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Odourant reception in the malaria mosquito *Anopheles gambiae*

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Summary

The mosquito *Anopheles gambiae* is the major vector of malaria in sub-Saharan Africa. It locates its human hosts primarily through olfaction, but little is known about the molecular basis of this process. Here we functionally characterize the *Anopheles gambiae* Odourant Receptor (AgOr) repertoire. We identify receptors that respond strongly to components of human odour and that may act in the process of human recognition. Some of these receptors are narrowly tuned, and some salient odourants elicit strong responses from only one or a few receptors, suggesting a central role for specific transmission channels in human host-seeking behavior. This analysis of the *Anopheles gambiae* receptors permits a comparison with the corresponding *Drosophila melanogaster* odourant receptor repertoire. We find that odourants are differentially encoded by the two species in ways consistent with their ecological needs. Our analysis of the *Anopheles gambiae* repertoire identifies receptors that may be useful targets for controlling the transmission of malaria.

Mosquitoes transmit many diseases, including malaria, which afflicts hundreds of millions of people each year[1]. The malaria burden is heaviest in sub-Saharan Africa, where the *An. gambiae* mosquito is the major vector. *An. gambiae* relies heavily on olfactory cues to identify its human hosts[2–4], but the molecular basis of host-seeking behavior is unknown.

Insects detect odours via olfactory receptor neurons (ORNs). The odourant specificities of many ORNs are conferred by the expression of individual odourant receptor genes[5]. A family of 79 *AgOr (Anopheles gambiae Odourant receptor)* genes has been identified bioinformatically in *An. gambiae*[6, 7]. Two of these receptors have been characterized functionally[8] using an *in vivo* heterologous expression system, the "empty neuron" system[9], which has also been used to decode the *D. melanogaster* odourant receptor repertoire[10–12]. These results invited a systematic, functional characterization of the AgOr repertoire and a

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Author contributions

Electrophysiology and computational analysis were performed by A.C. Molecular cloning was performed by A.C., G.W., and C.-Y.S. A.C. and J.C. wrote the manuscript. All authors contributed to the design and interpretation of the study.

Competing Interests

The authors declare no competing financial interests.

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comparison between the receptor repertoires of these two species, which exhibit different olfactory-driven behaviors. *D. melanogaster* consumes fruit and is considered a generalist. *An. gambiae* has evolved an anthropophilic host-seeking olfactory response that allows it to find human bloodmeals[4]. Little is known about how the odourant receptor repertoires of these species have adapted to meet their distinct ecological requirements.

Functional expression of the *AgOr* repertoire

To investigate the molecular basis of odour reception in *An. gambiae*, we amplified the coding regions of 72 *AgOr* genes from olfactory organ cDNA of adult mosquitoes. We then expressed each AgOr in the "empty neuron," a mutant ORN in *D. melanogaster* that lacks its endogenous odourant receptor[9]. Fifty of the 72 cloned AgOr receptors were functional in the empty neuron, conferring a regular, characteristic, spontaneous firing rate and exhibiting excitatory and/or inhibitory responses to odourant stimuli (Figure 1a). This success rate (69%) is comparable to that for *D. melanogaster* antennal *Or* genes (77%) expressed in the empty neuron[11].

The empty neuron system previously was demonstrated to be a high-fidelity expression platform for the *D. melanogaster Or* genes[11], referred to here as *DmOrs*. Since *An. gambiae* and *D. melanogaster* are separated by 250 million years of evolution[13], we wanted to determine whether the empty neuron is also a faithful expression system for *AgOr* genes. One of the few AgOrs that has been unequivocally mapped to a specific ORN in the mosquito is AgOr8, which in its endogenous neuron responded to seven- and eight-carbon-chain compounds among a panel of tested odourants[14]. We expressed AgOr8 in the empty neuron and found that its response profile closely resembled that of the endogenous neuron (Figure 1b). We also generated dose-response curves for two ligands of AgOr8, 1-octen-3-ol and 1-hepten-3-ol (Supplementary Figure 1), and found that the differential sensitivity to these ligands observed in the endogenous neuron[14] was maintained in the empty neuron. These results validate the empty neuron as a faithful heterologous expression system for AgOrs.

The 50 functional AgOrs were tested against a chemically diverse panel of 110 odourants, including components of human emanations and oviposition site volatiles (Supplementary Table 1). Fifty-three of the 110 odourants were previously tested against the *D. melanogaster* antennal receptor repertoire in the empty neuron system[12], permitting functional comparisons between the odourant receptor repertoires of the fruit fly and the mosquito.

We tested each of the functional AgOrs against the 110-odourant panel, generating a data set of 5500 odourant-receptor combinations, with each combination tested n 5 times (Figure 1c and Supplementary Table 2a–d). We found that individual receptors respond to subsets of odourants, and individual odourants activate subsets of receptors, consistent with a combinatorial model of odour coding[12, 15, 16]. Some receptors gave strong responses (defined as 100 spikes/second) to many odourants, while other receptors are more selective (Supplementary Table 2a–d). These differences were visualized by generating a tuning curve for each receptor (Figure 2). The breadths of the tuning curves were quantified

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according to their kurtosis value, a measure of the "peakedness" of the distribution (Supplementary Tables 3a, b). We found a continuum of AgOr tuning breadths ranging from broad to narrow, consistent with analysis of the *D. melanogaster* Or repertoire[12]. We then considered the receptors at each extreme for insight into the molecular basis of odour recognition.

Narrowly tuned receptors for salient odourants

Narrowly tuned receptors have been suggested to be specialist channels that carry information about odourants of high biological relevance[17]. Consistent with this hypothesis, the most narrowly tuned AgOrs are robustly excited by odourants with high biological salience. Among the receptors that respond strongly to at least one odourant of the panel, the most narrowly tuned are AgOr2, AgOr8, AgOr5, and AgOr65. AgOr2 is narrowly tuned to a small set of aromatics including indole, which was found to constitute nearly 30% of the volatile headspace of human sweat[18]. AgOr8 responds strongly to 1-octen-3-ol, a human volatile that is a strong attractant for several species of mosquito[4]. AgOr5 is tuned to 2,3-butanedione, is a metabolic byproduct of human skin microflora[19]. AgOr65 responds strongly to 2-ethylphenol, which is found in the urine of many animals[20, 21]. We found no mosquito receptors narrowly tuned to esters or aldehydes, odourants that dominate the headspace of many fruits[22, 23]. By contrast, among the most narrowly tuned receptors in the fruit fly, the strongest responses are in most cases to esters (DmOr85a, DmOr59b, DmOr67c) or to a terpene that contains an ester group (DmOr82a)[12] (Supplementary Table 3b).

Some of the narrowly tuned AgOrs, in addition to responding strongly to odorants with high biological salience, respond with high sensitivity to these odourants. AgOr8 and AgOr2 respond to concentrations of 1-octen-3-ol and indole that range over more than four orders of magnitude and have response thresholds that lie between a 10^{-7} and a 10^{-6} dilution (Supplementary Figure 2).

Broadly tuned receptors lie at the other end of the AgOr distribution. It is possible that these receptors act in signaling the presence of odourants but not in specifically identifying or discriminating among them. We found that in *An. gambiae*, all strong responses to esters and aldehydes are conferred by broadly tuned AgOrs (all have kurtosis values less than the mean; Supplementary Table 2b and Supplementary Table 3a).

Salient odourants that activate specific receptors

"Odourant tuning curves," the reciprocal of receptor tuning curves, were also generated (Figure 3, Supplementary Table 3c, Supplementary Figure 3). For each odourant we plotted the responses of the 50 receptors along the X-axis, placing the strongest response at the center. Interestingly, the five odourants with the most narrow response distributions are all highly relevant to mosquito ecology. 3-methylindole is an oviposition site volatile that induces egg-laying and ORN responses in *Culex* mosquitoes[24, 25]. Indole is another oviposition site volatile[26], in addition to being a major component of human emanations that is found both in sweat[18] and human breath[27]. Geranyl acetate and citronellal are emitted

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from plants that are repellant to *An. gambiae*[28, 29]. Dimethylsulfide is emitted in human breath[30] and is attractive to the *Aedes aegypti* mosquito[31].

D. melanogaster odourant tuning curves were constructed from earlier data[12] to identify odourants that likewise strongly activate a small number of receptors (Supplementary Table 3d). In contrast to the *An. gambiae* tuning curves, many of the odourants with high kurtosis values are esters, the dominant chemical class in fruit emanations[23]. Together, the mosquito and fruit fly odourant tuning curves support a model in which odourants of particular biological relevance are coded via a small number of channels.

Taken together, these results suggest that odourant and receptor tuning analyses provide complementary avenues to identify receptors and odourants that are important for innate insect behavioral responses. We note with special interest that in *An. gambiae,* one of the "narrowly tuned" odourants activates one of the narrowly tuned receptors. AgOr2 is strongly activated by indole, the odourant that constitutes almost 30% of the volatile headspace of human sweat[18].

Diverse temporal dynamics of receptor responses

DmOrs were previously shown to yield responses that vary widely in their temporal characteristics, and the temporal dynamics were specific to the odourant-receptor combination[11,12]. To investigate the temporal dynamics of responses from AgOrs, we generated peristimulus time histograms for a number of odourant-receptor combinations (Supplementary Figure 4). As was observed with DmOrs, we find a diversity of temporal dynamics. Some odourants, including the human volatiles 1-octen-3-ol and indole, were capable of generating tonic responses that persisted throughout the three-second analysis period. Linalool oxide also generated a prolonged response. These odourants generated phasic responses from other receptors. The diversity of these responses suggests that temporal features may be a rich source of information about odourant identity.

How the mosquito covers odour space

How is the chemical world represented by the AgOrs? Excitatory activity is not distributed evenly across the odourant panel (Figure 1c, Supplementary Table 2a). For example, 28% of the strong responses were generated by heterocyclics, which constitute only 8% of the odourants in the panel. However, chemical class is only one descriptor of molecular identity. To represent the structural diversity among odourants more fully we adapted a recently developed odourant metric[32]. This metric is based on an optimized set of 32 molecular descriptors, including functional group, carbon chain length, and other physicochemical properties, which provide the basis of a 32-dimensional coordinate system. Each odourant can be mapped to a unique location in this multidimensional space. Odourants that are structurally similar lie close together in the space, while odourants that are structurally dissimilar lie far apart. To visualize this space we applied principal components analysis (PCA) to project it into two dimensions (Supplementary Figure 5a). As shown by the aromatics, odourants can be of the same chemical class yet map far apart.

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Having mapped the odourants of the panel into this chemically defined odour space, we then asked how the AgOr repertoire covers the space. To illustrate the responses elicited by each odourant we generated a bubble plot, in which the location of each bubble indicates odourant identity, and the size of each bubble represents the magnitude of the response to that odourant, summed across all receptors and measured in total spikes/second (Figure 4a).

The AgOr repertoire is sensitive to a broad region of the odour space. However, the responses are not of uniform magnitude across the space. The odourants in the region of the space that is occupied by heterocyclics (purple), for example, elicit greater responses than the odourants in the region that is home to esters (dark green), and much greater responses than those in the region inhabited by carboxylic acids (pink). The AgOr repertoire may have evolved particular sensitivity to certain regions of odour space, such as the region containing aromatics, some of which are major components in human emanations [18, 33–39], and heterocyclics, a chemical class that includes volatiles proposed to promote oviposition behavior[40]. Such enhanced sensitivity could reflect the insect's investment in detecting and discriminating among chemicals of these classes.

We also investigated the distribution of inhibitory responses across the *An. gambiae* receptor repertoire, which are not visualized in the odour space described above. As was observed across the *D. melanogaster* receptor repertoire[12], most odourants elicit at least one inhibitory response, and most receptors are inhibited by at least one odourant (Supplementary Table 2a).

Differences between *An. gambiae* and *D. melanogaster* odour spaces

We next asked whether the *An. gambiae* and *D. melanogaster* Or repertoires differ in their coverage of odour space. As an initial means of addressing this issue, we considered the 53 odourants that were tested against both the AgOr and the DmOr repertoires[12] and constructed odour spaces of the type described above for each receptor repertoire.

The two species differed in their relative coverage of odour space. The mosquito allocated greater relative coverage to the aromatics (Figure 4b; dark blue in lower right quadrant). By contrast, the fly devoted greater relative activity to some of the esters (dark green) and one of the two aldehydes that were compared (gray) (Figure 4c, Supplementary Figure 5b). We then compared the two species with respect to the strong responses (100 spikes/second). In the mosquito, 15% of the receptor-aromatic combinations yielded strong responses, compared to 7% of the receptor-ester combinations. By contrast, in the fly, 9% of the receptor-aromatic combinations yielded strong responses, compared to 20% of receptor-ester combinations (Figure 4d).

We next considered whether the species differed with respect to another kind of odour space, a biological odour space that relates odourants based on the primary sensory signals they generate. We created a space in which each axis represents the response magnitude (in spikes/second) for one odourant receptor, as described previously[12]. Odourants that elicit similar patterns of activity across the receptor repertoire map close together. Odourants that are close may be similar in their perceptual qualities, and may be more difficult for the animal to discriminate because they generate similar patterns of ORN activity. Experiments

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conducted with *Drosophila* larvae have provided evidence to support a relationship between such odour space distances and perception[10].

We constructed such spaces for the mosquito and the fly and depicted them in three dimensions by applying PCA (Figure 5a). Odourants of the same chemical class tend to cluster together (Figure 5a), as previously observed in the fly[12]. However, some chemical classes were differentially distributed in the odour spaces of the two insects. Esters were more widely distributed in *D. melanogaster* space than in *An. gambiae* space, while aromatics were more widely distributed in *An. gambiae* space (Figures 5b and 5c). To quantify these differences, we calculated the Euclidean distance for every pair of odourants with the same functional group, within the odour space of each species. We found that the mean inter-odourant distance for esters is significantly higher for *D. melanogaster* than *An. gambiae,* while the mean inter-odourant distance for aromatics is higher for *An. gambiae* ($p<0.001$ for esters, $p=0.01$ for aromatics, Mann-Whitney); there were no differences in distances among alcohols or ketones, the other groups that could be compared. These results suggest that mosquitoes may be better able than fruit flies to discriminate among aromatics, while fruit flies may be better able to discriminate among esters, perhaps reflecting the biological relevance of these classes of compounds to the animals.

Are the functional differences between the mosquito and fruit fly Or repertoires due to a particular clade of mosquito or fly receptors? When the *AgOr* family was first identified, phylogenetic analysis revealed a clade of *An. gambiae* odourant receptors with no close *D. melanogaster* relatives, and a clade of *Drosophila* odourant receptors with no close *An. gambiae* relatives[6, 7] (see also Supplementary Figure 6). Do these species-specific clades of receptors respond to odourants of a particular kind? We used matrix analysis to generate a dendrogram of receptors (Supplementary Figure 7), and PCA to create a "receptor space," based on the responses of the receptors to odorants (Supplementary Figure 8). In neither case did we observe clustering of receptors of the species-specific clades. Thus, the observed functional differences between the *An. gambiae* and *D. melanogaster* odourant receptor repertoires may reflect evolutionary changes distributed across the receptor repertoires rather than concentrated in a specific branch of the phylogenetic tree.

We note finally that no AgOr showed even a modest response of 50 spikes/second to any carboxylic acid or to any amine in our system. By contrast, some DmOrs respond strongly to certain carboxylic acids[12, 41]. Recently, a set of variant-ionotropic glutamate receptors that respond to amines and carboxylic acids have been identified in *D. melanogaster*[42]. Receptors of this class may detect these compounds in *An. gambiae*.

Discussion

Here we have functionally characterized the AgOr repertoire of odourant receptors from the mosquito *An. gambiae*. The olfactory system of *An. gambiae* allows the insect to locate human blood-meal hosts, thereby facilitating the transmission of malaria. We have identified individual receptors that respond robustly to human volatiles and may be central to the process by which the mosquito identifies its human hosts.

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Strikingly, some receptors that respond to human odourants are narrowly tuned and highly sensitive. Reciprocally, some notable odourants elicit strong responses from one or a few receptors. These highly focused relationships between certain odourants and certain receptors suggest a role for specific transmission channels in guiding the animal's behavior. A recent study of the *D. melanogaster* antennal lobe documented lateral inhibitory interactions that increased with greater total ORN input[43]. Odourants that excite few receptors in the mosquito may produce signals that suffer less inhibition and enjoy more saliency. Another study found that ablation of either of two narrowly tuned ORN classes impaired behavioral attraction to their cognate odourants[44].

Since our analysis was conducted using the same expression system as our previous study of *D. melanogaster* odourant receptors, it provided a unique opportunity to compare the Or repertoires of two species that belong to the same order but that exhibit different olfactory-guided behaviors. An outstanding question in the field of olfaction is how an organism's ecology shapes the function of its odourant receptor repertoire. A full answer to this question requires the functional characterization of entire receptor repertoires. We found that the two species show different coverage of a chemically defined odour space. Certain classes of odourants are differentially distributed in a biologically defined odour space of each species. These differences suggest the evolution of olfactory acuity and discriminatory power consistent with the ecological needs of each species. These evolutionary changes appear to have occurred over the odourant receptor repertoire as a whole, as opposed to having been effected by the emergence of a species-specific receptor clade.

The results may have implications for the control of malaria, one of the world's most devastating diseases. Screens for activators and inhibitors of selected receptors may identify compounds that attract mosquitoes into traps, interfere with their navigation, or repel them.

Methods summary

AgOr cDNAs were cloned by standard procedures and expressed in the empty neuron as described previously[8, 14]. Extracellular single-unit physiology was performed as described previously[9, 11]. Physicochemical odor space was constructed using the set of 32 optimized DRAGON descriptors[32], which were normalized.

Methods

Cloning of the *AgOrs*

Five-day-old laboratory-maintained *An. gambiae* mosquitoes of the Suakoko strain were cold anesthetized and the antennae, maxillary palpae, and proboscises were dissected by hand on a chill table. RNA was prepared with RNeasy (QIAGEN) according to the manufacturer's instructions. The RNA preparation was used for oligo dT-primed cDNA synthesis with Superscript II Reverse Transcriptase (Invitrogen) for the generation of 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 with a Mastercycler Gradient (Eppendorf) under the following conditions: 94°C for 5 min; 40 cycles of 94°C for 30 s, 55°C to 60°C for 30 s (annealing temperature varied

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depending on primer pair), 72°C for 60 s; and 72°C for 7 min. PCR amplification products were separated on a 1.0% agarose gel and were cloned into the Gateway pENTR entry vector (Invitrogen) or pGEM-TEasy (Promega) and verified by sequencing. Sequences of at least two independent clones were obtained for each *Or* and compared to verify polymorphisms as such rather than PCR errors. Sequence discrepancies were resolved by a second PCR reaction. We amplified 72 *AgOrs*, in addition to *AgOr7*, the *An. gambiae* ortholog of the atypical *D. melanogaster* odourant receptor gene *Or83b*[45]. Four (*AgOr37*, *AgOr40*, *AgOr52*, *AgOr58*) of the remaining six *AgOr* genes that were not amplified from adult tissue are expressed in the larval stage[46], and the other two may be artifacts of gene annotation.

Drosophila stocks and transgenes

The ab3A mutant flies and *Or22a-GAL4* constructs were described previously [9], [11]. To generate *UAS-Or* constructs, the pUAST vector (C. G. Warr) was adapted to generate a Gateway (Invitrogen) compatible destination vector. LR recombination reaction was performed with the Gateway (Invitrogen) pENTR entry clones and the modified pUAST vector. For those amplification products cloned into pGEM-TEasy, subcloning into unadapted pUAST was performed using the restriction enzymes *Bgl*II, *Kpn*I, and *Not*I (New England Biolabs). An exception was AgOr53, which was cloned into pNmyc-UAST vector (C. G. Warr) in frame with the start codon and three copies of the myc tag coding sequence. The resulting protein had an N-terminal myc tag. No other receptors had epitope tags.

Electrophysiology

Extracellular single-unit recordings were performed essentially as described previously [11]. Odourant stimuli were prepared in Pasteur pipettes as described previously [11]. Chemicals were of the highest purity available (Sigma-Aldrich). Seven-octenoic acid and cis/trans-3-methyl-2-hexenoic acid were synthesized by Richman Chemical (Lower Gywnedd, PA). All chiral chemicals were racemic mixtures with the exception of (+)-carvone, (−)-carvone, (+)-fenchone, (−)-fenchone, and L(+)-lactic acid. Ammonia, cadaverine, putrescine, acetic acid, propanoic acid, butanoic acid, isobutyric acid, isovaleric acid, L(+)-lactic acid, and 2-oxohexenoic acid were diluted in H_2O. Hexadecanoic acid, octadecanoic acid, 5α-androsten-3α-ol, were diluted in ethanol. All other odourants were diluted in paraffin oil (Fluka). Liquid odourants were diluted in solvent to a concentration of 10^{-2} volume/volume, and solid odourants were dissolved, 50 mg in 5 ml solvent.

Stimuli were presented by placing the tip of the pipette through a hole in a tube carrying a purified air stream (32 ml/s) directed at the fly and administering a pulse of charcoal-filtered air (3.2 ml/s) through the pipette containing the odourant. Pulse duration was 500 ms. Stimuli were used for a maximum of four presentations. Responses were quantified by subtracting the number of impulses in 500 ms of unstimulated activity from the number of impulses in the 500 ms following odourant stimulation, after a 150 ms delay to allow the odourant to travel down the airstream. Responses to diluents were also subtracted. For each odourant, each recording was from a separate sensillum, with no more than three sensilla analyzed per fly. Recordings were obtained from flies between 4 and 14 days old.

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A panel of six odourants previously tested by E. Hallem against the ab3B neuron were retested in this study to control for possible differences between electrophysiology rigs; none of the responses were significantly different (p   0.12 in all cases, t-test).

Data analysis

Principal component analysis (PCA), and hierarchical cluster analysis were performed using PAST, a statistics program (http://folk.uio.no/ohammer/past/) as described previously [12]. Physicochemical odour space was constructed using the set of 32 optimized DRAGON descriptors[32] (Talete, srl, DRAGON for Windows, version 5.5, 2007, http://www.talete.mi.it/). Descriptors were normalized. The twelve odourants that generate net negative (inhibitory) responses (1-chlorododecane, 3-methyl-2-hexenoic acid, cadaverine, cis-9-octadecanoic acid, delta-decalactone, hexadecanoic acid, nonanoic acid, octadecanoic acid, octanal, octanoic acid, putrescine, and tridecanoic acid) are not shown in the bubble plots of Figure 4. To generate odour spaces and for cluster analyses, we removed from analysis receptors that did not respond to any odourant on the panel with a response   50 spikes/second and odourants that did not elicit any responses   50 spikes/second (before solvent responses were subtracted) unless otherwise noted. Error bars represent SEM, unless otherwise noted. Phylogenetic analysis was performed with MEGA 4.0.2, using a Neighbor-joining algorithm and 500 replications. Peristimulus time histograms were generated using IGOR Pro 6.0 (Wavemetrics).

Supplementary Material

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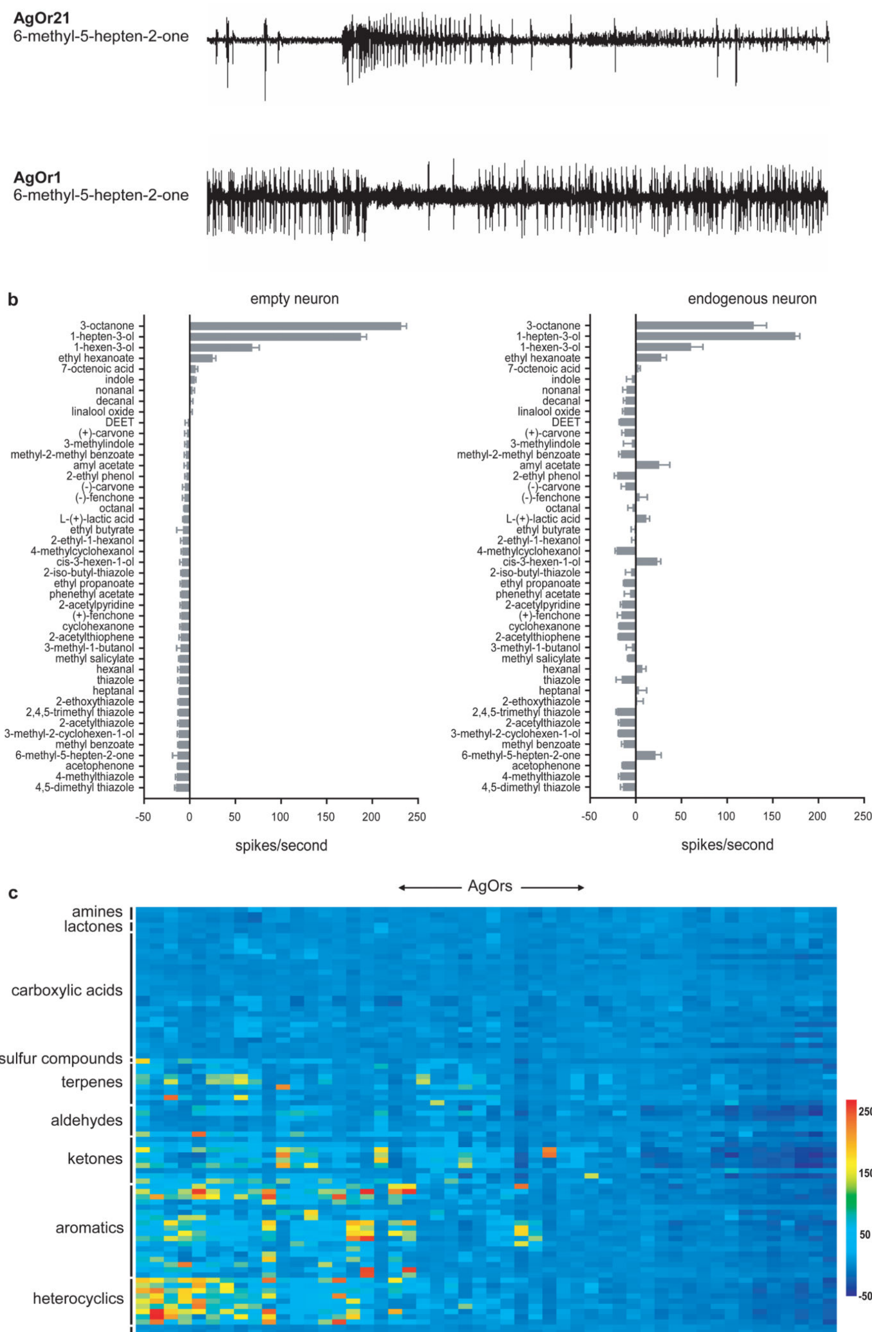

Figure 1. Functional characterization of the AgOrs

(a) Extracellular recordings from the empty neuron expressing an *AgOr*. Top, excitatory response of AgOr2 to 2-methylphenol; middle, excitatory response of AgOr21 to 6-methyl-5-hepten-2-one; bottom, inhibitory response of AgOr1 to 6-methyl-5-hepten-2-one. Action potentials from both neurons housed in the sensillum can be observed and distinguished by amplitude; the larger-amplitude action potential, from the 'A' neuron, expresses the *AgOr*, while the smaller-amplitude action potential, from the 'B' neuron, expresses its endogenous *D. melanogaster* odourant receptor[9]. (b) Left, odourant response

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profile of AgOr8 expressed in the empty neuron. Right, odourant response profile of the *An. gambiae* neuron that houses AgOr8 (adapted from Lu, *et al.*, 2007). All odourants were tested at a dilution of 10^{-2}. The spontaneous firing rate and the responses to the diluent are subtracted from odourant responses in each panel. Of the 28 odourants that inhibited the spontaneous firing rate by 50% or more in the empty neuron[47], 21 gave mean responses that were negative in the endogenous *An. gambiae* neuron. Error bars represent SEM. (c) Heat map of responses of the 50 functional AgOrs to 110 odourants. Response intensity is colour-coded according to the continuous colour scale on the right and represents the mean activity measured over a 0.5 second odourant stimulation period. Receptors, odourants, and numerical values are indicated in Supplementary Table 2. n = 5–6; for odourants that elicit responses   100 spikes/second, n = 6. Spontaneous activity and responses to diluent have been subtracted from response values. All odourants were tested at a 10^{-2} dilution. Odourants containing both a phenol ring and an ester moiety are classified as aromatics; terpenes containing an ester moiety are classified as terpenes.

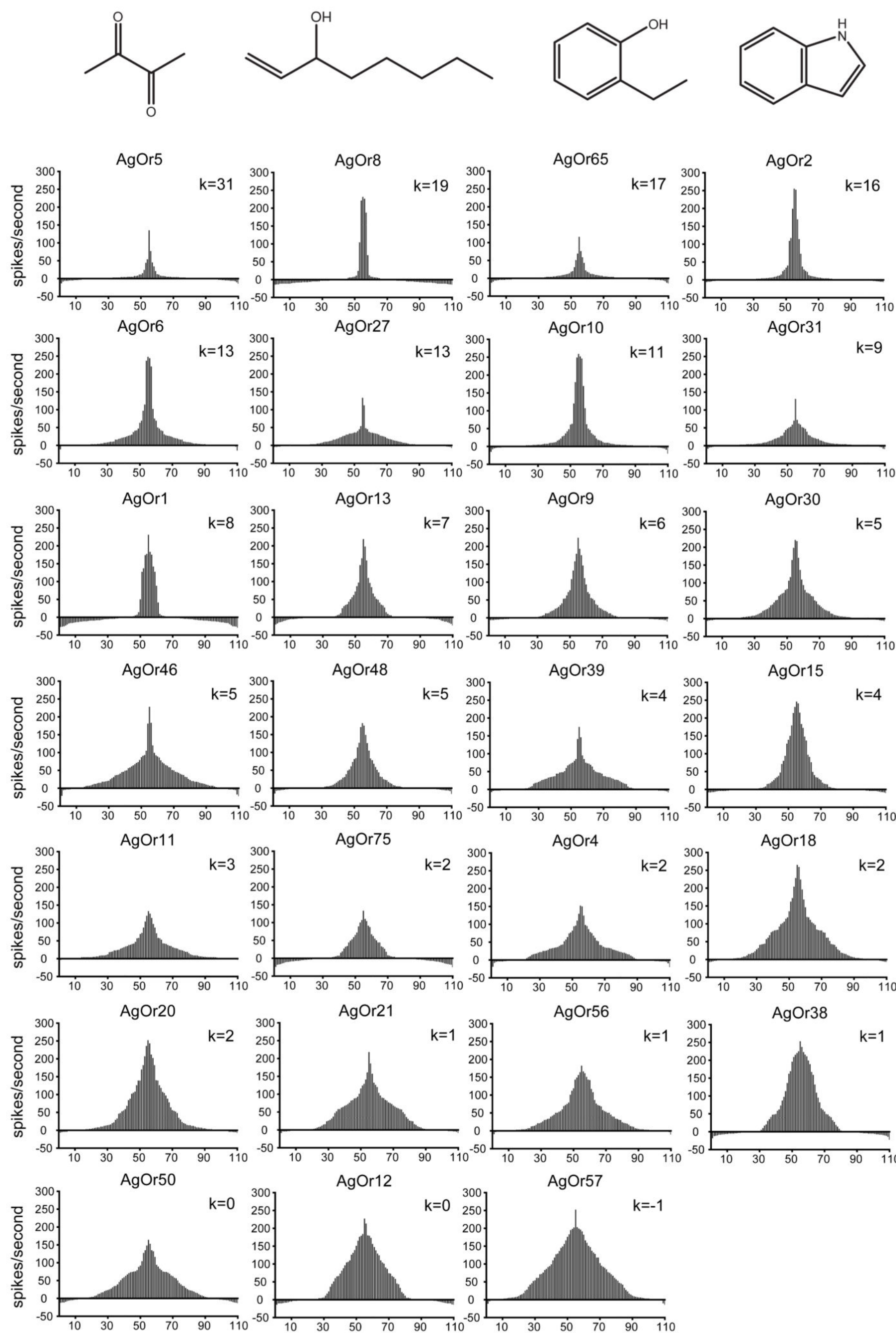

Figure 2. Tuning breadths of receptors

Tuning curves for the AgOrs that respond strongly (100 spikes/second) to at least one odourant on the panel. The 110 odourants are arranged along the X-axis according to the strength of the response they elicit from each receptor. The odourants that elicit the strongest responses are placed near the center of the distribution; those that elicit the weakest responses are placed near the edges. The order of odourants is therefore different for each receptor. The kurtosis value k, a statistical measure of “peakedness,” is located in the upper right corner of each plot. Structures of odourants that elicit strong responses from the most

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narrowly tuned AgOrs: 2,3-butanedione (AgOr5); 1-octen-3-ol (AgOr8); 2-ethylphenol (AgOr65); indole (AgOr2) are shown above the receptors they activate.

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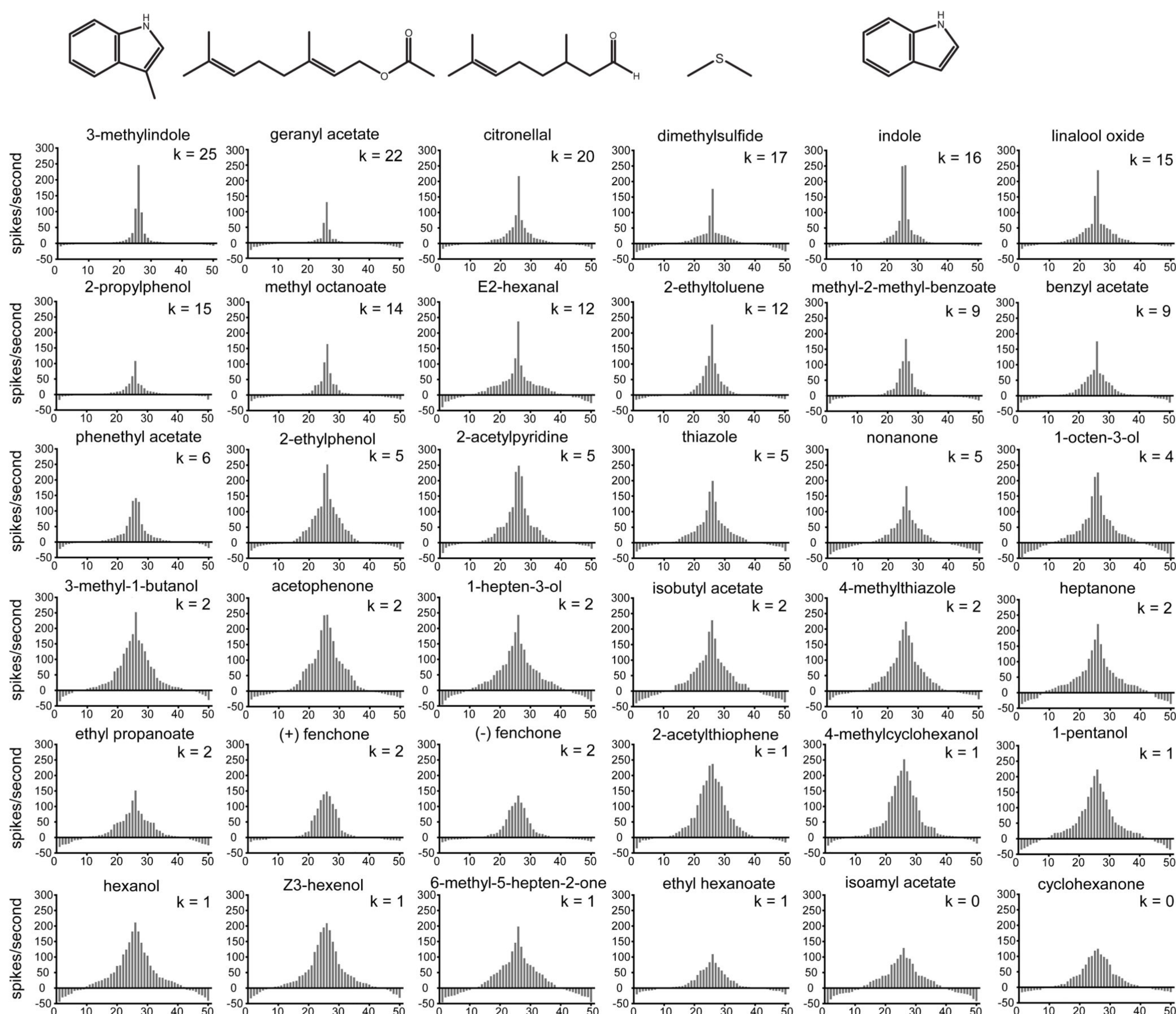

Figure 3. Odourant tuning curves

Tuning curves for odourants. The responses of the 50 AgOrs are ordered along the X-axis according to the magnitude of the response they generate for each odourant. The receptor with the strongest response is placed at the center of the distribution; those that have the weakest responses are at the edges. The order of receptors is therefore different for each odourant. The kurtosis value is indicated in each graph. Only odourants that generate a strong response (100 spikes/second) from at least one receptor are shown. At the top of the panel are the structures of several odourants that generate strong responses from just one or two receptors, shown above the corresponding graph. Shown here are tuning curves for the 12 most narrowly tuned odorants, the 12 most broadly tuned odorants, and 12 representative odorants of intermediate tuning breadth. Tuning curves for 25 additional odourants are shown in Supplementary Figure 3.

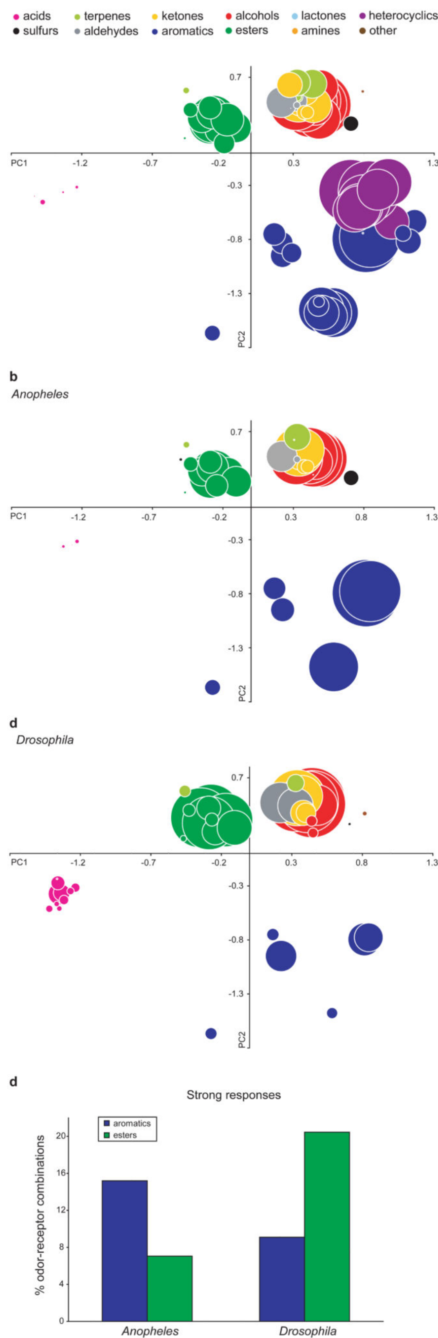

Figure 4. Distribution of responses across a physicochemical odour space

(a) Bubble plot of the responses generated by the AgOr repertoire to each of the 110 odourants. Size of the bubble scales with the sum of spikes across all receptors that exhibit at least one response 50 spikes/second from at least one odourant on the panel. Each odourant is plotted and colour-coded according to functional group except for 7-octenoic acid and 2-oxohexenoic acid. Shown are the first two principal components of the 32-dimensional physicochemical space (adapted from Haddad, *et al*., 2008). Descriptors were normalized. Comparison of responses generated by the AgOrs (b) and DmOrs (c) to the set

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of 53 odourants that were tested against both receptor repertoires. Area of the bubble corresponds to the sum of spikes across all receptors that exhibit at least one response 50 spikes/second from at least one odourant on the panel. Responses were normalized to the sum of spikes elicited by all 53 odourants across all AgOrs or all DmOrs that exhibit at least one response 50 spikes/second from at least one odourant on the panel. (d) Percent of odourant-receptor combinations that generate strong responses (100 spikes/second) within the ester and aromatic classes for *An. gambiae* and *D. melanogaster*. Only responsive receptors (those yielding a response 50 spikes/second to at least one odourant) were considered.

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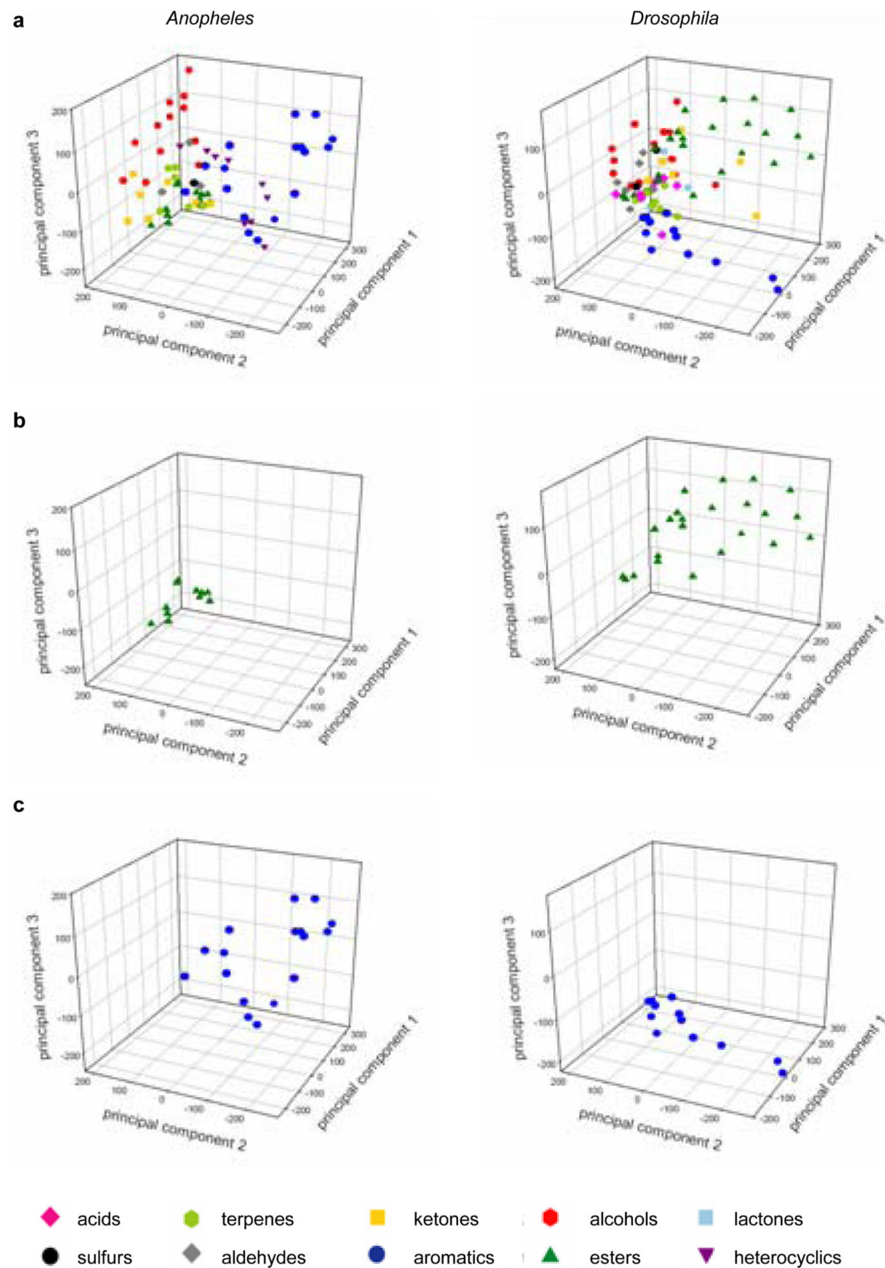

Figure 5. Distribution of odourants in a receptor activity-based odour space

First three principal components of a receptor activity-based odour space. (a) Left, *An. gambiae* odour space. Right, *D. melanogaster* odour space (adapted from Hallem and Carlson, 2006). All odourants tested against a receptor set were considered. Odourants are colour-coded according to functional group. (b) Only esters are shown. Left, *An. gambiae* odour space; right *D. melanogaster* odour space. (c) Only aromatics shown. Left, *An. gambiae* odour space; right *D. melanogaster* odour space. Mean inter-odourant distance for the set of esters tested against both receptor sets is 254±12 spikes/second for the AgOrs; 353±13 spikes/second for the DmOrs ($p<0.05$; t-test). Mean inter-odourant distance for the

set of aromatics tested against both receptor sets is 406±29 for the AgOrs; 321±17 for the DmOrs ($p<0.05$; t-test).

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