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Commentary

Smelling the difference: controversial ideas in insect olfaction

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Summary

In animals, the sense of smell is often used as a powerful way to attract potential mates, to find food and to explore the environment. Different animals evolved different systems to detect volatile odorants, tuned to the specific needs of each species. Vertebrates and nematodes have been used extensively as models to study the mechanisms of olfaction: the molecular players are olfactory receptors (ORs) expressed in olfactory sensory neurons (OSNs) where they bind to volatile chemicals, acting as the first relay of olfactory processing. These receptors belong to the G protein-coupled receptor (GPCR) superfamily; binding to odorants induces the production and amplification of second messengers, which lead to the depolarization of the neuron. The anatomical features of the insect olfactory circuit are similar to those of mammals, and until recently it was thought that this similarity extended to the ORs, which were originally annotated as GPCRs. Surprisingly, recent evidence shows that insect ORs can act like ligand-gated ion channels, either completely or partially bypassing the amplification steps connected to the activation of G proteins. Although the involvement of G proteins in insect olfactory signal transduction is still under question, this new discovery raises fascinating new questions regarding the function of the sense of smell in insects, its evolution and potential benefits compared with its mammalian counterpart.

Key words: olfactory receptors, ion channel, GPCR.

Introduction

The sense of smell allows insects to detect, discriminate and react to a broad range of different chemicals, even with similar molecular structure, found in the environment. Among thousands of compounds, each insect species has fine-tuned its olfactory system to those that are fundamental for its survival. As a consequence, insects show strong odor-evoked behaviors and change of physiological states in response to chemical cues.

For these reasons, insects have been used as models to study olfaction and olfaction-driven behavior for at least 100 years, since volatile signals influencing moths were first described by the French entomologist Fabre (Fabre, 1911).

Anatomy

Across the animal kingdom, olfactory systems are remarkably similar. Chemical cues are detected by olfactory sensory neurons (OSNs), which have access to the external environment. In *Drosophila*, these are located in sensory hairs (sensilla) located on a pair of head appendages, the antennae (~1200 OSNs each) and the maxillary palps (~120 OSNs each) (Fig. 1A). The sensilla are categorized into three distinct morphological types: basiconic, coeloconic and trichoid (Shanbhag et al., 1999) (Fig. 1B). Each sensillum is innervated by 2–4 OSNs (Fig. 1C). OSNs are bipolar neurons that extend dendrites into the lumen of the sensillum and project an axon to the antennal lobe (AL), the second relay center of the olfactory system located in the *Drosophila* brain. In the AL, OSNs expressing the same olfactory receptor (OR) gene make synaptic connections with projection neurons (PN) within globular regions called glomeruli, which are interconnected by inhibitory local interneurons (LNs) (Boeckh and Tolbert, 1993; Galizia and Menzel, 2000). The PNs send their axons to the mushroom body and the lateral horn of the protocerebrum; thus, translating the perception of each odor into a possibly unique temporal and spatial pattern of activity in the brain (Fig. 1D).

The molecular players: ORs

The discovery of the first ORs was elusive for many years due to the nature of the receptors themselves. The presence of a large number of ORs, their sequence divergence and the low expression level made them difficult to detect until, in 1991, the first mammalian ORs were cloned from the rat olfactory epithelium (Buck and Axel, 1991). The newly found proteins showed characteristics that were consistent with their classification as ORs: they are expressed specifically in the olfactory epithelium, they are members of the superfamily of G protein-coupled receptors (GPCRs) with seven membrane-spanning domains as hypothesized by previous studies (Jones and Reed, 1989) and their sequences are related. The protein's physiological function was indeed confirmed a few years later (Zhao et al., 1998), and genes with similar properties were soon described in other organisms (Freitag et al., 1995; Nef et al., 1996; Selbie et al., 1992; Sengupta et al., 1996).

Insect ORs were first identified in *Drosophila melanogaster* by three independent groups in 1999 (Clyne et al., 1999; Gao and Chess, 1999; Vosshall et al., 1999). Genomic data mining and an accurate analysis of low abundance genes expressed in the olfactory organ revealed a novel protein family with characteristics similar to those described in mammals. Like vertebrate ORs, they had seven predicted transmembrane domains but were surprisingly much more divergent in sequence from ORs described in other organisms, assigning them to a different evolutionary path. Moreover, even among themselves, OR sequences show wide divergence with only ~20% of similarity on average.

Most *Drosophila* OSNs co-express two different types of ORs: OR83b, a broadly expressed receptor, and one of the 61 ligand-specific ORs. OR83b is highly conserved among insect species

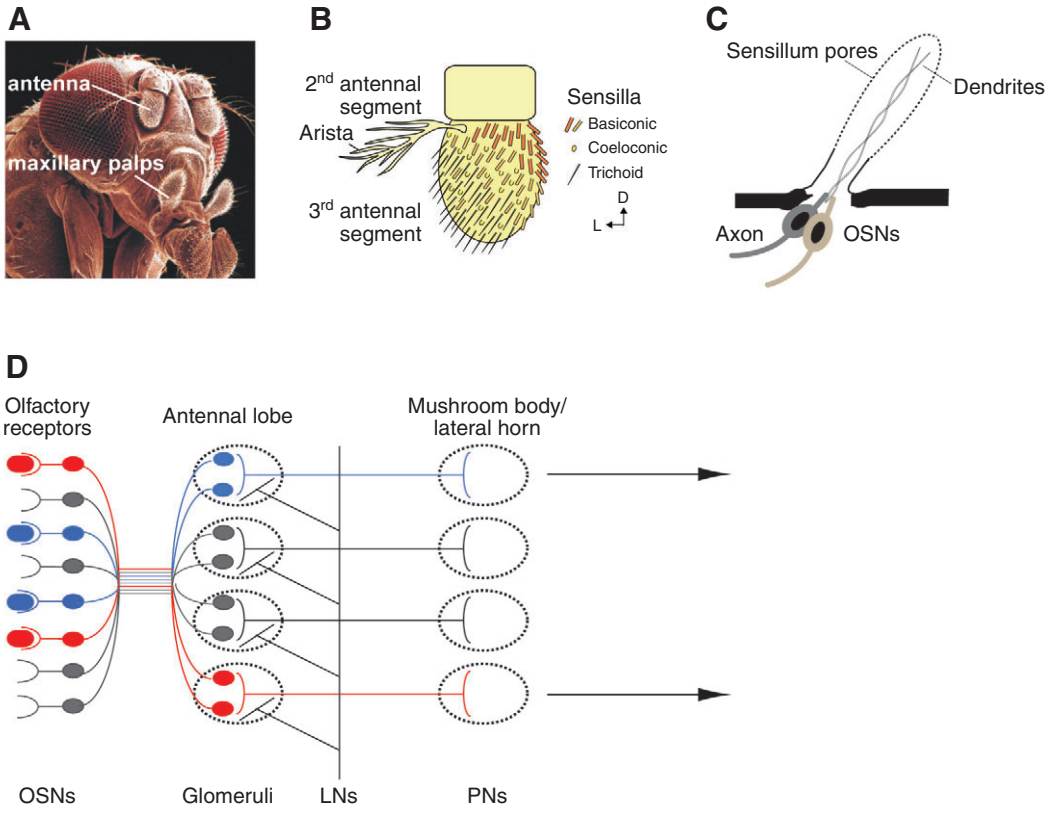

Fig. 1. The olfactory system of *Drosophila*. (A) *Drosophila melanogaster* head. The white lines point to the olfactory appendages, the antenna and the maxillary palps. SEM image is courtesy of Jürgen Berger, Max Planck Institute for Developmental Biology, Tübingen, Germany. (B) Drawing of the three different classes of sensilla present on the 3rd antennal segment. The drawing is adapted with permission from Benton et al. (Benton et al., 2006). (C) Anatomy of a sensillum. Each sensillum is innervated by 2–4 olfactory sensory neurons (OSNs), which project an axon to the antennal lobe and dendrites into the sensillum lumen. The sensillum has pores that allow odorants to reach the neurons. (D) Organization of the *Drosophila* olfactory system. OSNs bind to odorants and send the information to glomeruli, innervated by local interneurons (LNs). The information then travels to higher centers in the brain, the mushroom body and lateral horn, through projection neurons (PNs).

whereas the ligand-specific receptors are highly divergent. Electrophysiological and behavioral experiments in OR83b knock-out fruit flies revealed that OR83b is essential for the correct function of other ORs (Larsson et al., 2004). Benton and colleagues later demonstrated that not only is OR83b a chaperone that transports the ligand-binding ORs from the cell body to the dendrite where ORs can detect odorants but also that is a functional part of the receptor-complex (Benton et al., 2006). However, it still remains to be elucidated whether OR83b is involved at all in the binding to the odorants.

Functional characterization of insect ORs

Odorants that pass through pores on the sensillum bind to ORs expressed on the dendrite of OSNs and induce an action potential, which can be monitored using the single sensillum recording (SSR) technique (Bestmann et al., 1996; Stensmyr et al., 2003; Wojtasek et al., 1998); a recording electrode is placed in the desired sensillum and captures voltage changes due to the firing of the OSNs (Fig. 2A). Because the sensillum contains more than one OSN, the resulting trace represents the summed activity of all the neurons housed within the sensillum (Fig. 2B). In some sensilla, it is possible to distinguish the different OSNs because of the different amplitudes of their spikes. Electrophysiological recordings of antennal basiconic sensilla have revealed that OSNs are classified into distinct functional classes, each with a unique odorant response spectrum (de Bruyne et al., 2001). A fundamental step forward was achieved when John Carlson's group established a mutant fly strain with a deletion in the locus of the receptor OR22a/b, thereby abolishing odor-evoked responses in the OSN where the receptor is expressed without eliminating the OSN itself, the so-called 'empty neuron' (Hallem et al., 2004a). With this system, thanks to a combination of the SSR technique and the GAL4-UAS system (Hallem et al., 2004a), it is possible to express virtually any OR and study its properties *in vivo* and use it as a medium-throughput tool for OR de-orphanization, i.e. a simple way to assign ligands to each OR (Hallem et al., 2004b; Kurtovic et al., 2007). Based on this analysis, it was shown that not only is the OR responsible for the odorant response spectrum in OSNs but also for its spontaneous activity and response dynamics (Hallem et al., 2004a). Electrophysiological studies *in vivo* have been complemented by studies in cell culture: a limited number of insect ORs are in fact functionally expressed in human embryonic kidney 293 (HEK293) cells (Fig. 2C,D), HeLa cells and *Xenopus laevis* oocytes (Nakagawa et al., 2005; Neuhaus et al., 2004; Sato et al., 2008; Wetzel et al., 2001; Wicher et al., 2008). The functional characterization of insect ORs in heterologous expression systems has provided several new insights into the molecular mechanism of insect ORs, including functional interaction between OR subunits (Neuhaus et al., 2004), novel signaling properties of insect ORs (Sato et al., 2008; Smart et al., 2008; Wicher et al., 2008) and the role of OR83b (Nakagawa et al., 2005; Neuhaus et al., 2004).

Signal transduction cascades in olfactory systems

In mammalian and nematode OSNs the binding of odorants to ORs induces the activation of the G protein signaling cascade. Once activated, G_s proteins in mammals, also called G_{olf}, and G_i proteins in nematodes, increase the level of cyclic nucleotides (cAMP and cGMP, respectively) that directly bind and activate cyclic nucleotide-gated (CNG) channels, expressed on the membrane of OSNs. The opening of CNG channels lets cations enter into the neurons, producing an action potential that travels down the axon to the brain (Fig. 3A,B).

Before the identification of insect OR genes, there were several hints that pointed toward the involvement of GPCR-mediated second messenger pathways based on biochemical and electrophysiological evidence and the identification of the

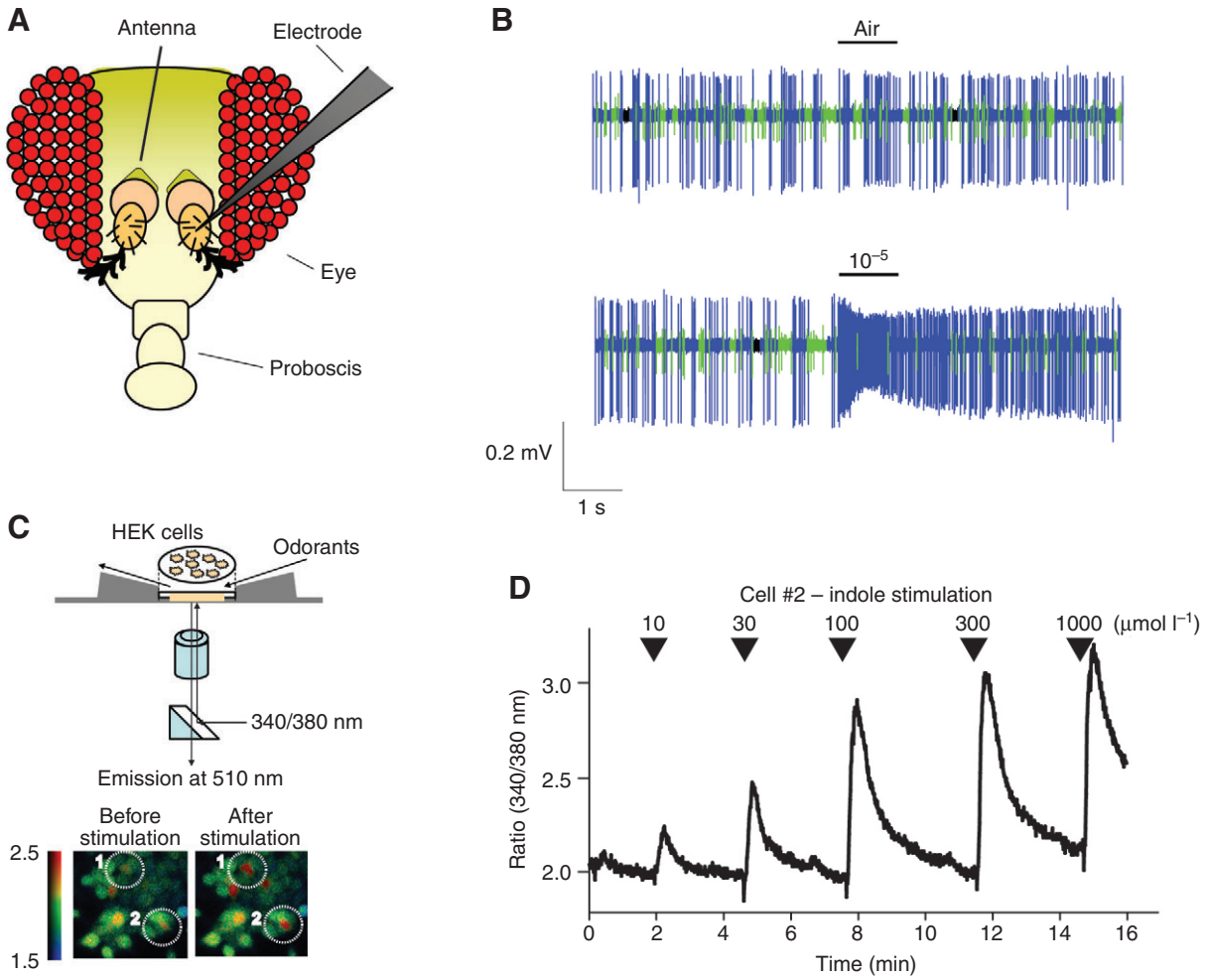

Fig. 2. Functional analysis of the olfactory receptor (OR)-evoked responses *in vivo* and *in vitro*. (A,B) Single sensillum recording (SSR) technique. (A) Drawing of a *Drosophila* head. During SSR, the recording electrode is positioned in the sensilla located on the 3rd segment of the antenna. (B) Responses of *Drosophila melanogaster* antennal basiconic sensilla to methyl acetate using SSR. The A cell (blue spikes) responds to increasing concentrations of the odorant, while the B cell (green spikes) is unaffected. (C,D) Ca^{2+}-imaging technique. (C) Schematic of Ca^{2+}-imaging assay of human embryonic kidney 293 (HEK293) cells. The cells, #1 and #2, are loaded with a Ca^{2+}-sensitive dye and the light emission of the dye is monitored through a microscope while the cells are stimulated with odorants. The cells marked with broken circles show an increase of Ca^{2+} concentration after odorant stimulation. (D) A representative trace showing the dose-dependent response to indole of cell #2 (panel C) expressing the mosquito *Anopheles gambiae* receptors GPROR10+GPROR7. Arrowheads indicate the increasing concentration of odorant delivered (μmol l^{-1}).

components of the cAMP and inositol 1,4,5-triphosphate (IP_3) signaling pathways in the *Drosophila* olfactory system. Stimulation with odorants or pheromones on isolated OSNs increases second messenger production like IP_3, and *in vivo* recordings from antennal neurons showed action potentials are generated when IP_3 is directly applied to the cells (Stengl, 1993; Talluri et al., 1995). In addition, the reduction of expression of the *Drosophila* $G_{\alpha q}$ gene, *dgq*, and other genes involved in phospholipid signaling induces a decrease of OSNs' odor-evoked responses but not their complete abolishment (Kain et al., 2008; Kalidas and Smith, 2002). These observations lead to the assumption that insect odorant responses were mediated by G_q-coupled GPCRs. However, other groups reported that altering the expression of the genes *rut* and *dnc*, affecting the cAMP transduction cascade, showed abnormal electrophysiological and behavioral responses to odorants, suggesting that $G_{\alpha s}$ is also involved in the transduction mechanism (Gomez-Diaz et al., 2004; Martin et al., 2001). Although the responses are abnormal, it is important to notice that no anosmic phenotypes have been found so far, as expected if G proteins were essential for the transduction mechanisms of odors in OSNs. Are G proteins necessary or sufficient for the correct functioning of the insect olfactory system? General neuronal sickness or the alteration of G protein-mediated signaling pathways downstream or independent of the olfactory receptors could be sufficient to explain the abnormal odor-evoked responses reported in these studies. Altogether, these observations suggest that multiple or alternative signaling cascades are present in the insect olfactory system.

New insights

Ion channel hypothesis

Structural analysis *in silico*, *in vitro* and *in vivo* surprisingly showed that insect ORs have an inverted topology compared with conventional GPCRs, presenting a cytoplasmic N-terminus and an extracellular C-terminus (Benton et al., 2006; Krogh et al., 2001; Lundin et al., 2007). Furthermore, electrophysiological analysis of moth receptors substantiated the idea of an atypical mechanism of

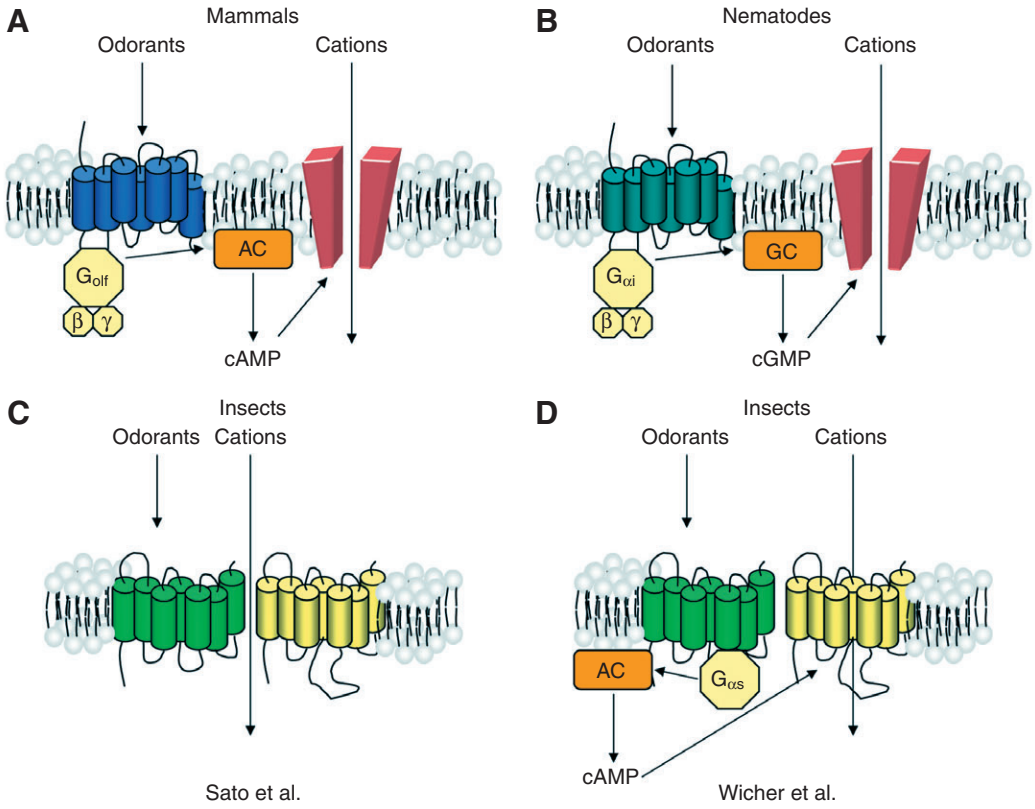

Fig. 3. Models of signal transduction mechanisms in olfactory sensory neurons (OSNs). (A,B) Signal transduction of non-insect olfactory receptors (ORs). (A) Mammalian ORs are G protein-coupled receptors (GPCRs) coupled to a stimulatory G protein, G_{olf}. After binding to an odorant, the G protein activates adenylate cyclase (AC), which increases the intracellular concentration of cAMP. This leads to the opening of a cyclic nucleotide-gated (CNG) channel and the depolarization of the neuron. (B) In nematodes, stimulation of ORs leads to the activation of guanylate cyclase (GC) and an increase in levels of cGMP. This leads to the opening of a CNG channel and the depolarization of the OSN. (C,D) Divergent views on signal transduction of insect ORs. (C) In Sato et al., evidence supports a model in which the OR83b/ORX complex forms an ion channel that is directly opened by the binding of the odorants and is permeable to cations (Sato et al., 2008). (D) By contrast, in Wicher et al.'s model the ligand-binding subunit (in green) is a GPCR that leads to the increase in cAMP through a stimulatory G protein (Wicher et al., 2008). This opens the CNG-like channel OR83b (in yellow).

olfactory signal transduction: cell culture expression of moth receptors is enough to confer ligand-dependent responses without the further addition of exogenous G proteins, and the electrophysiological properties of these responses are distinct from currents elicited by GPCRs, as observed for mammalian ORs activation (Katada et al., 2003; Nakagawa et al., 2005).

Further electrophysiological analysis recently carried out provided strong evidence for the idea that insect ORs are, in fact, ligand-gated non-specific cation channels (Sato et al., 2008) (Fig. 3C). Simultaneous measurements of whole-cell currents and Ca^{2+} influx in HeLa cells expressing insect ORs show that the onset of the response is ~10-fold faster than what is usually required by GPCRs. Furthermore, general pharmacological inhibition of G proteins does not impair ORs-evoked responses, as would be expected if they were GPCRs. Further experiments with single-channel recordings revealed that the response of insect ORs was not dependent on the cellular cytoplasmic components, including second messengers such as cAMP and cGMP. Finally, different subunit compositions of the OR complex are able to shift the ion selectivity of the measured current. This is an important finding because the ion selectivity is a direct property of ion channels. This makes it unlikely that ORs are associated with a separate ion channel and suggests that ORs themselves are necessary and sufficient to produce an odor-induced response (Sato et al., 2008).

Ion channel–GPCR hypothesis

An alternative hypothesis lies between the provocative ion-channel and classical GPCR theories (Wicher et al., 2008) (Fig. 3D). By electrophysiological recordings of insect ORs expressed in HEK293 cells, Wicher and colleagues show that activation of the *Drosophila* receptor OR22a is able to induce the opening of a cAMP-dependent CNG channel, suggesting the involvement of G_s proteins directly following OR22a activation. Moreover, the co-receptor OR83b alone can generate currents after an increase of intracellular cAMP/cGMP, similar to the currents recorded after ligand application. Finally, a mutation in OR83b can directly modulate the ion permeability of the OR complex, showing that this protein probably participates in the formation of the channel complex without the involvement of other ion channels (Wicher et al., 2008).

Taken together, the results from independent research groups show an unexpected mechanism of signal transduction in insect OSNs. Both groups focus on the new idea that, unlike the case in vertebrates, insect ORs can function as ligand-gated ion channels activated by odorants. However, there are still unanswered questions that need clarification. To what extent are G proteins and cyclic nucleotides involved in insect OSN signal transduction? The partially conflicting results could be explained by the time scale at which the two groups analyzed the OR activation in cells: while the first group looks at the early onset of OR activation (~1 s), the second group analyzes the characteristics of longer-lasting

dynamics after the fast response (~1.5 min). This behavior might be due to a double mechanism of ORs activation, where at first the G protein-independent channel component of the complex is activated but it is followed by a G protein-dependent response. The role played by cyclic nucleotides could then be different according to which mechanism is being considered, although there is no clear evidence of a cyclic nucleotide binding domain in the OR family (M.P., unpublished data).

In addition, the OR complexes in the two studies contained different ligand-specific subunits. It would be interesting to determine whether the OR studied by Wicher et al. (Wicher et al., 2008) have the same properties in other heterologous systems and *in vivo* and, *vice versa*, whether the long-lasting dynamics of the ORs used by Sato et al. (Sato et al., 2008) are similar to what observed for OR22a.

Finally, the possible dual nature of ORs as both functional GPCRs and CNG channels could raise interesting questions as to how substantially different functions developed within the same protein family.

Other types of ligand-gated ion channels in sensory perception

Although new in the field of olfaction, ligand-gated ion channels are used in other sensory systems for the perception of the outside world. Notable examples are the mammalian TRPM8 and TRPV1 channels, activated by cold/menthol (Dhaka et al., 2007) and heat/vanilloid (Caterina et al., 1997) compounds, respectively, both involved in nociception. Interestingly, both of these channels are regulated by Ca^{2+}-dependent and -independent pathways and cyclic nucleotides (Bhave et al., 2002; Daniels et al., 2008; De Petrocellis et al., 2007; Vanden Abeele et al., 2006). Other members of the TRP channel family, PKD1L3 and PKD2L1, have been recently implicated in the detection of sour compounds in mammals, while GPCRs are responsible for the detection of umami, sweet and bitter (Chandrashekar et al., 2006; Huang et al., 2006; Ishimaru et al., 2006). In the gustatory system of the fleshfly *Boettcherisca peregrina*, Murakami and Kijima have also suggested the presence of sugar-activated ion channels but their molecular identity is still unknown (Murakami and Kijima, 2000). Finally, the green alga *Chlamydomonas reinhardtii* has recruited the ion channel channelrhodopsin to sense photons (Nagel et al., 2002), unlike the GPCR rhodopsin employed by vertebrates. Remarkably, they both make use of retinal as their chromophore. Finally, one of the latest studies on insect olfaction has unraveled a new class of olfactory receptors in *Drosophila melanogaster* that belong to the ionotropic glutamate receptor family (iGluRs); therefore, adding one more dimension to the role of ion channels in the olfactory system (Benton et al., 2009). This study revealed that iGluR-like receptors (IRs) are expressed in antennal sensory neurons and confer odor-dependent responses to cells. IRs expression patterns are complementary to OR83b-expressing neurons and might explain the remaining olfactory-mediated responses in OR83b-null fruit flies. More importantly, this discovery highlights how multiple receptor families can be recruited to perform similar functions in the same organ but it is yet to be determined if IRs play a special role in fruit fly olfaction.

Open questions

The recent insights on insect olfactory signal transduction mechanisms open the way for new questions to be answered and offer a new way of thinking about old problems. What is the role of the co-receptor and the ligand-binding subunit within the complex? How can different odorants activate the same receptor complex? How can the same receptor complex be activated and inhibited by different odorants?

Structure–function analysis of insect ORs

Despite a weak similarity to known potassium channel pores (Wicher et al., 2008), there is not a clear consensus on where the pore of the channel is located and to what extent different subunits in the OR complex contribute to the pore itself. As a matter of fact, there is little data on the exact stoichiometry of the OR complex. Although we know it must include at least two subunits each of the co-receptor OR83b and the ligand-binding OR (Benton et al., 2006), the composition of the functional complex is still unknown and it might even vary for different OR83b/ORX combinations. Further research on these questions will help us understand how the ORs bind chemicals with different structures and how conformational changes within the proteins play a role in the transmission of the excitatory or inhibitory signal to the OSN.

Insect ORs are likely to undergo post-translational modifications that can modify their behavior, both pre- and post-stimulation. The possible outcomes of such modifications could affect several characteristics of the proteins and the channel activity: expression levels, internalization and turnover, ligand affinity, gating properties, the fraction of time it remains in an open conformation (open probability) and desensitization just to name some. In addition, the exact role of cyclic nucleotides and soluble second messengers needs to be further addressed, and possible differential effects on different OR complexes better explained.

Why do insects use ion channels as ORs?

One of the most interesting questions still remains: why are ion channels the better choice for insect olfaction compared with GPCRs? Bioinformatics analysis of ORs from different animal species suggests that olfactory receptors appeared multiple times during evolution (Dryer, 2000). Most animal species adopted GPCRs to respond to odorants: this involves a signaling cascade with several amplification steps before the neuron fires and the information that a chemical has been encountered is transmitted to higher centers in the brain. By contrast, insects have adopted ion channels that respond directly to environmental chemicals, although there is still an ongoing controversy regarding whether there is or is not G protein amplification. This type of response might lead to a more direct and quantitative correlation between the amount of molecules bound to the receptor and the activity of the neuron and a faster behavioral response by the animal.

Conclusions

Over the past few years, a novel paradigm on the molecular mechanisms underlying insect chemosensation has been revealed despite the common idea that the olfactory system is conserved across the animal kingdom, from its anatomy to the molecular level. Moreover, the discovery that a pair of seven transmembrane receptors functions as ligand-gated ion channels questions the assumption that seven transmembrane proteins belong to the GPCR superfamily. Finally, the discovery that insect ORs belong to a different class of proteins provides a new strategy to design better insect repellents that will specifically affect the insect olfactory system, with little or no effect on humans.

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Glossary

AL
The antennal lobe is the second relay center of the olfactory system in the insect brain. It receives information from olfactory sensory neurons and sends it to higher brain centers.

Anosmia
Anosmia is the inability to perceive odorants.

CNG
Cyclic nucleotide-gated channels are a class of ion channels opened by cyclic nucleotides. CNG channels are involved in the olfactory transduction mechanisms in mammals and nematodes.

Dendrites
Dendrites are branched projections of neurons. In olfactory neurons, they are responsible for the detection of odorants.

GAL4-UAS
GAL4 is a yeast transcription factor that is able to bind to specific upstream activating sequences (UAS) and drive the transcription of the gene downstream the sequence. The GAL4 protein can be expressed under tissue- or cell-specific promoters, specifying the expression of the genes of interest under control of the UAS sequences.

GPCR
A G protein-coupled receptor is a seven transmembrane receptor, which activates signaling transduction inside the cells *via* G proteins after being activated by its cognate ligand.

G proteins
Guanine nucleotide-binding proteins are involved in second messenger cascades. Mammals and nematodes employ trimeric G proteins in olfactory transduction mechanisms. G_{α} subunits are divided in different classes, depending on the effector protein they modulate. For example, $G_{\alpha s}$ activates adenylyl cyclase, $G_{\alpha i}$ inhibits it and $G_{\alpha q}$ activates phospholipase C.

HEK293
The human embryonic kidney cell line 293 is a heterologous cell line originally obtained from human embryonic kidney. It is often used as an expression system for GPCRs.

IP_3
Inositol 1,4,5-triphosphate is a secondary messenger molecule used in signaling transduction induced by the activation of phospholipase C.

LN
A local interneuron is a multipolar neuron, which modifies the output from the AL to higher brain centers through intra- and interglomerular communication.

Nociception
Nociception is the perception of pain.

OR
Olfactory receptors are proteins that bind odorants in the olfactory sensory neurons.

OSN
An olfactory sensory neuron, which is the primary center of the olfactory system, detects odorants through the ORs expressed on its dendrites and transmits the information to glomeruli.

PN
Projection neurons synapse with OSNs in the glomeruli and transmit the olfactory information to the AL and higher brain centers.

Sensillum
A sensillum is a sensory hair, which contains neurons surrounded by lymph; within a sensillum, a variable number of neurons can be housed. Olfactory sensilla found on the antenna of *Drosophila melanogaster* can be divided in three types, based on their shape and size: basiconic, ceoloconic and trichoid.

SSR
Single sensillum recording is an extracellular recording of voltage differences generated by the activation of ORs.

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