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Odor Coding in the Maxillary Palp of the Malaria Vector Mosquito *Anopheles gambiae*

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Summary

Background—Many species of mosquitoes, including the major malaria vector *Anopheles gambiae*, utilize carbon dioxide (CO₂) and 1-octen-3-ol as olfactory cues in host-seeking behaviors that underlie their vectorial capacity. However, the molecular and cellular basis of such olfactory responses remains largely unknown.

Results—Here, we use molecular and physiological approaches coupled with systematic functional analyses to define the complete olfactory sensory map of the *An. gambiae* maxillary palp, an olfactory appendage that mediates the detection of these compounds. In doing so, we identify three olfactory receptor neurons (ORNs) that are organized in stereotyped triads within the maxillary-palp capitata-peg-sensillum population. One ORN is CO₂-responsive and characterized by the coexpression of three receptors that confer CO₂ responses, whereas the other ORNs express characteristic odorant receptors (AgORs) that are responsible for their *in vivo* olfactory responses.

Conclusions—Our results describe 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. These results also facilitate the understanding of how *An. gambiae* mosquitoes sense olfactory cues that might be exploited to compromise their ability to transmit malaria.

Introduction

Because of its role in transmitting malaria, the Afrotropical vector mosquito *Anopheles 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 olfactory receptor neurons (ORNs) to respond to a myriad set of chemical cues with which it carries out odor-

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Note Added in Proof The data referred to throughout as “L.B. Kent and H.M. Robertson, personal communication” are now in press: Kent, L.B., Walden, K.K.O., and Robertson, H.M. (2007). The Gr family of candidate gustatory and olfactory receptors in the yellow fever mosquito *Aedes aegypti*. *Chem. Senses*, in press.

Supplemental Data Experimental Procedures and four figures are available at <http://www.current-biology.com/cgi/content/full/17/18/bxs/DC1/>.

mediated behaviors, such as host seeking, nectar feeding, and oviposition [1-3]. *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 [4, 5]. 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 [6]. Ultrastructural studies have revealed that each capitate-peg sensillum in *An. gambiae* is invariably innervated by three ORNs [6].

Carbon dioxide (CO₂) is emitted by all potential vertebrate hosts and serves as a universal attractant to many mosquito species [1, 7]. It has been reported that CO₂ stimulation synergizes with host body odor and induces take-off and sustained-flight behaviors in host-seeking anopheline mosquitoes [7-9]. In addition to CO₂, 1-octen-3-ol is another small molecule that emanates from large herbivores as well as from humans [10, 11] and has been identified as a behavioral attractant to tsetse flies [10].

The maxillary palp is the primary CO₂-sensitive organ of mosquitoes, and early studies observed a specific loss of response to CO₂ in palpectomized female *Culex* mosquitoes [12]. 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 CO₂ and 1-octen-3-ol in electrophysiological studies [13, 14]. 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 does *Ae. aegypti* in mediating host selection and location [1].

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) [15, 16]. Insect ORNs typically express one highly conserved and broadly expressed nonconventional DmOR83b-like OR together with one or two conventional odorant-binding ORs [16-19]. In *An. gambiae*, 79 putative odorant receptor (*AgOR*) genes have been identified [20], and, thus far, two of them have been demonstrated to encode functional ORs [21]. The anopheline *DmOR83b* ortholog, *AgOR7*, is widely expressed in olfactory organs [4].

Here we confirm that *An. gambiae* palpal ORNs respond to CO₂ 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*) [20] are coexpressed within the CO₂-sensitive ORN, and 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, whereas *AgOR28* is tuned to a broad panel of odorants. Lastly, we show that the coexpression of the *AgGRs* confers CO₂ 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.

Results

Responses to CO₂ 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 (Figures 1A and 1B). Capitate pegs, the single type of chemosensory sensilla in this appendage, are distributed on the ventral side of palpal segments two, three and four [6] (Figures 1A and 1B). Transmission

electron microscopic (TEM) studies confirmed that one neuron within *An. gambiae* capitata pegs had a uniquely lamellate dendritic structure (Figure 1C) that is characteristic of insect CO₂-responsive ORNs [6, 22].

In order to characterize the olfactory response profile of this appendage, we performed extensive single-sensillum electrophysiological recordings (SSRs) of 45 capitata-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 (Figures 2A–2C), indicating the presence of three ORNs (henceforth referred to as the capitata peg (cp) A, B, or C neuron) within each capitata-peg sensillum, which is in accord with previous ultrastructural studies [6]. The cpA neuron is characterized by the largest action potential amplitude, and the cpB and cpC neurons manifest intermediate and the smallest amplitudes, respectively (Figures 2A–2C).

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 capitata 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 3A).

The cpA neuron exhibited a spontaneous firing rate of 6.7 ± 63.1 spikes/s and responded strongly to stimulation by CO₂ (Figure 2D). We demonstrated the cpA neuron to be a highly sensitive CO₂ detector, with a dose-response curve exhibiting a steep slope of 90 spikes every ¹⁰log concentration and a detection threshold at around 600 ppm (Figure 3B). In addition to its CO₂ detection, this neuron manifested significant excitatory responses to thiazole, 4-methylthiazole, and cyclohexanone (Figures 3A and 3B). 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 [23], was unusually activated by the paraffin oil solvent, accounting for much of its response to other odorants (Figures 2E and 3A, arrow). Finally, the cpA neuron was significantly inhibited by seven compounds, with indole and 3-methylindole inducing the strongest inhibition (Figure 3A).

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 (Figures 2F, 2G, 3A, and 3C). The cpB neuron continued to increase its firing rate to 1-octen-3-ol at concentrations between 8.5×10^{-4} ng and 0.85 ng until the response reached its maximum at 0.85 ng and started to decline at higher concentrations (Figure 3C). 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 (Figures 2H and 2I), probably because of overstimulation; 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 (Figures 2J and 2K), 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 3C). Much weaker excitatory responses were evoked by 3-octanone and 1-hexen-3-ol and five other tested odorants (Figures 3A and 3C). Furthermore, the cpB neuron was significantly inhibited by 19 tested compounds, among which 4-methylcyclohexanol, 2,4,5-trimethylthiazole, and 2-ethylphenol elicited the strongest inhibitory effects (Figure 3A). Notably, the insect repellent DEET (N,N-diethyl-3-methylbenzamide) was one of the compounds that inhibited the firing of the cpB neuron (Figure 3A).

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 3A). 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 (Figures 2L, 2M, 3A, and 3D). A small number of compounds (e.g., 6-methyl-5-hepten-2-one) evoked excitatory responses in both cpB and cpC neurons (Figures 2B, 2C, and 3A).

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 polymerase chain reaction (RT-PCR) amplifications with primer pairs targeting all 79 *AgOR* genes, by 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 S1 in the Supplemental Data available online). Of these, *AgOR8* appears to be palp specific because we have never detected it with similar assays in other mosquito olfactory appendages (M.R. and L.J.Z., unpublished data); *AgOR28*, however, is also expressed in the proboscis that acts as another accessory olfactory appendage in female *An. gambiae* [5]. We next investigated the localization of these two *AgORs* in female *An. gambiae* mosquitoes through the use of fluorescent in situ hybridization (FISH) studies (Figures 4A–4D and Figure S3).

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 (Figures 4A, 4B, and 4D and data not shown). As was the case in previous studies in *Drosophila* and the silkworm *Bombyx mori* [17, 18, 24], the 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 colocalized in palpal ORNs along with *AgOR7* (Figures 4A and 4B). Antisense *AgOR8* and *AgOR28* riboprobes labeled one of the paired *AgOR7*-positive cell bodies (Figures 4A and 4B), 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 colocalization was never observed (Figure 4C). Indeed, it appeared that expressions of *AgOR8* and *AgOR28* were mutually exclusive of each other in these paired maxillary-palp ORNs (Figure 4C). Consistent with this hypothesis, a mixture of *AgOR8* and *AgOR28* FISH probes labeled an almost entirely overlapping ORN population with the *AgOR7* probe (Figure 4D).

A small fraction of *AgOR7*-positive ORNs (<10%), however, appeared to be devoid of *AgOR8* or *AgOR28* signals (Figure 4D, arrowheads). We cannot rule out the possibility that *AgOR8* or *AgOR28* might 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 might 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 CO₂ or 1-octen-3-ol? To address these questions, we conducted two-electrode, voltage-clamp recordings in *Xenopus* oocytes coinjected with complementary RNAs (cRNAs) encoding *AgOR8* or *AgOR28*, along with *AgOR7* and a *Gα₁₅* subunit. *Xenopus* oocytes respond to odorant stimuli by the release of Ca²⁺ from internal stores to activate an endogenous Ca²⁺-activated Cl⁻ channel. This system has been

successfully employed in previous studies so that functions of insect odorant and pheromone receptors could be characterized [18, 25].

We used a panel of 82 odorants in the *Xenopus* oocyte system. When *AgOR28* was coexpressed in oocytes with *AgOR7* and *Gα₁₅*, it conferred the strongest responses to 2,4,5-trimethylthiazole, acetophenone, and 2-acetylthiophene (Figures 5A–5C), as well as weaker responses to 16 other odorants (Figure 5B). Although *AgOR28* appeared to be a broadly tuned receptor (Figure 5B, 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 5B). Importantly, these data also demonstrated that odorant-induced increases in inward currents are consistently dose dependent (Figure 5C). The response spectrum of *AgOR28* matches nicely that of the cpC neuron in the SSR analyses, indicating that *AgOR28* is probably 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 5E, inset, and Figure 5F). 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 (Figures 5D and 5F). 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 5F). Because 1-octen-3-ol occurs as an optically active mixture of enantiomers in human and animal odors [14], 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 5E 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 Third ORN in the Capitate Peg Expresses Mosquito CO₂ Receptors

In *Drosophila*, one *GR* gene, *DmGR21a*, has been implicated in CO₂ detection [26, 27] and, more recently, it has been shown that *DmGR21a* together with a second *GR*, *DmGR63a*, mediate CO₂ sensitivity [28, 29]. Both *An. gambiae* and *Ae. aegypti* have three closely related homologs of *DmGR21a* and *DmGR63a* (Figure S2) (20, L.B. Kent and H.M. Robertson, personal communication). Although *AgGR22* and *AgGR24* are likely to be orthologs of *DmGR21a* and *DmGR63a*, with 68% and 65% identity, respectively [20], *AgGR23* is less conserved, sharing 38% and 26% identity with *DmGR21a* and *DmGR63a*, respectively (Figure S2). 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 S2), suggesting that *AgGR23* is likely to be the ortholog of a *DmGR* that has been lost subsequent to the divergence of these two species.

To investigate whether these putative mosquito CO₂ receptors are expressed in the maxillary palp, we carried out FISH experiments with riboprobes for all three *AgGR* genes (Figures 4E–4H and Figure S4). We reasoned that the CO₂-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 (Figures 4E and 4F and data not shown). This extends recently published data showing the colocalization of *DmGR21a* and *DmGR63a* in *Drosophila* and *AgGR22* and *AgGR24* in *An. gambiae* [28]. 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 4G). This is further supported by the observation that *AgGR22* and *AgOR28* labeled paired cell bodies in the maxillary palp (Figure 4H).

Moreover, we note that the *AgGR22*-positive ORN has a much larger cell volume than either of the *AgOR7*-positive ORNs (Figures 4G and 4H). This is in accordance with its more voluminous dendrite that displays considerably more branching than do the other two dendrites within the capitata peg (Figure 1C) [6].

Coexpression of *AgGR* Genes Confers a CO₂ Response

To test whether the *AgGR* genes are capable of conferring a response to CO₂, we expressed them in an in vivo expression system, the empty neuron system [30]. 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 CO₂. However, upon increasing the dosage of both transgenes by 2-fold, we observed a marked response to CO₂: 27 ± 5.6 spikes/s (standard error of the mean [SEM]; n = 8) over the spontaneous firing rate (Figures 6A and 6B). A steep dependence on gene dosage was also found for the *Drosophila* orthologs of these genes [29]. Cyclohexanone elicited only 4.7 ± 1.8 spikes/s (SEM; n = 6) (data not shown), suggesting the possibility that response to this odorant (Figures 3A and 3B) depended on *AgGR23* or another receptor.

To investigate whether *AgGR23* acts in detection of cyclohexanone or CO₂, we added an *AgGR23* transgene to the empty neuron containing a single copy of *AgGR22* and *AgGR24*. We found that the 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 CO₂ response from -1.4 ± 1.0 spikes/s (n = 10) to 14 ± 4.2 spikes/s (n = 8) (Figures 6A and 6B). Although the *AgGR23* transgene was a myc-tagged genomic clone, and although we have not tested the response of a neuron containing two copies of all three transgenes, the results clearly implicate *AgGR23* in the CO₂ response.

In summary, we have shown that coexpression of *AgGR* genes, orthologs of *Drosophila* CO₂ receptors, is sufficient to confer responses to CO₂ on CO₂-insensitive ORNs. These results, together with our FISH experiments, show that the ORN coexpressing *AgGR22*, *AgGR23*, and *AgGR24* is the CO₂-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 capitata-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 [17, 24], and this expression pattern might 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 labelum of adult females [5]. 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* [5]. 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 [3, 31], albeit with much lower sensitivity (threshold at circa 0.25 mg versus 0.85 pg) and response intensity (maximal response at 10 spikes/s versus 165 spikes/s) [31]. 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 [32]. This might 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 [17, 33].

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 the highly sensitive detection of two important mosquito kairomones, CO₂ and 1-octen-3-ol. Within our panel of tested compounds, the CO₂-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 to be 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 (Figures 3A, 5B, and 5E). 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 [18, 34].

AgOR8 and *AgOR28* were shown to vary sharply in their breadth of tuning: Although *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 [34, 35], *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 [36]. Because 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 might mediate responses to nectar-feeding or oviposition cues. It should, however, be noted that other formal possibilities exist because 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, and considerably weaker responses were elicited from 3-octanone and 1-hexen-3-ol. Furthermore, the native *AgOR8*-expressing cpB neuron as well as the CO₂-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 [35]. The monounsaturated eight-carbon alcohol 1-octen-3-ol is a well-established odorant cue for mosquitoes [11, 37] and has been documented to synergize the maxillary-palp-based attractiveness of CO₂ to several mosquito species [9]. Interestingly, *Ae. aegypti* also manifests a highly sensitive electrophysiological response to 1-octen-3-ol in its maxillary palp [14] and has a close ortholog of *AgOR8* (*AaOR8*, with 70% identity) that is likewise exclusively expressed in the maxillary palp [38].

The recent data for their *Drosophila* homologs [28, 29], 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 CO₂ detection in *An. gambiae*. This ORN reaches its maximal response level near 4000 ppm of CO₂ (Figure 3B), about one-tenth the concentration measured in human breath [7]. This concentration corresponds to an apparently important behavioral response threshold because *An. gambiae* and other mosquitoes do not show increased responses to CO₂ levels above 4000 ppm [39]. Fruit flies, however, use this odorant as an avoidance signal [26, 27], and, accordingly, the CO₂-sensitive neuron continues to increase its firing rate at CO₂ concentrations above 4% [28]. The *An. gambiae* cpA neuron, similar to the CO₂-responsive ORN in *Ae. aegypti* [13], is a highly sensitive CO₂ detector, exhibiting a steep slope of dose-response curves that facilitates detecting small increments in CO₂ concentration. However, when compared with the *Ae. aegypti* neuron, the CO₂-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 CO₂-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 CO₂ in favor of a greater reliance on human-specific cues as a basis for host-seeking behaviors [1, 9].

Like their orthologs in *Drosophila*, *AgGR22* and *AgGR24* encode functional receptors and produce a significant response to CO₂ when coexpressed in an “empty” olfactory neuron. This result indicates that the two AgGRs are capable of being functionally expressed in a nonnative ORN. Furthermore, as was the case for *DmGR21a* and *DmGR63a* [29], the level of response depends on the dosage of *AgGR22* and *AgGR24* genes. Two copies of both transgenes induced CO₂ sensitivity in the ab3A neuron, whereas one copy of both transgenes was insufficient. Interestingly, the ectopic CO₂ response in the ab3A neuron is lower than that measured in the *An. gambiae* cpA neuron. This might 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 might facilitate CO₂ chemosensation [6, 22], 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* CO₂ receptors and is coexpressed with *AgGR22* and *AgGR24* within the mosquito CO₂-sensitive ORN, enhances the response conferred by *AgGR22* and *AgGR24*.

Both FISH and SSR analyses reveal a stereotyped triad of ORNs within the capitata-peg-sensillum population of the maxillary palp: Two *AgOR7*-positive neurons always pair with a third ORN coexpressing *AgGR22*, *AgGR23*, and *AgGR24* (Figure 7). One possible biological implication for this stereotyped pairing of the CO₂-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 CO₂ 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 might ultimately be able to perceive the ratio between these two compounds within the host odor blend with higher fidelity and spatial resolution [40]. This is remarkably 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 [18, 41].

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 capitata 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, CO₂ and 1-octen-3-ol. The stereotyped organization of three ORNs within the capitata-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 [24, 42]. Although no apparent functional segregation of antennal and palpal ORNs has been observed in *Drosophila* [23, 42], one type of sensilla, the trichoid sensilla, is exclusively associated with pheromone detection in this insect [43-45]. Our results suggest that the *An. gambiae* capitata-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 CO₂ 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 CO₂ and 1-octen-3-ol among a complex odor blend. Perception of CO₂ 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 [1, 9].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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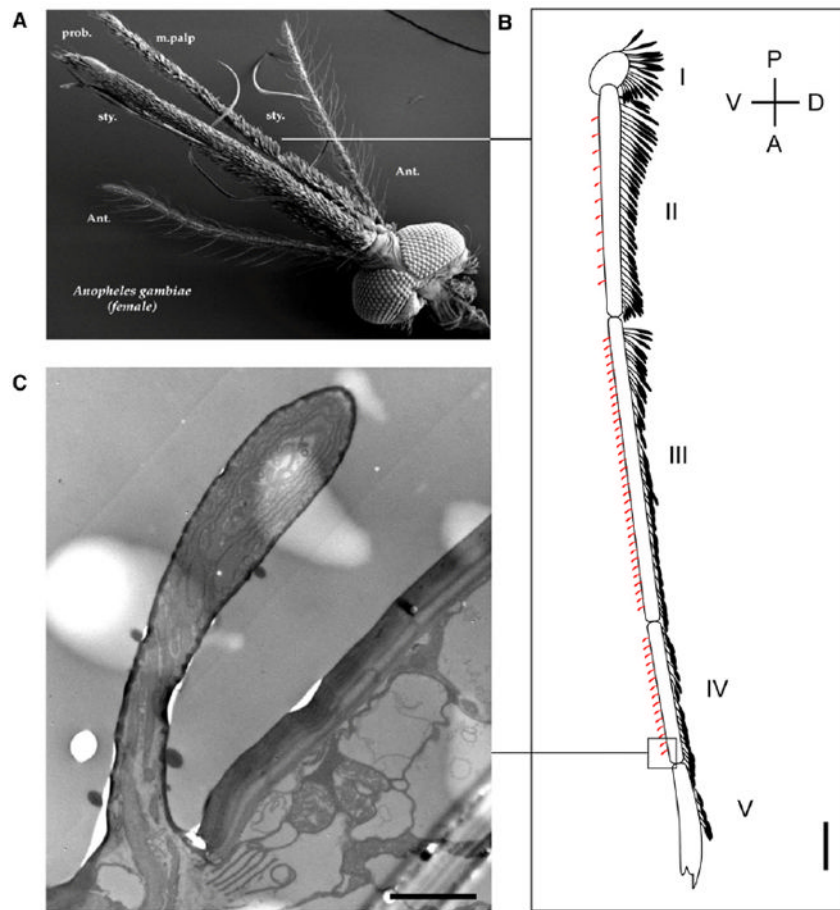


Figure 1. The Maxillary Palp in Female *An. gambiae* Mosquitoes

(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 μ m.

(C) Transmission electron micrograph showing the specialized lamellate dendritic structure of one neuron within the capitate-peg sensillum. The scale bar represents 2 μ m.

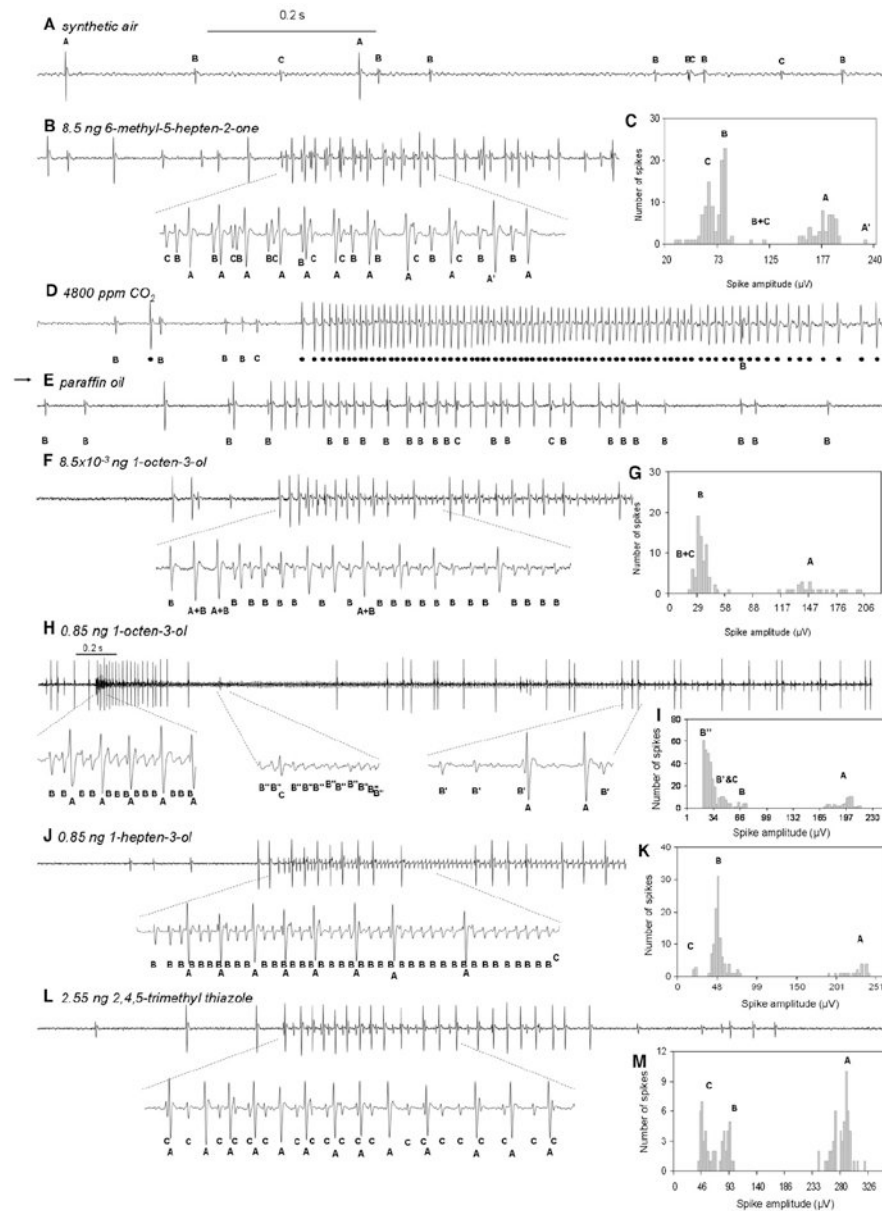


Figure 2. Single-Sensillum Electrophysiological Recordings from Capitrate-Peg Sensilla
 Representative extracellular recordings from capitrate-peg sensilla in response to air, CO₂, paraffin oil, and four other odorants. Individual action potentials (spikes) are labeled A, B, or C according to spike amplitude, 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 capitrate-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 CO₂.

- (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).

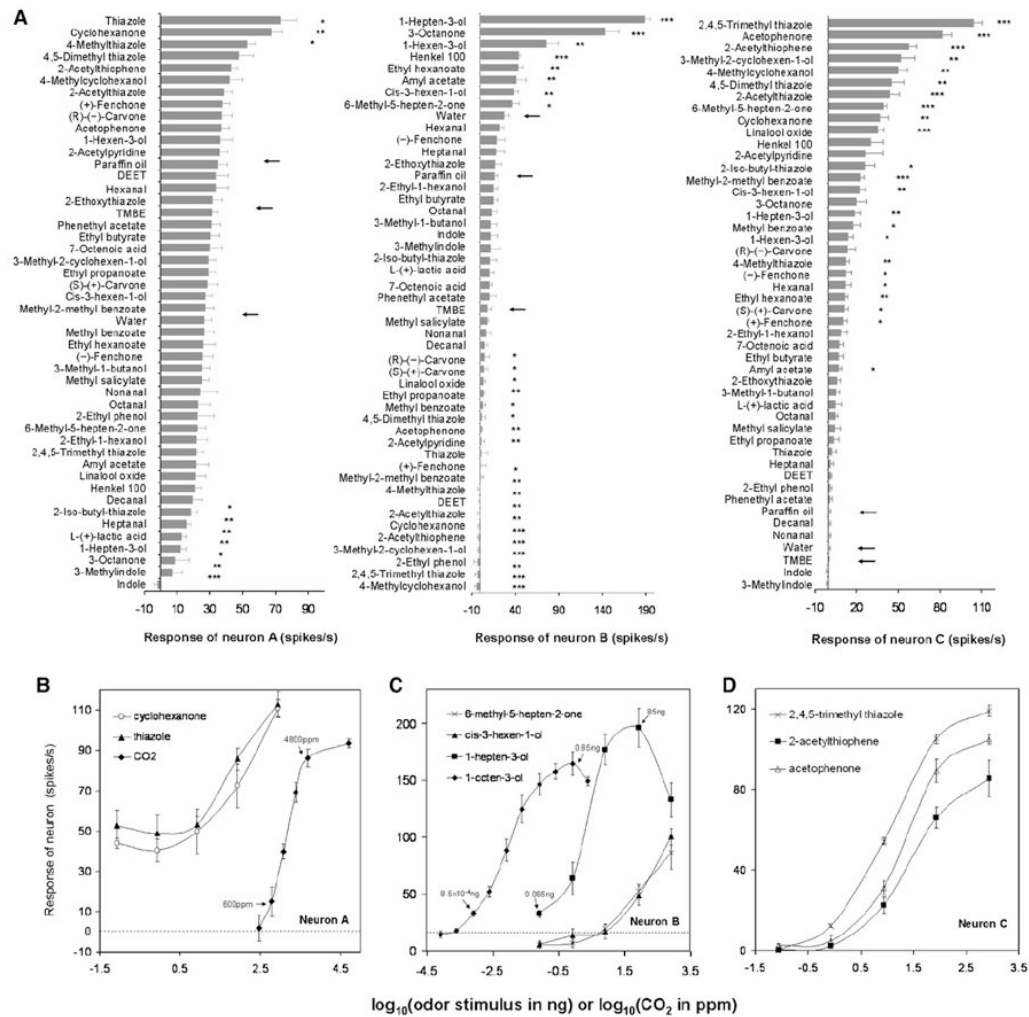


Figure 3. Response Spectra and Dose-Response Curves of the Three ORNs within the Capitate-Peg Sensillum

(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 ($n = 8\text{--}12$ 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 CO₂ are plotted as \log_{10} ppm, 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 CO₂, 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.

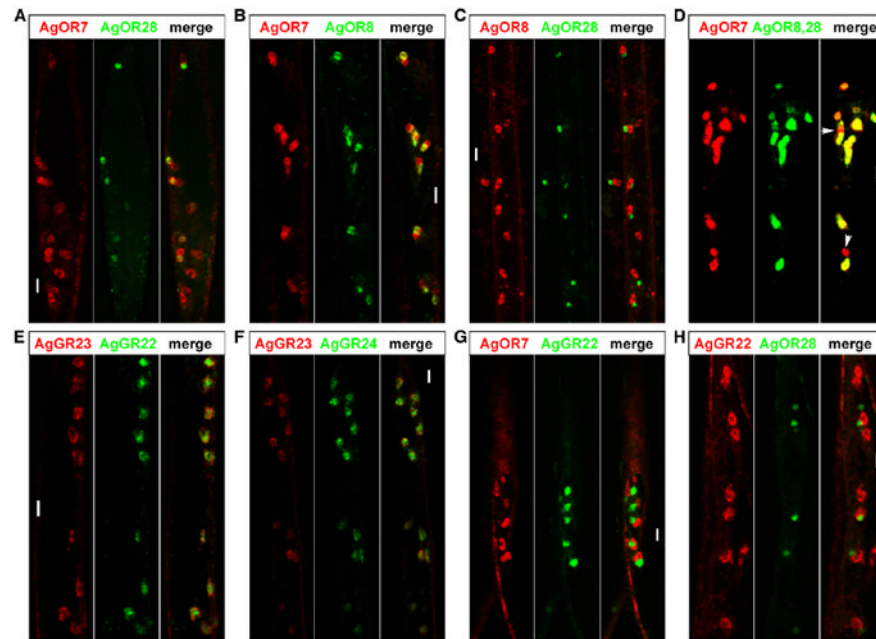


Figure 4. Expression of *AgOR* and *AgGR* Genes in the Maxillary Palp

- (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 μ m.

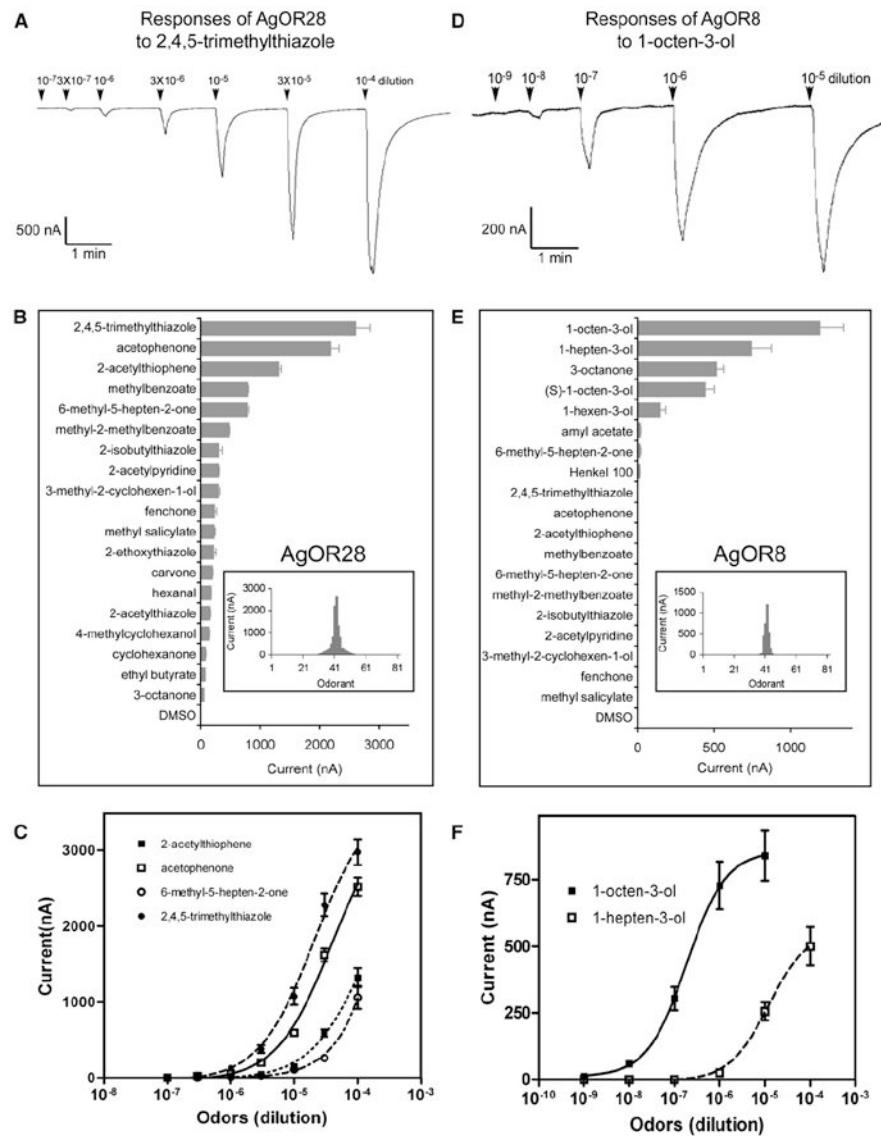


Figure 5. Functional Characterization of *AgOR8* and *AgOR28* Genes in *Xenopus Oocytes*
 (A) Response trace of *AgOR28* to the indicated concentrations of 2,4,5-trimethylthiazole. Odorants are applied for 10 s at the time point indicated by the arrows and induce inward currents in oocytes coinjected with cRNAs encoding *AgOR28*, *AgOR7* and a *Ga₁₅* 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 (\pm 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 (\pm SEM) of 5–6 independent oocytes.

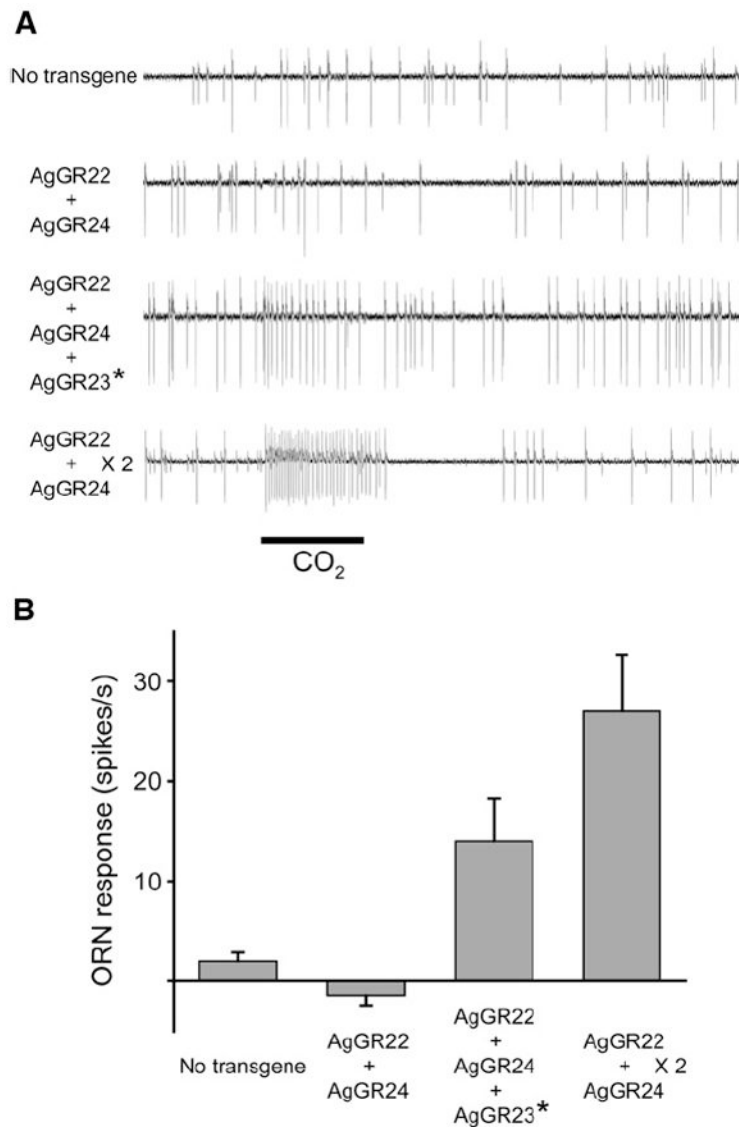


Figure 6. AgGR22, AgGR23, and AgGR24 Confer a Response to CO₂

(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 [30]. 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% CO₂.

(B) Mean responses to 100% CO₂ 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 CO₂ 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.

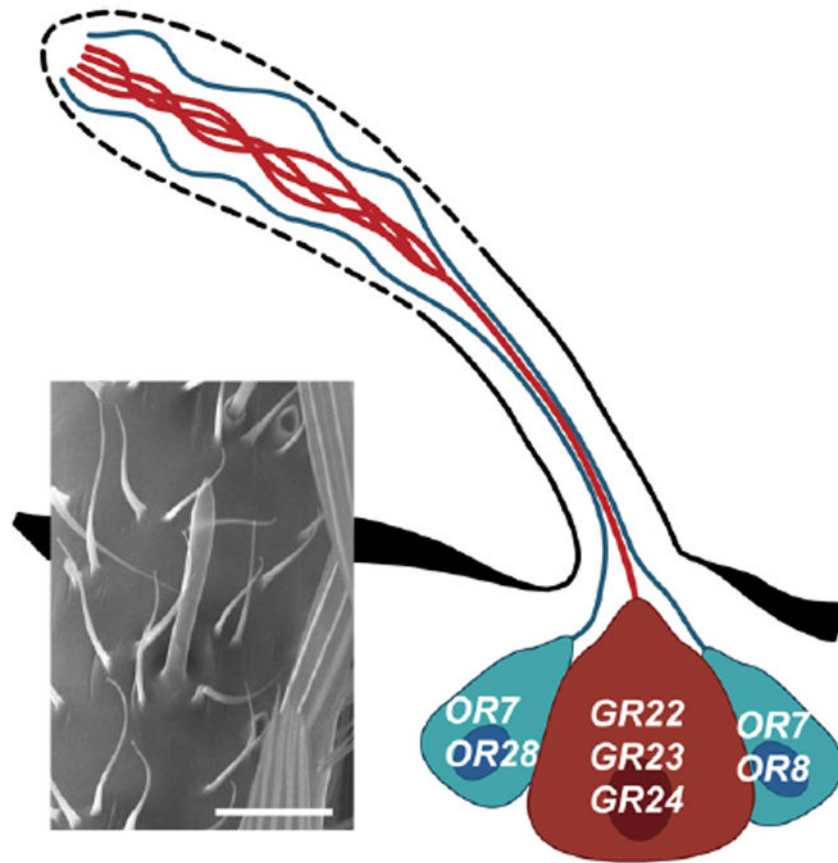


Figure 7. Spatial Organization of the Three Neurons within the Capitulate-Peg Sensillum
 Schematic drawing of three ORNs in a capitulate peg as opposed to a scanning electron micrograph of the sensillum (left). Three ORNs form stereotyped triads within each capitulate-peg sensillum. The neuron coexpressing *AgGR22*, *AgGR23*, and *AgGR24* (red) responds to CO₂. The other two ORNs (green) express *AgOR7* along with *AgOR8* or *AgOR28*, respectively. The scale bar represents 10 μm.