Insect Biochemistry and Molecular Biology 66 (2015) 31–41

Contents lists available at ScienceDirect

Insect Biochemistry and Molecular Biology

journal homepage: www.elsevier.com/locate/ibmb

Review

Towards an understanding of the structural basis for insect olfaction by odorant receptors

Colm Carraher [a], Julie Dalziel [b], Melissa D. Jordan [a], David L. Christie [c], Richard D. Newcomb [a, c], Andrew V. Kralicek [a, *]

[a] The New Zealand Institute for Plant & Food Research Limited, Private Bag 92169, Auckland 1142, New Zealand
[b] Food Nutrition & Health Team, Food & Bio-based Products Group, AgResearch Private Bag 11008, Palmerston North 4442, New Zealand
[c] School of Biological Sciences, University of Auckland, Private Bag 92019, Auckland 1142, New Zealand

ARTICLE INFO

Article history:
Received 10 May 2015
Received in revised form
16 September 2015
Accepted 16 September 2015
Available online 28 September 2015

Keywords:
Insect olfaction
Odorant receptor
Orco
Structure
Function

ABSTRACT

Insects have co-opted a unique family of seven transmembrane proteins for odour sensing. Odorant receptors are believed to have evolved from gustatory receptors somewhere at the base of the Hexapoda and have expanded substantially to become the dominant class of odour recognition elements within the Insecta. These odorant receptors comprise an obligate co-receptor, Orco, and one of a family of highly divergent odorant "tuning" receptors. The two subunits are thought to come together at some as-yet unknown stoichiometry to form a functional complex that is capable of both ionotropic and metabotropic signalling. While there are still no 3D structures for these proteins, site-directed mutagenesis, resonance energy transfer, and structural modelling efforts, all mainly on *Drosophila* odorant receptors, are beginning to inform hypotheses of their structures and how such complexes function in odour detection. Some of the loops, especially the second extracellular loop that has been suggested to form a lid over the binding pocket, and the extracellular regions of some transmembrane helices, especially the third and to a less extent the sixth and seventh, have been implicated in ligand recognition in tuning receptors. The possible interaction between Orco and tuning receptor subunits through the final intracellular loop and the adjacent transmembrane helices is thought to be important for transducing ligand binding into receptor activation. Potential phosphorylation sites and a calmodulin binding site in the second intracellular loop of Orco are also thought to be involved in regulating channel gating. A number of new methods have recently been developed to express and purify insect odorant receptor subunits in recombinant expression systems. These approaches are enabling high throughput screening of receptors for agonists and antagonists in cell-based formats, as well as producing protein for the application of biophysical methods to resolve the 3D structure of the subunits and their complexes.

Contents

* Corresponding author.
E-mail address: andrew.kralicek@plantandfood.co.nz (A.V. Kralicek).

http://dx.doi.org/10.1016/j.ibmb.2015.09.010
0965-1748/

1. Introduction

Olfaction is the most important of the senses for insects, being critical for feeding, oviposition, mate recognition and predator avoidance (Carey and Carlson, 2011). Insects detect odours using an array of receptors that fall into two major classes. Ionotropic Receptors (IRs) are ligand-gated ion channels that are sensitive to acid and amine odours (Rytz et al., 2013). Odorant Receptors (ORs) are a much larger class that are also ligand-gated ion channels (Kaupp, 2010) and are related to insect Gustatory Receptors (GRs) (Montell, 2009, 2013). The ORs are capable of discriminating amongst thousands of volatiles (Kaupp, 2010), detecting many compounds with great sensitivity (Angioy et al., 2003). These receptors are integral membrane proteins found in the dendritic membranes of olfactory sensory neurons (OSNs), housed within sensilla (often fine hair-like structures) located on the insect's antennae. Odorant molecules are thought to diffuse through pores in the walls of sensilla and enter a lymph, where they are transported by odorant binding proteins (OBPs) to membrane-bound ORs (Leal, 2013). Odorant binding by these receptors results in OSN depolarisation and a neuronal signal that is decoded by the insect brain, informing behavioural response decisions. In this review we will focus on the ORs and in particular on what is currently known about their structure and function.

Insect OR-mediated olfaction requires the co-expression of two OR genes in each OSN: a co-receptor *Orco*, previously known as *Or83b* (Vosshall and Hansson, 2011), which is broadly expressed across OSNs (Larsson et al., 2004), and an odorant-binding subunit (*OrX*) that is expressed in a specific subset of OSNs (Carey et al., 2010; Hallem et al., 2004; Wang et al., 2010). Orco protein interacts with OrXs early in the endomembrane system in OSNs, is necessary for correct trafficking of the complex to the dendritic membrane, and is essential to maintain the OR complex within the sensory cilia (Benton et al., 2006). However, Orco has not been found to have any olfactory function without the presence of an OrX (Elmore et al., 2003). The caveat to this assertion is the discovery of some allosteric agonists and antagonists for Orco that are proving useful in structure/function studies of the co-receptor (Jones et al., 2011, 2012; Kumar et al., 2013; Taylor et al., 2012). Orco orthologues from different species can rescue function in null mutants of *Drosophila melanogaster*, indicating a conserved functional role across insects (Jones et al., 2005), and that Orcos from different species have little impact on the tuning of the OrX partner (Nichols et al., 2011).

Vertebrate ORs are seven transmembrane helix (TMH) G protein-coupled receptors (GPCRs) (Kato and Touhara, 2009). Insect ORs are also seven TMH proteins; however, membrane topology analysis of the insect OR subunits both *in vivo* and expressed in cell lines revealed that they have the opposite orientation in the membrane compared with GPCRs, with an intracellular N-terminus and an extracellular C-terminus (Benton et al., 2006; Jordan et al., 2009; Lundin et al., 2007; Smart et al., 2008; Tsitoura et al., 2010). Furthermore, it is generally accepted that insect OrXs and Orco form a greatly expanded phylogenetic lineage that seems to be derived from insect GRs (Missbach et al., 2014; Robertson, 2009; Robertson et al., 2003) and are not related to GPCRs (Benton et al., 2006).

A number of lines of evidence support a stable heteromeric complex being formed between Orco and OrX subunits, including *in vivo* protein fragment complementation assays (PCA), resonance energy transfer (RET), and co-immunoprecipitation (Benton et al., 2006; German et al., 2013; Neuhaus et al., 2005; Tsitoura et al., 2010). None of these studies, however, has provided information on the stoichiometry of the receptor subunits required for these interactions or addressed Orco's ability to couple promiscuously with a large number of highly divergent OrX subunits (61–341 depending on the insect species) (Touhara and Vosshall, 2009). There is some evidence that this interaction is mediated through contacts between the third intracellular loops (ICL3s) of the subunits or the proximate TMH regions (Benton et al., 2006). However, sequence analysis of Orco and OrX subunits has failed to identify common oligomerisation motifs, despite the higher degrees of conservation found around these regions in other proteins (Clyne et al., 1999; Miller and Tu, 2008; Ray et al., 2014; Vosshall, 2003).

The Orco/OrX complex is believed to form an odorant-gated non-selective cation channel with ionic permeability for Ca^{2+}, Na^{+} and K^{+} (Nakagawa et al., 2012; Sato et al., 2008; Smart et al., 2008; Wicher et al., 2008; Yao and Carlson, 2010). Odorant binding induces ionotropic signalling by the complex. There is however, some additional *in vitro* evidence for the existence of a metabotropic signalling pathway, supporting a role for G protein/secondary messenger regulation of ORs (Chatterjee et al., 2009; Deng et al., 2011; Getahun et al., 2013; Jones et al., 2011; Kaupp, 2010; Raja et al., 2014; Sargsyan et al., 2011; Wicher, 2013; Wicher et al., 2008) (Fig. 1). A G protein-binding site, however, is yet to be definitively identified in either Orco or any OrX. For an overview and discussion concerning the mechanisms and relative importance of these different signalling pathways, please see the following review articles (Kaupp, 2010; Nakagawa and Vosshall, 2009; Silbering and Benton, 2010; Stengl and Funk, 2013; Wicher, 2013).

What little is known of the mechanism of activation and structural organisation of the insect Orco/OrX complex has so far come from studies on insect Orco and OrX subunits expressed in heterologous cell lines, *Xenopus* oocytes, and transgenic flies. However, knowledge on this topic remains poor in comparison to understanding of other membrane protein receptors, particularly for examples where structural data are available (Corringer et al., 2012; Kumar and Mayer, 2013; Moreira, 2014; Vaidehi et al., 2014; Zhang et al., 2013). The lack of a crystal structure from closely related receptor/channel families has also negated the use of homology modelling approaches on insect ORs. However, a recent analysis of amino acid covariation across insect Orcos and OrXs has been used to *de novo* construct the first 3D models of the *D. melanogaster* odorant receptors, DmOrco and DmOr85b (Hopf

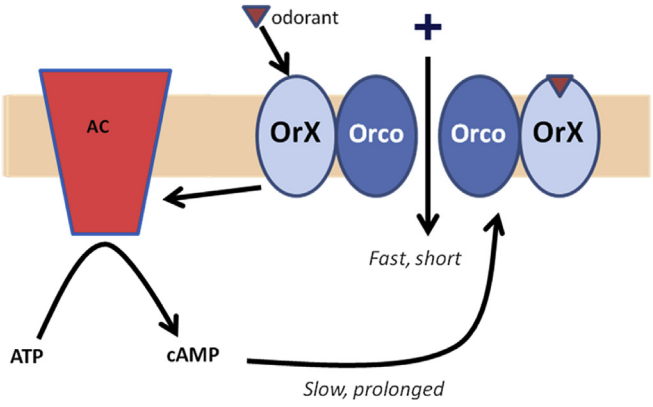

Fig. 1. Overview of insect odorant receptor signal transduction. Odorant binding to the OrX subunit in the Orco/OrX complex activates two signalling pathways, a fast short ionotropic pathway and a slow prolonged metabotropic pathway. The ionotropic pathway involves the direct odour activated opening of the ion channel pore (Sato et al., 2008). The metabotropic pathway involves the indirect opening of the channel pore, as odorant binding to the OrX subunit activates an adenylyl cyclise (AC) that causes cAMP production (Wicher et al., 2008). The increased concentrations of cAMP prolongs the opening of the channel pore (Sargsyan et al., 2011). Figure adapted from Kaupp (2010).

et al., 2015). This approach exploits the sequence variation within large protein families to identify pairs of amino acids that co-vary, with such evolutionary couplings serving as pairwise distance constraints for protein structure prediction (Hopf et al., 2012; Marks et al., 2012). However, this is an indirect method of determining protein structure, and as such the resulting model should be treated with an appropriate level of caution.

Whilst we realise that these models are unlikely to accurately represent the actual tertiary structure of these proteins, they provide us with the current best guess. In this review we use these *in silico* models as templates to integrate the current limited knowledge of structure and function of the insect Orco/OrX complex, and raise hypotheses on the structural nature of the Orco/OrX complex: how odorant selectivity is encoded, and how does the insect OR ion channel function. We also highlight biochemical techniques that could help further advance our understanding of how the Orco/OrX complex functions at the molecular level. Finally, we discuss the prospects of applying structural biology approaches to the Orco and OrX subunits as well as the complex.

2. What is known about the structural nature of the insect Orco/OrX ion channel complex?

Although the insect OR complex has been shown to function as a ligand-gated cation channel, it displays no obvious sequence similarity to the three major super families of ligand-gated ion channels: the pentameric cys-loop channels, the tetrameric/pentameric ionotropic glutamate receptor (iGluR) channels, or the trimeric/tetrameric ATP/purinergic receptor channels (Petkov, 2009). Most ion channels consist of a pore-forming α subunit assembled together most commonly as a homo-tetramer that has a central channel pore composed of membrane-spanning domains contributed by each subunit (Gonzalez et al., 2012; Petkov, 2009; Tian et al., 2014). If insect ORs are structured similarly, then there are two possible quaternary structures that might be adopted. The Orco subunits could form a stable ion channel pore to which OrX subunits couple externally (Fig. 2A). This hypothesis is supported by the fact that Orco can form homomeric ion channels activated by synthetic Orco agonists such as VUAA1 and its analogues (Chen and Luetje, 2012, 2013; Jones et al., 2011, 2012; Taylor et al., 2012), as well as intracellular cyclic nucleotides (Wicher et al., 2008). In a second model, the Orco and OrX subunits could both act as pore-forming units in a heteromeric channel pore (Fig. 2B). This hypothesis is supported by the fact that when different OrXs are expressed with Orco in *Xenopus* oocytes and HEK cells, the degree of VUAA1 agonist-induced ion permeability and levels of inhibition by ruthenium red (RR) are both affected, implying that each OrX contributes differently to the pore (Nakagawa et al., 2012; Nichols et al., 2011; Pask et al., 2011).

In the absence of high-resolution experimentally determined structures, only biophysical data obtained from analysing purified homomeric Orco and heteromeric Orco/OrX complexes will provide insights into the stoichiometry and oligomeric state of the complex. This might be achieved by applying techniques such as ultracentrifugation (Ebel, 2011) and size-exclusion chromatography (Korepanova and Matayoshi, 2012) to Orco/OrX complexes in detergent micelles. Surface Plasmon Resonance (SPR) could also be employed to investigate protein–protein interactions between detergent-solubilised Orco and OrX subunits, as well as the kinetics associated with the binding of odorant ligands (Patching, 2014).

3. What is known about how odorant selectivity is encoded by each OrX subunit?

The first detailed analysis of insect OrX selectivity was performed by Hallem and Carlson (2006), who measured the responses of 24 OrXs in the *D. melanogaster* adult fly, against a panel of 109 chemically diverse and ecologically relevant odorants. The selectivity of these receptors ranged from narrowly to broadly tuned, representing both specialist and generalist characteristics. This led to the hypothesis that insect olfaction is based on a combinatorial code, with distinct subsets of receptors responding

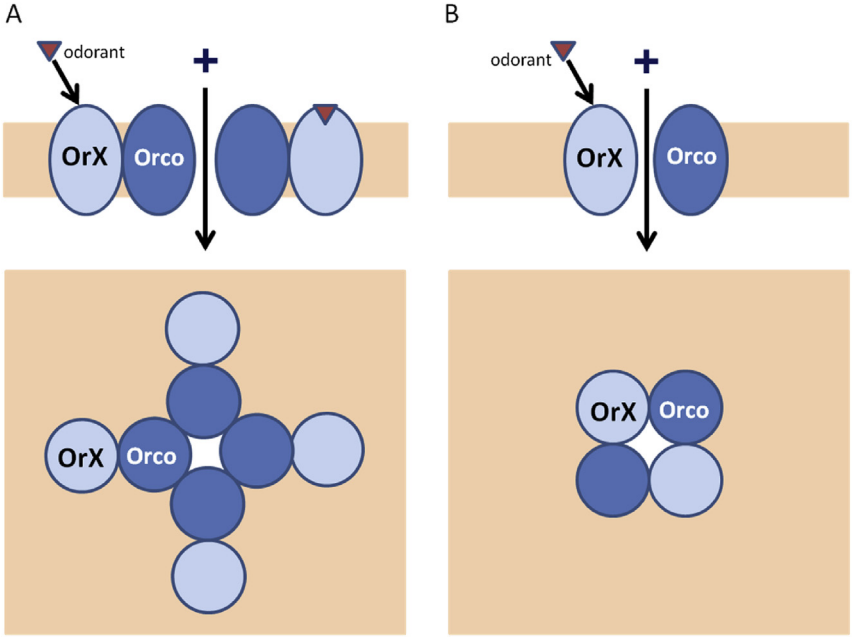

Fig. 2. Models of the Orco/OrX ion channel complex. (A) Orco subunits independently form a homomeric ion channel to which OrX subunits can couple extraneously. The channel pore is formed solely of Orco subunits, with a single OrX subunit bound externally to each Orco subunit. (B) A heteromer of Orco and OrX subunits form the ion channel, with both subunits contributing to the pore surface.

to the same ligand. Later studies in the mosquito, *Anopheles gambiae*, seemed to confirm this, with many AgOrXs observed to detect a range of different odorants, some of which overlap (Carey et al., 2010; Wang et al., 2010).

Recent research suggests that many insect ORs are selectively tuned to biologically relevant odorants rather than being part of a panel of receptors that recognises these compounds in a combinatorial manner. Analysis of the 21 DmOrXs expressed in *D. melanogaster* larvae showed that 19 of these could be strongly activated by a single unique odorant out of a panel of 479 tested at low concentrations, probably reflecting those typically encountered by the insect in their environment (Mathew et al., 2013). Dedicated OSN circuits have also been identified in *Drosophila* for geosmin (DmOr56a (Stensmyr et al., 2012)), citrus terpenes (DmOr19a (Dweck et al., 2013)), farnesol (DmOr83c (Ronderos et al., 2014)), polyphenolics (Dweck et al., 2015b), and for pheromones (DmOr47b, DmOr67d, and DmOr88a (Dweck et al., 2015a; Ha and Smith, 2006)). Boyle et al. (2013) used a computational approach to explore the pharmacophore space of OrXs predicting ~500 new activators and inhibitors for each of nine DmOrXs. A lack of receptor cross-activity was observed, suggesting that many of the insect's biologically relevant odours are detected by a small repertoire of receptors. For a more in depth overview of the topic of receptor tuning see Andersson et al. (2015) and Bohbot and Pitts (2015).

So how is odorant selectivity encoded within each OrX? Guo and Kim (2010) built a Quantitative Structure Activity Relationship (QSAR) model of DmOrXs that suggests that the OrX TMHs and extracellular loops (ECLs) form the ligand-binding pocket. Despite the low amino acid sequence similarity among OrXs, some site-directed mutagenesis studies have been performed to probe OrX specificity (Table 1, Figs. 3A and 4). These studies have relied on cell-based or OSN functional assays, which in essence measure ion channel function. As with most site-directed mutagenesis studies these results should be interpreted with caution (Colquhoun, 1998; Subramanyam and Colecraft, 2015), because it is not possible to be certain that a resulting phenotype is due to the amino acid change directly affecting ligand affinity as opposed to ligand-specific signal transduction differences that affect ion channel function, or even protein folding, membrane targeting or receptor stability.

Only those studies that investigate mutants that impact odorant preference alone are likely to be more robust to scrutiny, as at least we have some level of confidence that the receptor is folding correctly since the mutant receptor is still capable of responding to

Table 1
OrX mutations and structural features.

Reference number (Fig. 3A)	Mutation	OR85b equivalent position	OrX	Domain	Effect of modification	Reference
1	C23W	F11[a]	DmOr67d	N terminus	Abolishes receptor function *in vivo*.	Jin et al. (2008)
2	V91A	V77[a]	DmOr59b	TMH2	Shows a loss of odour inhibition by 1-octen-3-ol and insensitivity to DEET	Pellegrino et al. (2011)
3	A148T	L138[a]	OfumOR3	TMH3	Alters the pheromone selectively to that of OnubOR3.	Leary et al. (2012)
4	F142C	F142[a]	DmOr85b	TMH3	Alters 2-heptanone/2-heptanol preference ratio at 1 mM odorant concentration (in C146S background). Sensitivity to both odorants is also reduced.	Nichols and Leutje (2010)
5	N143C	N143[a]	DmOr85b	TMH3	Alters 2-heptanone/2-heptanol preference ratio at 1 mM odorant concentration (in C146S background).	Nichols and Leutje (2010)
6	C146S	C146[a]	DmOr85b	TM3	Sulfhydryl modification fails to inhibit responses to 2-heptanone.	Nichols and Leutje (2010)
7	V147C	V147[a]	DmOr85b	TMH3	Sulfhydryl-modifying agents enhances the receptors response to 2-heptanone (in C146S background).	Nichols and Leutje (2010)
8	M148C	M148[a]	DmOr85b	TMH3	The antagonist 2-nonanone protects M148C from a sulfhydryl-modifying agent (in C146S background).	Nichols and Leutje (2010)
9	E149C	E149[a]	DmOr85b	TMH3	Sulfhydryl-modifying agents enhances the receptors response to 2-heptanone (in C146S background).	Nichols and Leutje (2010)
10	Y150C	Y150[a]	DmOr85b	TMH3	Sulfhydryl-modifying agents enhances the receptors response to 2-heptanone (in C146S background).	Nichols and Leutje (2010)
11	M162V	W156[b]	AgOr15	ECL2	Ratio of acetophenone response to 4-methylphenol response (Ace/4-mp) was increased relative to WT AgOr15.	Hughes et al. (2014)
12	G181T	Q177[b]	AgOr15	ECL2	Ace/4-mp response ratio was increased relative to WT AgOr15.	Hughes et al. (2014)
13	A195I	G193[a]	AgOr15 & AgOr13	TMH4	Ace/4-mp response ratio was decreased relative to WT AgOr15 and close to AgOr13. Reverse mutation in AgOr13 increased the ratio relative to WT.	Hughes et al. (2014)
14	D299N	A257[a]	BmOR1	TMH5	Rectification index was decreased and reversal potentials were positively shifted compared with those of WT. P_K/P_{Na} ratio of ion selectivity was slightly decreased compared with WT.	Nakagawa et al. (2012)
15	A264V	P265[b]	AgOr15	TMH5	Ace/4-mp response ratio was decreased relative to WT AgOr15.	Hughes et al. (2014)
16	N289D	P289[b]	AgOr15	ECL3	Ace/4-mp response ratio was increased relative to WT AgOr15.	Hughes et al. (2014)
17	M293I	V294[b]	AgOr15	TMH6	Ace/4-mp response ratio was increased relative to WT AgOr15.	Hughes et al. (2014)
18	F296L	F297[b]	AgOr15	TMH6	Ace/4-mp response ratio was decreased relative to WT AgOr15.	Hughes et al. (2014)
19	F307I	Y308[b]	AgOr15	TMH6	Ace/4-mp response was increased relative to WT AgOr15.	Hughes et al. (2014)
20	E356Q	Q315[a]	BmOR1	TMH6	Rectification index was decreased and reversal potentials were positively shifted compared with those of WT. P_K/P_{Na} ratio of ion selectivity was slightly decreased compared with WT.	Nakagawa et al. (2012)
21	F369L	M370[b]	AgOr15	TMH7	Ace/4-mp response ratio was decreased relative to WT AgOr15.	Hughes et al. (2014)
22–24	Y405H, S406A, P501L	Y378[b], K379[b], L384[b]	AgOR1	TMH7/C terminus	SYSYLAVL mutated to SHAYLAVP showed a reduced response to 4-methylphenol.	Ray et al. (2014)
25	F379I	F380[b]	AgOr15	TMH7	Ace/4-mp response ratio was decreased relative to WT AgOr15.	Hughes et al. (2014)
26	F380Y	F381[b]	AgOr15	TMH7	Ace/4-mp response ratio was decreased relative to WT AgOr15.	Hughes et al. (2014)

WT = wild type.
[a] DmOr85b equivalent sites from Hopf et al. (2015).
[b] DmOr85b equivalent sites from an alignment constructed for this review.

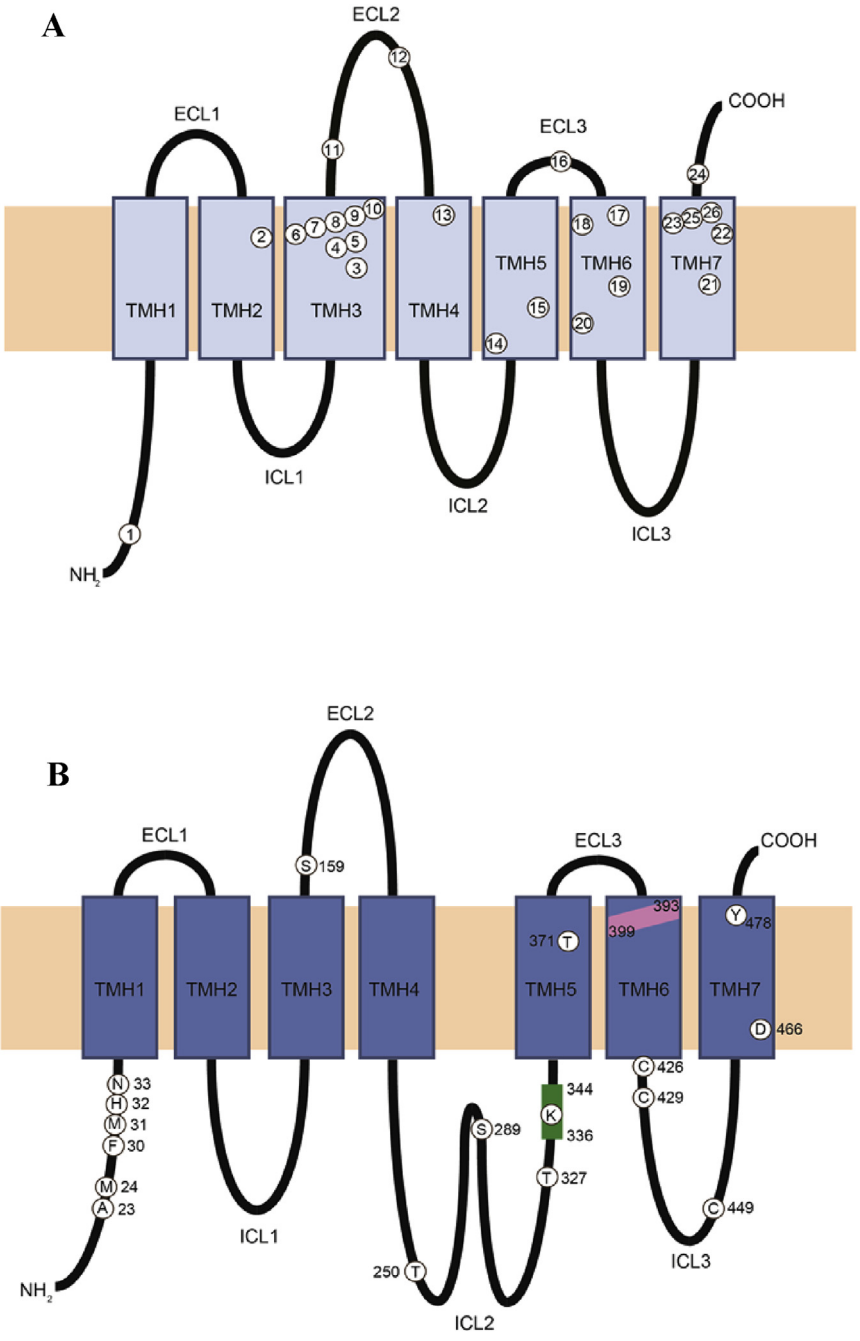

Fig. 3. Membrane topology of the DmOr85b and DmOrco subunits. Topology diagrams were generated on the basis of the membrane topologies derived from evolutionary couplings of the *D. melanogaster* OR (DmOr85b and DmOrco) structural models (Hopf et al., 2015) or an alignment performed for this review. (A) DmOr85b membrane topology showing key residues identified from site-directed mutagenesis studies; numbering relates to Table 1. TMH – Transmembrane Helix, ECL – extracellular loop, ICL – Intracellular loop. (B) Orco membrane topology showing key residues identified from mutagenesis studies; see Table 2 for a detailed description of each residue. Key domains are highlighted as follows: calmodulin (CaM) binding sequence (green), potassium channel-like selectivity filter sequence (pink). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).

target odorants. In light of this, the study of Leary et al. (2012) provides strong evidence for a region at the interface of TMH3 with ECL2 being involved in ligand recognition. A single A148T substitution (Fig. 4) in this region changed the preference of the *Ostrinia nubilalis* pheromone receptor OR3 from (*E*)-11-tetradecenyl acetate to (*E*)-12-tetradecenyl acetate (the pheromone for *Ostrinia furnacalis*). When the converse substitution was investigated in the *O. furnacalis* OR3, the preference of this mutant receptor was altered to (*E*)-11-tetradecenyl acetate, strongly indicating that residue 148 is involved in pheromone selectivity.

Hughes et al. (2014) investigated 37 amino acid differences between the paralogous *A. gambiae* receptors, AgOr13 and AgOr15 that are predicted to be located within the TMH and ECL regions. Analysis of the acetophenone/4-methyl phenol response ratio of AgOr15 identified five mutations (M162V, G181T, N289D, M293I, and F307I) that increased the odorant response ratio, and six mutations (A195I, A264V, F296L, F369L, F379I, and F380Y) that reduced it. The A195I mutation (Fig. 4), at the interface between ECL2 and TMH4, exhibited the greatest reduction in acetophenone/4-methyl phenol response ratio moving it closer to that of AgOr13.

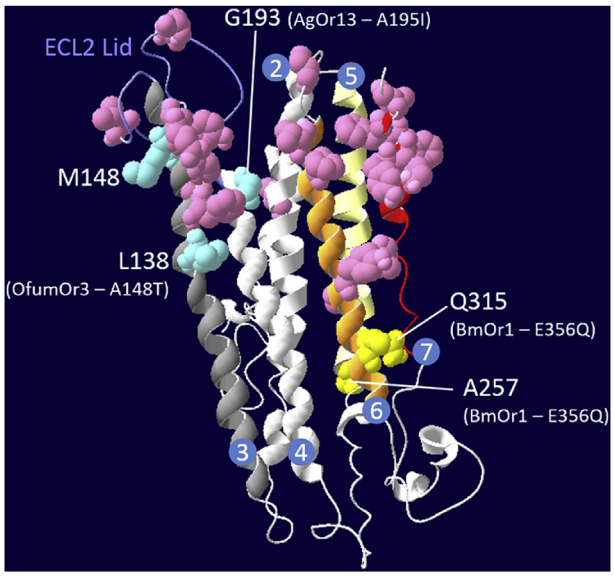

Fig. 4. Residues that may be important for ligand binding and modulating ion channel activity and selectivity mapped onto a model of DmOr85b. Positions of residues (pink and blue spheres) that have been proposed to be involved in ligand binding on the basis of site-directed mutagenesis studies of OrXs. These are mainly found on the extracellular ends of TMH3 (dark grey), TMH6 (orange), and TMH7 (red). The ECL2 region (purple) has been speculated to be a lid that covers the ligand binding pocket. The positions of two residues (yellow spheres) that appear to modulate ion channel activity and selectivity are located on TMH5 (pale yellow) and TMH6. Numbers in blue circles identify TMH regions. The DmOr85b model (model 140_12) was produced by Hopf et al. (2015) and was visualised and annotated here using DeepView/Swiss-PdbViewer v4.1.0. TMH domains of DmOr85b have been defined as follows: TMH1 (I35 to S57), TMH2 (L65 to E103), TMH3 (N116 to Y153), TMH4 (A192 to S222), TMH5 (A257 to T285), TMH6 (V293 to A320), and TMH7 (T360 to A382). Note: TMH1, TMH2 and TMH7 domains are not well defined in the model and contain semi-helical regions that we have chosen to assign as α-helix. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).

When the converse mutation was made in AgOr13, the response ratio was increased, suggesting that this residue is critical for ligand preference. The effect of different substitutions at this position in AgOr15 on odorant sensitivity depended on the odorant, implying A195 is located near the binding site. Sensitivity to acetophenone (but not 4-methyl phenol) correlated well with amino acid side chain length.

Two other major studies have identified residues that may play a role in controlling ligand accessibility to the binding pocket. Nichols and Leutje (2010) used the substituted cysteine accessibility method (SCAM) to identify residues 146 to 150 at the interface of the TMH3 and ECL2 regions of DmOr85b (Fig. 3A) as being functionally important for 2-heptanone binding. When these residues were individually mutated to cysteines, their covalent modification with ethylsulfonate-methanethiosulfonate (MTSES) reduced the potency of the response to 2-heptanone. This result suggests these residues play a role in the access pathway to the ligand binding site because if these residues directly interacted with the odorant then the addition of the large MTSES moiety would have eliminated odorant binding by blocking any interaction. The binding of 2-nonanone, a competitive antagonist, was found to protect the M148C mutation (Fig. 4) from covalent modification by MTSES, implying this residue is located physically near the binding site of DmOR85b. In another study, mutation of conserved proline residues in the ECL2 regions of AgOR10, *Culex quinquefasciatus* CquiOR10, and *Bombyx mori* BmOR1 receptors reduced the efficacy of these receptors in response to their odorant ligands (Xu and Leal, 2013). The authors speculate that these residues might help form β-sheets that create a lid covering the binding pocket that they propose is buried within the TMHs. The presence of strongly evolutionary constrained residues in this loop further highlights the possible functional importance of this region (Hopf et al., 2015).

In summary, the second extracellular loop possibly forms a lid over the binding pocket and the extracellular regions of some transmembrane helices, especially the third and to a less extent the sixth and seventh, are implicated in ligand recognition. As the above studies illustrate, it can be difficult to use functional assays to infer what roles particular residues play in OrX function. In the absence of a crystal structure of an OrX subunit with a bound odorant ligand, only techniques that can determine the effect of mutations on ligand-binding independently of channel function will shed light on residues which help define OrX specificity. Such techniques include scintillation proximity assays on cells expressing OrXs, their cell membranes, or purified OrXs (Berry et al., 2012; Glickman et al., 2008; Harder and Fotiadis, 2012). Application of "label-free" techniques such as SPR analysis (Bocquet et al., 2015; Heym et al., 2015), isothermal chemical denaturation (Mahendrarajah et al., 2011; Ross et al., 2015; Schon et al., 2013), and isothermal titration calorimetry (Draczkowski et al., 2014; Rajarathnam and Rosgen, 2014), to purified OrXs and their mutants will provide an understanding of ligand binding kinetics and the roles of individual residues.

4. What are the structural features of the Orco/OrX ion channel pore?

Ion channels generally have multiple pore-forming subunits that contribute to the pore's structure, or associated accessory subunits that help to regulate channel gating (Petkov, 2009). The pore-forming subunits typically contribute three key structural features to the ion channel: a selectivity filter to provide some degree of selectivity for ion permeability (which can vary from just selecting cations from anions to distinguishing between different ions); a pore domain (PD) for the passage of ions; and a gating mechanism (Petkov, 2009). Mutagenesis studies on Orco (Table 2, Fig. 3B) and the evolutionary coupling-based structural model for DmOrco (Fig. 5) together provide some assignments of regions within Orco that may contribute to these functions:

(i) *A selectivity filter to regulate ion channel permeability.* Wicher et al. (2008) identified the ^{393}TVVGYLG^{399} sequence in TMH6 of DmOrco (Fig. 3B) as being similar to the ion selectivity filter motif found in potassium ion channels (TTVGYGD) (McCoy and Nimigean, 2012). As insect ORs are not closely related to potassium channels it seems more likely that this sequence has arisen through convergence rather than a shared ancestor. The structural model for DmOrco (Fig. 5) indicates that this sequence is located in the extracellular half of TMH6. Wicher et al. (2008) deleted two residues to change the sequence to TVGYG, resulting in reduced K^+ ion permeability of the DmOrco channel in whole-cell patch clamp experiments. Whether this sequence actually has a role in ion selectivity is open to debate, as Orco is a non-selective cation channel and thus has no preference for potassium ions over other cations. In contrast, Nakagawa et al. (2012) demonstrated that a Y464A substitution in the highly conserved TMH7 region of BmOrco also alters ion selectivity. The corresponding substitution in DmOrco (Y478A, Fig. 5A and B) altered the spontaneous and odour-evoked action potentials in neurons. Nakagawa et al. (2012) also identified two mutations, D299N at the base of TM5 and E356Q in TM6 (Fig. 4)) in the *B. mori* pheromone receptor, BmOr1, that also

Table 2
DmOrco mutations and structural features (refer to Fig. 3B).

Mutation	Domain	Effect of modification	Reference
A23S/M24A/F30A/M31A/H32A/N33A	N terminus	Co-expression of DmOr47a or DmOr85b with DmOrco^{6mut} (all six mutations together) or DmOrco$^{\Delta 23-33}$ (deletion of 11 residues) in Xenopus oocytes reduced or abolished responses to odorants or VUAA1.	Hopf et al. (2015)
S159N/T250N/S289N	ECL2/ICL2	Mutation of three putative phosphorylation sites together reduced current relative to that of WT DmOrco in patch clamp experiments.	Sargsyan et al. (2011)
T327N/T371N	ICL2/TMH5	Mutation of two putative phosphorylation sites together reduced current relative to that of WT DmOrco in patch clamp experiments.	Sargsyan et al. (2011)
S336 – R344	ICL2	Putative calmodulin binding site – VUAA1 responses were reduced and prolonged by calmodulin inhibition with the potent antagonist W7 (green in Fig. 3B).	Mukunda et al. (2014)
K339N	ICL2	Mutation in the proposed calmodulin binding site reduced Ca^{+} response relative to that of WT DmOrco to the degree observed in the presence of the antagonist W7.	Mukunda et al. (2014)
V394, L398	TMH6	Deletion of these two residues within the putative potassium ion channel like selectivity sequence (^{393}TVVGYLG^{399}) affected ion permeability (Pink in Fig. 3B).	Wicher et al. (2008)
C429S	ICL3	Decreased sensitivity to methyl hexanoate but increased sensitivity to VUAA1 for DmOrco/DmOr22a complex.	Turner et al. (2014)
C449S	ICL3	Decreased sensitivity to methyl hexanoate but increased sensitivity to VUAA1 for DmOrco/DmOr22a complex.	Turner et al. (2014)
D466E	TMH7	Increased sensitivity of Orco to VUAA1 two-fold compared with that of WT, and increased sensitivity of DmOrco/DmOr22a complex to methyl hexanoate.	Kumar et al. (2013)
Y478A	TMH7	Affected spontaneous and odour-evoked action potentials in neurons containing DmOrco; however, the nature of these was variable and OrX dependent. The corresponding mutation (Y464A) in BmOrco altered ion selectivity of the BmOR1/BmOrco complex.	Nakagawa et al. (2012)

WT = wild type.

affected ion selectivity, supporting the possibility that OrXs may play a role in defining cation selectivity in the Orco/OrX complex.

(ii) *A pore domain to enable cations to flow through the channel.* The pores of non-selective cation channels contain negative charges to attract cations and exclude anions from entering the channel (Petkov, 2009). If the presence of the selectivity filter sequence in the extracellular half of TMH6 of Orco implies that TMH6 forms part of the pore domain, then there should also be a prevalence of negatively charged residues in this region. In the model of DmOrco (Fig. 5A) exposed negatively charged residues are located on the intracellular half of TMH6 (E417, E418, S419, S420, S421), and following on from this in ICL3 (Y432, D433, S435, E436). Could these residues form the inner lining of the channel's pore?

(iii) *A channel gate to control the rate of ion flow.* Ion channels open and close to regulate the movement of ions through their pores (daCosta and Baenziger, 2013; Petkov, 2009; Zhou and McCammon, 2010). In the closed state a narrow constriction or "gate" within the pore prevents ion flow. If TMH6 of Orco forms part of the pore domain, then the intracellular half of TMH6 and possibly ICL3 below it could form the channel

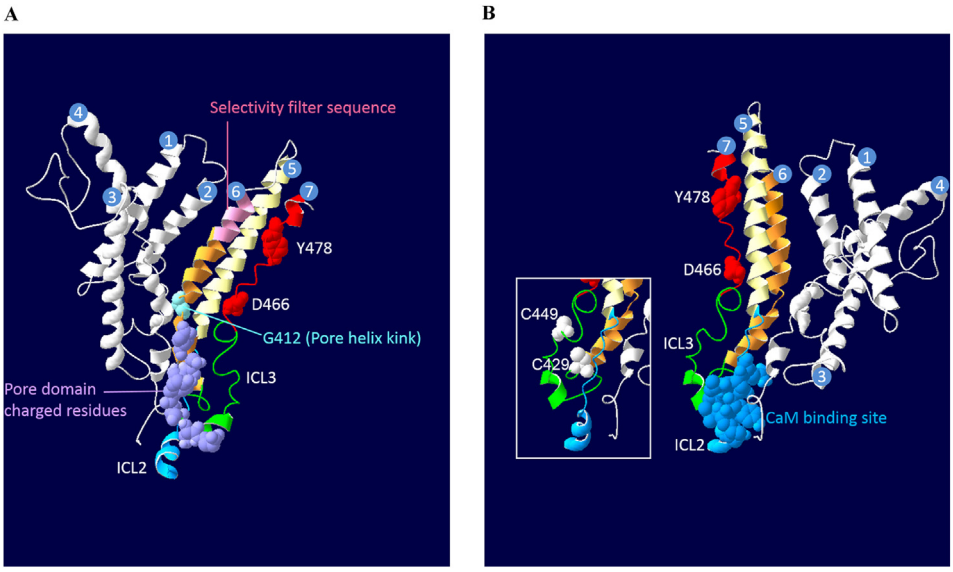

Fig. 5. Residues and regions proposed to be involved in channel function mapped onto a model of DmOrco. (A) The proposed pore domain in TMH6 is shown in orange, with the potassium selectivity filter related sequence highlighted in pink and the G412 residue proposed to act as a hinge shown in cyan. Negatively charged residues in TMH6 are shown as purple spheres and the proposed ICL3 gate region that have been suggested to line the inside surface of the pore is coloured green. The residues D466 and Y478 in TMH7 (red spheres) have been shown to regulate ion channel activity. (B) The model rotated 180° with respect to (A). The residues (blue spheres) that form the proposed calmodulin (CaM) binding domain are located in the ICL2 region, right behind the ICL3 loop. Inset shows the cysteine residues (white spheres) that have been proposed to form a disulphide bond. TMH regions are indicated by numbers in blue circles. The DmOrco model (model 310_2) was produced by (Hopf et al. (2015)), and was visualised and annotated here using DeepView/Swiss-PdbViewer v4.1.0. TMH domains of DmOrco have been defined as follows: TMH1 (F40 to M64), TMH2 (T78 to L94), TMH3 (S126 to T154), TMH4 (M193 to H233), TMH5 (A353 to A381), TMH6 (T393 to M423), and TMH7 (V463 to V483). Note: TMH4, TMH6 and TMH7 are not well defined in the model and contain semi-helical regions that we have chosen to assign as α-helix. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).

gate. One mode of gating motion that has been proposed for potassium ion channels involves forming a 'hinge' around a conserved glycine residue in the middle of the pore helix (Zhou and McCammon, 2010). Such a kink is found around the conserved G412 in TMH6 of Orco (Fig. 5A), raising the possibility that gating might occur through flexibility of TMH6 at this residue. If this kink mechanism proves legitimate then it is likely to have arisen independently in insect odorant receptors.

5. How might channel gating be regulated in the Orco/OrX ion channel?

The opening and closing of an ion channels gate can be regulated by a number of signals (e.g. ligand binding, protein–protein interactions, post-translational modifications, and membrane electrochemical gradients) that are believed to cause a conformational change in the ion channel that widens or constricts the gate (daCosta and Baenziger, 2013; Petkov, 2009; Zhou and McCammon, 2010). So far, three signals for regulating the Orco/OrX ion channel gating have been proposed:

(i) **Odorant binding**. How odorant binding to the OrX subunit induces opening of the "gate" and ion flow through the Orco/OrX ion channel pore is yet to be determined. There is an indication, however, that the ICL3 and TMH7 regions of Orco and OrXs may play a role in transmitting the odorant binding signal within the complex. A yeast two-hybrid study suggested that the ICL3 regions of Orco and OrXs interact together (Benton et al., 2006), which may explain why they are the most conserved regions in both Orco and OrXs (Hopf et al., 2015). A cysteine-replacement mutagenesis study identified two mutations (C429S and C449S) that individually and together decrease the sensitivity of the response of DmOrco/DmOr22a to an odorant, while increasing sensitivity to the Orco agonist VUAA1 (Turner et al., 2014). Similar effects of substitution of either C429 or C449 (Fig. 5B inset), and the absence of additive effects from double mutations, may indicate the presence of a disulphide bond between these residues. Such a feature may aid the structural integrity of the loop, enabling the transduction of conformational changes to the transmembrane pore, as observed in the epithelial Na^+ channel (ENaC) (Jasti et al., 2007).

The TMH7 region is also highly conserved in both Orco and OrXs (Hopf et al., 2015). The functional importance of TMH7 in DmOrco has been demonstrated by a D466E substitution (Fig. 5A and B) that causes increased sensitivity to VUAA1 in homomeric DmOrco channels and odorants in heteromeric DmOrco/DmOr22a channels, suggesting that this mutation favours the opening of the channel gate (Kumar et al., 2013). The importance of TMH7 in OrXs is highlighted by a number of mutations (F369L, F379I, and F380Y) that affect the response of AgamOr15 to acetophenone and 4-methyl phenol (Table 1) (Hughes et al., 2014).

If the ICL3 and TMH7 regions do form the molecular interface between subunits, it might explain how different OrXs could change the gating properties of the Orco/OrX ion channel (Nakagawa et al., 2012; Nichols et al., 2011; Pask et al., 2011). If ICL3 forms part of the gate, then each OrX ICL3 domain would interact differently with the Orco ICL3 domain, affecting the nature of the cytoplasmic side of the channel. Likewise, if TMH6 from Orco forms part of the pore domain, then each OrX TMH7 might interact differently with the complementary TMH7 of DmOrco affecting the position of the adjacent pore domain helix (TMH6).

(ii) **Calmodulin (CaM) binding**. In some ion channels the association of CaM with the channel gate can act as a calcium sensor that opens or closes the gate in response to intracellular calcium concentrations (Mruk et al., 2012; Reichow et al., 2013). A putative CaM binding site (^{336}SAIKYWVER^{344}) has been identified in ICL2 of Orco (Fig. 2B) (Mukunda et al., 2014). A point mutation (K339N) affects ion flow, at least in the homomeric Orco ion channel. The effect of this substitution on the gating of the heteromeric Orco/OrX ion channel appears to be dependent on the OrX involved, providing further evidence for the involvement of the OrX in the channel pore (Mukunda et al., 2014). The location of the CaM-binding site in the evolutionary couplings model of DmOrco is directly below the cytoplasmic end of TMH5 (Fig. 5). This region effectively covers the back of the end of TMH6 and ICL3, therefore it is in a prime position to influence gating of Orco if TMH6 and ICL3 form the channel gate.

(iii) **Phosphorylation**. Phosphorylation is commonly employed by ion channels to regulate their gating dynamics (Antz et al., 1999; Barros et al., 2012; Kanelis et al., 2010). According to the predicted topology of DmOrco, there are three putative intracellular protein kinase C (PKC) phosphorylation sites (T250, S289 and T327) present in the ICL2 region (Fig. 2B) (Sargsyan et al., 2011). Mutation of the PKC phosphorylation sites in Orco to Asn residues inhibit Orco activation by 8-bromo-cAMP and also reduces Orco's constitutive ion channel activity (Sargsyan et al., 2011), suggesting that ICL2 phosphorylation may play a role in controlling the sensitivity of Orco to intracellular cAMP concentrations. However, direct evidence of the phosphorylation of the Orco is still to be obtained. Strategies to do this include using phospho-specific antibodies (Stoevesandt and Taussig, 2013), flow cytometry (Willinger et al., 2005) or mass spectrometry to detect the presence of phosphorylated Orco (Brill et al., 2004).

6. Prospects for determining the structural nature of the Orco/OrX ion channel

Further site-directed mutagenesis studies in combination with the biochemical and biophysical techniques mentioned above and recent evolutionary coupling-based models of Orco and an OrX will all help the generation of new hypotheses concerning how the OrX/Orco complex functions. The difficulty we face is being able to test these hypotheses conclusively. To do this we need an experimentally determined structural model of insect OR subunits and the complex they form. One of the most important steps towards this goal has been overcome with insect Orco and OrXs having been recently over-expressed and purified in milligram quantities using the baculovirus expression system in insect cells, and wheat germ and *Escherichia coli* cell-free expression systems (Carraher et al., 2013; Tegler et al., 2015). Correct membrane protein expression and folding can often be a challenge, so it is heartening to see that such diverse expression systems can be applied to the production of insect ORs.

The techniques of X-ray and electron crystallography (Carpenter et al., 2008; Fujiyoshi, 2013) could provide a means to resolving the structures of OR subunits and their complexes. The application of these two techniques is high risk/high reward, as they have the major challenge of obtaining crystals that diffract to high resolution. The chances of success might be improved through the addition of odorant ligands, Orco agonists and inhibitors, or antibodies generated against Orco or OrX subunits, to help lock the protein/s into a single conformation to allow crystallisation. In the

case of the Orco subunit, it may also be possible to screen a panel of Orco proteins from different insect species, in the hope that one of these proteins is more prone to crystallization. Increasing each subunits stability by mutagenesis (Serrano-Vega et al., 2008; Warne et al., 2008), or with antibody fragments (Rasmussen et al., 2007) or lysozyme fusions (Cherezov et al., 2007; Rosenbaum et al., 2007) could also aid crystallization.

An alternative atomic resolution technique is Nuclear Magnetic Resonance (NMR) spectroscopic analysis (Maslennikov and Choe, 2013). This approach is likely to be extremely challenging due to the large size of the OR subunits (>40 kDa) and the need to find conditions in which they are stable at >0.1 mM concentrations. However, NMR spectroscopy could be applied to study the structures of Orco and OrX TM-loop-TM fragments stabilised in micelles (Neumoin et al., 2009); or cyclic loops with N- and C-termini covalently linked by intein-mediated protein splicing (Tae et al., 2011). For example, NMR spectroscopy might be used to determine whether the Orco and OrX TMH7 and/or ICL3 loops associate in solution, and if so then define the Orco motif that enables the promiscuous interaction of Orco with such a large number of diverse OrXs. Finally, the development of amphipols and lipid bilayer mimetic nanodiscs could provide a means to resolving the solution structures of Orco and OrX homomeric and heteromeric complexes by cryo-Electron Microscopy (cryo-EM) (Popot, 2010). A comparison of the 3D reconstructions of these complexes would help to determine critical features that underpin ion channel pore function and mediate Orco/OrX interaction. Major advances continue to be made in the field of cryo-EM. Recently the structure of a TRPV1 channel was resolved to 3.4 Å resolution through the use of direct electron detection cameras and improved image-processing algorithms (Cao et al., 2013; Liao et al., 2013).

Structural information from any of the above techniques will be a major step forward for the insect olfaction, receptor biology, and ion channel communities, as these structures will provoke hypotheses that can inform further structural and functional studies. In particular, we will finally be in a position to address major questions for the field including: (1) How can Orco partner with a large number of divergent OrXs? (2) What is the structural nature of the ion channel? (3) What is the molecular mechanism behind ion channel activation in response to odorant binding? and (4) How is odorant selectivity encoded by each OrX subunit? Knowledge of how odorant binding is transduced into channel activity will be critical for understanding how insect ORs mediate the vast set of interactions observed between insects and their environment.

Acknowledgements

Funding for this work was provided by the Marsden Fund (PAF0901), the Ministry of Business, Innovation and Employment New Zealand (C06X0701) and PFR CORE funding (P/261012/01).

References

Andersson, M.N., Löfstedt, C., Newcomb, R.D., 2015. Insect olfaction and the evolution of receptor tuning. Front. Ecol. Evol. 3.
Angioy, A.M., Desogus, A., Barbarossa, I.T., Anderson, P., Hansson, B.S., 2003. Extreme sensitivity in an olfactory system. Chem. Senses 28, 279–284.
Antz, C., Bauer, T., Kalbacher, H., Frank, R., Covarrubias, M., Kalbitzer, H.R., Ruppersberg, J.P., Baukrowitz, T., Fakler, B., 1999. Control of K+ channel gating by protein phosphorylation: structural switches of the inactivation gate. Nat. Struct. Biol. 6, 146–150.
Barros, F., Dominguez, P., de la Pena, P., 2012. Cytoplasmic domains and voltage-dependent potassium channel gating. Front. Pharmacol. 3, 49.
Benton, R., Sachse, S., Michnick, S.W., Vosshall, L.B., 2006. Atypical membrane topology and heteromeric function of *Drosophila* odorant receptors *in vivo*. Plos Biol. 4, e20.
Berry, J., Price-Jones, M., Killian, B., 2012. Use of scintillation proximity assay to measure radioligand binding to immobilized receptors without separation of bound from free ligand. Methods Mol. Biol. 897, 79–94.
Bocquet, N., Kohler, J., Hug, M.N., Kusznir, E.A., Rufer, A.C., Dawson, R.J., Hennig, M., Ruf, A., Huber, W., Huber, S., 2015. Real-time monitoring of binding events on a thermostabilized human A2A receptor embedded in a lipid bilayer by surface plasmon resonance. Biochim. Biophys. Acta 1848, 1224–1233.
Bohbot, J.D., Pitts, R.J., 2015. The narrowing olfactory landscape of insect odorant receptors. Front. Ecol. Evol. http://dx.doi.org/10.3389/fevo.2015.00039.
Boyle, S.M., McInally, S., Ray, A., 2013. Expanding the olfactory code by *in silico* decoding of odor-receptor chemical space. eLife 2, e01120.
Brill, L.M., Salomon, A.R., Ficarro, S.B., Mukherji, M., Stettler-Gill, M., Peters, E.C., 2004. Robust phosphoproteomic profiling of tyrosine phosphorylation sites from human T cells using immobilized metal affinity chromatography and tandem mass spectrometry. Anal. Chem. 76, 2763–2772.
Cao, E., Liao, M., Cheng, Y., Julius, D., 2013. TRPV1 structures in distinct conformations reveal activation mechanisms. Nature 504, 113–118.
Carey, A.F., Carlson, J.R., 2011. Insect olfaction from model systems to disease control. Proc. Natl. Acad. Sci. U. S. A. 108, 12987–12995.
Carey, A.F., Wang, G.R., Su, C.Y., Zwiebel, L.J., Carlson, J.R., 2010. Odorant reception in the malaria mosquito *Anopheles gambiae*. Nature 464, 66–U77.
Carpenter, E.P., Beis, K., Cameron, A.D., Iwata, S., 2008. Overcoming the challenges of membrane protein crystallography. Curr. Opin. Struct. Biol. 18, 581–586.
Carraher, C., Nazmi, A.R., Newcomb, R.D., Kralicek, A., 2013. Recombinant expression, detergent solubilisation and purification of insect odorant receptor subunits. Protein Expr. Purif. 90, 160–169.
Chatterjee, A., Roman, G., Hardin, P.E., 2009. Go contributes to olfactory reception in *Drosophila melanogaster*. BMC Physiol. 9, 22.
Chen, S., Luetje, C.W., 2012. Identification of new agonists and antagonists of the insect odorant receptor co-receptor subunit. Plos One 7, e36784.
Chen, S., Luetje, C.W., 2013. Phenylthiophenecarboxamide antagonists of the olfactory receptor co-receptor subunit from a mosquito. Plos One 8, e84575.
Cherezov, V., Rosenbaum, D.M., Hanson, M.A., Rasmussen, S.G.F., Foon, S.T., Kobilka, T.S., Choi, H.J., Kuhn, P., Weis, W.I., Kobilka, B.K., Stevens, R.C., 2007. High-resolution crystal structure of an engineered human β2-adrenergic G protein-coupled receptor. Science 318, 1258–1265.
Clyne, P.J., Warr, C.G., Freeman, M.R., Lessing, D., Kim, J., Carlson, J.R., 1999. A novel family of divergent seven-transmembrane proteins: candidate odorant receptors in Drosophila. Neuron 22, 327–338.
Colquhoun, D., 1998. Binding, gating, affinity and efficacy: the interpretation of structure-activity relationships for agonists and of the effects of mutating receptors. Br. J. Pharmacol. 125, 924–947.
Corringer, P.J., Poitevin, F., Prevost, M.S., Sauguet, L., Delarue, M., Changeux, J.P., 2012. Structure and pharmacology of pentameric receptor channels: from bacteria to brain. Structure 20, 941–956.
daCosta, C.J., Baenziger, J.E., 2013. Gating of pentameric ligand-gated ion channels: structural insights and ambiguities. Structure 21, 1271–1283.
Deng, Y., Zhang, W., Farhat, K., Oberland, S., Gisselmann, G., Neuhaus, E.M., 2011. The stimulatory Galpha(s) protein is involved in olfactory signal transduction in Drosophila. Plos One 6, e18605.
Draczkowski, P., Matosiuk, D., Jozwiak, K., 2014. Isothermal titration calorimetry in membrane protein research. J. Pharm. Biomed. Anal. 87, 313–325.
Dweck, H.K., Ebrahim, S.A., Kromann, S., Bown, D., Hillbur, Y., Sachse, S., Hansson, B.S., Stensmyr, M.C., 2013. Olfactory preference for egg laying on citrus substrates in Drosophila. Curr. Biol. 23, 2472–2480.
Dweck, H.K., Ebrahim, S.A., Thoma, M., Mohamed, A.A., Keesey, I.W., Trona, F., Lavista-Llanos, S., Svatos, A., Sachse, S., Knaden, M., Hansson, B.S., 2015a. Pheromones mediating copulation and attraction in Drosophila. Proc. Natl. Acad. Sci. U. S. A. 112, E2829–E2835.
Dweck, H.K.M., Ebrahim, S.A.M., Farhan, A., Hansson, B.S., Stensmyr, M.C., 2015b. Olfactory proxy detection of dietary antioxidants in Drosophila. Curr. Biol. 25, 455–466.
Ebel, C., 2011. Sedimentation velocity to characterize surfactants and solubilized membrane proteins. Methods 54, 56–66.
Elmore, T., Ignell, R., Carlson, J.R., Smith, D.P., 2003. Targeted mutation of a Drosophila odor receptor defines receptor requirement in a novel class of Sensillum. J. Neurosci. 23, 9906–9912.
Fujiyoshi, Y., 2013. Future directions of electron crystallography. Methods Mol. Biol. 955, 551–568.
German, P.F., van der Poel, S., Carraher, C., Kralicek, A.V., Newcomb, R.D., 2013. Insights into subunit interactions within the insect olfactory receptor complex using FRET. Insect Biochem. Mol. Biol. 43, 138–145.
Getahun, M.N., Olsson, S.B., Lavista-Llanos, S., Hansson, B.S., Wicher, D., 2013. Insect odorant response sensitivity is tuned by metabotropically autoregulated olfactory receptors. Plos One 8.
Glickman, J.F., Schmid, A., Ferrand, S., 2008. Scintillation proximity assays in high-throughput screening. Assay Drug Dev. Technol. 6, 433–455.
Gonzalez, C., Baez-Nieto, D., Valencia, I., Oyarzun, I., Rojas, P., Naranjo, D., Latorre, R., 2012. K(+) channels: function-structural overview. Compr. Physiol. 2, 2087–2149.
Guo, S., Kim, J., 2010. Dissecting the molecular mechanism of *drosophila* odorant receptors through activity modeling and comparative analysis. Proteins 78, 381–399.
Ha, T.S., Smith, D.P., 2006. A pheromone receptor mediates 11-cis-vaccenyl acetate-induced responses in Drosophila. J. Neurosci. 26, 8727–8733.
Hallem, E.A., Carlson, J.R., 2006. Coding of odors by a receptor repertoire. Cell 125, 143–160.

Hallem, E.A., Ho, M.G., Carlson, J.R., 2004. The molecular basis of odor coding in the *Drosophila* antenna. Cell 117, 965–979.
Harder, D., Fotiadis, D., 2012. Measuring substrate binding and affinity of purified membrane transport proteins using the scintillation proximity assay. Nat. Protoc. 7, 1569–1578.
Heym, R.G., Hornberger, W.B., Lakics, V., Terstappen, G.C., 2015. Label-free detection of small-molecule binding to a GPCR in the membrane environment. Biochim. Biophys. Acta 1854, 979–986.
Hopf, T.A., Colwell, L.J., Sheridan, R., Rost, B., Sander, C., Marks, D.S., 2012. Three-dimensional structures of membrane proteins from genomic sequencing. Cell 149, 1607–1621.
Hopf, T.A., Morinaga, S., Ihara, S., Touhara, K., Marks, D.S., Benton, R., 2015. Amino acid coevolution reveals three-dimensional structure and functional domains of insect odorant receptors. Nat. Commun. 6, 6077.
Hughes, D.T., Wang, G., Zwiebel, L.J., Luetje, C.W., 2014. A determinant of odorant specificity is located at the extracellular loop 2-Transmembrane domain 4 interface of an *Anopheles gambiae* odorant receptor subunit. Chem. Senses 39, 761–769.
Jasti, J., Furukawa, H., Gonzales, E.B., Gouaux, E., 2007. Structure of acid-sensing ion channel 1 at 1.9A resolution and low pH. Nature 449, 316–323.
Jin, X., Tal, S.H., Smith, D.P., 2008. SNMP is a signaling component required for pheromone sensitivity in Drosophila. Proc. Natl. Acad. Sci. U. S. A. 105, 10996–11001.
Jones, P.L., Pask, G.M., Rinker, D.C., Zwiebel, L.J., 2011. Functional agonism of insect odorant receptor ion channels. Proc. Natl. Acad. Sci. U. S. A. 108, 8821–8825.
Jones, P.L., Pask, G.M., Romaine, I.M., Taylor, R.W., Reid, P.R., Waterson, A.G., Sulikowski, G.A., Zwiebel, L.J., 2012. Allosteric antagonism of insect odorant receptor ion channels. Plos One 7, e30304.
Jones, W.D., Nguyen, T.A.T., Kloss, B., Lee, K.J., Vosshall, L.B., 2005. Functional conservation of an insect odorant receptor gene across 250 million years of evolution. Curr. Biol. 15, R119–R121.
Jordan, M.D., Anderson, A., Begum, D., Carraher, C., Authier, A., Marshall, S.D., Kiely, A., Gatehouse, L.N., Greenwood, D.R., Christie, D.L., Kralicek, A.V., Trowell, S.C., Newcomb, R.D., 2009. Odorant receptors from the light brown apple moth (*Epiphyas postvittana*) recognize important volatile compounds produced by plants. Chem. Senses 34, 383–394.
Kanelis, V., Hudson, R.P., Thibodeau, P.H., Thomas, P.J., Forman-Kay, J.D., 2010. NMR evidence for differential phosphorylation-dependent interactions in WT and DeltaF508 CFTR. EMBO J. 29, 263–277.
Kato, A., Touhara, K., 2009. Mammalian olfactory receptors: pharmacology, G protein coupling and desensitization. Cell. Mol. Life Sci. 66, 3743–3753.
Kaupp, U.B., 2010. Olfactory signalling in vertebrates and insects: differences and commonalities. Nat. Rev. Neurosci. 11, 188–200.
Korepanova, A., Matayoshi, E.D., 2012. HPLC-SEC characterization of membrane protein-detergent complexes. Curr. Protoc. Protein Sci. Chapter 29. Unit 29 25 21–12.
Kumar, B.N., Taylor, R.W., Pask, G.M., Zwiebel, L.J., Newcomb, R.D., Christie, D.L., 2013. A conserved aspartic acid is important for agonist (VUAA1) and odorant/tuning receptor-dependent activation of the insect odorant co-receptor (Orco). Plos One 8, e70218.
Kumar, J., Mayer, M.L., 2013. Functional insights from glutamate receptor ion channel structures. Annu. Rev. Physiol. 75, 313–337.
Larsson, M.C., Domingos, A.I., Jones, W.D., Chiappe, M.E., Amrein, H., Vosshall, L.B., 2004. Or83b encodes a broadly expressed odorant receptor essential for *Drosophila* olfaction. Neuron 43, 703–714.
Leal, W.S., 2013. Odorant reception in insects: roles of receptors, binding proteins, and degrading enzymes. Annu. Rev. Entomol. 58, 373–391.
Leary, G.P., Allen, J.E., Bunger, P.L., Luginbill, J.B., Linn Jr., C.E., Macallister, I.E., Kavanaugh, M.P., Wanner, K.W., 2012. Single mutation to a sex pheromone receptor provides adaptive specificity between closely related moth species. Proc. Natl. Acad. Sci. 109, 14081–14086.
Liao, M., Cao, E., Julius, D., Cheng, Y., 2013. Structure of the TRPV1 ion channel determined by electron cryo-microscopy. Nature 504, 107–112.
Lundin, C., Kall, L., Kreher, S.A., Kapp, K., Sonnhammer, E.L., Carlson, J.R., von Heijne, G., Nilsson, I., 2007. Membrane topology of the *Drosophila* OR83b odorant receptor. FEBS Lett. 581, 5601–5604.
Mahendrarajah, K., Dalby, P.A., Wilkinson, B., Jackson, S.E., Main, E.R., 2011. A high-throughput fluorescence chemical denaturation assay as a general screen for protein-ligand binding. Anal. Biochem. 411, 155–157.
Marks, D.S., Hopf, T.A., Sander, C., 2012. Protein structure prediction from sequence variation. Nat. Biotechnol. 30, 1072–1080.
Maslennikov, I., Choe, S., 2013. Advances in NMR structures of integral membrane proteins. Curr. Opin. Struct. Biol. 23, 555–562.
Mathew, D., Martelli, C., Kelley-Swift, E., Brusalis, C., Gershow, M., Samuel, A.D.T., Emonet, T., Carlson, J.R., 2013. Functional diversity among sensory receptors in a Drosophila olfactory circuit. Proc. Natl. Acad. Sci. U. S. A. 110, E2134–E2143.
McCoy, J.G., Nimigean, C.M., 2012. Structural correlates of selectivity and inactivation in potassium channels. Biochim. Biophys. Acta-Biomembr. 1818, 272–285.
Miller, R., Tu, Z.J., 2008. Odorant receptor c-terminal motifs in divergent insect species. J. Insect Sci. 8.
Missbach, C., Dweck, H.K.M., Vogel, H., Vilcinskas, A., Stensmyr, M.C., Hansson, B.S., Grosse-Wilde, E., 2014. Evolution of insect olfactory receptors. eLife 3.
Montell, C., 2009. A taste of the Drosophila gustatory receptors. Curr. Opin. Neurobiol. 19, 345–353.
Montell, C., 2013. Gustatory receptors: not just for good taste. Curr. Biol. 23, R929–R932.
Moreira, I.S., 2014. Structural features of the G-protein/GPCR interactions. Biochim. Biophys. Acta 1840, 16–33.
Mruk, K., Shandilya, S.M.D., Blaustein, R.O., Schiffer, C.A., Kobertz, W.R., 2012. Structural insights into neuronal K+ channel-calmodulin complexes. Proc. Natl. Acad. Sci. 109, 13579–13583.
Mukunda, L., Miazzi, F., Kaltofen, S., Hansson, B.S., Wicher, D., 2014. Calmodulin modulates insect odorant receptor function. Cell Calcium 55, 191–199.
Nakagawa, T., Pellegrino, M., Sato, K., Vosshall, L.B., Touhara, K., 2012. Amino acid residues contributing to function of the heteromeric insect olfactory receptor complex. Plos One 7, e32372.
Nakagawa, T., Vosshall, L.B., 2009. Controversy and consensus: noncanonical signaling mechanisms in the insect olfactory system. Curr. Opin. Neurobiol. 19, 284–292.
Neuhaus, E.M., Gisselmann, G., Zhang, W., Dooley, R., Stortkuhl, K., Hatt, H., 2005. Odorant receptor heterodimerization in the olfactory system of *Drosophila melanogaster*. Nat. Neurosci. 8, 15–17.
Neumoin, A., Cohen, L.S., Arshava, B., Tantry, S., Becker, J.M., Zerbe, O., Naider, F., 2009. Structure of a double transmembrane fragment of a G-protein-coupled receptor in micelles. Biophys. J. 96, 3187–3196.
Nichols, A.S., Chen, S.S., Luetje, C.W., 2011. Subunit contributions to insect olfactory receptor function: channel block and odorant recognition. Chem. Senses 36, 781–790.
Nichols, A.S., Leutje, C.W., 2010. Transmembrane segment 3 of *Drosophila melanogaster* odorant receptor subunit 85b contributes to ligand-receptor interactions. J. Biol. Chem. 285, 11854–11862.
Pask, G.M., Jones, P.L., Rutzler, M., Rinker, D.C., Zwiebel, L.J., 2011. Heteromeric *Anopheline* odorant receptors exhibit distinct channel properties. Plos One 6, e28774.
Pellegrino, M., Steinbach, N., Stensmyr, M.C., Hansson, B.S., Vosshall, L.B., 2011. A natural polymorphism alters odour and DEET sensitivity in an insect odorant receptor. Nature 478, 511–514.
Patching, S.G., 2014. Surface plasmon resonance spectroscopy for characterisation of membrane protein-ligand interactions and its potential for drug discovery. Biochim. Biophys. Acta 1838, 43–55.
Petkov, G., 2009. Ion Channels, Pharmacology: Principles and Practice. Elsevier, pp. 387–427.
Popot, J.L., 2010. Amphipols, nanodiscs, and fluorinated surfactants: three nonconventional approaches to studying membrane proteins in aqueous solutions. Annu. Rev. Biochem. 79, 737–775.
Raja, J.S.I., Katanayeva, N., Katanaev, V.L., Galizia, C.G., 2014. Role of G(o/i) subgroup of G proteins in olfactory signaling of *Drosophila melanogaster*. Eur. J. Neurosci. 39, 1245–1255.
Rajarathnam, K., Rosgen, J., 2014. Isothermal titration calorimetry of membrane proteins – progress and challenges. Biochim. Biophys. Acta 1838, 69–77.
Rasmussen, S.G.F., Choi, H.J., Rosenbaum, D.M., Kobilka, T.S., Thian, F.S., Edwards, P.C., Burghammer, M., Ratnala, V.R.P., Sanishvili, R., Fischetti, R.F., Schertler, G.F.X., Weis, W.I., Kobilka, B.K., 2007. Crystal structure of the human β2 adrenergic G-protein-coupled receptor. Nature 450, 383–387.
Ray, A., van der Goes van Naters, W., Carlson, J.R., 2014. Molecular determinants of odorant receptor function in insects. J. Biosci. 39, 555–563.
Reichow, S.L., Clemens, D.M., Freites, J.A., Nemeth-Cahalan, K.L., Heyden, M., Tobias, D.J., Hall, J.E., Gonen, T., 2013. Allosteric mechanism of water-channel gating by Ca2+-calmodulin. Nat. Struct. Mol. Biol. 20, 1085–1092.
Robertson, H.M., 2009. The insect chemoreceptor superfamily in Drosophila pseudoobscura: molecular evolution of ecologically-relevant genes over 25 million years. J. Insect Sci. 9.
Robertson, H.M., Warr, C.G., Carlson, J.R., 2003. Molecular evolution of the insect chemoreceptor gene superfamily in *Drosophila melanogaster*. Proc. Natl. Acad. Sci. U. S. A. 100, 14537–14542.
Ronderos, D.S., Lin, C.C., Potter, C.J., Smith, D.P., 2014. Farnesol-detecting olfactory neurons in Drosophila. J. Neurosci. 34, 3959–3968.
Rosenbaum, D.M., Cherezov, V., Hanson, M.A., Rasmussen, S.G.F., Foon, S.T., Kobilka, T.S., Choi, H.J., Yao, X.J., Weis, W.I., Stevens, R.C., Kobilka, B.K., 2007. GPCR engineering yields high-resolution structural insights into β2-adrenergic receptor function. Science 318, 1266–1273.
Ross, P., Weihofen, W., Siu, F., Xie, A., Katakia, H., Wright, S.K., Hunt, I., Brown, R.K., Freire, E., 2015. Isothermal chemical denaturation to determine binding affinity of small molecules to G-protein coupled receptors. Anal. Biochem. 473, 41–45.
Rytz, R., Croset, V., Benton, R., 2013. Ionotropic receptors (IRs): chemosensory ionotropic glutamate receptors in Drosophila and beyond. Insect Biochem. Mol. Biol. 43, 888–897.
Sargsyan, V., Getahun, M.N., Llanos, S.L., Olsson, S.B., Hansson, B.S., Wicher, D., 2011. Phosphorylation via PKC regulates the function of the *Drosophila* odorant co-receptor. Front. Cell. Neurosci. 5, 1–8.
Sato, K., Pellegrino, M., Nakagawa, T., Nakagawa, T., Vosshall, L.B., Touhara, K., 2008. Insect olfactory receptors are heteromeric ligand-gated ion channels. Nature 452, 1002–1006.
Schon, A., Brown, R.K., Hutchins, B.M., Freire, E., 2013. Ligand binding analysis and screening by chemical denaturation shift. Anal. Biochem. 443, 52–57.
Serrano-Vega, M.J., Magnani, F., Shibata, Y., Tate, C.G., 2008. Conformational thermostabilization of the β1-adrenergic receptor in a detergent-resistant form. Proc. Natl. Acad. Sci. U. S. A. 105, 877–882.
Silbering, A.F., Benton, R., 2010. Ionotropic and metabotropic mechanisms in chemoreception: 'chance or design'? EMBO Rep. 11, 173–179.
Smart, R., Kiely, A., Beale, M., Vargas, E., Carraher, C., Kralicek, A.V., Christie, D.L.,

Chen, C., Newcomb, R.D., Warr, C.G., 2008. *Drosophila* odorant receptors are novel seven transmembrane domain proteins that can signal independently of heterotrimeric G proteins. Insect Biochem. Mol. Biol. 38, 770–780.
Stengl, M., Funk, N.W., 2013. The role of the coreceptor Orco in insect olfactory transduction. J. Comp. Physiol. A Neuroethol. Sens. Neural Behav. Physiol. 1–13.
Stensmyr, M.C., Dweck, H.K., Farhan, A., Ibba, I., Strutz, A., Mukunda, L., Linz, J., Grabe, V., Steck, K., Lavista-Llanos, S., Wicher, D., Sachse, S., Knaden, M., Becher, P.G., Seki, Y., Hansson, B.S., 2012. A conserved dedicated olfactory circuit for detecting harmful microbes in *Drosophila*. Cell 151, 1345–1357.
Stoevesandt, O., Taussig, M.J., 2013. Phospho-specific antibodies by design. Nat. Biotechnol. 31, 889–891.
Subramanyam, P., Colecraft, H.M., 2015. Ion channel engineering: perspectives and strategies. J. Mol. Biol. 427, 190–204.
Tae, H.S., Cui, Y., Karunasekara, Y., Board, P.G., Dulhunty, A.F., Casarotto, M.G., 2011. Cyclization of the intrinsically disordered alpha1S dihydropyridine receptor II-III loop enhances secondary structure and in vitro function. J. Biol. Chem. 286, 22589–22599.
Taylor, R.W., Romaine, I.M., Liu, C., Murthi, P., Jones, P.L., Waterson, A.G., Sulikowski, G.A., Zwiebel, L.J., 2012. Structure-activity relationship of a broad-spectrum insect odorant receptor agonist. ACS Chem. Biol. 7, 1647–1652.
Tegler, L.T., Corin, K., Hillger, J., Wassie, B., Yu, Y., Zhang, S., 2015. Cell-free expression, purification, and ligand-binding analysis of *Drosophila melanogaster* olfactory receptors DmOR67a, DmOR85b and DmORCO. Sci. Rep. 5, 7867.
Tian, C.A., Zhu, R.X., Zhu, L.X., Qiu, T.Y., Cao, Z.W., Kang, T.G., 2014. Potassium channels: structures, diseases, and modulators. Chem. Biol. Drug Des. 83, 1–26.
Touhara, K., Vosshall, L.B., 2009. Sensing odorants and pheromones with chemosensory receptors. Annu. Rev. Physiol. 71, 307–332.
Tsitoura, P., Andronopoulou, E., Tsikou, D., Agalou, A., Papakonstantinou, M.P., Kotzia, G.A., Labropoulou, V., Swevers, L., Georgoussi, Z., Iatrou, K., 2010. Expression and membrane topology of *Anopheles gambiae* odorant receptors in lepidopteran insect cells. Plos One 5, e15428.
Turner, R., Kumar, B., Newcomb, R., Brittain, T., Christie, D., 2014. Mutational Analysis of Cysteine Residues of the Insect Odorant Co-receptor (Orco) from *Drosophila melanogaster* Reveals Differential Effects on Agonist- and Odorant/tuning Receptor-dependent Activation. In preparation.
Vaidehi, N., Bhattacharya, S., Larsen, A.B., 2014. Structure and dynamics of G-protein coupled receptors. Adv. Exp. Med. Biol. 796, 37–54.
Vosshall, L.B., 2003. Diversity and expression of odorant receptors in *Drosophila*. In: Blomquist, G., Vogt, R. (Eds.), Insect Pheromone Biochemistry and Molecular Biology. Elsevier Academic Press, pp. 567–591.
Vosshall, L.B., Hansson, B.S., 2011. A unified nomenclature system for the insect olfactory coreceptor. Chem. Senses 497–498.
Wang, G., Carey, A.F., Carlson, J.R., Zwiebel, L.J., 2010. Molecular basis of odor coding in the malaria vector mosquito *Anopheles gambiae*. Proc. Natl. Acad. Sci. U. S. A. 107, 4418–4423.
Warne, T., Serrano-Vega, M.J., Baker, J.G., Moukhametzianov, R., Edwards, P.C., Henderson, R., Leslie, A.G.W., Tate, C.G., Schertler, G.F.X., 2008. Structure of a β1-adrenergic G-protein-coupled receptor. Nature 454, 486–491.
Wicher, D., 2013. Sensory receptors-design principles revisited. Front. Cell. Neurosci. 7, 1.
Wicher, D., Schafer, R., Bauernfeind, R., Stensmyr, M.C., Heller, R., Heinemann, S.H., Hansson, B.S., 2008. *Drosophila* odorant receptors are both ligand-gated and cyclic-nucleotide-activated cation channels. Nature 452, 1007–1011.
Willinger, T., Freeman, T., Hasegawa, H., McMichael, A.J., Callan, M.F., 2005. Molecular signatures distinguish human central memory from effector memory CD8 T cell subsets. J. Immunol. 175, 5895–5903.
Xu, P.X., Leal, W.S., 2013. Probing insect odorant receptors with their cognate ligands: insights into structural features. Biochem. Biophys. Res. Commun. 435, 477–482.
Yao, C.A., Carlson, J.R., 2010. Role of G-proteins in odor-sensing and CO_2-sensing neurons in Drosophila. J. Neurosci. 30, 4562–4572.
Zhang, J., Xue, F., Liu, Y., Yang, H., Wang, X., 2013. The structural mechanism of the cys-loop receptor desensitization. Mol. Neurobiol. 48, 97–108.
Zhou, H.X., McCammon, J.A., 2010. The gates of ion channels and enzymes. Trends Biochem. Sci. 35, 179–185.