RESEARCH ARTICLE

Trace amines inhibit insect odorant receptor function through antagonism of the co-receptor subunit [v1; ref status: indexed, http://f1000r.es/35u]

Sisi Chen, Charles W. Luetje

Department of Molecular and Cellular Pharmacology, University of Miami Miller School of Medicine, Miami, FL, 33101, USA

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Abstract

Many insect behaviors are driven by olfaction, making insect olfactory receptors (ORs) appealing targets for insect control. Insect ORs are odorant-gated ion channels, with each receptor thought to be composed of a representative from a large, variable family of odorant binding subunits and a highly conserved co-receptor subunit (Orco), assembled in an unknown stoichiometry. Synthetic Orco directed agonists and antagonists have recently been identified. Several Orco antagonists have been shown to act via an allosteric mechanism to inhibit OR activation by odorants. The high degree of conservation of Orco across insect species results in Orco antagonists having broad activity at ORs from a variety of insect species and suggests that the binding site for Orco ligands may serve as a modulatory site for compounds endogenous to insects or may be a target of exogenous compounds, such as those produced by plants. To test this idea, we screened a series of biogenic and trace amines, identifying several as Orco antagonists. Of particular interest were tryptamine, a plant-produced amine, and tyramine, an amine endogenous to the insect nervous system. Tryptamine was found to be a potent antagonist of Orco, able to block Orco activation by an Orco agonist and to allosterically inhibit activation of ORs by odorants. Tyramine had effects similar to those of tryptamine, but was less potent. Importantly, both tryptamine and tyramine displayed broad activity, inhibiting odorant activation of ORs of species from three different insect orders (Diptera, Lepidoptera and Coleoptera), as well as odorant activation of six diverse ORs from a single species (the human malaria vector mosquito, *Anopheles gambiae*). Our results suggest that endogenous and exogenous natural compounds serve as Orco ligands modulating insect olfaction and that Orco can be an important target for the development of novel insect repellants.

Article Status Summary

Referee Responses

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1 **Joseph Dickens**, United States Department of Agriculture USA

2 **Coral Warr**, Monash University Australia

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Corresponding author: Charles W. Luetje (cluetje@med.miami.edu)

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Introduction

Insects have positive and negative impacts on humans, in terms of health, economy, and food stores. Insects pollinate plants to increase global food production, with 35% of global production of crops depending on animal pollinators[1,2]. Insects also cause significant destruction of crops and food stores[3–5]. Insects can also transmit fatal diseases such as dengue fever[6], malaria[7], yellow fever and epidemic typhus[8]. Insects use olfaction to sense their surroundings and to guide important activities, including feeding, mating and oviposition. This makes the insect olfactory system receptors an attractive target for the chemical control of deleterious insect species.

Insects use odorant receptors (ORs) to recognize and distinguish a diverse range of odorants[9,10]. Each OR is composed of two functionally essential parts: a highly conserved co-receptor subunit (Orco) and one of a large number of variable odorant-binding (or "tuning") subunits[11–17]. These subunits associate in an unknown stoichiometry to form an odorant-gated ion channel[18,19]. ORs have also been proposed to initiate, or be modified by, second messenger cascades[13,18]. While the odorant-binding subunit is responsible for interacting with odorants[9,20,21], both the odorant-binding subunits and Orco are involved in forming the ion channel pore[21,22]. Insect ORs are not related to the receptors and channels of humans and other tetrapods[15], suggesting that control of detrimental insect activity may be possible through the development of insect OR selective compounds. A current approach to developing these compounds is to identify the particular odorant binding subunits that recognize behaviorally important odorants[10,23–26] and then conduct large scale ligand screens[27,28], but high diversity among the odorant binding subunit repertoires of different species makes this approach exceptionally labor intensive[29,30].

The recent identification of the synthetic compound VUAA1 as a novel OR agonist that acts directly on Orco[27], suggests that manipulation of insect behavior might be achieved by targeting Orco. Based on the VUAA1 structure, several additional synthetic Orco agonists and a larger, more diverse series of synthetic Orco antagonists have been identified[31–33]. Importantly, several of these Orco antagonists were shown to inhibit odorant activation of ORs through a non-competitive mechanism[31–33]. These findings suggest that Orco antagonists might be useful in altering insect behavior.

Orco subunits are highly conserved across insect species, suggesting that Orco serves an essential function common to all insect ORs[15,34]. This high conservation underlies observations that Orco subunits from different species are functionally interchangeable; an Orco subunit from one species can form functional ORs with an odorant-binding subunit from a different species[21,22]. As the "pharmacology" of synthetic Orco agonists and antagonists has expanded, it has also become clear that Orco subunits from disparate insect species have very similar sensitivities to known Orco ligands[27,31–33,35]. This suggested to us that the binding site for Orco ligands may serve as a modulatory site for compounds endogenous to the insects or may be a target of exogenous compounds, such as those generated by plants. Insects use a variety of amines as neurotransmitters and neuromodulators[36–39]. Plants also generate a variety of amines that may play a role in resistance to insect herbivores[40–42]. For these reasons, we screened a panel of biogenic and trace amines for agonist and antagonist activity at insect Orco subunits. We found tryptamine to be a potent Orco antagonist with broad activity at Orco subunits from different species. Tyramine and phenethylamine also function as Orco antagonists, but were substantially less potent than tryptamine. Importantly, we found that tryptamine, acting through Orco, could inhibit odorant activation of a wide range of ORs from a variety of insect species. Our findings suggest a role for Orco as a modulatory site common to all insect ORs and support the development of Orco-directed compounds that can be used to manipulate insect behavior.

Methods

Materials

Xenopus laevis frogs were purchased from Nasco (Fort Atkinson, WI). The care and use of *Xenopus laevis* frogs in this study were approved by the University of Miami Animal Research Committee (Animal Welfare Assurance #A-3224-01, Protocol #13-056) and meet the guidelines of the US National Institutes of Health. All experimentation was conducted on cultured oocytes after surgical removal from the frogs (see below). The amines screened in this study (Figure 1), odorants (L-fenchone, acetophenone, geranyl acetate, 6-methyl-5-hepten-2-one, 2-nonanone and eugenol), OLC12 and other chemicals were from Sigma-Aldrich. Cqui\Orco (from *Culex quinquefasciatus*), Onub\Or6, Onub\Orco (from *Ostrinia nubilalis*), Mcar\Or5 and Mcar\Orco (from *Megacyllene caryae*) were cloned and inserted into the pGEMHE vector[43] as previously described[23,24,44,45]. Dmel\Or35a and Dmel\Orco (from *Drosophila melanogaster*) were generously provided by J. Carlson and L. Vosshall, respectively. Agam\Or27, Agam\Or28, Agam\Or31, Agam\Or39, Agam\Or48, Agam\Or65 and Agam\Orco (from *Anopheles gambiae*) were generously provided by L. Zweibel.

Expression of insect ORs in *Xenopus* oocytes

Mature *Xenopus laevis* frogs were anesthetized by submersion in 0.1% 3-aminobenzoic acid ethyl ester. Depth of anesthesia was judged by loss of nasal flare and swallow reflexes. Oocytes were surgically removed. The incision was treated with gentamicin sulfate (two subcutaneous injections of 0.1 mL 10 mg/mL gentamycin at the surgical site) and sutured. Immediately following surgery (and before recovery from anesthesia), as an analgesia agent, one subcutaneous injection of Meloxicam solution (0.1 mg/mL) (0.1 mg/kg body weight) was administered to the dorsal lymph sac of the frogs. The frogs were allowed to recover from surgery in a humid chamber before being placed back in the holding tank. Surgeries were performed on individual frogs no more often than once every 3 months. Following the fourth surgery, frogs were anesthetized as described above and then pithed.

Follicle cells were removed by treatment with collagenase B (Boehringer Mannheim) for 2 hours at room temperature. Capped cRNA encoding each OR subunit was generated using mMessage mMachine kits (Ambion). For heteromeric ORs, 25 ng of cRNA encoding each OR subunit was injected into Stage V-VI *Xenopus* oocytes. For expression of Orco homomers, 50 ng of cRNA was injected. Oocytes were incubated at 18°C in Barth's saline (in mM: 88 NaCl, 1 KCl, 2.4 $NaHCO_3$, 0.3 $CaNO_3$, 0.41 $CaCl_2$, 0.82 $MgSO_4$, 15 HEPES, pH 7.6, and 150μg/ml ceftazidime) for 2–5 days prior to electrophysiological recording.

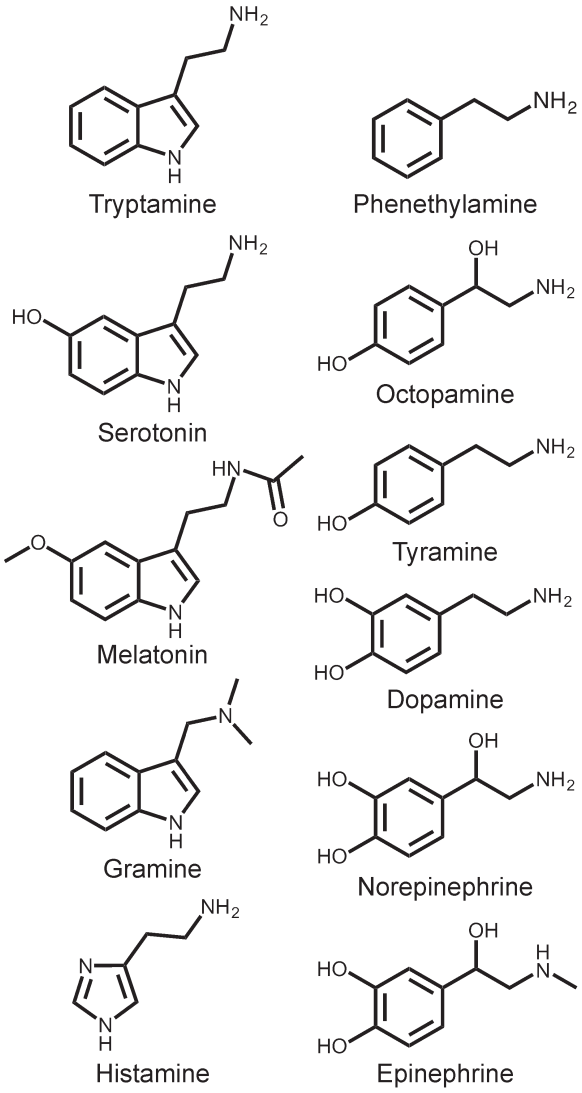

Figure 1. Structures of amines tested in this study.

Electrophysiology and data capture

Odorant and Orco ligand induced currents were recorded under two-electrode voltage clamp, using an automated parallel electrophysiology system (OpusExpress 6000A, Molecular Devices). Oocytes were perfused with ND96 (in mM: 96 NaCl, 2 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 5 HEPES, pH 7.5). Orco ligands were prepared as 50 or 100 mM stock solutions in DMSO and then diluted into ND96 on the day of the experiment. Odorants were prepared as 100 mM stock solutions in DMSO and then diluted into ND96. Unless otherwise noted, applications were for 60 sec at a flow rate of 1.0 ml/min, with extensive washing in ND96 at 4.6 ml/min between applications. Micropipettes were filled with 3 M KCl and had resistances of 0.2–2.0 MΩ. The holding potential was -70 mV. Current responses, filtered (4-pole, Bessel, low pass) at 20 Hz (-3 db) and sampled at 100 Hz, were captured and stored using OpusXpress 1.1 software (Molecular Devices).

Experimental protocols and data analysis

To screen for agonist activity, oocytes were exposed to 30 sec applications of candidate compounds with 5 min washes between applications (Figure 2A). For the concentration-response protocol (Table 1), applications were for 20 sec at a flow rate of 1.65 ml/min.

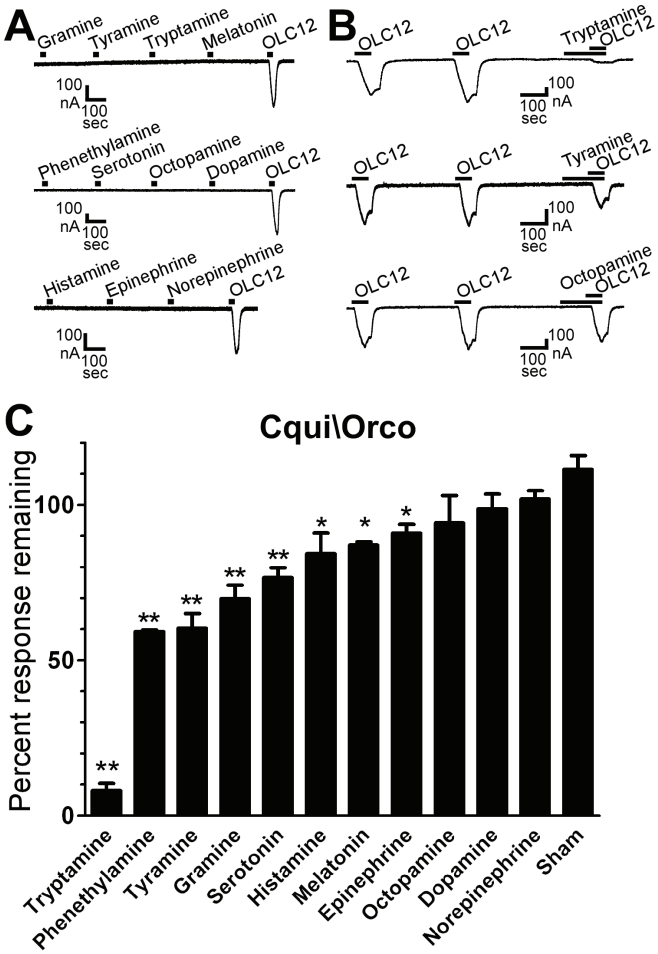

Figure 2. Tryptamine and several other amines are antagonists of Cqui\Orco. A) The tested amines do not display Orco agonist activity. Oocytes expressing Cqui\Orco were challenged with 30 sec applications of 100μM gramine, tyramine, tryptamine and melatonin (top trace), phenethylamine, serotonin, octopamine and dopamine (middle trace), or histamine, epinephrine and norepinephrine (bottom trace), with 5 min washes between applications. 30μM OLC12 (Orco agonist) was applied at the end of each trace. **B**) Tryptamine and tyramine are antagonists of Cqui\Orco. Oocytes expressing Cqui\Orco were exposed to 60 sec applications of 30μM OLC12 with 4 min washes between applications. 100μM tryptamine (top trace), tyramine (middle trace), or octopamine (bottom trace) were applied and incubated for 90 sec preceding the third application of OLC12 and then co-applied during the OLC12 application. **C**) Screen of 11 amines for Orco antagonism. Responses of Cqui\Orco to 30μM OLC12 (~EC_5) in the presence of 100μM of each compound are presented as a percentage of the average of two preceding responses to OLC12 alone (mean ± SEM, n = 3-10). Statistical significance was assessed by one-way ANOVA, followed by Dunnett's post-test comparing to sham treated oocytes (*p<0.01; **p<0.001).

Table 1. Odorant and Orco agonist concentration-response curve values for Orco homomers and heteromeric ORs from several insect species. Concentration-response data was fit as described in Methods. n_H is the apparent Hill coefficient. Values are presented as mean ± SEM (n = 3-14).

Receptor	Ligand (Normalizing Conc.)	EC_{50} μM	n_H	Fit Max
Agam\Orco	OLC12 (30μM)	124 ± 9	2.4 ± 0.3	47 ± 2
Agam\Orco + Agam\Or31	Geranyl Acetate (30μM)	65 ± 23	1.0 ± 0.3	2.9 ± 0.3
Agam\Orco + Agam\Or65	Eugenol (1μM)	0.08 ± 0.01	0.9 ± 0.1	1.0 ± 0.03
Agam\Orco + Agam\Or65	OLC12 (30μM)	67 ± 6	2.0 ± 0.3	5.7 ± 0.3
Cqui\Orco	OLC12 (30μM)	95 ± 6	2.5 ± 0.3	48 ± 2
Dmel\Orco	OLC12 (10μM)	36 ± 4	3.9 ± 1.9	36 ± 4
Dmel\Orco + Dmel\Or35a	OLC12 (10μM)	20 ± 5	1.9 ± 0.8	4.7 ± 0.4
Onub\Orco + Onub\Or6	OLC12 (100μM)	100 ± 4	2.1 ± 0.2	2.0 ± 0.1

To measure antagonist activity at Orco (Figure 2B, 2C, Figure 3, Figure 4A and Figure 5A), oocytes were exposed to two 60 sec applications of the synthetic Orco agonist OLC12 (2-((4-Ethyl-5-(4-pyridinyl)-4H-1,2,4-triazol-3-yl)sulfanyl)-N-(4-isopropylphenyl)acetamide) with 4 min washes between applications. Oocytes were then exposed to a 90 sec application of antagonist candidate, immediately followed by a 60 sec co-application of antagonist candidate and OLC12. The current response in the presence of antagonist candidate was compared to the mean of the preceding two responses to OLC12 alone and is presented as a percentage.

To measure inhibition of odorant activation of heteromeric ORs (Figures 4B, 4C, Figure 5B and Figure 6), oocytes were exposed to a 30 sec application of odorant followed by a 10 min wash. Oocytes were then exposed to a 90 sec application of tryptamine or tyramine, immediately followed by a 30 sec co-application of tryptamine or tyramine and odorant. The current response in the presence of antagonist candidate was compared to the preceding response to odorant alone and expressed as a percentage. In our previous work, we found that repeated odorant applications to some ORs could cause a progressive decrease in response amplitude[31,33]. For this reason, we then re-normalized antagonism data to the value obtained when the assay was run in the absence of antagonist candidate (sham). In the "sham" assay, oocytes were exposed to a 30 sec application of odorant followed by a 10 min wash and then exposed to a 90 sec application of ND96 (no antagonist candidate), immediately followed by a 30 sec application of odorant. The second odorant response was compared to the first response and expressed as a percentage. In Figure 4B, 4C, Figure 5B and Figure 6, the sham value for 100nM eugenol was 57 ± 3% (mean ± SEM, n = 3). In Figure 5B, the sham value for 10nM eugenol was 93 ± 4% (n = 4). In Figure 4B and C, the sham value for 1μM Z11-14:OAc was 82 ± 6% (n = 6) and the sham value for 150μM 2-phenylethanol was 92 ± 2% (n = 3). In Figure 6, the sham value for 3μM L-fenchone was 83 ± 1% (n = 3), the sham value for 40μM acetophenone was 92 ± 1% (n = 3), the sham value for 70μM geranyl acetate was 97 ± 1% (n = 3), the sham value for 10μM 6-methyl-5-hepten-2-one was 94 ± 2% (n = 3) and the sham value for 3μM 2-nonanone was 81 ± 1% (n = 3).

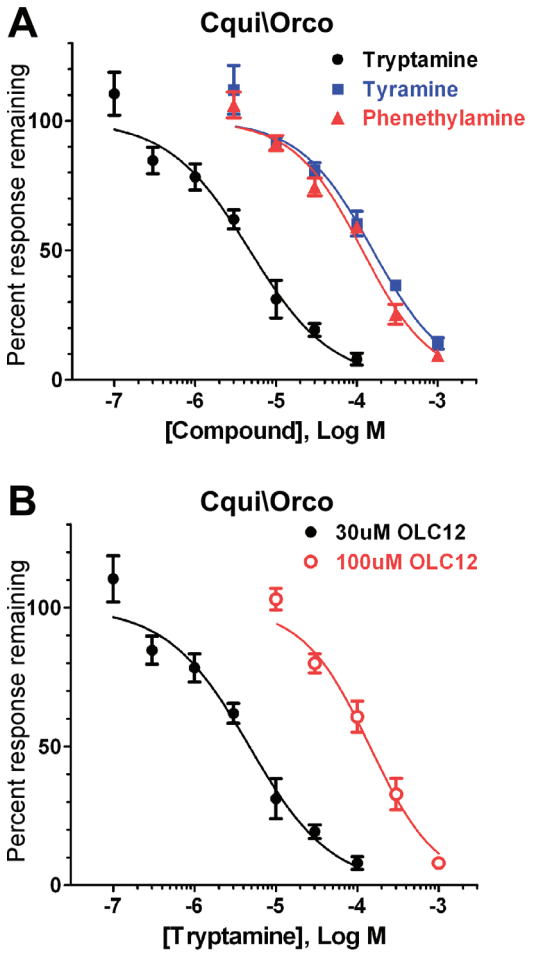

Figure 3. Trace amine antagonists of Cqui\Orco. A) Concentration-inhibition curves for tryptamine, tyramine and phenethylamine inhibition of Cqui\Orco activated by 30μM OLC12. **B**) Altering the concentration of Orco agonist (OLC12) shifts the tryptamine inhibition curve. The IC_{50} for tryptamine inhibition of Cqui\Orco activation by 30μM OLC12 (4.7 ± 0.7μM, n = 5) is significantly different ($p<0.0001$, F-test) from the IC_{50} for tryptamine inhibition of Cqui\Orco activation by 100μM OLC12 (143 ± 18μM, n = 6).

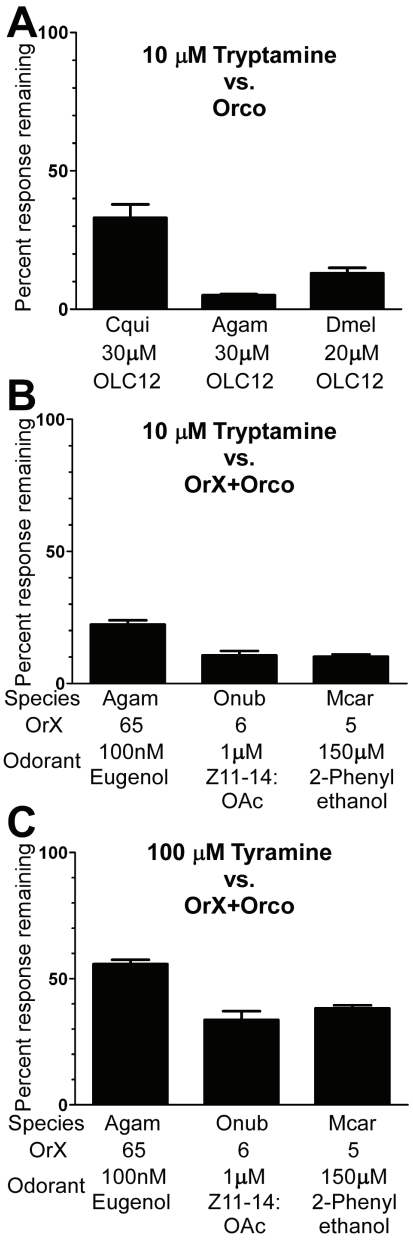

Figure 4. Tryptamine and tyramine inhibit odorant activation of ORs from different insect species. A) Oocytes expressing Orco from each of three different species were activated by the indicated concentration of OLC12. For Cqui\Orco from *Cx. quinquefasciatus*, 30μM is the ~EC_5; for Agam\Orco from *An. gambiae*, 30μM is the ~EC_3; for Dmel\Orco from *D. melanogaster*, 20μM is the ~EC_{10}. Current responses in the presence of 10μM tryptamine were compared to the average of two preceding responses to OLC12 and are presented as mean ± SEM (n = 4-9). **B**–**C**) Tryptamine and tyramine inhibit odorant activation of heteromeric ORs from different insect species. Oocytes expressing an OR from *An. gambiae* (Agam\Orco+Agam\Or65) were activated by 100nM eugenol, oocytes expressing an OR from *O. nubilalis* (Onub\Orco+Onub\Or6) were activated by 1μM Z11-14:OAc, oocytes expressing an OR from *M. caryae* (Mcar\Orco+Mcar\Or5) were activated by 150μM 2-phenylethanol. Current responses in the presence of 10μM tryptamine (**B**) or 100μM tyramine (**C**) were compared to the preceding response to odorant alone and are presented as mean ± SEM (n = 3).

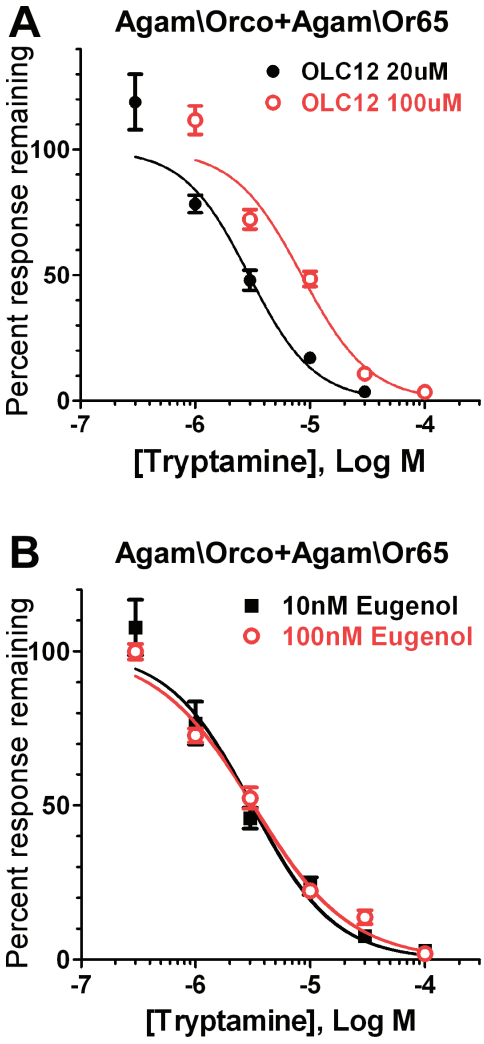

Figure 5. Tryptamine antagonism of odorant activation of an Agam\OR is non-competitive. A) Tryptamine competitively inhibits OLC12 activation of Agam\Orco+Agam\Or65. Altering the concentration of Orco agonist (OLC12) shifts the tryptamine inhibition curve. The IC_{50} for tryptamine inhibition of Agam\Orco+Agam\Or65 activation by 20μM OLC12 (2.9 ± 0.5μM, n = 3) is significantly different (p<0.0001, F-test) from the IC_{50} for tryptamine inhibition of Agam\Orco+Agam\Or65 activation by 100μM OLC12 (8.5 ± 1.1μM, n = 3). **B**) Tryptamine non-competitively inhibits odorant activation of Agam\Orco+Agam\Or65. Altering odorant (eugenol) concentration fails to shift the tryptamine inhibition curve. The IC_{50} values for tryptamine inhibition of responses to 10nM eugenol (3.1 ± 0.4μM, n = 4), and 100nM eugenol (3.2 ± 0.3μM, n = 3) did not differ (p=0.7172, F-test).

Initial analysis of electrophysiological data was done using Clampfit 9.1 software (Molecular Devices). Curve fitting and statistical analyses were done using Prism 5 (Graphpad). Concentration-inhibition data were fit to the equation: $I = I_{max}/(1+ (X/IC_{50})^n)$ where I represents the current response at a given concentration of inhibitor, X; I_{max} is the maximal response in the absence of inhibitor; IC_{50} is the concentration of inhibitor present that still allows a half maximal response from odorant; n is the apparent Hill coefficient. Concentration-response data were fit to the equation: $I = I_{max}/(1+(EC_{50}/X)^n)$

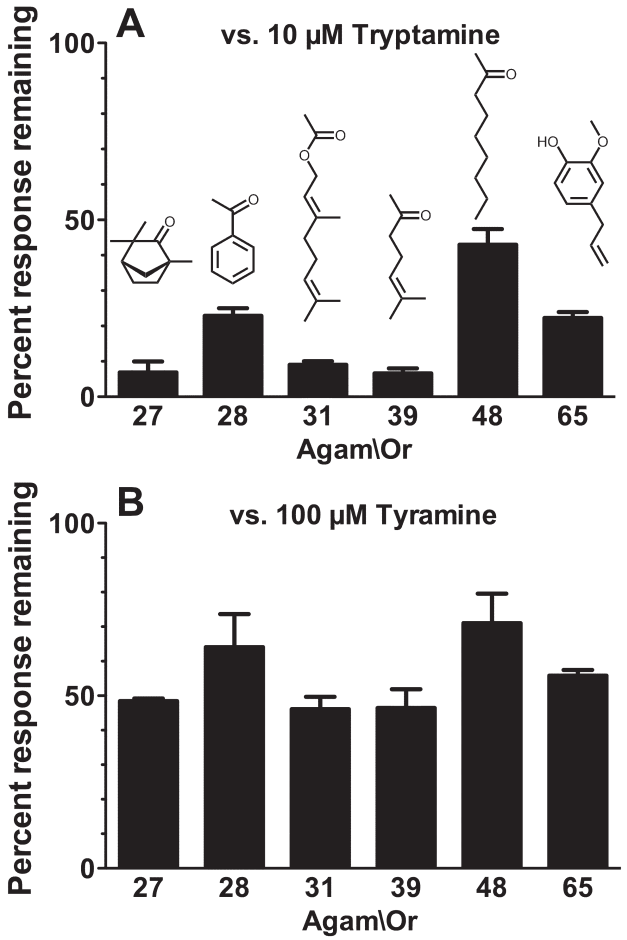

Figure 6. Tryptamine and tyramine inhibit odorant activation of multiple Agam\ORs. Current responses of oocytes expressing Agam\Orco+Agam\Or27 (activated by 3μM L-fenchone), Agam\Orco+Agam\Or28 (activated by 40μM acetophenone), Agam\Orco+Agam\Or31 (activated by 70μM geranyl acetate), Agam\Orco+Agam\Or39 (activated by 10μM 6-methyl-5-hepten-2-one), Agam\Orco+Agam\Or48 (activated by 3μM 2-nonanone), or Agam\Orco+Agam\Or65 (activated by 100nM eugenol) in the presence of 10μM tryptamine (**A**) or 100μM tyramine (**B**) were compared to the preceding response to odorant alone and are presented as mean ± SEM (n = 3). Odorant structures are shown.

where I represents the current response at a given concentration of odorant, X; I_{max} is the maximal response; EC_{50} is the concentration of agonist yielding a half maximal response; n is the apparent Hill coefficient. Statistical significance ($p<0.05$) was assessed using a two-tailed unpaired *t* test, an F test, or a one-way analysis of variance followed by the Dunnett's post-test, as appropriate.

Results

Inhibition of odorant and Orco agonist initiated current responses of oocytes expressing insect odorant receptors by various amines

13 Data Files

http://dx.doi.org/10.6084/m9.figshare.977791

To screen a panel of biogenic and trace amines (Figure 1), we expressed Orco from *Culex quinquefasciatus* (Southern House Mosquito) in *Xenopus* oocytes and recorded ligand-induced current responses using two-electrode voltage clamp electrophysiology (see Methods). Orco subunits from several species, including Cqui\Orco, have been shown to form homomeric channels when heterologously expressed in the absence of odorant-binding subunits[27,33]. This convenient property of Orco allowed us to perform the initial screen without potentially confounding interactions with odorant-binding subunits. Successful functional expression of Cqui\Orco was confirmed by application of OLC12, a previously identified Orco specific agonist[31]. While OLC12 elicited robust current responses, none of the amines displayed agonist activity at Cqui\Orco (Figure 2A). Next we screened the amines for antagonist activity by applying 30μM OLC12 (~EC_5) to activate Cqui\Orco and co-applying 100μM of each amine (Figure 2B, C). Several amines were able to inhibit OLC12 activation of Cqui\Orco. Tryptamine was the most effective antagonist, blocking more than 90% of the OLC12 response (92 ± 2% inhibition). Highly significant inhibition ($p<0.001$) was also observed for phenethylamine (41 ± 1%), tyramine (40 ± 5%), gramine (30 ± 4%) and serotonin 23 ± 3%), but the extent of inhibition was less than 50%, suggesting relatively low affinity interactions. Histamine (16 ± 8%), melatonin (13 ± 1%) and epinephrine (9 ± 3%) also displayed significant ($p<0.01$), but modest, inhibition of the OLC12 current. Octopamine, dopamine and norepinephrine were inactive in this assay.

In Figure 3A, we constructed concentration-inhibition curves for block of Cqui\Orco activity in order to quantitatively evaluate the inhibitory potency of tryptamine, as well as phenethylamine and tyramine, representing the less effective amines. Tryptamine was clearly the most potent of these antagonists, inhibiting Cqui\Orco with an IC_{50} of 4.7 ± 0.7μM, a value similar to that of the most potent synthetic Orco antagonists that we identified in our previous work[33]. Phenethylamine (IC_{50} = 117 ± 12μM) and tyramine (IC_{50} = 157 ± 22μM) were substantially less potent than tryptamine (25-fold and 33-fold, respectively). Previously identified Orco antagonists inhibited OLC12 activation of Orco through a competitive mechanism[31,33]. To determine whether tryptamine was also a competitive antagonist of Orco, we measured blockade of Cqui\Orco achieved by tryptamine when the OLC12 concentration was increased from 30μM to 100μM (Figure 3B). Tryptamine was significantly less effective at inhibiting responses to 100μM OLC12 (IC_{50} = 143 ± 18μM, $p<0.0001$, F-test), indicating that tryptamine is a competitive antagonist of Cqui\Orco.

We next asked whether tryptamine could also inhibit Orco from other insect species. In addition to Cqui\Orco, we tested Agam\Orco from *An. gambiae* (human malaria vector mosquito) and Dmel\Orco from *D. melanogaster*. Co-application of 10μM tryptamine inhibited OLC12 activation of Orco from each of these three insect species (Figure 4A). We then wondered whether tryptamine could also inhibit odorant activation of heteromeric insect ORs containing both Orco and odorant binding subunits. We chose ORs from three insect orders: Agam\Orco+Agam\Or65 from *An. gambiae* (Order Diptera) that responds to the eugenol[25]; Onub\Orco+Onub\Or6 from *O. nubilalis* (European Corn Borer, Order Lepidoptera) that responds to the pheromone Z11-14:OAc[45]; and

Mcar\Orco+Mcar\Or5 from *M. caryae* (Long-Horned Beetle, Order Coleoptera) that responds to 2-phenylethanol[44]. We chose to proceed with an OR from *An. gambiae* instead of *Cx. quinquefasciatus* for two reasons. The best characterized of the Cqui\Or subunits respond to indoles[23,24], which are structurally related to tryptamine and might confound our experiments. Also, the Agam\Or subunit family has been more extensively characterized[10,25], offering more options for OR expression (see below). Each odorant was applied at or near the EC_{50} concentration (Table 1,[44,45]). Co-application of 10μM tryptamine resulted in substantial inhibition of each receptor (Figure 4B). We also examined tyramine. While tyramine is a low potency Orco antagonist (Figure 3A), it is a major neurotransmitter in insects[37]. Tyramine was also able to reduce odorant activation of these ORs, but was less effective than tryptamine (Figure 4C). These results suggest that tryptamine and tyramine are broadly active antagonists of insect ORs.

Several previously identified Orco antagonists have been shown to inhibit odorant activation of insect ORs through a non-competitive mechanism[31–33]. To determine whether the tryptamine inhibition of odorant activation that we observed in Figure 4 was also non-competitive, we examined the effect of tryptamine on activation of the heteromeric Agam\Orco+Agam\Or65 in more detail (Figure 5). When the concentration of Orco directed agonist (OLC12) was increased, the tryptamine inhibition curve was significantly shifted to the right (Figure 5A). However, when the concentration of odorant agonist (eugenol) was increased, the tryptamine inhibition curve did not shift (Figure 5B). These results indicate that, similar to previously identified synthetic Orco antagonist compounds, tryptamine is a competitive antagonist of direct activation of Orco and a non-competitive antagonist of odorant activation of the OR.

The ability of tryptamine to interact with Orco and exert a non-competitive inhibitory effect on odorant activation of a heteromeric OR (Figure 5) suggests that tryptamine should be able to inhibit activation of a variety of ORs activated by diverse odorants. To examine this possibility, we tested the ability of tryptamine to inhibit odorant activation of ORs formed by Agam\Orco and each of six different odorant-binding subunits chosen from across the *An. gambiae* OR gene family[46]. We activated each OR with a previously identified cognate odorant[25] at a concentration at or near the EC_{50} (Table 1,[25]). In addition to Agam\Orco+Agam\Or65 (activated by eugenol), we tested Agam\Orco+Agam\Or27 (activated by L-fenchone), Agam\Orco+Agam\Or28 (activated acetophenone), Agam\Orco+Agam\Or31 (activated by geranyl acetate), Agam\Orco+Agam\Or39 (activated by 6-methyl-5-hepten-2-one) and Agam\Orco+Agam\Or48 (activated by 2-nonanone). With the exception of Agam\Or39 and Agam\Or48, which display overlapping odorant specificities at 4 odorants, there is little or no similarity among the odorant specificities of these six odorant-binding subunits[25]. In each case, 10μM tryptamine was able to inhibit odorant activation of the receptor, despite the disparate odorant-binding subunits and diverse odorant structures (Figure 6). Tyramine was also able to inhibit odorant activation of each of these receptors, but was less effective than tryptamine (note that tyramine is applied at 100μM). We conclude that tryptamine and tyramine are general antagonists of insect ORs.

Discussion

Animals use a variety of biogenic and trace amines as neurotransmitters and neuromodulators. These include compounds derived from tyrosine (dopamine, norepinephrine, epinephrine, tyramine, octopamine and phenethylamine), tryptophan (serotonin, melatonin and tryptamine) and histidine (histamine)[47]. Dopamine and serotonin play a variety of roles in the insect nervous system[48–50]. In addition, insects use octopamine, histamine and tyramine as neurotransmitters[48–53]. Melatonin also appears to exert neuromodulatory effects in insects[54,55]. Interestingly, many of these amines modulate the olfactory system[56–58].

Recent reports[27,32], together with our previous findings[31,33], have revealed the existence of a ligand-binding site on the Orco subunit and that inhibition of odorant activation through a non-competitive mechanism may be a general property of Orco-directed antagonists. Our current results suggest that endogenous and exogenous natural compounds serve as Orco ligands and modulate insect olfaction. While tyramine is a major neurotransmitter in insects[53], its low potency in our assay (Figure 3) suggests that it might not serve as an endogenous OR modulator. However, the function of an endogenous Orco antagonist is unlikely to be the complete block of OR function. Rather, an endogenous Orco antagonist might be used to diminish olfactory sensitivity by inhibiting a fraction of the available receptors. For tyramine, such inhibition could occur at concentrations ranging from 10μM to 30μM. Alternatively, there may be additional, more potent, but as yet uncharacterized, endogenous Orco antagonists that can decrease olfactory sensitivity at lower concentrations.

In contrast to the low potency of tyramine, we found tryptamine to be a high potency Orco antagonist. Tryptamine inhibited odorant activation of an OR with an IC_{50} in the low micromolar range (Figure 5). While it is currently unclear whether tryptamine is endogenous to insects, tryptamine and similar compounds, such as gramine, are produced by a variety of plants and are thought to serve as a defense against insect herbivores[42,59]. Various tryptamine analogs have been proposed as larvicides[60] and when tryptamine is caused to accumulate in poplar and tobacco, through ectopic expression of tryptophan decarboxylase, the feeding behavior of insects that target these plants is altered[41]. Tryptamine-based structures also act on various receptors and transporters, particularly those involved in serotonergic neurotransmission, exerting psychedelic effects in humans. Indeed, many plant derived and synthetic hallucinogens are based on the tryptamine and phenethylamine scaffolds[61–63]. Interestingly, the potency that we observed for tryptamine inhibition of odorant activation of an insect OR (Figure 5) is similar to the potency for tryptamine inhibition of the *D. melanogaster* serotonin transporter[64].

Might there also be natural endogenous or exogenous Orco agonists? An endogenous Orco agonist could serve to increase olfactory sensitivity, perhaps in a circadian fashion, to alter behavior during critical foraging or mating periods. An exogenous, plant-derived Orco agonist would, by activating all ORs through Orco, serve as an olfactory “confusant” and might alter the feeding behavior of insect herbivores. The limited screen of 11 compounds

that we conducted here did not identify any Orco agonists, but more extensive screening is clearly warranted.

Several synthetic Orco antagonists have been shown to inhibit odorant activation of ORs through an allosteric mechanism[31–33]. The ability of these compounds to inhibit multiple ORs from a variety of species is likely due to the high conservation of Orco across the insects[12]. Similarly, we found that tryptamine and tyramine, acting as Orco antagonists, could inhibit odorant activation of ORs from insect species chosen from three different orders: Diptera (*An. gambiae*), Lepidoptera (*O. nubilalis*) and Coleoptera (*M. caryae*). Furthermore, when we examined multiple ORs from a single species (*An. gambiae*), we found that tryptamine and tyramine blocked odorant activation of each receptor. The action of these compounds through Orco allowed blockade to occur despite the highly diverse odorant-binding subunits used to form the receptors and the different odorant structures used to activate the receptors. Interestingly, while all six receptors were inhibited, the extent of inhibition varied depending on the odorant-binding subunit present and the pattern of variation was similar for tryptamine and tyramine. This suggests differences in allosteric coupling between Orco and the various odorant-binding subunits. Also, while we showed that tryptamine is a potent inhibitor of odorant activation of Agam\Or65+Agam\Orco, the results we present in Figure 6 suggest that tryptamine is even more potent at other ORs, such as those formed by Agam\Or27, Agam\Or31 and Agam\Or39. Our current results with naturally occurring amines, together with previous reports with synthetic compounds[27,31–33,35] strongly suggest that: 1) allosteric antagonism of odorant activation of ORs is a general property of Orco antagonists; 2) Orco antagonists are broadly active at ORs of many insect species; and 3) Orco is an important target for the development of novel insect repellants. The broad activity of Orco directed compounds across many insect species that has been observed to date suggests that these compounds may have limited agricultural utility, since both pests and pollinators could be affected. Determining whether species-specific Orco ligands can be developed will require further effort. What is clear, however, is that the pursuit of new, synthetic Orco directed ligands (both agonists and antagonists) is a promising direction for the development of new, more effective insect repellants that can aid in controlling the spread of insect-borne diseases.

Data availability

figshare: Inhibition of odorant and Orco agonist initiated current responses of oocytes expressing insect odorant receptors by various amines, doi: 10.6084/m9.figshare.977791[65]

Author contributions

SC and CWL conceived the study. SC and CWL designed the experiments. SC performed the experiments. SC and CWL analyzed the data. SC and CWL wrote the manuscript.

Competing interests

No competing interests were disclosed.

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Referee Responses for Version 1

Coral Warr
School of Biological Sciences, Monash University, Clayton, Victoria, Australia

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In this study the authors report the identification of new antagonists for the Orco protein, a co-receptor for the ligand binding Olfactory receptor (Or) family in insects. This is of interest as they suggest Orco antagonists might be a useful approach to modifying insect behaviour. The authors screened a panel of biogenic amines and found several that functioned as Orco antagonists, of which tryptamine, naturally produced in plants, was the most effective. They further showed that this activity was conserved against Orco from a number of insect species. The paper is well written, the data is solid and well presented, and is appropriately analysed and interpreted. The caveat to the use of currently identified Orco antagonists in biocontrol, namely that they appear to affect Orco in all insects and are not species-specific, is appropriately acknowledged. My one feedback comment is that the justification for screening just amines was not really clear, and I wondered if the authors had also in fact screened other types of compounds? If so it would be valuable to other researchers to also mention any screened compounds that did not have any effect on Orco function.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Competing Interests: No competing interests were disclosed.

Joseph Dickens
Invasive Insect Biocontrol and Behaviour Laboratory, United States Department of Agriculture, Beltsville, MD, USA

Approved: 07 April 2014

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This is a well-written paper that documents the discovery of modulatory effects of biogenic amines on the responses of odorant receptor assemblages (OR = odorant receptor + Orco = odorant receptor co-receptor) in insects. Both plant- and insect-produced amines are shown to antagonize responses of OR/Orco complexes to known agonists through interactions suggested to be with Orco. The potential role of the amines in regulating insect chemosensory behavior is suggested, and Orco is proposed as an important target for development of novel insect repellents.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Competing Interests: No competing interests were disclosed.