Inhibition of *Anopheles gambiae* Odorant Receptor Function by Mosquito Repellents

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*Running title: *Mosquito repellents inhibiting Orco function*

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Background: The mode of action of insect repellents on odorant receptor (OR) function remains unclear.
Results: *Anopheles gambiae* OR function *in vitro* is inhibited by specific repellents.
Conclusion: The identified inhibitory effects are due to functional blocking of Orco, the common subunit of OR heteromers.
Significance: The specific mechanism of action is distinct from the proposed modes of DEET function.

ABSTRACT

The identification of molecular targets of insect repellents has been a challenging task, with their effects on odorant receptors (ORs) remaining a debatable issue. Here, we describe a study on the effects of selected mosquito repellents including the widely used repellent DEET, on the function of specific ORs of the African malaria vector *Anopheles gambiae*. The study, which has been based on quantitative measurements of a Ca^{2+}-activated photoprotein biosensor of recombinant OR function in an insect cell-based expression platform and a sequential compound addition protocol, revealed that heteromeric OR (ORx/Orco) function was susceptible to strong inhibition by all tested mosquito repellents except DEET. Moreover, our results demonstrated that the observed inhibition was due to efficient blocking of Orco function. This mechanism of repellent action, which is reported for the first time, is distinct from the mode of action of other characterized insect repellents including DEET.

Insect odorant receptors (ORs), which constitute a novel family of heteromeric ligand-gated cation channels (1-3), have been traditionally regarded as the main if not sole molecular targets for insect repellents. The great progress made recently in our understanding of the molecular mechanism(s) of insect olfactory function (4-6) has thus created hope for rational development of improved repellents and/or attractants that could effect a significant reduction in the rate of transmission of malaria and other infectious diseases transmitted by different insect and other arthropod vectors (4,7).

Prominent among the insect repellents is DEET. This was one of the first to be tested for effects on various insect ORs, with the relevant studies yielding rather contradictory results. Specifically, different mechanisms of DEET action on ORs have been suggested

(summarised in (8,9), which include activation of specific ORs, inhibition of specific ORs responding to attractants and/or modulation of multiple ORs causing olfactory «confusion». In addition, the effects of a small number of other repellents, such as IR3535, picaridin and others whose action on insect ORs has been characterized in a more limited fashion, have also been reported (10,11).

More recently, the modulation of ORs by a number of other compounds, such as amiloride derivatives (12,13), trace amines (14) and synthetic Orco agonists and antagonists (15-18) has suggested that, because Orco ligands and modulators affect OR function and Orco is highly conserved across insect species, Orco might be a potential target for broadly active insect repellents. Despite their importance for the pharmacological characterization of the receptors, however, most synthetic compounds are expected to have limited usefulness in field application tests due to their low solubility and lack of volatility (15-18). Consequently, more studies are needed in order to understand the modulation of insect olfactory function by physiologically active compounds, especially repelling ones and develop new classes of repellents that may work effectively in the field.

The functional characterization and deorphanisation of insect ORs, including most of the *Anopheles gambiae* repertoire, has been largely achieved through the use of two major test systems, functional receptor expression in *Xenopus laevis* oocytes (19) or an "empty neuron" of transgenic *Drosophila melanogaster* (20), in conjunction with electrophysiological methods. Besides these assay systems, a number of insect odorant receptors including pheromone ones, have also been expressed in mammalian or insect cells (17,21-28), which employed as main reporter probes fluorescent calcium indicators and were coupled to imaging of individual cells or measurements of fluorescence changes in multiple well formats. While rather complex preparation, instrumentation and handling requirements are needed in the case of the *Xenopus* and *Drosophila* systems, the assays involving tissue culture cells with fluorescent probes impose other types of limitations including susceptibility to photobleaching, narrow dynamic range and potential for interference by some compounds that either quench the fluorescent signals or autofluoresce thus causing low signal-to-noise ratios. Consequently, development and use of alternative cell-based systems employing reporting tools that may provide more robust and quantitative readouts of insect odorant receptor activity while being amenable to miniaturization is highly desirable.

Here we are reporting on an alternative, lepidopteran insect cell-based assay system for functional expression of mosquito ORs that we have used for characterizing the effects of specific mosquito repellents on receptor function. By reconstituting *An. gambiae* odorant receptors in the specific heterologous expression system together with a Ca^{2+}-activated photoprotein biosensor allowing quantitative assessments of receptor function, we were able to characterize the effects of specific mosquito repellents including DEET on the function of specific ORx/Orco heteromer combinations. We show that the specific repellents we tested but not DEET block the function of multiple ORs by inhibiting the function of the common co-receptor subunit Orco.

EXPERIMENTAL PROCEDURES

Chemicals- Odorants, Orco agonists, repellents and OR inhibitors used in the current study are summarized in Table 1. Specifically, benzaldehyde, 2-, 3-, and 4- methylphenol, ethyl butyrate, 2-ethylphenol, cyclohexanone, DEET and ethyl *trans*-cinnamate were from Sigma Aldrich, indole and cuminic alcohol from Acros Organics, isopropyl cinnamate from Alfa Aesar and carvacrol from Beauvilliers Flavors SAS (Peynier, France). VUAA1 (17) was generously provided by Dr. Richard Newcomb (New Zealand Institute for Plant & Food Research, Auckland, New Zealand), while OrcoRAM2 (11) was obtained from hit2lead (Chembridge Corporation, San Diego, California, USA) and Vitas-M Laboratory, Ltd (Apeldoorn, the Netherlands). OX3a (16) was purchased from Vitas-M Laboratory. Coelenterazine was from Promega, BIOMOL GmbH (Hamburg, Germany) and Biosynth (Staad, Switzerland), while DPDPE ([D-Pen^2,D-Pen^5]Enkephalin) was obtained from Tocris Bioscience (Bristol, UK). Ruthenium red was a gift of Dr. Dimitrios Kontogiannatos (Agricultural University of Athens, Greece). Initial stock solutions and dilutions for OrcoRAM2 and OX3a (50mM), as well as for all repellents were prepared in DMSO, with subsequent working dilutions prepared freshly

in Ringer solution, such as the final concentration of DMSO did not exceed the range of 0.2-0.35%. For all remaining odorants, initial stock solutions were prepared in methanol or ethanol (Table 1).

Plasmids- The plasmid vector pIE1/153A (henceforth pEIA, Figure 1A) was used for expression of *An. gambiae* ORs, thereafter termed ORx and Orco (for AgamOR7) (29) and the reporter calcium photoprotein in lepidopteran insect cells. This vector ensures high levels of expression, by double-enhancing the silkworm cytoplasmic actin promoter with two baculovirus-derived elements, the *hr3* enhancer and the IE1 trans-activator (30-32). The construction of plasmids pEIA.OR1, pEIA.OR2, pEIA.Orco as well as pEA.Gα16 and pEA.DOR used for expression of human Gα16 and murine δ-opioid receptor, respectively, has been reported (33-35). For expression of the Ca^{2+}-activated luminescent photoprotein, the mito i-Photina ORF (36) was excised from pcDNA3neo-mito i-Photina K16 (Axxam SpA, Milan, Italy, www.axxam.com) with *Hind*III-*Xho*I as a 702bp fragment and subcloned in the *Sma*I site of pEIA (30) after blunt-ending. For expression of OR9, PCR amplification and subcloning in the pEIA vector was as described (34) using primers OR9-FA/C [GAATGGATCCCACC**ATG**GTTAGGCTTTT CTTCAGC] and OR9-RA/N [GATAGGATCC*CTA*ATCCGTCATCGATCTC] (*Bam*HI restriction sites are underlined; initiation and termination codons are in bold and italics, respectively).

Cell culture and transfection- Trichoplusia ni BTI-Tn 5B1-4 HighFive™ cells ((37); thereafter termed Hi5) were used throughout this study. The cells were maintained at 28°C and were grown in IPL-41 insect cell culture medium (Genaxxon Bioscience GmbH), supplemented with 10% fetal bovine serum (Sigma or Biosera). Transfection was performed with Escort IV reagent (Sigma) according to standard protocols.

Expression of mosquito ORs and bioluminescence assays- To monitor olfactory receptor activation, Hi5 cells were transfected with pEIA plasmids expressing Orco, ORx and Photina at ratios of 1:1:2, with 2 μg of total plasmid DNA per 10^6 cells. In experiments involving analyses of single subunits (Orco or ORx), the ratio of plasmids expressing Orco or ORx and Photina was 1:1. The functional assay was performed 2-4 days post-transfection. Briefly, the cells were washed and resuspended in Ringer solution [140 mM NaCl, 2 mM KCl, 2.5 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM Hepes, 10 mM glucose, pH 7.2] and native coelenterazine was added at a concentration of 5 μM. This was followed by transfer of the cell suspension to a white 96-well plate (200,000-300,000 cells/well) and further incubation at RT in the dark for at least 2 h. Luminescence was measured in an Infinite M200 microplate reader (Tecan Group Ltd). Addition of chemicals was either by using the autoinjector allowing rapid injection and simultaneous reading or manually outside the plate reader. In the latter case, baseline luminescence was usually recorded for 20 sec, after which the compounds were added with the change in luminescence recorded every 3-7 sec for a further period of up to 120 sec.

To test the suitability of the expression system for use as screening platform for olfactory receptor agonists and antagonists in a single compound screen, as was previously reported for other ion channels (38), olfactory receptors (28) and nuclear receptors (39), cells co-expressing selected *An. gambiae* ORs with Orco and Photina were transferred to 96-well plates. Following coelenterazine loading, the cells were subjected to 2 cycles of compound additions (Figure 1B). In the first cycle, compounds screened for effects on olfactory receptors were added to the cells, while in a second application, the cognate ligand for each receptor (odorant for ORx-Orco and Orco agonist for Orco alone) was added to all wells. Between the two applications cells were allowed to return to baseline luminescence levels (15-20 min). The same design was used for testing the effects of the selected repellents.

Data analysis and curve fitting- Initial data acquisition and analysis were performed using i-Control 1.3 (Tecan), while curve fitting and EC_{50}/IC_{50} calculations were done using GraphPad Prism 4.0 for Windows (graphpad.com). Specifically, concentration-response data were fitted to the equation for non-linear regression, sigmoidal dose-response: Y=Bottom + (Top-Bottom)/(1+10^(LogEC_{50}-X)), where Y: % response at a given concentration for a given odorant; X: logarithm of concentration, with Top and Bottom values being the maximal and minimal % responses for the given odorant, as normalized to 100%, set for the maximal response for a specific OR against all tested odorants, and the EC_{50} being the odorant concentration yielding a half maximal response.

Luminescence value comparisons between independent experiments were made relative to normalization standards with cognate ligands (i.e., 100μM of 4-methylphenol and indole, for OR1 and OR2 respectively) applied in every given experiment and considered to provide 100% of maximal response for the specific set of experiment. Unless otherwise stated, results represent the means of two to three independent experiments.

RESULTS

Mosquito ORs produce Orco-dependent and odorant-specific ionotropic responses in lepidopteran cells- We have previously reported on the expression of *An. gambiae* ORs in a lepidopteran insect cell-based system that directs efficient synthesis and correct localization of recombinant receptors in the expressing cells (34). To establish the functionality of the expressed receptors in this system and develop a platform suitable for quantitative assessments of receptor activity, we introduced into the cells a reporter construct for a Ca^{2+}-activated photoprotein, Photina® (36) (Figure 1A), which functions similarly to aequorin, but has enhanced quantum yield (36,40). Upon activation of OR ligand-gated cation channels, which cause Ca^{2+} influx into the cells, Photina® undergoes a conformational change leading to oxidation of coelenterazine and emission of blue light proportional to the Ca^{2+} influx (Figure 1A).

Following co-expression of OR1 and OR2 with Orco and Photina, cellular luminescence responses were monitored after addition of specific ligands known to activate each OR (19,20,41,42). As shown in Figure 2A, specific responses could be detected in cells expressing OR1+Orco upon administration of 4-methylphenol, while cells expressing OR2+Orco responded, as expected, to indole. On the other hand, cells expressing OR9+Orco responded, as expected, to the addition of 2-ethylphenol (data not shown). The presence of Orco was obligatory for functional responses to occur, as no responses were obtained in its absence (Figure 2A). Co-expression of Gα16 was also not able to substitute for a functional Orco in stimulating agonist-dependent activity of OR2 and OR1 to any appreciable degree (Figure 2B and C).

To further deduce OR response specificity and potency differences, luminescence responses were monitored after addition of selected compounds known to activate each OR (19,20,41,42). As shown by the examples presented in Figure 2D and were further quantified in Figure 2E, specific responses could be detected in cells expressing OR1+Orco upon administration of 4-methylphenol (and to lesser extent to 3-methylphenol), while cells expressing OR2+Orco responded, as expected, to indole and, to a lesser extent, benzaldehyde. In contrast, no responses could be detected in cells expressing either OR1+Orco or OR2+Orco following administration of ethyl butyrate (Figure 2D). With a higher concentration of 4-methylphenol, a small response could also be detected in OR2+Orco-expressing cells (data not shown and Figure 3A). Besides the specific odorants discussed here, other chemicals have also been tested with comparable results relative to previously reported studies (data not shown).

The dose response curves and derived EC_{50} values from the agonist-induced luminescence for OR1/Orco and OR2/Orco heteromers distinguish agonists of varying efficacies and potencies (Figure 2E). Thus, EC_{50} values of 2.8 and 3.4 μM were determined for OR1/Orco against 4-methyl phenol and OR2/Orco against indole, respectively (Figure 2E and Table 2). Additionally, the dose response curves of Orco to Orco agonists such as Orco RAM2 revealed values in the order of 60 μM (Figure 2E and Table 2). In general, when expressed in lepidopteran cells, all tested mosquito receptors were found to be functional and display the same basic specificities as those determined using the *Xenopus* oocyte and *Drosophila* empty neuron systems (19,20,41). Specifically, OR1 was found to respond to 4- and 3-methylphenol, and OR2 to indole, benzaldehyde and 2-methylphenol, with the overall response patterns and dose-response profiles for the examined ligands and receptors (4MP>3MP for OR1, and IN>BA>2MP for OR2) being in good general agreement with those obtained for the same receptors from frog oocytes with two-electrode voltage-clamp (19).

A convenient assay for initial assessment of specific chemical compound effects on the functionality of olfactory receptors- To investigate the effects of specific chemical compounds on the functionality of olfactory receptors, we employed an assay that permitted the easy classification of examined compounds into agonists, partial agonists, antagonists or inert, i.e., not displaying any activity against the tested receptors, in one round of compound testing. The assay, which is shown diagrammatically in Figure 1B, relies on two

sequential additions of compounds to cells expressing specific receptor heteromers and the reporter Photina construct, and recordings of the differential luminescence responses obtained after each addition, which depend on the nature of the chemical of the first addition. Specifically, the compound of unknown function under examination is added first at a relatively high concentration (100 μM), and this is followed 10-20 minutes later by the addition of the specific agonist at the same concentration of 100 μM, which usually corresponds to an EC_{90} or greater, in the same microtiter well. This assay, performed in 96-well format with no wash steps in between, allows the distinction of compounds under investigation into a receptor agonists [+/- for primary/secondary receptor responses, respectively; Figure 1B, #1], non-active (-/+ responses; Figure 1B, #2) or antagonists (-/- responses; Figure 1B, #3). It should be noted that following a maximal primary response triggered by a receptor-specific agonist in cells expressing the corresponding receptor heteromer, the cells do not respond to a second addition of the same or a different agonist of similar specificity and potency (Figure 1B, #1 and Figure 3A), presumably due to temporary receptor inactivation that is gradually reversed over time following removal of the agonist (data not shown). On the other hand, partial agonists producing a lower than maximal primary response, produce a significantly lower secondary response upon addition of the receptor-specific agonist [(+)/(+) responses; Figure 3A], apparently because of partial receptor occupancy and correspondingly reduced receptor inactivation.

Typical system validation examples employing the OR2/Orco heteromer are presented in Figure 3A. Indole, the specific OR2 agonist triggers a primary but no secondary functional response upon a new addition of indole to the cells. This effect appears to be specific for the cognate pair of OR heteromer/ligand used rather than being caused by exhaustion of the photoprotein, as cells co-transfected with OR2+Orco and delta-opioid receptor respond positively to the addition of DPDPE, the specific delta-opioid receptor ligand, following the primary indole addition (Figure 3B). On the other hand, benzaldehyde, 2-methylphenol and 4-methylphenol, partial OR2 agonists (Figure 2E and data not shown), trigger partial agonist primary and secondary responses (Figure 3A), while ethyl butyrate and cyclohexanone, which do not represent ligands for the specific receptor, do not trigger a primary functional response but allow the opening of the olfactory channel upon secondary addition of indole (Figure 3A). Controls were included both at the beginning and end of each set of experiments to ascertain the stability of OR2 responses to indole during the course of the experiments (Figure 3A).

Given the absence of known antagonists for any ORx receptors, the case of inhibition in the context of a receptor heteromer was tested through the use of one of the recently reported synthetic phenyl-thiophene-carboxamide Orco antagonists, OX3a (16), and ruthenium red (43). OX3a has been shown to inhibit non-competitively odorant activation of a heteromeric OR of *Culex quinquefasciatus* and was assumed to block general odorant-dependent OR activation as the originally characterised chemical of this class of Orco antagonists (16). On the other hand, ruthenium red, a non-specific cation channel blocker, inhibits the function of many insect odorant receptors (1,44,45). In the case of these inhibitors, their addition to the cells did not induce any functional responses, but did reduce receptor activation upon secondary addition of indole (Figure 3C). Similar results were obtained from other tested ORx/Orco heteromers involving ORx subunits of known ligand specificities (data not shown).

Olfactory receptor inhibition by specific mosquito repellents- A number of repellents previously reported to be active against different *Anopheles* and *Aedes* species were subsequently examined for their possible effects on various olfactory receptors of *An. gambiae*. Prominent amongst the initially examined repellents were DEET (46,47), ethyl cinnamate (EC; (47,48), isopropyl cinnamate (IPC; (47-49), cuminic alcohol (CMA; (47,50) and carvacrol (CRV; (47,51) (Table 1). Except for DEET, which has been studied extensively, to the best of our knowledge no information concerning the molecular mechanism of action has been available for these repellents.

The chosen repellents were tested initially at a concentration of 100 μM on cells expressing OR1/Orco and OR2/Orco heteromers. As is shown in Figure 4A, none of the tested repellents displayed agonist activity upon addition to the cells. Judging from the results of the secondary addition of 4-methylphenol or indole to the cells expressing OR1+Orco or OR2+Orco, respectively, EC, CRV and IPC caused

essentially complete inhibition of OR1/Orco and OR2/Orco receptor function at this concentration (Figure 4A). The effect of CMA on both receptor heteromers was considerably less pronounced with the inhibition amounting to 30-40%, while no noticeable inhibitory action was exerted by DEET on the tested receptors. Similar inhibitory effects were also recorded with other mosquito receptor heteromers examined (data not shown).

To ensure that the observed inhibition in Ca^{2+} influx into the cells was due to specific inhibition of olfactory receptor function, rather than general off-target effects, e.g., at the level of the cellular membranes or the calcium photoprotein, the same compounds were examined for their effects on cells transfected with the murine delta opioid receptor (52,53) along with the human Gα16 (54) (presented in Figures 2C and 3B). As shown in Figure 4B, administration of the delta opioid agonist DPDPE to cells expressing the specific receptor in the presence of a high concentration of the tested repellents, did not affect plasma membrane-anchored opioid receptor signalling and the ensuing Ca^{2+} release from intracellular endoplasmic reticulum membrane stores (Figure 4B).

Dose response curves for the inhibition exerted by two of the repellents displaying highly inhibitory actions, ethyl cinnamate and isopropyl cinnamate, against OR1/Orco and OR2/Orco heteromers were constructed using 100 μM concentrations (>EC_{90}) of the cognate ligands 4-methylphenol and indole, respectively, as agonists for the secondary additions. For ethyl cinnamate, the rates of inhibition (IC_{50} values) of the two heteromers were found to be very similar, 25.9 and 28.9 μM, respectively (Figure 4C). Inhibition by isopropyl cinnamate was also found to be in the same order of magnitude, with IC_{50} values of 22.2 and 34 μM, respectively, for OR1/Orco and OR2/Orco (Figure 4C).

Orco antagonism is a cause of interference with mosquito olfactory receptor function- Given the similarity of the inhibitory effects of the tested repellents on different olfactory receptor heteromers, we examined the possibility that the inhibition may be exerted at the level of Orco, the common subunit of olfactory receptor heteromers. While we found Orco to be similarly responsive to both Orco agonists tested, VUAA1 (17) and OrcoRAM2 (11) (data not shown), the latter was used for detailed investigations (shown also in Figures 2E). Indeed, as shown in Figure 5A, when 100 μM of each repellent was added to cells expressing Orco, all examined repellents, except DEET, exerted some level of inhibitory effect on Orco function, as the responses to the secondary addition of 100 μM of the Orco agonist OrcoRAM2 to the cells were considerably reduced (Figure 5A).

The dose response curves for the inhibition exerted on Orco homomers by the tested repellents (Figure 5B) revealed IC_{50} values of 64.5 and 41.7 μM for ethyl cinnamate and isopropyl cinnamate, respectively, somewhat higher than those exerted on the function of OR1/Orco and OR2/Orco heteromers (Figure 4C). This finding suggests that the repellent action observed in the context of the heteromer (Figure 4) likely originates from interference with Orco function. Interestingly, DEET did not conform to the behavior of the rest of the repellents at the tested concentration.

DISCUSSION

In this report, we present the use of a lepidopteran insect cell-based assay for quantitative assessments of the functional properties of *An. gambiae* olfactory receptors. Besides high levels of receptor expression achieved in the insect cells (30,34) through the use of plasmid-based expression vectors employing genetic control elements of the silkmoth and its baculovirus (31,32), the major functional component of the assay system is a construct directing robust expression of Photina®, a Ca^{2+}-activated photoprotein (36). The activation of this photoprotein provides a luminescence-based readout reporting quantitatively on increases of intracellular Ca^{2+} ions hence, in this study, ion channel activity upon administration of cognate olfactory receptor agonists.

Our system may be considered as effective alternative to the two major systems used for studying insect olfactory receptor function, the *Drosophila* empty neuron (20) and *Xenopus* oocytes (19) as well as other heterologous cell-based expression systems employing cultured insect (21,24-26) or mammalian cells (22,23,27,28) in conjunction with fluorescent calcium indicator dyes. In contrast to these systems, which have some important drawbacks (see Introduction), the new system combines simplicity of handling with a reporter photoprotein that only luminesces when the levels of intracellular Ca^{2+} increase as a result of olfactory channel activation. Because it allows automated luminescence measurements of total

cell populations seeded in microtiter plates in a mix-and-measure fashion and with high signal-to-noise ratios in the absence of wash steps, this system is very suitable for functional screens for ligand discovery at a medium to high throughput scale.

As already mentioned, a version of Photina targeted to the mitochondria via a specific leader sequence (36) was employed in our optical cell-based assay. The reasons for choosing the mitochondrially-targeted version of Photina over the cytoplasmic were, first, the ~10-fold higher responses reported for the mitochondrial photoprotein over the cytoplasmic one, at least for the case of studied GPCRs and, secondly, the slower, thus more accurate, detection of channel activation reaction kinetics due to the longer time assumed to be needed for the Ca^{2+} wave to reach the mitochondria-localized Photina. Moreover, we have not noticed any artefactual responses related to metabolic stress in our system, as functional responses from cells expressing specific receptors were obtained only after administration of cognate ligands at functionally relevant concentrations.

The specificities of OR responses determined in the lepidopteran insect cell system have been in good agreement with those determined using the *Drosophila* empty neuron and *Xenopus* oocytes expression systems (19,20). As far as relevant potencies and efficacies are concerned, our results can be compared directly to those determined using the frog oocyte system (19), which can be considered as more accurate and quantitative descriptors of receptor pharmacology, as in the *Drosophila* system only one very high, thus not physiologically relevant, concentration was applied. Briefly, the tested ORs were found to recognize the same ligands, from the panel of chemicals used for confirmation, with similar dose-dependent responses, 4MP>3MP for OR1 and IN>BA>2MP for OR2, respectively (Figure 2E). The calculated EC_{50} values (Table 2) were somewhat higher than the ones reported with the electrophysiological methods, which were in the high nanomolar range (19). The Orco homomer was also found to respond to Orco agonists, as expected.

The sequential addition protocol employed in this study, initially validated using known odorants, ruthenium red and an Orco antagonist, made possible the classification of compounds added in the first instance to cells expressing specific receptors into partial or full receptor agonists, antagonists or inert in terms of ligand activities (Figure 1B and 3A). Using this assay, it has been possible to deduce that all but one of several examined compounds known to represent powerful mosquito repellents act as olfactory receptor inhibitors reducing receptor activation upon subsequent agonist addition (Figure 4A). Interestingly, the exception has been the most widely used repellent DEET, which was not found to act as agonist or antagonist for any of the examined *An. gambiae* olfactory receptors in the tested concentration of 100 μM (Figure 4A).

The fact that the tested repellents were found to inhibit the function of all receptors examined in this study led to the examination of the possibility and demonstration that Orco, the common receptor subunit of the functional receptor complex, was a target of inhibition (Figures 5A and B). Although further studies are warranted, this finding and, most importantly, the dose response curves for the inhibition, suggest that the inhibitory action of the tested repellents on the receptor heteromers under examination is likely due to Orco inhibition with the ORx constituents contributing little, if any, to it. Whether the somewhat lower IC_{50} values of the repellents for the receptor heteromers (Table 2) indeed reflect enhanced affinities for Orco owing to structural changes of the latter in the context of the heteromers should be investigated further.

It should be noted further that while the synthesis of several compounds acting as Orco antagonists has been reported recently (15,16,18), to our knowledge this is the first report demonstrating antagonist action on an anopheline Orco associated with powerful repellency on anopheline and other mosquitoes. Likely important factors that contribute to the repelling capacity of the Orco antagonists reported here are their physical properties such as volatility and efficient recognition by several mosquito odorant binding proteins (55), at least *in vitro*, which has been demonstrated for some of these compounds in the *An. gambiae* system (K.I., K.K., Maria Konstantopoulou, Spyros E. Zographos and Georgia Kythreoti, unpublished data). The fact that a highly conserved protein such as Orco is a major target of the tested repellent compounds may also explain why the specific repellents are effective against mosquito species other than *An. gambiae*, as well as other insects (47,48,50,51). The demonstrated Orco involvement, also leads us to postulate that, at least in the case of the two repellents studied in

more detail here, their mosquito "repellence" action should probably be interpreted as a passive disorientation effect, which reflects an essential anosmia caused by the blocking of a large complement of olfactory receptors rather than an active one causing repulsion to the approaching mosquitoes.

Finally, the finding that DEET behaves differently than the remaining repellents tested in this study was not unexpected, as a number of reports have suggested different modes of function for this repellent. These include activation of specific ORs – the excito-repellent hypothesis (56-58); inhibition of specific ORs responding to attractants (59,60); and/or modulation of many ORs causing an olfactory «confusion» similar in effect but not in molecular terms to the one postulated above for the repellents tested in this study (10,61). In addition, the involvement of ionotropic (62) and gustatory receptors (63) in *Drosophila* was also demonstrated. Therefore it seems that, in contrast to the case of the Orco function-inhibiting repellents presented in this report, the molecular targets for DEET are fundamentally different. Infact, no inhibition of *Ae. aegypti* Orco could be observed even at 10mM of DEET (11). Nevertheless, inhibitory effects of DEET on a *Drosophila* OR heteromer, OR47a/OR83b, with a calculated IC_{50} value of 929 μM in the *Xenopus* oocyte system have been reported (13). In the same assay system, some inhibitory effects of DEET were also documented for a number of insect olfactory receptors including *An. gambiae* OR1/Orco and OR2/Orco (59). In these latter cases, the inhibition ranged from a low of 30% to a maximum of approximately 55% at a DEET concentration of 1mM (59).

In view of the fact that effects of repellents with secondary (allosteric) recognition sites may be observed at high repellent concentrations (9), we consider questionable whether modulations of odorant receptor activity by high concentrations of DEET are physiologically relevant. Such reservations notwithstanding, however, it should also be of interest to investigate whether the repellents studied here have additional sensory targets, in analogy to DEET or citronellal, which has been reported to target both TRPA1 and Orco-dependent pathways in *Drosophila* (64).

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FOOTNOTES

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TABLE AND FIGURE LEGENDS

TABLE 1. Structural formulae of odorants and other chemicals tested in the present study. CAS numbers are in brackets, common names in parentheses; the corresponding abbreviations used throughout in the figures or in text are presented in the middle. ND, not determined; [C], concentration; *, mM doses employed for dose response curve derivation.

TABLE 2. EC_{50} and IC_{50} values from concentration-dependent response curves. Summarised are values from curves presented in figures 2E, 4C and 5B. EC_{50}, half maximal effective concentration; IC_{50}, half maximal inhibitory concentration; pEC_{50}, negative logarithm of the EC_{50} ($-logEC_{50}$); pIC_{50}, negative logarithm of the IC_{50} ($-logIC_{50}$); Std. error, standard error (SE) reported by GraphPad Prism for calculated $logEC_{50}$/$logIC_{50}$; R^2, measure of goodness of fit.

FIGURE 1. Assays for functional analysis of mosquito ORs and analysis of compound effects on OR function. (**A)** The pEIA vector is used for the heterologous expression of ORx, Orco and Photina in lepidopteran insect cells. Upon addition of specific ligands (solid circles) to cells expressing the two subunits of the ion channel complex (depicted is an oversimplified consensus model based on current knowledge) and the photoprotein, increased luminescence is measured. **(B)** The two-step addition protocol: in the first addition cycle, different compounds (in blue) are added to each well containing cells expressing specific ORs, while in the second cycle the specific agonist (in red) of the receptor is applied. As exemplified here, compound #1 can be classified as agonist, compound #3 as antagonist, while #2 is not recognized by this particular OR heteromer.

Figure 2. Functional expression and characterization of mosquito ORs. (A) Selective responses to 4-methylphenol and indole can be monitored from cells expressing OR1/Orco and OR2/Orco, respectively, upon challenging with the cognate ligand at 10μM concentration (shown are time course of luminescence changes over time; RLU, relative light units). No responses are measured from ORx alone or Orco alone-expressing cells, even at higher agonist concentration, showing that coexpression of ORx and Orco is required. **(B)** The presence of Gα16 protein does not stimulate agonist-dependent activity of OR2 to any appreciable degree in cells expressing OR2/Gα16 challenged with indole at 10 μM. **(C)** Responses of cells expressing OR1/δOR/Gα16 to sequential administration of 4-methylphenol at 100 μM (arrowhead 1) and the δ-opioid receptor ligand DPDPE at 1 μM (arrowhead 2) are shown. In the latter case, Gα16-dependent PLC coupling of the co-expressed murine δ-opioid receptor was clearly observed, as expected. The later calcium response is also much slower than direct calcium ion flux through ion channels, as expected for GPCR downstream signaling, whereas lower concentrations of agonist, in the nM range (data not shown), were sufficient for activation of the GPCR relative to those required for OR activation. **(D)** Responses are ORx-specific and odorant-selective. Cells expressing OR1/Orco or OR2/Orco were challenged with 4-methylphenol, indole, benzaldehyde and ethyl butyrate at 100 μM concentration. **(E)** Dose-response curves for OR1, OR2 and Orco. The red curves correspond to the most potent ligands, 4-methylphenol for OR1 (EC_{50} = $2.8x10^{-6}$, n=2) and indole for OR2 (EC_{50} = $3.4x10^{-6}$, n=2). The maximal response of each receptor to the administration of its cognate agonist was used for normalization of all other responses for the same receptor. OA denotes Orco Agonist (OrcoRAM2).

FIGURE 3. Analysis of compound effects on *Anopheles* OR function. (A) Responses of cells expressing OR2/Orco to two applications of compounds, as described in Figure 1B. In the first application [blue bars], cells in different wells were challenged with solvent, indole, benzaldehyde, 2-methylphenol, 4-methylphenol, cyclohexanone and ethyl butyrate, each at a concentration of 100 μM. The second addition (red bars; specific agonist indole at 100 μM added to same cells) was performed after the luminescence has returned to baseline levels (n=3). **(B)** Cells expressing OR2, Orco, δOR

and Gα16 were initially challenged with indole (100 μM), and subsequently, in the second cycle, either with 100 μM indole or 1 μM of the δ-opioid receptor agonist DPDPE. The secondary response was only abolished in the former but not the latter case (n=3). **(C)** Partial inhibition of responses to indole of OR2/Orco expressing cells by 100 μM of ruthenium red or OX3a could be demonstrated with this design (n=3 and 5, respectively).

FIGURE 4. Screening for effects of selected mosquito repellents on *An. gambiae* ORs. (A) OR1/Orco and OR2/Orco heteromer-expressing cells were tested for primary responses to 100 μM of each repellent (ethyl cinnamate, carvacrol, cumin alcohol, DEET and isopropyl cinnamate) [blue bars], and inhibition of secondary responses to the cognate agonists 4-methylphenol and indole, respectively, added in the same wells at 100 μM [red bars]. Responses are presented as percentages of the response of each receptor heteromer to the cognate ligand in the absence of any candidate antagonist (n=2-8). **(B)** Effects of same repellents at 100 μM on the functionality of the δ-opioid receptor and its downstream PLC-coupled signaling responses to 10 μM of DPDPE (n=2). **(C)** Dose response inhibition curves for ethyl cinnamate and isopropyl cinnamate against OR1/Orco and OR2/Orco-expressing cells following stimulation with 100 μM of the respective cognate agonists, 4-methylphenol and indole. (n=2 and 3, respectively).

FIGURE 5. Effects of selected mosquito repellents on *An. gambiae* Orco. (A) Responses of Orco homomer expressing cells to 100 μM of the tested repellents, ethyl cinnamate, carvacrol, cumin alcohol, DEET and isopropyl cinnamate [blue bars], and 100μM of OrcoRAM2 as secondary addition in the same wells [red bars]. Responses are presented as percentages of the response to the Orco agonist in the absence of any candidate antagonist (n=2). **(B)** Dose-dependent inhibition curves for Orco expressing cells, in the presence of increasing concentrations of ethyl cinnamate and isopropyl cinnamate, to 100 μM of the Orco agonist OrcoRAM2 (n=3 and 4, respectively).

Table 1. Structural formulae of odorants and other chemicals tested in the present study.

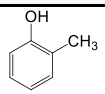
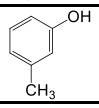
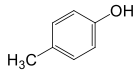
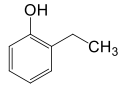
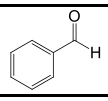
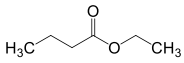
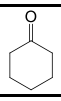
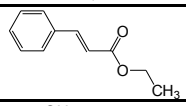
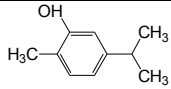
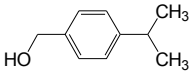
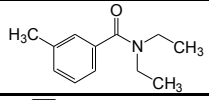
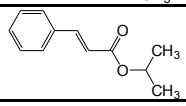
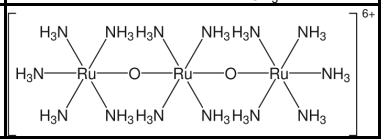
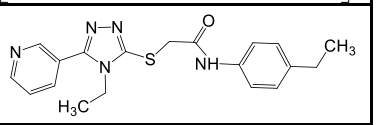
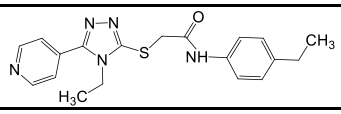
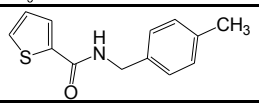
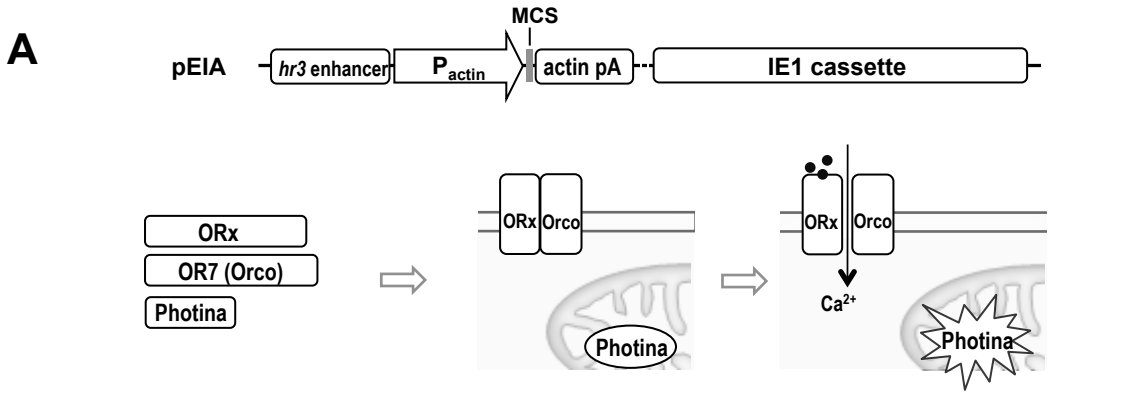
Chemical [CAS number]	Abbreviation	Purity (%)	Solvent-highest [C] used*	Structural formula
2-Methylphenol (*o*-Cresol) [95-48-7]	2MP	99+	Methanol-1mM	
3-Methylphenol (*m*-Cresol) [108-39-4]	3MP	99	Methanol-1mM	
4-Methylphenol (*p*-Cresol) [106-44-5]	4MP	99	Methanol-1mM	
2-Ethylphenol [90-00-6]	2EP	98.5	Methanol-1mM	
Benzaldehyde [100-52-7]	BA	>99	Ethanol-1mM	
Indole [120-72-9]	IN	99+	Methanol-300µM	
Ethyl butyrate [105-54-4]	EB	99	Ethanol-100µM	
Cyclohexanone [108-94-1]	CH	≥99.9	Methanol-100µM	
Ethyl *trans*-cinnamate [103-36-6]	EC	99	DMSO-2mM	
Carvacrol [499-75-2]	CRV	>98	DMSO-100µM	
Cuminic alcohol[536-60-7]	CMA	97	DMSO-100µM	
N,N-Diethyl-*m*-toluamide[134-62-3]	DEET	98	DMSO-100µM	
Isopropyl cinnamate [7780-06-5]	IPC	98	DMSO-1mM	
Ruthenium red [11103-72-3]	RR	ND	Water-100µM	
N-(4-ethylphenyl)-2-((4-ethyl-5-(3-pyridinyl)-4H-1,2,4-triazol-3-yl)thio)acetamide [525582-84-7]	VUAA1	ND	Methanol-100µM	
N-(4-ethylphenyl)-2-((4-ethyl-5-(4-pyridinyl)-4H-1,2,4-triazol-3-yl)thio)acetamide [618427-06-8]	OrcoRAM2 (OA)	>90	DMSO-500µM	
N-(4-methylbenzyl)-2-thiophenecarboxamide	OX3a	>90	DMSO-100 µM	

Table 2. EC_{50} and IC_{50} values from concentration-dependent response curves.

I. Activation		
Receptor	**Chemical**	**EC_{50}** (pEC_{50}±Std.error) **R^2**
OR1+Orco	4-Methylphenol	2.8μM (5.55±0.198) 0.9416
OR2+Orco	Indole	3.4μM (5.47±0.119) 0.9829
Orco	OrcoRAM2	58.5μM (4.23±0.034) 0.9655
II. Inhibition/blockade		
Receptor	**Chemical**	**IC_{50}** (pIC_{50}±Std.error) **R^2**
OR1+Orco	Ethyl cinnamate	25.9μM (4.59±0.263) 0.859
	Isopropyl cinnamate	22.2μM (4.66±0.32) 0.8309
OR2+Orco	Ethyl cinnamate	28.9μM (4.54±0.079) 0.9814
	Isopropyl cinnamate	34μM (4.47±0.253) 0.9162
Orco	Ethyl cinnamate	64.5μM (4.19±0.054) 0.9903
	Isopropyl cinnamate	41.7μM (4.38±0.061) 0.9883

Fig. 1


A
pEIA
MCS
hr3 enhancer
P actin
actin pA
IE1 cassette
ORx
OR7 (Orco)
Photina
ORx
Orco
Photina
ORx
Orco
Ca2+
Photina

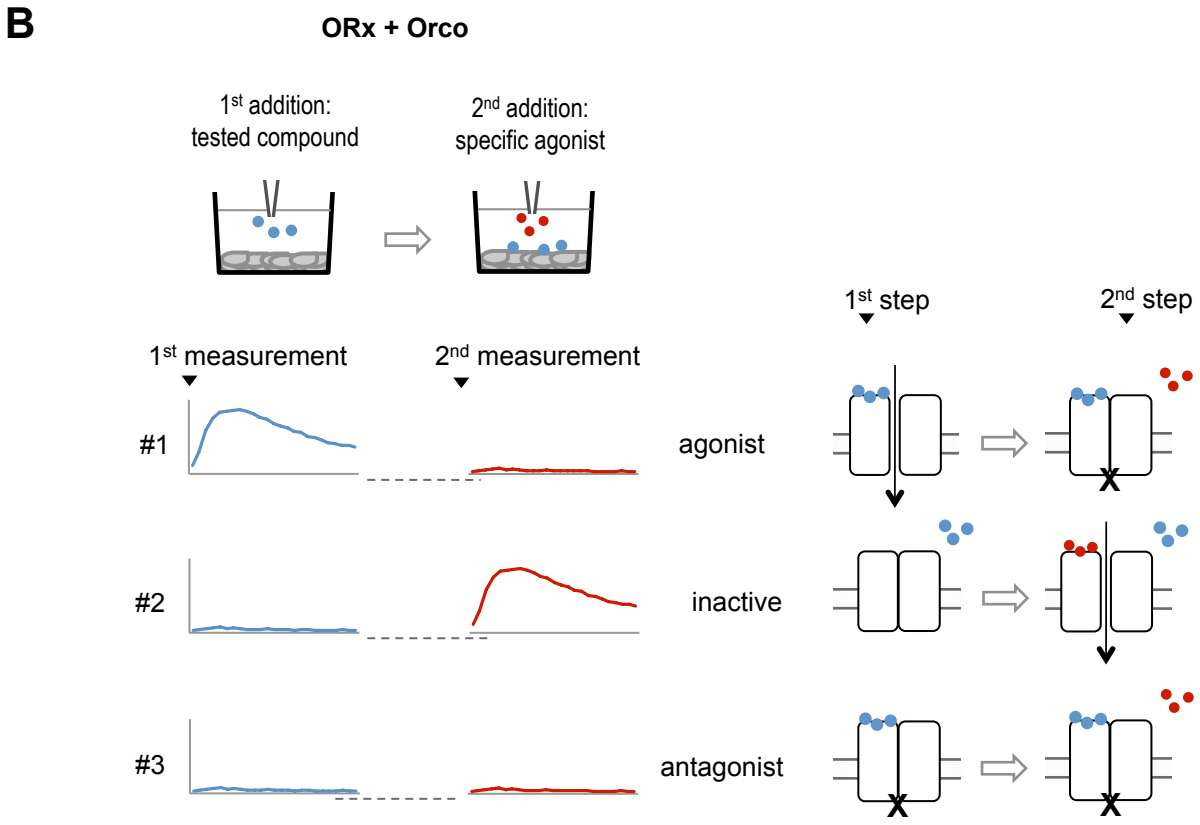

B
ORx + Orco
1st addition:
tested compound
2nd addition:
specific agonist
1st measurement
2nd measurement
1st step
2nd step
#1
agonist
#2
inactive
#3
antagonist

Fig. 2

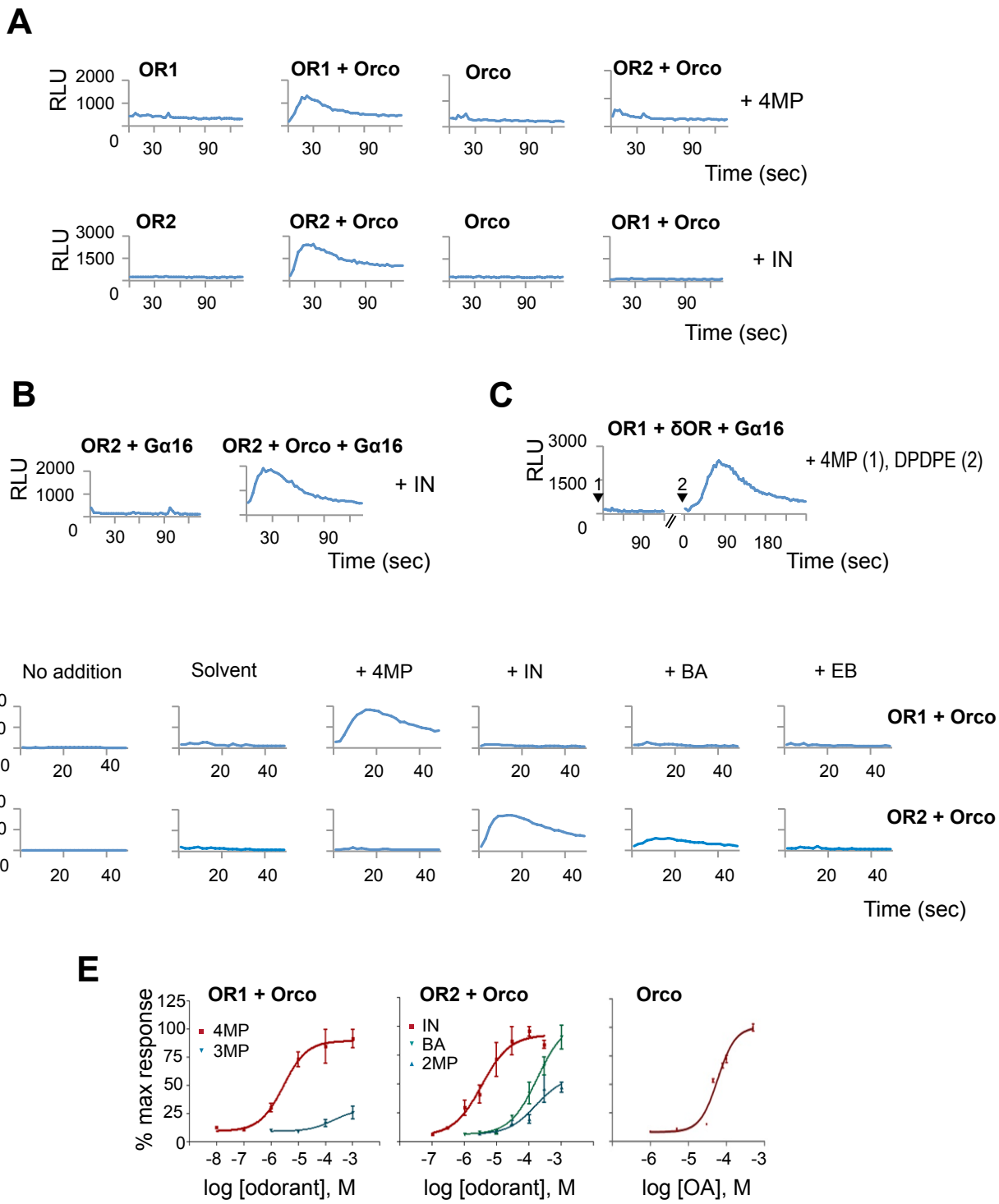

A
OR1
OR1 + Orco
Orco
OR2 + Orco
+ 4MP
RLU
2000
1000
0
30
90
Time (sec)
OR2
OR2 + Orco
Orco
OR1 + Orco
+ IN
3000
1500
B
OR2 + Gα16
OR2 + Orco + Gα16
C
OR1 + δOR + Gα16
+ 4MP (1), DPDPE (2)
1
2
90
0
180
D
No addition
Solvent
+ 4MP
+ IN
+ BA
+ EB
OR1 + Orco
OR2 + Orco
5000
2500
7000
3500
20
40
E
% max response
125
100
75
50
25
4MP
3MP
IN
BA
2MP
-8 -7 -6 -5 -4 -3
log [odorant], M
log [OA], M

Fig. 3

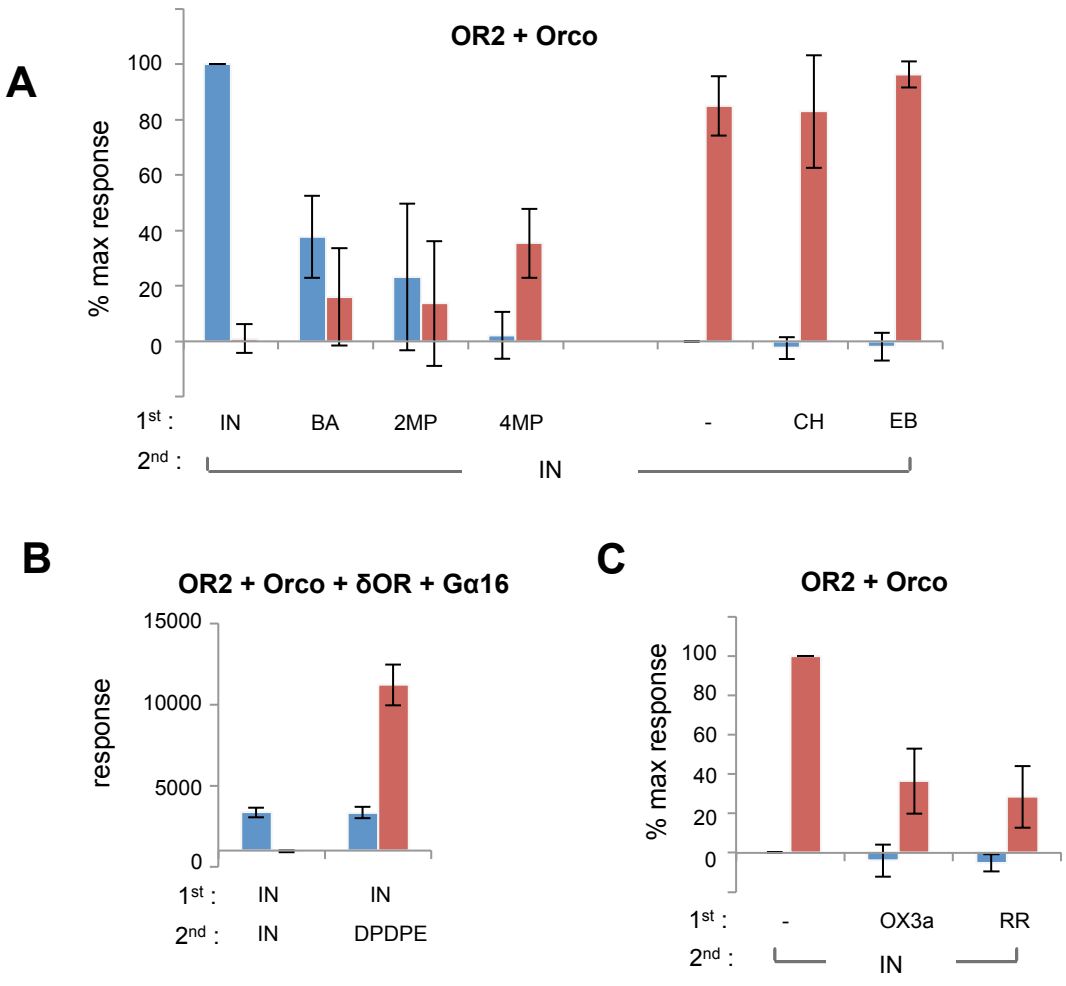
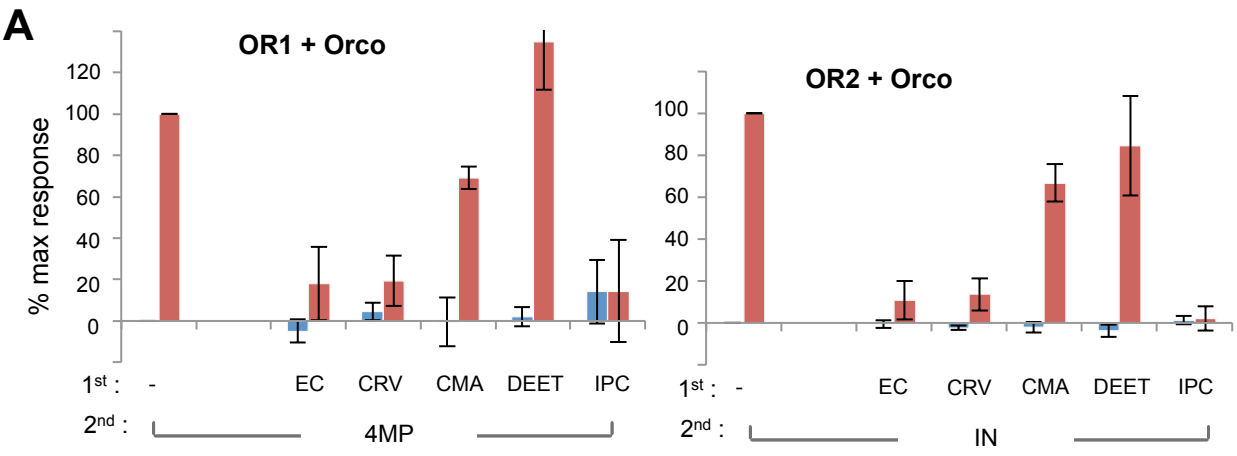
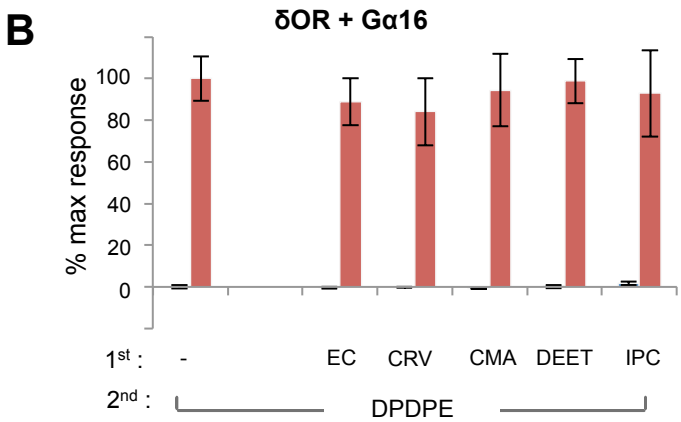

A
OR2 + Orco
% max response
100
80
60
40
20
0
1st : IN BA 2MP 4MP - CH EB
2nd : IN
B
OR2 + Orco + δOR + Gα16
response
15000
10000
5000
0
1st : IN IN
2nd : IN DPDPE
C
OR2 + Orco
% max response
100
80
60
40
20
0
1st : - OX3a RR
2nd : IN

Fig. 4


A
OR1 + Orco
% max response
120
100
80
60
40
20
0
1st : -
EC
CRV
CMA
DEET
IPC
2nd :
4MP
OR2 + Orco
100
80
60
40
20
0
1st : -
EC
CRV
CMA
DEET
IPC
2nd :
IN


B
δOR + Gα16
% max response
100
80
60
40
20
0
1st : -
EC
CRV
CMA
DEET
IPC
2nd :
DPDPE

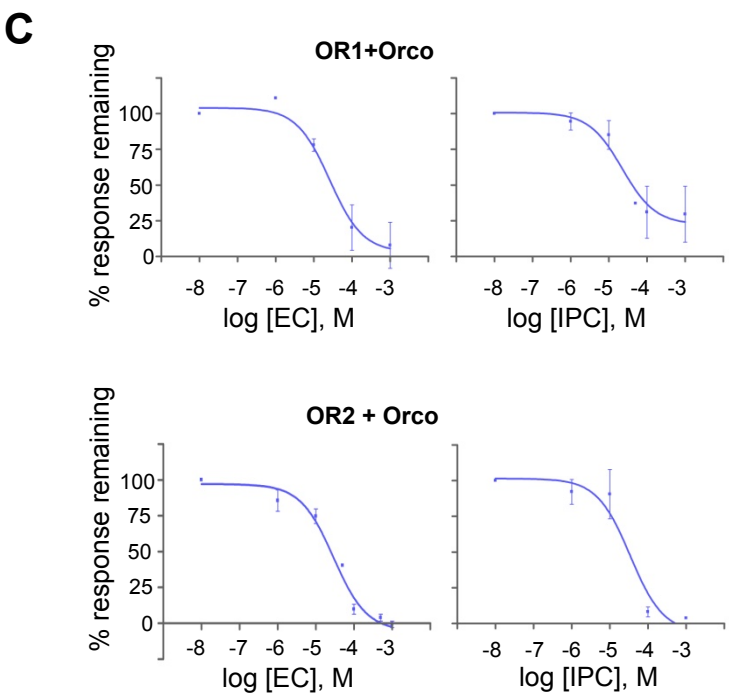
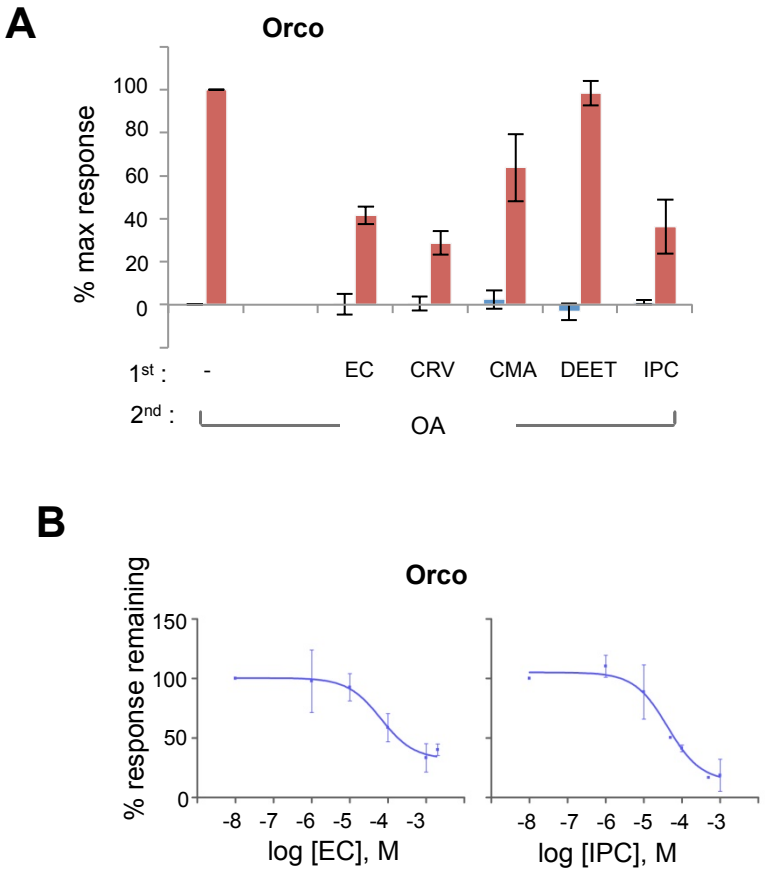

C
OR1+Orco
% response remaining
100
75
50
25
0
-8 -7 -6 -5 -4 -3
log [EC], M
-8 -7 -6 -5 -4 -3
log [IPC], M
OR2 + Orco
% response remaining
100
75
50
25
0
-8 -7 -6 -5 -4 -3
log [EC], M
-8 -7 -6 -5 -4 -3
log [IPC], M

Fig. 5


A
Orco
% max response
100
80
60
40
20
0
1st :
-
EC
CRV
CMA
DEET
IPC
2nd :
OA
B
Orco
% response remaining
150
100
50
0
-8 -7 -6 -5 -4 -3
log [EC], M
-8 -7 -6 -5 -4 -3
log [IPC], M

Neurobiology:

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