# MAPPING OF ODOR AND TEMPERATURE RECEPTORS IN THE MALARIA VECTOR MOSQUITO *ANOPHELES GAMBIAE*

By

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To my parents,

Jinming Lu and Yongrui Guo

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LIST OF ABBREVIATIONS

| | |
|---|---|
| ab1 | Antennal basiconic type 1 |
| ab3 | Antennal basiconic type 3 |
| AgGR | *An. gambiae* gustatory receptor |
| AgOR | *An. gambiae* odorant receptor |
| AL | Antennal lobe |
| AP | Alkaline phosphatase |
| $CO_2$ | Carbon dioxide |
| cp | Capitate peg |
| cRNA | Complementary RNA |
| DEET | *N*,*N*-diethyl-3-methylbenzamide |
| FISH | Fluorescent *in situ* hybridization |
| GPCR | G protein-coupled receptor |
| $H_2CO_3$ | Carbonic acid |
| $HCO_3^-$ | Bicarbonate |
| LPO | Labial pit organ |
| OR | Odorant receptor |
| ORN | Odorant receptor neuron |
| RT-PCR | Reverse-transcriptase polymerase chain reaction |
| SSR | Single-sensillum recording |
| TEM | Transmission electron microscopy |
| TRP | Transient receptor potential |

# CHAPTER I

# INTRODUCTION

## Transmission of Malaria by Anopheline Mosquitoes

One of the most devastating infectious diseases in the world, malaria causes more than a million deaths each year, mostly in children in Africa. An arthropod-borne disease, malaria is transmitted when an infected female anopheline mosquito takes a blood meal from and injects *Plasmodium* sporozoites into a vertebrate host. Originally, these mosquitoes get infected with *Plasmodium* parasites when they take a previous blood meal from an infected vertebrate host.

The level of malaria transmission is largely reflected in a quantitative term known as vectorial capacity (Macdonald, 1957). This reflects vector interactions with both the pathogen and the vertebrate host and is directly related to the human-biting frequency of vector mosquitoes (Spielman, 1994). The Afrotropical vector mosquito, *Anopheles gambiae* sensu stricto, is highly anthropophilic (prefer to feed on humans), endophagic (prefer to feed indoors) and endophilic (prefer to rest indoors). As a result of these behaviors, *An. gambiae* is likely to remain in close proximity with humans and serves as the most efficient malaria vector in Africa.

*An. gambiae* mosquitoes find their human hosts primarily through olfactory cues, although heat, humidity as well as visual cues also play a role in host location (Bowen, 1991; Takken and Knols, 1999; Zwiebel and Takken, 2004).

Female *An. gambiae* are sensitive to various compounds emanated by humans, including carbon dioxide ($CO_2$), 1-octen-3-ol, lactic acid, ammonia and carboxylic acids, and rely on these compounds in their host-seeking behavior (Bowen, 1991; Takken and Knols, 1999; Zwiebel and Takken, 2004). Some of these compounds, together with host-derived heat, act synergistically to elicit an attractive response (Smallegange et al., 2005). Therefore, an in-depth understanding of the mechanism by which *An. gambiae* mosquitoes detect human odorants would be instrumental in designing efficient mosquito repellents and might ultimately lead to a significant reduction in the incidence of malaria.

**Structure of the Mosquito Olfactory System**

*An. gambiae* and other mosquitoes have three olfactory appendages, the antenna, the proboscis, and the maxillary palp (Figure 1), and all are populated by porous sensory hairs called sensilla (Kwon et al., 2006; Lu et al., 2007; Pitts et al., 2004). Within each sensillum, odorant receptor neurons (ORNs) project ciliated dendrites into the aqueous lymph where odorants are diffused and come into contact with the dendrites. This leads to activation of odorant receptors (ORs) that are distributed along the dendritic surface of ORNs. The ORNs then project their axons to a brain region called the antennal lobe and ORNs expressing the same OR converge on globular structures called glomeruli (Stocker, 1994; Vosshall and Stocker, 2007). The glomeruli are where ORNs form synaptic connections with dendrites of the projection neurons, which then

project to higher olfactory processing centers such as the mushroom body and lateral horn of the protocerebrum (Stocker, 1994; Vosshall and Stocker, 2007).

**Olfactory Organs of Adult Mosquitoes**

*An. gambiae* and other mosquitoes have three olfactory appendages, the antenna, the proboscis, and the maxillary palp (Figure 1) (Kwon et al., 2006; Lu et al., 2007; Pitts et al., 2004). Among the olfactory appendages, the antenna contains the largest quantity and variety of olfactory sensilla and expresses the majority of 79 *An. gambiae* odorant receptor (AgOR) genes (Hill et al., 2002; McIver, 1982; Pitts and Zwiebel, 2006). Female *An. gambiae* antenna possesses 13 segments, and segments 2-13 are covered with numerous olfactory sensilla containing the sensory dendrites of ORNs. Antennal ORNs respond to a vast variety of host compounds including ammonia, indole, 4-methylphenol, lactic acid and several other carboxylic acids, reflecting their functional diversity (Cork and Park, 1996; Ghaninia et al., 2007b; Meijerink et al., 2001; Meijerink and van Loon, 1999; Qiu et al., 2006; van den Broek and den Otter, 1999). These ORNs fasciculate into the antennal nerve and project along with hygro- and thermoreceptive neurons to the antennal lobe (Ghaninia et al., 2007a; Ignell et al., 2005).

The maxillary palp is a simpler organ than the antenna, harboring a single type of chemosensory sensillum, the capitate peg. In *An. gambiae*, the maxillary palp consists of five segments and only the three mid-segments contain capitate pegs. Electrophysiological recordings revealed the existence of a single

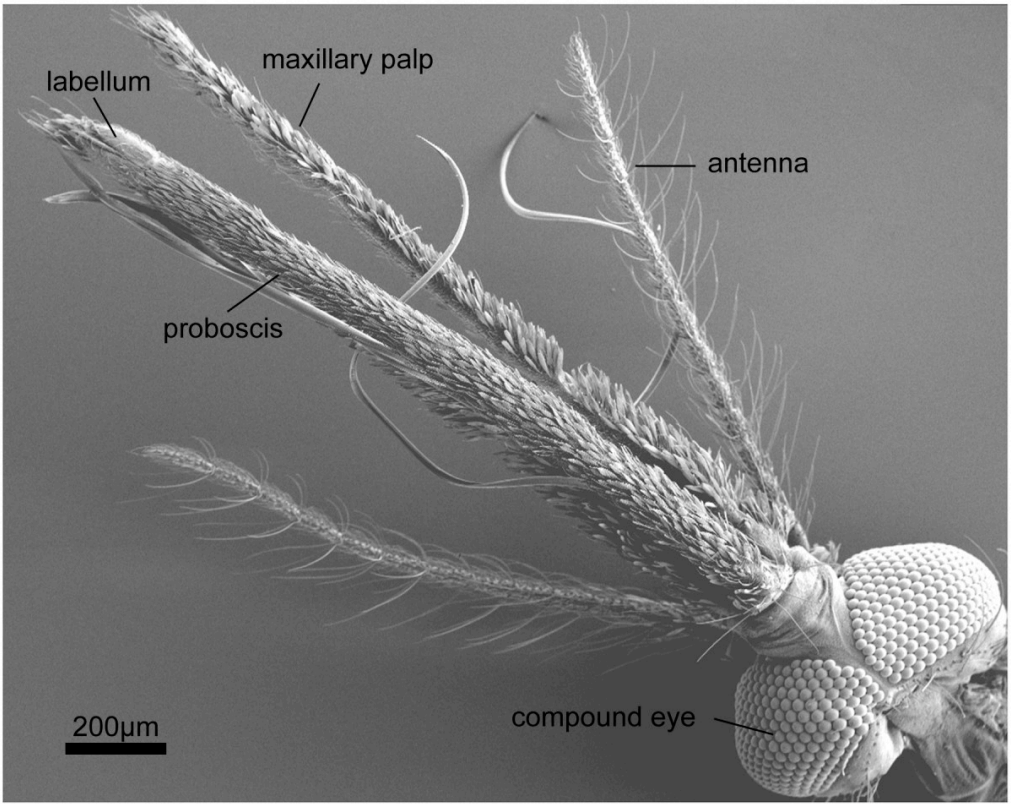


**Figure 1. Female Head (Ventral View).** Scanning electron micrograph showing the sensory appendages of an adult female *An. gambiae* s.s. Eyes, antennae, and maxillary palps occur in pairs, although the second palp is hidden below the proboscis in this micrograph. The proboscis is a single appendage that encloses the blood-feeding stylets, which appear as ribbon-like tentacles here. At the distal end of the proboscis is the labellum, or labellar lobes (Pitts and Zwiebel, 2006).

functional class of capitate pegs across the maxillary palp and three ORNs form stereotyped triads within each capitate peg (Lu et al., 2007). Notably, the capitate peg (cp) A and B neurons respond with high sensitivity to $CO_2$ and 1-octen-3-ol, respectively, both of which are established mosquito attractants (Lu et al., 2007). A distinctive set of *AgORs* and *An. gambiae* gustatory receptors (*AgGRs*) are expressed in the triad of ORNs in the maxillary palp of *An. gambiae* (Jones et al., 2007; Lu et al., 2007). While the cpA neuron coexpresses three $CO_2$ receptors, *AgGR22*, *AgGR23* and *AgGR24*, the other two ORNs express *AgOR7* along with *AgOR8* or *AgOR28*, respectively (Jones et al., 2007; Lu et al., 2007). These palpal ORNs innervate five to six dorso-medial glomeruli in the antennal lobe (Anton and Rospars, 2004; Anton et al., 2003; Ghaninia et al., 2007a).

The proboscis is a long slender organ housing the feeding stylets of the mosquito and harboring numerous chemosensory sensilla at its bulbous tip called the labellum. A primarily gustatory organ, the labellum of *An. gambiae* has been demonstrated to respond to odorants including butylamine and aliphatic carboxylic acids, both of which are components of human skin emanations (Kwon et al., 2006). One of the labellar Type 2 (T2) sensilla harbors at least two ORNs and responds to butylamine, aliphatic acids and several ketone compounds including acetophenone (Kwon et al., 2006). Furthermore, *AgOR6*, an *AgOR* that is coexpressed with AgOR7 in a subset of labellar ORNs, is expressed in this sensillum (Kwon et al., 2006). More than 20 additional *AgORs* were also detected by reverse-transcriptase polymerase chain reaction (RT-PCR) in the labellar cDNAs of *An. gambiae* (Kwon et al., 2006). Labellar ORNs

fasciculate with gustatory neurons in the labial nerve and project through the suboesophageal ganglion to reach 1-2 glomeruli in the medial region of the antennal lobe (Ghaninia et al., 2007a; Kwon et al., 2006).

**Mosquito Odorant Receptors (ORs)**

The complete *An. gambiae* genome is available and has provided an opportunity to study mosquito olfaction at the level of ORs (Fox et al., 2001; Fox et al., 2002; Hill et al., 2002). *An. gambiae* has a large family of 79 *AgOR* genes, which are dispersed throughout the three chromosomes (Hill et al., 2002). RNA in situ hybridization analysis reveals that each *AgOR* gene is expressed in a spatially conserved subpopulation of ORNs in either adult or larval olfactory organs, while each ORN normally expresses one conventional *AgOR* (Kwon et al., 2006; Lu et al., 2007; Xia et al., 2008). Although the majority of *AgOR* genes do not have close orthologs in *Drosophila* and are more closely related to other *AgORs* than any *Drosophila ORs*, one *AgOR* gene, *AgOR7*, is well conserved and is expressed in most ORNs (Hill et al., 2002; Pitts et al., 2004). AgOR7 and DmOR83b proteins share 78% identity, and like DmOR83b, AgOR7 is likely to form functional heterodimers with conventional AgORs and be required for normal ciliary targeting of AgOR proteins (Benton et al., 2006; Larsson et al., 2004; Pitts et al., 2004).

Multiple *AgORs* have had their identities confirmed as odorant receptor genes. When expressed in *Xenopus* oocyte, mammalian cell lines and a mutant *Drosophila* antennal basiconic type 3A (ab3A) ORN lacking the endogenous *OR*

gene, these AgORs confer responses to a distinct subset of tested odors (Ditzen et al., 2008; Hallem et al., 2004a; Lu et al., 2007; Xia et al., 2008). Similar to *Drosophila* ORs, response profiles of AgORs expressed in heterologous systems closely mirror that of the endogenous ORNs (Lu et al., 2007), and this suggests that insect ORs provide the primary determinant of response spectra of ORNs (Hallem et al., 2004b; Lu et al., 2007; Nakagawa et al., 2005). As expected, the "odor tunings" of AgORs vary drastically, as they range along a continuum from broadly tuned to narrowly tuned ORs, reflecting the difference in their functional roles from broad odor samplers to specific and sensitive odor detectors (Hallem et al., 2004a; Lu et al., 2007; Xia et al., 2008).

Although they are likely to be seven-transmembrane proteins, insect ORs have no significant homology to G protein-coupled receptors (GPCRs) and appear to have evolved independently of mammalian chemoreceptors (Benton et al., 2006; Vosshall et al., 1999). Furthermore, multiple *Drosophila* ORs, including DmOR83b, have their N-termini located intra-cellularly and adopt an inverted membrane topology compared to that of GPCRs (Benton et al., 2006; Lundin et al., 2007; Wistrand et al., 2006). Most AgORs share the atypical membrane topology with *Drosophila* ORs, and these data suggest that insect ORs do not function as GPCRs, which was confirmed by recently studies showing that insect ORs are ligand-gated ion channels and their responses to odorants are comprised of two independent components (Sato et al., 2008; Wicher et al., 2008). While insect ORs function as ligand-gated channels that respond to odorant stimulation in an ionotropic pathway; the DmOR-DmOR83b heterodimers

also form complexes of odorant-sensing units and cyclic-nucleotide-activated non-selective cation channels that mediate responses to odorants in a metabotropic pathway (Figure 2) (Sato et al., 2008; Wicher et al., 2008).

One class of ORN stands out as an exception to the AgOR-AgOR7 paradigm, as it expresses three AgGRs, AgGR22, AgGR23 and AgGR24, which, as indicated in a subsequent chapter in this thesis, have been shown to function as $CO_2$ receptors in *An. gambiae* and do not require AgOR7 for function (Lu et al., 2007). The sequences of the three AgGRs are highly related to $CO_2$ receptors of *Drosophila* and other insects (Jones et al., 2007; Lu et al., 2007). *Drosophila* has lost its ortholog of *AgGR23* subsequent to the divergence of the two species (Lu et al., 2007), and ectopic expression of either AgGR22 and AgGR24 or their *Drosophila* orthologs, DmGR21a and DmGR63a, is sufficient to induce $CO_2$ sensitivity in an otherwise $CO_2$-insensitive *Drosophila* ORN (Jones et al., 2007; Kwon et al., 2007; Lu et al., 2007). AgGR23 is also implicated in $CO_2$ detection. But its exact function remains poorly understood (Lu et al., 2007), it might act to modulate the responses of the core receptors of AgGR22 and AgGR24.

**Temperature Detection in the Mosquito Antenna**

In addition to olfactory stimuli, *An. gambiae* and other blood-feeding mosquitoes rely on crucial temperature cues for host location (Bowen, 1991; Takken and Knols, 1999). Heat is emitted by all vertebrate hosts, serves as a universal

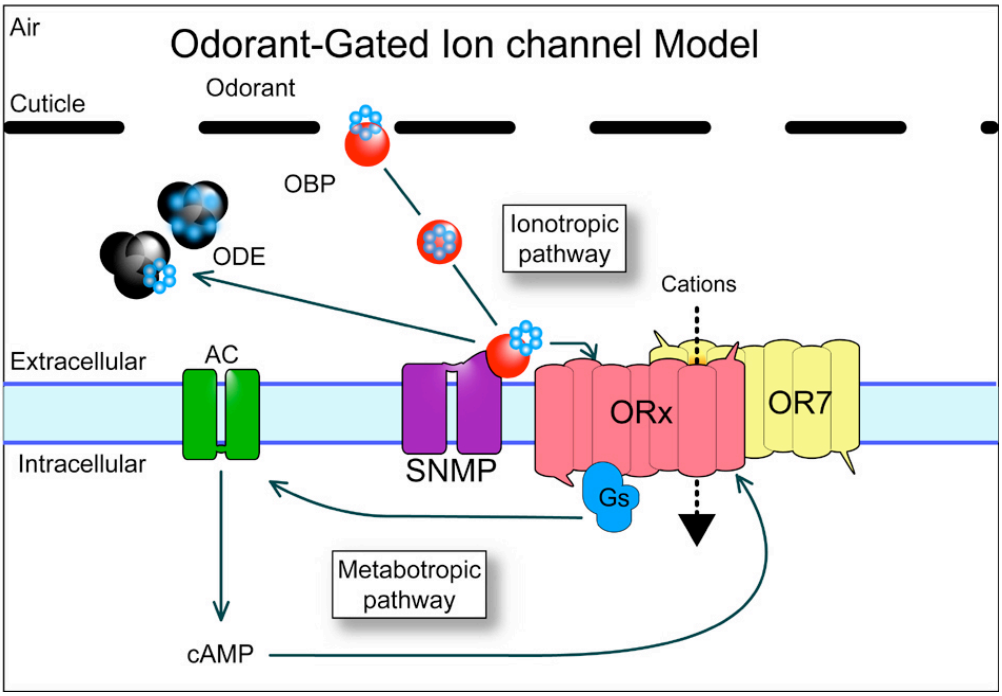


**Figure 2. Proposed Model of Insect Olfactory Signal Transduction.** Insect ORs are ligand-gated ion channels and their responses to odorants are comprised of two independent components. While insect ORs function as ligand-gated channels that respond to odorant stimulation in an ionotropic pathway; the DmOR-DmOR83b (AgOR-AgOR7 shown here) heterodimers also form complexes of odorant-sensing units and cyclic-nucleotide-activated non-selective cation channels that mediate responses to odorants in a metabotropic pathway (Sato et al., 2008; Wicher et al., 2008). (Figure courtesy of Dr. Jonathan Bohbot).

attractant to many mosquito species (Bowen, 1991; Takken and Knols, 1999) and synergizes with host odor to increase the efficiency of host-seeking behaviors (Kline and Lemire, 1995; Laarman, 1958). Early antennal ablation studies suggested that the antenna might harbor the thermoreceptive neurons that underlie the heat-evoked behaviors of mosquitoes (Ismail, 1962). In addition, ultrastructural studies characterized antennal small coeloconica and ampullacea sensilla as probable sites of temperature detection (Boo and McIver, 1975; McIver, 1973). In accord with this, electrophysiological studies in the yellow-fever vector mosquito, *Aedes aegypti*, identified an antagonistic pair of thermoreceptive neurons within the small coeloconica sensilla on the antennal tip (Davis and Sokolove, 1975; Gingl et al., 2005).

Despite some knowledge of the physiology of antennal thermoreceptive neurons, little is known about genes that are involved in peripheral temperature detection in mosquitoes. The *Drosophila* genome possesses a large family of 13 transient receptor potential (TRP) genes (Montell, 2005a; Montell, 2005b). These genes encode six-transmembrane nonselective cation channels and several of them, including DmTRPA1, DmTRPA2 and Painless, have been demonstrated as thermoreceptors (Lee et al., 2005; Rosenzweig et al., 2005; Tracey et al., 2003; Viswanath et al., 2003). *An. gambiae* has close homologs of all 13 *Drosophila* TRP channels, and some of these genes might also play a role in temperature detection.

**Relevance of This Work**

The work of this thesis is carried out in the context when the complete *An. gambiae* genome has become available. The identification of 79 *AgOR* genes and homologs of *Drosophila* TRP channels has provided an unprecedented opportunity to reexamine olfaction and thermoreception in *An. gambiae*, and to approach earlier morphological, electrophysiological and behavioral studies with a fresh molecular input. This work aims at an in-depth study of the three sensory appendages of *An. gambiae* and has offered insight into the molecular underpinnings of olfaction and thermoreception within three functional classes of mosquito sensilla. As a result, this information will greatly promote our understanding of the host-seeking behavior of *An. gambiae*, and will likely offer new opportunities for malaria control.

# CHAPTER II

## OLFACTORY RESPONSES IN A GUSTATORY ORGAN OF THE MALARIA VECTOR MOSQUITO *ANOPHELES GAMBIAE*

### Preface

The following publication by Kwon et al. (for which I was listed as the second author) appeared in the *Proceedings of the National Academy of Sciences USA* (*PNAS* 103(36): 13526-31). My contribution to this work included the identification of *AgORs* whose expression are associated with one labellar olfactory sensillum and conducting the *in situ* hybridization studies of *AgOR6* on the labellum of *Anopheles gambiae*. Michael Rutzler did RT-PCR survey of the whole proboscis and Hyung-Wook Kwon guided me through the *in situ* hybridization experiments, performed the immunolabeling and took the confocal images.

### Introduction

In the Afrotropical malaria vector mosquito *Anopheles gambiae* and in other insects, olfactory signal transduction is initiated by G protein-coupled receptors (GPCRs) on the dendrites of olfactory receptor neurons (ORNs), which have, thus far, been characterized in several insect species (Clyne et al., 1999; Fox et al., 2001; Krieger et al., 2002; Vosshall et al., 1999). In *An. gambiae*, 79 GPCR genes hypothesized to encode odorant receptors (AgORs) have been identified (Hill et al., 2002). Genes encoding candidate odorant receptors (ORs) are

diverse, with one notable exception comprising AgOR7 and other members of a highly conserved nonconventional OR subfamily that is widely expressed throughout insect olfactory organs (Hill et al., 2002; Krieger et al., 2003; Krieger et al., 2002; Vosshall et al., 2000). In addition to widespread expression in olfactory organs such as the antennae and maxillary palps of *An. gambiae* and Dengue virus vector mosquito *Aedes aegypti*, Ag/AaOR7 has recently been localized to around 25 distinct type-2 (T2) sensilla on the proboscis (Melo et al., 2004; Pitts et al., 2004). In contrast to Ag/AaOR7, their *Drosophila* ortholog, DOr83b (Vosshall et al., 1999), is not expressed in the proboscis of the adult fruit fly. This result suggests that mosquitoes and perhaps other bloodfeeding insects may contain a set of olfactory inputs derived from the proboscis that is absent in other arthropods.

Recent studies have demonstrated that *Drosophila* DOr83b is not directly responsive to odorants but, rather, is a general component of the olfactory signal transduction machinery (Elmore et al., 2003; Larsson et al., 2004; Nakagawa et al., 2005; Neuhaus et al., 2005). In *An. gambiae*, AgOR7 expression in the proboscis may, therefore, support similar olfactory capacity, leading to the prediction that the proboscis would be responsive to odorant stimuli and that other conventional odorant-activated AgORs would also be expressed in the proboscis. To test this hypothesis, we conducted electrophysiological experiments to characterize the olfactory responses from the proboscis of *An. gambiae* to a diverse panel of odorants that included several human sweat compounds (Cork and Park, 1996; Meijerink et al., 2001). Furthermore, we

characterized AgOR gene expression in the proboscis and axonal projections to antennal lobes (ALs), a primary olfactory processing center in the insect brain (Stocker, 1994). Taken together, the resulting data strongly support the view that the proboscis is an accessory olfactory organ in the malaria vector mosquito.

## Methods

**Insect Preparations.** *An. gambiae* sensu stricto (G3 strain) were reared as described (Fox et al., 2001). Nonbloodfed 3- to 4-day-old female mosquitoes were used for electrophysiological recordings and neuroanatomical studies. Before the electrophysiological experiments described below, adult mosquitoes were cooled at 4°C and restrained in a pipette tip, holding head and appendages in place.

**RT-PCR of Whole Proboscis Tissue.** Cold anesthetized mosquitoes were dissected over dry ice, and 100 proboscises from both sexes were collected by hand dissection. Frozen materials were homogenized with a glass grinder in RLT buffer (Qiagen, Valencia, CA), and RNA was isolated by using RNeasy procedures according to the manufacturers instructions (Qiagen). For each experiment, 40% of each RNA preparation was used for first-strand cDNA synthesis with Superscript II reverse transcriptase and Oligo dT primers (Invitrogen, Carlsbad, CA) to generate templates for individual PCR reactions. The complete set of oligonucleotide primers for investigating tissue-specific *An.*

*gambiae* odorant receptor (AgOR) expression is available online at www.cas.vanderbilt.edu/zwiebel/primers.htm PCR conditions were as follows: 94°C for 2 min; 45 cycles of 94°C for 10 s, 55°C for 30 s, a slow ramp of 0.4°C/s to 72°C, 72°C for 30 s; 72°C for 4min in a PTC-200 (MJ Research). PCR products were subjected to agarose gel electrophoresis subsequent to amplification and monitored following ethidium bromide staining by UV-light illumination. DNA bands of the expected product sizes were cut out of the gels and cloned into Topo-PCRII (Invitrogen) and sequenced.

**Single Sensillum RT-PCR.** After electrophysiological recordings using a diagnostic panel of odorants, the base of the S1 sensillum was impaled with a sharp tungsten electrode. The material was then carefully collected and transferred into a tube containing 10 μl of RNase-free water before RNA extraction and amplification. Total RNA was extracted with the TRIzol reagent (Invitrogen), and 5 μg of linear polyacrylamide (GenElute; Sigma, St. Louis, MO) was added as a carrier. First-strand synthesis was carried out in a 4-μl volume with 40 units of SuperScript III (Invitrogen) and 20 ng of (dT)-T7 primer [GCATTAGCGGCCGCGAAATTAA TACGACTCACTATAGGGAGA(T)21V] in 1× first-strand buffer (Invitrogen) with 90-min incubation at 50°C. The first-strand cDNA was made double-stranded as described (Wetzel et al., 1999) in a 30-μl reaction volume [8 units of DNA polymerase I, 0.4 units of *Escherichia coli* RNase H, and 2 units of E. coli DNA ligase (Invitrogen)] by incubating at 16°C for 2 h; the reaction was polished by adding 4 units of T4 DNA polymerase (NEB)

and incubating for 15 min at 16°C. The second-strand cDNA was purified by proteinase K (Sigma, 100 μg/ml) treatment at 50°C for 35 min, followed by phenol-chloroform extraction, Micro Bio-Spin 30 RNase-free column (Bio-Rad) wash, and isopropanol precipitation. In vitro transcription was carried out in 20-μl volumes by incubating at 37°C for 4 h [80 units of T7 RNA polymerase (Promega), 7.5 mM NTP, 1× AmpliScribe buffer (Epicentre)]. Subsequently, aRNA was purified by phenol-chloroform extraction, Micro Bio-Spin 30 RNase-free column (Bio-Rad) wash, and isopropanol precipitation. Purified aRNA was incubated in a 10-μl reaction volume at 50°C for 90 min with 150 ng of random primers (Invitrogen) and 100 units of SuperScript III (Invitrogen) in 1× first-strand buffer to generate the corresponding cDNA population. Ten microliters of cDNA was diluted to 120 μl before going into a LightCycler real-time PCR machine (Roche) for PCR screen (2 μl of cDNA for each reaction). All primers/probes were designed to target within the last 400 bp of the coding sequence (primer/probe sequences can be accessed at www.cas.vanderbilt.edu/zwiebel/primers.htm). cDNA samples positive for the *AgOR7* signal were considered reflecting the original messages in the olfactory neurites and a valid template for the subsequent PCR screen of 26 *AgORs* that showed consistent presence in the aforementioned proboscis screen. Thermal cycling was carried out in a LightCycler machine for its sensitivity. The conditions were: for the *AgOR7*, 95°C for 15 min, followed by 55 cycles, each consisting of denaturation at 95°C for 0 s and annealing/extension at 64°C for 1 min; for the other *AgORs*, 95°C for 15 min, followed by 55 cycles, each consisting of

denaturation at 94°C for 15 s, annealing at 61°C for 20 s, and extension at 72°C for 20 s. The PCR reactions were monitored real-time through the LightCycler machine, and the positive reaction products were run on a 2% agarose gel and TA-cloned into pCR II-TOPO vectors (Invitrogen), followed by sequencing analysis.

***In Situ* Hybridization and Immunolabeling.** Paraffin-embedded preparations were sectioned at 10 to 12-μm thickness by using a sliding microtome (HM340E; Microm), subsequently dewaxed with Citri-Solv (Fisher BioSciences, Rockville, MD), and rehydrated in an ethanol series to PBS. *In situ* hybridization and probe preparation were carried out as described (Goldman et al., 2005; Vosshall et al., 2000), with digoxigenin (DIG)-labeled RNA probes comprising approximately 800 bp of *AgOR6* coding sequence. Signals were visualized by alkaline phosphatase (AP) coupled to anti-DIG antibodies (Roche, Indianapolis, IN) at 1:1,000 dilution. AP signals were detected by using Fast Red tablets (Roche) according to the manufacturer's instructions. Anti-AgOR7 immunostaining was carried out as described (Pitts et al., 2004). Images were captured with confocal microscopy as described above.

## Results

**Expression and Localization of the *ORs* in the Proboscis and Single Sensillum.** Diverse olfactory responses should correlate with the expression of

several *AgOR* family members. To examine this relationship, we dissected whole proboscises, including shafts from male and female heads, and performed a series of nonquantitative RT-PCR analyses using a set of primers specific to each candidate *AgOR* (Hill et al., 2002). In a total of eight experiments (four for each gender), 16 *AgORs* were reproducibly identified in cDNAs prepared from both male and female proboscises (shaded rows, Table 1), and an additional 9 *AgORs* (shaded rows with asterisk, Table 1), were reproducibly amplified exclusively from female tissue.

In an attempt to further dissect *AgOR* expression within the proboscis, we used a single sensillum RT-PCR approach to identify *AgORs* within an individual S1 sensillum on the labellum of the proboscis. Antisense RNA amplification (Baugh et al., 2001) was used to generate sufficient material for cDNA synthesis from individual S1 sensilla after SSR analyses, and *AgOR7* expression was used as an assessment of ORN cDNA integrity. In this manner, only cDNA samples positive for *AgOR7* were subsequently screened for the presence of other *AgORs* identified from the aforementioned whole-proboscis RT-PCR studies (Table 1). Of 10 *AgOR7*-positive S1 sensillum preparations, one *OR* in particular (*AgOR6*) was consistently amplified in 6 preparations (arrow in Table 1). In addition, *AgOR53* was detected in 3 preparations, *AgOR12* and *18* were each detected twice, and seven other *AgORs* were detected only once (Table 1). Taken together, these data strongly suggest that *AgOR6* is expressed in ORNs associated with S1 sensillum on the proboscis of *An. gambiae*. Double-labeling studies using *AgOR6 in situ* hybridization coupled with AgOR7 immunostaining

| Target *AgOR* Gene | Detection of *AgOR* Expression by RT-PCR in Whole Proboscis Tissue (4 Experiments, unless indicated otherwise) | | Detection of *AgOR* Expression by Single-Sensillum RT-PCR in S1 Sensillum (10 Experiments) |
|---|---|---|---|
| | Male | Female | |
| *AgOR1* | 0 | 0 | ND |
| *AgOR2* | 0 | 0 | ND |
| *AgOR3* | 4 | 4 | 1 |
| *AgOR4* | 4 | 4 | 0 |
| *AgOR5* | ND | ND | ND |
| ***AgOR6*** | **4** | **4** | **6** |
| *AgOR7* | 4 | 4 | 10 |
| *AgOR8* | 0 | 0 | ND |
| *AgOR9* | 0 | 0 | ND |
| *AgOR10* | 0 | 1 | ND |
| *AgOR11* | 1 | 0 | ND |
| *AgOR12* | 4 | 4 | 2 |
| *AgOR13* | 4 | 4 | 0 |
| *AgOR14* | 3 | 3 | 0 |
| *AgOR15* | 4 | 4 | 1 |
| *AgOR16** | 0 | 3 | 0 |
| *AgOR17* | 2 | 4 | 0 |
| *AgOR18* | 4 | 4 | 2 |
| *AgOR19* | 4 | 4 | 1 |
| *AgOR20* | 4 | 4 | 0 |
| *AgOR21* | 4 | 3 | 0 |
| *AgOR22* | 0 | 0 | ND |
| *AgOR23* | 0 | 1 | ND |
| *AgOR24** | 0 | 2 | ND |
| *AgOR25* | 1 | 1 | ND |
| *AgOR26* | 0 | 0 | ND |
| *AgOR27* | 0 | 0 | ND |
| *AgOR28** | 0 | 3 | 1 |
| *AgOR29* | ND | ND | ND |
| *AgOR30* | 0 | 1 | ND |
| *AgOR31* | 0 | 0 | ND |
| *AgOR32* | 0 | 0 | 0 |
| *AgOR33* | 0 | 0 | ND |
| *AgOR34* | 0 | 0 | ND |
| *AgOR35* | 0 | 0 | ND |
| *AgOR36* | 0 | 0 | ND |
| *AgOR37* | 0 | 0 | ND |
| *AgOR38* | 0 | 0 | ND |
| *AgOR39* | 1 | 3 | 0 |

| | | | |
|---|---|---|---|
| *AgOR40* | 0 | 0 | ND |
| *AgOR41* | 0 | 0 | ND |
| *AgOR42* | 0 | 0 | ND |
| *AgOR43* | 0 | 0 | ND |
| *AgOR44* | 0 | 1 | ND |
| *AgOR45* | 0 | 0 | ND |
| *AgOR46* | 0 | 0 | ND |
| *AgOR47* | 0 | 0 | ND |
| *AgOR48* | 0 | 0 | ND |
| *AgOR49* | 0 | 0 | ND |
| *AgOR50* | 0 | 0 | ND |
| *AgOR51* | 0 | 0 | ND |
| *AgOR52* | 0 | 0 | ND |
| *AgOR53* | 4 | 3 | 3 |
| *AgOR54* | 0 | 0 | ND |
| *AgOR55* | 3 | 4 | 1 |
| *AgOR56/57* | 2 | 3 | 0 |
| *AgOR58* | 0 | 0 | ND |
| *AgOR59* | 0 | 1 | ND |
| *AgOR60* | 0 (3 experiments) | 0 (3 experiments) | ND |
| *AgOR61* | 0 | 0 | ND |
| *AgOR62* | 1 | 4 | 1 |
| *AgOR63* | 0 | 0 | ND |
| *AgOR64* | 0 | 0 | ND |
| *AgOR65* | 0 | 1 | ND |
| *AgOR66/67* | 0 (3 experiments) | 2 | ND |
| *AgOR68** | 0 (3 experiments) | 2 | ND |
| *AgOR69* | 0 | 0 | ND |
| *AgOR70** | 0 | 3 | 1 |
| *AgOR71** | 0 | 2 | ND |
| *AgOR72* | 0 (3 experiments) | 0 (3 experiments) | ND |
| *AgOR73** | 0 (3 experiments) | 2 | ND |
| *AgOR74* | 0 (3 experiments) | 0 | ND |
| *AgOR75/76* | 0 (3 experiments) | 1 | ND |
| *AgOR77* | 0 | 0 | ND |
| *AgOR78/79* | 0 (3 experiments) | 0 | ND |

**Table 1. Expression Profiles of *AgORs* in the Labellum of *An. gambiae*.**
16 *AgORs* were reproducibly detected in the whole-proboscis tissue from male and female mosquitoes (shaded rows). Expression of nine *AgORs* (shaded rows with asterisks) was reproducibly detected exclusively in female mosquitoes. *AgOR56* and *AgOR57* are 95.6% identical in their predicted cDNA sequence and could not be distinguished in the amplified region. Expression profiles of *AgORs* from the S1 sensillum on the labellum of *An. gambiae* were detected by single-sensillum RT-PCR. In all 10 preparations of the single S1 sensillum, *AgOR7* mRNA expression was detected. *AgOR6* was detected in 6 of 10 S1 preparations (indicated by boldface type), whereas *AgOR53* was detected in 3 of 10 S1 preparations. *AgOR12* and *AgOR18* were detected twice and another 7 *AgORs* were detected only once in 10 S1 preparations. ND, not determined.

were also carried out to more precisely localize *AgOR6* transcripts in the labellum of *An. gambiae*. Here, *AgOR6* mRNA was detected in multiple cells throughout the medial portion of the labellum along with AgOR7 protein (Figure 3). These studies also confirm that *AgOR6* mRNA is consistently coexpressed with AgOR7 protein in a subset of labellum ORNs (Figure 3C, arrowheads), whereas the remainder of ORNs presumably expresses other conventional *AgORs*. *AgOR6* transcripts were also localized to ORNs in the adult antennae (data not shown).

## Discussion

**Expression and Function of *ORs* in the Labellum.** Previous studies in *Heliothis virescens* using whole-appendage expression surveys have revealed the presence of candidate *OR* transcripts in the proboscis (Krieger et al., 2004; Krieger et al., 2003). This result is reminiscent of physiological studies in another lepidopteran, where the labial pit organ (LPO) and its projections of *Manduca sexta* have defined accessory olfactory pathways that are exclusively responsive to $CO_2$ (Kent et al., 1986), where they play a key role in finding host plants at a distance (Guerenstein et al., 2004; Thom et al., 2004). Interestingly, the LPO is not responsive to other volatile odorants, suggesting that this structure may be functionally more closely related to the maxillary palps in mosquitoes, which are the site of $CO_2$ sensitivity in these insects (Grant et al., 1995). In this report, we provide a demonstration that fully functional *AgORs* are expressed in

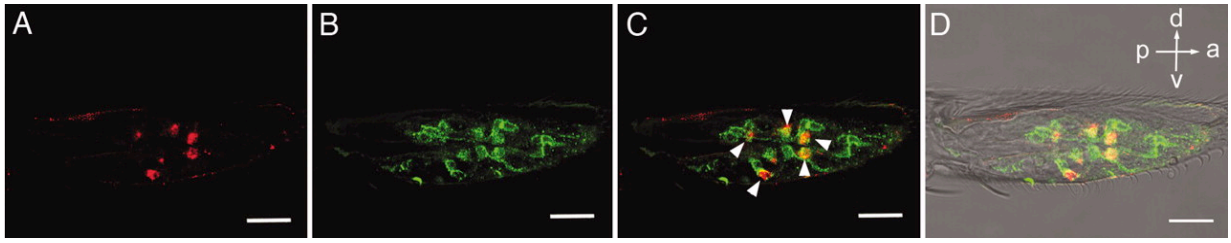


**Figure 3. Colabeling of *AgOR6* and AgOR7 by *in situ* Hybridization and Immunostaining on a Sagittal Section of the Labellum.** (A) *In situ* hybridization with antisense *AgOR6* probes (red), where *AgOR6*-positive cells are evident. (B) AgOR7-positive cells detected with antibody immunostaining (green). (C) Merged image of A and B; colocalization of *AgOR6* and AgOR7 is observed (arrowheads). (D) Merged image from C together with bright-field illumination. *AgOR6*-positive cells are located in a medial region of the labellum. v, ventral; d, dorsal; a, anterior; p, posterior. (Scale bars, 20 μm.)

chemosensory sensilla located on the labial portion of the proboscis, which is a predominantly gustatory appendage in *An. gambiae*. Importantly, *OR* expression in the proboscis has, until now, not been described in any other dipteran insects, consistent with electrophysiological studies presented here, demonstrating a lack of olfactory sensitivity in the *D. melanogaster* labellum. These findings imply that there may be important functional and organizational differences between the chemosensory processes of *An. gambiae* and *D. melanogaster* that may reflect significantly different life-cycle characteristics, including feeding habits, oviposition demands, and other elements. In this light, it is especially tempting to focus on the anautogenous requirement for vertebrate blood that is characteristic of *An. gambiae* and other hematophagous mosquitoes as a critical distinction between the life cycles of these dipterans.

In *An. gambiae*, the presence of 24 conventional *AgORs* has been detected from whole-proboscis RT-PCR screens (Table 1). Among the *AgORs* identified in the S1 sensillum, *AgOR6* is observed in the majority (6 of 10) of these assays, indicating that it is highly expressed in one or more ORN associated with individual S1 sensilla and, moreover, may reasonably be expected to be tuned to one or more of the odorants that evoke the strongest responses in these assays. Analyses of SSR spike train amplitudes suggest that two ORNs are likely to be located in the S1 sensillum of the proboscis labellum. Furthermore, our *in situ* hybridization and RT-PCR data are consistent with the view that *AgOR6* is expressed in a subset of AgOR7-positive labellum ORNs,

and AgOR6 is one of several AgORs that facilitate olfactory responses in this appendage.

These data argue against our earlier hypothesis that AgOR7 may also function in a gustatory role on the labellum of *An. gambiae* (Pitts et al., 2004) and, instead, provide compelling evidence for the presence of cryptic ORNs on this mosquito chemosensory appendage. Although these data do not formally rule out a role for AgOR7 in gustation, we favor the hypothesis that AgOR7 is a true homolog to the nonconventional *Drosophila* DOr83b protein and that, accordingly, its expression defines the majority of ORNs in this system. The strong olfactory responses recorded in this study from the labellum of *An. gambiae* may convey information that is critical to the later-stage events in bloodfeeding, host preference, and other behaviors of this mosquito and, therefore, may have profound effects on its vectorial capacity.

# CHAPTER III

## ODOR CODING BY MAXILLARY PALP NEURONS OF THE MALARIA VECTOR MOSQUITO *ANOPHELES GAMBIAE*

### Preface

The following publication by Lu et al. appeared in *Current Biology* (17: 1533-44). My contribution to this work included mapping of *AgORs* and *AgGRs* to the maxillary palp of *Anopheles gambiae* and some cloning of palpal *AgORs* and *AgGRs* from mosquito tissues. Mosquito single-sensillum electrophysiology was performed by Yu Tong Qiu. AgOR expression in *Xenopus laevis* oocytes and two-electrode voltage clamp recording were performed by Guirong Wang. Functional studies of AgGRs were done by Jae Young Kwon. RT-PCR survey was done by Michael Rutzler and transmission electron microscopic analysis by Hyung-Wook Kwon. Identification of putative insect $CO_2$ receptor sequences and phylogenetic analysis were performed by R. Jason Pitts with some help from me.

### Introduction

Because of its role in transmitting malaria, the Afrotropical vector mosquito *An. gambiae* represents one of the most significant threats to global health. As is the case for other mosquitoes, *An. gambiae* uses a large and divergent population of ORNs to respond to a myriad set of chemical cues with which it carries out odor-mediated behaviors such as host seeking, nectar feeding and oviposition (Qiu et al., 2006; Takken and Knols, 1999; Zwiebel and Takken, 2004). *An. gambiae* and

other mosquitoes have three olfactory appendages: the antenna, the proboscis and the maxillary palp, and all are populated by ORN-containing porous sensilla (Kwon et al., 2006; Pitts et al., 2004). In contrast to the antenna, which contains the largest quantity and variety of olfactory sensilla, the maxillary palp is much less complex, harboring a single morphological type of chemosensory sensillum, the capitate peg (McIver and Siemicki, 1975). Ultrastructural studies have revealed that each capitate peg sensillum in *An. gambiae* is invariably innervated by three ORNs (McIver and Siemicki, 1975).

$CO_2$ is emitted by all potential vertebrate hosts and serves as a universal attractant to many mosquito species (Gillies, 1980; Takken and Knols, 1999). It has been reported that $CO_2$ stimulation synergizes with host body odor and induces take-off and sustained flight behaviors in host-seeking anopheline mosquitoes (Dekker et al., 2001; Gillies, 1980; Mboera and Takken, 1997). In addition to $CO_2$, 1-octen-3-ol is another small molecule which emanates from large herbivores as well as from humans (Cork and Park, 1996; Hall et al., 1984) and has been identified as a behavioral attractant to tsetse flies (Hall et al., 1984).

The maxillary palp is the primary $CO_2$-sensitive organ of mosquitoes, and early studies observed a specific loss of response to $CO_2$ in palpectomized female *Culex* mosquitoes (Omer and Gillies, 1971). Moreover, in the yellow-fever vector mosquito, *Aedes aegypti*, two palpal ORNs in the capitate peg sensillum were shown to exhibit high sensitivity to both $CO_2$ and 1-octen-3-ol in electrophysiological studies (Grant and O'Connell, 1996; Grant et al., 1995). In

contrast, relatively little is known about olfactory responses of the maxillary palp in *An. gambiae*, which uses a different spectrum of odor cues than *Ae. aegypti* in mediating host selection and location (Takken and Knols, 1999).

At the molecular level, odor coding in insects is thought to rely on the activation of a large family of highly divergent seven-transmembrane domain odorant receptor proteins (ORs) (Hallem et al., 2006; Rutzler and Zwiebel, 2005). Insect ORNs typically express one highly conserved and broadly expressed non-conventional DmOR83b-like OR together with one or two conventional odorant-binding ORs (Couto et al., 2005; Hallem et al., 2006; Larsson et al., 2004; Nakagawa et al., 2005). In *An. gambiae*, 79 putative *AgOR* genes have been identified (Hill et al., 2002) and, thus far, two of them have been demonstrated to encode functional ORs (Hallem et al., 2004a). The anopheline *DmOR83b* ortholog, *AgOR7*, is widely expressed in olfactory organs (Pitts et al., 2004).

Here we confirm that *An. gambiae* palpal ORNs respond to $CO_2$ and 1-octen-3-ol with high sensitivity. We characterize palpal chemosensory receptors, and map them to ORN triads that are invariably compartmentalized within each capitate peg sensillum. The three receptors that were previously classified as gustatory receptors (*AgGRs*) (Hill et al., 2002) are coexpressed within the $CO_2$-sensitive ORN, while the two *AgORs* are coexpressed with *AgOR7* in their respective ORNs. We demonstrate that *AgOR8* and *AgOR28* genes encode functional receptors that confer odorant-induced responses when heterologously expressed in *Xenopus* oocytes. AgOR8 mediates specific and sensitive responses to 1-octen-3-ol while AgOR28 is tuned to a broad panel of odorants.

Lastly, we show that coexpression of the AgGRs confers $CO_2$ responses in the "empty neuron" *in vivo* expression system of *Drosophila*. Taken together, these data elucidate the complete molecular and cellular basis of odor coding in the maxillary palp, which *An. gambiae* uses for detecting olfactory cues that are crucial in establishing its vectorial capacity.

## Experimental Procedures

### Insects

*An. gambiae sensu stricto*, originated from Suakoko, Liberia, was reared as described (Fox et al., 2001; Qiu et al., 2004) and 5- to 7-day-old females that had not been blood fed were used for experiments. For extracellular recordings, females with legs removed were mounted on a transparent Perspex block (1.1x1.1x1.5 cm) via a piece of transparent Scotch double-sided sticky tape. To immobilize the mosquito, the wing, the antenna, the proboscis and the junctions between palpal segments were pressed gently against the tape. The maxillary palp surface was viewed at 750x magnification through an Olympus IX50 inverted microscope. All recordings were made from capitate pegs on segments two to four of the maxillary palp.

### Transmission Electron Microscopy

Head with appendages were dissected from five-day old female mosquitoes and fixed with 4% glutaraldehyde for 4-5 days at 4°C. After washing in 2.5%

potassium dichromate for 2 hr, tissues were post-fixed with 1% osmium tetroxide for 4-5 days at 4°C. Tissues were later dehydrated in a graded alcohol series, embedded in Spurr's resin (Spurr, 1969) within BEEM capsules (Electron Microscopy Sciences) and sectioned at 50 nm using a Leica Ultracut UCT ultramicrotome. Sections were stained with 1% uranyl acetate followed by lead citrate before viewed in a Philips CM10 transmission electron microscope.

**Mosquito Single-Sensillum Electrophysiology**

Extracellular recordings of capitate pegs from female *An. gambiae* were made as described (Qiu et al., 2006) using an USB-IDAC analog-digital conversion interface with the software Autospike (Syntech). All odorants were of the highest purity grade commercially available except 90% aqueous solution of L(+)-lactic acid (pharmaceutical grade; >95% L-isomer), 7-octenoic acid (>99%, a gift from M. Birkett) and Henkel 100 (a gift from T. Gerke). Odorants were dissolved and diluted in paraffin oil, except that ammonia was diluted in water and 7-octenoic acid was dissolved in tertyl-butyl methyl ether (TBME). In the latter case TBME was allowed to evaporate for 15 min from samples before use. Hundred-fold dilutions of 92 compounds in paraffin oil were prepared gravimetrically on a Cahn C-33 microgram balance (Cahn Instruments) and used to screen the response spectra of palpal ORNs.

Odorants were tested in random order; for the same odorant, lower concentrations were tested first to prevent possible adaptation to higher concentrations. Ten μl of diluted odorant was added onto a 1x1.5 cm piece of

filter paper, which was placed into a Pasteur pipette. A 200-ms odorant stimulus was added to an air stream, which was set at 11.1 ml/s, using a stimulus controller (Syntech) so that the odorant-carrying air stream was bolstered up to a constant flow rate of 40 ml/s with charcoal-filtered and humidified air. For controls, paraffin oil, water or TBME were used instead of diluted odorant.

For stimulations with $CO_2$, the maxillary palp surface was initially exposed to a stream of synthetic air with monitored quality (21% oxygen and 79% nitrogen, Linde Gas) at a flow rate of 5 ml/s, as regulated by a flow meter and a C5-01/b stimulus controller (Syntech). Different concentrations (300, 600, 1200, 2400 and 4800 ppm) of $CO_2$ were produced by mixing 5% $CO_2$ (Linde Gas) with synthetic air in a 25-l Tedlar bag and later injected into the synthetic air stream at 5 ml/s via a 224-PCXR4 pump (SKC Gulf Coast Inc.). A second flow meter and stimulus controller were used to keep the total flow constant.

**Reverse Transcriptase PCR**

Five-day old mosquitoes were cold anesthetized and dissected by hand on a chill table. For each sex, 140 maxillary palps were used for RNA preparation. Total RNA was prepared using RNeasy (Qiagen) according to the manufacturer's instructions. One third of each RNA preparation was used for oligo dT-primed cDNA synthesis using Superscript II Reverse Transcriptase (Invitrogen) to generate templates for subsequent PCR reactions. Negative control samples with no reverse transcriptase were included in each cDNA synthesis and subsequent PCR analysis.

PCR was performed using a DNA Engine Dyad (MJ Research) under the following conditions: 94ºC for 2 min; 40 cycles of 94ºC for 10 s, 55ºC for 30 s, a slow ramp at 0.4ºC/s to 72ºC, 72ºC for 30 s; 72ºC for 4 min. Primer pairs that span introns were used in order to distinguish cDNA amplicons from those amplified from remaining genomic DNA. The complete set of *AgOR* primers can be accessed at www.cas.vanderbilt.edu/zwiebel/primers2.htm. PCR amplification products were run on a 1.5% agarose gel and verified by DNA sequencing.

### *In situ* Hybridization

Procedures for fluorescent double *in situ* hybridization were modified from previous work (Goldman et al., 2005; Kwon et al., 2006; Vosshall et al., 2000) as follows: heads with maxillary palps attached were dissected from five-day old female mosquitoes and collected in 4% paraformaldehyde in PBS with 0.05% Tween-20 on ice. 20 min after dissection was over, fixative was replaced with 4% paraformaldehyde in PBS without Tween-20 and fixation continued on ice for additional 5 hr. Tissues were dehydrated in an ethanol series, cleared with xylenes and embedded in Paraplast plus (Kendall) before being sectioned at 8-10 μm on a Microme HM-340E microtome (Carl Zeiss). After 21-hr drying at 40ºC, sections were de-waxed using Citri-Solv (Fisher) and rehydrated in an ethanol series to PBS. Post-fixation, acetylation, pre-hybridization and the washes in between were performed as previously described (Vosshall et al., 2000). Templates for the *in situ* probes were 700-850 bp fragments PCR amplified from female maxillary palp cDNA and these fragments were cloned into

pCRII-TOPO (Invitrogen). Digoxigenin-labeled and fluorescein-labeled RNA probes were generated and spot tested following the manufacturer's directions (Roche). Hybridization was performed in a Boekel InSlide-Out hybridization oven for 21 hr, followed by post-hybridization washes. Fluorescein-labeled probes were visualized with anti-fluorescein-AP (Roche) followed by incubation with Fast Red substrates (Roche). Digoxigenin-labeled probes were visualized with anti-DIG-POD (Roche) followed by incubation with fluorescein-labeled tyramides (Perkin Elmer). No hybridization was observed with sense probes (data not shown). Sections were mounted in Vectashield (Vector Laboratories) and visualized using an LSM510 confocal microscope (Carl Zeiss).

**Receptor Expression in *Xenopus laevis* Oocytes and Two-Electrode Voltage Clamp Electrophysiological Recording**

Full-length coding sequences of *AgOR7*, *AgOR8* and *AgOR28* were PCR amplified from female *An. gambiae* maxillary palp cDNA. *AgOR7* coding sequence was cloned into pT7TS (a gift from G. Lepperdinger with permission of D. Melton); coding sequences of *AgOR8* and *AgOR28* were first cloned into pENTR/D-TOPO (Invitrogen) and then sub-cloned into pSP64DV by means of the Gateway LR reaction. The pSP64DV vector was a Gateway-compatible destination vector converted from pSP64T-Oligo (a gift from A. George) using the Gateway Vector Conversion System (Invitrogen). cRNAs were synthesized from linearized vectors using mMESSAGE mMACHINE or mMESSAGE mMACHINE T7 Ultra (Ambion). Human $G\alpha_{15}$ RNA was transcribed from pSGEM-$G\alpha_{15}$ (a gift from H. Hatt) (Wetzel et al., 2001).

Mature healthy oocytes (stage V-VII) were treated with 2 mg/ml collagenase S-1 in washing buffer (96 mM NaCl, 2 mM KCl, 5 mM $MgCl_2$ and 5 mM HEPES, pH 7.6) for 1-2 h at room temperature. Oocytes were later micro-injected with 27.6 ng *AgOR8* or *AgOR28* cRNA, 27.6 ng *AgOR7* cRNA and 0.276 ng $G\alpha_{15}$ cRNA. After injection, oocytes were incubated for 3-5 days at 18°C in 1XRinger's solution (96 mM NaCl, 2 mM KCl, 5 mM $MgCl_2$, 0.8 mM $CaCl_2$ and 5 mM HEPES, pH 7.6), supplemented with 5% dialyzed horse serum, 50 μg/ml tetracycline, 100 μg/ml streptomycin and 550 μg/ml sodium pyruvate.

Whole cell currents were recorded from the injected *Xenopus* oocytes with a two-electrode voltage clamp. Odorants were dissolved in DMSO at a 1:10 ratio to make stock solutions. Prior to recording, stock solutions were diluted in 1XRinger's solution to the indicated concentrations before being applied to *Xenopus* oocytes in an RC-3Z oocyte recording chamber (Warner Instruments). Odorant-induced currents were recorded using an OC-725C oocyte clamp (Warner Instruments) at a holding potential of –80 mV. Data acquisition and analysis were carried out using Digidata 1322A and pCLAMP software (Axon Instruments).

**Identification of Putative Insect $CO_2$ Receptor Sequences**

The public DNA database of all available insect genome sequences at National Center for Biotechnology Information and the *Culex pipiens quinquefasciatus* (Johannesburg strain) whole genome sequencing database at the Broad Institute (http://www.broad.mit.edu/annotation/genome/culex_pipiens) were queried using

sequences of *AgGR22*, *AgGR23* and *AgGR24* or *AaGR1*, *AaGR2* and *AaGR3* (Kent et al., 2008) by tBLASTn (Altschul et al., 1990). Putative insect $CO_2$ receptor sequences were manually annotated using Sequencher v4.5 (Gene Codes, Inc.). Specifically, the first 247 amino acid sequences of the *Bombyx mori BmGR23* were extracted from contig 17455 while the remaining 108 amino acid sequences were from contig 8952. In addition, the *Bombyx BmGR24* is likely to be a pseudogene and therefore renamed *BmGR24pse* because there are several in-frame stop codons in its N-terminal portion, although it remains possible that there is an intact version of the exon that has not yet been identified in the genome project.

**Phylogenetic Analysis**

Deduced amino acid sequences of putative insect $CO_2$ receptors were aligned using ClustalX (Thompson et al., 1997) and phylogenetic analysis was performed using distance methods in $_{PAUP}$* v4.0b10 (Swofford, 1998). The details of corrected distance phylogenetic methods can be found in Figure 10 legend (Hill et al., 2002). Bootstrap analysis was used to assess statistical support for relationships via uncorrected neighbor-joining analysis of 1000 pseudo-replicated data sets.

***Drosophila* Transgenes and Electrophysiology**

All constructs were injected into $w^{1118}$ flies. cDNAs of *AgGR22* and *AgGR24* were amplified using the following primers: 5'-

TGCGGCCGCATGATTCACACACAGATGGAA-3' and 5'-GGGTACCTTAGGTGTTCACTTTGTCTG-3' for *AgGR22*, 5'-TGCGGCCGCATGAGTCTCTACTTCAACGCG-3' and 5'-GGGTACCCTAAGAATGAGACGAATTAC-3' for *AgGR24*.

The predicted protein sequences encoded by these cDNAs correspond to the amino acid sequences shown in Figure 10. For the *AgGR23* genomic clone, 5'-TGCGGCCGCACGCTGGAACGGTTGTCGCTA-3' and 5'-GGGTACCTTACTGTTTCTGTAGCAGCT-3' were used.

Electrophysiology was performed as previously described (Kwon et al., 2007).

## Results

### Responses to $CO_2$ and 1-Octen-3-ol in the Maxillary Palp

The maxillary palp of female *An. gambiae* is comprised of five segments, all of which are densely covered with flattened scales on their dorsal side (Figure 4A and 4B). Capitate pegs, the single type of chemosensory sensilla in this appendage, are distributed on the ventral side of palpal segments two, three and four (McIver and Siemicki, 1975) (Figure 4A and 4B). Transmission electron microscopic (TEM) studies confirmed that one neuron within *An. gambiae* capitate pegs had a uniquely lamellate dendritic structure (Figure 4C) that is characteristic of insect $CO_2$-responsive ORNs (McIver and Siemicki, 1975; McIver, 1972).

In order to characterize the olfactory response profile of this appendage, we performed extensive single-sensillum electrophysiological recordings (SSRs) of 45 capitate peg sensilla at various positions from all three segments of the maxillary palp. Based on response amplitudes and additional sorting criteria, action potentials in most recordings could be resolved into three distinct populations (Figure 5A-5C), indicating the presence of three ORNs (henceforth referred to as the capitate peg (cp) A, B or C neuron) within each capitate peg sensillum, which is in accord with previous ultrastructural studies (McIver and Siemicki, 1975). The cpA neuron is characterized by the largest action potential amplitude, while the cpB and cpC neurons manifest intermediate and the smallest amplitudes, respectively (Figure 5A-5C).

To test the response profiles of the ORN population in the maxillary palp, we adopted a broad panel of 97 compounds that included specific odorants implicated in mosquito behavior. All analyzed capitate pegs fell into a uniform functional class, each housing three distinct types of ORNs. Of the 97 compounds tested, we found that 36 were able to elicit responses from at least one of the three neurons (Figure 6A).

The cpA neuron exhibited a spontaneous firing rate of 6.7±3.1 spikes/s and responded strongly to stimulation by $CO_2$ (Figure 5D). We demonstrated the cpA neuron to be a highly sensitive $CO_2$ detector, with a dose-response curve exhibiting a steep slope of 90 spikes every $^{10}$log concentration and a detection threshold at around 600 ppm (Figure 6B). In addition to its $CO_2$ detection, this neuron manifested significant excitatory responses to thiazole, 4-methylthiazole

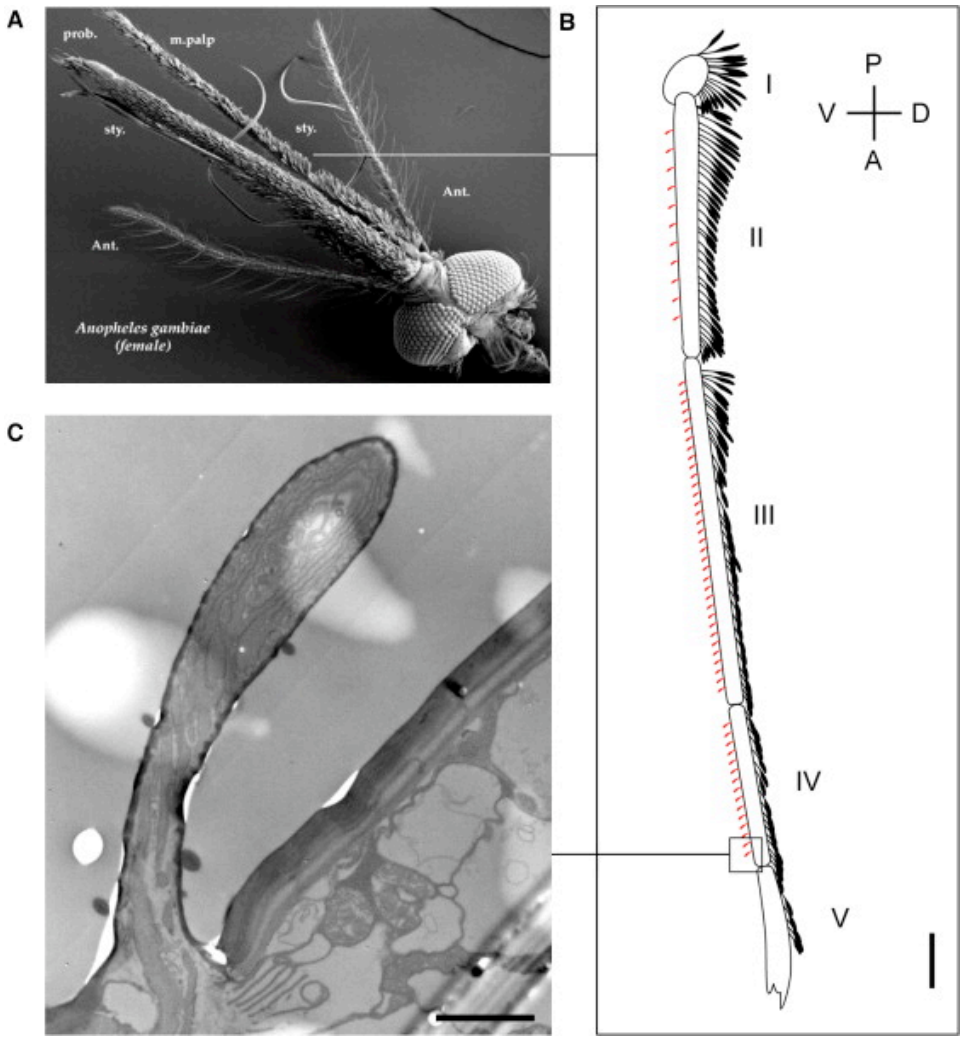


**Figure 4. The Maxillary Palp in Female *An. gambiae* Mosquitoes.**
(A) Scanning electron micrograph showing the olfactory appendages of a female *An. gambiae* mosquito: the antenna, the maxillary palp, and the proboscis.
(B) A schematic drawing based on a bright field photograph showing that the female maxillary palp is comprised of five segments that are densely covered with scales on their dorsal side. Capitate pegs (red) are distributed on the ventral side of palpal segments two, three, and four. The scale bar represents 100 μm.
(C) Transmission electron micrograph showing the specialized lamellate dendritic structure of one neuron within the capitate-peg sensillum. The scale bar represents 2 μm.

and cyclohexanone (Figure 6A and 6B). We also note that the cpA neuron, as was observed in the case of an individual ORN within the *Drosophila* antennal basiconic type 1 (ab1) sensillum (de Bruyne et al., 2001), was unusually activated by the paraffin oil solvent, accounting for much of its response to other odorants (Figure 5E and 6A, arrow). Finally, the cpA neuron was significantly inhibited by 7 compounds, with indole and 3-methylindole inducing the strongest inhibition (Figure 6A).

We showed the cpB neuron, displaying a spontaneous firing rate of 3.2±2.5 spikes/s, to be a highly specific and sensitive detector of 1-octen-3-ol, being able to detect 0.1 ppm or 0.85 pg of this odorant in our delivery system (Figure 5F, 5G, 6A and 6C). The cpB neuron continued to increase its firing rate to 1-octen-3-ol at concentrations between $8.5X10^{-4}$ ng and 0.85 ng until the response reached its maximum at 0.85 ng and started to decline at higher concentrations (Figure 6C). Moreover, we found that, at higher concentrations, stimulation with 1-octen-3-ol suppressed the amplitude of the cpB neuron to a level that was not distinguishable from background noise (Figure 5H and 5I), likely due to over-stimulation; thus, the response could no longer be reliably quantified at concentrations higher than 2.55 ng. This neuron also responded strongly to 1-hepten-3-ol (Figure 5J and 5K) and, although the dose-response curve of 1-hepten-3-ol has a shape similar to that of 1-octen-3-ol, its response threshold is about 100 times higher (Figure 6C). Much weaker excitatory responses were evoked by 3-octanone and 1-hexen-3-ol and 5 other tested odorants (Figure 6A and 6C). Furthermore, the cpB neuron was significantly inhibited by 19 tested

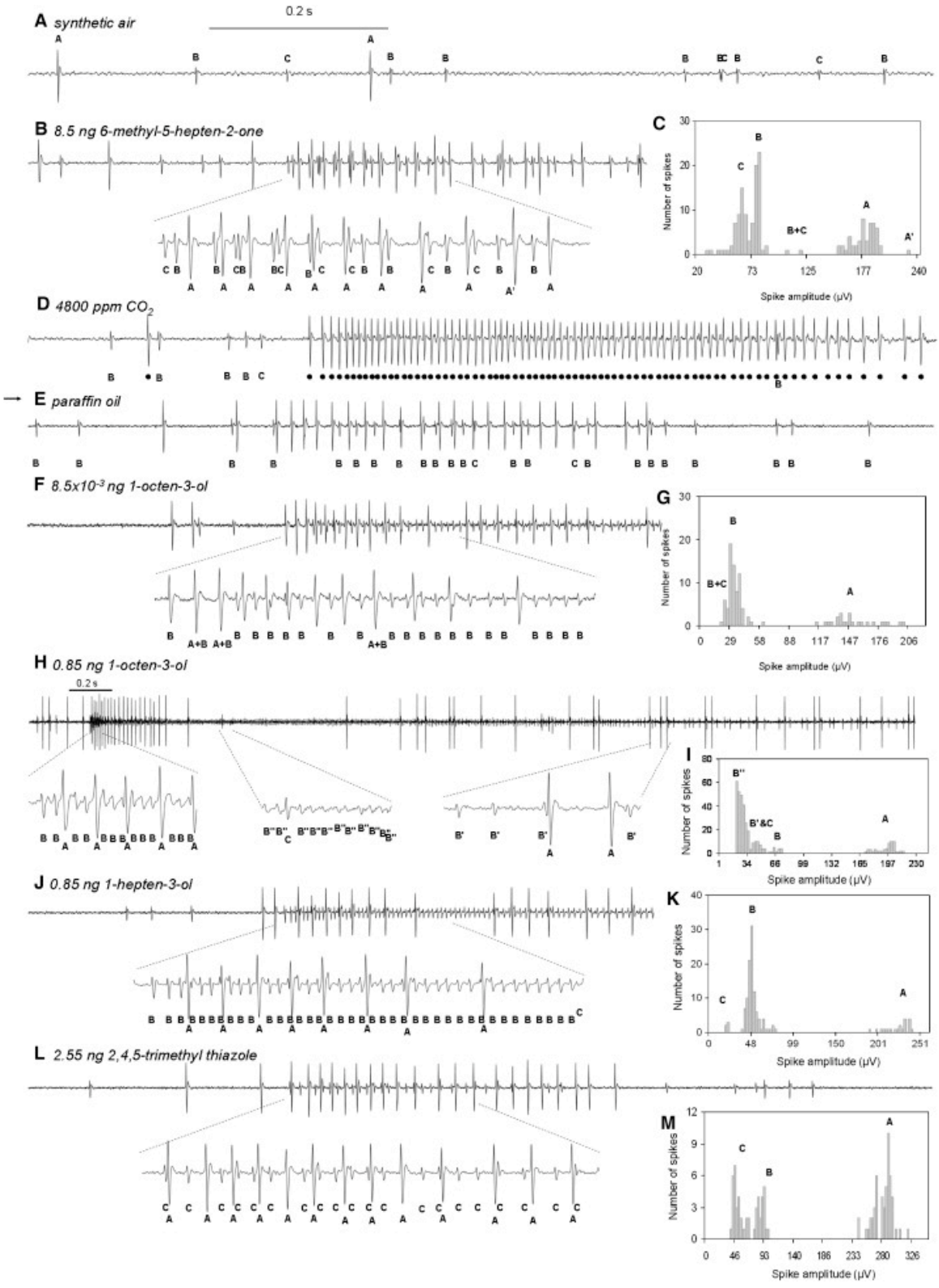
A synthetic air
0.2 s
B 8.5 ng 6-methyl-5-hepten-2-one
C
Number of spikes
Spike amplitude (µV)
D 4800 ppm CO2
E paraffin oil
F 8.5x10-3 ng 1-octen-3-ol
G
H 0.85 ng 1-octen-3-ol
I
J 0.85 ng 1-hepten-3-ol
K
L 2.55 ng 2,4,5-trimethyl thiazole
M

**Figure 5. Single-Sensillum Electrophysiological Recordings from Capitate-Peg Sensilla.**

Representative extracellular recordings from capitate-peg sensilla in response to air, $CO_2$, paraffin oil (arrow), and four other odorants. Individual action potentials (spikes) are labeled A, B, or C according to spike amplitude, spike shape, and interspike intervals. The time scales of all presented traces (except for H) are the same, and the 0.2 s odor stimulation for all is indicated above the recording in (A) (horizontal bar). For (H), a different time scale was adopted, and odor stimulation is indicated with a separate bar.

(A) Recording from a capitate-peg sensillum that is exposed to synthetic air.

(B) Both the cpB and cpC neurons are moderately activated by 8.5 ng of 6-methyl-5-hepten-2-one. One large spike marked as A' represents the superposition of a cpA spike with a cpB or cpC spike.

(C) Spike amplitude distribution of 156 spikes from 2.5 s of the recording in (B).

(D) Large action potentials from the cpA neuron (dots) increase in frequency in response to 4800 ppm of $CO_2$.

(E) The cpA neuron is also activated by paraffin oil (arrow).

(F, H, and J) The cpB neuron responds sensitively to the stimuli of 1-octen-3-ol and 1-hepten-3-ol. As shown in (H), the stimulus of 0.85 ng 1-octen-3-ol suppresses the spike amplitudes of the cpB neuron to a level (marked as B'') smaller than the cpC spikes before the cpB spike amplitudes recover (marked as B').

(G) Spike amplitude distribution of 96 spikes from 1 s of the recording in (F).

(I) Spike amplitude distribution of 270 spikes from 5 s of the recording in (H).

(K) Spike amplitude distribution of 129 spikes from 1.8 s of the recording in (J).

(L) Response of the cpC neuron to its strongest ligand, 2,4,5-trimethylthiazole.

(M) Spike amplitude distribution of 102 spikes from 5 s of the recording in (L).

compounds, among which 4-methylcyclohexanol, 2,4,5-trimethylthiazole and 2-ethylphenol elicited the strongest inhibitory effects (Figure 6A). Notably, the insect repellent DEET (*N*,*N*-diethyl-3-methylbenzamide) was one of the compounds that inhibited firing of the cpB neuron (Figure 6A).

The cpC neuron displayed a low spontaneous frequency of 0.8±0.8 spikes/s and was not significantly inhibited by any of the tested odorants (Figure 6A). In contrast to the other two neurons, the cpC neuron appeared to be a more broadly tuned ORN that was excited by 24 compounds of our odor panel; the strongest excitatory responses were found upon stimulation with 2,4,5-trimethylthiazole, acetophenone and 2-acetylthiophene (Figure 5L, 5M, 6A and 6D). A small number of compounds (*e.g.*, 6-methyl-5-hepten-2-one) evoked excitatory responses in both cpB and cpC neurons (Figure 5B, 5C and 6A).

**Expression of *AgOR8* and *AgOR28* Genes in the Maxillary Palp**

To search for conventional *AgOR* genes expressed in the maxillary palp, we initially carried out reverse transcriptase-PCR (RT-PCR) amplifications with primer pairs targeting all 79 *AgOR* genes, using RNA extracted from either hand-dissected male or female whole maxillary palps. In analyses of three independently prepared RNAs from each sex, we consistently found amplification products of only *AgOR7* and two conventional *AgORs*, *AgOR8* and *AgOR28* (Figure 7). Of these, *AgOR8* appears to be palp-specific as we have never detected it using similar assays in other mosquito olfactory appendages [M.R. and L.J.Z., unpublished data]; *AgOR28*, however, is also expressed in the

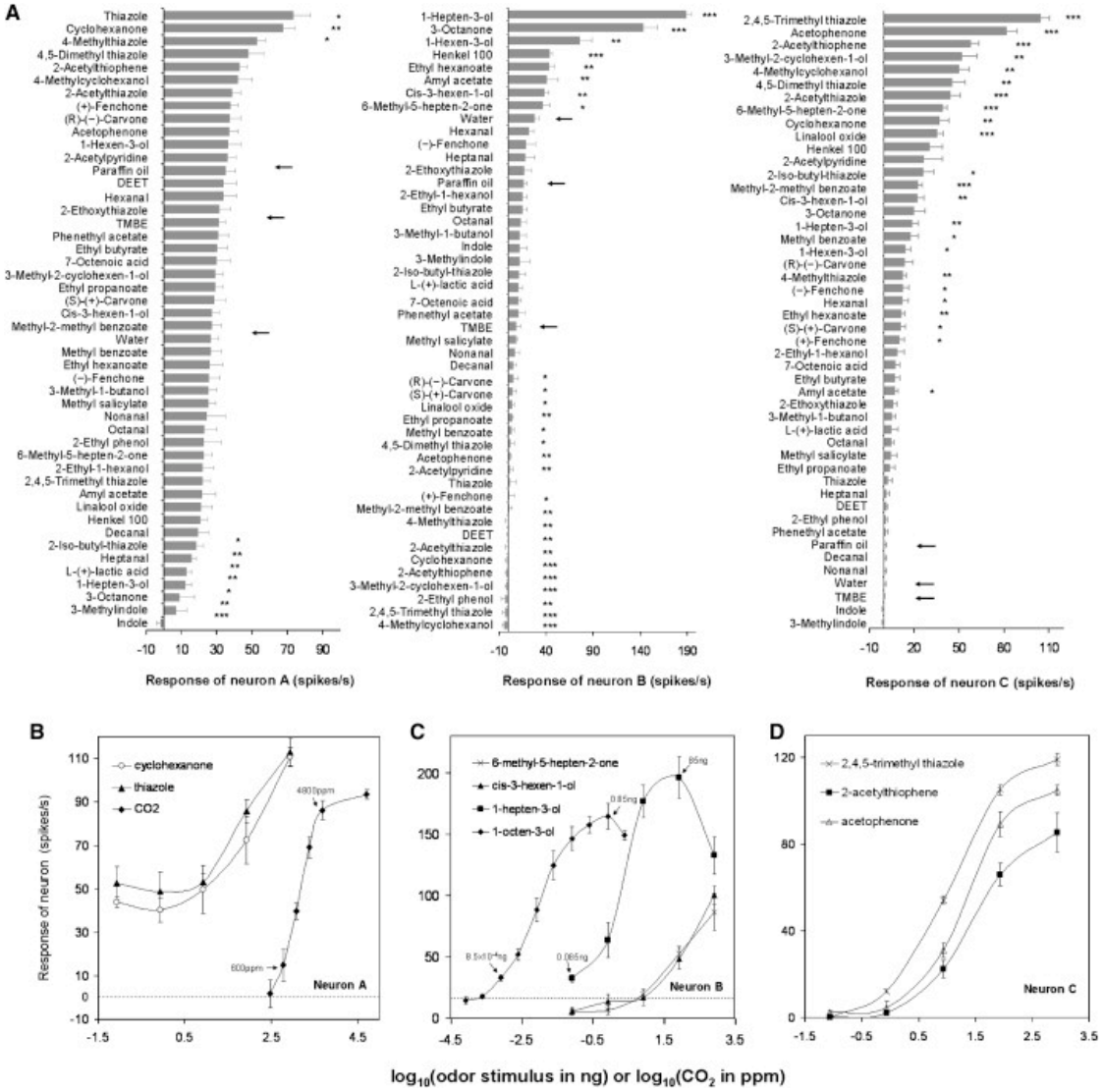
A
Response of neuron A (spikes/s)
Response of neuron B (spikes/s)
Response of neuron C (spikes/s)
B
C
D
Response of neuron (spikes/s)
cyclohexanone
thiazole
CO2
480ppm
600ppm
Neuron A
6-methyl-5-hepten-2-one
cis-3-hexen-1-ol
1-hepten-3-ol
1-octen-3-ol
0.85ng
0.065ng
Neuron B
2,4,5-trimethyl thiazole
2-acetylthiophene
acetophenone
Neuron C
log10(odor stimulus in ng) or log10(CO2 in ppm)

**Figure 6. Response Spectra and Dose-Response Curves of the Three ORNs within the Capitate-Peg Sensillum.**

(A) Response spectra to 48 odorants are shown for the three neurons within the capitate-peg sensillum. The odorants are diluted $10^{-2}$ (85 ng) in paraffin oil. Responses indicate increases or decreases over spontaneous frequency (see text) and are presented as spikes/s. Error bars represent the SEM (n = 8–12 recordings). Asterisks indicate a significant increase or decrease in spike frequency of the neuron in response to the odorant compared with the corresponding solvent control (t test, "*" indicates $p < 0.05$; "**" indicates $p < 0.01$; and "***" indicates $p < 0.001$). Responses to the three solvents are indicated by arrows.

(B–D) Dose-response curves of the three neurons in capitate-peg sensilla to their most-potent ligands. Concentrations of $CO_2$ are plotted as log10ppm, and concentrations of other odorants are plotted as $\log_{10}$ng. Error bars represent the SEM (n = 6). For the dose-response curve of the cpA neuron to $CO_2$, response threshold and saturation level are marked (small arrows) (B). Dose-response curves of the cpB neuron are shown in (C). The dashed line indicates the mean response of this neuron to paraffin oil. Small arrows indicate response thresholds and maximal response concentrations of the cpB neuron to 1-octen-3-ol and 1-hepten-3-ol.

proboscis which acts as another accessory olfactory appendage in female *An. gambiae* (Kwon et al., 2006). We next investigated the localization of these two *AgORs* in female *An. gambiae* mosquitoes through the use of fluorescent *in situ* hybridization (FISH) studies (Figure 8A-8D).

We included antisense *AgOR7* probes in our FISH experiments as a marker for ORNs and found *AgOR7* labeling was consistently restricted to paired ORN cell bodies in palpal segments two to four (Figure 8A, 8B and 8D, and data not shown). As was the case in previous studies in *Drosophila* and the silkmoth *Bombyx mori* (Couto et al., 2005; Goldman et al., 2005; Nakagawa et al., 2005), labeling of paired cell bodies strongly supports that two adjoining *AgOR7*-positive ORNs are present within a single capitate peg sensillum.

FISH analyses of *AgOR8* and *AgOR28* indicated that both *AgORs* were co-localized in palpal ORNs along with *AgOR7* (Figure 8A and 8B). Antisense *AgOR8* and *AgOR28* riboprobes labeled one of the paired *AgOR7*-positive cell bodies (Figure 8A and 8B), suggesting that these *AgORs* characteristically map to only one of the two ORNs. This was subsequently confirmed by *AgOR8*-*AgOR28* double-labeling experiments in which co-localization was never observed (Figure 8C). Indeed, it appeared that expressions of *AgOR8* and *AgOR28* were mutually exclusive of each other in these paired maxillary palp ORNs (Figure 8C). Consistent with this hypothesis, a mixture of *AgOR8* and *AgOR28* FISH probes labeled an almost entirely overlapping ORN population with the *AgOR7* probe (Figure 8D).

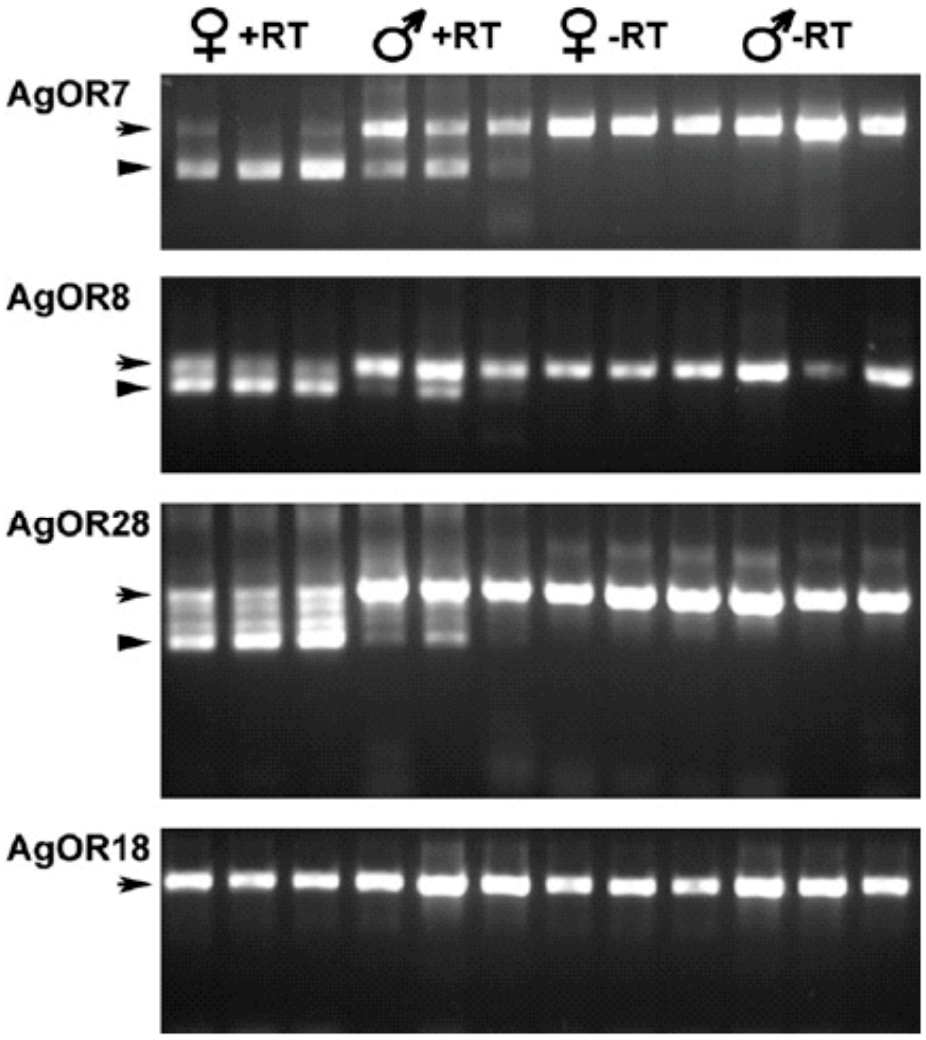


**Figure 7. Expression of *AgOR* Gene in the Maxillary Palp as Determined by RT-PCR.**
Lanes are denoted as follows: Products from three independent female maxillary-palp RNA samples with RT, three independent male maxillary-palp RNAs with RT, triplicates of female maxillary-palp RNA with no RT, and triplicates of male maxillary palp RNA with no RT. Genomic DNA products are indicated by arrows, and cDNA products are indicated by arrowheads. *AgOR7*, *AgOR8*, and *AgOR28* are constantly detected in both male and female maxillary-palp RNA, whereas a different *AgOR*, *AgOR18*, is not detected.

A small fraction of *AgOR7*-positive ORNs (<10%), however, appeared devoid of *AgOR8* or *AgOR28* signal (Figure 8D, arrowheads). We cannot rule out the possibility that *AgOR8* or *AgOR28* may not be uniformly expressed across the entire ORN population such that, at a given time point, a subset of ORNs can express either *AgOR* at a level below the detection threshold of our FISH protocols. Alternatively, the minor fraction of unlabeled ORNs observed here may have expressed another chemosensory receptor gene.

### *AgOR8* and *AgOR28* Both Encode Functional Odorant Receptors

Do *AgOR8* and *AgOR28* encode functional OR proteins and, if so, do they respond to maxillary palp stimuli such as $CO_2$ or 1-octen-3-ol? To address these questions, we conducted two-electrode, voltage-clamp recordings in *Xenopus* oocytes co-injected with complementary RNAs (cRNAs) encoding *AgOR8* or *AgOR28*, along with *AgOR7* and a $G\alpha_{15}$ subunit. *Xenopus* oocytes respond to odorant stimuli by release of $Ca^{2+}$ from internal stores to activate an endogenous $Ca^{2+}$-activated $Cl^-$ channel. This system has been successfully employed in previous studies to characterize functions of insect odorant and pheromone receptors (Nakagawa et al., 2005; Wetzel et al., 2001).

We used a panel of 82 odorants in the *Xenopus* oocyte system. When *AgOR28* was coexpressed in oocytes with *AgOR7* and $G\alpha_{15}$, it conferred the strongest responses to 2,4,5-trimethylthiazole, acetophenone and 2-acetylthiophene (Figure 9A-9C), as well as weaker responses to 16 other odorants (Figure 9B). While AgOR28 appeared to be a broadly tuned receptor

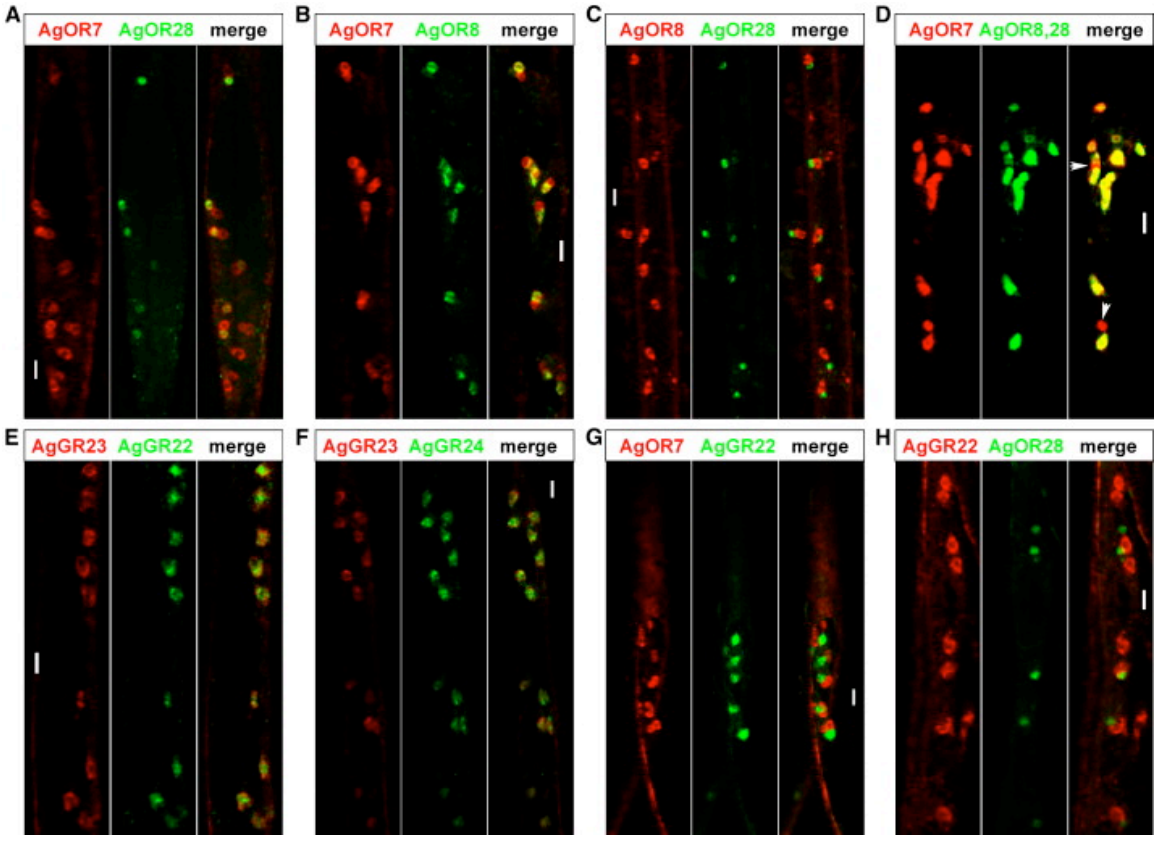


**Figure 8. Expression of *AgOR* and *AgGR* Genes in the Maxillary Palp.**
(A) FISH analyses reveal that two *AgOR7*-positive neurons are paired within the same sensillum, and the *AgOR28* probe consistently labels one of the paired neurons.
(B) *AgOR8* likewise labels one of the paired *AgOR7*-positive neurons.
(C) *AgOR8* and *AgOR28* are not coexpressed and are mapped to paired neurons.
(D) A mixture of *AgOR8* and *AgOR28* probes labels almost the entire population of *AgOR7*-positive neurons. However, two neurons in this section are not labeled (arrows).
(E) Probes for *AgGR23* and *AgGR22* label the same neurons.
(F) *AgGR23* and *AgGR24* also colabel the same neurons.
(G) *AgGR22* is not coexpressed with *AgOR7*, and the *AgGR22*-positive neuron forms neuronal triads with the paired *AgOR7*-positive neurons.
(H) *AgGR22* and *AgOR28* label paired neurons.
The scale bar represents 10 μm.

(Figure 9B, inset), it should be noted that three of its five strongest ligands were shown to be acetyl-containing compounds, one of which, acetophenone, elicits a large inward current of over 2000 nA at a $10^{-4}$ dilution (Figure 9B). Importantly, these data also demonstrated that odorant-induced increases in inward currents are consistently dose-dependent (Figure 9C). The response spectrum of AgOR28 matches nicely that of the cpC neuron in the SSR analyses, indicating that AgOR28 is likely expressed in this ORN to determine its odorant response properties.

In contrast to AgOR28, AgOR8 was more narrowly tuned when expressed in *Xenopus* oocytes, displaying strong responses to 1-octen-3-ol, 1-hepten-3-ol, 3-octanone and 1-hexen-3-ol (Figure 9E, inset and 9F). Among these, 1-octen-3-ol was clearly the most effective ligand, with as low as a $10^{-8}$ dilution of this odorant eliciting a significant response (Figure 9D and 9F). Interestingly, despite the fact that both 1-hepten-3-ol and 1-hexen-3-ol are structurally related to 1-octen-3-ol, AgOR8 displayed strikingly lower sensitivity to these two odorants than to 1-octen-3-ol (Figure 9F). Since 1-octen-3-ol occurs as an optically active mixture of enantiomers in human and animal odors (Grant and O'Connell, 1996), we next tested whether AgOR8 responded to (S)-1-octen-3-ol and found that, at all concentrations, currents induced by (S)-1-octen-3-ol were considerably smaller than currents induced by the racemic mixture (Figure 9E and data not shown). These data closely reflect the response profiles of the cpB neuron of the *An. gambiae* maxillary palp, strongly suggesting that the expression of *AgOR8* in this ORN mediates its highly sensitive response to 1-octen-3-ol.

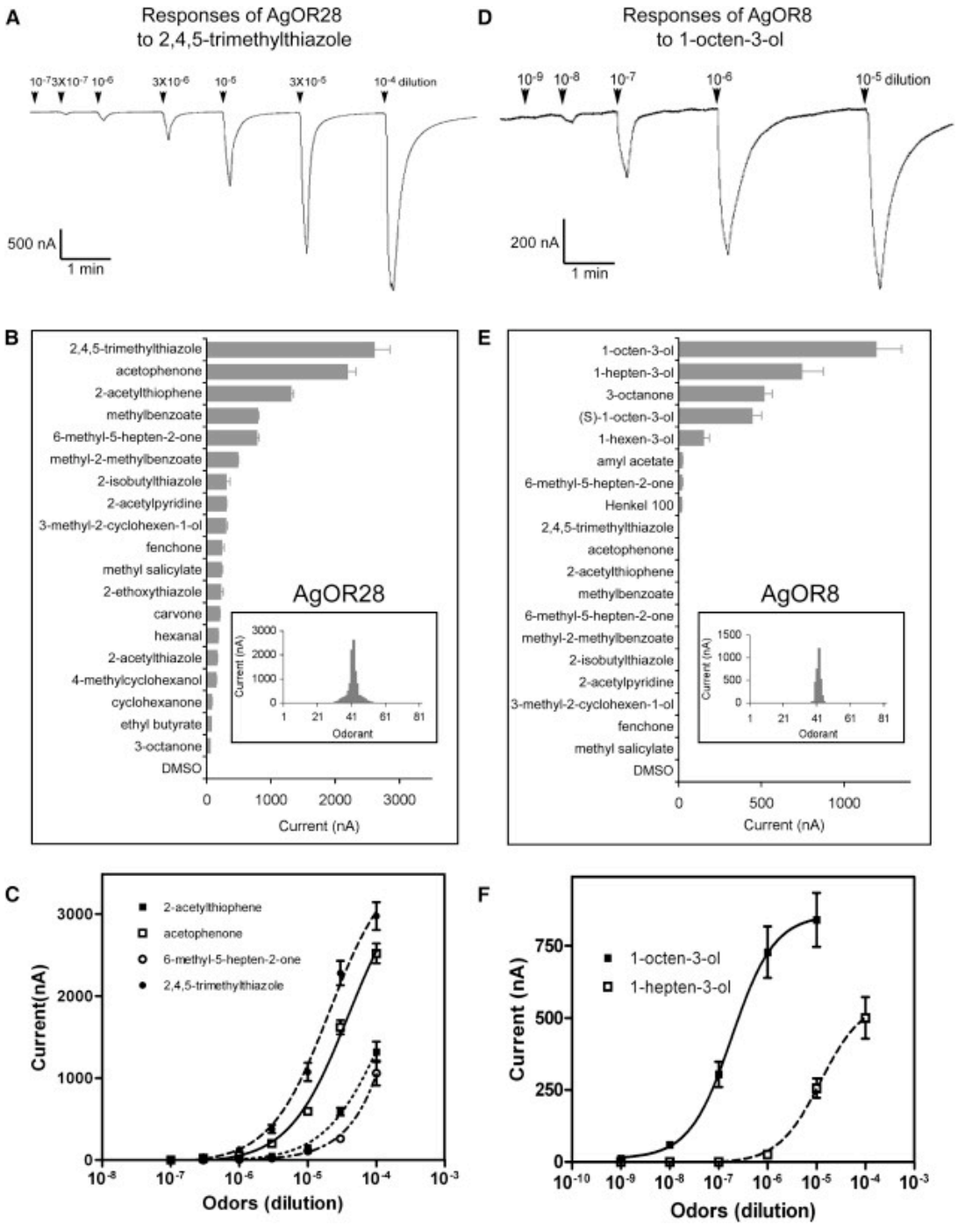
A
Responses of AgOR28
to 2,4,5-trimethylthiazole
10-7 3X10-7 10-6 3X10-6 10-5 3X10-5 10-4 dilution
500 nA
1 min
D
Responses of AgOR8
to 1-octen-3-ol
10-9 10-8 10-7 10-6 10-5 dilution
200 nA
1 min
B
2,4,5-trimethylthiazole
acetophenone
2-acetylthiophene
methylbenzoate
6-methyl-5-hepten-2-one
methyl-2-methylbenzoate
2-isobutylthiazole
2-acetylpyridine
3-methyl-2-cyclohexen-1-ol
fenchone
methyl salicylate
2-ethoxythiazole
carvone
hexanal
2-acetylthiazole
4-methylcyclohexanol
cyclohexanone
ethyl butyrate
3-octanone
DMSO
AgOR28
Current (nA)
Odorant
0 1000 2000 3000
E
1-octen-3-ol
1-hepten-3-ol
3-octanone
(S)-1-octen-3-ol
1-hexen-3-ol
amyl acetate
6-methyl-5-hepten-2-one
Henkel 100
2,4,5-trimethylthiazole
acetophenone
2-acetylthiophene
methylbenzoate
6-methyl-5-hepten-2-one
methyl-2-methylbenzoate
2-isobutylthiazole
2-acetylpyridine
3-methyl-2-cyclohexen-1-ol
fenchone
methyl salicylate
DMSO
AgOR8
Current (nA)
Odorant
0 500 1000
C
2-acetylthiophene
acetophenone
6-methyl-5-hepten-2-one
2,4,5-trimethylthiazole
Current(nA)
Odors (dilution)
F
1-octen-3-ol
1-hepten-3-ol
Current (nA)
Odors (dilution)

**Figure 9. Functional Characterization of *AgOR8* and *AgOR28* Genes in *Xenopus* Oocytes.**

(A) Response trace of AgOR28 to the indicated concentrations of 2,4,5-trimethylthiazole. Odorants are applied for 10 s at the time point indicated by the arrows and induce inward currents in oocytes coinjected with cRNAs encoding AgOR28, AgOR7 and a $G\alpha_{15}$ subunit.

(B) Odorant response spectrum of AgOR28 heterologously expressed in *Xenopus* oocytes. Response is measured as induced currents in nA. Error bars show the SEM (n = 5–6). The tuning curve for this receptor is placed in the inset. The 82 odorants are displayed along the x axis with the odorants eliciting the strongest responses being placed near the center and the odorants eliciting the weakest responses being placed near the edges.

(C) Dose-response curves of AgOR28 to four of its most-effective ligands. Every point represents the means (±SEM) of 5–6 independent oocytes.

(D) Response trace of AgOR8 to the indicated concentrations of 1-octen-3-ol.

(E) Odorant response spectrum of AgOR8 heterologously expressed in *Xenopus* oocytes. Error bars show the SEM (n = 5–6). The tuning curve for this receptor is placed in the inset.

(F) Dose-response curves of AgOR8 to two most effective ligands. Every point represents the means (±SEM) of 5–6 independent oocytes.

**A Third ORN in the Capitate Peg Expresses Mosquito $CO_2$ Receptors**

In *Drosophila*, one *GR* gene, *DmGR21a*, has been implicated in $CO_2$ detection (Faucher et al., 2006; Suh et al., 2004) and, more recently, it has been shown that *DmGR21a* together with a second *GR*, *DmGR63a*, mediate $CO_2$ sensitivity (Jones et al., 2007; Kwon et al., 2007). Both *An. gambiae* and *Ae. aegypti* have three closely related homologs of *DmGR21a* and *DmGR63a* (Figure 10) (Hill et al., 2002; Kent et al., 2008). Although *AgGR22* and *AgGR24* are likely orthologs of *DmGR21a* and *DmGR63a*, with 68% and 65% identity, respectively (Hill et al., 2002), *AgGR23* is less conserved, sharing 38% and 26% identity with *DmGR21a* and *DmGR63a*, respectively (Figure 10). Although *Drosophila* species do not have a close homolog of *AgGR23*, two non-Dipteran species, the silkworm *Bombyx mori* and the beetle *Tribolium castaneum* do (Figure 10), suggesting that *AgGR23* is likely the ortholog of a *DmGR* that has been lost subsequent to the divergence of these two species.

To investigate whether these putative mosquito $CO_2$ receptors are expressed in the maxillary palp, we carried out FISH experiments with riboprobes for all three *AgGR* genes (Figure 8E-8H). We reasoned that the $CO_2$-sensitive ORN might not express *AgOR7* and thus would not have been labeled by the *AgOR7* probe in our previous FISH experiments. Interestingly, in the female maxillary palp, *AgGR22*, *AgGR23* and *AgGR24* were all coexpressed in the same *AgOR7*-negative neurons that are restricted to segments two to four (Figure 8E, 8F and data not shown). This extends recently published data showing co-localization of *DmGR21a* and *DmGR63a* in *Drosophila* and *AgGR22* and *AgGR24* in *An.*

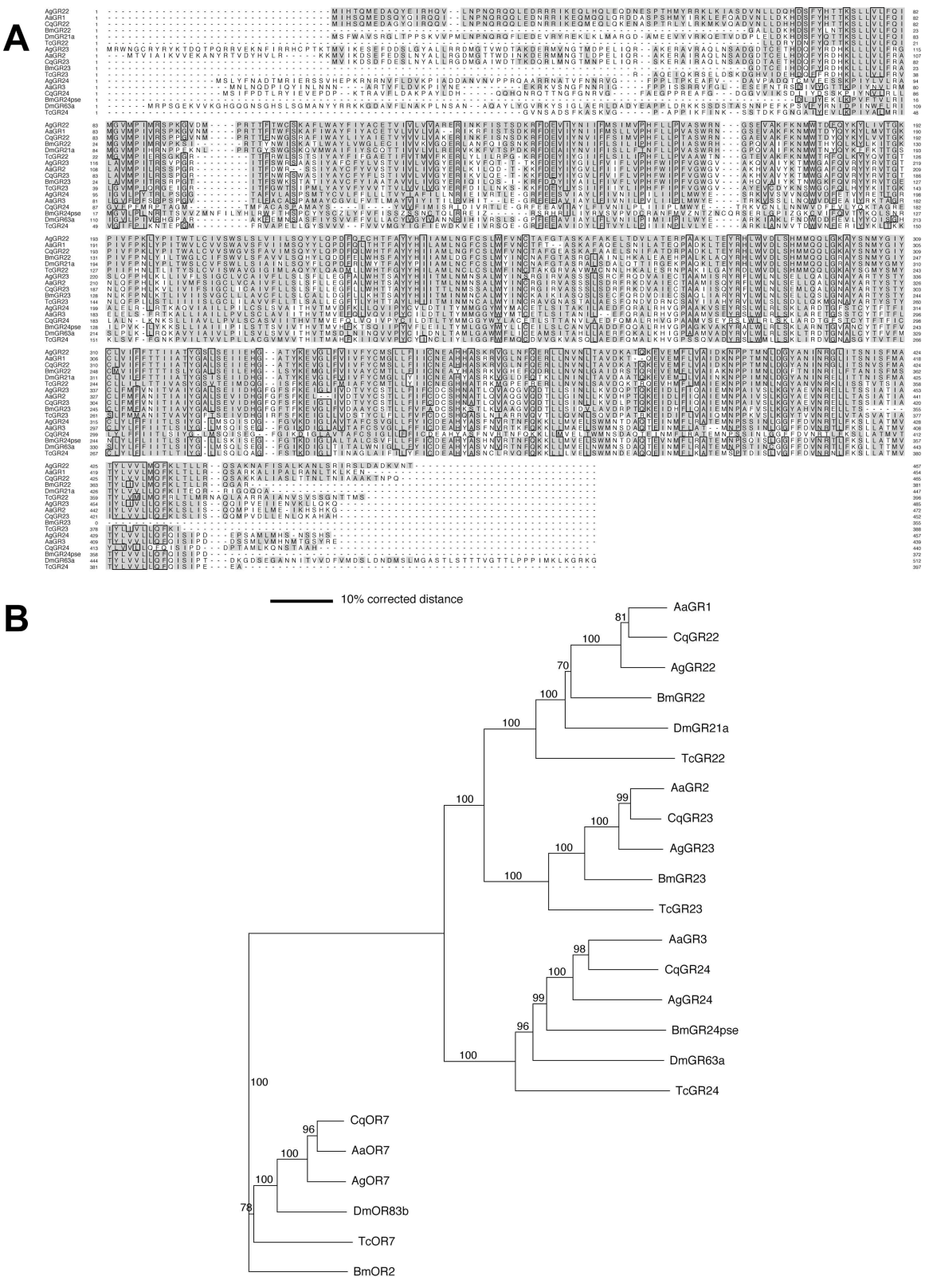
A
B
10% corrected distance
AaGR1
CqGR22
AgGR22
BmGR22
DmGR21a
TcGR22
AaGR2
CqGR23
AgGR23
BmGR23
TcGR23
AaGR3
CqGR24
AgGR24
BmGR24pse
DmGR63a
TcGR24
CqOR7
AaOR7
AgOR7
DmOR83b
TcOR7
BmOR2

**Figure 10. The Putative Insect $CO_2$ Receptors Are Highly Conserved.**
(A) Deduced amino acid sequences were aligned with the ClustalX algorithm (Thompson et al., 1997) and viewed in SeqVu (The Garvan Institute of Medical Research). For all alignments, similarity shading is based upon an 85% Goldman-Engelman-Steitz scale, and identity boxing is based on a 65% scale (Riek et al., 1995) with SeqVu.
(B) Phylogenetic relationship of putative insect $CO_2$ receptors. The corrected distance tree was generated with PAUP* v4 with distances corrected in TreePuzzle v5 with a maximum-likelihood model and the BLOSUM62 amino acid matrix (Bohbot et al., 2007; Hill et al., 2002; Robertson and Wanner, 2006; Robertson et al., 2003). The tree is rooted with the group of *DmOR83b* orthologs. Bootstrap support from 1000 replications of neighbor joining with uncorrected distances is shown on the relevant branch points. Although these putative insect $CO_2$ receptors are closely related to each other, they form three distinct phylogenetic clusters with *AgGR22*, *AgGR23*, and *AgGR24*, respectively. Although the three mosquito species, *An. gambiae*, *Ae. aegypti*, and *Culex pipiens quinquefasciatus*, the silkworm *Bombyx mori*, and the red flour beetle *Tribolium castaneum* all seem to have three homologous $CO_2$ receptor genes, *Drosophila*, on the other hand, does not have a close homolog of *AgGR23*.

*gambiae* (Jones et al., 2007). Furthermore, *AgOR7-AgGR22* double-labeling studies also showed that the *AgGR22*-positive cell always forms a triad with a set of paired *AgOR7*-positive ORN cell bodies in the maxillary palp (Figure 8G). This is further supported by the observation that *AgGR22* and *AgOR28* labeled paired cell bodies in the maxillary palp (Figure 8H).

Moreover, we note that the *AgGR22*-positive ORN has a much larger cell volume than either of the *AgOR7*-positive ORNs (Figure 8G and 8H). This is in accordance with its more voluminous dendrite that displays considerably more branching than the other two dendrites within the capitate peg (Figure 4C) (McIver and Siemicki, 1975).

**Coexpression of *AgGR* Genes Confers a $CO_2$ Response**

To test whether the *AgGR* genes are capable of conferring a response to $CO_2$, we expressed them in an *in vivo* expression system, the "empty neuron" system (Dobritsa et al., 2003). This system is based on a mutant ORN of the *Drosophila* antenna, ab3A, which lacks its endogenous receptor and does not respond to odors.

When we coexpressed one copy of both *AgGR22* and *AgGR24* cDNAs in the empty neuron, we did not observe a response to $CO_2$. However, upon increasing the dosage of both transgenes by two fold, we observed a marked response to $CO_2$: 27$\pm$5.6 spikes/s (SEM; n=8) over the spontaneous firing rate (Figure11A and 11B). A steep dependence on gene dosage was also found for the *Drosophila* orthologs of these genes (Kwon et al., 2007). Cyclohexanone elicited

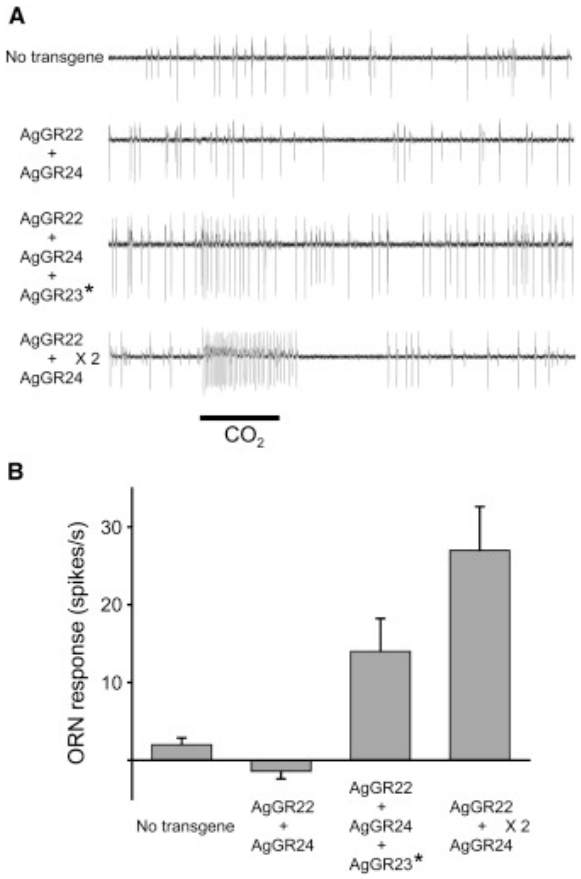


**Figure 11. AgGR22, AgGR23, and AgGR24 Confer a Response to $CO_2$.**
(A) Representative traces from a mutant, transgenic ab3 sensillum, which contains two ORNs, ab3A and ab3B. The large spikes represent the activity of the ab3A neuron and are counted; the small spikes represent the activity of the neighboring ab3B neuron (Dobritsa et al., 2003). Flies contain a *DmOR22a* promoter-*GAL4* construct and a variable number of *UAS-AgGR* transgenes (from top: no transgenes, one copy each of *AgGR22* and *AgGR24*, one copy each of *AgGR22*, *AgGR23**, and *AgGR24*, and two copies each of *AgGR22* and *AgGR24*). The scale bar represents a 0.5 s stimulus period of 100% $CO_2$.
(B) Mean responses to 100% $CO_2$ of the ab3A neuron expressing the indicated transgenes. The number of spikes in the 0.5 s prestimulus period was subtracted from the number in the 0.5 s $CO_2$ stimulation period. Error bars represent the SEM; n = 8–20. All transgenes are cDNA clones except the *AgGR23** transgene, which is marked by an asterisk to indicate that it is a myc-tagged genomic clone.

only 4.7±1.8 spikes/s (SEM; n=6) (data not shown), suggesting the possibility that response to this odorant (Figure 6A and 6B) depended on AgGR23 or another receptor.

To investigate whether AgGR23 acts in detection of cyclohexanone or $CO_2$, we added an *AgGR23* transgene to the empty neuron containing a single copy of *AgGR22* and *AgGR24*. We found that addition of the *AgGR23* transgene had no effect on response to cyclohexanone (3.0±2.7 spikes/s; n=8) (data not shown). However, addition of *AgGR23* increased the $CO_2$ response from -1.4±1.0 spikes/s (n=10) to 14±4.2 spikes/s (n=8) (Figure 11A and 11B). Although the *AgGR23* transgene was a myc-tagged genomic clone, and we have not tested the response of a neuron containing two copies of all three transgenes, the results clearly implicate AgGR23 in the $CO_2$ response.

In summary, we have shown that coexpression of *AgGR* genes, orthologs of *Drosophila* $CO_2$ receptors, is sufficient to confer responses to $CO_2$ on $CO_2$-insensitive ORNs. These results, together with our FISH experiments, show that the ORN coexpressing *AgGR22*, *AgGR23* and *AgGR24* is the $CO_2$-sensitive ORN (the cpA neuron) identified in our electrophysiological studies.

## Discussion

### A Topographic Map of ORNs and Chemosensory Receptors in the Maxillary Palp

We have described a complete and highly concordant map of both the molecular and cellular olfactory components on the maxillary palp of the adult female *An.*

*gambiae* mosquito, which is the principal Afrotropical vector for malaria. We have used *in vivo* SSR analyses to identify and physiologically characterize three functional types of ORNs within each capitate peg sensillum as well as RT-PCR, FISH and heterologous expression to fully elucidate the expression patterns and functional characteristics of chemosensory genes throughout the maxillary palp. These studies suggest that a relatively small number of *AgOR* and *AgGR* genes are responsible for encoding the entire functional repertoire of chemosensory receptors in palpal ORNs.

In *Drosophila*, a discrete subset of *DmOR* genes is expressed in the maxillary palp as compared to those found in the antenna (Couto et al., 2005; Goldman et al., 2005), which may represent a form of organotypic segregation of sensory inputs across the peripheral olfactory apparatus. In *An. gambiae*, however, *AgOR28* has been detected by RT-PCR amplifications in both the maxillary palp (this study) and the labellum of adult females (Kwon et al., 2006). This is consistent with observations that acetophenone, one of the strongest ligands for AgOR28, has also been shown to activate a labial sensillum associated with the expression of *AgOR28* (Kwon et al., 2006). Furthermore, 1-octen-3-ol, the most effective ligand for the *AgOR8*-expressing ORN in the maxillary palp, also elicits responses from a subset of antennal ORNs (Qiu et al., 2006; van den Broek and den Otter, 1999) albeit with much lower sensitivity (threshold at ca. 0.25 mg vs. 0.85 pg) and response intensity (maximal response at 10 spikes/s vs. 165 spikes/s) (van den Broek and den Otter, 1999). These data support a model whereby differential expressions of AgORs across the three olfactory

appendages of *An. gambiae* manifest a range of affinities for overlapping sets of odorant ligands, reflecting a topographic ordering of both high-sensitivity and low-sensitivity receptors. This, in turn, could afford the mosquito a more precise ability to locate and respond to odorant cues across biological space.

Recent anterograde labeling and three-dimensional reconstruction studies of the antennal lobe (AL) from female *An. gambiae* have revealed convergence of antennal and maxillary palpal projections onto three dorsal-medial glomeruli (Ghaninia et al., 2007a). This may represent a fundamental difference from *Drosophila*, in which sensory inputs of antennal and palpal ORNs fail to converge to common AL glomeruli and remain organotopically distinct (Couto et al., 2005; Fishilevich and Vosshall, 2005).

**Odor Coding in the *An. gambiae* Maxillary Palp**

We conducted physiological measurements of palpal ORNs against a broad panel of odorants and demonstrated that there were only three types of ORNs (cpA, cpB and cpC) within the capitate peg sensillum repertoire exhibiting partly overlapping but nevertheless distinct response spectra. Of these, two types mediate highly sensitive detection of two important mosquito kairomones, $CO_2$ and 1-octen-3-ol. Within our panel of tested compounds, the $CO_2$-sensitive cpA neuron was shown to be narrowly tuned to a few odorants. The cpB neuron was also narrowly tuned to a few odorants, including its best ligand 1-octen-3-ol, whereas the cpC neuron appeared more broadly tuned.

We successfully used *Xenopus* oocytes to recapitulate the response profiles of AgOR8 and AgOR28, the two conventional AgORs expressed on the maxillary palp of *An. gambiae* and found that the response spectrum measured in oocytes largely corresponded to that of the native ORNs (Figure 6A, 9B and 9E). These data foster the assignment of *AgOR8* and *AgOR28* to the cpB and cpC neurons in the maxillary palp, respectively, and are consistent with current paradigms suggesting that insect ORs provide the primary determinant of response spectra of ORNs (Hallem et al., 2004b; Nakagawa et al., 2005).

AgOR8 and AgOR28 were shown to vary sharply in their breadth of tuning: while AgOR8 appeared to be a narrowly tuned receptor, AgOR28 responded to a broad panel of odorants, albeit most strongly to only a few ligands such as 2,4,5-trimethylthiazole and acetophenone. Consistent with previous studies in which some *Drosophila* ORs show reduced promiscuity when tested with odorants at lower concentrations (Hallem and Carlson, 2006; Hallem et al., 2004b), AgOR28 remained responsive to a smaller subset of odorants at lower concentrations. Furthermore, a substantial fraction of AgOR28's ligands were demonstrated to be structurally related and fall into two categories: acetyl-containing compounds and thiazole derivatives. Of the two most effective AgOR28 ligands, 2,4,5-trimethylthiazole is a naturally occurring odorant, and acetophenone is a plant volatile that evokes physiological or behavioral responses in many insect species (Wright et al., 2005). Since these odorants are not associated with human hosts, these data support a hypothesis that AgOR28 is not directly involved in the host-seeking behaviors of *An. gambiae*; instead, it may mediate responses to nectar-

feeding or oviposition cues. It should, however, be noted that other formal possibilities exist as the odor panel employed here is hardly exhaustive, and AgOR28 might respond with high sensitivity to untested compounds of particular biological significance.

AgOR8 provided the molecular basis for the sensitive palpal response to 1-octen-3-ol. In addition, it was also strongly excited by 1-hepten-3-ol, while considerably weaker responses were elicited from 3-octanone and 1-hexen-3-ol. Furthermore, the native *AgOR8*-expressing cpB neuron as well as the $CO_2$-sensitive cpA neuron exhibited strikingly prevalent inhibitory responses to a large number of tested odorants; they might use inhibition as a mechanism to suppress noise, as suggested of several *Drosophila* specialist ORNs (Hallem and Carlson, 2006). The mono-unsaturated eight-carbon alcohol 1-octen-3-ol is a well-established odorant cue for mosquitoes (Cork and Park, 1996; Mboera et al., 2000), and has been documented to synergize the maxillary palp-based attractiveness of $CO_2$ to several mosquito species (Mboera and Takken, 1997). Interestingly, *Ae. aegypti* also manifests a highly sensitive electrophysiological response to 1-octen-3-ol in its maxillary palp (Grant and O'Connell, 1996) and has a close ortholog of *AgOR8* (*AaOR8*, with 70% identity) that is likewise exclusively expressed in the maxillary palp (Bohbot et al., 2007).

The recent data for their *Drosophila* homologs (Jones et al., 2007; Kwon et al., 2007), together with the SSR, FISH and functional data presented here, strongly support the hypothesis that the palpal cpA neuron coexpressing *AgGR22*, *AgGR23* and *AgGR24* mediates $CO_2$ detection in *An. gambiae*. This

ORN reaches its maximal response level near 4000 ppm of $CO_2$ (Figure 6B), about 1/10th the concentration measured in human breath (Gillies, 1980). This concentration corresponds to an apparently important behavioral response threshold as *An. gambiae* and other mosquitoes do not show increased responses to $CO_2$ levels above 4000 ppm (Costantini et al., 1996). Fruit flies, however, use this odorant as an avoidance signal (Faucher et al., 2006; Suh et al., 2004) and, accordingly, the $CO_2$-sensitive neuron continues to increase its firing rate at $CO_2$ concentrations above 4% (Jones et al., 2007). The *An. gambiae* cpA neuron, similar to the $CO_2$-responsive ORN in *Ae. aegypti* (Grant et al., 1995), is a highly sensitive $CO_2$ detector, exhibiting a steep slope of dose-response curves that facilitates detecting small increments in $CO_2$ concentration. However, when compared with the *Ae. aegypti* neuron, the $CO_2$-sensitive ORN in *An. gambiae* has a higher detection threshold (around 600 ppm) and the slope of its dose-response curve is not as steep, indicating that $CO_2$-chemosensation is somewhat less acute in this mosquito. This is in agreement with behavioral studies in which this highly anthropophilic mosquito shows less dependency on $CO_2$, in favor of a greater reliance on human-specific cues as a basis for host-seeking behaviors (Mboera and Takken, 1997; Takken and Knols, 1999).

Like their orthologs in *Drosophila*, *AgGR22* and *AgGR24* encode functional receptors and produce a significant response to $CO_2$ when coexpressed in an “empty” olfactory neuron. This result indicates that the two AgGRs are capable of being functionally expressed in a non-native ORN. Furthermore, as was the case for *DmGR21a* and *DmGR63a* (Kwon et al., 2007), the level of response depends

on the dosage of *AgGR22* and *AgGR24* genes. Two copies of both transgenes induced $CO_2$ sensitivity in the ab3A neuron whereas one copy of both transgenes was insufficient. Interestingly, the ectopic $CO_2$ response in the ab3A neuron is lower than that measured in the *An. gambiae* cpA neuron. This may be accounted for by lower levels of gene expression achieved with the *GAL4-UAS* system, by lack of the uniquely lamellate dendritic structure that characterizes the cpA neuron and may facilitate $CO_2$ chemosensation (McIver and Siemicki, 1975; McIver, 1972), or by lack of additional components. In this regard, we have shown that the third *AgGR* gene, *AgGR23*, which is also a close homolog of *Drosophila* $CO_2$ receptors and is coexpressed with *AgGR22* and *AgGR24* within the mosquito $CO_2$-sensitive ORN, enhances the response conferred by *AgGR22* and *AgGR24*.

Both FISH and SSR analyses reveal a stereotyped triad of ORNs within the capitate peg sensillum population of the maxillary palp: two *AgOR7*-positive neurons always pair with a third ORN coexpressing *AgGR22*, *AgGR23* and *AgGR24* (Figure 12). One possible biological implication for this stereotyped pairing of the $CO_2$-sensitive ORN and the 1-octen-3-ol-sensitive ORN is that the *An. gambiae* mosquito might use a complex host odor blend as a crucial host-seeking cue, of which $CO_2$ and 1-octen-3-ol are two components. As a consequence of having the two ORNs responsive to these two components linked together in the same sensilla, the mosquito may ultimately be able to perceive the ratio between these two compounds within the host odor blend with higher fidelity and spatial resolution (Baker et al., 1998). This is remarkably

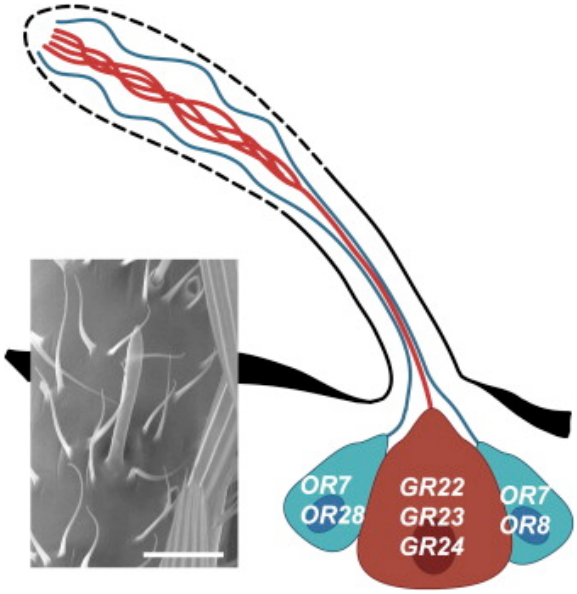


**Figure 12. Spatial Organization of the Three Neurons within the Capitate-Peg Sensillum.**
Schematic drawing of three ORNs in a capitate peg as opposed to a scanning electron micrograph of the sensillum (left). Three ORNs form stereotyped triads within each capitate-peg sensillum. The neuron coexpressing *AgGR22*, *AgGR23*, and *AgGR24* (red) responds to $CO_2$. The other two ORNs (green) express *AgOR7* along with *AgOR8* or *AgOR28*, respectively. The scale bar represents 10 μm.

similar to several cases of insect pheromone-sensing ORNs, in which neurons responsive to individual components of the pheromone blend are compartmentalized within the same sensillum (Nakagawa et al., 2005; Wojtasek et al., 1998).

**The Maxillary Palp as an Accessory Olfactory Organ**

The *An. gambiae* maxillary palp is an olfactory organ with a relatively simple spatial and functional organization. It has but a single type of chemosensory sensillum, the capitate peg, which is distributed along the three middle segments and invariably innervated by three physiologically and molecularly distinct ORNs. Remarkably, two of these ORNs respond with extreme sensitivity to two crucial signal molecules for mosquitoes, $CO_2$ and 1-octen-3-ol. The stereotyped organization of three ORNs within the capitate peg sensillum, as defined through our studies, is reminiscent of the *Drosophila* maxillary palp, in which six functional classes of ORNs are housed in stereotyped pairs within three sensillum types (de Bruyne et al., 1999; Goldman et al., 2005). Although no apparent functional segregation of antennal and palpal ORNs has been observed in *Drosophila* (de Bruyne et al., 1999; de Bruyne et al., 2001), one type of sensilla, the trichoid sensilla, is exclusively associated with pheromone detection in this insect (Kurtovic et al., 2007; van der Goes van Naters and Carlson, 2007; Xu et al., 2005). Our results suggest that the *An. gambiae* capitate peg sensillum is another type of functionally specialized olfactory sensillum in insects. The lower sensitivity of *AgOR28*-positive ORNs in this sensillum might reflect the

relative abundance of plant or bacterial odors in the atmosphere and a correspondingly low level of noise from other irrelevant cues. In contrast, host-seeking cues such as $CO_2$ and 1-octen-3-ol are likely to be encountered at much higher noise levels, and the mosquito ORNs responsive to these odorants would be required to detect them at extremely low concentrations that are naturally mixed with a myriad of irrelevant cues. This chemosensory organization might be crucial in enabling the host-seeking *An. gambiae* mosquito to detect and recognize $CO_2$ and 1-octen-3-ol among a complex odor blend. Perception of $CO_2$ activates mosquitoes and elicits oriented flight behavior, during the course of which they will detect additional cues, including more host-specific odorants, and integrate these signals to drive sustained host-seeking flight (Mboera and Takken, 1997; Takken and Knols, 1999).

# CHAPTER IV

## AGTRPA1, A CANDIDATE THERMORECEPTOR OF HOST BODY TEMPERATURE

### Preface

The following manuscript is in preparation. My contribution to this work included expression analysis of *AgTRP* genes by using RT-PCR, *in situ* hybridization and immunolabeling.

### Introduction

The malaria vector *Anopheles gambiae* and other blood-feeding mosquitoes rely on crucial temperature cues in addition to olfactory stimuli for host location (Bowen, 1991; Takken and Knols, 1999). Heat is emitted by all vertebrate hosts, serves as a universal attractant to many mosquito species (Bowen, 1991; Takken and Knols, 1999) and synergizes with host odor to increase the efficiency of host-seeking behaviors (Kline and Lemire, 1995; Laarman, 1958). The antenna might harbor the thermoreceptive neurons that underlie the heat-evoked behaviors of mosquitoes as suggested by early antennal ablation studies (Ismail, 1962). Indeed, later electrophysiological studies in the yellow-fever vector mosquito, *Aedes aegypti*, identified an antagonistic pair of thermoreceptive neurons within the small coeloconica sensilla on the antennal tip (Davis and Sokolove, 1975; Gingl et al., 2005). One thermoreceptive neuron is warm sensitive and increases its spike frequency to temperature rises; whereas the other neuron is cold

sensitive and increases its spike frequency to temperature drops (Davis and Sokolove, 1975; Gingl et al., 2005).

Despite some knowledge of the physiology of antennal thermoreceptive neurons, little is known about genes that are involved in peripheral temperature detection in mosquitoes. The *Drosophila* genome possesses a large family of 13 transient receptor potential (TRP) genes (Montell, 2005a; Montell, 2005b). These genes encode six-transmembrane nonselective cation channels and several of them have been demonstrated as thermoreceptors (Lee et al., 2005; Rosenzweig et al., 2005; Tracey et al., 2003; Viswanath et al., 2003). Of these, DmTRPA1 is activated by warm temperatures with a threshold of ~27°C and regulates thermotaxis in *Drosophila* (Rosenzweig et al., 2005; Viswanath et al., 2003).

## Experimental Procedures

### Insects

*An. gambiae sensu stricto*, originated from Suakoko, Liberia, was reared as described (Fox et al., 2001; Qiu et al., 2004) and 5- to 7-day-old females that had not been blood fed were used for experiments.

### Reverse Transcriptase PCR

Five-day old female mosquitoes were cold anesthetized and 100 antennae were dissected by hand on a chill table. RNA preparation and cDNA synthesis were carried out as described (Kwon et al., 2006; Lu et al., 2007).

PCR was performed using a DNA Engine Dyad (MJ Research) under the following conditions: 94ºC for 2 min; 35 cycles of 94ºC for 15 s, 60ºC for 20 s, 72ºC for 20 s; 72ºC for 5 min. Primer pairs that span introns were used in order to distinguish cDNA amplicons from those amplified from remaining genomic DNA. The complete set of *AgTRP* primers can be accessed at www.cas.vanderbilt.edu/zwiebel/primers3.htm. PCR amplification products were run on a 1.5% agarose gel and verified by DNA sequencing.

### *In Situ* Hybridization and Immunolabeling

*In situ* hybridization and probe preparation were carried out as described (Kwon et al., 2006; Lu et al., 2007), with digoxigenin-labeled RNA probes comprising approximately 800 bp of *AgTRPA1* coding sequence. Anti-horseradish peroxidase immunostaining was carried out as described (Pitts et al., 2004). Alternatively, nuclei were labeled by using YOYO-1 (Molecular Probes). Sections were mounted in Vectashield (Vector Laboratories) and visualized using an LSM510 confocal microscope (Carl Zeiss).

## Results

We asked whether TRP channels play any significant roles in peripheral temperature detection in *An. gambiae*. All 13 *Drosophila* TRP channels have close homologs in the genome of *An. gambiae*, so in order to focus on those genes specifically associated with peripheral processes, *AgTRP* genes were screened for antennal expression using RT-PCR. In this manner, we were able to

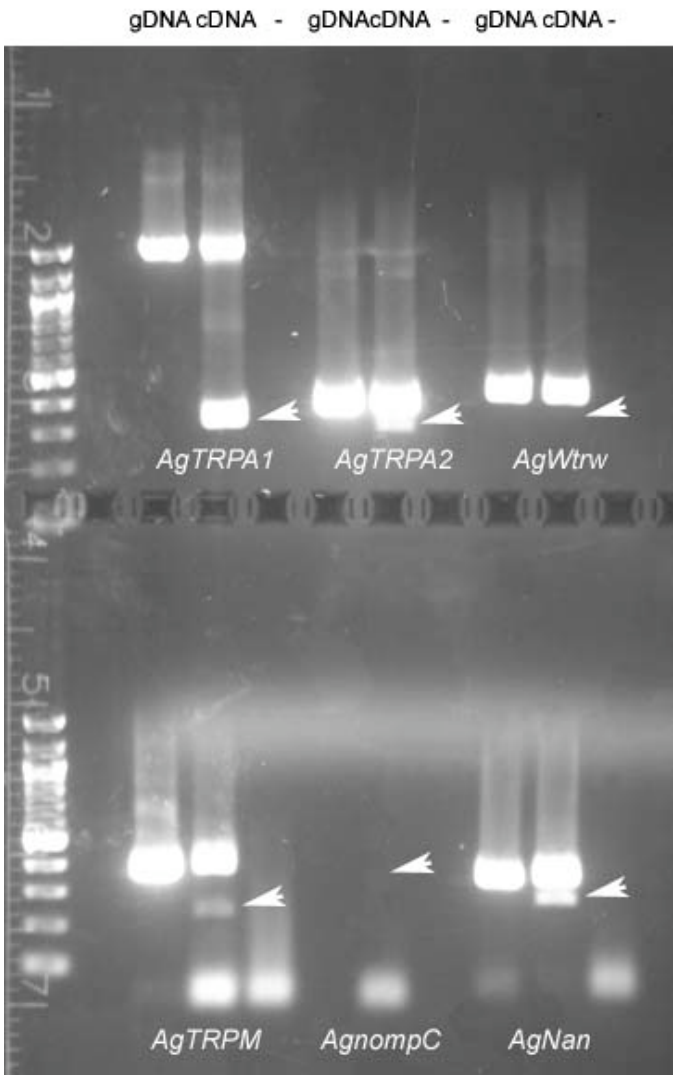


**Figure 13. Expression of *AgTRP* Genes in the Antenna as Determined by RT-PCR.**
A representative gel is shown and lanes are denoted as follows: Amplification products from genomic DNA, female antennal RNA samples with RT, and minus control. cDNA products are indicated by arrowheads. *AgTRPA1*, *AgTRPA2*, *AgTRPM* and *AgNan* are constantly detected in female antennal RNA, among them *AgTRPA1* appears to be robustly amplified.

detect amplification products of multiple *AgTRP* genes. Of these, *AgTRPA1* appears to be robustly expressed in the antenna, whereas several other *AgTRPs* are expressed at much lower levels (Figure 13). This led us to a more detailed spatial expression analysis of *AgTRP* genes in female *An. gambiae* mosquitoes via fluorescent in situ hybridization (FISH) and immunostaining.

FISH studies indicate that the expression of *AgTRPA1* is, surprisingly, restricted to a limited number of neurons in the antenna. In paraffin sections, we consistently saw one neuron in the 1$^{st}$ antennal segment and up to two neurons in the 2$^{nd}$ and the 13$^{th}$ segments that are labeled by antisense *AgTRPA1* riboprobes (Figure 14). On the other hand, the ten mid-segments (from the 3$^{rd}$ to the 12$^{th}$ antennal segments) are entirely devoid of *AgTRPA1* signals (data not shown). We find that each *AgTRPA1*-positive neuron is strongly labeled (Figure 14) and this accounts for the robust amplification of *AgTRPA1* in the aforementioned RT-PCR assay. Furthermore, the antennal localization of *AgTRPA1* labeling correlates well with that of the small coeloconica sensilla as revealed by early morphological studies (Ismail, 1964; Pitts and Zwiebel, 2006). Specifically, *AgTRPA1* labeling in the 13$^{th}$ antennal segment closely matches the position of the paired small coeloconica sensilla on the antennal tip (Figure 14A and 14B), which have been to shown through electrophysiological recordings to harbor thermoreceptive neurons (Davis and Sokolove, 1975; Gingl et al., 2005). Taken together, these data suggest that antennal *AgTRPA1*-positive neurons are temperature sensitive and thus AgTRPA1 is a good candidate for peripheral thermoreceptors in *An. gambiae*.

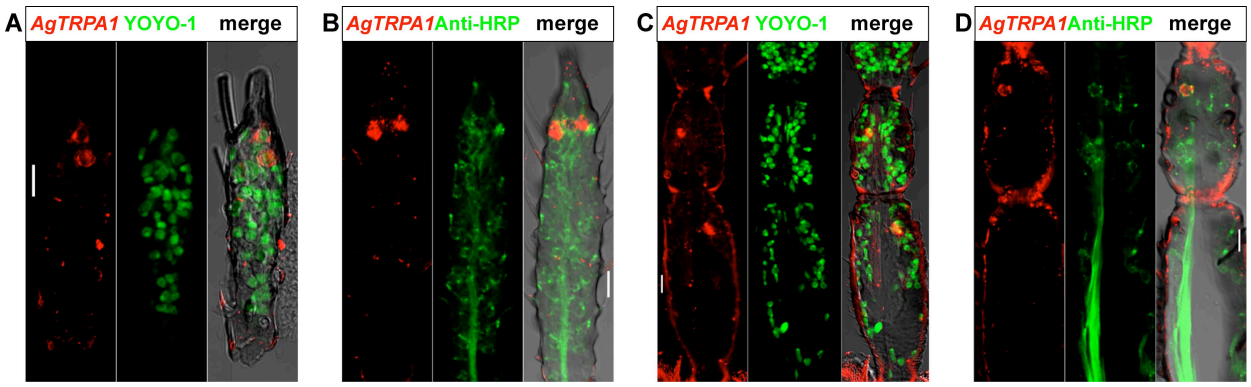


**Figure 14. Expression of *AgTRPA1* in the Antenna.**
(A) FISH analyses reveal that two *AgTRPA1*-positive cells are located in the 13$^{th}$ antennal segment.
(B) Two *AgTRPA1*-positive neurons extend their dendrites into the tip of the antenna.
(C) FISH analyses reveal one *AgTRPA1*-positive cells in the 1$^{st}$ antennal segment and another in the 2$^{nd}$ segment.
(D) *AgTRPA1* and anti-horseradish peroxidase co-labels a neuron in the 2$^{nd}$ antennal segment.
The scale bar represents 10 μm.

## Discussion

These data raise questions about the molecular identity of the thermoreceptor functioning in the other temperature sensitive neuron within the small coeloconica sensilla, which is likely to be another TRP channel. Although we failed to detect several other *AgTRP* (*AgTRPA2* and *AgTRPM*) genes in the antenna by our FISH experiments (data not shown), it is possible that any of these genes are expressed below the detection threshold of our FISH protocol. In addition, ultrastructural studies suggest that there is a third neuron within the small coeloconica sensilla (Boo and McIver, 1975; McIver, 1973). It remains to be resolved whether this neuron is also temperature sensitive and what receptor molecules underlie its response properties.

Taken together, these studies suggest that a TRP channel, *AgTRPA1*, is expressed in the antenna and functions as a peripheral thermoreceptor in *An. gambiae* (data not shown). This is in stark contrast to earlier studies in *Drosophila* showing that DmTRPA1 mainly functions in the brain (Rosenzweig et al., 2005). In *An. gambiae*, the expression of a thermoreceptor in a predominantly olfactory organ suggests that temperature, an important sensory cue in host location and other behaviors, is perceived along with olfactory cues and the two types of stimuli might be integrated in the antenna or at higher brain levels such as the antennal lobe (Zeiner and Tichy, 2000). Behavioral experiments demonstrated that temperatures at 32°-34°C significantly enhance the attraction of host odors to host-seeking *An. gambiae* mosquitoes (Y.T. Qiu and W. Takken, personal communication), and it will be of interest to elucidate the mechanism by

which the two types of sensory inputs are coupled and transformed into an ultimate sensory percept on the host.

Physiological studies in *Ae. aegypti* have previously demonstrated that antennal small coeloconica sensilla respond much more sensitively to convective than radiant heat (Davis and Sokolove, 1975; Gingl et al., 2005). It is likely that heat, emitted from a host's breath and body, is incorporated into host odor blends resulting in an odor/heat plume that is detected by the mosquito antenna. As heat is a universal stimulus for mosquitoes and most other blood-feeding insects, the identification of a peripheral mosquito thermoreceptor provides a potential target for the design of useful insect repellents. And this, in turn, would facilitate the ongoing fight against malaria by reducing the vectorial capacity of *An. gambiae* mosquitoes.

# CHAPTER V

# CONCLUSIONS AND FUTURE DIRECTIONS

## Summary

By using a combination of molecular and physiological approaches, we have studied the three head appendages of female *An. gambiae* mosquitoes, the antenna, the maxillary palp and the proboscis, and have characterized their respective functions in olfaction or thermoreception that might impact the host-seeking behavior of *An. gambiae*. Specifically, we have described three types of sensilla, the labellar T2 sensilla, the palpal capitate pegs and the antennal small coeloconica sensilla, and have characterized their responses to olfactory or temperature stimulation and have identified the underlying molecular and cellular components of these sensilla.

The labellum at the tip of the proboscis has been traditionally thought to function primarily as a gustatory organ in *An. gambiae*. Electrophysiological studies described in Chapter II have uncovered a set of labelllar olfactory responses to a small spectrum of human-related odorants, such as butylamine, several ketones and several carboxylic acids. One of the labellar T2 sensilla harbors at least two ORNs and responds to butylamine, aliphatic acids and several ketone compounds including acetophenone. Furthermore, *AgOR6*, an *AgOR* that is coexpressed with AgOR7 in a subset of labellar ORNs, is expressed and likely contributes to the responses of one of the ORNs within this

sensillum, as suggested by recent functional studies in *Xenopus* oocytes indicating that AgOR6 responds to acetophenone and several other ketones (G. Wang and L.J. Zwiebel, unpublished results).

The maxillary palp is another important olfactory appendage of *An. gambiae*, harboring neurons that detect $CO_2$ and 1-octen-3-ol. By using molecular and physiological approaches coupled with systematic functional analysis, we define the complete olfactory sensory map of the *An. gambiae* maxillary palp and have identified three ORNs that are organized in stereotyped triads within the palpal capitate-peg population. The cpA and cpB neurons respond with high sensitivity to $CO_2$ and 1-octen-3-ol, respectively; and the cpC neuron responds to a broad panel of odorants including 2,4,5-trimethylthiazole and acetophenone. These ORNs express a distinctive set of *AgORs* and *AgGRs*. While the cpA neuron coexpresses *AgGR22*, *AgGR23* and *AgGR24* that confer $CO_2$ responses, the other two ORNs express *AgOR7* along with *AgOR8* or *AgOR28*, respectively. Furthermore, the response spectra of AgOR8 and AgOR28, when coexpressed with AgOR7 in *Xenopus* oocytes, closely mirror that of the native cpB and cpC neurons.

The antennal small coeloconica sensilla house an antagonistic pair of thermoreceptive neurons: one is warm-sensitive and increases its spike frequency to temperature rises; the other is cold sensitive and increases its spike frequency to temperature drops. In situ hybridization analysis revealed that *AgTRPA1* is expressed in one of the paired thermoreceptive neurons and likely contributes to temperature detection in that neuron.

**Future Directions**

*An. gambiae* has a third $CO_2$ receptor, AgGR23, that does not have a close homolog in *Drosophila* species (Lu et al., 2007) and its function in the cpA neuron remains cryptic. Whereas one copy of *AgGR22* and *AgGR24* transgenes is not sufficient to induce $CO_2$ sensitivity in the *Drosophila* ab3A neuron, adding an *AgGR23* transgene further to this ORN will make it $CO_2$-sensitive (Lu et al., 2007). However, compared to the *An. gambiae* cpA neuron, the response of three *AgGR* transgenes to $CO_2$ is weak and its sensitivity is low (Lu et al., 2007), and this limitation hampers further interpretation of the result.

It might be worthwhile to explore the role of AgGR23 in other heterologous expression systems. Mammalian cell lines prove a reliable platform for such assays. Transfections of AgGR22, AgGR23 and AgGR24 or combinations of any two of the three AgGRs coupled with calcium imaging will offer insight into the respective contribution of each AgGR receptor to $CO_2$ detection. The aqueous medium also permits testing with different ligands such as gaseous $CO_2$, carbonic acid ($H_2CO_3$) and bicarbonate ($HCO_3^-$) so that it will be possible to resolve which ligand is actually binding to the AgGR receptors. Furthermore, by employing the protein-fragment complementation assay or the fluorescence resonance energy transfer assay and tagging the AgGR receptors with fluorophores, it will be of interest of elucidate the functional interaction of AgGRs within the $CO_2$-receptive hetero-dimers/trimers.

In situ hybridization analysis revealed that *AgTRPA1* is expressed in one of the paired thermoreceptive neurons within the antennal small coeloconica

sensilla and likely contributes to temperature detection in that neuron. Our results raise questions about the molecular identity of the thermoreceptor functioning in the other temperature sensitive neuron within the small coeloconica sensilla. It might as well be another TRP channel. Although we failed to detect several other *AgTRP* (*AgTRPA2* and *AgTRPM*) genes in the antenna by our FISH experiments (data not shown), it remains possible that this gene is expressed at a level below the detection threshold of our FISH protocol. Alternatively, several other *AgTRP* (*AgNan*, *AgIav* and *AgWtrw*) genes might be involved in temperature detection despite their roles in hearing and hygrosensation (Gong et al., 2004; Kim et al., 2003; Liu et al., 2007). In addition, ultrastructural studies suggest that there is a third neuron within the small coeloconica sensilla (Boo and McIver, 1975; McIver, 1973), and it remains to be resolved whether this neuron is also temperature sensitive and what receptor molecules underlie its response properties.

Physiological studies in *Ae. aegypti* demonstrated that antennal small coeloconica sensilla respond much more sensitively to convective than radiant heat, and both cells within the sensilla respond more sensitively to temperature changes than actual temperatures (Davis and Sokolove, 1975; Gingl et al., 2005). It remains to be resolved whether the antennal small coeloconica sensilla in *An. gambiae* function in a similar way. It will also be of interest to recapitulate the temperature responses of AgTRPA1 in heterologous expression systems. Although DmTRPA1 and mouse TRPA1 channel share a high level of sequence homology, they are activated by warm and cold temperatures, respectively (Story

et al., 2003; Viswanath et al., 2003). AgTRPA1, a sequence ortholog of DmTRPA1, might not be a functional ortholog of its *Drosophila* counterpart.

The identification of molecular components underlying olfactory and thermoreceptive behaviors of *An. gambiae* offers new opportunities to alter or control the host-seeking behavior of this deadly mosquito. The isolation of odor and thermoreceptors that detect crucial host-seeking cues allows screening for potent compounds that strongly excite, block or inhibit them, and this could, in turn, lead to identification of efficacious mosquito repellents, attractants or odor-masking compounds. Some compounds do not elicit a response from an OR but have been shown to block responses in this receptor elicited by other compounds (Araneda et al., 2000; Oka et al., 2004; Spehr et al., 2003), and therefore a large-scale cell-based screen could yield interesting candidate compounds that block the responses of AgORs, AgGRs or AgTRPA1 to crucial host-seeking cues such as $CO_2$, lactic acid, indole or heat.

The widely-used insect repellent DEET (*N*,*N*-diethyl-meta-toluamide) was identified in a large-scale behavioral assay (Gilbert et al., 1955) and its mechanism of action was unknown. Recent studies suggest that DEET partially exerts its effect as an odor-masking compound to several AgORs including the palpal 1-octen-3-ol receptor AgOR8 (Ditzen et al., 2008) and an understanding of how DEET acts on these AgORs would allow screening for more efficient insect repellents.

The identification of novel potent mosquito attractants could be very useful in developing the next generation of more efficient mosquito traps. Most current

mosquito traps are baited with well-established mosquito attractants such as $CO_2$ and 1-octen-3-ol, and it remains one of the ultimate goals in the field to develop a mosquito bait more attractive to female *An. gambiae* mosquitoes than (or as attractive as) a human host. To this end, molecular efforts to screen for potent mosquito attractants are coupled with behavioral assays to build an efficacious odor blend that can be used in the next generation of mosquito traps. This strategy, together with efforts to mask and reduce olfactory sensitivity in *An. gambiae*, will likely contribute to the control of the blood-feeding behavior of this deadly insect and lead to reduction in the incidence of malaria (van der Goes van Naters and Carlson, 2006).

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