neuron" where it is possible to misexpress ORs and study their function (Dobritsa et al., 2003) without interference from the endogenous OR. The ab3A neuron is especially appealing because the native receptors OR22a/b receptors do not function when expressed with OR83b W431 and Y432 mutants. If cell-specific factors independent of OR83b are necessary for OR trafficking, expression of OR85a/OR83b^{MUT} in this neuron will not result in functional activity.

While the precise reason for OR83b retention in the ER is unknown, it may not be ascribed to inability of OR83b to homodimerize, based on preliminary *in vitro* results (Figure 4.7).

It would be interesting to investigate whether the other residues conserved between OR83b and the endomannosidase family also lead to similar phenotypes, and define a common motif that is involved in OR trafficking. Targeted mutations in similar residues in the ligand-binding subunits might also identify their role in the interface among OR subunits. An alternative approach might involve random mutagenesis of ligand-binding subunits to produce compensatory mutations that might revert the phenotypes observed with OR83b^{W431A}.

Overall, more experimental evidence is needed to establish what the exact role of OR83b is within the OR complex, what are the functional domains within this protein, and how they influence the behavior of the OR protein complex.

5 Implications of the current study and future directions

This dissertation describes the mechanisms underlying the function of insect olfactory receptors and how their activity is modified by the insect repellent DEET.

Specific protein families are utilized throughout the animal kingdom to detect particular stimuli: TRP channels sense temperature (Bautista et al., 2007; Caterina et al., 1997; Chung and Caterina, 2007; Dhaka et al., 2007; Dhaka et al., 2006; Gracheva et al., 2010; Liman, 2006; Smith et al., 2002; Xu et al., 2002) and noxious chemicals (Bandell et al., 2004; Peier et al., 2002) in organisms as diverse as mammals, insects, and reptiles. On the other hand, multiple protein families can also be adopted within the same sensory modality: in mammals, the taste of umami, sweet, and bitter chemicals is GPCR-dependent (Chandrashekar et al., 2006), while sour compounds (Huang et al., 2006; Ishimaru et al., 2006) and sodium (Chandrashekar et al., 2010) are probably sensed by ion channels. Different species can, however, recruit different protein families to detect the same stimulus: while the green alga *Chlamydomonas reinhardtii* senses photons through the channel channelrhodopsin (Nagel et al., 2002), vertebrates employ the GPCR rhodopsin (Wald, 1935).

In olfaction, several unrelated receptor families have been described in insects, mammals, and nematodes. A surprising and fascinating distinction separates insects from other organisms, in that they specifically adopted ion channels to sense odorants in the environment, unlike GPCRs employed by mammals and nematodes. Is there a particular reason for using ion channels? Although we can only speculate, this might reflect an adaptation of the olfactory system to the specific needs of insects and their

high rate of movement when in flight. Since ion channels do not rely on intermediate amplification steps, they are faster than most GPCRs in producing neuronal depolarization, which may lead to faster behavioral responses. When leaving their source, odorants do not follow a linear path but are dispersed within plumes that change very rapidly with time. When approaching an odorant source, the insect brain may need to quickly compute whether it is located within or outside the plume. Having a fast switch at the periphery through the use of odorant-gated ion channels might have represented an evolutionary advantage.

In the future, it will be fascinating to investigate the role of G proteins in insect signal transduction. Although not necessary for initiation of the response, several lines of evidence indicate that G proteins may play a role in modulating OSN activity *in vivo* (Boekhoff et al., 1993; Boekhoff et al., 1990; Breer et al., 1990; Chatterjee et al., 2009; Dolzer et al., 2008; Gomez-Diaz et al., 2004; Gomez-Diaz et al., 2006; Kain et al., 2008; Martin et al., 2001; Stengl, 1994; Ziegelberger et al., 1990; Zufall and Hatt, 1991). In the genome of *Drosophila melanogaster* there are six genes encoding Gα, three β, and two γ subunits (Boto et al., 2010). Given the limited repertoire, it may be feasible to identify putative interacting G proteins by co-expressing both insect ORs and G protein subunits in cell culture, and screen for enhancement of OR activity. To further test for direct interaction, both directed mutagenesis of ORs and biochemical assays will be necessary.

Given the lack of similarity with any known protein, future research is needed to identify structural motifs within insect ORs. Although we have shown that some conserved residues may play a role in localization of the receptor complex to the dendrite, additional amino acids are likely to be involved. Moreover, targeted mutagenesis of OR83b and ligand-binding subunits would help identify residues forming the channel pore. This discovery could determine whether both subunits contribute to the formation of the channel, as we have suggested (Sato et al., 2008), or if the ligand-binding protein is dispensable for channel function, as proposed by Wicher and colleagues (Wicher et al., 2008). Further work could also focus on defining the stoichiometry of the OR complex. What is the number of subunits necessary to form a functional complex, and does it change for different OR complexes? And, finally, in the cases where two or more ligand-binding ORs are expressed in a given OSN, are different ORs incorporated in the same complex or is there a mechanism that keeps them separate?

Once formed and localized at the dendrite tip, the OR complex might require post-translational modifications to modulate its activity. Therefore, identifying additional components of the signaling pathways, such as protein kinases and phosphatases, will reveal mechanisms for desensitization, inactivation, or turnover of the olfactory complex.

A fascinating line of research could also delve into the mechanisms of interaction between odorant ligands and ORs. Discoverig the basis for ligand specificity and how different odorants trigger activation or inhibition in the same OR complex could be comparable to the finding of polyspecificity in antigen recognition by receptors within the immune system (Cohn, 2008; O'Callaghan and Bell, 1998). In both systems, the

receptors employed as detectors need to recognize a large variety of molecules. While the versatiliy of the immune system relies on somatic recombination events, the basis for odorant recognition by olfactory receptors is still unkown.

Eventually, a crystal structure of the OR complex would be fundamental to answer these questions, but studies in heterologous systems can provide preliminary insights. While it is possible to employ cell cultures to study insect ORs, functional expression is limited to ~50% of the genes tested (data not shown and T. Nakagawa, personal communication). Even when successful, insect OR expression levels are generally poor. Future research would therefore greatly benefit from improvements in OR expression efficiency. Mammalian olfactory receptors have also suffered from the same impediment, until two accessory proteins present in olfactory neurons were shown to facilitate cell surface expression in HEK293T cells (Saito et al., 2004). For this reason, it is possible that proteins with similar functions are present in insect OSNs and may improve surface expression in heterologous systems. To identify possible candidates, a cDNA library from OSNs could be transfected in HEK293T cells along with an insect OR, and the ability of the gene products to enhance odorant-evoked responses could then be used as a screening readout. Alternatively, enriching OSN-specific transcripts through targeted translational profiling (Heiman et al., 2008) could reveal potential accessory proteins, that would then be tested in a heterologous system.

Besides elucidating interesting cellular and molecular mechanisms of insect olfaction, more in depth knowledge of insect OR structure-function and better

expression systems would greatly improve ongoing studies that utilize heterologous expression for the discovery of new insect repellents.

The findings presented in this dissertation are likely to have an impact beyond the field of olfaction. In fact, it is interesting to note that insect gustatory receptors (GRs), sensing mostly water-soluble chemicals, are related to the OR family, therefore suggesting that they might be ligand-gated ion channels as well. Although there is still little evidence supporting this hypothesis, sugar-activated ion channels have been described in the gustatory sensilla of the flesh fly *Boettcherisca peregrine* (Murakami and Kijima, 2000). However, recent work has proposed that $G\alpha_q$ and/or $G\alpha_s$ signaling pathways participate in sugar detection in *Drosophila melanogaster* gustatory neurons (Kain et al., 2010; Ueno et al., 2006).

GRs are more than just sensors of water-soluble chemicals: members of this protein family in *Drosophila melanogaster* and *Anopheles gambiae* also detect external CO_2 levels (Jones et al., 2007; Lu et al., 2007). Activation of CO_2-responsive cells, likely mediated by the G protein $G\alpha_q$ (Yao and Carlson, 2010), is necessary to trigger the CO_2-evoked avoidance behavior in the vinegar fly (Jones et al., 2007).

Based on these results, it is likely that homologous proteins in mosquitoes mediate CO_2-evoked behavioral attraction of these hematophagous insects to their hosts. Since DEET does not block neuronal responses to CO_2 in *Anopheles gambiae* (Figure 3.3E), future research is needed to identify compounds that target the receptors expressed in these cells. Used in combination with DEET, this new "repellent" could have the benefit of potentiating the confusant effects of DEET by blocking an additional sensory channel through which hematophagous insects detect humans.

Our discovery that insect ORs are structurally different from mammalian ORs and that they are directly modulated by the repellent DEET is fascinating *per se*. However, in combination with the fact that some insect species are disease vectors and use olfaction to hone in to the host, it gives us a unique head start in the battle against insect-borne diseases. Insect ORs are, in fact, an excellent Trojan horse that may be exploited to specifically disrupt the host-seeking mechanisms that insects use to find humans, with the potential of decreasing disease transmission. To achieve this goal, a clear understanding of the functional OR domains that mediate dimerization, trafficking, and modulation of receptor subunits will be essential.

With this knowledge in hand, it will be possible to design repellents that could specifically target the insect olfactory system and impair the proper function of the OR complex.

6 Materials and methods

6.1 Bioinformatics

The snake plots in Figure 1.7, Figure 4.1, and Figure 4.2 were manually composed based on a ClustalW (Thompson et al., 1994) alignment of OR83 protein orthologues performed through Jalview (Clamp et al., 2004).Transmembrane domains were predicted by the PredictProtein algorithm (Rost et al., 2004). The amino acid sequences were derived from mRNA clones obtained from Genbank (accession numbers in parenthesis): *Drosophila melanogaster* (NM_079511.4), *Ceratitis capitata* (AY843206.1), *Anopheles gambiae* (AY363725.1), *Culex pipiens* (DQ231246.1), *Bombyx mori* (AB100454.1), *Helicoverpa zea* (AY843204.1), *Antheraea pernyi* (AJ555486.1), *Spodoptera exigua* (AY862142.1), *Ceratosolen solmsi* (EU281848.1), *Apis mellifera* (NM_001134943.1), *Apocrypta bakeri* (EU281849.1), *Microplitis mediator* (EF141511.1), *Philotrypesis pilosa* (EU281850.1), *Tribolium castaneum* (XM_968103). The sequence of *Schistocerca Americana* OR83b was provided by Dr. Leslie Vosshall.

Putative phosphorylation sites were identified using PredictProtein (Rost et al., 2004), PROSITE (Hulo et al., 2008), NetPhos 2.0 (Blom et al., 1999), and YingOYang 1.2 (Gupta, 2001).

Snake plots and positions of variant amino acids of OR59b in Figure 3.13 and Figure 3.14 were manually composed using transmembrane domain predictions generated with the PredictProtein (Rost et al., 2004) algorithm.

Structure of odorant molecules in Figure 3.5, Figure 3.6, Figure 3.7, Figure 3.8, and Figure 3.12 were drawn with the PubChem editor (Ihlenfeldt et al., 2009).

6.2 Genomic DNA and cDNA preparation

DNA was prepared according to the Quick Fly Genomic DNA Prep protocol from the Berkeley *Drosophila* Genome Project (http://www.fruitfly.org/about/methods/inverse.pcr.html). 1.5 µl of DNA were used for amplification using the KOD PCR Kit (Novagen, Madison, WI, USA). For experiments conducted in Chapter 3, *Or59b* primers were designed to anneal to the 5' and 3' UTR of the w^{1118} *Or59b* locus:

Forward: 5'-gaattcTCCGGGTATAAAGTGCAGGTGCTGGCACCG-3'

Reverse 5'-ctcgagGCTCTTTTTTGCGGGGGGCTCATGGGTGCAG-3'

Or83b was amplified using primers that amplify the complete coding region:

Forward: 5'-gaattcATGACAACCTCGATGCAG-3'

Reverse: 5'-caattgCTTGAGCTGCACCAGCACCA-3'

PCR products were cloned into pGEM-T Easy (Promega Corporation, Madison, WI, USA), sequenced (GENEWIZ, Inc., South Plainfield, NJ, USA), and analyzed using SeqMan software (DNASTAR Inc., Madison, WI, USA). For each strain, at least four independent samples were analyzed, derived from at least two different genomic preparations and two different PCR reactions. These were sequenced and compared to NCBI reference sequences for each gene (*Or59b*: NM_079098.1; *Or83b*: NM_079511.4).

For cDNA preparation, total RNA was extracted from w^{1118} and Boa Esperança antennae using the RNeasy Mini Kit (QIAGEN, Valencia, CA, USA). cDNA was prepared with SuperScript^{TM} III First-Strand Synthesis System (Invitrogen, Carlsbad, CA,

USA) using oligo(dT) primers. *Or59b* cDNA from both *w^1118* and Boa Esperança was amplified using the following primers:

Forward: 5'-gaattcATGGCGGTGTTCAAGCTAATCAAACCG-3'

Reverse: 5'-ctcgagTTACTGGAACTGCTCGGCCAGATTCA-3'

PCR products representing full-length *Or59b^NCBI REF* and Or59b^Boa Esperança cDNAs were cloned into pGEM-T Easy, completely sequenced, and subcloned into the pUAST attB vector (Bischof et al., 2007) using EcoRI and XhoI restriction sites.

6.3 Generation of *Or59b* and *Or83b* transgenes

Amino acid mutations were introduced into the *w^1118 Or59b* cDNA or *Or83b* (from (Benton et al., 2006)) by two rounds of PCR reactions. Briefly, the *Or59b* gene cloned into pGEMT-EZ or *Or83b* gene cloned in pUAST were used as templates for a first round of PCR amplification. Two independent reactions were prepared: one contained the forward primer with the desired mutation and the reverse primer annealing to the vector backbone; the second contained the reverse mutating primer and the forward primer annealing to the vector. The PCR products from the reactions were purified and 1 μl of each was used as a template and mixed in a second round of amplification with the forward and reverse vector primers to obtain the full gene.

Primers annealing on the vector backbone:

OR59b

Forward SP6 (5'-ATTTAGGTGACACTATAG-3')

Reverse T7 (5'-TAATACGACTCACTATAGGG-3')

OR83b

       Forward 5'-CCAGCAACCAAGTAAATCAACTGC-3'

       Reverse 5-'TACACAAACAATTAGAATCAGTAG-3'

Mutating primers:

OR59b^{V41F}

       Forward: 5'-CCGCCGAAGGAGGGATTCCTGCGCTACGTGT-3'

       Reverse: 5'-ACACGTAGCGCAGGAATCCCTCCTTCGGCGG-3'

OR59b^{V91A}

       Forward: 5'-AGGTGTGCATCAATGCGTATGGCGCCTCGG -3'

       Reverse: 5'-CCGAGGCGCCATACGCATTGATGCACACCT -3'

OR59b^{T376S}

       Forward: 5'-TGAACAGCAACATAAGCGTGGCCAAGTTCGC-3'

       Reverse: 5'-GCGAACTTGGCCACGCTTATGTTGCTGTTCA-3'

OR59b^{V388A}

       Forward: 5'-GCATCATTACAATAGCGCGACAAATGAATCT-3'

       Reverse: 5'-AGATTCATTTGTCGCGCTATTGTAATGATGC-3'

OR83b^{W431A}

       Forward 5'-GCCTACTCGTGCCACGCCTACGATGGCTCCGAG-3'

       Reverse 5'-CTCGGAGCCATCGTAGGCGTGGCACGAGTAGGC-3'

OR83b^{W431F}

       Forward 5'-GCCTACTCGTGCCACTTCTACGATGGCTCCGAG-3'

       Reverse 5'-CTCGGAGCCATCGTAGAAGTGGCACGAGTAGGC-3'

OR83b^{Y432A}

Forward 5'- TACTCGTGCCACTGGGCCGATGGCTCCGAGGA-3'

Reverse 5'- TCCTCGGAGCCATCGGCCCAGTGGCACGAGTA-3'

OR83b^{D433A}

Forward 5'- CGTGCCACTGGTACGCTGGCTCCGAGGAGGC-3'

Reverse 5'- GCCTCCTCGGAGCCAGCGTACCAGTGGCACG-3'

OR59b$^{V41F\ V91A}$ was generated using OR59b^{V41F} as a template and the primers used to generate the OR59b^{V91A} mutant. Similarly, OR59b$^{T376S\ V388A}$ was generated using OR59b^{T376S} as a template and the primers used to generate the OR59b^{V388A} mutant.

All PCR products were amplified using the KOD polymerase Kit (Novagen, Madison, WI, United States), T:A cloned into pGEM-T Easy (Promega, Madison, WI, United States) cut at the EcoRI (5`) and XhoI (3`) restriction sites (New England Biolabs, Ipswich, MA, United States), subcloned into the transgenic expression vector pUAST (OR83b) or pUAST attB (OR59b), and sequenced.

Constructs for transgenic animals were injected into w^{1118} embryos by Genetic Services (Cambridge, MA, United States). The phiC 31 integrase system (Bateman et al., 2006; Bischof et al., 2007) was used to insert all the *Or59b* constructs and the mutated *Or83b* into the attp2 and attp40 insertion sites on the third and second chromosome, respectively. Wild type *Or83b* was inserted in a random location on the second chromosome as described in (Benton et al., 2006). Single transformants were isolated and balanced according to standard fly genetic methods.

6.4 Fly stocks

Drosophila melanogaster stocks were maintained on conventional cornmeal-agar-molasses medium under a 12 hour light:12 hour dark cycle at 25°C.

In Chapter 3, the *w^{1118}* strain was used as wild type control. In addition to *w^{1118}*, the following wild type strains were used for experiments described in Figure 3.10: Akayu (*Drosophila* Genetic Resource Center (DGRC) #103389, origin: Japan); Algeria (isogenic for II and III chromosomes, DGRC #103390, origin: Algeria); Alma-Ata (DGRC #103391, origin: Kazakstan); Canton-S (isogenic for II and III, lab stock, origin: Ohio, USA); CA1 (Bloomington *Drosophila* Stock Center #3846, origin: Cape Town, South Africa); Coffs Harbour (DGRC #103411, origin; New South Wales, Australia); Kericho-7B (DGRC #103428, origin: Kericho, Kenya); Manago (isogenic for II and III, DGRC #103433, origin: Hawaii, USA); Oregon-R (isogenic for II and III, lab stock, origin: Oregon, USA); San Miguel (isogenic for II and III, DGRC #103450, origin: Buenos Aires, Argentina); WT Berlin (isogenic for II and III, Heisenberg laboratory, Würzburg, Germany, origin: Berlin, Germany); Batumi-L (DGRC #103396, origin: Batumi, Georgia); Boa Esperança (DGRC #103400, origin: Minas Gerais, Brazil); BOG 2 (Bloomington #3842, origin: Bogota, Colombia); CO 3 (Bloomington #3848, origin: Commack, New York, USA); EV (Bloomington #3851, origin: Ellenville, New York, USA); Medvast-21 (DGRC #103435, origin: Finland); VAG 2 (Bloomington #3876, origin: Athens, Greece).

Mutant alleles used for experiments in Chapter 3: *Or22a/b$^{\Delta halo}$* (Dobritsa et al., 2003), *Or22a-Gal4* (Fishilevich and Vosshall, 2005). Mutant alleles in the *Or59b* gene used in experiments of Figure 3.15 are based on the OR59b protein derived from the NCBI reference mRNA sequence (NCBI REF number NM_079098.1). Fly genotypes:

Or22a/b^{Δhalo}; Or22a-Gal4/UAS-Or59b (labelled Or59b^{NCBI REF} in the figure), *Or22a/b^{Δhalo}; Or22a-Gal4/UAS-Or59b^{V41F}* (V41F), *Or22a/b^{Δhalo}; Or22a-Gal4/UAS-Or59b^{V91A}* (V91A), *Or22a/b^{Δhalo}; Or22a-Gal4/UAS-Or59B^{V41F V91A}* (V41F V91A), *Or22a/b^{Δhalo}; Or22a-Gal4/UAS-Or59b^{T376S}* (T376S), *Or22a/b^{Δhalo}; Or22a-Gal4/UAS-Or59b^{V388A}* (V388A), *Or22a/b^{Δhalo}; Or22a-Gal4/UAS-Or59b^{T376S V388A}* (T376S V388A), *Or22a/b^{Δhalo}; Or22a-Gal4/UAS-Or59b^{V41F V91A T376S V388A}* (Boa Esperança).

Mutant alleles and transgenic flies used for experiments in Chapter 4: *Or22a/b^{Δhalo}* (Dobritsa et al., 2003), *Or22a-Gal4* (Fishilevich and Vosshall, 2005). Genotypes of the flies used for Figure 4.4, Figure 4.5, and Figure 4.6: *CyO/Bl; Or83b²*, *Or83b-Gal4/UAS-Or83b; Or83b¹/Or83b²*, *Or83b-Gal4/UAS-Or83b^{W431A}; Or83b¹/Or83b²*, *Or83b-Gal4/UAS-Or83b^{W431F}; Or83b¹/Or83b²*, *Or83b-Gal4/UAS-Or83b^{Y432A}; Or83b¹/Or83b²*, *Or83b-Gal4/UAS-Or83b^{D433A}; Or83b¹/Or83b²*, *Or83b-Gal4/UAS-Or83b^{W431A}; +/Or83b²*, *Or83b-Gal4/UAS-Or83b^{W431F}; +/Or83b²*.

6.5 Histology

Antibody staining was performed on 14 µm frozen antennal sections of *w^{1118}* and transgenic *Drosophila* animals. Five to seven day old flies were collected and fly heads were fixed in frozen OCT. 14 µm sections were cut on a cryostat (Microm HM 550, Thermo Fisher Scientific, Waltham, MA, United States) and collected on SuperFrost Plus slides (Thermo Fisher Scientific, Waltham, MA, United States). Sections were fixed for 7 min in 4% paraformaldehyd/1x PBS and washed twice for 10 min in 1x PBS. Sections then were permeabilized for 30 min in P/T (1x PBS/0.1% Triton-100) and blocked horizontally for 30 min in 500 µl P/T/S (P/T/5% heat inactivated normal goat

serum). Primary antibodies were diluted in P/T/S and 100 µl of the antibody dilutions were applied per slide. For the OR83b/ER double staining the following antibodies were used: α-2nd EC loop of *Drosophila* OR83b (dilution 1:5000; Benton et al., 2006) and Guinea pig α-Boca (Culi and Mann, 2003). For the OR22a staining, a rabbit α-OR22a/b was used (Dobritsa et al., 2003). To prevent evaporation during the overnight incubation at 4°C, cover slips were placed on each slide. The next day, sections were washed 3 times for 10 min in P/T and blocked for 30 min with 500 µl P/T/S. The following secondary fluorescent antibodies were used: Cy3-conjugated goat α-rabbit IgG (Jackson ImmunoResearch, West Grove, PA, United States, dilution 1:200) and FITC-conjugated goat α-guinea pig IgG (Jackson ImmunoResearch, West Grove, PA, United States, dilution 1:200). 100 µl of the antibodies diluted in P/T/S were applied per slide, a cover slip was placed on each slide and slides were incubated at 25°C in the dark. Sections were then washed 3 times for 5 min in P/T. 60 µl Vectashield (Vector Labs, Burlingame, CA, United States) were applied, microscope cover glasses were placed on each slide and slides were stored at 4°C in the dark. Visualization was performed using a Zeiss LSM510 confocal microscope (Carl Zeiss Jena GmbH, Jena, Germany).

6.6 Single sensillum electrophysiology

Female transgenic flies were recorded at 5 days after adult eclosion. All other flies were recorded at 5-10 days after adult eclosion. Single sensillum recordings were performed as described (Ditzen et al., 2008; Pellegrino et al.). Odorants were obtained from Sigma-Aldrich at high purity and diluted (v/v) in paraffin oil as indicated. DEET was obtained from Alfa Aesar (Ward Hill, MA, USA) and was applied undiluted. Chemical

Abstracts Service (CAS) numbers: paraffin oil (8012-95-1); 1-octen-3-ol (3391-86-4); pentanal (110-62-3); pentanoic acid (109-52-4); 2-heptanone (110-43-0); 1-octanol (111-87-5); (-)linalool (126-91-0); methyl acetate (79-20-9); 2,3-butanedione (431-03-8); ethyl hexanoate (123-66-0); butyraldehyde (123-72-8); ethyl-3-hydroxybutyrate (5405-41-4); ethyl acetate (141-78-6); hexanol (111-27-3); DEET (134-62-3). 30 µl of the desired odor dilution was pipetted onto a filter paper strip (3 x 50 mm) and 30 µl of undiluted DEET or paraffin oil solvent was pipetted onto a second filter paper strip. Both filter paper strips were then carefully inserted into a glass Pasteur pipette. Prior to any recordings, charcoal-filtered air was forced through the pipette for 1-3 s to remove dead space in the odorant delivery system. For actual recordings, charcoal-filtered air was continuously applied to the insect antenna, with odorant delivered through the pipette to the fly antennae for 1 s. Sensilla types were identified by size, location on the antenna, and responsiveness to known preferred odorants (Hallem and Carlson, 2006).

Data were collected using Autospike (Syntech) and analyzed by custom spike sorting algorithms (Ditzen et al., 2008). Spike trains were grouped in 200 ms bins and responses were calculated by subtracting the average spontaneous activity in 15 s before odorant application from activity during the first 500 ms (excitatory odorants) or 1 s (inhibitory odorants) of odorant delivery. The onset of odorant-evoked responses varied due to slight variations in the position of the odorant delivery system relative to the sensillum being recorded. To correct for this, we calibrated the inferred odorant onset for each sensillum recorded based on excitatory responses elicited by control stimuli applied at the beginning of each trial (ab2: 10^{-5} methyl acetate; ab3: 10^{-5} 2-heptanone).

6.7 Statistics

Statistical analysis in Figure 3.4 and the peri-stimulus plots in Figure 4.4 were performed in R2.3.1 (http://www.r-project.org/) or using Microsoft Excel statistical functions. The statistical tests performed are indicated in the figure legends under each figure.

Dose-response curves in Figure 3.5, Figure 3.6, Figure 3.7, Figure 3.8, and Figure 3.12 were fitted using OriginPro 8 (OriginLab, Northampton, MA, USA) by a logistic function, except responses to 1-octen-3-ol in Figure 3.7D, which are fitted by a biphasic function. Comparisons of paired dose-response curves in the same figures are performed by an F-test to assess statistical significance of differences between the two curve fits, followed by Bonferroni correction for multiple comparisons. A two-tailed paired t-test was performed to assess statistical significance of all comparisons in Figure 3.10, Figure 3.12, and Figure 3.15, followed by Bonferroni correction for multiple comparisons applied to each set of experiments. Data in Figure 3.11 were fitted with a linear regression analysis.

6.8 Yeast two-hybrid assay

A yeast two-hybrid analysis was performed according to the Matchmaker^{TM} GAL4 Two-Hybrid System 3 User Manual (Clontech, Mountain View, CA, United States). The following OR fragments were used (amino acid codon number): OR43a IC3 (298–342), OR47a (305-355), OR83b IC3 (412–459), OR83b^{W431A} IC3 (412-459), OR83b^{W431F} IC3 (412-459), OR83b^{Y432A} IC3 (412-459), OR83b^{D433A} IC3 (412-459).

All *Or83b* mutant fragments were amplified by PCR using the respective pUAST vectors described before as template, and the following primers:

Forward 5'- gaattcGGCAATCGTCTGATTGAAGAGAGTTCATCCGT -3'

Reverse 5'- cccgggttaTTTCGCTCCCGATATGCTCATCGCCTTCTG -3'.

The OR47a (305-355) fragment was amplified by PCR using an *Or47a* cDNA clone (from T. Nakagawa) as template, and the following primers:

Forward 5'-gaattcTGCGGGGAGAACCTGAAGACGGAG -3'

Reverse 5'-cccgggAATGCGGAATCCCCGATGAGCCCG -3'.

The OR43a (298–342) fragment was obtained as described in (Benton et al., 2006).

All PCR products, amplified using the KOD polymerase Kit (Novagen, Madison, WI, United States), were T:A cloned into pGEM-T Easy (Promega, Madison, WI, United States) cut at the EcoRI (5') and XmaI (3') restriction sites (New England Biolabs, Ipswich, MA, United States) and subcloned into GAL4 DNA-binding domain or activation domain vectors pGBK-T7 and pGAD-T7 (Clontech, Mountain View, CA, United States). All constructs were sequenced at GENEWIZ, Inc (South Plainfield, NJ, United States) and analyzed using the program SeqMan Pro from DNASTAR Lasergene 8.

All constructs were transformed into yeast strain AH109 (Clontech, Mountain View, CA, United States) using a standard protocol for the LiAc/SS carrier DNA/PEG transformation method. Briefly, frozen competent cells were thawed in a 37ºC water bath for 15-30 s. They were centrifuged for 2 min at 13,000 g and the supernatant was removed. The following transformation mix was prepared: 260 µl PEG 3350 (50% (w/v)), 36 µl LiAc 1.0 M, 50 µl single-stranded carrier DNA (2mg/ml), 14 µl plasmid DNA plus

sterile water (1 µg of pGAD vector and 1 µg of pGBKT vector). The mix was added to the cells and cells were incubated in a 42ºC water bath for 20 min. Cells were then centrifuged for 30 s at 13,000g, the supernatant was removed and cells were washed with water. 1 ml of water was added and 200 µl of the resuspended cells were plated on selective plates (SD/-Leu/-Trp).

Three days after transformation, single colonies were re-streaked on selective plates (SD/-Leu/-Trp) to allow colonies to grow. Two days later colonies were re-streaked on media selecting for the expression of the HIS3 and ADE2 reporter genes. Interactions were scored for growth 1 week after re-streaking. In cases were the DNA/bait produced background growth on selective plates due to leaky HIS3 expression, colonies were re-streaked on plates containing 2.5 mM, 5 mM or 7.5 mM of 3-AT (3-Amino-1,2,4-triazole). Interactions were scored for growth 2 weeks after re-streaking.

6.9 *Xenopus* oocyte electrophysiology

CNGs and CFTR DNA clones were provided by T.-Y. Chen and A. Kovacs, respectively. Full length cDNAs of fruit fly ORs [OR47a and OR83b], mosquito ORs (GPROR1, GPROR2, GPROR8, and GPROR7), mouse TRP channel (mTRPM8) and OR (mOR-EG), were cloned into the *Xenopus laevis* oocyte expression vector pXpress-X and linearized with XbaI. Full length cDNAs of CFTR and rat olfactory CNGs (CNGA2, CNGA4, and CNGB1) were cloned into pGEMHE and linearized with NheI. Full length cDNA of the fruit fly K-channel ether-a-go-go (EAG) was cloned into pGH19 and

linearized with NotI. All plasmids were transcribed in vitro with mMessage mMachine (Ambion, Inc.).

Oocytes were microinjected with 25 ng of complementary RNA (cRNA) for a conventional OR and 25 ng of cRNA for the OR83b family. Whole-cell currents were recorded with a two-electrode voltage-clamp filled with 3 M KCl, and were amplified with an OC-725C amplifier (Warner Instruments), low-pass filtered at 50 Hz and digitized at 1 kHz. Odorants were applied to the recording chamber using a gravity driven perfusion system. Subtracted I-V curves were acquired with a step protocol ranging from -80 mV to +40 mV (20 mV step), and the currents were normalized to the +40 mV data point in the presence of ligand only. Electrodes were filled with a 3 M KCl solution, while the extracellular oocyte Ringer's solution contained (in mM): 82.5 NaCl, 2 KCl, 1 $MgCl_2$, 5 HEPES, 1.8 $CaCl_2$ (pH 7.5), except for mTRPM8 experiments where no $CaCl_2$ was added.

Outside-out patch-clamp recordings were performed 18–26 h after injection. After removal of the vitelline layer, oocytes were transferred to a Petri dish with a bath solution of oocytes Ringer's solution. Pipettes (4–7 MΩ) were covered with Sylgard (Dow Chemical Company) and filled with intracellular solution containing (in mM): 100 KOH, 10 HEPES, 1 EGTA, 100 sulphamic acid (pH 7.6). After contact of the pipette tip with the oocyte membrane and seal formation (more than 5 GΩ), patches were excised and transferred to the recording chamber, where the extracellular side was continuously superfused with extracellular solution containing (in mM): 100 NaOH, 10 HEPES, 1

$MgCl_2$, 100 sulphamic acid (pH 7.5); this was supplemented, where indicated, with the odorants. Solutions were switched by computer-driven electric valves (General Valve Corp.). Currents were recorded with an Axopatch 200A amplifier (Axon Instruments, Inc.), low-passed at 1 kHz (eight-pole Bessel; Frequency Devices), digitized at 10 kHz by means of an ITC-16 interface (Instrutech Corporation) and saved to a PC hard disk with PULSE v8.11 acquisition software (HEKA Elektronic). Data were analyzed with Clampfit 9.0 (Axon Instruments, Inc.) and Origin PRO 7 (Origin Lab). The I–V curves showing ion permeability were produced with low-Na^+ solution (Na^+ in oocyte Ringer's solution replaced by the impermeable cation $NMDG^+$) and Cl^--free solution (Cl^- in oocyte Ringer's solution replaced by sulphamic acid). These experiments used bath application of ligands, precluding any measurement of the response latency of these currents.

Stock solutions of pentyl acetate (1 M, CAS number: 628-63-7), 2-methyl phenol (1 M, CAS: 95-48-7), 4-methyl phenol (1 M, CAS: 8001-28-3), 1-octen-3-ol (1 M, CAS: 3391-86-4), forskolin (40 mM, CAS: 66575-29-9), and menthol (1 M, CAS: 15356-60-2) were prepared in DMSO, and then added to Ringer's solution. DEET (CAS: 134-62-3) was diluted directly to the extracellular solution.

6.10 Images copyright

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6.11 Experiments performed by others

cAMP assays in HEK293T cells (Figure 2.7A), Ca^{2+}-imaging experiments (Figure 2.7B), *Xenopus* experiments in Figure 2.8, and patch-clamp experiments in mammalian cell lines (Figure 2.9) were performed by Dr. Koji Sato or Dr. Takao Nakagawa as specified in the figures and described in (Sato et al., 2008).

Drosophila behavioral assays (Figure 3.1, Figure 3.2, and Figure 3.3C) and electrophysiology experiments (Figure 3.3A, B, D, and E) were performed by Dr. Mathias Ditzen as described in (Ditzen et al., 2008).

Immunostaining and yeast two-hybrid assays in Figure 4.5, Figure 4.6, and Figure 4.7, and sequencing of *Or59b* alleles summarized in Figure 3.14 were performed by Nicole Steinbach, a Master student from the Ludwig-Maximilians-University of Munich, under my supervision.

7 Publications

The original findings described in this dissertation were reported in the following publications:

Ditzen M, **Pellegrino M**, Vosshall LB.

Insect odorant receptors are molecular targets of the insect repellent DEET.

Science. 2008 Mar 28;319(5871):1838-42.

Sato K, **Pellegrino M**, Nakagawa T, Nakagawa T, Vosshall LB, Touhara K.

Insect olfactory receptors are heteromeric ligand-gated ion channels.

Nature. 2008 Apr 24;452(7190):1002-6.

And in the following review articles:

Pellegrino M, Nakagawa T, Vosshall LB.

Single sensillum recordings in the insects *Drosophila melanogaster* and *Anopheles gambiae*.

J Vis Exp. 2010 Feb 17;(36):1-5.

Pellegrino M, Nakagawa T.

Smelling the difference: controversial ideas in insect olfaction.

J Exp Biol. 2009 Jul;212(Pt 13):1973-9.

The following manuscript is in preparation:

Pellegrino M, Steinbach N, Vosshall LB

A natural polymorphism in an insect odorant receptor confers pharmacological resistance to DEET.

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