Response Dynamics in Drosophila Olfaction

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“The act of smelling something, anything, is remarkably like the act of thinking. Immediately at the moment of perception, you can feel the mind going to work, sending the odor around from place to place, setting off complex repertories through the brain polling one center after another for signs of recognition, for old memories and old connection”.

Lewis Thomas

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Introduction

Why study insect olfaction?

By most measures of evolutionary success, insects are incomparable due to their biodiversity and numerous ecological adaptations (Labandeira and Sepkoski, 1993; Grimaldi and Engel, 2005). Their economic and ecological impact also makes them relevant and important organisms to study. Half of all insect species are dependent on plants, consuming a significant amount of annual production in natural habitats such as forest and agricultural crops (Hill, 1996). When attempting to increase agricultural production, insight gained from insect-plant interactions is indispensable. Most flowering plants (80 %) also require insect pollinators (Mitter et al., 1991; Bernays, 1998) and thus crop production can also benefit from better knowledge of insect-plant interactions. In addition to phytophagous species, blood-feeding insects are known to transmit many of the world's deadliest diseases, such as malaria, which is responsible for infection of an estimated 219 million people annually and results to the death of 660,000 in 2010 alone (WHO, 2012). Similarly, vector born diseases in livestock continue to hold back development in large parts of the world (Kabayo, 2002).

Insects are highly dependent on olfaction (Hildebrand and Shepherd, 1997; Krieger and Breer, 1999; Takken and Knols, 1999). In addition to conventional measures of pest control, topically applied insect repellents and traps loaded with attractant play a crucial role in protecting humans and animals from blood-feeding insects (Rowland et al., 2004, Gikonyo et al., 2003). Therefore, there is an irrefutable need to better understand the factors governing the olfactory relationships between insects and their hosts, and a detailed investigation of insect olfaction is a key for the development of successful control strategies. The mechanisms underlying odor signaling are also of interest to biosensor developers. The incredible sensitivity and working range of the insect nose (Glatz and Hill, 2010) makes it one of the best models to consider. These tiny nervous systems also present tractable neural networks with similar properties to vertebrates (Lin et a., 2000; Matsutani et al., 2000; Lo´pez-Mascaraque et al., 2005; Blanchart et al., 2006; Prieto-Godino et al., 2012).

Insect Olfaction

The olfactory system tracks a moving world of volatile cues by detecting changes in concentration and molecular type in space and time. This process requires adaptation to the dynamic odor landscape and constant adjustment of coding strategies according to the insect's lifestyle. Insects show robust and extremely sensitive behaviors that are elicited by chemical cues in a species-specific manner (Schneider, 1969). Odorant stimulation also triggers a rapid behavioral response in less than 500 ms (Budick and Dickinson , 2006; Bhandawat et al., 2010), which demands fast neuronal processing. Olfaction is designed in such a way that it is processed at various levels, starting with reception of semio-chemicals at the periphery (Hildebrand and Shepherd, 1997), processing of signals in a specialized neuropil of the brain (the antennal lobe in insects) (Couto et al., 2005), integration of olfactory and sensory modalities in higher processing centers of the brain (Galizia et al., 1999; Mori et al., 1999), and ultimately translation of olfactory signals into behavior (Semmelhack and Wang 2009; Knaden et al., 2012). The basis of sophisticated olfactory behavior thus depends on the ability of the insect peripheral system to selectively and efficiently detect and rapidly process olfactory information, suggesting that olfactory sensory neurons (OSN) need to be sensitive and fast.

There are a number of reasons why insect sensory neurons need to be sensitive. For instance, the emission of odor molecules in nature is very low, e.g. below 0.1 ng/minute for most odors from host plants of *Manduca sexta* (Späthe et al., 2013). Similarly, the amount of pheromone released by a female oriental fruit moth is as low as 0.5 ng/hr (Lacey and Sanders, 1992). Furthermore, emitted odors are dispersed, mixed with background noise, and diluted by the ambient motion of air to form a shifting and filamentous plume, making the olfactory world an arena of constant movement and flux (Murlis et al., 1992; Vicker et al., 2001; Koehl, 2006). Insects must thus perform odor-mediated navigation in a turbulent and unpredictable plume. Insects are the first invertebrates to have developed powered flight and took the sky match earlier than their vertebrate counterparts such as birds (Grimaldi and Engel, 2005). Flight contributes to the enormous success of insects through facilitated dispersion. However, the high-speed requirements for sensory detection in flight can also make olfaction more challenging. Beside the

small amount of odor present, the odor plume is transient, making odor tracking more challenging especially in high-speed flight (Kaissling et al., 1987; Vickers et al., 2001). This might necessitate the modification of the olfactory system in flying insects (Edward and Palka, 1991; Edwards, 1997). Thus the olfactory periphery of flying insects should be sensitive enough to detect low concentration and briefly repeated stimuli of turbulent odor plumes. This thesis addresses how the insect OSNs are adapted for speed and sensitivity.

The Insect Olfactory Periphery: The Antenna

Sensilla are the structural units of the insect olfactory periphery and are found on the antennae and palps (Steinbrecht, 1997; Keil, 1999; Shanbhag et al., 1999; Stocker, 2001) (Figure 1). Olfactory sensilla must maximize exposure of the OSNs to the outside environment while protecting the delicate cells inside the sensillum. The sensillum morphology itself could thus be a limiting factor for odor reception. The large numbers of long sensilla trichodea (up to 60,000 pheromone selective sensilla per antenna) in moths are suggested to result from the demand for extreme sensitivity in moth pheromone communication (Schneider and Kaissling, 1957; Boeckh et al., 1960 Steinbrecht, 1970; Keil, 1984; Meng et al., 1989). Olfactory sensilla show a large diversification in types even in the same species (Steinbrecht, 1997; Keil, 1999; Shanbhag et al., 1999) and are broadly classified in flies as sensilla basiconica, trichodea and coeloconica (Steinbrecht, 1997; Keil 1999; Shanbhag et al., 1999). Coeloconic sensilla are one of the most common sensillum types in insects and assumed to be the ancestral type (Meinecke, 1975). Beside morphological differences, there are also structural differences between the neurons housed in these different sensillum types that could potentially affect response dynamics. For instance, OSNs housed in basiconic sensilla possess dendrites split into 5 to as many as 150 parallel terminal branches (Shanbhag et al., 1999; Stocker, 2001). However, the dendritic segments of OSNs housed in coeloconic and trichoid sensilla extend unbranched into the sensillum lumen (Shanbhag et al., 1995; 1999; Stocker, 2001). Dendrite diameter has also been suggested as a factor affecting an OSN's response dynamics (Baker et al., 2012).

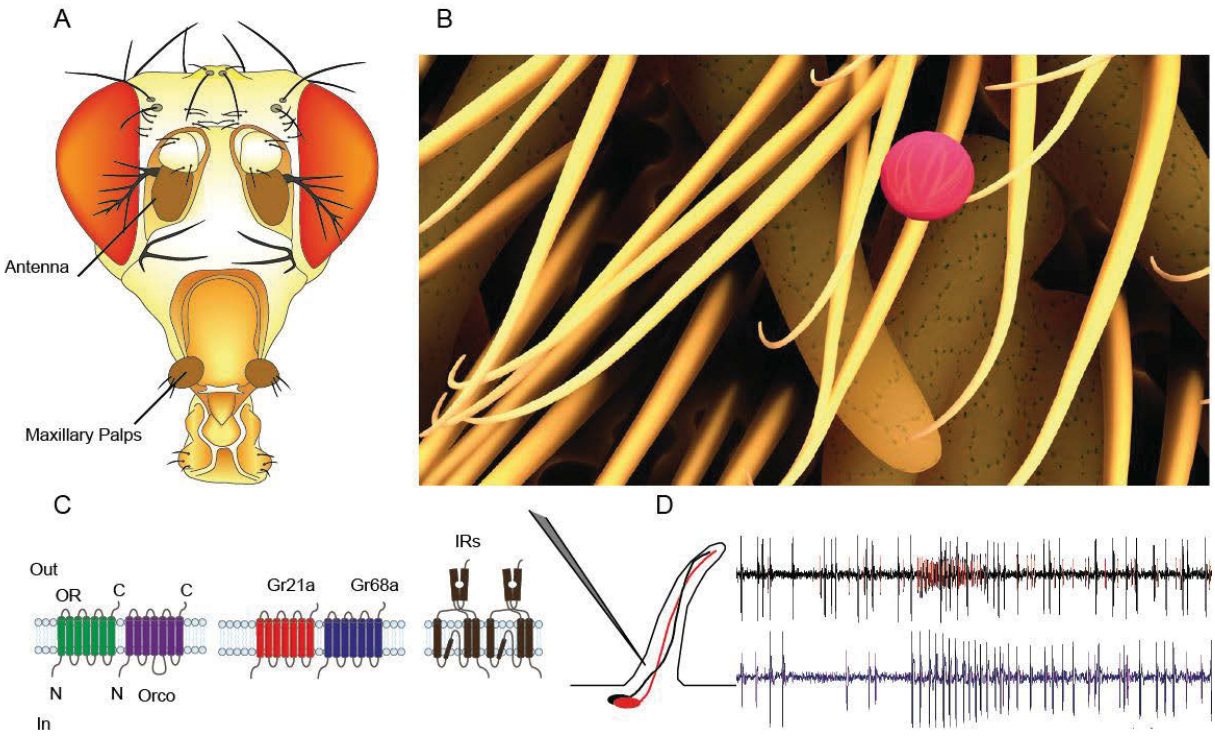

Figure 1. Peripheral olfactory system of Drosophila. Olfactory sensilla are located on the third antennal segments and maxillary palps (A). Depiction of an antennal basiconic type olfactory sensillum showing pores through which odors (red circle) can approach the internal sensory neuron dendrites. (C). Three different receptor families are expressed on the dendrites of antennal OSNs, from left to right: odorant receptors, gustatory receptors, and ionotropic receptors. (D). Single sensillum electrophysiological technique used to record OSN activity during odorant stimulation. Sample OSN traces shown on right. Individual cells can be discriminated by amplitude. B is from Kimberly Falk (2012).

Each insect sensillum houses several types of proteins involved in odorant detection, including odorant-binding proteins (OBPs) (Kaissling et al., 1989; Rogers et al., 2001; Forstner et al., 2006; Pelosi et al., 2006; Jin et al., 2008; Laughlin et al., 2008; Vogt et al., 2009; Anholt et al., 2010; Stengl, 2010; Ziemba et al., 2012; Sun et a., 2013), odorant-degrading enzymes (ODEs) (Durand et al., 2011, Ishida et al., 2005; Ishida and Leal, 2008), sensory neuron membrane proteins, (SNMPs; Benton et al., 2007; Jin et al., 2008), and three types of chemoreceptors - odorant receptors (ORs), ionotropic receptors (IRs), and gustatory receptors (GRs) (Clyne et al., 1999; Vosshall et al., 1999; Jones et al*.,* 2007; Kwon et al., 2007; Benton et al., 2009). The sensillum lymph bathes and protects the dendrite of OSNs and provides the necessary ionic milieu for the OSNs (Thurm and Kiippers, 1980; Keil and Steinbrecht, 1984). Functionally, OBPs contained in the sensillum lymph are the liaison between the external world and the chemoreceptors housed on the OSN dendrites (Leal, 2005). The predominantly lipophilic volatiles are believed to be bound and solubilized by OBPs and transported through the hydrophilic sensillar lymph where they finally activate the membrane-bound ORs (Steinbrecht, 1987, 1997, 1999; Leal, 2005; Maitani et al., 2010, Leal, 2013).

There is convincing evidence in the literature that OBPs are transporters, while the odorants activate the receptors themselves. However, it has also been postulated that the odor forms an odor-OBP complex that activates the receptor (Xu et al., 2005; Laughlin et al., 2008), though this hypothesis has been challenged recently (Gomez-Diaz et al., 2013). ORs have been shown to be functional in both heterologous and *ex vivo* systems where the natal perireceptor protein environment is not present, however, the response kinetics is less sensitive and delayed, suggesting that OBPs may indeed play a role in the odor response (Stengl et al., 1992; Steinbrecht et al., 1995; Ziegelberger, 1995; Mohl et al., 2002; Pophof, 2004; Leal et al., 2005; Xu et al., 2005; Grosse-Wilde et al., 2006; Syed et al., 2006; van Naters and Carlson, 2007; Laughlin et al., 2008; Carey et al., 2010; Biessmann et al., 2010; Pelletier et al., 2010). Furthermore, OBPs are also important for proper behavioral response in *Drosophila* to odorants (Swarup et al., 2011). Another group of proteins found in the sensillum lymph and suggested to be important for odor detection through rapid degradation of odorants is odor-degrading enzymes (ODE). The kinetics of the olfactory system requires that odorant molecules are rapidly inactivated (Ishida et al., 2004; Ishida and Leal, 2005; Ishida and Leal, 2008) and we have seen

that OSNs can resolve fast repeated stimulation physiologically (up to 5 Hz Chapter I), and behaviorally (up to 2Hz) (Krishnan et al., 2011). This fast response to odors is crucial for flying insects during navigation towards an odor source; however, the mechanism for such fast response termination is not clear as the ODEs dynamic is much slower.

Once odor molecules have passed through the receptor lymph of the sensillum, they are detected by membrane-bound protein receptors localized on the dendritic surface of OSNs (Clyne et al., 1999; Vosshall et al., 1999; Benton et al., 2009). Despite the sensillum morphology and perireceptor factors mentioned above, a seminal paper studying odor response kinetics of olfactory receptors ectopically expressed in the "empty neuron" system suggest that the olfactory receptor itself confers the majority of response characteristics for the sensory neuron, including response profile, sensitivity, and firing kinetics (Hallem et al., 2004). Three different kinds of chemosensory receptors have been identified in *D. melanogaster* (shown in Figure 1C). Ionotropic receptors, IRs, (a total of 61 genes in *Drosophila*) are three trans-membrane proteins expressed with co-receptors Ir8a and Ir25a (Benton et al., 2009; Croset et al., 2010; Abuin et al., 2011). IRs are the only receptors found in basal insects and are conserved among multicellular plants and unicellular organisms (Croset et al., 2010). IRs are localized in coeloconic sensilla, but are also found in arista and in the sacculus (Yao et al., 2005; Benton et al., 2009; Croset et al., 2010). However, the role of IRs expressed outside the antennal sensillum is unknown (Rytz et al., 2013). IRs are specifically activated by acids and amines (Yao et al., 2005; Ai et al., 2011; Silbering et al., 2011) and have few ligands (Yao et al., 2005; Silbering et al., 2011). Their conservation across organisms also suggests that they are the ancestral chemosensory receptor (Croset et al., 2010). Gustatory receptors (GRs), for which there are 73 GR genes in *Drosophila* are seven transmembrane proteins (Jones et al*.,* 2007; Kwon et al., 2007). The only olfactory GR expressed in the *Drosophila* antenna is a heterodimer of Gr21a and Gr63a and is CO_2 sensitive (Kwon et al., 2007). The chemoreceptor gene for CO_2 is highly conserved in mosquitoes, moths and beetles (Robertson and Kent, 2009), but the overall function of CO_2 detection appears to be species specific (Guerenstein et al., 2004; Suh et al., 2004; Dekker et al., 2005; Hu et al., 2007).

Odorant receptors (ORs), for which there are ~60 genes in *Drosophila* (Clyne et al., 1999; Vosshall et al., 1999) are also seven transmembrane proteins (Benton et al., 2006; Lundin et al.,

2007; Smart et al., 2008). ORs appear to have derived from the gustatory receptor family (Robertson et al., 2003; Nordström et al., 2011). The OR proteins form heterodimers composed of at least one OrX and a conserved coreceptor Orco of unknown stoichiometry (Neuhaus et al., 2004; Benton et al., 2006). Changing the OrX subunit in the complex alters the receptor specificity to odorants (Hallem and Carlson, 2006), suggesting that the OrX is responsible for ligand-binding specificity (Nichols and Luetje, 2010; Nichols et al., 2011). The Orco coreceptor is essential for trafficking ORs to the membrane (Larsson et al., 2004), and its expression varies across antennal OSNs (Larsson et al., 2004; Benton et al., 2006). During odorant activation, two kinds of protein interactions occur: homomeric, i.e. OrX vs OrX, and heteromeric interaction, i.e. between OR and Orco (Neuhaus et al., 2005; Benton et al., 2006; Tsitoura et al., 2010; German et al., 2012). Although Orco has not been shown to respond to any natural odorants on its own (Wanner, et al., 2007; Nichols et al., 2011), it is essential for odor-induced ion flux (Sato et al., 2008; Wicher et al., 2008), suggesting that Orco directly contributes to the structure of the channel pore.

Olfactory Signal Transduction in Insects

Signal transduction takes place in the dendrite of the OSNs housed in the sensillum (Stengl, 2010; Wicher, 2012). There are generally two major types of chemoreceptors, ionotropic and metabotropic receptors. Ionotropic receptors (IRs) are ligand-gated ion channels activated by ligand binding (Sato et al., 2008; Wicher et al., 2008). In ligand-gated transduction, the receptor functions both as receptor and ion channel. The activation of the receptor results in opening of the ion channel. The associated ion influx changes the membrane potential and elicits the subsequent generation of action potentials that carry the signal to the brain. Membrane depolarization also activates various voltage-gated cation channels, which leads to further depolarizion of the OSNs (Hodgkin and Huxley, 1952; Hille, 1984) and amplifies the signal. By contrast, activation of a metabotropic receptor stimulates an intracellular signaling cascade that elicits ion exchange, action potential generation, and signal amplification. This cascade may include enzyme activation, second messenger production or activation of ion channels (Breer et a., 1990; Firestein et al., 1991).

Insect odorant receptors are also ligand-gated ion channels (Sato et al., 2008; Smart et al., 2008; Wicher et al., 2008), However, the dendrites of insect OSNs are enriched with intracellular signaling molecules such as G-proteins ($G_{\alpha s}$ and $G_{\alpha q}$; Talluri et al., 1995; Laue et al., 1997; Miura et al., 2005; Kain et al., 2008; Deng et al., 2011), and IP_3 signaling pathways (Stengl, 1994). Diverse PKCs and PKAs iso-types are also co-expressed in insect olfactory organs (Rosenthal et al., 1987; Schaeffer et al., 1989; Tunstall et al., 2012). Furthermore, it has been shown that odorant stimulation activates intracellular signaling molecules both *in vivo* and *in vitro* (Boekhoff et al., 1990; Breer et al., 1990; Ziegelberger et al., 1990; Zufall and Hatt, 1991; Maida et al., 2000; Wicher et al., 2008; Deng et al., 2011). These lines of evidence suggest that complex intracellular signaling cascades could be involved in insect olfaction, but the mechanisms by which insect odorant receptor neurons convert chemical signals into electrical codes are still under investigation and debate.

Why study insect OSN signal transduction?

When I started my PhD in 2009 there was an intense debate stemming from two papers, Sato et al., and Wicher et al., 2008, that concerned the molecular basis of signal transduction in insect olfaction. The debate focused on whether insect olfactory signals were transduced by ionotropic or metabotropic cascades. Most of the investigations on insect odorant receptors are performed in heterologous expression systems (*in vitro*) to allow investigations on the function of isolated receptor proteins. However, such systems remove the receptors from their endogenous cellular environment. It is thus necessary to decipher the mechanism by which insect ORs transduce odor signals in the natural environment, especially concerning the molecular players and their contribution to response dynamics.

I therefore chose to investigate insect olfactory signal transduction in the fly itself. As a man grown witnessing the significant impact of insects as agricultural crop pests and vectors for various humans and livestock diseases, it was my interest to investigate how insects smell in order to develop and improve environmentally friendly olfaction-based insect pest and vector management, as well as to exploit the beneficial aspect of insects. As mentioned previously chemoreception is carried out by three different distantly related receptor families, which gives rise to a couple of questions: How does the diversification of chemoreceptors add to the odor

coding of the olfactory system? How do these diverse families contribute to the sensitivity, speed and capacity to detect chemical signals?

In my thesis, I investigated how OSNs convert chemical signals into electric signals and factors that affect their response dynamics. The model organism I use is *Drosophila*. *Drosophila* is a preeminent organism for studies of olfaction due to its broad base of genetic tools and well characterized receptors and ligands. To address how OSNs transform chemical signals present in the environment into electric signals *in vivo*, we used electrophysiology, pharmacology, biochemistry, molecular techniques and performed behavioral experiments.

Chapter I compares the response kinetics of the different chemoreceptor families in *Drosophila* to determine how the olfactory system has evolved to detect the diverse odor world. I found that OSNs expressing IRs are less sensitive and require longer stimulus durations to elicit a response, while OSNs expressing ORs are more sensitive to brief odor stimuli and their response dynamics changes with stimulus duration, unlike IR-OSNs. These dynamics are also dependent on response polarity (i.e. excitation or inhibition). Chapter II introduces the method for a drug delivery system we developed to inject pharmaceuticals into antennal sensilla to enable us to study the role of intracellular signaling in *Drosophila* olfaction *in vivo*. Our results show that injection of agents that mimic intracellular signaling modulate the response dynamics accordingly. In chapter III we investigate the functionality of Orco as ion channels when expressed alone and we show that its sensitivity to cAMP is regulated by phosphorylation via PKC. We further supplement the result using microinjection of specific pharmaceuticals into sensilla, such as inhibition of PKC reduced the OSNs response. In Chapter IV, we used the microinjection technique (Chapter II) and the results from Chapter III to investigate the role of intracellular signaling in/on OSN response dynamics. Our results show that pre-exposure of OSNs expressing ORs to low dose odorant stimulation sensitizes the response to subsequent stimulations, but this modulation is not present in IR-expressing OSNs. Injection of agents that disrupt the intracellular signaling cascade, demonstrate that sensitization is dependent on intracellular signaling. Specifically, inhibition of cAMP production or genetic modification of Orco phosphorylation sites abolish the sensitization in subsequent stimulations. In Chapter V, I attempt to determine the underlying molecular, structural and biochemical mechanisms that determine the response kinetics observed in Chapters I-IV. I show that odorant stimulation

activates PKC-mediated intracellular signaling and modulates the OSN's sensitivity and its response to brief, intermittent stimuli. I find that mutation of PKC significantly affects both the sensitivity and speed of odor reception and behavioral response. Our results indicate that signal transduction in ORs involves both metabotropic and ionotropic signaling. I thus hypothesize that this complex signaling mechanism evolved to optimize sensitivity and speed in insect olfaction, which could be useful for plume following by flying insects. Finally, in my general discussion, I discuss the relevance of my thesis work to the understanding of insect olfaction in general, including limitations of my approaches and directions for future studies.

Overviews of manuscripts

Chapter I

Temporal Response dynamics of Drosophila OSNs is receptor type and response polarity dependent

Merid N Getahun, Dieter Wicher, Bill S Hansson and Shannon Olsson.

Published: (2012) Front. Cell.Neurosci. 6:54.

In this manuscript, we compared the response kinetics of the different chemoreceptor families in *Drosophila* to determine how the olfactory system has evolved to detect the divers odor world.we found that odor sensory neurons (OSNs) expressing ionotropic receptors (IRs) are less sensitive, and require longer stimulus durations to elicit a response, while OSNs expressing odorant receptors (ORs) are more sensitive to brief odor stimuli and their response dynamics changes with stimulus duration, unlike IR-OSNs. Furthermore, we found that the response dynamics is dependent on response polarity (i.e. excitation or inhibition)

The study was conceived and designed together with Bill S Hansson, Dieter Wicher and Shannon Olsson who participated at all stages. I executed the experiments described in the manuscript (100 %), analyzed the data (75 %) and wrote the manuscript (80 %). The manuscript was refined in consultation with the authors of the paper.

Chapter II

Title: Piezo controlled microinjection: An in vivo complement for in vitro sensory studies in insects.

Shannon B. Olsson, Merid Negash Getahun, Dieter Wicher and Bill S Hansson

Published (2011) Journal of Neuroscience Methods 201:385-389

This manuscript is about the overviews the methodology for a drug delivery system we developed to inject pharmaceuticals into antennal sensilla to enable us to study the role of intracellular signaling in *Drosophila* olfaction *in vivo*. Our results show that injection of agents that mimic intracellular signaling modulate the response dynamics accordingly.

The study was conceived and designed together with Bill S Hansson, Dieter Wicher and Shannon Olsson who participated at all stages. I executed the experiments described in the

manuscript (60 %), analyzed the data (50 %). The manuscript was refined in consultation with the authors of the paper.

Chapter III

Title: Phosphorylation via PKC regulates the function of the Drosophila odorant co-receptor

Vardanush Sargsyan, Merid Negash Getahun, Sofía Lavista Llanos, Shannon B. Olsson, Bill S. Hansson and Dieter Wicher

In chapter III we investigate the functionality of Orco as ion channels when expressed alone and we show that its sensitivity to cAMP is regulated by phosphorylation via PKC. We further supplement the result using microinjection of pharmaceuticals into sensilla where inhibition of PKC activation has reduced the OSNs response. The study was conceived and designed together Vardanush Sargsyan, Sofía Lavista-Llanos, Shannon B. Olsson, Bill S. Hansson and Dieter Wicher. I executed the experiments described in the manuscript (15 %), analyzed the data (15 %). The manuscript was refined in consultation with the authors of the paper.

Published (2011) Front Cell Neurosci 5: 5

Chapter IV

Title: Insect Odorant Response Sensitivity Is Tuned by Metabotropically Autoregulated Olfactory Receptors

Merid N. Getahun, Shannon B. Olsson, Sofia Lavista-Llanos, Bill S. Hansson., Dieter Wicher.
Published: 2013 PLoS One 8(3):

In this manuscript, I use the microinjection technique to investigate the role of intracellular signaling in OSN response dynamics. My results indicate that pre-exposure of OSNs expressing ORs to low dose odorant stimulation sensitizes the response to subsequent stimulations, but this modulation is not present in IR-expressing OSNs. By injecting agents that disrupt the intracellular signaling cascade, I found that this sensitization is dependent on intracellular signaling. Specifically, inhibition of cAMP or genetic modification of Orco phosphorylation sites abolished the sensitization in subsequent stimulation.

The study was conceived and designed together with, Bill S. Hansson, Dieter Wicher, Shannon Olsson and Sofia Lavista-Llanos, who participated at all stages. I executed the experiments described in the manuscript (85 %) analyzed the data (50 %). The manuscript was refined in consultation with the authors of the paper.

Chapter V

The molecular basis for temporal resolution by insect olfactory receptors and its impact on odor-guided behavior

Merid N. Getahun, Michael Thoma, Sofia Lavista-Llanos, Markus Knaden, Shannon B. Olsson, Dieter Wicher and Bill S. Hansson.

Under review

In this manuscript, I attempt to determine the underlying biochemical mechansisms that determine the response kinetics observed in Chapters I-III. I show that odorant stimulation activate diverse intracellular signaling that are involved in modulating the OSNs response to optimize its sensitivity and fast odorant response. Our results depict signal transduction in ORs is much more complicated than currently thought, i.e simple ligand gated. Interestingly, a single genetic mutation of the intracellular signaling molecule can alter the sensitivity of OR-expressing OSNs to make them functionally similar to IR-expressing OSNs. However, the genetic alterations effecting ORs did not affect the IR response, and the transduction was independent of neuronal environment.

The study was conceived and designed together with Bill, S. Hansson, Dieter Wicher, Shannon Olsson, Sofia Lavista-Llanos, Thoma Michael, Markus Knaden, who participated at all stages. I executed the experiments described in the manuscript (90 %), analyzed the data (90 %) and wrote the manuscript (90 %). The manuscript was refined in consultation with the authors of the paper.

Chapter I

Temporal response dynamics of *Drosophila* OSNs is receptor type and response polarity dependent

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Temporal response dynamics of *Drosophila* olfactory sensory neurons depends on receptor type and response polarity

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Insect olfactory sensory neurons (OSN) express a diverse array of receptors from different protein families, i.e. ionotropic receptors (IR), gustatory receptors (GR) and odorant receptors (OR). It is well known that insects are exposed to a plethora of odor molecules that vary widely in both space and time under turbulent natural conditions. In addition to divergent ligand specificities, these different receptors might also provide an increased range of temporal dynamics and sensitivities for the olfactory system. To test this, we challenged different *Drosophila* OSNs with both varying stimulus durations (10–2000 ms), and repeated stimulus pulses of key ligands at various frequencies (1–10 Hz). Our results show that OR-expressing OSNs responded faster and with higher sensitivity to short stimulations as compared to IR- and Gr21a-expressing OSNs. In addition, OR-expressing OSNs could respond to repeated stimulations of excitatory ligands up to 5 Hz, while IR-expressing OSNs required ∼5x longer stimulations and/or higher concentrations to respond to similar stimulus durations and frequencies. Nevertheless, IR-expressing OSNs did not exhibit adaptation to longer stimulations, unlike OR- and Gr21a-OSNs. Both OR- and IR-expressing OSNs were also unable to resolve repeated pulses of inhibitory ligands as fast as excitatory ligands. These differences were independent of the peri-receptor environment in which the receptors were expressed and suggest that the receptor expressed by a given OSN affects both its sensitivity and its response to transient, intermittent chemical stimuli. OR-expressing OSNs are better at resolving low dose, intermittent stimuli, while IR-expressing OSNs respond more accurately to long-lasting odor pulses. This diversity increases the capacity of the insect olfactory system to respond to the diverse spatiotemporal signals in the natural environment.

Keywords: odorant receptors, ionotropic receptors, pulse resolution, single sensillum recording

INTRODUCTION

Insect olfactory sensory neurons (OSN) express a large number of receptor proteins of different types. These receptor types include ionotropic receptors (IR), gustatory receptors (GR), and odorant receptors (OR) (Clyne et al., 1999; Vosshall et al., 1999; Benton et al., 2009). IRs are composed of three trans-membrane proteins and co-receptors, while GRs and ORs are seven trans-membrane proteins (Vosshall et al., 1999; Benton et al., 2006, 2009). ORs are co-expressed with the ubiquitous co-receptor Orco, while Gr21a, a CO_2 sensor, is co-expressed with Gr63a (Benton et al., 2006; Jones et al., 2007). All OSNs are housed within different morphological types of olfactory hairs, known as sensilla. There appear to be important organizational differences between OSNs that express IRs, GRs, or ORs. Multiple IRs and GRs can be co-expressed per neuron, while OR expression generally follows a one neuron-one receptor rule (Thorne et al., 2004; Wang et al., 2004; Couto et al., 2005; Benton et al., 2009). Receptors from different protein families can also be co-localized in the same sensillum (Couto et al., 2005; Song et al., 2012). For example, in *Drosophila*, the ab1 sensillum houses four OSNs, three expressing ORs and one expressing Gr21a. Also, in the *Drosophila* coeloconic sensillum ac3 an OSN expressing Or35a is co-localized with an OSN expressing Ir75abc (Yao et al., 2005; Silbering et al., 2011).

These diverse receptors have evolved at different points in evolutionary time (Robertson et al., 2003; Croset et al., 2010). Recent research also suggests that many have broad affinity to different chemical classes (Hallem et al., 2004; Yao et al., 2005; Benton et al., 2009; Ai et al., 2010). Yet specificity might not be the only reason for receptor diversification. In the natural environment, insects are constantly challenged with odors not only of diverse molecular types, but with diverse spatio-temporal dynamics. At some distances, odor plumes can present brief and intermittent stimuli (Kaissling et al., 1987; Vickers et al., 2001) with low molecular flux, while at close range or high molecular flux, odors could present a nearly continuous stimulus (Murlis et al., 2000; Louis et al., 2008; Gomez-Marin et al., 2011). These spatiotemporal factors could also be a significant driving force for diversification. The behavior of an insect is a result of the integration of responses from several OSNs expressing a variety of receptor

types (Silbering et al., 2011). Thus it is worthwhile to characterize the response dynamics across the OSN repertoire.

To address whether these different receptor types exhibit differences in temporal response kinetics, we assess the response dynamics of *Drosophila* OSNs expressing various receptor types to both different stimulus durations and frequencies. We evaluate the temporal dynamics of antennal OSNs expressing ORs (Or59b and Or35a), IRs (Ir84a, Ir75abc, and Ir41a), and GRs (Gr21a). Or59b-OSNs and Ir41a-OSNs respond with either excitation or inhibition to different ligands, and were chosen to assess the effect of response polarity on temporal kinetics. Or35a- and Ir75abc-OSNs are housed in the same sensillum, and are tested to control for the effects of the perireceptor environment on the temporal response. Finally, Gr21a-expressing OSNs are the only GR-expressing OSNs found on the antenna. Here we show that sensory neurons expressing receptors from different protein families also exhibit different dynamics to brief and intermittent stimuli.

MATERIALS AND METHODS

Both male and female flies at 2–6 days of age were used. Stocks were maintained on conventional cornmeal agar medium under a 12 h light: 12 h dark cycle at 25°C.

ELECTROPHYSIOLOGY

A fly was mounted in a cut pipette tip with the head protruding and small amount of wax placed into the tip end to prevent movement. The pipette was then fixed onto a microscope slide with wax and the antennae fixed on a cover slip with a sharpened glass micropipette, similar to (Hallem et al., 2004; Yao et al., 2005; Pellegrino et al., 2010). An electrolytically sharpened tungsten electrode was placed in the eye for grounding and a sharpened tungsten recording electrode was brought into contact with the base of the sensillum using a Luigs and Neumann, SM-59 manipulator (Ratingen, Germany) at 1000× magnification with an Olympus BX-51 microscope (Olympus Corporation, Tokyo, Japan).

ODOR STIMULI

Methyl acetate (>98%), citral (>95%), phenyl acetaldehyde (>90%), butyric acid (>99%), 1, 4-diaminobutane and isoamylamine (>98%), 1-hexanol (>99%), and ethyl hexanoate (>99%) were purchased from Sigma Aldrich Germany. Phenyl acetaldehyde, methyl acetate, 1-hexanol and ethyl hexanoate were diluted in mineral oil (BioChemika Ultra, Fluka), and butyric acid, 1, 4-diaminobutane, and isoamylamine were dissolved in water. Citral was dissolved in hexane (>99%, Fluka Analytical, Buchs, Switzerland). We chose odor concentrations within the linear portion of the dose response curve and the tested concentrations are indicated with circles (**Figure 1**). All concentrations are reported as log [odor] v/v. For Gr21a stimulation, a 1.5 ml glass vial was filled with pure CO_2 and placed into the stimulus system similar to the other stimuli. After each frequency set (1–10 Hz), the CO_2 was refilled.

For frequency stimulation, we used a custom-built multicomponent stimulus system similar to (Olsson et al., 2011). Briefly, 400 ul of appropriate dilutions of each odorant was added

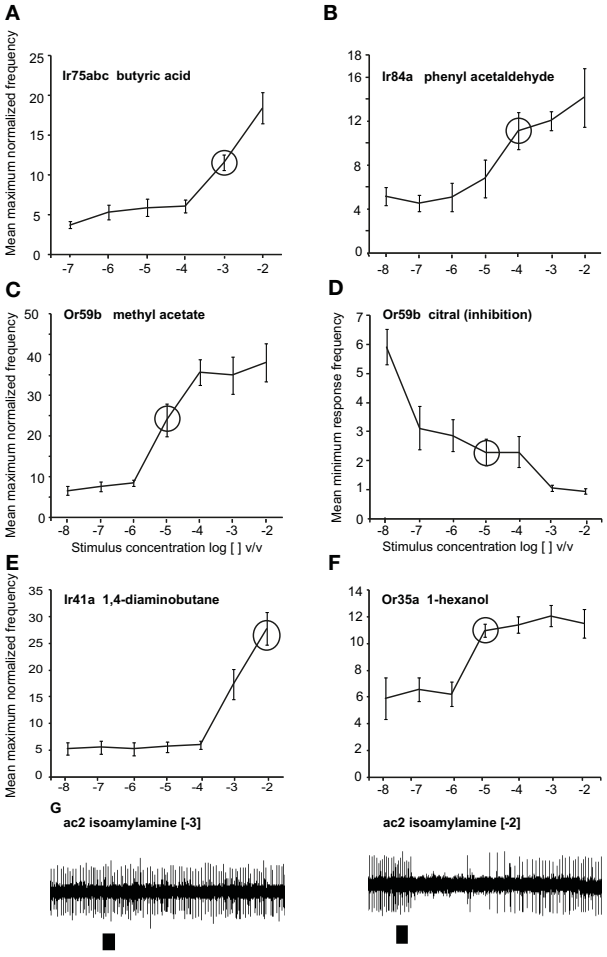

FIGURE 1 | Responses to odors at different doses. Dose-response curves presented as normalized maximum frequency response for **(A)** Ir75abc-expressing neurons to butyric acid $n = 8$–13 **(B)** Ir84a-expressing neurons to phenylacetaldehyde, $n = 9$–12. **(C)**, Or59b-expressing neurons to methyl acetate, $n = 8$–17 **(D)** Or59b-expressing neurons to citral presented as the minimum frequency, $n = 6$–10. **(E)** Ir41a-expressing neurons to 1, 4-diaminobutane $n = 6$–8 **(F)**. Or35a-expressing OSNs to 1-hexanol, $n = 6$–8. **(G)** Representative traces showing the response of OSNs of ac2 sensilla to isoamylamine at two different concentrations (responses to lower concentrations were not observed). Please note that while only Ir41a-expressing neurons are excited by 1, 4-diaminobutane in this sensillum (ac2), all neurons are inhibited by isoamylamine, and we thus label the inhibitory responses with the entire sensillum label.

to an Eppendorf tube and placed in the bottom of a PEEK vial (4.6 cm × 2.5 cm × 2.5 cm dimensions). Each vial was sealed with a stainless steel plug (Olsson et al., 2011). The pulse duration, inter-stimulus interval and number of pulses were adjusted through a custom built Labview program (Olsson et al., 2011). The odors were delivered from the headspace via Teflon tubing 150 cm long with an inner diameter of 1 mm and positioned as close as possible (∼1.5 cm) to the antennae. The flow rate of air was 0.5 L/min. For stimulation, the stimulus system was connected to the IDAC (Syntech, Ockenfels, Germany) and through USB connection to a PC. Stimulation was controlled by an OEM (EDP 0504, thinXXS) pump control system and DAQ (USB 6008

data acquisition hardware, National Instruments, Austin, TX, USA) with custom-built Labview 8.5 software (built by Daniel Veit; National Instruments). For frequency stimulation the on time was 50 ms for OR- and Gr21a-OSNs and the off time was adjusted from 950 ms or 50 ms for 1–10 Hz, respectively. For IR-OSNs stimulated with [−4] and [−3] stimulus concentrations, the pump on time was 200 ms and off time 800, 300, or 50 ms for 1–4 Hz, respectively. At [−2], the protocol was identical to the OR-OSNs and Gr21a-OSNs. The consistency of odor delivery for different pulse durations and frequencies was confirmed using PID (200a, Aurora Scientific Ontario, Canada).

DATA ANALYSIS

All raw spike data were acquired and converted to digital spikes using Autospike 3.7 (Syntech). Co-localized neurons were identified based on spike amplitude. Peri-stimulus time histograms (PSTHs) were obtained by averaging spike activities in 25 ms bins from the start of the stimulation and normalized to the average frequency for 2 s before stimulation (Olsson et al., 2011; Sargsyan et al., 2011). The OSN responses between consecutive pulses were compared using repeated measure ANOVA by assessing the normalized mean of area under curve (AUC) spike frequency per each stimulus duration, i.e. pump on time + off time. Consecutive pulses were normalized to the response of 1st pulse. Between treatments, a Mann-Whitney U test or *t*-test was used depending on the normality of the data. To evaluate the capacity of receptors to resolve pulsed stimuli, we visualized the response using normalized peri-stimulus histograms and quantified the % return to the spontaneous activity (baseline), using the ratio between the first value in the 2nd pulse and the maximum peak value of the first (previous) pulse converted to a percentage: Percent return to baseline = 1−(1st value of the 2nd pulse/maximum frequency of the 1st pulse) × 100 (Bau et al., 2002). A One-Way ANOVA followed by a Tukey *post-hoc* test was performed to determine if the return to baseline was significantly reduced between the different stimulation frequencies. Latency was measured as the time from the onset of the odor stimulus to the maximum response frequency (mechanical delay was not considered). Response width was calculated as the time between half-maximal response for excitation and half-minimal response in the case of inhibition. Spearman's correlation was used to assess the relationship between repeated pulses and latency as well as between response width and intensity with stimulus duration. All analyses were performed using SPSS version 17 (IBM Corporation, Armonk, New York, US).

RESULTS

RESPONSE DYNAMICS OF DIFFERENT SENSORY NEURONS TO VARYING STIMULUS DURATIONS

We first assessed the response of OSNs carrying ORs, Gr21a, or IRs to key ligands presented with varying stimulus durations at concentrations found in the linear portion of the dose-response curve for each OSN (**Figure 1**). OSNs expressing Or59b housed in basiconic sensillum type ab2 were stimulated with methyl acetate at [−5] concentration, with stimulus durations varying from 10 ms to 2 s. At 20 ms, the mean normalized frequency of Or59b-expressing OSNs was greater than the spontaneous activity ($t = 3.482$, $P = 0.005$), indicating that a 20 ms stimulation was sufficient to elicit a response (**Figure 2A** asterisk right). A maximal stimulus response was obtained with a 50 ms stimulation ($P < 0.05$), however, stimulations of 1 s or more significantly reduced the OSN response maximum ($t = 3.482$, $P = 0.005$, mean normalized maximum frequency for 500 ms vs. 1 s stimulation and $t = 5.047$, $P < 0.001$ for 500 ms vs. 2 s, Student's *t*-test). Similar response dynamics were observed in Or35a-expressing OSNs ($t = 5.007$, $P < 0.001$ mean normalized maximum frequency for 500 ms vs. 2 s stimulation; **Figure 2B**). Adaptation to long stimulus durations (>1 s) was also apparent for Or22a-OSNs (data not shown). There was also a positive and significant correlation between response width at half-maximal response and stimulus duration for both OR-expressing OSNs ($r = 0.853$, $P < 0.001$ for Or59b-OSNs and $r = 0.93$, $P < 0.001$ for Or35a-OSNs; both Spearman's correlation **Figures 2A,B** left panels).

OSN expressing Ir84a (**Figure 2C**) were stimulated with [−4] phenyl acetaldehyde and a significant response was obtained at 100 ms ($P < 0.001$, Mann–Whitney *U*-test; **Figure 2B**). A maximal response was reached at 500 ms ($P = 0.001$, Mann–Whitney *U*-test, as compared to 100 ms), and the maximum response intensity did not decrease at longer stimulation durations ($t = 0.605$, $P = 0.554$ at 500 ms stimulation vs. 1 s, and $t = 0.394$, $P = 0.699$ for 500 ms vs. 2 s; Student's *t*-test). The response of Ir75abc-expressing neurons was similar when stimulated with [−3] butyric acid, (significant response at 100 ms; Mann–Whitney U, $P = 0.016$, **Figure 2D**), and reached a maximum response at 500 ms ($t = 2.286$, $P = 0.036$ compared to 100 ms). Furthermore, the response did not change at longer stimulus durations ($t = 0.096$, $P = 0.924$, 500 ms vs. 1 s, $t = 0.068$, $P = 0.946$, 500 ms vs. 2 s; **Figure 2D** right panel). There was also a positive and significant correlation between stimulus duration and response width at half maximal response ($r = 0.905$, $P < 0.001$ for Ir84a-OSNs, and $r = 0.917$, $P < 0.001$ for Ir75abc-OSNs, Spearman's correlation; **Figures 2C** and **D** left panel). Similarly, the Ir41a-OSN response to 1,4-diaminobutane at [−2] did not show adaptation at longer stimulus durations ($t = 0.073$, $P = 0.944$ for 500 ms vs. 1 s stimulations; $t = 0.01$, $P = 0.992$ for 500 ms vs. 2 s stimulations).

OSNs expressing Gr21a, which are housed in ab1 sensilla on the *Drosophila* antenna, respond to pure CO_2 beginning at a 20 ms stimulation (Mann–Whitney *U*-test, $P = 0.009$ **Figure 2E**). Peak response was obtained at 1 s ($t = 4.641$, $P = 0.002$, Student's *t*-test compared to 20 ms), while at a 2 s stimulation the maximum response frequency decreased significantly ($t = 2.63$, $P = 0.02$, Student's *t*-test, 1 s vs. 2 s). However, the response latency also became shorter with stimulus duration, decreasing from the 20 ms duration (with a mean half-maximal response on set time of 400 ± 26.35 ms), to 1 s (with a mean half maximal response on set time 300 ± 17.67 ms, $t = 3.028$, $P = 0.016$, Student's *t*-test; **Figure 2E** left panel). This is opposite to both OR- and IR-expressing OSNs, where there was no difference (**Figures 2A–D**). Similarly, the response width also increased with stimulus duration ($r = 0.781$, $P < 0.001$, Spearman's correlation, **Figure 2E** left panel).

Increasing stimulus concentrations reduced the duration required to elicit a response regardless of the receptor expressed.

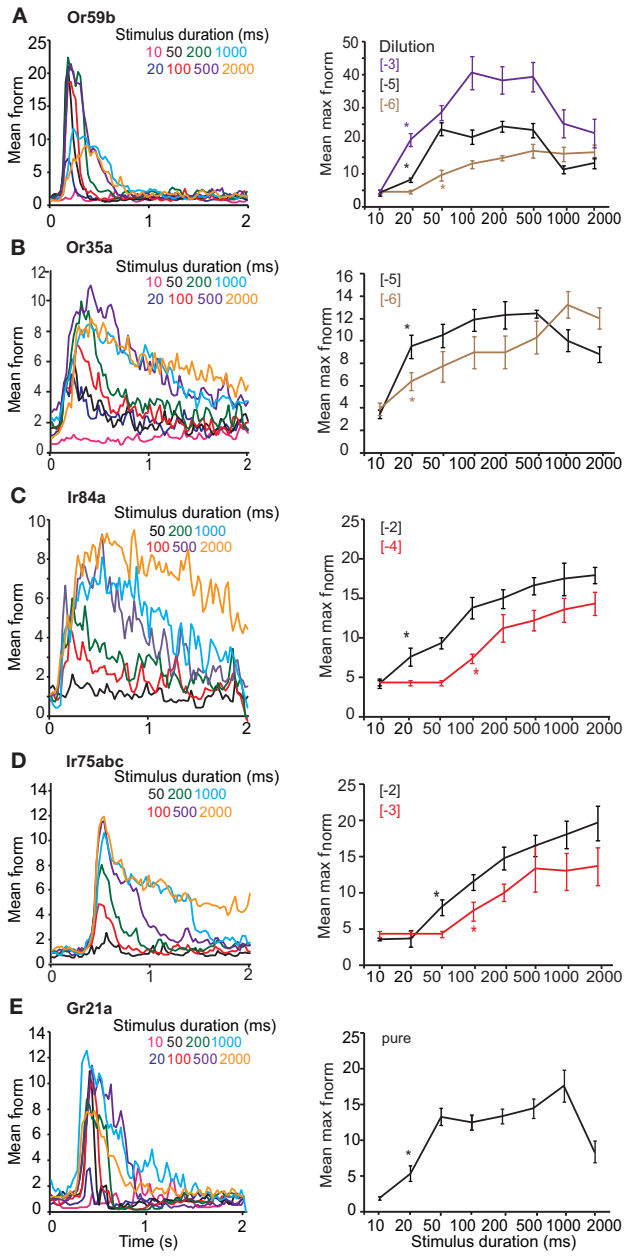

FIGURE 2 | Response of OSNs to varying stimulus durations. (**A**, left) Mean peri-stimulus time histograms (PSTHs, 25 ms bins) showing the response of Or59b-expressing OSNs to various stimulus durations of log [−5] v/v methyl acetate. (**A**, right) Mean normalized maximum frequency for Or59b-expressing neurons plotted vs. stimulus duration ($n = 8$–15) for three different concentrations. Asterisks indicate the minimum stimulus duration that elicited a significant response, $P < 0.05$. (**B**, left) Mean peri-stimulus time histograms as in **(A)** showing the response of Or35a-expressing OSNs to various stimulus durations of log [−5] v/v 1-hexanol. (**B**, right) Mean normalized maximum frequency for Or35a-expressing neurons plotted versus stimulus duration for log [−5] and [−6] v/v of 1-hexanol ($n = 6$–14). (**C**, left) Response of Ir84a-expressing neurons to various durations of log [−4] v/v phenyl acetaldehyde as in **(A)**, $n = 8$–10 (**C**, right) as in A for two different concentrations. (**D**, left) Response of Ir75abc-expressing neurons to various durations of log [−3] v/v butyric acid ($n = 6$–15) and **(D**, right) as in **(C)**. **(E)** Response of Gr21a-expressing neurons to pure CO_2 at different stimulus durations ($n = 6$–10).

For example, Or59b-OSNs required 50 ms at [−6] to elicit a significant response ($t = 2.486$, $P = 0.025$; **Figure 1A** right), but only 20 ms at [−3] ($P < 0.001$, Mann–Whitney *U*-test, asterisk in **Figure 2A** right). Similarly, Ir84a-expressing OSNs stimulated with phenyl acetaldehyde at [−2] required only 20 ms to elicit a significant response (Mann–Whitney U, $P = 0.02$, **Figure 2C**), while Ir75abc-expressing OSNs required a 50 ms stimulation when the concentration of butyric acid increased by 10× [−2] (Mann–Whitney U, $P = 0.002$, **Figure 2D**, asterisk right).

However, the dose-dependency of OSN adaptation to long stimulus durations was dependent on the receptor expressed. At [−6] long stimulus durations did not reduce the response of Or59b-expressing OSNs ($t = 0.292$, $P = 0.776$ for 500 ms vs. 1 s; $t = 0.33$, $P = 0.745$ for 500 ms vs. 2 s) or Or35a-expressing OSNs ($t = 1.151$, $P = 0.147$ for 500 ms vs. 1 s; $t = 0.948$, $P = 0.356$ for 500 ms vs. 2 s; **Figures 2A,B** right). However, at [−3] concentration, stimulations of 1 s or more significantly reduced the Or59b-expressing OSN response maximum ($t = 2.235$, $P = 0.045$ for 500 ms vs. 1 s; $t = 2.658$, $P = 0.021$ for 500 ms vs. 2 s, **Figure 2A**). In contrast, longer stimulus durations did not reduce the response of IR-expressing OSNs regardless of concentration (Ir84a-expressing OSNs at [−2]: Mann–Whitney U, $P = 0.847$ for 500 ms vs. 1 s; Ir75abc-expressing OSNs at [−2]: $t = 0.644$, $P = 0.531$ for 500 ms vs. 1 s; Ir41a-expressing OSNs at [−2], $t = 0.073$, $P = 0.944$ for 500 ms vs. 1 s; **Figures 2C,D** right panels).

PULSE RESOLUTION OF DIFFERENT SENSORY NEURONS

After investigating the response of OSNs to various stimulus durations, we presented the neurons with repeated stimulations of varying frequency. The latency to repeated stimulations at 1 Hz increased for all OSN types ($r = 0.742$, $P < 0.001$ for Or59b-OSNs; $r = 0.94$, $P < 0.001$ for Gr21a-OSNs; $r = 0.787$, $P < 0.001$ for Ir75abc-OSNs; $r = 0.652$, $P < 0.001$ for Ir84a-OSNs; Spearman's correlation; **Figures 3A–E**). However, a variability in latency was observed between the tested OSNs; e.g., Ir75abc-OSNs showed more delayed time to maximum than all other neurons tested, $P < 0.001$, ANOVA followed by Tukey *post-hoc* test (**Figure 3E**). At 100× stimulus concentrations or a 5 s interstimulus interval, the latency for Or59b-expressing OSNs did not change with repeated stimulation ($r = 0.09$, $P = 0.475$; $r = -0.006$, $P = 0.952$, respectively, Spearmans's correlation; **Figure 3F**). Similarly, Ir75abc-expressing OSN response onset recovered with a higher concentration ($r = 0.01$, $P = 0.90$, Spearmans's correlation). However, at 5 s interstimulus intervals the response onset became significantly faster for the later pulses ($r = -0.885$, $P < 0.001$, Spearmans's correlation; **Figure 3G**). The latency also decreased with subsequent stimulations of CO_2 for Gr21a-expressing OSNs at 5 s interstimulus intervals ($r = -0.976$, $P < 0.001$, Spearmans's correlation; **Figure 3H**). In summary, this shows that changes in response onset kinetics to repeated stimuli are similar across all tested OSNs and response latencies can be regulated either by altering stimulus concentrations or inter-stimulus intrervals.

Ir84a and Ir75abc-OSNs, housed in ac4 and ac3 sensilla respectively, could resolve repeated 200 ms pulses of [−4] and [−3] stimulus concentrations, respectively, up to 4 Hz (the maximum testable frequency due to stimulation length). The mean

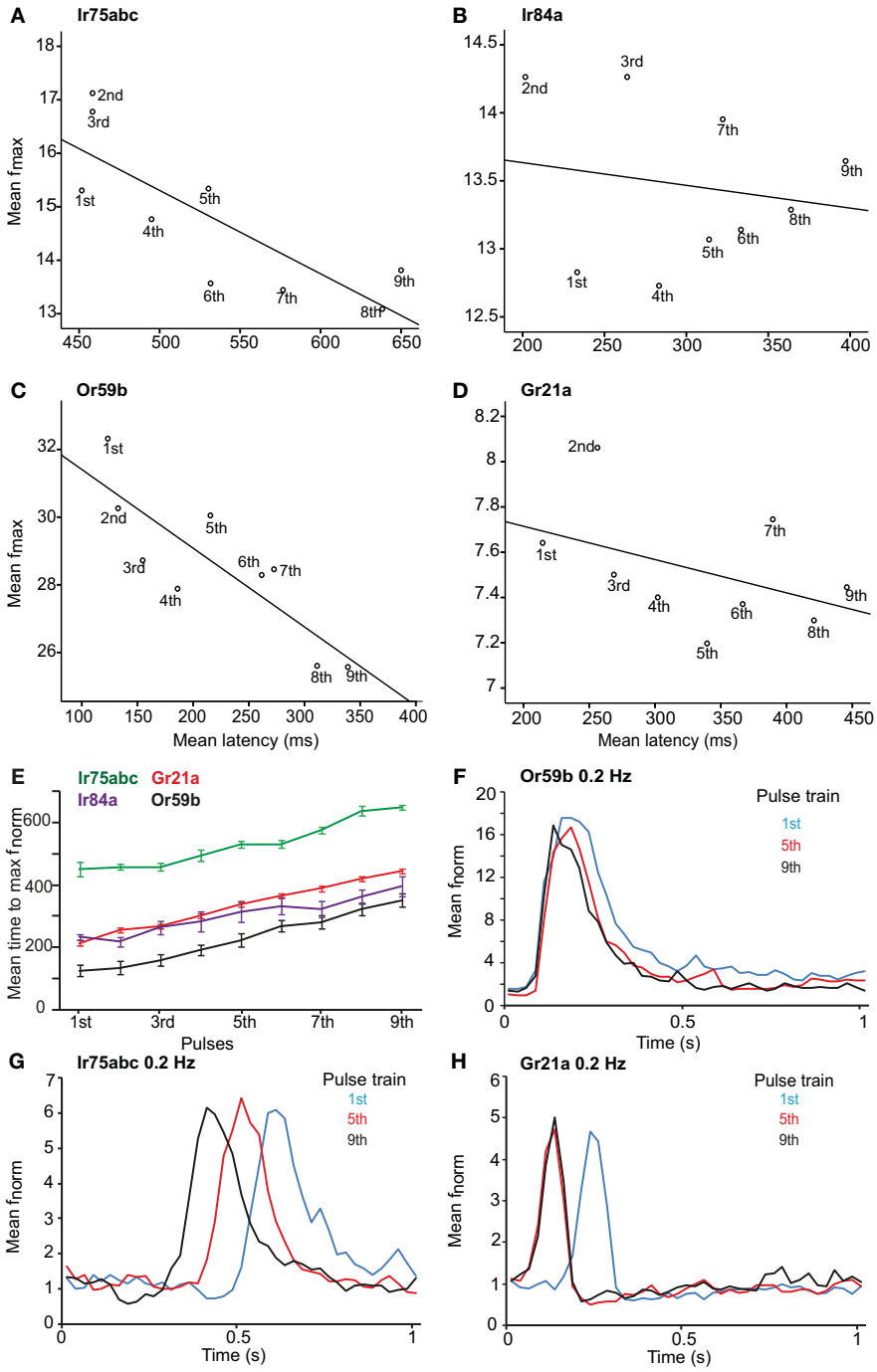

FIGURE 3 | Latency and maximum response of OSNs to repeated stimuli. Maximum response frequency vs. time to peak (latency), with best fit line, for OSNs carrying various receptors in response to repeated 1 Hz stimulations. **(A)** Ir75abc, **(B)** Ir84a, **(C)** Or59b and **(D)** Gr21a. Pulse number (1–9) indicated below each point. **(E)** Mean time to maximum response frequency for neurons in **(A–D)** at a 1 Hz repeated stimulation. **(F)** The response onset recovery of Or59b-expressing OSNs when stimulated at 0.2 Hz, $n = 10$ **(G)** as in **(F)** for Ir75abc-expressing OSNs, $n = 7$ and **(H)** as in **(F)** for Gr21a-expressing OSNs, $n = 10$.

return to base line during repeated stimulation was significantly reduced at 4 Hz as compared to 1 and 2 Hz stimulation, ($P < 0.05$ ANOVA followed by Tukey *post-hoc* test; **Figures 4A,B**). At an increased concentration of [−2], Ir75abc OSNs could resolve pulsed stimuli up to 5 Hz at a 50 ms pulse duration (**Figure 4C**).

Gr21a-expressing OSNs housed in ab1 sensilla resolved intermittent pulses of CO_2 as fast as 8 Hz with no significant difference in return to baseline between 1 Hz and 5 Hz stimulations. At 8 Hz, the mean return to base line was significantly reduced, and at 10 Hz only 2.4% recovery to the base line occurred

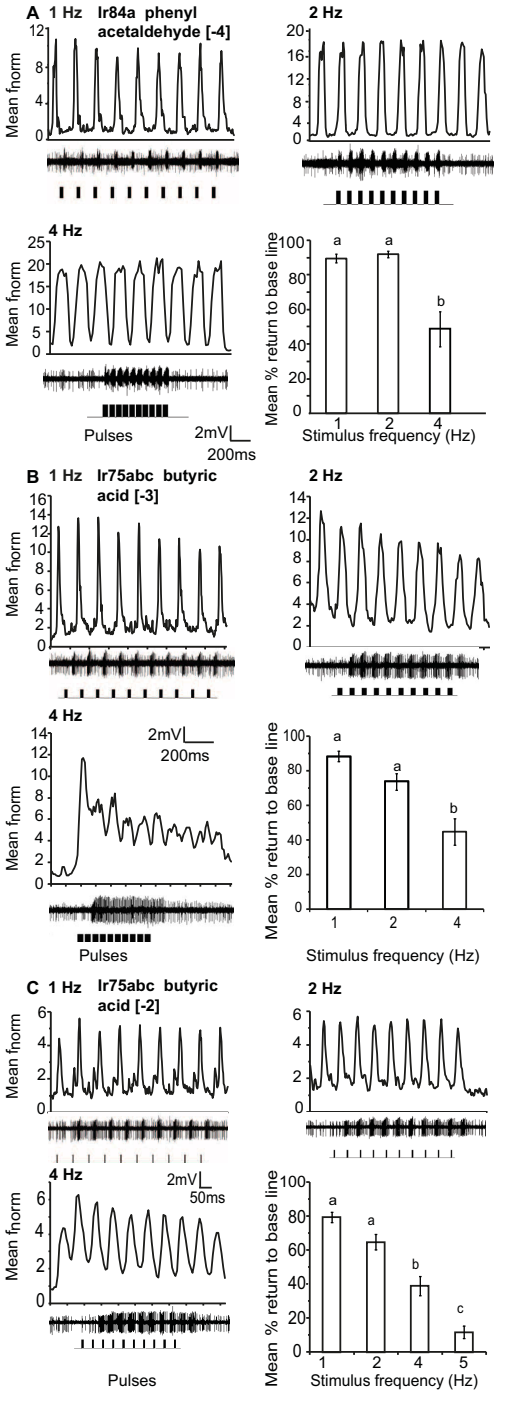

FIGURE 4 | Response of OSNs to repeated stimulus pulses at varying frequencies. (A) Average normalized PSTH responses for Ir84a-expressing neurons in response to repeated pulses of log [−4] v/v phenyl acetaldehyde at listed frequencies. Traces below each panel show sample 200 ms recordings. Square pulses indicate stimulus presentation. The final panel shows the mean percent return to base line across all pulses at listed frequencies; error bars indicate SEM (ANOVA, $P < 0.05$, followed by Tukey *post-hoc*, $n = 7$–9). **(B)** Response of Ir75abc-expressing neurons to repeated stimulations of log [−3] v/v butyric acid stimulation as in **(A)** (ANOVA, $P < 0.05$, followed by Tukey *post-hoc* ($n = 8$–10). **(C)** Response as in **(B)** to a 10× concentration of butyric acid (log [−2]); ANOVA, $P < 0.05$, followed by Tukey *post-hoc*, $n = 14$–15).

($P < 0.001$, ANOVA followed by Tukey *post-hoc* test; **Figures 5A** and **B**). Gr21a-expressing OSNs also exhibited short term adaptation based on AUC (see "Materials and methods") that was frequency dependent, i.e. at 1 Hz stimulation the 9th pulse resulted in a significantly reduced response compared to the 1st pulse (repeated measure ANOVA, $P < 0.001$), while at 2 Hz the 4th pulse was reduced ($P = 0.039$), at 4 Hz the 5th ($P = 0.001$), and at 5 and 8 Hz the 2nd ($P < 0.01$, repeated measure ANOVA; **Figure 5** asterisks).

PULSE RESOLUTION OF STIMULI ELICITING OPPOSITE RESPONSE POLARITY

We also tested the pulse following capacity to single excitatory and inhibitory odor ligands in Or59b-expressing OSNs. We applied [−5] methyl acetate as an excitatory and [−5] citral as an inhibitory ligand. Or59b-expressing OSNs could resolve the excitatory stimulus up to 5 Hz (**Figure 6A**). The mean return to base line was significantly reduced at 5 Hz stimulation as compared to 1 and 2 Hz ($P < 0.05$, ANOVA followed by Tukey *post-hoc* test; **Figure 6C**). However, the pulse resolution was also affected by concentration, as a 100× increase in concentration reduced the pulse resolution to 2 Hz ($P < 0.05$). In contrast to the excitatory responses, Or59b-cells were able to resolve pulses of the inhibitory ligand citral only up to 2 Hz, and at 4 and 5 Hz the OSNs showed total inhibition and did not recover when stimulated repeatedly with the inhibitory ligand ($P < 0.05$ ANOVA followed by

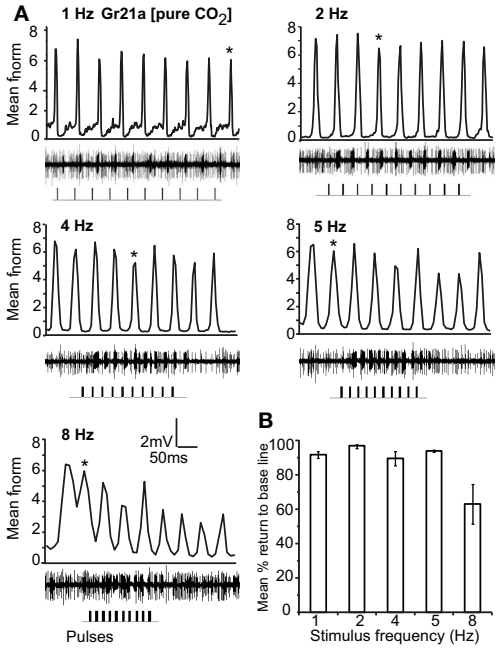

FIGURE 5 | Response of Gr21a-expressing OSNs to repeated stimulus pulses at varying frequencies. (A) Average normalized PSTH responses of Gr21a-expressing neurons to repeated pulses of pure CO_2 at listed frequencies. Traces below each panel show sample 50 ms recordings. Square pulses indicate stimulus presentation. **(B)** Mean percent return to base line across all pulses of listed frequencies, error bars indicate SEM (ANOVA, $P < 0.05$, followed by Tukey *post-hoc* test, $n = 11$–12).

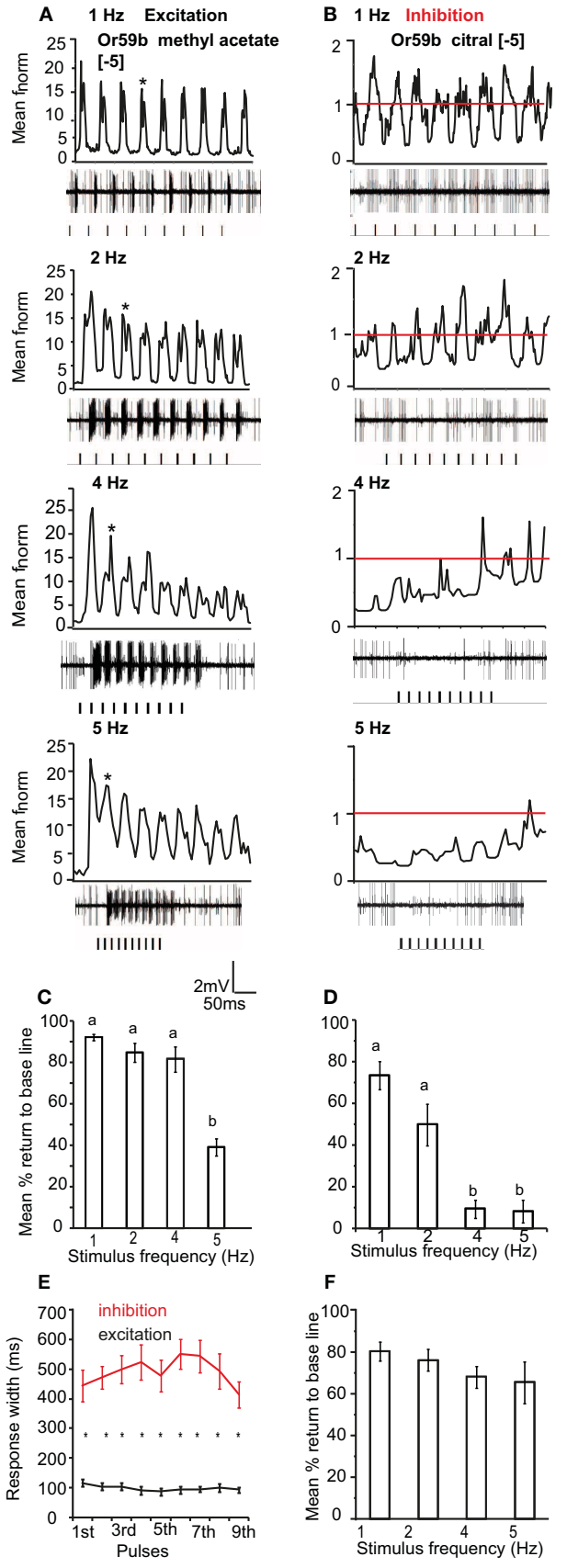

FIGURE 6 | Continued

FIGURE 6 | OR-expressing OSN response polarity and pulse resolution. **(A)** Mean normalized PSTH response of Or59b-expressing OSNs to repeated pulses of log [−5] v/v methyl acetate at listed frequencies. Traces below each panel show sample 50 ms recordings. Square pulses indicate stimulus presentation. **(B)** Mean normalized PSTH response of Or59b-expressing OSNs to repeated pulses of log [−5] v/v citral (an inhibitory odor) at listed frequencies as in **(A)**. Red line indicates baseline frequency. **(C)** Mean percent return to base line across all pulses for Or59b-OSN response to methyl acetate, error bars indicate SEM (ANOVA, $P < 0.05$, followed by Tukey *post-hoc* test, $n = 9$–13) and **(D)** as in **(C)** for citral (ANOVA, $P < 0.05$, followed by Tukey *post-hoc* test, $n = 13$–15). **(E)** Mean response width of Or59b-expressing OSNs for excitation and inhibition. **(F)** Mean percent return to base line in response to a pulsed binary mixture of methyl acetate and citral, error bars indicate SEM, ($P > 0.05$ ANOVA, $n = 8$–9).

Tukey *post-hoc* test; **Figures 6B** and **D**). The inhibitory ligand also resulted in a larger response width as compared to the excitatory ligand, even though both ligand concentrations were at similar points in the dose response curve (see **Figure 1**). This indicates that a given OSN response to an inhibitory or excitatory ligand can differ not only in polarity but also in temporal dynamics (**Figure 6E**). Furthermore, Or59b-OSNs showed short-term adaptation to the excitatory ligand that was frequency dependent (repeated measure ANOVA, $P < 0.05$). At increasing frequencies, short-term adaptation occurred earlier in the stimulus train (**Figure 6A** asterisk). In contrast, we did not find short-term adaptation based on response width to the inhibitory ligand (repeated measure ANOVA, $P > 0.05$).

We also asked if the total inhibition of the neuron at high frequencies of citral (>4 Hz) could interfere with odor coding of the excitatory ligand when presented simultaneously to the OSN. We thus stimulated the neurons with the binary mixture of the two ligands at the concentrations listed above. Stimulation with the two component blend resulted in an improved pulse resolution over either separate odor, with no significant difference in pulse resolution between 1 and 5 Hz ($P > 0.05$ ANOVA (**Figure 6F**). The effect of response polarity on pulse resolution was also observed in OSNs that express IRs. Ir41a-OSNs exhibited an excitatory response to 50 ms pulses of [−2] 1,4-diaminobutane and resolved pulsed stimuli as fast as 2 Hz, (ANOVA, $P < 0.05$; **Figures 7A** and **D**). However, the pulse resolution to the inhibitory ligand isoamylamine at [−2] (the concentration at which the neurons are inhibited by the ligand, **Figure 1G**) was only maintained at 1 Hz (ANOVA $P < 0.05$; **Figures 7B** and **E**). In addition, the binary mixture of 1, 4-diaminobutane and isoamylamine at the same concentration [−2], sharpened the response of Ir41a-OSNs especially at 4 Hz (**Figures 7C** and **F**).

DISCUSSION

Odor stimuli contain three elements of information: odor identity; odor intensity, and a temporal component (Hallem et al., 2004). To respond to these stimuli, insect OSNs express a wide variety of receptors. Here we investigate the response dynamics of OSNs expressing receptors from different protein families to stimuli of both different durations and frequencies. We find that ORs, IRs, and Gr21a exhibit distinct response characteristics that could increase the response range of the insect to the temporally dynamic natural odor environment.

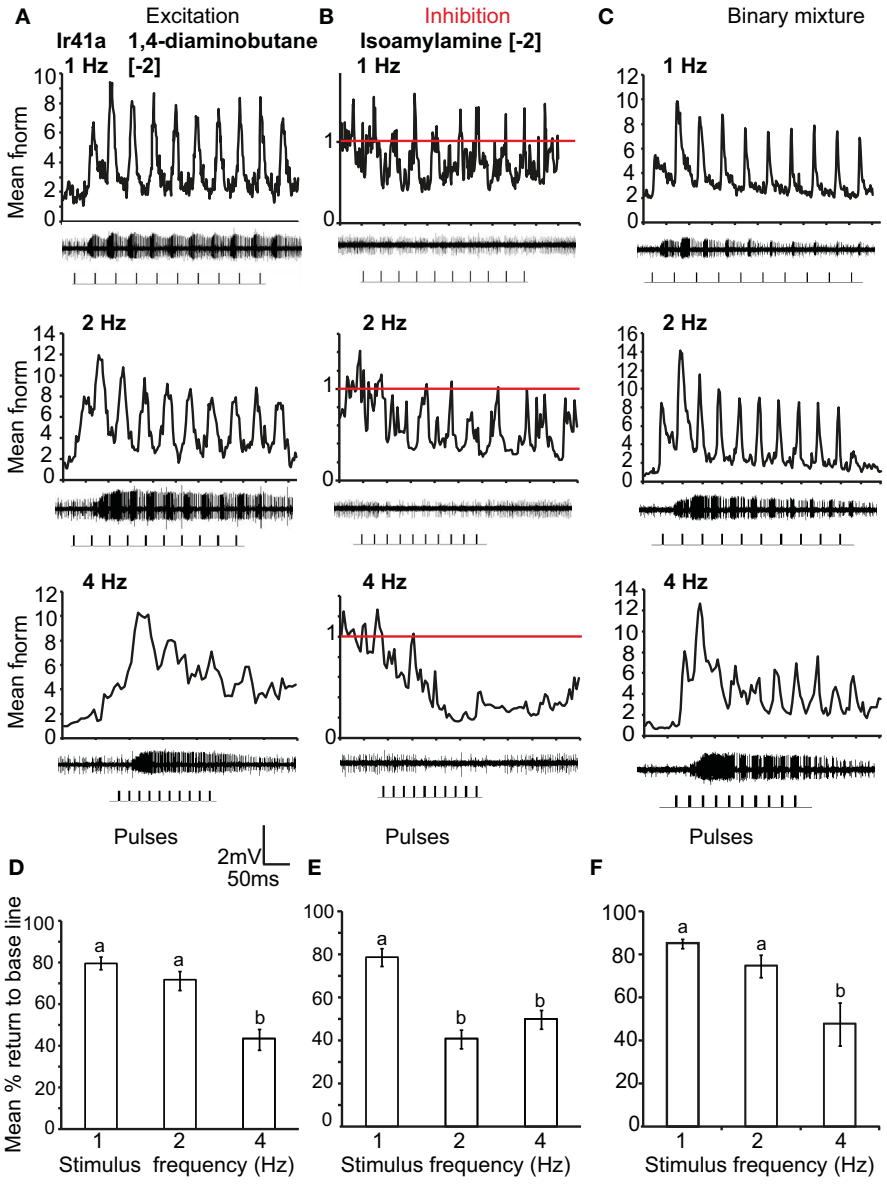

FIGURE 7 | IR-expressing OSN response polarity and pulse resolution. **(A)** Mean normalized PSTH response of Ir41a-expressing OSNs to repeated pulses of log [−2] v/v 1, 4-diaminobutane at listed frequencies. Traces below each panel show sample 50 ms recordings. Square pulses indicate stimulus presentation. **(B)** As in **(A)** for log [−2] v/v of the inhibitory odor isoamyl amine. Red line indicates baseline frequency. **(C)** Mean normalized PSTH response of Ir41a-expressing OSNs to a binary mixture of 1, 4-diaminobutane and isoamyl amine at [−2] v/v. **(D)** Mean percent return to base line to the excitatory ligand across all pulses at listed frequencies, error bars indicate SEM (ANOVA, $P < 0.05$, followed by Tukey *post-hoc* test, $n = 7$–8) **(E)**, as in **(D)** for log [−2] v/v of the inhibitory odor isoamyl amine (ANOVA, $P < 0.05$, followed by Tukey *post-hoc* test; $n = 7$–9). **(F)** as in **(D)** for the binary mixture (ANOVA, $P < 0.05$, followed by Tukey *post-hoc* test, $n = 7$–9).

RESPONSE DYNAMICS TO DIFFERENT STIMULUS DURATIONS ARE A FUNCTION OF RECEPTOR TYPE

We found that the response of *Drosophila* OSNs to varying stimulus durations (**Figure 2**) depends on the type of receptor expressed in that neuron. OR-expressing OSNs showed adaptation to higher concentrations of long stimulus pulses (>1 s), both in maximum frequency and latency. This response feature was also independent of ligand (data not shown). In contrast, when IR-expressing OSNs were tested with the same protocol, they required longer stimulus durations to respond, and there was no desensitization even up to 2 s stimulation either in response intensity or latency regardless of stimulus concentration. As a consequence, OSNs that express IRs are able to transmit information concerning the presence of long-lasting odors in their environment better than OR-expressing OSNs. However, this could also present a trade off, because the signal transduction in these OSNs appears to be slower, as seen in **Figure 3E**, where the time to maximum frequency was

longer in IR-expressing OSNs as compared to OR-expressing OSNs.

The difference in response between IR- and OR-expressing OSNs to longer pulses was not a function of stimulus presentation, which was assessed by PID (see "Materials and methods"). It is therefore a property of the OSNs themselves. Are these differences a function of the peri-receptor environment, or rather a property of internal OSN kinetics? To test this, we assessed the response of Or35a-OSNs, which are housed in coeloconic sensillum ac3 together with Ir75abc-OSNs. As with other OR-expressing OSNs, Or35a-OSNs also responded to stimulations as brief as 20 ms and showed desensitization at longer pulses (2 s) in maximum response frequency (**Figure 2B**). The response kinetics of these OSNs is therefore less influenced by the environment where they are expressed and rather by intrinsic properties of the neurons themselves.

The broad protostome conservation of IRs contrasts sharply with the restriction of ORs to insect genomes. This phylogenetic evidence suggests that IRs were the first olfactory receptor repertoire in insects (Robertson et al., 2003; Croset et al., 2010). IRs are also restricted to coeloconic sensilla, whereas ORs are found in several morphological sensillum types (Gupta and Rodrigues, 1997; Goulding et al., 2000; zur Lage et al., 2003; Benton et al., 2009). Our results show that IR-expressing OSNs required longer stimulation times to respond to key odorants, and responded with lower response intensities. This could imply that IRs are less efficient and less sensitive in detecting and transducing a chemical signal. OR activation results in both ionotropic and metabotropic signaling (Wicher et al., 2008; Deng et al., 2011), while IRs are thought to be purely ionotropic (Benton et al., 2009). Iontropic signaling is also known to be less sensitive (Sato et al., 2008, 2011; Wicher et al., 2008). The requirement for higher concentrations in IR-expressing OSNs has been also shown in Yao et al. (2005). The signal transduction in Gr21a has been shown to involve $G\alpha_q$ protein, but not $G\alpha_s$ (Yao and Carlson, 2010; Deng et al., 2011). Thus, it is possible that the transduction cascade itself leads to these differences in response to varying stimulus durations.

The desensitization/adaptation at longer stimulus durations could affect the temporal accuracy of OR-expressing OSNs in reporting long-lasting odor strands, but it may also enrich the coding possibilities for odor discrimination (DeBruyne and Baker, 2008; Nagel and Wilson, 2011) by allowing the neuron to return to its resting state more quickly. This could provide additional possibilities for odor discrimination such as under background odor, or for resolution of intermittent pulsed stimuli. Adaptation extends the operating range of sensory systems, in some cases over an enormous span of stimulus intensities (Torre et al., 1995). It may also play a role in complex functions of neuronal systems such as stimulus location (Kaissling et al., 1987). Similar results were reported in the locust where the electrophysiological response of projection neurons also depended on stimulus duration (Brown et al., 2005; Mazor and Laurent, 2005). In contrast, the long-lasting response of IR-expressing OSNs could allow for close range detection while on or very near the stimulus source where stimulus durations could persist for much longer periods of time (Murlis et al., 2000; Louis et al., 2008; Gomez-Marin et al., 2011).

PULSE RESOLUTION IS RECEPTOR TYPE DEPENDENT

The different classes of OSNs also showed differences in their pulse resolution to repeated stimuli. Brief intermittent stimuli were not detected by IR-expressing OSNs, in contrast to those expressing ORs (which could respond up to 5 Hz). This response characteristic was mainly due to a difference in sensitivity, as increasing the stimulus concentration for IR-expressing OSNs improved the detection and resolution to 5 Hz. In contrast, a 100× increase in concentration actually reduced the OR-OSNs pulse resolution. The accuracy of encoding rapidly fluctuating intermittent odorant stimuli above 5 Hz was significantly reduced for all OSNs regardless of receptor type. Similarly, other insects resolved up to 5 Hz pulses of general odors or pheromones (e.g., Lemon and Getz, 1997; Barrozo and Kaissling, 2002; Bau et al., 2002), even at the antennal lobe (e.g., Christensen and Hildebrand, 1997; Lei and Hansson, 1999; Lei et al., 2009).

Short term adaptation and latency to peak response to repeated stimuli were independent of the receptor expressed in the OSN (**Figures 3A–E**). In addition, the time to peak response and the response intensity were recovered in all OSNs either by increasing the inter-stimulus interval to 5 s or by increasing the concentration. This suggests that adaptation to repeated stimulation is a general feature of all OSNs, regardless of the receptor expressed. Adaptation is assumed to be an early step in information processing and decision making (Kaissling et al., 1987; Baker et al., 1988; Dolzer et al., 2003; Theodoni et al., 2011), and appears to affect the response of all OSN types in a similar manner.

RESPONSE POLARITY AFFECTS PULSE RESOLUTION

Both OR- and IR-expressing OSNs were unable to resolve pulsed inhibitory ligands at frequencies as high as excitatory ligands (**Figures 6B** and **7B**). This could be because the response inhibition lasted longer than excitation (**Figure 6E**), even though the concentrations tested were at the same point in the dose response curve (**Figure 1**). According to Ghatpande and Reisert (2011), fast response termination improves pulse resolution. Similarly, Su et al. (2011) showed that the inhibitory responses of OSNs lasted much longer than their excitatory responses, but the reason for this difference is not clear. Interestingly, a mixture of both excitatory and inhibitory odors improved pulse resolution at high frequencies (**Figures 6F** and **7F**). As a consequence, OSNs may respond to intermittent blends at faster rates, which may increase their ability to track complex natural stimuli.

The fast-terminating biphasic response exhibited by Gr21a-OSNs in response to CO_2 stimulation could be the reason why Gr21a-OSNs resolved more rapid stimulations as compared to OR- and IR-expressing OSNs (**Figure 5**). A biphasic response improved pulse resolution in antennal lobe neurons (Lei and Hansson, 1999). Besides the OSN itself, the chemistry of CO_2 could also contribute to

better pulse resolution as it will readily hydrate to bicarbonate (Kwon et al., 2007), and the degree of odor clearing is one of the challenges for resolving rapidly fluctuating odorant stimuli (Ishida and Leal, 2005; Ghatpande and Reisert, 2011).

CONCLUSION

Terrestrial olfaction requires the tracking of brief, intermittent airborne stimuli in a turbulent and dynamic environment. Fast reaction times to pockets of clean air are suggested to be behaviorally important for successful and rapid source location; hence, the selection over evolutionary time for sensitive and high-fidelity odor strand detection and resolution in the insect olfactory system is crucial (Baker and Vickers, 1997). Equally, the temporal structure of olfactory information has been shown to be critical for odor coding in a variety of systems (Laurent et al., 2001). Here we show that IR-expressing OSNs are better in detecting long-lasting odor pulses, but they are less sensitive. That could suggest that they are better at close range odor detection where odor-OR interaction time is not a limiting factor (high molecular flux). In contrast OR-expressing neurons are more sensitive and better at resolving brief (low molecular flux) pulsed stimuli. This diversity in temporal characteristics could provide a broad palette of response kinetics for the insect olfactory system to respond to the high-dimensional temporal input found in an insect's odor environment.

IRs are the only receptors found in basal insects and conserved between unicellular and multicellular organisms (Croset et al., 2010). ORs appear to have derived from the gustatory receptor family (Robertson et al., 2003; Nordström et al., 2011), which is present in insects as well as in aquatic arthropods such as water fleas (Peñalva-Arana et al., 2009). Besides increasing the diversity of chemicals that could be detected, OR-OSNs also allow the olfactory system to rapidly detect and transduce brief airborne odor information. This is especially important for flying insects, for which stimulus contact is brief and fast response in time is most critical. OR-expressing OSNs were indeed more sensitive to intermittent stimuli than IRs and Gr21a. The sensitive and fast neuronal response observed in OR-expressing OSNs could result from Orco-dependent transduction, which may have evolved through selective pressure to increase sensitivity and speed of odor detection while in flight.

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Chapter II

Piezo controlled microinjection: An in vivo complement for in vitro sensory studies in insects

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Short communication

Piezo controlled microinjection: An *in vivo* complement for *in vitro* sensory studies in insects

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ABSTRACT

Recent insights into insect olfactory signaling based on *in vitro* analyses have created an urgent need for equivalent *in vivo* analyses using living organisms. Here, we present a microinjection system that establishes a "virtual petri dish" within sensory structures for the application of agents to sensory neurons. Our system uses a series of pumps to inject chemical agents via air pressure into the surrounding lymph. We show using tetrodotoxin and forskolin application that robust effects on response dynamics of *Drosophila melanogaster* olfactory sensory neurons could be observed within 200 s, and suggest data analysis techniques to improve estimation of pharmacological effects on response kinetics. This approach provides an improved *in vivo* method to investigate questions in sensory neuron physiology as a complement to heterologous expression systems.

1. Introduction

Olfactory systems utilize a variety of olfactory receptors, signaling pathways, and subsystems to detect an astonishing variety of chemical cues in their environment (Kaupp, 2010). In particular, two basic mechanisms for olfactory sensory neuron (OSN) signaling in insects have been reported, one ionotropic (Sato et al., 2008; Wicher et al., 2008) and one metabotropic (Wicher et al., 2008). The first describes the ligand-gated opening of an ion channel formed by insect odorant receptors. In the second mechanism, ligand-binding to odorant-specific receptor proteins activate stimulatory G-proteins, akin to mammalian olfactory signaling. This leads to enhanced production of cAMP, which subsequently activates the co-receptor also forming an ion channel.

These two studies utilized heterologous expression of olfactory receptors in systems such as human embryonic kidney cells (Wicher et al., 2008) or *Xenopus* oocytes (Sato et al., 2008), giving rise to concerns regarding the translation of heterologous *in vitro* analyses to the natal system. Recent *in vivo* analyses using transgenic *Drosophila* strains with various G-protein mutations have produced similarly divergent results (cf. Deng et al., 2011; Kain et al., 2008; Yao and Carlson, 2010). Overall, these studies indicate an urgent need for a method allowing OSN signal transduction to be assessed in an *in vivo* setting.

We have developed a novel piezo-controlled microinjection system that combines the simultaneous injection of chemical agents into microscale structures with electrophysiological recording. This approach establishes a "virtual petri dish" inside the insect sensillum, allowing for pharmacological manipulation and direct observation of sensory physiology without the use of heterologous systems or genetic manipulation. The concept of microinjection or perfusion into insect sensilla and other microscale structures is not new. However, previous studies using diffusion as the mechanism for chemical application have required at least 30 min (Laughlin et al., 2008) or up to 3 h (Flecke et al., 2006) for effects to become apparent. Such prolonged recording sessions can make it difficult to discriminate pharmacological effects from neuronal damage due to extended recording or compound degradation. Other studies that also show robust and rapid effects on neural response kinetics have required incision of tissue ("cut tip" method, e.g. Kaissling et al., 1991; Pophof, 2004; Van den Berg and Ziegelberger, 1991), microiontophoresis (Storer and Goadsby, 1997), or unspecified methods of pressure delivery (1.8 kPa; Maïbèche-Coisne et al., 2004) to apply agents in question.

In our system, controlled injection of chemical agents using a series of mechanical pumps produces qualitative pharmacological effects within 200 s (Fig. 1A–C). A small, peristaltic tubing pump (Fig. 1C.1) coupled to a piezoelectric micropump (Fig. 1C.2) was used to generate and control the necessary air pressure to inject agents into an insect sensillum through glass capillaries housed within a modified microelectrode holder (Fig. 1C.3). An Ag/AgCl coated wire electrode connected to a high-impedance AC/DC amplifier (Fig. 1C.4) was used to simultaneously record extracellular neuronal signals via connection to computer (Fig. 1C.6) both before and after olfactory stimulation (Fig. 1C.5). The novel piezo-electric

Abbreviations: OSN, Olfactory sensory neuron; OR, Olfactory receptor.

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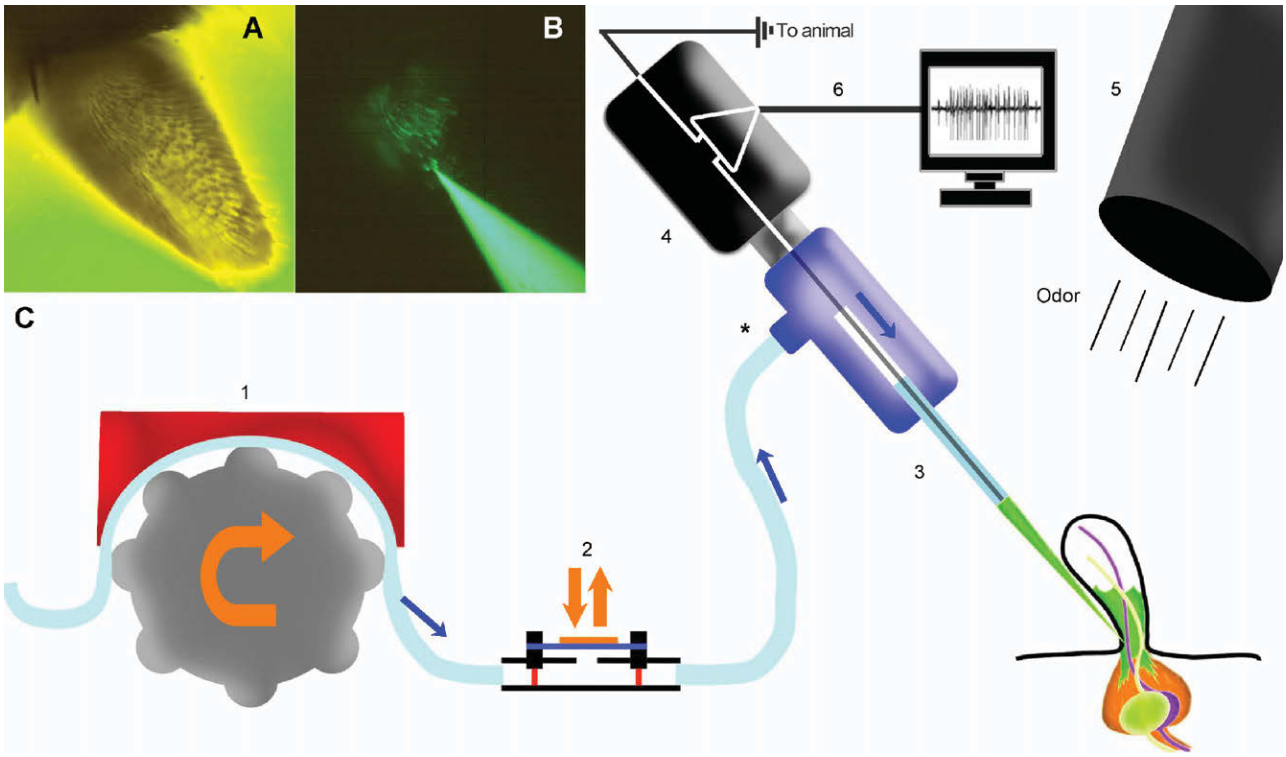

Fig. 1. Design of the microinjection system. (A and B) Photomicrographs of the third antennal segment of a *Drosophila* antenna showing the placement of the micropipette in an olfactory sensillum under bright-field (A) and fluorescent (B) light. Use of the Or22a-Gal4; UAS-CD8-GFP *D. melanogaster* transgenic line and fluorescein in the micropipette tip facilitated contact with fluorescent Or22a-carrying cells in ab3 basiconic sensilla. (C) System schematic. Peristaltic (1) and piezo micropumps (2, piezo element in blue and orange) were used to generate sufficient air pressure to overcome internal sensillum pressure and drive pharmacological agents through a microelectrode (3) and into the sensillum. A chloridized silver wire electrode connected to a 10× amplifier (4) was used to extracellularly record action potentials in response to olfactory stimulation (5). The ground electrode was placed in the eye. Signals were recorded and analyzed via computer connection (6). Asterisk indicates point of air pressure measurement.

control of injection through the microcapillary allows for adjustable injection of agents and presents a simple, yet significant improvement over previous methods that use diffusion or tissue incision to present agents.

2. Materials and methods

The Or22a-Gal4; UAS-CD8-GFP *Drosophila melanogaster* transgenic line was obtained from the L. Vosshall lab (Rockefeller U., New York). Flies were reared at 18 or 25 °C and maintained under a 12 h light:dark cycle on a cornmeal–molasses–agar medium. 2–5-day-old adults were fixed dorsally to a microscope slide as described (Dekker et al., 2006; Pellegrino et al., 2010; Fig. 1A and B). Electrophysiological recordings were analyzed from 50 *D. melanogaster* sensilla (11 for tetrodotoxin injection, 18 for forskolin injection/diffusion, 21 for control saline injection/diffusion). Saline injection data were obtained from (Sargsyan et al., 2011) for comparison.

2.1. Piezo microinjection system

A peristaltic pump and a piezoelectric micropump with a passive check valve were connected and used to generate air pressure through silicon tubing and into a customized microelectrode holder. Silicon tubing (1.6 mm O.D. and 0.8 mm I.D.) passed through a peristaltic pump (ISM852, ISMATEC, Wertheim-Mondfeld, Germany) and connected to a piezo micropump (MDP1304; thinXXS Microtechnology AG, Zweibruken, Germany or mp5; Bartels Mikrotechnik, Dortmund, Germany; used interchangeably). A passive check valve (mp-cv: Bartels Mikrotechnik) was used to prevent backflow. Siltube TR60 (3.0 mm O.D., 1.0 mm I.D.) connected the check valve to the pump and microelectrode holder (MTE007, HEKA, Lambrecht, Germany). An OEM pump control system (EDP 0504, thinXXS) was used to drive the pump via DAQ (USB 6008 data acquisition hardware, National Instruments, Austin, TX, USA) connection to a PC. Piezo micropump frequency and amplitude were controlled by custom-built software via Lab View 8.5 (built by Daniel Veit; National Instruments, Austin, TX, USA). The micropump operated at 5 Hz and 220 V amplitude.

2.2. Micropump measurements

Airflow rates at the inlet of the microelectrode holder (see Fig. 1C, asterisk) for the combined two-pump system (calculated with a 0.01 s piezo stroke time) were measured over various piezo frequencies using a mass flow meter (200 Hz sampling rate, CMOSens EM1, Sensirion, Staefa, Switzerland). Air pressure was measured at the holder inlet for the peristaltic, the piezo micropump, and the combined system using a 50 kPa On-Chip Temperature Compensated & Calibrated Silicon Pressure Sensor (MPX2053 series, Motorola, Denver, CO, USA).

2.3. Extracellular single sensillum recording and microinjection

GFP expressing Or22a neurons were localized in ab3-type basiconic sensilla at 1000× magnification using an Olympus BX-51 microscope equipped with fluorescent filters and illumination and a 3D motorized micromanipulator system (Luigs-Neumann, Ratingen, Germany). Sharpened glass-capillary electrode tips (<3 μm tip, 4–5 MΩ, borosilicate; 1.5 mm O.D. 0.841 mm I.D., World Precision Instruments, Sarasota, FL, USA) were filled with injection stimuli (tetrodotoxin or forskolin) dissolved in saline (171.9 mM

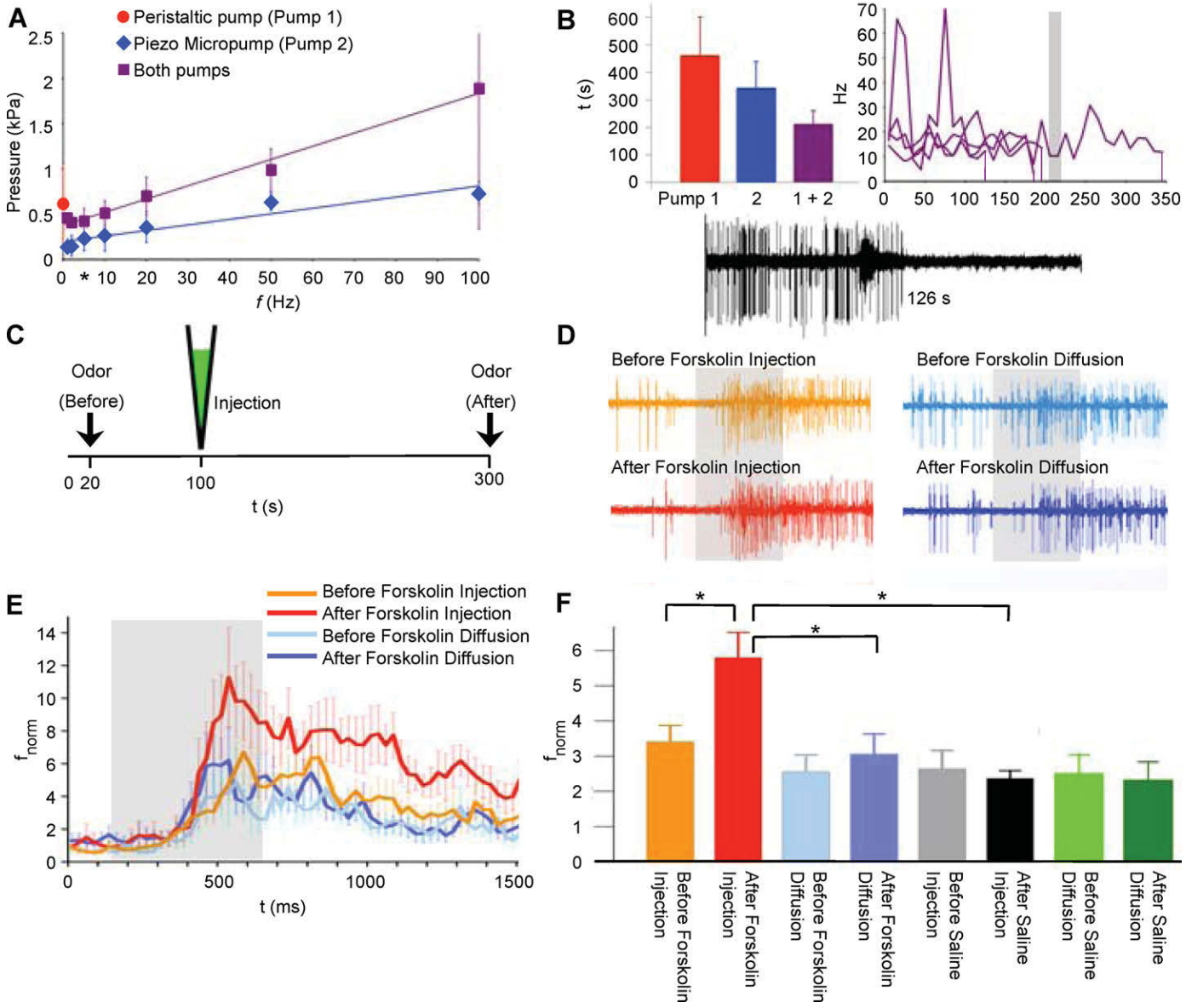

Fig. 2. Development and assessment of the microinjection technique. (A) Total air pressure using one or both pumps measured at the entrance to the microelectrode holder at various piezo frequencies. Asterisk indicates the 5 Hz frequency used in subsequent experiments. Both peristaltic (B, top left; Pump 1, $t = 463 \pm 139$ s, s.e.m., $n = 5$) and piezo micropumps (Pump 2, $t = 344$ s, $n = 2$) were required in tandem to reduce injection time to roughly 200 s as measured by time-to-disruption of the OSN action potentials by 10 μM tetrodotoxin (Pumps 1 + 2, $t = 213 \pm 48$ s, s.e.m., $n = 4$). (B, top right) Time course for 4 sensilla showing the variation in time-to-disruption by TTX using the two-pump system, with an average of roughly 200 s. (B, bottom) 30 s sample trace showing disruption of spontaneous activity by tetrodotoxin in 126 s using the microinjection technique. (C) Microinjection protocol. Olfactory stimuli were presented at roughly 20 s (before injection) and active injection commenced at 100 s. A second olfactory stimulation was performed at roughly 300 s while injection continued (after injection). (D) 1.5 s sample traces comparing Or22a OSN olfactory responses to ethyl butyrate. Before injection, odorant stimulation induced a 20.5 (orange) and 18.5 (light blue) Hz increase in spike frequency. After injection, 1 mM forskolin injection increased the spike frequency change to 29 Hz (red) following odorant stimulation while it remained relatively unchanged at 20.5 Hz (dark blue) for diffusion alone. Gray shading denotes stimulus period, including the mechanical delay. (E) Kinetics of olfactory responses to a 100 ng loading of ethyl butyrate before (20 s) and after (300 s) 1 mM forskolin injection. Normalized frequency ratios are presented as peri-stimulus time histogram lines in 25 ms bins for 1350 ms following stimulus onset (gray). Traces indicate odor responses before and after microinjection (orange/red) or diffusion (light/dark blue) of forskolin. Error bars, s.e.m., $n = 9$ for both treatments. (F) Normalized frequencies for stimulus (forskolin injection, red) and controls [diffusion of forskolin only, blue, saline injection (Sargsyan et al., 2011), black, $n = 11$, and saline diffusion only, green, $n = 10$] calculated from areas of the peri-stimulus time histograms for the total response window (1350 ms; $P \leq 0.01$ within and between treatments); Student's paired t-tests (within treatment) or two-tailed t-tests (between treatments). Error bars, s.e.m. $^*P \leq 0.01$.

KCl, 9.2 mM KH_2PO_4, 10.8 mM K_2HPO_4, 3 mM $MgCl_2$, 1 mM $CaCl_2$, 1.5 mM HCl, 22.5 mM glucose, 25.0 mM NaCl, pH 6.5 after Kaissling and Thorson, 1980), or the saline alone. Fluorescein was also added at the tip to aid visualization under fluorescence (Fig. 1A and B). The shaft was then backfilled with saline. An Ag/AgCl coated silver wire was used to detect the extracellular analog signals originating from the OSNs, and extracellular analog signals were amplified (10×; Syntech Universal AC/DC Probe), sampled (10,667.029 samples/s) and filtered (100–3000 Hz with 50/60 Hz suppression) via USB-IDAC connection to a computer (Syntech). Action potentials were extracted as digital spikes from the analog signal according to top–top amplitudes using Syntech Auto Spike 32 software. Co-localized OSNs were distinguished by amplitude in ab3 sensilla (Hallem et al., 2004). At time 0 s, cells were contacted extracellularly in the shaft of the sensillum lymph cavity (Fig. 1) and recorded for approximately 20 s before an initial 0.5 s stimulation

with odor. Consistent and pulsed pressure injection (5 Hz, 0.2 kPa) commenced at 100 s, and cells were again stimulated with 0.5 s odor at approximately 300 s while injection continued at the same rate and pressure (Fig. 2C).

2.4. Odor stimulation

Ethyl butyrate (99%, Sigma, Munich, Germany) was dissolved in hexane (10 ng/μl; 99%, Fluka Analytical, Buchs, Switzerland). A 100 ng loading was pipetted onto roughly 1 cm diameter filter paper and placed in disposable Pasteur pipettes. This concentration is detected by Or22a OSNs in the linear portion of the dose–response curve (Dobritsa et al., 2003). Filtered and humidified air passed over the antenna from a stimulus air controller at approximately 1 l min^{-1} and through an aluminum tube (6 mm I.D.) with an outlet roughly 10 mm from the antenna. During stimulation, airflow bypassed a complementary air stream (0.5 l min^{-1} during 0.5 s) and passed through the stimulus pipette and into the tube approximately 2 cm from the tube outlet.

Photoionization detection (miniPID Fast Response Miniature Photo-Ionization Detector, Model 200A, Aurora Scientific Inc, Aurora, Ontario, CA) was used with ethyl butyrate to measure the mechanical delay between electronic stimulus onset and physical onset at the antenna. During measurements, the PID sensor was placed at the approximate location of the antenna during olfactory experiments. A 200 ms mechanical delay as determined by photoionization detection was subtracted from all analyses of response kinetics.

2.5. Data analysis

OSN responses were analyzed by raw spike counts in Hz and normalized frequency ratio (peri-stimulus spike count frequency/average pre-stimulus frequency before stimulus onset over the same time period). Responses were analyzed for 500 and 1350 ms after stimulus onset. These time periods were chosen to encompass the stimulus (500 ms) and total OSN response (approximately 1350 ms) windows. For response kinetics, normalized spike frequency ratios were analyzed in 25 ms bins by dividing each 25 ms instantaneous spike frequency by the average pre-stimulus frequency over 1.5–2 s. Areas under the curve (Matthews et al., 1990) were measured for each peri-stimulus time histogram using the trapezoid rule for each data point and subsequent areas divided by the time to establish a normalized frequency average for each response. Student's paired *t*-tests compared responses before and after injection, and two-tailed Student's *t*-tests compared treatments after injection. Only cells contacted for at least 300 s were used for analysis. All analyses performed using PASW (SPSS) v. 18 software.

3. Results and discussion

The piezo micropump produces a consistent, pulsed flow of input air pressure at the microelectrode (Fig. S1) that can be adjusted over a range of values (Fig. 2A; 0.1–0.7 kPa air pressure at 1–100 Hz) via piezo frequency adjustment. The efficacy of this method was tested at a continuous 5 Hz piezo frequency (0.2 kPa air pressure; Fig. 2A, asterisk) using 10 μM tetrodotoxin (TTX) placed in the tip of micropipettes. The inhibition of neuronal activity by this sodium channel blocker was used as a measure for the efficiency of compound injection. Both spontaneous activity and response to odorant stimulation in ab3 type basiconic sensilla were eliminated following action potential disruption by TTX. The two-pump pulsed-pressure system both doubled the input pressure (Fig. 2A) and roughly halved the time to action potential disruption over the micropump itself (Fig. 2B; $t = 463 \pm 139$ s, s.e.m. and 344 s for pumps 1 and 2, respectively, total $n = 7$; $t = 213 \pm 48$ s, s.e.m. for both pumps, $n = 4$). For this reason, an injection protocol was adopted using these settings requiring 200 s of injection before odor stimulation and assessment of pharmacological effects (Fig. 2C and D). *Drosophila* express a single voltage-gated sodium channel, para (Zhang et al., 2011), which is maximally reduced by TTX at 10 nM (Warmke et al., 1997). As our injection technique required 10 μM TTX for disruption within 200 s (lower concentrations were not effective in this timeframe), this equates to less than 0.1 fl injected into the 2.75 μm × 9.5 μm *Drosophila* sensillum (56 fl volume; Venkatesh and Naresh Singh, 1984). We thus recommend that the concentration of injected pharmacological agents be at least 100–1000 fold higher than required for isolated cell preparations.

Using the two-pump protocol developed with TTX, the adenylyl cyclase activator forskolin was injected into *D. melanogaster* ab3 sensilla to simulate experiments performed *in vitro* (Wicher et al., 2008). Forskolin application in HEK293 cells expressing *Drosophila* OR Or22a and/or Orco (the ubiquitous insect olfactory coreceptor) was observed to activate a cation current. After 200 s of forskolin injection, a 500 ms pulse of ethyl butyrate (100 ng loading) was presented to the sensillum and the response of the Or22a-carrying cell recorded while injection continued. This response was then compared with the response to an equivalent stimulus presented before injection (Fig. 2D and E). Forskolin only induced an increase in odor response if there was injection of the agent using both pumps (Fig. 2E, red trace; Fig. 2F; $P = 0.011$, $n = 9$, within treatment, Student's paired *t*-test). Without injection using the two-pump system, passive diffusion of forskolin into the sensilla was insufficient to induce a change in response to ethyl butyrate compared to initial conditions (Fig. 2E, dark blue trace). This shows that the microinjection system significantly enhances the application of pharmacological agents into the sensillum over techniques that utilize diffusion (Fig. 2F; after diffusion, blue vs. after injection, red: $P = 0.009$ between treatments, two-tailed Student's *t*-test). The odorant response after forskolin injection was also significantly greater than after injection of the saline alone (after forskolin injection, red vs. after saline injection, black: $P < 0.001$, $n = 11$, two-tailed Student's *t*-test).

This method was recently used to assess the role of phospholipase C (PLC) and protein kinase C (PKC) activity in the *Drosophila* odorant response (Fig. S2B; Sargsyan et al., 2011). Microinjection of the PLC inhibitor U73122 or the PKC inhibitor Gö6976 into ab3 sensilla significantly reduced the OSN response to odor pulses in comparison to control saline injection. In contrast, injection of the PKC activator phorbol myristate acetate (PMA) significantly increased the odor response firing frequency (Sargsyan et al., 2011). The robust effect of these pharmacological agents in both the heterologous system and the fly itself shows that this technology can be used as a suitable *in vivo* replicate for *in vitro* studies of OSNs.

To assess changes in OSN frequency following olfactory stimulation, it is common practice to count the number of spikes (action potentials) fired during the stimulus period, often 500 ms (Hallem et al., 2004; Kain et al., 2008; Yao and Carlson, 2010). Spikes can be recorded as is (Fig. S2C; Kain et al., 2008; Yao and Carlson, 2010), or normalized to an equivalent pre-stimulus period (Fig. S2E; Hallem et al., 2004). Although these methods are effective for detecting the magnitude of a response, they do not necessarily reflect the temporal dynamics (Fig. 2E) exhibited during the response window (Fig. S2A). Recent studies show that OSNs exhibit complex dynamics shaped by both signal transduction and subsequent spike generation (e.g. French et al., 2011; Nagel and Wilson, 2011). To reflect response kinetics, the area of the peri-stimulus time histogram can be used as a simple measure to incorporate the shape of the response for each trial. Using this method, a significant difference in temporal response pattern was measured in OSNs

only when forskolin was injected (Fig. 2E and F). In addition, the analysis of odor response dynamics following injection of compounds acting on phospholipase C and protein kinase C (Sargsyan et al., 2011), revealed an increased resolution of agent effects when using the area under curve (Fig. S2B) as compared to spike counts (Fig. S2C and E). Although spontaneous activity was not affected by microinjection (Fig. S2D), responses should also be normalized to control for any within-treatment changes in resting activity. Thus, studies that potentially alter response dynamics genetically or pharmacologically (such as the technique described here) should take both firing kinetics and spontaneous activity into account.

4. Conclusions

This microinjection system provides a significant improvement in the application of agents to insect sensilla over previous methods using diffusion or tissue incision. Pharmacological agents are injected at the site of electrode penetration eliminating the need for tissue incision, which is impractical for very small structures such as *Drosophila* antennal sensilla. The injection of agents is rapid in comparison to diffusion-based techniques [Fig. 2E and F, a 10× (Laughlin et al., 2008) to up to 50× (Flecke et al., 2006) reduction in treatment time], and the addition of the piezo micropump allows for controlled application of agents into tissues such as insect sensilla (Figs. 2B and S1). Higher or lower injection rates can be accomplished using different input pressures (Fig. 2A and B). The system is also simple and portable, and does not induce noise when placed near the site of electrophysiological recording (Fig. 2D and E).

This method was developed for qualitative assessment of the effect of injected agents on microscale tissues *in vivo*. Demonstration of the technique via forskolin injection showed robust effects on odor response kinetics after 200 s of injection that were not observed with diffusion or injection of saline alone. This microinjection technique provides a natural setting to address a variety of questions typically pursued using heterologous or transgenic platforms, such as the role of peri-receptor events and aspects of the signal transduction cascade. The current injections were performed under 1000× magnification in a sensillum roughly 3 μm in diameter. The system is thus appropriate for fine-scale injection into living tissue, and provides an improved technique to assess typically *in vitro* studies of sensory signaling *in vivo*. This method provides a significant catalyst to unraveling the questions and controversies currently surrounding insect olfactory signaling, and other systems as well.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jneumeth.2011.08.015.

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Chapter III

Phosphorylation via PKC regulates the function of the *Drosophila* odorant co-receptor

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Phosphorylation via PKC regulates the function of the *Drosophila* odorant co-receptor

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Insect odorant receptors (ORs) have a unique design of heterodimers formed by an olfactory receptor protein and the ion channel Orco. Heterologously expressed insect ORs are activated via an ionotropic and a metabotropic pathway that leads to cAMP production and activates the Orco channel. The contribution of metabotropic signaling to the insect odor response remains to be elucidated. Disruption of the G_q protein signaling cascade reduces the odor response (Kain et al., 2008). We investigated this phenomenon in HEK293 cells expressing *Drosophila* Orco and found that phospholipase C (PLC) inhibition reduced the sensitivity of Orco to cAMP. A similar effect was seen upon inhibition of protein kinase C (PKC), whereas PKC stimulation activated Orco even in the absence of cAMP. Mutation of the five PKC phosphorylation sites in Orco almost completely eliminated sensitivity to cAMP. To test the impact of PKC activity *in vivo* we combined single sensillum electrophysiological recordings with microinjection of agents affecting PLC and PKC function and observed an altered response of olfactory sensory neurons (OSNs) to odorant stimulation. Injection of the PLC inhibitor U73122 or the PKC inhibitor Gö6976 into sensilla reduced the OSN response to odor pulses. Conversely, injection of the PKC activators OAG, a diacylglycerol analog, or phorbol myristate acetate (PMA) enhanced the odor response. We conclude that metabotropic pathways affecting the phosphorylation state of Orco regulate OR function and thereby shape the OSN odor response.

Keywords: insect odorant receptor, *Drosophila*, Or83b, orco, G protein, cAMP, phosphorylation, single sensillum recording

INTRODUCTION

Olfaction in nematodes and vertebrates utilizes G protein-coupled receptor (GPCR) signaling. In insects, odorant receptor (OR) proteins share the seven-transmembrane topology of GPCRs but retain no sequence-similarity (Benton et al., 2006). Furthermore, they form heterodimers of a ligand-binding OR and an ubiquitous co-receptor such as Dmel/Orco (previously Or83b in *Drosophila*; Vosshall and Hansson, 2011), both of which are inversely oriented in the membrane compared to GPCRs (Benton et al., 2006; Lundin et al., 2007). On the other hand, G proteins are expressed in the dendrites of olfactory sensory neurons (OSNs) bearing the ORs (Boto et al., 2010), and *Drosophila* mutants with disturbed G protein signaling cascades show impaired odor processing (reviewed in Hansson et al., 2010). Here we concentrate on OR22a as ligand-binding receptor. This is probably the most well-investigated OR of *D. melanogaster*. It is expressed in large basiconic sensilla and is tuned to fruit volatiles (ethyl butyrate, ethyl hexanoate) emitted by, e.g., pineapple (Stensmyr et al., 2003; Hallem and Carlson, 2006).

Studies on insect OR function in heterologous expression systems provided evidence for a G protein-independent, ionotropic mode of action (Sato et al., 2008; Wicher et al., 2008). However, an additional metabotropic pathway has been shown to stimulate cAMP production, which in turn activates the ion channel-forming co-receptor protein Orco (Wicher et al., 2008). Manipulating the cAMP level changed the kinetics of the odor response in heterologous cells (Smart et al., 2008). Genetic manipulation of G protein signaling in flies produced comparatively mild effects on odor responses (Yao and Carlson, 2010). However, the important role of stimulatory G proteins and subsequent cAMP signaling was recently demonstrated both in flies and in the heterologous expression system (Deng et al., 2011). A consensus model of these controversial results suggests a modulation of the ionotropic response by metabotropic pathways (Nakagawa and Vosshall, 2009).

In addition to cAMP signaling, DAG/IP_3 signaling may also play a role in insect olfaction (Krieger and Breer, 1999). Mutations in the *Drosophila dgq* gene encoding the G_q α subunit produces flies with reduced responses to odor stimulation (Kain et al., 2008). The responses were further attenuated by additional mutations in *plc21C*, a gene encoding for a PLCβ. In the present study, we investigate the effect of G_q protein downstream signaling on heterologously expressed Orco proteins and demonstrate the relevance of these results using single sensillum electrophysiology in *Drosophila* OSNs combined with microinjection of compounds affecting the G_q protein signaling cascade.

MATERIALS AND METHODS

PKC MUTANT ORCO

Or83b protein kinase C (PKC) phosphorylation mutants M1, M2, and Orco PKC synthetic genes were generated and subcloned into *Eco*RI/*Xho*I sites of pcDNA3.1(+) plasmid (eurofins MWG operon, Ebersberg, Germany) and directly used for cell transfections. Sequences were analyzed by doublestrand DNA sequencing (eurofins MWG operon) and point mutations for M1 (S159N, T250N, S289N), M2 (T327N, T371N), and Orco PKC (S159N, T250N, S289N, T327N, T371N) verified. Expression and membrane targeting of Orco PKC was demonstrated by immunofluorescence. HEK293 cells were cultured on glass coverslips, transfected, and fixed in 4% PFA. Membranes were labeled with Texas Red-X conjugated wheat germ agglutinin (WGA; 5 μg/ml, Invitrogen). Cells were subsequently incubated with a primary rabbit polyclonal antibody (1:1000) against Orco (kindly provided by Leslie Vosshall) and an Alexa488 (1:1000) secondary antibody. Confocal images were taken and analyzed by LSM 510 Meta (Carl Zeiss, Germany).

CELL CULTURE AND TRANSFECTION

Transient transfection

HEK293 cells were cultured at a density of $\sim 2 \times 10^4$ per 35-mm dish and transfected with 1 μg Or83b-pcDNA3.1(−) or 1 μg Or83b PKC-pcDNA3.1(+) using Roti-Fect transfection kit (Roth, Karlsruhe, Germany). In some experiments the partial Orco PKC mutants M1 and M2 were used (**Figure 4A**). To test for the role of G_s and G_q protein activation on Orco, cells were co-transfected with 1 μg hβ_2 adrenergic receptor (β_2 AR)/pCMV6-XL5 (Origene, Rockville, MD, USA) and with 1 μg hα_1 adrenergic receptor (α_1 AR)/pCMV6-XL4 (Origene). In all preparations with transient transfection cells were co-transfected with 0.5 μg EGFP; for electrophysiological experiments we only used cells showing GFP fluorescence (when illuminated at 470 nm) as indicator of putative OR expression.

Stable transfection

The open reading frame of Orco was PCR-amplified using gene specific primers with restriction sites for XhoI and HindIII and cloned into the pcrII TA-cloning vector (Invitrogen, Carlsbad, CA, USA). The identity of the insert was sequenced in full length to verify identity and integrity and subcloned into the pcDNA3.1(+) expression vector via the integrated restriction sites. The resultant construct was verified by sequencing. Flp-In™-T-Rex™ 293 cells held in DMEM (high glucose, with l-Glutamine)/Ham's F12 (with l-Glutamine; PAA, Pasching, Austria) +10% FCS were transfected with OR 83b pc DNA 3.1 using Fugene (Invitrogen) according to the manufacterer's protocol. 24-h post-transfection 800 mg/l of G418 were added to the medium. After 12 weeks, clones were separated and tested for activity using measurements of channel activity as described (Wicher et al., 2008). The clone exhibiting the highest sensitivity to 8-bromo-cAMP was maintained under antibiotic selection and used for this study.

PATCH-CLAMP ELECTROPHYSIOLOGY

Ion currents in HEK293 cells were measured at room temperature using whole-cell patch-clamp with appropriate compensation of series resistance and of capacitive currents. Additional experiments were performed in the inside-out configuration. Current measurements and data acquisition were performed using an EPC9 patch-clamp amplifier controlled by PatchMaster software (both HEKA Elektronik, Lambrecht, Germany). Patch-clamp pipettes were fabricated from borosilicate capillaries. Pipettes for whole-cell recordings had resistances of 2–4 MΩ for excised-patch recordings the pipette resistance was up to 15 MΩ.

The pipette solution contained (in mM) 140 KCl, 4 NaCl, 2.2 $CaCl_2$, 2 Mg-ATP, 0.05 Na-GTP, 5 EGTA, 10 HEPES (pH 7.3), and the bath solution contained (in mM) 135 NaCl, 5 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, 10 glucose (pH 7.4). For recordings of inside-out patches the pipette solution was used for the bath and vice versa.

With the exception of GTP-γ-S, and GDP-β-S, which were applied via the patch pipette, all substances were applied to the bath using either a bath perfusion system (BPS4 from ALA, NY, USA) or a rapid solution changer (RSC160 from Biologic, Claix, France) which were controlled by the PatchMaster software (HEKA Elektronik).

For data analysis the software IgorPro (WaveMetrics, Lake Oswego, OR, USA) or Prism 4 (Graph Pad Software, San Diego, CA, USA) were used.

EXTRACELLULAR SINGLE SENSILLUM RECORDING AND MICROINJECTION

Recording and injection protocols were performed on Or22a-GAL4; UAS-CD8-GFP *Drosophila melanogaster* flies expressing membrane tagged GFP in Or22a-OSNs. Two- to 5-day-old adults were fixed dorsally to a microscope slide. Compounds and concentrations for injection were diluted in receptor lymph solution (Kaissling and Thorson, 1980) as follows: U73122 (0.5 mM), Gö6976 (0.5 mM), OAG (0.1 mM), PMA (0.1 mM). Note that due to a dilution effect, concentrations of injected agents were 100× the concentration used in whole-cell preparations. A microinjection setup consisting of a dual-pump system was used to inject agents via air pressure through the microelectrode holder and into the sensillum lymph. For odor stimulation, 10 μl of ethyl butyrate (99%, Sigma, Munich, Germany) in hexane (10 ng/μl; 99%, Fluka Analytical, Buchs, Switzerland) was pipetted onto 1 cm diameter filter paper disks and placed in disposable Pasteur pipettes. Odor stimuli were delivered at 0.5 l/min into a 1.0 l/min humidified air stream.

Sensilla were localized at 1000× magnification and an Ag/AgCl coated silver wire inserted into a sharpened glass capillary used to detect the extracellular analog signals originating from the OSNs. Action potentials were extracted digitally according to top–top amplitudes using Syntech Auto Spike 32 software. Cell activities were recorded for approximately 20 s before an initial 0.5 s stimulation with ethyl butyrate. Microinjection commenced at 100 s, and cells were again stimulated with an 0.5-s odor pulse after approximately 300 s. Responses of the larger amplitude Or22a-carrying cell were analyzed for 1500 ms after stimulus onset. For response kinetics, spike frequency ratios were analyzed as peri-stimulus time histograms (PSTHs) in 25 ms bins by dividing each 25 ms instantaneous spike frequency by the average pre-stimulus frequency over 2 s to give a normalized ratio for each time point. Areas under the PSTH curve were calculated for the stimulus (500 ms)

and total response (1350 ms) windows respectively, adjusting for a 150 ms mechanical stimulus delay. These values were divided by time to establish a normalized frequency average for each response. Mann–Whitney *U* tests compared treatments with the control (receptor lymph ringer) after injection. All analyses were performed using PASW (SPSS) v. 18 software.

CHEMICALS

8-Bromo-cAMP, 8-bromo-cGMP, dl-isoproterenol hydrochloride (ISO), dl-Norepinephrine hydrochloride (NE), ethyl butyrate (Etb), forskolin, GTP-γ-S, GDP-β-S, phorbol 12-myristate 13-acetate (PMA), and 9-(tetrahydro-2-furanyl)-9H-purin-6-amine (SQ22536) were obtained from Sigma (Taufkirchen, Germany); U73122, U73343 and Gö6976 from Calbiochem (Darmstadt, Germany); 1-oleoyl-2-acetyl-sn-glycerol (OAG) from Alexis (Lörrach, Germany).

RESULTS

Mutant flies with disrupted G_q protein/phospholipase C (PLC) signaling cascade show reduced odor responses (Kain et al., 2008). When seeking the molecular mechanism by which PLC inhibition affects the odor response, the most parsimonious assumption is that PLC targets the OR complex itself. As multiple receptors are affected by G_q protein disruption (Kain et al., 2008), the ubiquitous Orco would be a good target candidate. In human embryonic kidney (HEK293) cells co-expressing Or22a and Orco, we have previously observed that intracellular application of the non-hydrolysable G protein inhibitor GDP-β-S reduces the sensitivity of the receptor dimers to ethyl butyrate, a key ligand (Wicher et al., 2008). G protein inhibition prevented odor-induced cAMP production and consequent activation of Orco via the metabotropic pathway. Here, we asked whether inhibition of G proteins could affect the sensitivity of Orco to cAMP. Using the voltage-clamp technique in the whole-cell configuration, we perfused HEK293 cells expressing Orco with the non-hydrolysable GDP analog GDP-β-S via a patch pipette. Stimulation of Orco by bath application of the membrane-permeable cAMP analog 8-bromo-cAMP could – even at the highest concentrations – induce only a weak membrane current (**Figure 1B**). Without GDP-β-S in the pipette, 8-bromo-cAMP induced a current in a concentration-dependent manner (**Figure 1B**). Unexpectedly, permanent stimulation of G proteins with the non-hydrolysable GTP analog GTP-γ-S induced a current even in the absence of 8-bromo-cAMP (**Figures 1A,B**). Subsequent application of 8-bromo-cAMP further enhanced this current. However, there was less current production by 8-bromo-cAMP than under control conditions (287 pA vs. 550 pA; **Figure 1B**), indicating that the pool of channels available for activation by 8-bromo-cAMP is reduced due to pre-activation by GTP-γ-S.

To assess whether G proteins have a direct effect on Orco and whether there is any subtype-specificity, we co-expressed Orco together with the β_2-adrenergic receptor (β_2-AR), which activates G_s proteins, and with the α_1-adrenergic receptor (α_1-AR), which activates G_q proteins. Stimulation of β_2-AR with 10 μM isoproterenol induced currents of comparable size to those obtained after application of 1 μM 8-bromo-cAMP (relative current: 1.2 ± 0.2; $n = 5$). Preincubation of cells with the adenylyl cyclase inhibitor

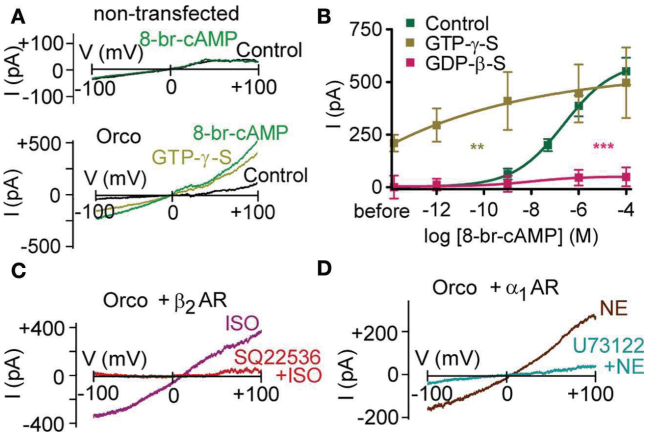

FIGURE 1 | G protein activity regulates the cAMP effect on Orco. (A) Whole-cell current response in a non-transfected HEK293 cell on a voltage ramp from −100 to +100 mV after breaking into the cell (Control) and 2 min after application of 100 μM 8-br-cAMP (top), and a HEK293 cell expressing Orco after breaking into the cell (Control), after perfusion with GTP-γ-S (500 μM) to activate G proteins, and 2 min after application of 100 μM 8-br-cAMP (bottom). Note that 8-br-cAMP fails to produce a current in non-transfected cells. **(B)** Concentration–response curve for 8-br-cAMP-induced Orco currents, measured at −100 mV in the whole-cell mode with standard pipette solution (Control) and a solution containing GTP-γ-S (500 μM) to activate or GDP-β-S (500 μM) to inactivate G proteins, respectively. The current "before" is the difference between GTP-γ-S and Con described in **(A)**. (Control, $n = 11$; GTP-γ-S, $n = 8$, $^{**}P$ to Control < 0.01; GDP-β-S, $n = 9$, $^{***}P < 0.001$). **(C)** Test for the role of G_s protein activation on Orco. Current responses in a cell expressing Orco and the β_2 adrenergic receptor (β_2 AR) to isoproterenol stimulation (10 μM) in the absence (ISO) and 6 min after application of SQ22536 (200 μM). Curves represent differences (2 min ISO – before ISO). **(D)** Test for the role of G_q protein activation on Orco. Currents evoked in a cell expressing Orco and the α_1 adrenergic receptor (α_1 AR) by norepinephrine (NE) stimulation (1 μM) in the absence and 8 min after application of the PLC inhibitor U73122 (10 μM). Curves represent differences (2 min NE – before NE).

SQ22536 (200 μM) prevented current production by isoproterenol (relative current: -0.1 ± 0.1; $n = 5$; **Figure 1C**). Thus, neither the $G_s\alpha$ subunit nor the βγ subunit complex was able to activate Orco in the absence of cAMP.

Stimulation of α_1-AR with 1 μM norepinephrine induced currents of 1.6 ± 0.3 ($n = 5$), normalized to the 1 μM 8-bromo-cAMP response. To test for a direct G protein effect, PLC was inhibited with U73122 (10 μM). Under these conditions, norepinephrine failed to elicit a current (relative current: -0.2 ± 0.2; $n = 7$; **Figure 1D**), indicating that neither the $G_q\alpha$ subunit nor the βγ subunit complex could activate Orco alone. The activation of co-expressed Orco upon stimulation of α_1-AR is remarkable as it for the first time demonstrates that Orco can be metabotropically activated independent of cyclic nucleotides. There are thus at least two independent signaling pathways capable of producing Orco currents.

To examine whether inhibition of PLC activity could account for the reduced cAMP-sensitivity of Orco with GDP-β-S, we tested the effect of 8-bromo-cAMP in the presence of U73122. We then observed only marginal responses, even at the highest concentration of 8-bromo-cAMP (**Figures 2A,C**). U73343, an

inactive analog of U73122, did not suppress the 8-bromo-cAMP effect ($P = 0.91$; $n = 8$). The G protein activity-dependence of the Orco response to cAMP is therefore related to either PLC activity or a downstream process, i.e., the response of Orco to cAMP requires some basal PLC activity. To maintain catalytic activity of PLC, physiologically free Ca^{2+} levels are necessary (Rebecchi and Pentyala, 2000). We thus tried to stimulate Orco using a Ca^{2+}-free pipette solution, and we recorded only marginal responses to 8-bromo-cAMP (5 μM; 31 ± 17 pA; $n = 9$) or forskolin (10 μM; 95 ± 41 pA; $n = 8$) compared with those obtained with standard pipette solution (cAMP: 398 ± 67 pA; $n = 11$; forskolin: 697 ± 67 pA; $n = 9$).

Inhibition of PLC activity prevents PIP_2 cleavage and subsequent IP_3 and DAG production. We asked which of these effects could account for the observed depression of current production by cAMP. Mimicking PIP_2 accumulation due to PLC inhibition using bath application of PIP_2 with inside-out patches did not significantly reduce the cAMP effect (5 μM cAMP enhanced the background current of 3.6 ± 0.7 pA by 10 ± 2.7 pA; $n = 8$). Thus, the PIP_2 cleavage products may instead be critical for the cAMP-sensitivity of Orco. Even though IP_3 activated a tiny current (~0.5 pA at 5 μM), this current was too weak to rescue the U73122-inhibited current, and it likely reflects the activation of an endogeneous HEK293 cell channel (Bugaj et al., 2005). By contrast, the DAG analog OAG enhanced the membrane current upon PLC inhibition (at 100 μM from 0.8 ± 0.2 pA to 2.6 ± 0.7 pA; $n = 6$), and partially restored sensitivity to cAMP (at 100 μM to 5.4 ± 1 pA; $n = 6$). DAG levels thus appear to control the response of Orco to cAMP. As DAG activates PKC, we assessed whether inhibition of PKC would mimic the effect of PLC inhibition. Application of 8-bromo-cAMP after preincubation of cells with the PKC inhibitor Gö6976 produced only weak whole-cell current responses that did not differ significantly from those obtained with U73122 (**Figure 2C**). On the other hand, activation of PKC with phorbol myristate acetate (PMA) induced an Orco current, while subsequent application of 8-bromo-cAMP caused only a mild further current increase (**Figures 2B,C**).

The concentration–response curve for whole-cell current activation by 8-bromo-cAMP indicates that Orco is highly sensitive to cAMP at physiological Ca^{2+} levels (**Figures 2B,C**). To demonstrate this directly we tested the effect of cAMP on inside-out patches. Intriguingly, current activation even after fast application of cAMP develops slowly and with a delay (**Figure 3**). The activation process thus differs from classical gating such as in CNG channels. The concentration–response curve obtained was characterized by $EC_{50} = 0.7$ nM cAMP and a Hill coefficient = 0.40 (**Figure 2D**). Therefore, Orco is at least four orders of magnitude more sensitive to cAMP than the most cAMP-sensitive CNG channel (Dhallan et al., 1990). As in the whole-cell experiments, GDP-β-S, U73122, and Gö6976 drastically reduced the responses to cAMP (**Figure 2D**).

These results suggest that PKC activity plays a central role in controlling Orco function, and especially the sensitivity to cAMP. The Orco protein bears five PKC phosphorylation sites (**Figure 4A**), three in the intracellular loop 2 (IC2), T250, S289 and T327, and two with putative extracellular localization (S159 in EC2; T371 in EC3). To confirm the role of PKC-mediated phosphorylation, we produced an Orco PKC mutant with serine/threonine to asparagine replacements at all PKC sites (**Figure 4A**). This mutant was expected to mimic the Orco wild type in the presence of PLC or PKC inhibitors for both background activity and stimulation by cAMP. Odorant receptor heterodimers such as Or22a/Orco show some background activity even in the absence of odor stimuli (Wicher et al., 2008). Inside-out patches from HEK293 cells expressing Orco conducted a significantly higher resting current than those from non-transfected cells

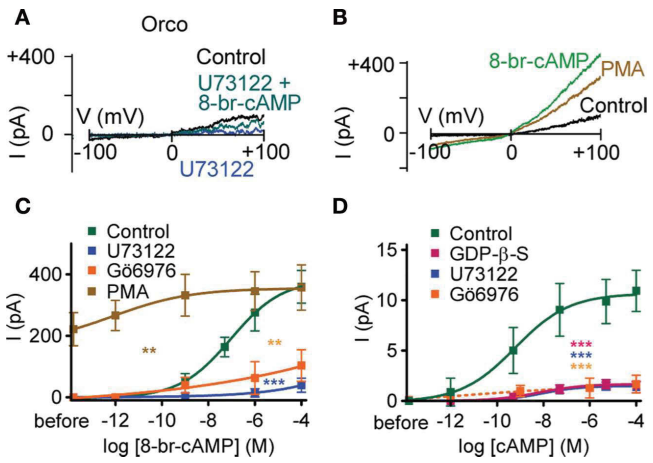

FIGURE 2 | Phospholipase C and protein kinase C activity regulates the cAMP effect on Orco. (A) Current responses in a HEK293 cell expressing Orco on a voltage ramp from −100 to +100 mV after breaking into the cell (Control), after application of the PLC inhibitor U73122 (10 μM), and after application of 8-br-cAMP (100 μM) in presence of U73122. **(B)** Current responses in a cell expressing Orco before (Control) and after PMA (1 μM) and 8-br-cAMP stimulation (100 μM). **(C)** Concentration–response for 8-br-cAMP-induced Orco currents, measured as described in **(B)** with a standard bath solution (Control) and a solution containing U73122 (10 μM), the PKC inhibitor Gö6976 (2 μM) or the PKC activator PMA (1 μM), respectively. (Control, $n = 10$; U73122, $n = 7$, $^{***}P < 0.001$; Gö6976, $n = 11$, $^{**}P < 0.01$; PMA, $n = 11$, $^{**}P < 0.01$). **(D)** Concentration–response curves for cAMP-induced currents in inside-out patches from cells expressing Orco. Data represent maximum mean currents at −60 mV produced under control conditions and with 500 μM GDP-β-S, 10 μM U73122 or 1 μM Gö6976 in the bath. (Control, $n = 13$; GDP-β-S, $n = 17$, $^{***}P < 0.001$; U73122, $n = 16$, $^{***}P < 0.001$; Gö6976, $n = 10$, $^{***}P < 0.001$). The continuous curves are Hill fits described by EC_{50} values of 677 pM, 33 nM and 10 nM and Hill coefficients of 0.40, 0.33, and 0.51 for the control, GDP-β-S and U73122, respectively.

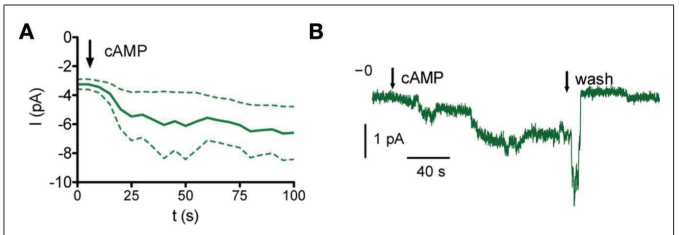

FIGURE 3 | Time course of Orco current activation. (A) Current response upon cAMP stimulation (bold, mean of four excised patches, inside-out configuration, dashed, SEM). **(B)** Sample trace of an inside-out patch. Arrows mark application or wash of 500 pM cAMP as indicated.

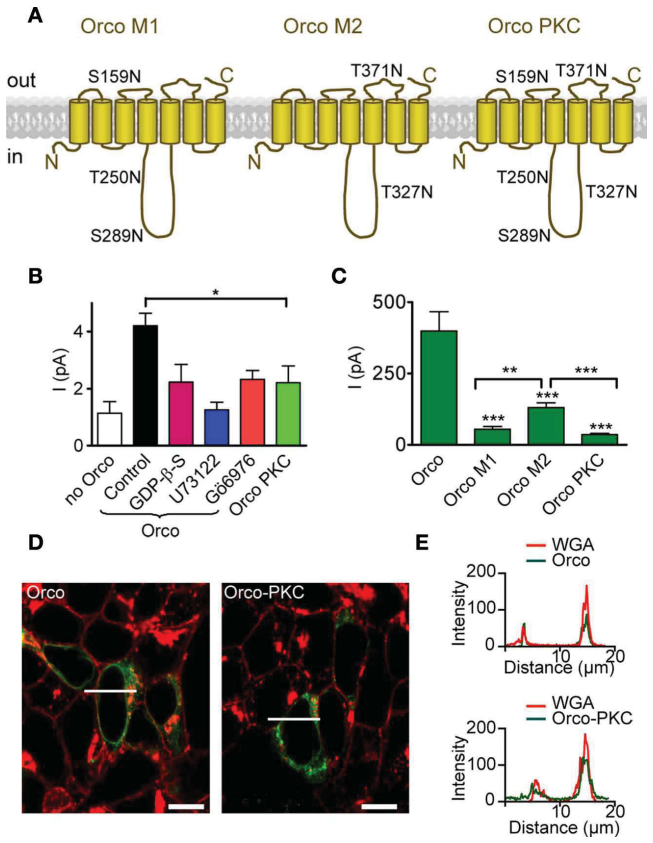

FIGURE 4 | Mutation of PKC phosphorylation sites in Orco reduces the resting current and cAMP responses. (A) Scheme of Orco topology with mutations of PKC sites indicated in the three mutants Orco M1, Orco M2, and Orco PKC. Two sites are predicted to be in extracellular loops (ECL2 and ECL3) while three sites are in the intracellular loop 2. **(B)** Mean inward currents in inside-out patches from non-transfected HEK293 cells ($n = 12$), cells expressing Orco and Orco PKC, measured at −60 mV without stimulation. The constitutive current through Orco (Control, $n = 43$) was reduced by GDP-β-S; (500 μM, $n = 13$, $^{*}P < 0.05$, Student's t-test.), U73122 (10 μM; $n = 10$, $^{**}P < 0.01$), and Gö6976 (1 μM; $n = 8$, $^{*}P < 0.05$), and it is significantly larger than in patches from non-transfected HEK293 cells ($^{***}P < 0.001$). Compared with Orco PKC containing patches ($n = 15$), only the Orco current in the control is significantly larger ($^{*}P = 0.02$). **(C)** Whole-cell current responses to 5 μM 8-br-cAMP in cells expressing Orco (WT) or the three PKC mutants, measured at −100 mV. (WT, $n = 11$; M1, $n = 10$; M2, $n = 15$; Orco PKC, $n = 11$; −4, $^{***}P < 0.001$; Student's t-test). Currents in M2 expressing cells are significantly larger than in M1 ($^{**}P = 0.002$) or Orco PKC ($^{***}P < 0.001$) expressing cells. Error bars represent SEM. **(D)** Confocal micrographs of HEK293 cells transfected with Orco and Orco PKC. Green, immunofluorescence; red, Texas-red fluorescence of wheat germ agglutinine (WGA) labeled plasma membrane; bar, 10 μm; line indicates position of intensity profile shown in **(E)**. **(E)** Intensity profile of Orco and Orco PKC immunfluorescence and membrane staining (WGA-Texas-red) in two cells displayed in **(D)**. Colocalization of fluorescence signals indicates membrane insertion of the Orco proteins.

(**Figure 4B**), indicating that Orco is spontaneously active even in the absence of cAMP. The resting current was reduced by GDP-β-S, U73122, and Gö6976 (**Figure 4B**). For the PKC phosphorylation mutant Orco PKC, the resting current was similar to non-transfected cells and Orco-expressing cells in the presence of these inhibitors, and was significantly lower than for Orco-expressing cells under control conditions (**Figure 4B**). Compared to native Orco stimulation, activation of the mutant with 8-bromo-cAMP in the whole-cell configuration or cAMP in inside-out patches produced very weak responses (**Figures 4C, 5** and **6**). Similarly, PMA failed to elicit a current in the absence of 8-bromo-cAMP (**Figure 5C**).

To confirm that impaired membrane targeting of Orco PKC did not account for the small current production by cAMP, we tested the distribution of Orco and Orco PKC immunofluorescence in the HEK293 cells (**Figure 4D**). Comparison of immunostaining with plasma membrane staining indicated that both Orco and Orco PKC were localized within the membrane (**Figures 4D,E**).

We also designed two partial mutants (**Figure 4A**), mutant 1 (S159N, T250N, S289N) and mutant 2 (T327N, T371N) which both showed no resting activity. We next asked how their response to 8-bromo-cAMP compared to that of Orco and Orco PKC (**Figure 4C**). The weakest response was seen in the complete mutant Orco PKC; the response obtained with mutant 2 was significantly stronger than that of mutant 1 and Orco PKC itself (**Figure 4C**). This indicates that the various

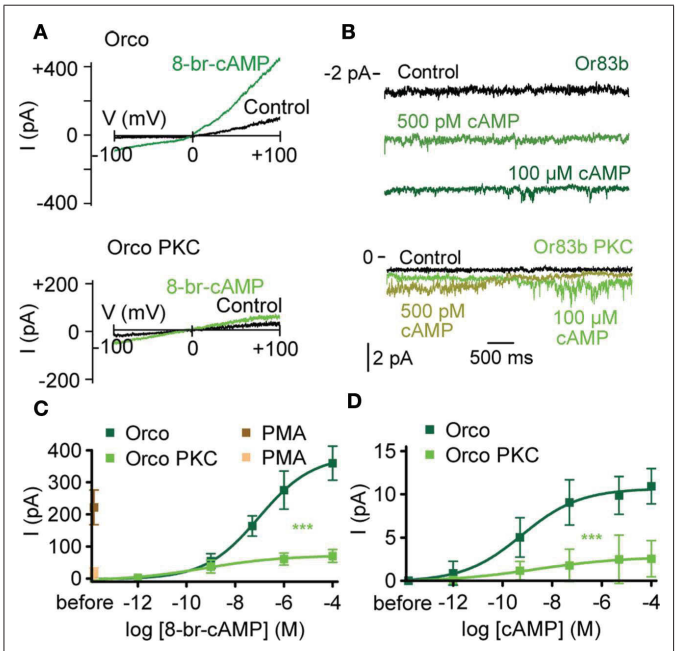

FIGURE 5 | Complete mutation of PKC phosphorylation sites in Orco disrupts cAMP responses. (A) Whole-cell current response in a cell expressing Orco (top) and Orco PKC (bottom) on a voltage ramp from −100 to +100 mV after breaking into the cell (Con) and 2 min after application of 8-br-cAMP (100 μM). **(B)** Current recordings from excised patches (inside-out configuration) of a cell expressing Orco (top) and Orco PKC (bottom) after breaking into the cell (Control) and 2 min after application of cAMP at 500 pM and 100 μM, respectively. Patches were held at −60 mV, bars indicate current level. **(C)** Concentration–response curve for 8-br-cAMP-induced Orco and Orco PKC whole-cell currents, measured at −100 mV. (Orco, $n = 10$; Orco PKC, $n = 13$, $^{***}P < 0.001$). PMA (1 μM) was applied in the absence of 8-br-cAMP. (Orco, $n = 11$; Orco PKC, $n = 10$). **(D)** Concentration–response curve for cAMP-induced currents in inside-out patches from cells expressing Orco and Orco PKC. Data represent maximum mean currents, measured at −60 mV. (Orco, $n = 13$; Orco PKC, $n = 13$, $^{***}P < 0.001$).

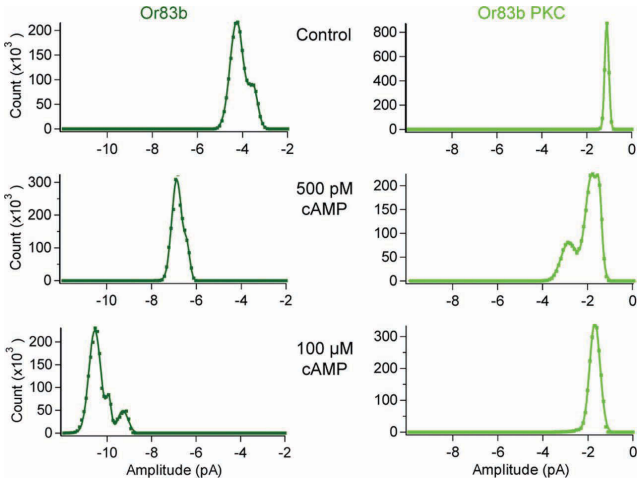

FIGURE 6 | cAMP responses in Orco and Orco PKC. All-point histograms for the data shown in **Figure 5B**. Currents from inside-out patches from a cell expressing Orco (top) and Orco PKC (bottom) after breaking into the cell (Control) and 2 min after application of cAMP at 500 pM and 100 µM, respectively. Patches were held at −60 mV, currents were recorded for 5 s (20 repetitions).

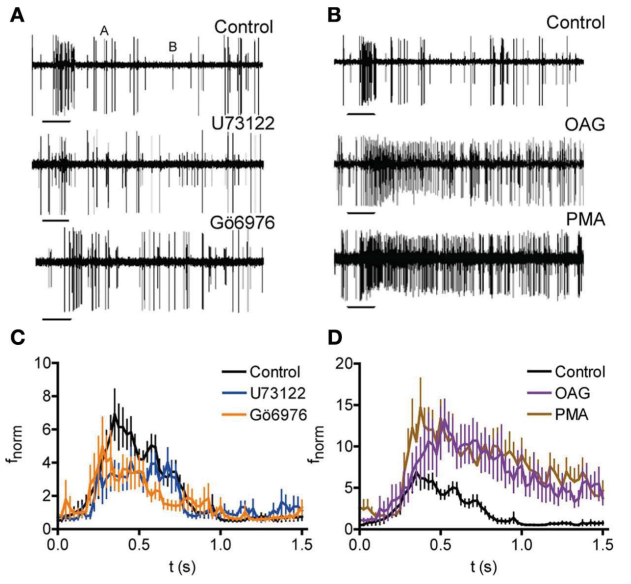

FIGURE 7 | Modulation of PKC-mediated phosphorylation affects the odor response of *Drosophila* OSNs. (A,B) Recordings of neuronal activity before and after Etb stimulation (−5 v/v; 0.5 s, bars) following injection of indicated compounds (Control, injection of receptor lymph solution). Etb stimulation enhances the activity of the A neuron but not of the B neuron. Inhibition of PLC (U73122) and PKC (Gö6976) reduces the Etb response of the A neuron **(A)** whereas activation of PKC (OAG, PMA) enhances the Etb response **(B)**. **(C)** Normalized spike frequency (f_{norm}) of the A neuron upon Etb stimulation (0–0.5 s, at −5 v/v) after injection of receptor lymph solution (Control; $n = 11$), U73122 ($n = 13$), or Gö6976 ($n = 12$). Both treatments reduce the odor response (for P see text). **(D)** f_{norm} as described in **(C)** after injection of ringer solution (Control; $n = 11$) OAG ($n = 8$) or PMA ($n = 10$).

phosphorylation sites contribute to cAMP-sensitivity in additive manner, and the extent of Orco response to cAMP stimulation may thus be regulated by the degree of its phosphorylation via PKC.

To test whether PKC phosphorylation affects the odor response of OSNs in the fly, we combined extracellular recording of OSN activity upon odor stimulation with injection of compounds affecting PLC/PKC activity. For these experiments, a microelectrode was inserted into the antenna near the base of large basiconic ab3 sensilla housing OSNs expressing the receptor protein Or22a. These neurons were localized under fluorescence using the GAL4-UAS system to drive GFP expression in Or22a expressing neurons (Dobritsa et al., 2003). Stimulation with ethyl butyrate (Etb; log[−5] dilution) accelerated the firing frequency of the Or22a expressing neuron (A in **Figures 7A,B**; $f_{norm} = 3.72 \pm 0.73$, stimulus window, $f_{norm} = 2.37 \pm 0.22$, total response, $n = 11$) without affecting the other neuron B), as shown by Hallem et al. (2004). Injection of the PLC inhibitor U73122 into sensilla diminished the response to Etb in comparison to sham injection (**Figures 7A,C**; $f_{norm} = 2.52 \pm 0.43$, stimulus window, $n = 13$; $P = 0.014$, Mann–Whitney U). By contrast, the inactive analog U73343 had no effect ($f_{norm} = 4.67 \pm 1.04$, stimulus window, $n = 11$, $P = 0.870$, Mann–Whitney U). The PKC inhibitor Gö6976 reduced the odor response in a manner similar to U73122 (**Figures 7A,C**; $f_{norm} = 2.57 \pm 0.43$, stimulus window, $n = 12$; $P = 0.023$, Mann–Whitney U). By contrast, injection of the PKC activators OAG or PMA caused a robust increase of the Etb response (**Figures 7B,D**; $f_{norm} = 7.06 \pm 1.45$ and 7.91 ± 1.17, respectively, total response, $n = 8$ and 10; $P = 0.003$ and <0.001, Mann–Whitney U).

DISCUSSION

Here we present evidence that the function of the odorant co-receptor Orco is controlled by its phosphorylation state via PKC. Regulation of ligand sensitivity by PKC phosphorylation has earlier been observed in, e.g., CNG channels. PKC activity can either enhance cGMP sensitivity (Müller et al., 1998) or reduce it (Müller et al., 2001). In the latter case the phosphorylation site was localized within the cGMP binding domain. Under normal physiological conditions the basal activity of PLC and PKC in HEK293 cells is sufficient to maintain Orco sensitivity to cAMP stimulation. Conditions leading to inhibition of these enzymes such as low free Ca^{2+} concentration suppress the activation of Orco by cAMP and thus may affect the odorant response. By contrast, high PLC/PKC activity would activate Orco independently of cAMP.

Inhibition of PLC function by GDP-β-S in HEK293 cells expressing Orco indicates constitutive activity of Orco–G_q protein pairs. Constitutive activity of GPCR-G protein pairs was, for example, reported in other receptors such as thromboxane receptors (Chillar et al., 2010) and mutations in adrenergic receptors (Cotecchia, 2010). In insect ORs, constitutive activity causing a receptor current in the absence of stimuli occurs in various heterologously expressed receptor heterodimers (Sato et al., 2008; Wicher et al., 2008), as well as in solely expressed Orco (**Figure 4B**). For Orco, background activity of PLC or PKC seems to be sufficient to maintain a phosphorylation state required for a constitutive activity (**Figure 4B**).

Odor stimulation of Or22a, either solely expressed or co-expressed with Orco in HEK293 cells, activated G_s proteins but not G_q proteins (Wicher et al., 2008). If this is a general rule

(which remains to be shown), odorant responses would not induce G_q downstream signaling. This signaling cascade would thus be available to modulate OR function, for example by neuromodulators. The experiments using combined single sensillum recordings and microinjection demonstrate the significance of up- and down-regulation of PLC/PKC activity for the odor response of OSNs. Enzyme inhibition reduced the frequency dynamics of the OSN response, while stimulation of PKC produced a more robust and prolonged OSN response (**Figure 7**). A recent study utilizing similar extracellular recordings in transgenic flies with various G protein mutations failed to see any effect of G proteins on the *in vivo* olfactory response (Yao and Carlson, 2010). However, genetically manipulated animals could contain some counter-regulation of the metabotropic effects such as adaptation or up-regulation of regulating enzymes (e.g., phosphodiesterases). This can even occur during transient expression of constitutively active G proteins.

An unexpected result of the experiments with excised patches from HEK293 cells expressing Orco was the slow activation kinetics of the current after fast cAMP stimulation (**Figure 3**). The heterologous system may lack components that *in vivo* accelerate its activation. However, in the case that this slow time course of Orco activation would be similar in the OSNs, metabotropic signaling would not be able to contribute to the fast odor response of these neurons. For example, a 0.5-s odor pulse gives rise to a response terminating after 2 s (**Figure 7**), whereas the response of Orco to cAMP took tens of seconds to develop (**Figure 3**). It must be noted that the compound microinjection mimicked metabotropic signaling processes initiated in a temporal domain before the odor stimulation.

Our study was not designed to determine whether insect ORs are mixed ionotropic and metabotropic receptors (Wicher, 2010) or metabotropically modulated ionotropic receptors (Nakagawa and Vosshall, 2009). However, the PKC mutant of Orco provides a useful tool to address this question in future investigations that assess the relationship between ionotropic and metabotropic signaling.

CONCLUSION

We have demonstrated a significant impact of the metabotropic pathway on olfactory response both in heterologous *in vitro* studies as well as in the *Drosophila* fly itself. Orco phosphorylation via PKC regulates OR sensitivity to cAMP, and therefore to odorants, and it may activate the receptor even in the absence of cAMP. This provides a powerful mechanism to adapt OR sensitivity not only via G_q proteins but also via free $[Ca^{2+}]_i$ levels.

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Chapter IV

Insect Odorant Response Sensitivity Is Tuned by Metabotropically Autoregulated Olfactory Receptors

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Insect Odorant Response Sensitivity Is Tuned by Metabotropically Autoregulated Olfactory Receptors

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Abstract

Insects possess one of the most exquisitely sensitive olfactory systems in the animal kingdom, consisting of three different types of chemosensory receptors: ionotropic glutamate-like receptors (IRs), gustatory receptors (GRs) and odorant receptors (ORs). Both insect ORs and IRs are ligand-gated ion channels, but ORs possess a unique configuration composed of an odorant-specific protein OrX and a ubiquitous coreceptor (Orco). In addition, these two ionotropic receptors confer different tuning properties for the neurons in which they are expressed. Unlike IRs, neurons expressing ORs are more sensitive and can also be sensitized by sub-threshold concentrations of stimuli. What is the mechanistic basis for these differences in tuning? We show that intrinsic regulation of Orco enhances neuronal response to odorants and sensitizes the ORs. We also demonstrate that inhibition of metabotropic regulation prevents receptor sensitization. Our results indicate that Orco-mediated regulation of OR sensitivity provides tunable ionotropic receptors capable of detecting odors over a wider range of concentrations, providing broadened sensitivity over IRs themselves.

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Introduction

Insects, for which olfaction is of primary importance for survival [1,2] possess remarkable chemosensory capabilities. Male silkworm moths, for example, are able to respond behaviourally to 3000 molecules/ml air [3]. Nevertheless, the cellular and molecular mechanisms underlying the outstanding sensitivity of the insect olfactory system are not well understood.

Insects are known to possess three different types of chemosensory receptors: odorant receptors (ORs), ionotropic glutamate-like receptors (IRs), and gustatory receptors (GRs) [4–6]. IRs are three-transmembrane proteins, whereas GRs and ORs are seven-transmembrane proteins [5–7]. Insect odorant receptors (ORs) also exhibit a unique configuration of heterodimers composed of an odorant-specific olfactory receptor protein (OrX) and a ubiquitous coreceptor (Orco) [7] which operate as ligand-gated ion channels [8,9].

The independent evolution [10,11] of these two different ionotropic receptor families (ORs/GRs and IRs) has become a great topic of speculation for the field (e.g. [2,12]). Why do these multiple families persist among all higher insect orders? And why do they possess such radically different molecular conformations? Initially, it was suggested that these multiple families expand the affinity of the olfactory palette to different chemical classes [6,13–15]. However, a recent study also revealed that olfactory sensory neurons (OSNs) expressing ORs, GRs, or IRs exhibit intrinsic differences in temporal kinetics to brief or intermittent stimuli [16]. Specifically, OR-expressing neurons respond faster and with higher sensitivity to brief stimulation, while IR-expressing neurons do not adapt to long stimulations. This implies that OR-expressing neurons are more accurate at detecting the low-concentration, punctate plume packets received at long distances from the odor source [17], while IR-expressing neurons can better track the high-concentration, long lasting stimulation received when on or near the source [16]. This diversity offers both broader ligand specificity and expanded spatiotemporal dynamics with which to parse the odor world, and is particularly important for insects challenged by the high-speed performance of flight [16]. Interestingly, the purported evolution of ORs [11,18] corresponds well to the evolution of flight during the Carboniferous Era (see [19]).

Given that ORs appear to offer mechanistic differences to IRs (c.f. [12,20]), what aspects of the OR molecular structure and/or function generate these advantages? Indeed ORs are ionotropic receptors, although their inverted 7-transmembrane topology is considerably different in structure to the 3-transmembrane IRs. In addition, the involvement of G proteins in the olfactory signal transduction of insect ORs remains controversial [21–23]. In heterologously expressed insect ORs, ligand application elicited a fast ionotropic current [8,9] that was accompanied by a slow, metabotropic current. Ligand binding to OrX led to enhanced cAMP production and activated an ion channel formed by the Orco protein [9]. We previously demonstrated that activators of phospholipase C (PLC) or protein kinase C (PKC) can stimulate Orco channel activity, while inhibition of PLC or PKC abolishes Orco sensitivity to cAMP [24].

Given the relatively low sensitivity exhibited by ionotropic receptors alone [16], might this suggested metabotropic activity contribute to the high olfactory sensitivity of insect ORs? To

address this question, we combined extracellular recording of OSN activity upon odor stimulation with simultaneous microinjection of compounds affecting metabotropic signalling [25]. This technique has been shown to mimic results obtained with in vitro manipulation of second messenger pathways [24,25]. We also address whether manipulation of the metabotropic pathway affects OSN sensitivity, response range, or sub-threshold sensitization of the neuron to repeated odorant stimulation. Finally, using a genetically manipulated fly with impaired Orco function we independently demonstrate the intrinsic nature of intracellular signaling for sensitizing ORs.

Materials and Methods

Extracellular Single Sensillum Recording and Microinjection

Recording and injection protocols performed on *Drosophila melanogaster* flies were as described [25]. 2–5 day old adults were fixed dorsally to a microscope slide [26,27]. For odor stimulation 10 µl of appropriate concentration was pipetted onto approximately 1 cm filter paper in disposable Pasteur pipettes. Charcoal-filtered and humidified air (approximately 1 l/min) passed over the antenna from a stimulus air controller (Syntech, CS-5, Hilversum, NL) through an aluminium tube approximately 10 mm from the antenna. During stimulation, airflow bypassed a complementary air stream (0.5 l/min during 0.5 s) through the stimulus pipette placed roughly 3 cm from the preparation. Compounds and concentrations for injection were diluted in saline [28] as follows: 8-br-cAMP (1 mM), U73122 (0.5 mM), Gö6976 (0.5 mM), SQ22536 (20 mM), OAG (0.1 mM), PMA (0.1 mM). Note that due to a dilution effect, concentrations of injected agents were 100x the concentration used in isolated cell preparations [25]. To check whether the injected compounds reach the outer OSNs dendrites where the ligand-receptor interaction occurs, we injected the Or22a agonist ethyl butyrate (Etb) at threshold concentration (−9 v/v) into the base of ab3 sensilla. During the 200 s injection period, Etb enhanced the spontaneous activity of the ab3A neuron expressing Or22a, but there was no change in activity for the ab3B neuron (Fig. S1A). To exclude mechanical artifacts that may affect OSNs during long lasting injection, we also tested the effect of saline and 8-br-cAMP microinjection which did not change OSN spontaneous activity over the 300 s recording period (Fig. S1B).

Recordings were performed in Or22a-GAL4; UAS-CD8-GFP flies expressing membrane tagged GFP in 22a-OSNs, and in flies whose endogenous Orco was replaced either with Orco or Orco mut in all Ors expressing OSNs.

Responses were analyzed between 500 and 1350 ms after stimulus onset, accounting for mechanical stimulus delay (150 ms). For response kinetics, spike frequency ratios were analyzed as peri-stimulus time histograms (PSTHs) in 25 ms bins by dividing each 25 ms frequency by the average pre-stimulus frequency over 2 s to give a normalized ratio for each time point. The PSTHs presented in the figures show the normalized means ± standard error of mean (s.e.m.) for *n* cells. Areas under the PSTH curve were measured for each response profile using the trapezoid rule and divided by the time to establish a normalized frequency average for each response.

Orco Mut and Transgenic Flies

Molecular biology and fly genetics. The Orco phosphorylation mutant "Orco mut" was generated as described for "Orco PKC" in [24]. Full-length Orco PKC (now named Orco mut) was digested from Orco PKC-pcDNA3.1(+) and subcloned into pUAST [29] using matching restriction sites. *Drosophila melanogaster* UAS-Orco mut transformants were generated at Aktogen Ltd (University of Cambridge, UK). Two independent lines were used in our experiments (UAS-Orco mut(1) and UAS-Orco mut(2)) with identical results. We generated Orco homozygote null mutant flies (Orco1) expressing either Orco mut (UAS-Orco mut(1) or UAS-Orco mut(2)) or Orco wild-type (UAS-Orco) in Orco22a OSNs (Or22a-Gal4). Control flies were Orco1 homozygote null mutant carrying UAS-Orco mut or UAS-Orco wild type insertions, but no Or22a-Gal4 driver. Antennae mRNA expression was confirmed by RT-PCR and *in situ* hybridization with specific primers and antisense digoxigenin-labeled RNA probe corresponding to Orco cDNA, respectively (not shown). Specific genotypes of flies used in this study were "no Orco": w/w; +/UAS-Orco mut; Orco1/Orco1; "Orco": w/w; UAS-Orco/UAS-Orco; Orco1-Or22a-GAL4/Orco1-Or22a-GAL4; "Orco mut": w/w; UAS-Orco mut/UAS-Orco mut; Orco1-Or22a-GAL4/Orco1-Or22a-GAL4.

Insect strains. *Drosophila* stocks were maintained on conventional cornmeal-agar-molasses medium under a 12 h light: 12 h dark cycle at 18°C or 25°C. Mutant alleles and transgenic lines used were: Or22aGAL4; UAS-CD8mGFP (Silke Sachse), Orco1, Orco2 (Bloomington Stock center, [30]), Orco-GAL4 (Bloomington Stock center, [30]), UAS-OrcoPKC(1), UAS-OrcoPKC(2) (this reference).

Immunofluorescences. Antennae sections were immunolabeled with primary antibodies against *Drosophila* Orco (1:1000) and Or22a (1:100) ([31]; kindly provided by Leslie Vosshall), and secondary anti-antibody conjugated to Alexa Fluor 568 (1:200, Invitrogen). Confocal images were obtained at 1-µm intervals over 20 µm Z-stack using a LSM510 Meta confocal microscope (Zeiss, Jena, Germany).

Data Presentation and Statistics

Results were given as means ± standard error of mean (s.e.m.), *n* = number of cells. The evaluation of statistical significance of differences was performed with two-way ANOVA for testing two variables. Mann-Whitney U tests (between treatments) and paired Wilkoxon Signed Ranks tests (within-treatment) compared responses using summary statistics calculated from areas under the peri-stimulus time histogram curve [26] using PASW (SPSS) v. 18 software.

Chemicals

All odors were purchased from Sigma (Taufkirchen, Germany). Ethyl acetate (Eta, >99%), ethyl butyrate (Etb, 99%), and methyl acetate (Mea, >98%) were dissolved in hexane (99%, Fluka Analytical, Buchs, Switzerland). Phenyl acetaldehyde (PAA >90%) and 1-hexanol (>99%) were diluted in mineral oil (BioChemika Ultra, Fluka); butyric acid (Ba, >99%) and 1,4-diaminobutane (Dab, >98%) were dissolved in water.

8-bromo-cAMP, forskolin, phorbol 12-myristate 13-acetate (PMA), and 9-(tetrahydro-2-furanyl)-9H-purin-6-amine (SQ22536) were obtained from Sigma; U73122, and Gö6976 from Calbiochem (Darmstadt, Germany); 1-oleoyl-2-acetyl-sn-glycerol (OAG) from Alexis (Lörrach, Germany).

Results

Repetitive Subthreshold Odor Stimulation Sensitizes ORs but not IRs

We inserted a glass pipette microelectrode into the base of large basiconic ab3 sensilla housing OSNs ab3A expressing the receptor protein Or22a, previously characterized in cultured cells [9] and stimulated the animal with the Or22a ligand [32], ethyl butyrate

(Etb). While an initial application of Etb at subthreshold concentration (log −10 dilution) failed to increase OSN activity (Fig. 1A, B), a second or third stimulation presented after at least 10 seconds produced significant odorant responses (Fig. 1A–C). With a 3 min interstimulus period, this sensitization was absent (Fig. 1B). Sensitization by repeated subthreshold odor stimuli were also observed in OSNs ac3B and ab2A expressing Or35b and Or59b, respectively (Fig. 1E, F), as well as in ab1A expressing Or42b (not shown).

However, repetitive subthreshold stimulation of ac3 OSNs expressing Ir75abc did not lead to an increased response after a second or third stimulation for interstimulus intervals ranging from 10 s to 3 min (Fig. 2A–D). In addition, ac2 and ac4 OSNs expressing Ir41a and Ir84a, respectively, could not be sensitized by repeated stimulation (Fig. 2E, F).

Metabotropic Signalling Shapes the Odorant Response of OSNs

We then asked whether manipulation of intracellular signalling in Or-expressing OSNs could affect the odor response. Injection of

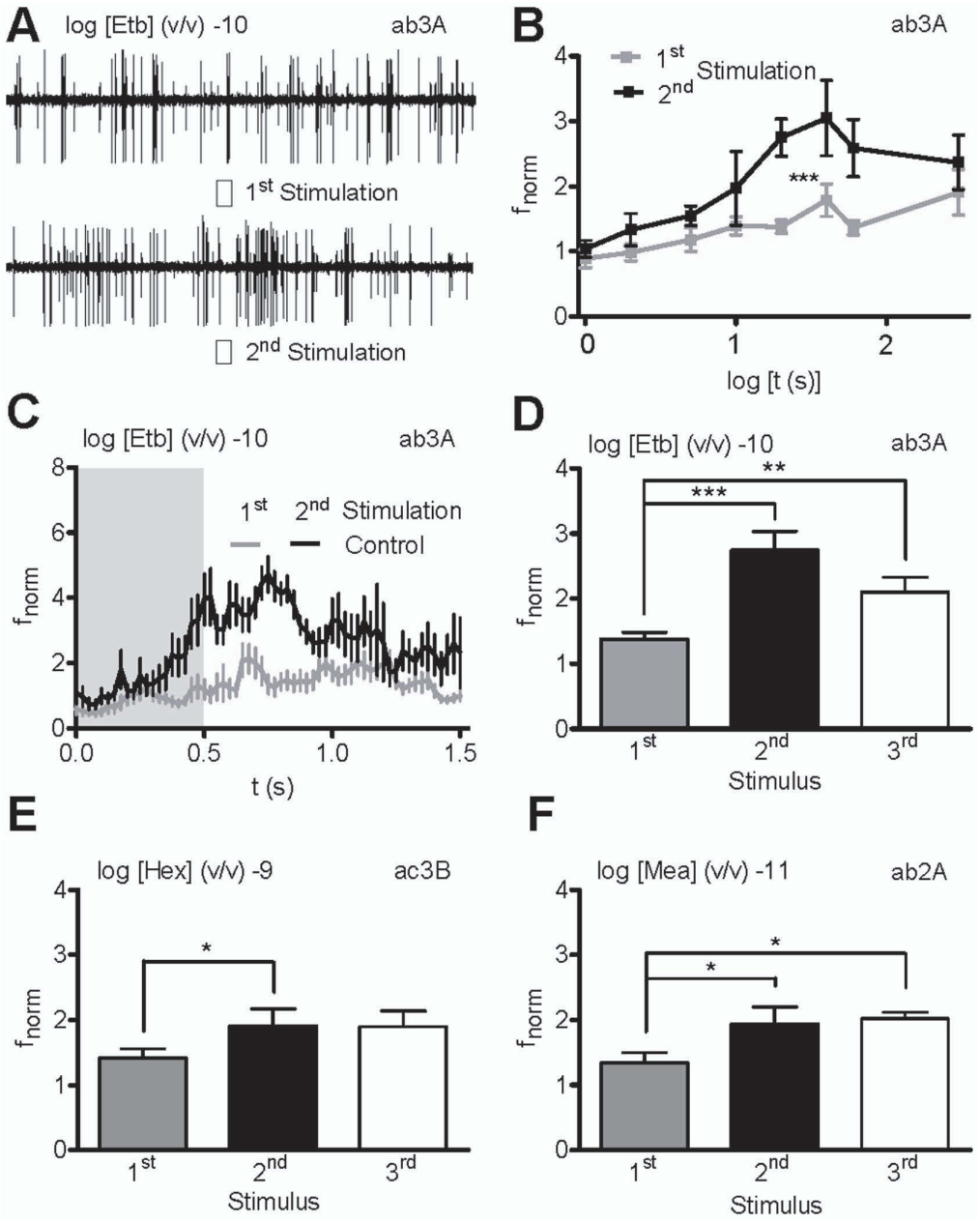

Figure 1. Repeated subthreshold stimulation sensitizes odorant receptors. A, Recordings of neuronal activity from ab3 sensilla (large action potentials, ab3A neuron expressing Or22a; small action potentials, ab3B neuron expressing Or85b) upon before and after 20 s repeated ethyl butyrate (Etb) stimulation (−10 v/v; 0.5 s, shaded area). The first stimulation fails to elicit a response while the second does so. **B**, Dependence of normalized ab3A neuron spike frequency (f_{norm}) upon 1st and 2nd subthreshold Etb stimulation (−10 v/v; 0.5 s) on the interval between stimulations ($n = 12$). **C**, Time course of f_{norm} for 1st and 2nd stimulation (interval 20 s, $n = 12$). **D–F**, Mean f_{norm} for ab3A (**D**), ac3B (**E**) and ab2A (**F**) neuron to repetitive subthreshold Etb (**D**), ethyl acetate (Eta, **E**) and methyl acetate (Mea, **F**) stimulations (interval 20 s, $n = 12$). $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$; Paired Wilcoxon Signed Ranks test.
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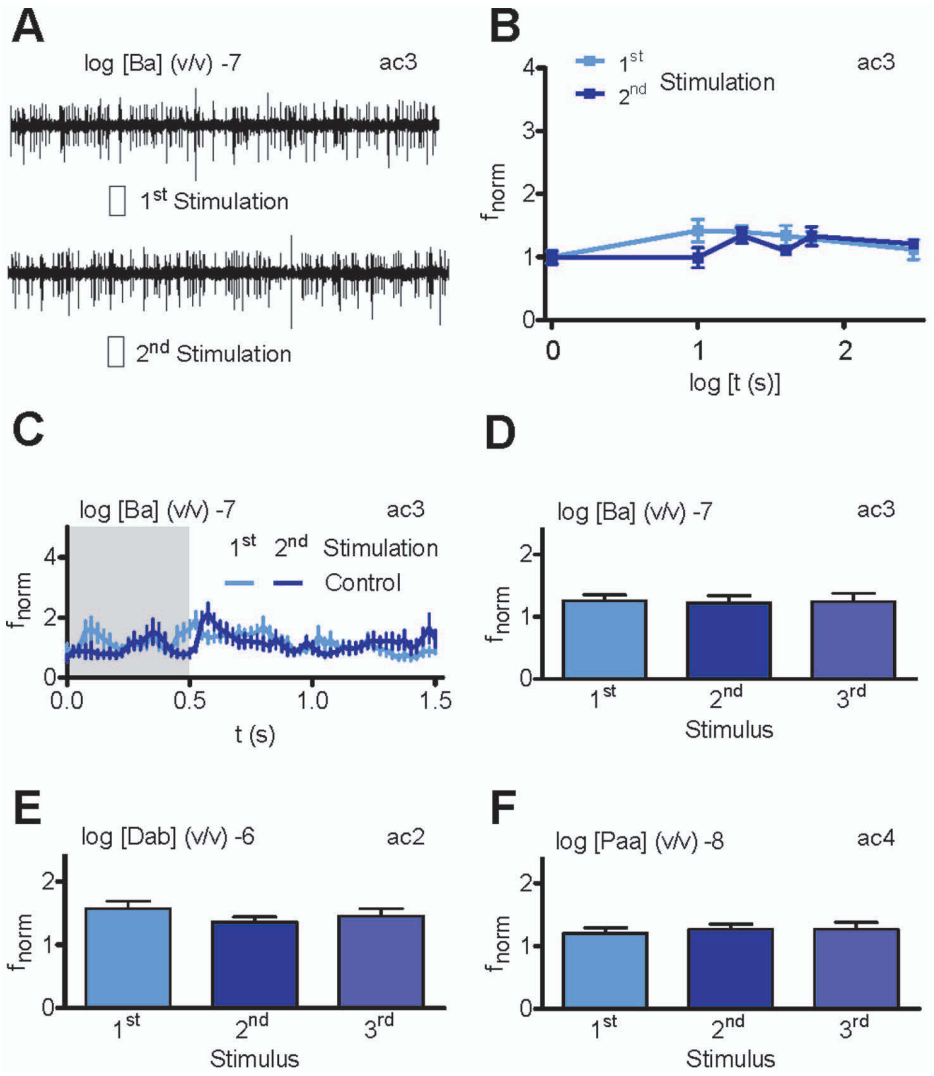

Figure 2. Repeated subthreshold stimulation does not sensitize ionotropic receptors (IRs). A, Recordings of neuronal activity from ac3 sensilla (large action potentials, Ir75abc neuron; small action potentials, Or35a neuron) upon before and after 20 s repeated butyric acid (Ba) stimulation (−7 v/v; 0.5 s, shaded area). Both stimulations fail to elicit a response. **B**, Dependence of normalized Ir75abc neuron spike frequency (f_{norm}) during 1st and 2nd subthreshold Etb stimulation (−7 v/v; 0.5 s) on the interval between stimulations ($n = 12$). **C**, Time course of f_{norm} for 1st and 2nd stimulation (interval 20 s, $n = 12$). **D–F**, Mean f_{norm} for Ir75abc (**D**), Ir41a (**E**) and Ir84a (**F**) neuron to repetitive subthreshold Ba (**D**), Dab (**E**) and Paa (**F**) stimulations (interval 20 s, $n = 12$). N.s.; Paired Wilcoxon Signed Ranks test.
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the adenylyl cyclase inhibitor SQ22536 into the base of ab3A sensilla reduced the response to Etb (Fig. 3A–C). In contrast, injection of 8-bromo-cAMP, a membrane-permeable cAMP analog shown to activate OR dimers such as Or22a/Orco and Orco alone [9], enhanced the OSN response upon Etb stimulation (Fig. 3A, B). In line with this result, microinjection of the adenylyl cyclase activator forskolin enhanced the Etb response and shifted the concentration-dependence curve towards lower Etb concentrations (Fig. 3C). Taken together, inhibition of cAMP production weakened odor responses whereas enhancement of cAMP levels, either by direct injection or by adenylyl cyclase activation via forskolin or cholera toxin (Fig. 3E) augmented them.

The sensitivity of the Orco channel mediating this metabotropic response to cAMP is regulated by protein kinase C (PKC)-dependent phosphorylation [24]. Inhibition of phospholipase C (PLC) or PKC reduced the odor response in the fly whereas PKC activation enhanced it [24]. We thus asked whether inhibition of PLC or PKC could counteract the response potentiation by cAMP. Co-injection of 8-bromo-cAMP with the PLC inhibitor U73122 or the PKC inhibitor Gö6976 not only prevented any cAMP effect, but even diminished the Etb response with respect to the Control injection (Fig. 3D). The sensitivity of the odor response is thus influenced by secondary regulation of Orco channel activity.

Regulation of OR Function is Intrinsic

Manipulation of intracellular signalling cascades may affect cellular targets other than ORs. Raising the cAMP concentration can, for example, activate cyclic nucleotide gated channels [33]. We thus inhibit Orco sensitivity to cAMP to assess whether the effect of intracellular signalling is intrinsic to the Or/Orco complex. The activation of Orco by cAMP requires a basal PKC-mediated phosphorylation [24]. We previously created an Orco mutant (called Orco mut) with excluded phosphorylation by

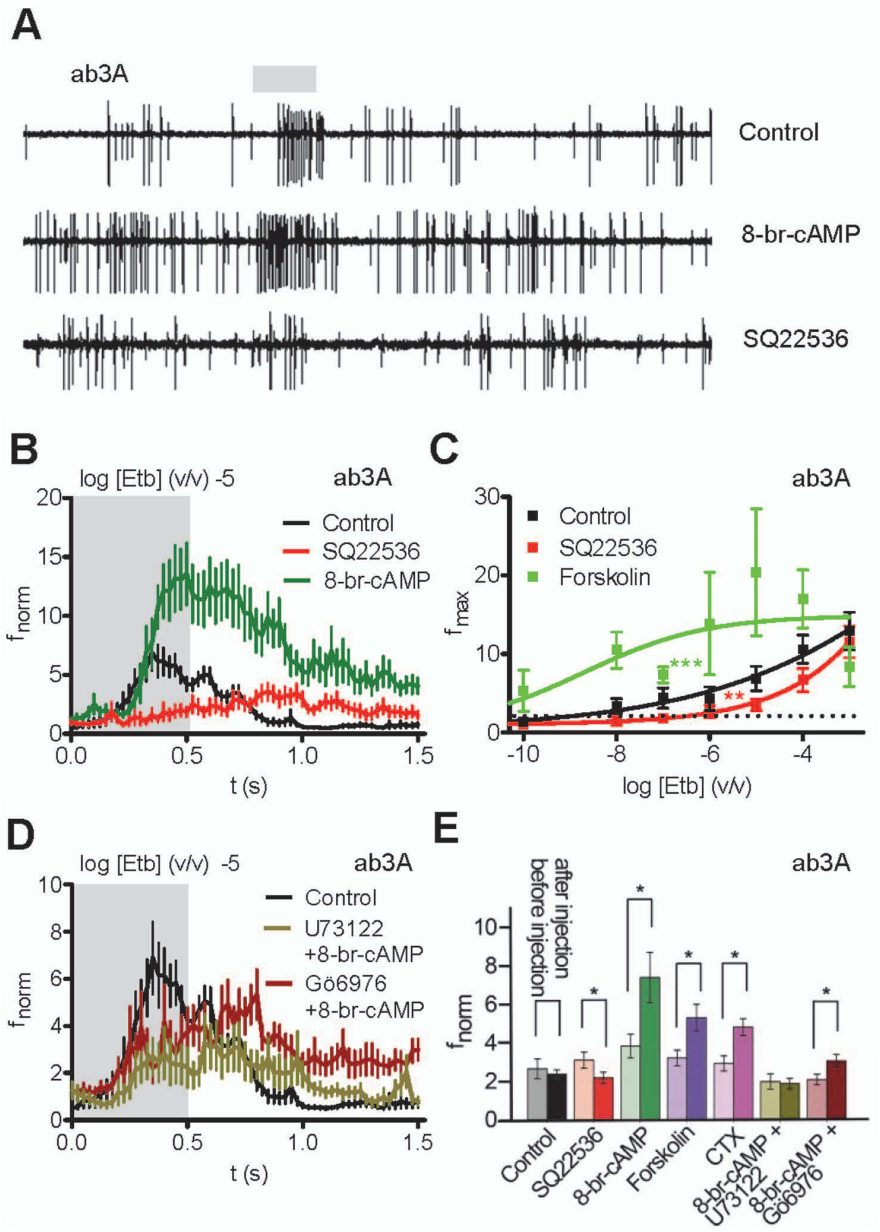

Figure 3. Manipulation of cAMP signalling in *Drosophila* ab3 sensilla affects the odorant response. A, Recordings of neuronal activity (large action potentials, Or22a neuron; small action potentials, Or85b neuron) before and after Etb stimulation (−5 v/v; 0.5 s, shaded area) in the presence of indicated compounds. While 8-br-cAMP enhances the Etb response, inhibition of adenylyl cyclase with SQ22536 attenuates it. **B**, Normalized spike frequency (f_{norm}) of ab3A upon Etb stimulation (0 to 0.5 s, shaded area) at indicated dilution after injection of saline solution (Control; $n = 11$), of 8-bromo-cAMP ($n = 11$; $P < 0.05$, Mann-Whitney U test) and of the adenylyl cyclase inhibitor SQ22536 ($n = 17$; $P < 0.01$, U test). **C**, Concentration dependence of the maximum frequency f_{max} of f_{norm} to Etb stimulation after saline, forskolin and SQ22536 injection (**$P < 0.01$, ***$P < 0.001$, ANOVA). **D**, f_{norm} as described in (B) after injection of saline solution (Control; $n = 11$), U73122 plus 8-br-cAMP ($n = 10$; $P = 0.18$, U test), and Gö6976 plus 8-br-cAMP ($n = 17$; $P = 0.16$, U test). In the presence of the PLC or PKC inhibitors 8-br-cAMP fails to enhance the odor response. **E**, Comparison of treatment effects on Etb response before and after microinjection. f_{norm} on Etb stimulation (0.5 s) as determined from area under the curve measurements of the total response (1.35 s). Responses to Etb were measured 20 s after commencement of recording (before injection) and 200 s after injection (after injection) of the control ($n = 11$), SQ22536 ($n = 17$), 8-br-cAMP ($n = 11$), forskolin ($n = 9$; data from Olsson et al., 2011), cholera toxin (CTX; $n = 12$), 8-br-cAMP plus U73122 ($n = 10$), and 8-br-cAMP plus Gö6976 ($n = 17$). Error bars represent s.e.m. Asterisks indicate significant differences ($P < 0.05$, Paired Wilcoxon Signed-Rank Test).
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S/T to N exchanges in all five PKC sites, which is virtually insensitive to cAMP [24]. By replacing the expression of Orco with Orco mut, we produced a fly line with an inactive metabotropic pathway. In Orco null mutant flies we rescued Orco or Orco mut (Fig. 4A) in all Or-expressing OSNs [31]. If our observed effect of intracellular signalling is extrinsic to the OR complex, then cAMP production should enhance the OR response even when Orco is insensitive to cAMP.

Antennal sections immunostained against Orco and Or22a (Fig. 4A) showed appropriate expression of Orco mut and Or22a proteins in the dendrites of "Orco mut flies", indicating that the chaperone function of Orco required to transfer the odorant-

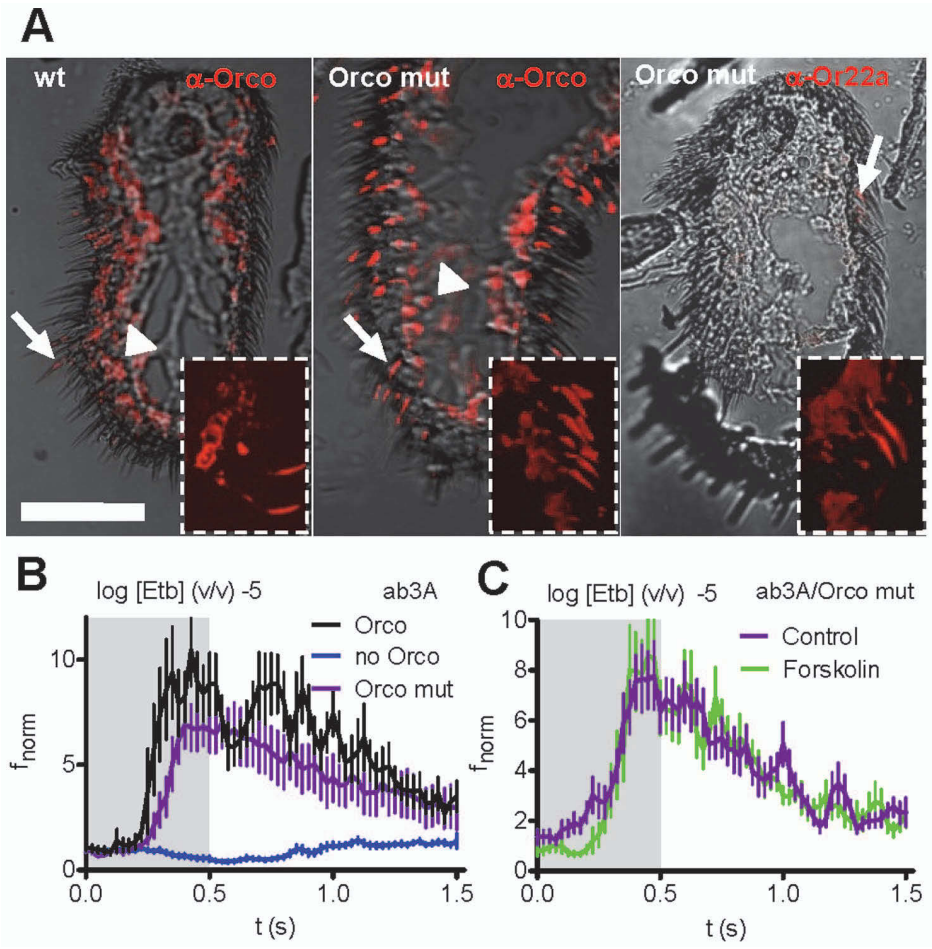

Figure 4. Regulation of OR response by cAMP signaling is intrinsic. A, Orco (left), Orco mut (middle) and Or22a (right) proteins visualized in adult antennal sections with specific antibodies (red). The proteins show expression in cell bodies (arrowhead) and dendrites (arrow). Or22a-expressing cells are housed in few sensilla opposite to arista (a). Scale bar 50 mm. **B**, Normalized ab3A neuron spike frequency (f_{norm}) upon Etb stimulation wild type flies (Orco, $n=12$), for Orco null mutants (no Orco, $n=15$), and mutants rescued with Orco mut ("Orco mut flies"; $n=14$; $P=0.016$ vs. Control, Mann-Whitney U test). **C**, f_{norm} as in **B** upon Etb stimulation in Orco mut flies ($n=17$) before (Control) and after forskolin injection.
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specific OR proteins into the plasma membrane [31] was not affected in Orco mut flies. Accordingly, these OSNs also responded to odorant stimulation (Fig. 4B). Nevertheless, injection of forskolin into ab3 sensilla did not change the Etb response (Fig. 4C; $f_{norm}=4.17\pm0.43$ before and 4.04 ± 0.55 after injection at log −5 Etb; $P=0.41$, paired Wilcoxon signed ranks test). To exclude a saturation of the odorant response at log −5 Etb in Orco mut flies, we also tested lower Etb concentrations. For log −6 Etb to log −8 Etb, forskolin injection also did not significantly change the maximum f_{norm} (Student's t test). This indicates that forskolin injection, and therefore intracellular signalling, acts on the OR complex intrinsically.

Orco Activation Sensitizes ORs and Orco Inactivation Prevents Sensitization

As repetitive subthreshold odorant stimulation was seen to elicit an OSN response, we asked whether cAMP production could sensitize ORs (Fig. 1). Adenylyl cyclase stimulation via microinjection of forskolin prior to subthreshold Etb stimulation (log −10 dilution) of Or22a-expressing OSNs induced a response already at the initial odor pulse (Fig. 5A). A similar effect was observed upon PKC stimulation with OAG or PMA microinjection (Fig. 5B). Thus, activation of Orco through intracellular signalling sensitizes the OR to respond to subthreshold odor concentration.

Inhibition of adenylyl cyclase via SQ22536 prevented receptor sensitization (Fig. 5C, D), and repeated subthreshold Etb stimulations failed to elicit a response in Orco mut flies, further indicating that receptor sensitization requires metabotropic signalling (Fig. 5E, F). In these flies, the essential role of Orco function for OSN sensitization was also shown for ab1 sensilla housing Or42b expressing OSNs and ab2 sensilla with Or59b expressing OSNs (Fig. 5G, H).

It should be mentioned that, although injection of cAMP for 200 s strongly enhanced the Etb response (Fig. 3B), it did not increase the spontaneous activity of the ab3A neuron (Fig. S1B). Thus, the stimulation of the odor response by Orco activation need not be accompanied by Orco pacemaker activity.

Discussion

Although both insect ORs and IRs operate as ionotropic receptors, their tuning properties differ fundamentally. While prolonged stimulation leads to adaptation of ORs, there is no adaptation of IRs [16]. On the other hand, ORs but not IRs

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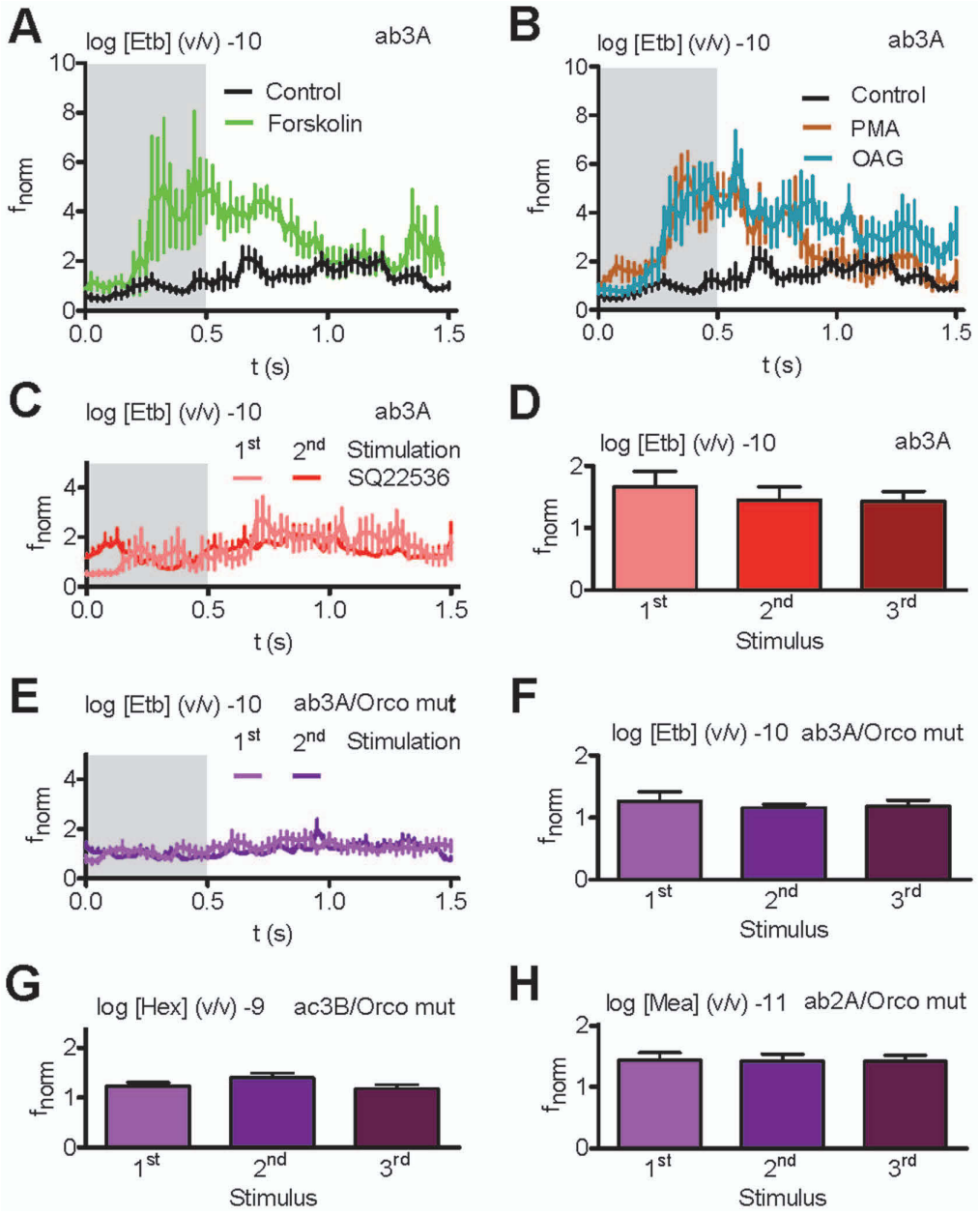

Figure 5. OR sensitization is mimicked by Orco activation and disrupted by Orco inhibition. A, **B**, f_{norm} for ab3A neurons expressing Or22a upon initial subthreshold Etb stimulations (log [Etb] −10) after injection of saline (Control;, forskolin (**A**, $n = 8$; $P < 0.05$, Mann-Whitney U test), and the protein kinase C activators PMA (**B**, $n = 7$; $P < 0.001$, U test) and OAG (**B**, $n = 7$; $P = 0.001$, U test). **C**, Time course of f_{norm} upon 1st and 2nd subthreshold Etb stimulation (log [Etb] −10, interval 20 s) after injection of SQ22536 ($n = 13$). **D**, Mean f_{norm} for Or22a neurons to repetitive subthreshold Etb stimulations (interval 20 s) after injection of SQ22536 ($n = 13$). **E**, Time course of f_{norm} for neurons expressing Orco mut upon 1st and 2nd subthreshold Etb stimulation (log [Etb] −10, interval 20 s, $n = 12$). **F – H**, Mean f_{norm} for ab3A (**F**), ac3B (**G**) and ab2A (**H**) neurons expressing Orco mut to repetitive subthreshold Etb (**F**), Eta (**G**) and Mea (**H**) stimulations (interval 20 s, $n = 8$–14). N.s.; Paired Wilcoxon Signed Ranks test.
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expand their dynamic range through intrinsic sensitization. This difference in sensitization is apparent even between ORs and IRs expressed in co-localized sensilla (c.f. Fig. 1E, Fig. 2B–D). Thus, sensitization must result from intrinsic, rather than extrinsic neuronal properties that are unique to ORs. The most parsimonious explanation for the mechanistic differences between these families, is the use of intracellular signalling to modulate OR activity [34]. Given the previous *in vivo* evidence for a role of metabotropic signalling in OR function [21,23,35–38], we first pursue the metabotropic regulation of Orco in mediating OR activity.

OR sensitization could be mimicked by manipulations enhancing cAMP production or PKC activity and depressed by inhibition of cAMP production or PLC/PKC activity (Fig. 5). These intracellular signalling systems not only influence the OR sensitivity at weak odor stimuli, they also modulate the OR response for stronger stimuli (Fig. 3). In detail, microinjection of cAMP or adenylyl cyclase activators into sensilla increased the odorant response and shifted the dose-response curve toward

lower odorant concentrations. A previous study has revealed that Orco sensitivity to cAMP is regulated by protein kinase C (PKC)-dependent phosphorylation [24]. Our results show that inhibition of PLC or PKC also inhibited any effect of cAMP, indicating that the enhanced sensitivity caused by cAMP is regulated by Orco activity. The metabotropic regulation of Orco also lead to sensitization of the OSN to repeated subthreshold odor responses, which is abolished by adenylyl cyclase inhibition. Furthermore, the sensitization of the odor response was blocked in mutant flies with impaired Orco phosphorylation (Orco mut) further indicating that metabotropic regulation of Orco activity is required for the enhanced odorant response. It cannot be excluded that cAMP and PKC activation may regulate OR sensitivity to odors via other mechanisms, such as through modulation of membrane traffick. Nevertheless, the lack of response modulation following injection of forskolin into PKC flies, indicates that the metabotropically-enhanced odor sensitivity is intrinsic to the OR complex and does not result from extrinsic cellular processes.

Our results thus suggest that intracellular signalling, and in particular metabotropic regulation of Orco, plays a vital role in conferring the mechanistic differences between ORs and IRs. Although we cannot yet confirm the mechanistic basis of intracellular signalling in these OSNs, we can conclude that modulations that activate Orco when heterologously expressed enhance the odor sensitivity of ORs *in vivo* and, vice versa, modulations that inhibit Orco reduce OR sensitivity. It must also be kept in mind that the ORs are Ca^{2+}-permeable, constitutively active ion channels [8,9], the background activity of which is also able to activate enzymatic activity. Future studies should characterize the composition of the respective signalling subsystems, e.g. those involved in sensitizing receptors vs. those involved in terminating the odorant response.

The evolution of a highly sensitive and adaptable olfactory system is believed to be a key factor allowing insects to radiate into more or less every environment on earth [2]. Given the importance of OSN dynamics in tracking turbulent odor plumes [39], olfactory sensitization via Orco regulation can enhance an insect's ability to accurately detect and respond to intermittent, low concentration stimuli [16]. Insect ORs are thought to have evolved from ionotropic gustatory receptors [40], which detect millimolar ligand concentrations [41]. Our results imply that the special heterodimeric design of ORs has likely evolved to quickly detect and respond to volatile compounds at very low concentrations, such as those encountered by flying insects. Regardless of the source of this difference, it is clear that the OR expansion of ionotropic receptors offers the insect olfactory system both broadened ligand affinity as well as expanded spatiotemporal dynamics with which to navigate the olfactory world.

Acknowledgments

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Supporting Information

Figure S1 Effect of compound injection on spontaneous activity of OSNs. A, Recordings of spontaneous spike frequency (f_{norm}, normalized to first 15 s of recording) for ab3A and ab3B neurons with injection of saline (Control, ab3A, $n = 5$) or ethyl butyrate (Etb, ab3A, ab3B, $n = 11$) at 100 s. **B**, Recordings of f_{norm} for ab3A neurons with injection of saline (Control, $n = 5$) or 8-br-cAMP ($n = 12$) at 100 s.
(TIF)

Author Contributions

Conceived and designed the experiments: SBO BSH DW. Performed the experiments: MNG SLL. Analyzed the data: SBO DW. Contributed reagents/materials/analysis tools: SLL. Wrote the paper: DW.

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Chapter V

The molecular basis for temporal resolution by insect olfactory receptors and its impact on odor-guided behavior

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Abstract

Insects are well-known for their exquisitely sensitive olfactory systems as well as their ability to localize odor information over long distances. The resolution of brief, repeated filaments of an odor plume is essential for flying insects to locate an odor source. However, the molecular mechanisms underlying plume resolution remain poorly defined. In the present study, we applied genetic, molecular, biochemical, physiological and behavioral analyses to investigate the molecular mechanisms underlying temporal resolution by *Drosophila* OSNs. Here, we show that disrupted phosphorylation by PKC in the ubiquitous coreceptor Orco impairs pulse resolution of OSNs at low odor concentrations. We further confirm that PKC activity is important for the OSN response, as a single mutation of either PKC53E or PKCδ abolishes the response of Orco-expressing cells to brief odor stimuli. In contrast, mutation of PKC genes does not affect the odor response of IR-expressing cells, which do not possess Orco. Finally, flies with Orco or PKC gene mutations do not respond to brief odor pulses in walking behavioral assays and have difficulty locating an odor source in free flight assays. Using our comprehensive approach, we conclude that a modulation of the OSN response by PKC in cells expressing Orco optimizes both sensitivity and speed of odor detection. This mechanism enables insects to quickly track dynamic

odor plumes, particularly while challenged by the high-speed performance of flight. We thus hypothesize that the unique Orco receptor complex found in insects may have resulted from the necessity for fast-odor tracking following the evolution of flight.

Introduction

Natural odors are structurally, quantitatively and spatiotemporally complex (Bruce et al., 2005). Once emitted from their source, odors are dispersed, mixed with background noise, and further diluted by the ambient motion of air to form a shifting and filamentous plume (Murlis et al., 1992, Vickers et al., 2001, Koehl, 2006). During odor localization, OSNs are then confronted with rapidly fluctuating, intermittent and repetitive odorant stimuli (Murlis et al., 1992, Nagel and Wilson, 2011) where the interaction of odorants and OSNs is brief (Kaissling et al., 1987, Vickers et al., 2001, Baker, 2009, Kaupp, 2010). It is known that pulsed odor stimuli increase the targeting efficiency of insects (Baker et al., 1985). However, the molecular processes that contribute to this sensitive and fast temporal resolution are not known.

The dendritic membranes of insect OSNs contain diverse families of chemosensory receptors including; odorant receptors (OR), gustatory receptors (GR) and ionotropic receptors (IR) (Stocker, 1994, Vosshall and Stocker, 2007, Benton et al., 2009) that transform chemical signals from the outside world into electrochemical signals. These receptors differ in their topology. While ORs and GRs are composed of 7-transmembrane proteins analogous to metabotropic receptors, IRs are related to ionotropic glutamate receptors. We have previously shown that OR-expressing OSNs were more sensitive to brief odor pulses than IR-expressing cells (Getahun et al., 2012). ORs also form heterodimers of an odorant-specific OR protein and a ubiquitous coreceptor (Orco) and both sub-units contribute to ion channel activity (Nichols, 2011, Pask et al., 2011, Nakagawa et al., 2012) however, the molecular mechanism still not fully understood.

The dendrites of insect OSNs are enriched with co-localized G-proteins such as $G_{\alpha}s$ and $G_{\alpha}q$ (Talluri et al., 1995, Laue et al., 1997, Miura et al., 2005, Kain et al., 2008, Boto et al., 2010,

Deng et al., 2011). Diverse PKC enzymes are also expressed in the olfactory organs of *Drosophila* (Rosenthal et al., 1987, Schaeffer et al., 1989), suggesting that a signaling cascade involving diverse PKC could be involved. Furthermore, there is evidence that OSN signal transduction is modulated by intracellular signaling in arthropods (Boekhoff et al., 1990, Breer et al., 1990, Ziegelberger et al., 1990, Stengl, 1993, Stengle, 1993, Maida et al., 2000, Martín et al., 2001, Gomez-Diaz et al., 2004, Kain et al., 2008, Wicher et al., 2008, Stengl, 2010, Deng et al., 2011, Getahun et al., 2013, Nolte et al., 2013).

Here, we investigate the molecular mechanisms underlying the detection of brief and intermittent odor pulses that insects encounter in odor plumes. We use a combination of biochemical, molecular, and physiological tools accompanied by behavioral experiments. Our data demonstrate that the temporal resolution of OSNs expressing ORs is regulated by PKC phosphorylation of Orco, while the response of OSNs expressing IRs is not affected by modifications to PKC. We further show that this signaling mechanism affects insects abilities to exhibit efficient odor tracking in behavioral assays. We discuss these results as well as their implication for optimizing sensitivity and speed of plume resolution, particularly for flying insects.

Materials and Methods

Drosophila Stocks. All experiments were performed on adult 2-6 day old wild type *D. melanogaster* CS male and female flies. Stocks were maintained on conventional cornmeal agar medium under a 12 h light: 12 h dark cycle at 25°C. The Orcomut flies were as described in (Getahun et al., 2013): endogenous Orco (Orco wt) was replaced with a mutated version of Orco with excluded phosphorylation by S/T to N exchanges in all five PKC sites, which is virtually insensitive to cAMP (Sargsyan et al., 2011) (Orcomut) in all Orco cells, using the GAL4/UAS system (Brand and Perrimon, 1993). For that we rescued an Orco null mutation (Larsson et al., 2004) using Orco-Gal4 and UAS-Orcomut, or UAS-Orco wt as a control. The independent insertions Orco-GAL4/+, UAS-Orco/+ and UAS-Orcomut/+ had no effects on the physiology of OSNs, as previously characterized (Getahun et al., 2013). PKC53E EY14093 (BL 20790) mutants were obtained from Bloomington at Indiana University, USA. This mutation was previously characterized by (Murillo-Maldonado et al., 2011) and homozygous are viable. PKCδ e04408 mutants were also obtained from the Bloomington (BL18258) *Drosophila* stock center but the mutation was characterized in our laboratory. PKC δ homozygous mutants are viable and fertile, and there is no apparent effect of the mutation on antennal morphology, OSN neuronal amplitude, or spontaneous activity. The absence of PKC δ gene was confirmed using PCR using the following primers (5'-GTACCTGAATGGCGGTGATC-3' FOR and 5'-CAAACGACCACCAATCCACA-3' REV). We also used the RNAi interference technique to turn down the expression of PKC δ and PKC53E specifically in the OSNs. Lines BL28355 for PKCδ and BL27491for PKC53E RNAi were obtained from the Bloomington *Drosophila* stock center. To get a stable line for behavioral experiments as well as to increase expression of RNAi both fly lines were made homozygous.The genotype of the flies generated are listed as follow:

PKCδ RNAi are w/w; Orco-Gal4/Orco-Gal4; UAS-ds RNA (PKCδ)/UAS-ds RNA (PKCδ) and PKC53E RNAi are w/w; Orco-Gal4/Orco-Gal4; UAS-ds RNA (PKC53E)/UAS-ds RNA (PKC53E). Control lines UAS-ds RNA (PKCδ)/+ and UAS-ds RNA (PKC53E)/+ had no effect both on the physiological and behavioral response (data not shown).

Odor Stimuli. All the odors were obtained from Sigma Aldrich at highest purity: ethyl butyrate (> 98 %), 2-heptanone (> 98 %), ethyl hexanoate (> 98 %), methyl acetate (> 98 %), ethyl acetate (> 99 %), 1-hexanol (> 99 %), ethyl-3-hydroxybutyrate (> 98 %), 2, 3-butane diol (> 97%), pentyl acetate (> 99 %); phenyl acetaldehyde (> 90 %), these compounds were diluted in mineral oil (BioChemika Ultra, Fluka). However, the following odors 1, 4-diaminobutane (> 98 %); Propionic acid (> 99.5 %), butyric acid (> 99 %) all dissolved in water). For frequency stimulation, we used a custom-built multicomponent stimulus system similar to (Olsson et al., 2011, Getahun et al., 2012). The consistency of odor delivery for different frequencies at log [-3] dilution was confirmed using PID 200a (Aurora Scientific Ontario, Canada). The PID sensor was placed at the outlet of the odor delivery tube at the same position as the insect.

Electrophysiology. A fly was mounted in a cut pipette tip with wax as in (de Bruyne et al., 1999, Hallem et al., 2004, Yao et al., 2005, Getahun et al., 2012). An electrolytically sharpened tungsten electrode was placed in the eye for grounding and a sharpened tungsten-recording electrode was brought into contact with the base of the sensillum using a Luigs and Neumann SM-59 manipulator (Ratingen, Germany) at 1000x magnification with an Olympus BX-51 microscope (Olympus Corporation, Tokyo, Japan). OSN action potentials were amplified, recorded, and analyzed using Syntech equipment as in (Getahun et al., 2012). All physiological data were acquired using Auto spike 3.7 (Syntech Ockenfels, Germany), and OSN spikes were detected using the same software.

Biochemistry. PKC analyses were performed according to (Ziegelberger et al., 1990, Maida et al., 2000). Briefly, olfactory antennae of *Drosophila* were cut under binocular light microscope using fine forceps and the isolated sensillum was immediately stimulated with a headspace mixture of odors (ethyl hexanoate, ethyl butyrate, ethyl acetate, methyl acetate, 2,3-butane diol, 2-heptanone, 1-hexanol, pentyl acetate, E-3-hydroxybutyrate). All odors were diluted in mineral oil to [-5] dilution v/v with 100 µl of each mixed in a single vial and stimulated using a custom stimulus device (Olsson et al., 2011). The antennae were stimulated with two 500 ms pulses of the odor mixture and then immediately frozen in liquid nitrogen. For each analysis approximately 200 antennae were used per replicate, and stored at -80 °C until for further analysis. The antennae were crushed under liquid nitrogen and homogenized in 20 mM Tris-HCL (pH 7.2) in the presence of a protease inhibitor cocktail (from Sigma Aldrich, Germany), and 2.5 % octylglucoside at 4 °C for 20 minutes. The homogenate was centrifuged at 10000 rpm for 15 minute at 4 °C and the supernatant used for ELISA. PKC kinase kits were obtained from Enzo Life science (Lörrach, Germany) using synthetic peptides as a substrate for PKC and a polyclonal antibody that recognizes the phosphorylated form of the respective substrate. The activity of the kinase was measured with color development and absorbance measured at 450 nm. The protein concentrations were determined using BRADFORD BSA. The relative kinase activity was calculated as follows [Average absorbance (sample)-Average absorbance (blank)]/amount of crude protein used per assay.

Immunofluorescence. Frozen antennae sections were fixed 10 minutes in 4% paraformaldehyde, washed three times in phosphate buffer (1XPBS) and permeabilized 30 minutes in 0.02% triton-X100-PBS (PT). After 1 hour blocking with 5% normal goat serum (NGS, Invitrogen) in PT the antennae sections were immunolabeled with primary antibodies

against *Drosophila* PKC (goat) (dn-16) Sc-15726, lot K 1102, Santa Cruz Biotechnology, Germany) (1:500) and Orco (rabbit, 1:1000) provided by L. Vosshall; and incubated at 4° C overnight. Samples were washed three times in PT during 10 minutes and blocked during 30 minutes with 5% NGS before incubating with the secondary anti-antibodies conjugated to Alexa Fluor 488 (anti-rabbit) and Alexa Fluor 568 (anti-goat) (1:200, Invitrogen). Samples were washed three times in PT before mounting in Vectashield (Sigma). Confocal images were obtained at 1-mm intervals over 20 mm Z-stack using a LSM510 Meta confocal microscope (Zeiss, Jena, Germany).

Behavioral assays. A glass Y-tube olfactometer was used under ambient laboratory conditions. 30-40 starved individual female *Drosophila* were tested with a single 100 or 500 ms pulse of ethyl acetate log [-5] dilution v/v) released into one of the arms. Flies were given 1 min to respond once the fly reached the tube junction (Figure 7A left). Fisher's exact test (SPSS, Inc.) was applied to compare responses to odor vs. control between genotypes and pulse durations and Chi-square goodness of fit test used within groups. Flywalk experiments were performed and raw data was pre-processed as described (Steck et al., 2012). Briefly, 15 individual female flies, starved for 24 h, were placed in parallel aligned glass tubes and their positions recorded under red-light conditions (λ = 630 nm) over a period of ~8 h. In every experiment, both wild type (wt) and Orco-mut flies were tested to avoid technical artifacts. Flies were continuously exposed to a humidified airflow (~20 °C, ~75 % rh) of 0.3 l/min (20 cm/s in the glass tubes). Repeated odor pulses (inter-stimulus interval 90 s) were released from a multicomponent stimulus device (Olsson et al., 2011) loaded with 100 µl of odor dilutions in mineral oil. Responses were calculated as the mean distance flies covered within 4 s after encounter with the odor pulse. In the trap assay thirty 24 h starved female flies were released in a 50 cm x 50 cm x 50 cm mesh

cage (Faucher et al., 2013). From preliminary tests, fly catches using either vinegar alone or single odor were low. For that reason 100 µl of vinegar was used as a background for 100 µl ethyl acetate at [-2] dilution, and number of flies caught was counted after 1, 12 and 24 H.

Data Analysis. Co-located neurons were identified based on spike amplitude. Peri-stimulus time histograms (PSTHs) were obtained by averaging spike activities in 25 ms bins. Spike frequencies after stimulus onset were normalized to the average spontaneous frequency 2 seconds before stimulation. Sigmoidal concentration response curves were fitted using Graph Pad Prism 4 with variable slope parameter (Graph Pad Software Inc. La Jolla California USA). Independent t-test was used to compare two treatments. To evaluate the capacity of the OSNs to resolve pulsed stimuli, we quantified the % return to spontaneous activity (base line) between consecutive pulses as: Percent return to baseline = 1- (1^{st} response frequency value of the 2^{nd} stimulus pulse / maximum response frequency of the 1^{st} stimulus pulse)*100 (Bau et al., 2002, Getahun et al., 2012). A one-way ANOVA followed by Tukey Post Hoc test was performed to see if the return to the base line was significantly reduced between the frequencies of stimulation. Latency was measured as the time from the onset of the odor stimulus to the maximum response frequency (mechanical delay was not considered). The kinase activity in stimulated homogenate was normalized as a ratio to the un-stimulated samples and PKC activity was compared using an independent t-test. Dose response curves were compared using independent t-tests between individual dilutions. Behavioral data was analyzed using independent t-tests (Free flight assay) and Fisher's exact test (Y-maize) and Mann-Whitney test (Fly walk). All statistical calculations were performed using SPSS version 17(SPSS Inc. Chicago IL USA.)

Results

Orco phosphorylation by PKC is required for temporal resolution of brief and intermittent odor stimuli.

Wild type OSNs expressing the olfactory receptor 22a (Or22a-OSNs) exhibited a significant response already when challenged with a 20 ms odor pulse of ethyl butyrate [-5] dilution; $P = 0.02$; Figure 1 A). In the absence of PKC phosphorylation of Orco (Orco-mut flies), the response of Or22a Orcomut OSNs to brief odor pulses was significantly reduced (Figure 1 B) and 10x longer stimulations were required to elicit a significant response ($P < 0.05$ up to 200 ms duration; Figure 1 B, C). The response to brief, repeated stimulations was also abolished in Orcomut OSNs (Figure 1 D). Although Orcomut OSNs could resolve longer odor pulses, they also showed a delayed response to these compared to the wild type (Figure 1 E). This indicates that the pulse-following capacity of OR-expressing OSNs to brief, low-concentration odor stimuli, as encountered in a natural odor plume, is reduced in the absence of Orco-mediated phosphorylation. To confirm that the above physiological effects were attributable to the mutations in Orco itself, we rescued Orco-wt using the Orco GAL4/UAS system (Brand and Perrimon, 1993). In these rescued flies, both response speed and sensitivity were recovered, and displayed no significant difference as compared to wild type OSNs ($P > 0.05$; Figure 1 C). Interestingly, the response to brief stimulations and pulse resolution of Orco-mut flies could also be recovered by increasing the stimulus concentration by 100x (Figure 1F), further indicating that the effects observed are a result of reduced sensitivity in the absence of PKC-mediated phosphorylation of Orco. This effect of PKC-mediated phosphorylation was also observed for other ORs expressed in both antenna and palp OSNs (Figure 2). In addition, the concentration-response curves of Orcomut cells expressing various receptors was also shifted to the right at

brief stimulations (50 ms; Figure 3 left and Table 1; note higher EC 50 values), while at longer stimulations (500 ms) Orco mut cells showed saturation at lower concentrations.

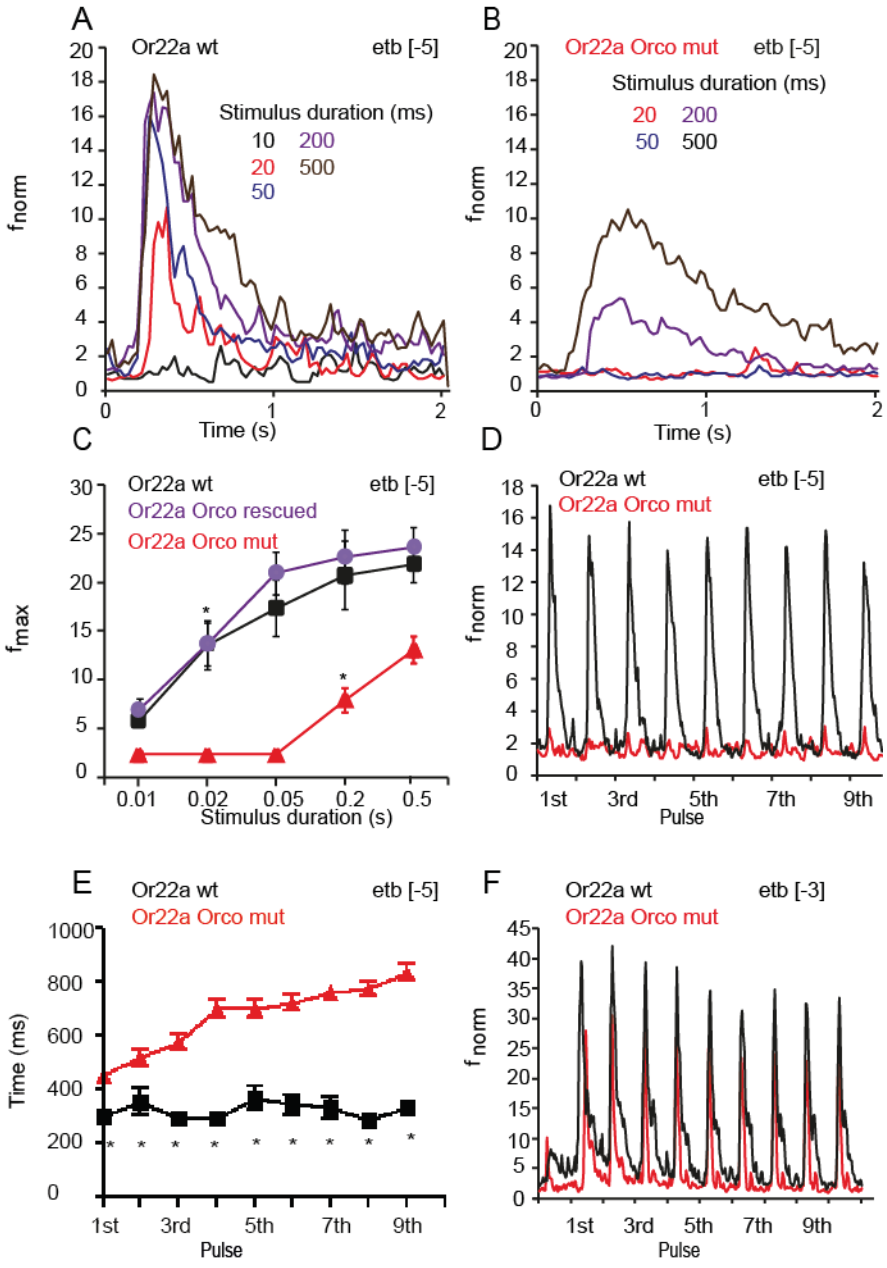


Figure 1. Odorant-induced responses of OSNs in wild type and Orco-mut flies. (A) Mean peri-stimulus time courses of normalized responses (Hz) for Or22a wt OSNs responding to ethyl butyrate [-5] dilutions at varying pulse durations (10 ms to 500 ms). Each trace represents the average of 7 - 13 trials. (B) Response of Or22a Orco mut OSNs to the same stimulus regime as

in (A) (n = 11 - 15). (C) Average maximum response frequencies (Hz) for Or22a wt and Or22a Orco mut OSNs in (A) and (B), and Orco rescued OSNs to the same stimulus regime as in (A) (n = 8 - 9 for Orco rescue flies; error bars indicate SEM). (D) Mean peri-stimulus time courses of normalized responses frequencies of Or22a wt and Or22a Orco mut OSNs to repeated 50 ms pulses of ethyl butyrate [-5] at 1 Hz (n = 12 for wt and n = 22 for Or22a Orco mut OSNs). (E) Time to maximum frequency in ms to nine consecutive 500 ms pulses of ethyl butyrate [-5] (n = 9 for wt, and 21 for Or22a Orco mut OSNs), * $P < 0.05$ independent t-test; error bars, SEM. (F) Mean peri-stimulus time courses of normalized responses frequencies of Or22a wt and Or22a Orco mut OSNs to repeated 50 ms pulses of ethyl butyrate [-3] at 1 Hz (n = 10 for wt and n = 11 for Or22a Orco mut OSNs).

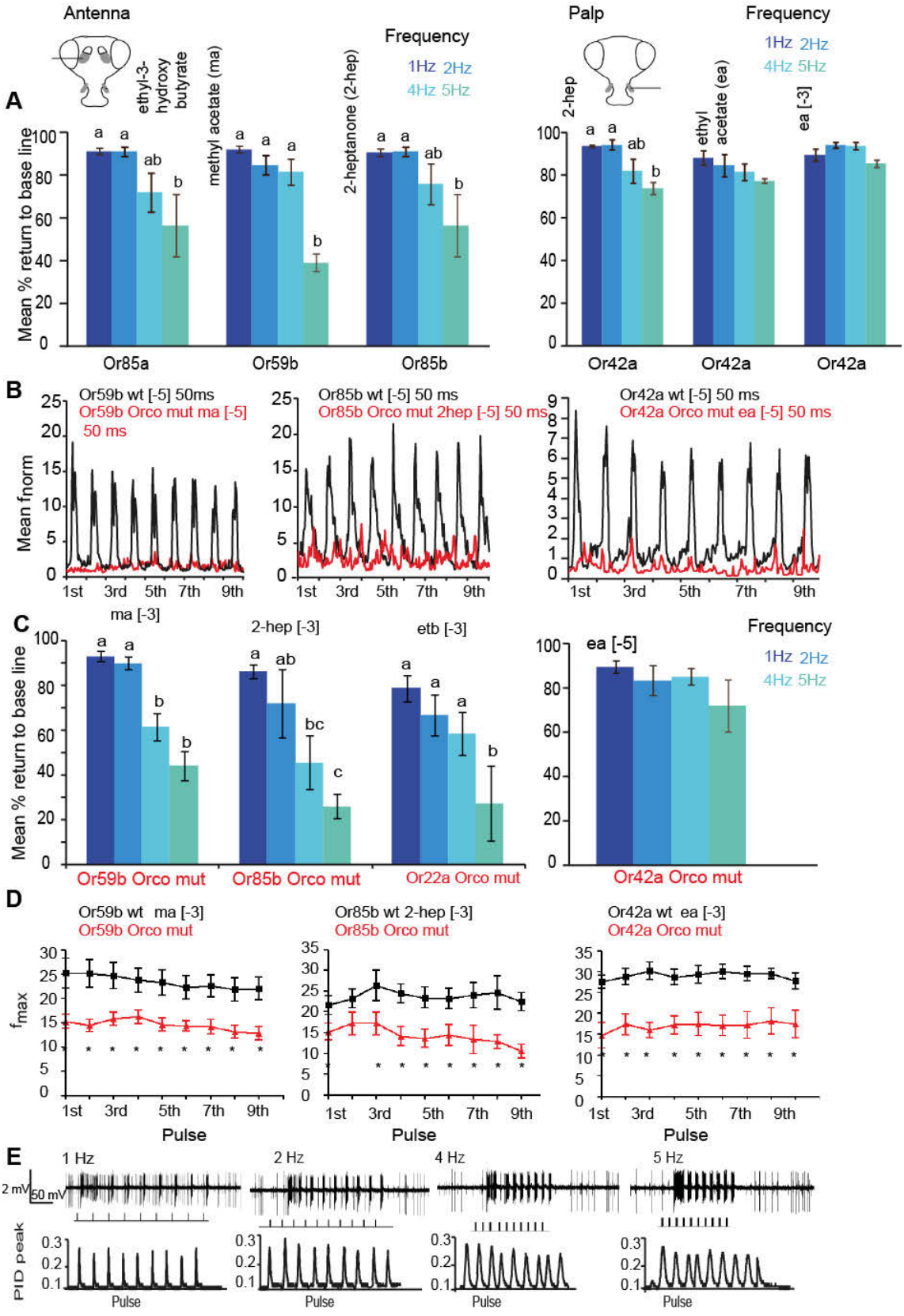

A
Antenna
ethyl-3-hydroxy butyrate
methyl acetate (ma)
2-heptanone (2-hep)
Frequency
1Hz 2Hz
4Hz 5Hz
Mean % return to base line
Or85a
Or59b
Or85b
Palp
2-hep
ethyl acetate (ea)
ea [-3]
Or42a
Or42a
Or42a
B
Or59b wt [-5] 50ms
Or59b Orco mut ma [-5] 50 ms
Or85b wt [-5] 50 ms
Or85b Orco mut 2hep [-5] 50 ms
Or42a wt [-5] 50 ms
Or42a Orco mut ea [-5] 50 ms
Mean fnorm
1st 3rd 5th 7th 9th
C
ma [-3]
2-hep [-3]
etb [-3]
ea [-5]
Frequency
1Hz 2Hz
4Hz 5Hz
Mean % return to base line
Or59b Orco mut
Or85b Orco mut
Or22a Orco mut
Or42a Orco mut
D
Or59b wt ma [-3]
Or59b Orco mut
Or85b wt 2-hep [-3]
Or85b Orco mut
Or42a wt ea [-3]
Or42a Orco mut
fmax
1st 3rd 5th 7th 9th
Pulse
E
1 Hz
2 Hz
4 Hz
5 Hz
2 mV
50 mV
PID peak
Pulse

Figure 2. Response of diverse OR-OSNs and Orco-mut OSNs to repeated pulsed stimuli (A) Pulse resolution of four OSNs expressing different receptors both on antenna and palp expressed as mean % return to baseline across 9 consecutive odor pulses (see Methods for odor concentrations; n = 7 - 10 for Or85a, n = 9 - 13 for Or59b, 9 - 10 for Or85b, and 10 - 12 for Or42a; error bars indicate SEM; letters indicate significant differences between frequencies, ANOVA followed by Tukey Post Hoc test). (B) Mean normalized frequency of OSNs tested in (A) as well as their Orco mut to 1 Hz, 50 ms repetitive stimulations of odors (n = 6 - 8). (C) Mean % return to baseline across 9 consecutive odor pulses for Orco mut OSNs at 10x higher concentration (n = 6 for Or59b and 6 - 7 for Or85b, n = 10-12 for Or22a, n = 5 - 8 for Or42a, error bars indicate SEM; letters indicate significant differences between frequencies, ANOVA followed by Tukey Post Hoc test). (D) Mean maximum normalized frequencies for wt and Orco mut OSNs responding to 1 Hz, 50 ms stimulations of respective ligands at [-3] concentration. Orco mut data as in (C) Rrror bars indicate SEM; asterisks show significant difference, $P < 0.05$, independent t-tests. (E) Sample traces of Or22a Orco mut showing response to 50 ms pulse of etb [-3]. Square pulses indicate stimulus presentation. Below each frequency traces shows the PID measurement of etb [-3] pulses at specified frequency (n = 5).

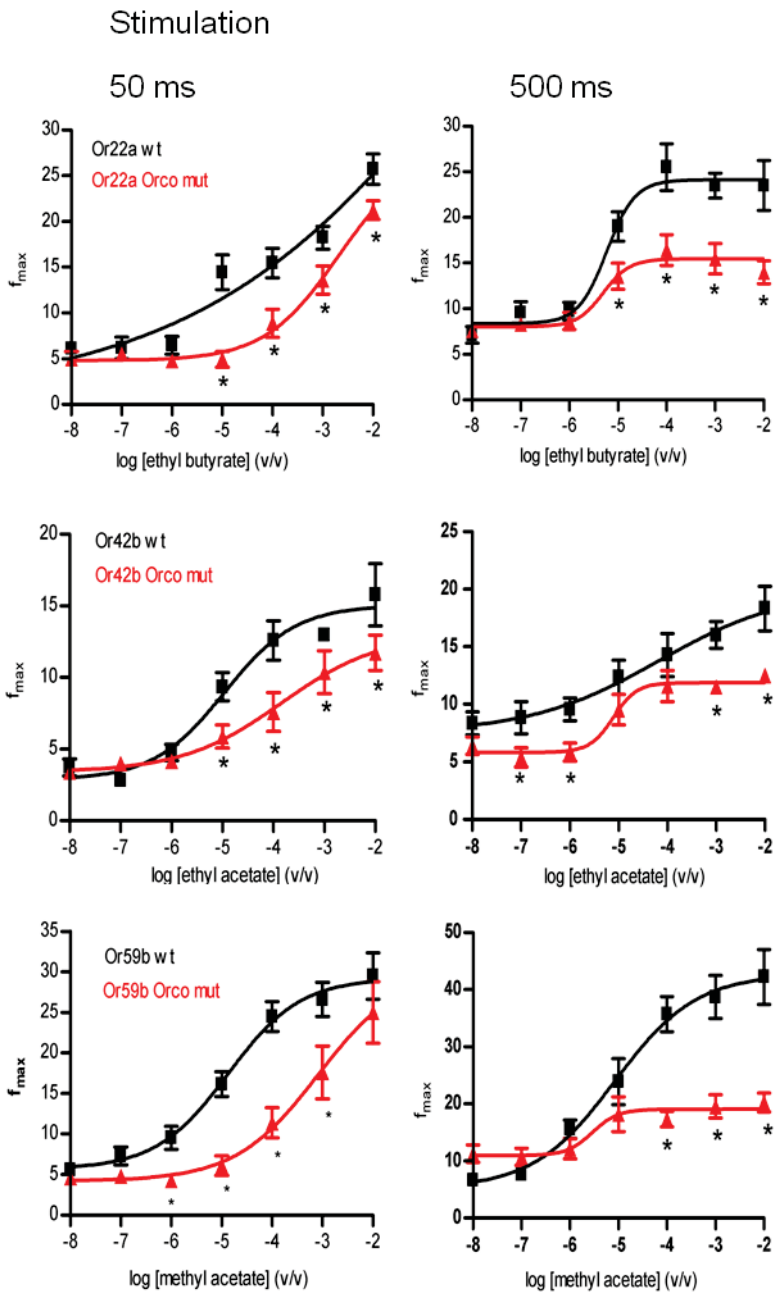


Figure 3. Concentration-response curves of the maximum normalized frequency (fmax) for Or22a wt and Or22a Orco mut OSNs responding to 50 ms pulses of ethyl butyrate). (right) concentration dependence of the maximum frequency (fmax) of fnorm for Or22a neuron to ethyl butyrate 500 ms stimulation in wild type and orco mut flies (error bars indicate SEM; asterisks

indicate significant differences, independent t-test n = 7 - 18). (B) Concentration dependence of the maximum frequency (fmax) of fnorm for Or42b wt and Or42b Orco mut and for 500 ms pulse duration (n = 6 - 12) (C). Concentration dependence of the maximum frequency (fmax) of fnorm in Or59b neuron to methyl acetate to 50 ms stimulation for wild type flies and orco mut flies and for 500 ms stimulus (n = 8 - 15).

Odorant stimulation increases PKC activity in Orco-expressing cells

To confirm the presence of PKC activity in OSNs, we first used immunofluorescence to show that PKC protein expression was localized to the sensillum and extending into the sensillum shafts where the OSN dendrites are located (Figure 4 A). We then stimulated the antenna with a mixture of established OR ligands (see Methods). Upon stimulation, PKC activity increased by 3.6 fold as compared to unstimulated controls (P = 0.001; Figure 4 B). In contrast, odor stimulation in Orco-/- mutants did not affect PKC activity (P = 0.75, Figure 4 C). These results show that activation of odorant receptors by OR ligands is necessary to increase PKC activity in this context.

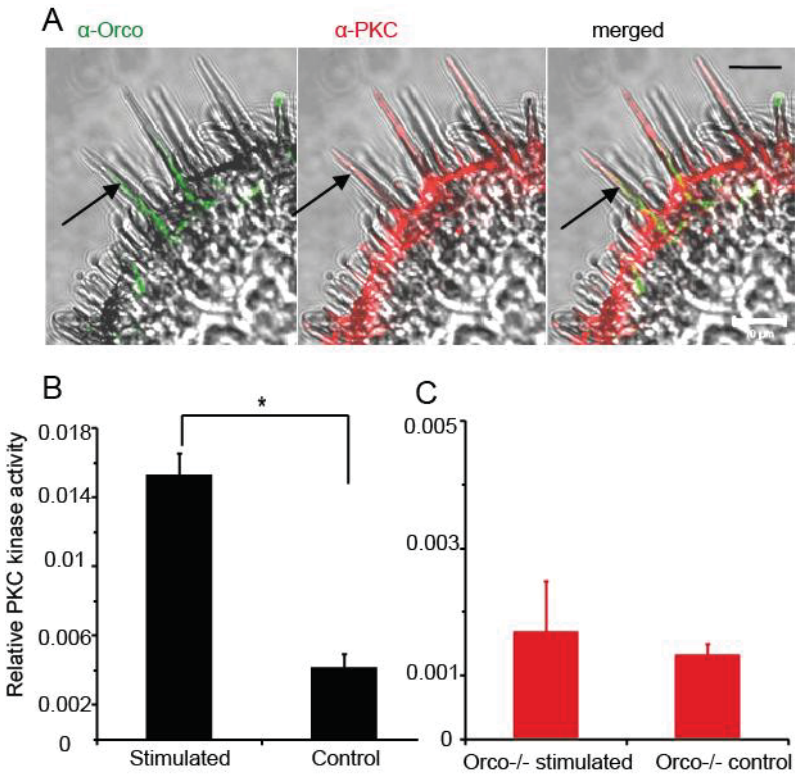


Figure 4. PKC expression and its activity due to odorant stimulation. (A) Protein kinase C protein localization in adult antennal sections with *Drosophila* anti-PKC antibodies (red). (B) Relative PKC activity in the wild type *Drosophila* antenna due to odorant stimulation verses unstimulated control, n = 3 show significant differences, P = 0.001, independent t-test. (C) PKC activity upon stimulation of the Orco-/- null mutant fly antenna as in (B).

PKC is required for temporal resolution of brief and intermittent odor stimuli.

Orco has been shown to be necessary for trafficking receptors to the dendrite (Larsson et al., 2004) and this function could potentially be PKC phosphorylation dependent. To confirm that the observed reduction in Orco-mut OSN sensitivity was not a result of reduced OR trafficking, we also generated various *Drosophila* flies with mutations in PKC genes rather than Orco PKC

phosphorylation sites. We found that both null mutation of conventional PKC53E and novel PKCδ, and suppression of PKC53E and PKCδ genes using RNAi interference abolished OSN responses to brief pulses in a manner similar to the Orcomut cells ($P < 0.05$; Figure 5 A, B). These mutations also shifted the concentration-response of the OSNs to higher concentrations as compared to the wild type ($P < 0.05$; Figure 5 C-E and table 1). PKC mutant OSNs also displayed a significantly reduced response to brief repeated odor stimuli ($P < 0.05$; Figure 5 C-E right). Nevertheless, the response to brief or repeated odor pulses could be recovered at 100x concentrations in a similar manner to Orcomut OSNs. These results confirm that PKC mediated phosphorylation regulates the ligand sensitivity of OSN.

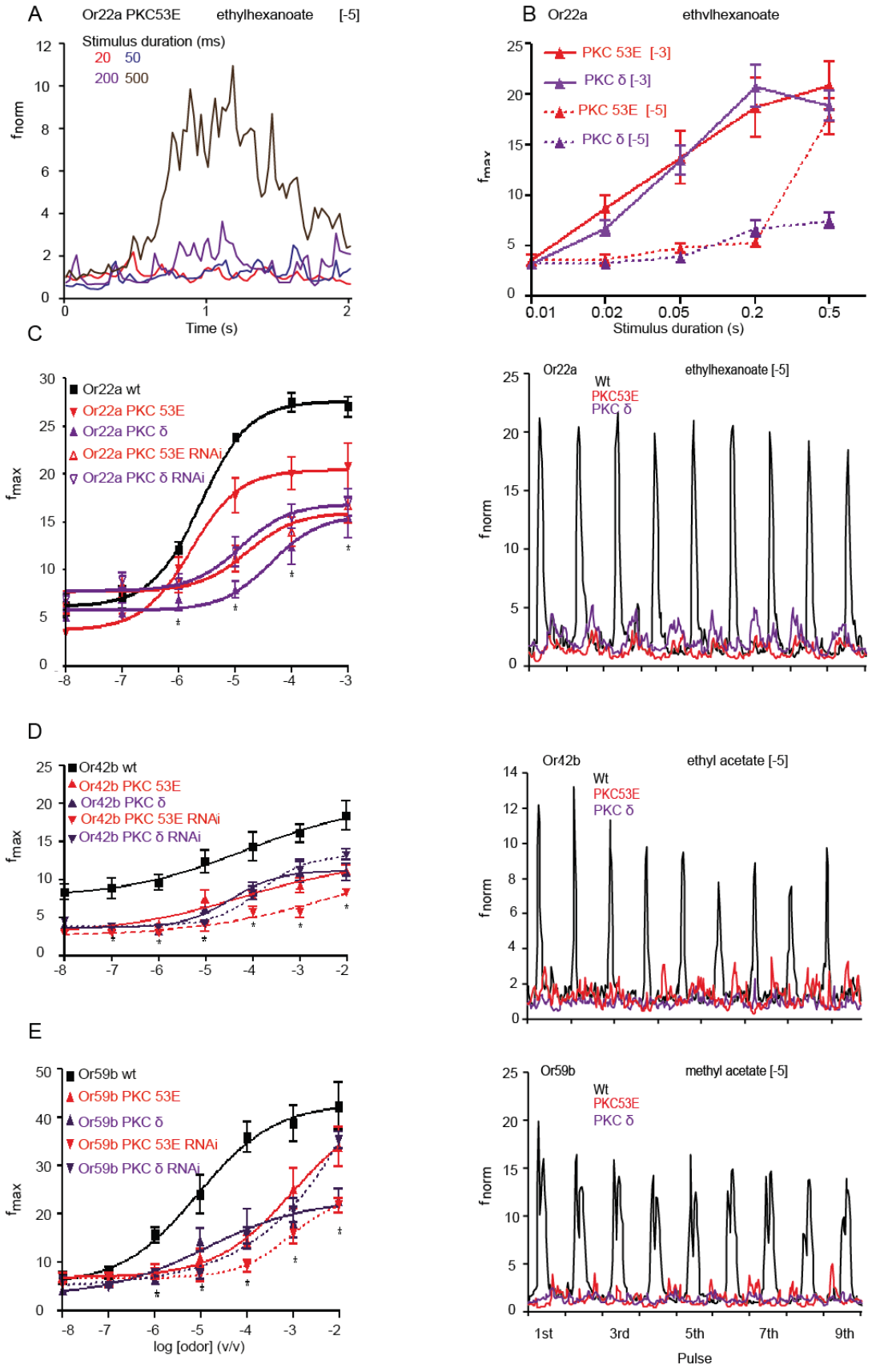

A
Or22a PKC53E
ethylhexanoate
[-5]
Stimulus duration (ms)
20 50
200 500
fnorm
Time (s)
B
Or22a
ethylhexanoate
PKC 53E [-3]
PKC δ [-3]
PKC 53E [-5]
PKC δ [-5]
fmax
Stimulus duration (s)
C
Or22a wt
Or22a PKC 53E
Or22a PKC δ
Or22a PKC 53E RNAi
Or22a PKC δ RNAi
fmax
Or22a
ethylhexanoate [-5]
Wt
PKC53E
PKC δ
fnorm
D
Or42b wt
Or42b PKC 53E
Or42b PKC δ
Or42b PKC 53E RNAi
Or42b PKC δ RNAi
fmax
Or42b
ethyl acetate [-5]
Wt
PKC53E
PKC δ
fnorm
E
Or59b wt
Or59b PKC 53E
Or59b PKC δ
Or59b PKC 53E RNAi
Or59b PKC δ RNAi
fmax
log [odor] (v/v)
Or59b
methyl acetate [-5]
Wt
PKC53E
PKC δ
fnorm
1st
3rd
5th
7th
9th
Pulse

Figure 5. Response of OSNs mutant for PKCδ and PKC53E as compared to wild type OSNs. (A). Mean peri-stimulus time curves of normalized responses (Hz) for Or22a PKC53E-OSNs responding to ethyl hexanoate [-5] dilutions at varying pulse durations (10 ms to 500 ms) (n = 7 - 10 trials). (B).The mean maximum normalized frequencies for Or22a-PKCδ and Or22a-PKC53E OSNs responses to [-5] and [-3] dilutions of ethyl hexanoate at various stimulus duration (error bars indicate SEM; n = 8 - 12). (C) (left) Dose-response curves of the maximum normalized frequency (fmax) for Or22a OSNs of different genotypes tested with 500 ms pulses of ethyl hexanoate (n = 8 - 12; error bars indicate SEM; asterisks indicate significant differences, $P < 0.05$, independent t-test). (Right) Mean peri-stimulus time histogram of normalized responses frequencies Or22a OSNs with listed PKC mutations to repeated pulsed 50 ms pulses of ethyl hexanoate [-5] at 1 Hz (n = 8 - 12 (D). Dose response of the maximum normalized frequency (fmax) for Or42b-OSNs and mutants to ethyl acetate as in (C) (n = 6 - 12) asterisks indicate significant differences, $P < 0.05$, independent t-test. (E) Dose response of Or59b to methyl acetate as in (C), (n = 8-11) asterisks indicate significant differences, $P < 0.05$, independent t-test

PKC-mediated signalling is specific to Orco-expressing OSNs and independent of perireceptor environment

To verify whether the effects of PKC activity on the OSN response are due to intracellular or perireceptor events, we also assessed the response of OR-expressing OSNs (OR-OSNs) co-localized in the same sensillum as IR-expressing OSNs (IR-OSNs), which do not express Orco (Ir75abc). First, we confirmed the function of IR-expressing OSNs in the Orco-/- mutant (see figure 6 A). The SSR trace shows that the Ir75abc-OSN was functional, while the co-localized

and Orco-dependent Or35a-OSN did not respond to its key ligand (Figure 6 A right). We then checked the activity of PKC in IR-OSNs by stimulating the antenna of (Orco-/-) mutants with a mixture of IR ligands. As seen in Figure 6B, the activity of PKC did not change ($P = 0.39$), suggesting that, unlike OR-OSNs, PKC activity is not required for an optimal IR-OSN response. Furthermore, we stimulated IR-OSNs in the mutant PKC flies used above (PKC53, PKCδ) and did not observe any significant change in the dose-response curve ($P > 0.05$; Figure 6 C, D) unlike OR-OSNs (Figure 5 C,D,E). We then tested the response of Or35a-OSNs, co-localized in the ac3 coeloconic sensillum along with Ir75abc-OSNs (Yao et al., 2005). As with other OR-OSNs (Figure 5), the concentration-response curve for PKC53E and PKCδ mutants was shifted to higher concentrations ($P < 0.05$) (Figure 6 E). This result shows that PKC-mediated signalling in OSNs is independent of the perireceptor environment surrounding the dendrite and is specific to Orco-expressing OSNs.

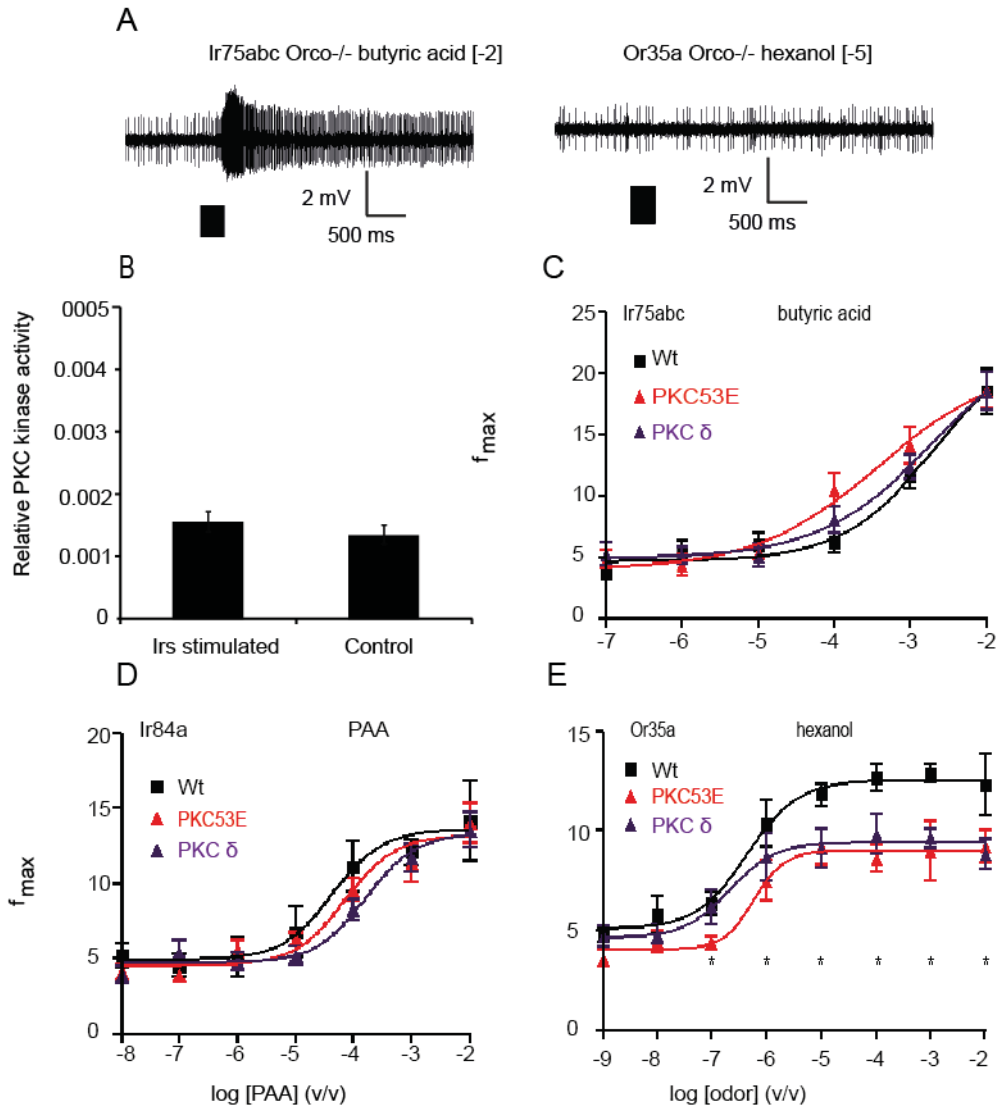


Figure 6. PKC activity and the IR-OSN response. (A) (Left) 10 s sample trace depicting the response of an IR75abc-OSNs to butyric acid [-2] in an Orco-/- mutant fly. (Right) 10 s sample trace indicating the absence of an Or35a-OSNs response to hexanol [-5] in an Orco-/- mutant fly. (B) Relative PKC activity due to odorant stimulation of IRs-OSNs vs. unstimulated control, n = 3. (C) Concentration-response curves for Ir75abc mutant for PKCδ and PKC53E OSNs tested with 500 ms pulses of butyric acid (mean maximum normalized frequency; n = 8 - 13 wt, n = 7 - 10 for PKC53E and PKCδ; error bars indicate SEM; * $P < 0.05$, independent t-test). (D)

Concentration-response curves as in (C) for Ir84a-OSNs and mutants responding to phenyl acetaldehyde (n = 7 - 12 for wt, n = 7 - 11 for PKCδ and PKC53E). (E) Concentration-response curves as in (C) for Or35a OSNs and mutants co-localized with Ir75abc OSNs in response to hexanol (n = 7 - 8 for wt, n = 8 - 17 for PKCδ and n = 7 - 11 for PKC53E; * $P < 0.05$, independent t-tests).

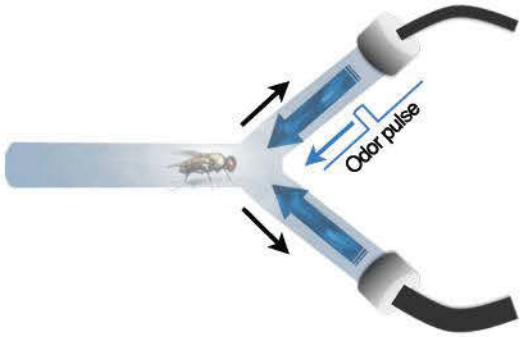
Table 1. Parameters described from dose response curve (Figure 3, Figure 5C-E, Figure 6 C-E)

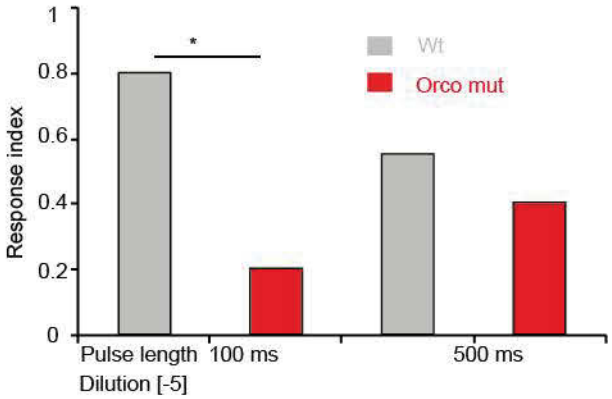
Receptor	Odor	Tstim (ms)	EC50 log [odor]	Hill	R^2
Or42b wt	Ethyl acetate	50	-4.9	0.6	0.7
Or42b Orco mut	Ethyl acetate	50	-3.8	0.4	0.6
Or59b wt	Methyl acetate	50	-4.9	0.5	0.5
Or59b orco mut	Methyl acetate	50	-3.1	1.7	0.3
Or22a wt	Etyl butyrate	500	-5.2	1.5	0.5
Or22a Orco mut	Etyl butyrate	500	-5.3	1.6	0.3
Or42b wt	Ethyl acetate	500	-4.2	0.3	0.4
Or42b Orco mut	Ethyl acetate	500	-5.1	1.8	0.5
Or59b wt	Methyl acetate	500	-5.1	0.5	0.5
Or59b Orco mut	Methyl acetate	500	-5.5	1.7	0.3
Or22a wt	Ethyl hexanoate	500	-5.6	1.1	0.9
Or22a PKC 53E	Ethyl hexanoate	500	-5.8	0.8	0.7
Or22a PKC δ	Ethyl hexanoate	500	-4.2	0.6	0.5
Or22a PKC 53E RNAi	Ethyl hexanoate	500	-4	0.3	0.4
Or22a PKC δ RNAi	Ethyl hexanoate	500	-4.8	0.6	0.6
Or42b wt	ethyl acetate	500	-4.2	0.3	0.4
Or42b PKC 53E	ethyl acetate	500	-4.2	0.3	0.7
Or42b PKC δ	Ethyl acetate	500	-4.5	0.9	0.7
Or42b PKC 53E RNAi	ethyl acetate	500	-2.1	0.3	0.6
Or42b PKC δ RNAi	ethyl acetate	500	-3.9	0.9	0.7
Or59b wt	Methyl acetate	500	-5.1	0.5	0.5
Or59b PKC 53E	Methyl acetate	500	-3	0.5	0.7
Or59b PKC δ	Methyl acetate	500	-4.9	0.4	0.5
Or59b PKC 53E RNAi	Methyl acetate	500	-3.1	0.8	0.7
Or59b PKC δ RNAi	Methyl acetate	500	-0.5	0.3	0.8
Or35a wt	Hexanol	500	-6.4	1.5	0.5
Or35a PKC 53E	Hexanol	500	-6.3	0.9	0.6
Or35a PKC δ	Hexanol	500	-6.7	1.0	0.2
Ir75abc wt	Butyric acid	500	-2.7	0.7	0.7
Ir75abc PKC 53E	Butyric acid	500	-3.4	0.5	0.7
Ir75abc PKC δ	Butyric acid	500	-2.7	0.6	0.7
Ir84a wt	Phenyl acetaldehyde	500	-4.3	0.7	0.4
Ir84a PKC 53E	Phenyl acetaldehyde	500	-3.7	0.4	0.7
Ir84a PKC δ	Phenyl acetaldehyde	500	-3.7	0.8	0.7

PKC activity is required for efficient odor tracking

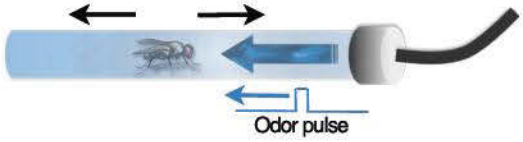
We finally investigated the effect of PKC-mediated signaling on the behavioral response of flies using three different behavioral paradigms (Y-tube, flywalk, and trap assays). We observed that unlike wild type flies, Orco-mut flies did not respond in Y-tube assays to a single 100 ms pulse of [-5] dilution of ethyl acetate (($P = 0.02$, Figure 7 A). However, both mutants and wild-type flies responded similarly when stimulated with 500 ms pulses (Figure 7 A, $P = 0.6$). We also tested the behavioral response of flies to repeated odor stimulation while monitoring their movement in a high-throughput behavioral assay, the Flywalk (Steck et al., 2012). Similarly, the Orco-mut fly response was significantly reduced when stimulated with 50 ms pulses of [-5] dilution ($P = 0.005$; Figure 7 B). In addition, wild type flies showed lower responses to 500 ms pulses of [-5] than Orco-mut flies ($P = 0.02$; Figure 7 B), which could reflect adaptation due to the higher olfactory input over the total stimulus course. Finally; we compared the behavioral response of Orcomut, PKC53E-RNAi, PKCδ-RNAi and wild type flies in a 24 h free flight trap assay. After 1 hour, more wild type flies (and parental controls) were trapped than any of the mutants. The response of Orco mut, PKC53E-RNAi and PKCδ-RNAi flies remained significantly reduced ($P < 0.05$) over the entire 24 h period (Figure 7 C). Taken together, our results show that PKC-mediated signaling in insect OSNs is required for a fast and sensitive behavioral response to odor stimuli.

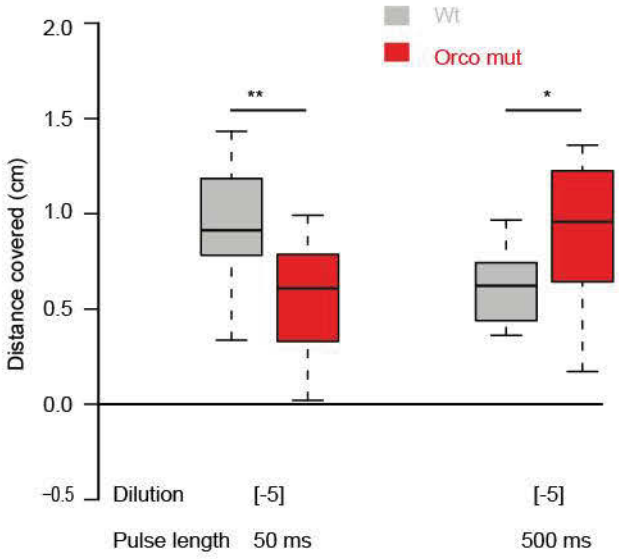
A


Odor pulse

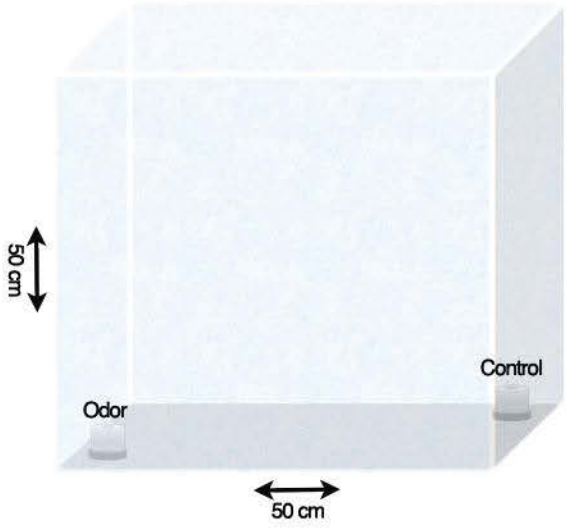

Response index
1
0.8
0.6
0.4
0.2
0
*
Wt
Orco mut
Pulse length 100 ms
500 ms
Dilution [-5]

B


Odor pulse


Distance covered (cm)
2.0
1.5
1.0
0.5
0.0
-0.5
**
*
Wt
Orco mut
Dilution [-5] [-5]
Pulse length 50 ms 500 ms

C


50 cm
Control
Odor
50 cm

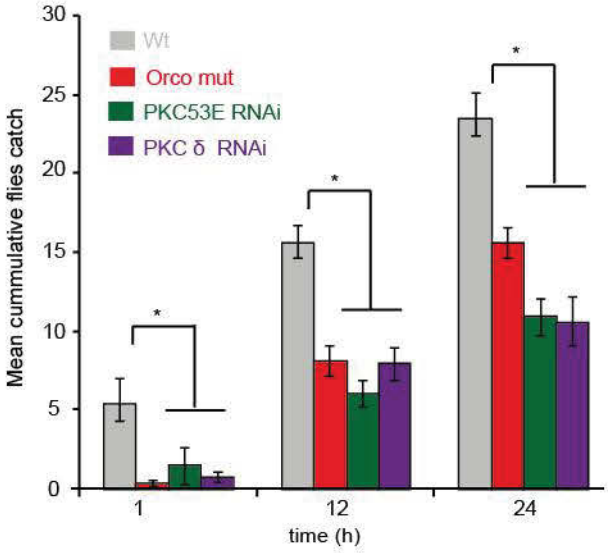

Mean cummulative flies catch
30
25
20
15
10
5
0
*
*
*
Wt
Orco mut
PKC53E RNAi
PKC δ RNAi
1
12
24
time (h)

Figure 7. PKC signaling and olfactory behavioral response. (A) Response index of wild type and Orco mut flies to single 100 ms or 500 ms pulses of ethyl acetate [-5] (n = 30 - 40; * P = 0.02, Fisher's exact test) (B). The median movement of wild type and Orco mut flies to repeated ethyl acetate stimulations at listed dilutions and two pulse length durations (50 ms and 500 ms; n = 15; * P = 0.005 and 0.02, respectively, Wilcoxon signed-rank test. (C). Mean cumulative wild type and the diverse PKC mutant flies trapped in free flight assays at listed hours (n = 6 replicates with 30 flies each; * $P < 0.05$, independent t-test).

Discussion

Here, we show that Orco phosphorylation through PKC is required for the temporal resolution of brief, repeated and low concentration stimuli. Our results indicate that mutations in PKC genes or PKC phosphorylation sites in Orco result in reduced sensitivity of *Drosophila* OSNs both at physiological and behavioral levels. In line with this, modulation of intracellular PKC levels also result in decreased neuronal sensitivity. These phenomena were only observed in Orco-expressing OSNs, suggesting a particular role of Orco-mediated signalling for the resolution of brief, intermittent pulses found in natural odor plumes.

Our immunofluorescence analyses show that PKC protein is expressed in the sensillum shaft where OSNs are located and PKC activity increases in OR-OSNs after odorant stimulation. PKC phosphorylation is required for catalytic and regulatory activity (Edwards and Newton, 1997, Newton, 2001, Adams, 2003, Kain et al., 2008), and our PKC activity assays subsequently indicated an increase in phosphorylated PKC due to odorant stimulation. Although various PKC

proteins are co-expressed in the *Drosophila* olfactory system (Rosenthal et al., 1987, Schaeffer et al., 1989), single mutations of either PKC53E or PKCδ genes were sufficient to decrease the sensitivity of OSNs to brief odor pulses by a factor of 10x. These results suggest that multiple PKCs could contribute to OSN signal amplification. Similarly, three PKC enzymes have been shown to work together for maximum ethanol sensitivity in *Drosophila* (Chen et al., 2010). Our results also illustrate that mutation of PKC phosphorylation sites in Orco significantly reduces the OSN response to brief pulses, again supporting the view that PKC targets Orco (Sargsyan et al., 2011). These results demonstrate the importance of PKC activity for brief pulse sensitivity and resolution.

Chemoreception in insects occurs through a diverse class of chemoreceptors including IRs, ORs, and GRs (Benton et al., 2009). Stimulation of IR-OSNs in an Orco-null environment (Orco-/-) did not increase the activity of PKC, and mutations of PKC53E and PKCδ genes did not change the response dynamics of IR-OSNs. This suggests that IR-OSNs do not depend on these particular intracellular PKCs for the odor response. However, the response of OR-OSNs co-localized with these IR-OSNs (Or35a) was reduced, indicating that the effect of PKC modulation is independent of the perireceptor environment where the neurons are expressed, and is also specific to Orco expressing cells. Of course, these results cannot exclude modulation of the IR-OSN response by other intracellular signaling molecules. Interestingly, however, the reduced sensitivity of OR-OSNs in PKC mutants so closely resembles the wild-type IR-OSN phenotype (Getahun et al., 2012) that PKC activity is likely a major factor contributing to the enhanced sensitivity and speed of the odor response in OR-OSNs.

It has been suggested that peripheral sensitivity to odors is not a necessity for behavioral sensitivity (Kaupp, 2010) as the high level of signal convergence in the insect brain could provide the necessary signal amplification (Couto et al., 2005, Olsen et al., 2010). However, our study indicates that modification of a single PKC enzyme or modification of a small number of Orco-phosphorylation sites is sufficient to significantly reduce the behavioral response of flies in Y-tube choice, no-choice, and free-movement choice assays. Specifically, these mutant flies required longer odor stimulations and/or more time to respond to odor cues. This provides strong evidence that activation of PKC is required for fast and sensitive olfactory behavior. The reduced behavioral response to brief stimulation also implies that PKC signaling is important for plume tracking by flying insects, where the plume is comprised of brief, punctuate filaments of odor molecules (Carde and Willis, 2008). Indeed, our free-flight trap assay illustrates a significantly reduced ability of PKC-mutant flies to locate the odor source. These results show that modification of intracellular signaling at the periphery produces a significant effect on the behavioral response.

Insects are the first invertebrates that have developed powered flight and took the sky much earlier than their vertebrate counterparts such as birds (Carpenter, 1953). Flight also contributes to the enormous success of insects (Carpenter, 1953, Edwards, 1997). However, the high-speed requirements of flight can also make sensory information detection more challenging (Edwards and Palka, 1991, Edwards, 1997), as it requires adjustment both at the receptor and downstream processing levels. Here, we show the important role of Orco-mediated signaling for temporal resolution of odor information. Interestingly, Orco also seems to be present only in higher insects (Krieger et al., 2003, Pitts et al., 2004, Smadja et al., 2009, Yang et al., 2012). We therefore

suggest that the sensitization mechanism found in Orco-expressing OSNs reflects an evolutionary adaptation of the olfactory system to meet the specific challenge of fast and sensitive plume resolution during flight. Future studies comparing olfactory signaling in pterogote (winged) vs. apterogote insects, as well as detailed ethological studies of plume tracking, could elucidate the evolution of Orco and its role in odor plume following.

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Author contribution

MNG, SLL, SBO, DW, BSH, conceived and designed the study; MNG performed the experiments, analyzed the data and wrote the manuscript, MT, MK performed and analyzed the Flywalk experiment. All authors discussed and agreed on the content of the paper.

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General discussion

Olfaction is a central sensory modality for insects for most activities (Krieger and Breer, 1999). From an evolutionary perspective, there is thus a strong selection pressure on insects to develop an efficient olfactory system for detecting and locating food sources, oviposition sites and mates. Furthermore, odors (pheromones, host plant odors, etc.) are emitted in trace amounts (Lacey and Sanders, 1992; Späthe et al., 2013), which demands the evolution of a sensitive olfactory system. Finally, flying insects must track and localize these trace stimuli in a filamentous odor plume while challenged by the high performance of flight. The ability of the olfactory system to quickly process odor information arriving at the periphery is thus essential for the entire odor response of the organism (Vickers, 2000; Budick and Dickinson, 2006; Cardé and Willis, 2008). The main objective of the current thesis was to investigate response dynamics of diverse chemoreceptors and attempt to find the mechanism by which the insect olfactory system has optimized sensitivity, speed, and dynamic range for efficient odor detection.

Receptor design affects OSN response kinetics

Departure from the aquatic environment correlates with a massive expansion of odorant receptor genes (Glusman, et al 2000, Robertson, et al., 2003) to accommodate the new air borne volatiles. This transition still can be detected in ancestral chemoreceptors that detect water soluble chemicals (e.g. acids and amines), such as IRs in insects (Croset et al., 2010; Rytz et al., 2013), class I “fish-like receptors” in frogs (Freitag et al., 1995) and other land vertebrates (Glusman et al., 2000). Likewise, fish lack the class II receptors in land vertebrates that detect air borne volatiles (Niimura and Nei, 2005), showing that olfactory receptors have evolved according to the life style of the organisms. Nevertheless, diversification of chemoreceptors might not occur simply to detect diverse chemicals. We show that OSNs expressing ancestral IRs are less sensitive to brief pulses (Chapter I), suggesting that IR-OSNs could have difficulty detecting odors under field conditions where odor concentration is low (Lacey and Sanders, 1992; Späthe et al., 2013) and interactions with odor filaments are brief (Vickers et al., 2001). However, IRs could be important for detecting odorants that are not detected by other receptors (Silbering et al., 2011). In addition, IR-OSNs are less desensitized when stimulated for longer periods of time (Chapter I), which could enable the OSNs to process and transmit quantitative information about

the presence or duration of odor in their environment at very close range, where concentrations are high and stimulus durations are long. These conditions cause adaptation in GR- and OR-expressing OSNs (Chapter 1). Our experiments show that this difference is not due to the sensillar environment, and thus is a property of the unique signal transduction properties within the OSNs themselves. (Chapters III-V).

In many instances, odor molecules diffuse from their point of origin and are transported away from their source by prevailing fluid currents (Vickers, 2006, Koehl, 2006). This creates a brief, intermittent odor source. As shown in Chapter I, regardless of the odorant receptor expressed, the response dynamics to repeated intermittent stimuli is similar. Pulse resolution, latency to repeated stimuli, short-term adaptation and also recovery in the response onset is a common neuronal property for all OSNs and not receptor-specific. The accuracy of encoding rapidly fluctuating intermittent odorant stimuli is significantly reduced in *Drosophila* under rapid stimulation above 5 Hz as in peripheral (e.g. Barrozo and Kaissling, 2002; Bau et al., 2002; Lemon and Getz, 1997), and central (e.g. Christensen and Hildebrand, 1997; Lei and Hansson, 1999; Lei et al., 2009) neurons of other insects. However, there is no evidence whether this adaptation affects plume tracking ability (Kaissling et al., 1987). Rather, adaptation is assumed to be an early step in information processing and decision-making (Kaissling et al., 1987; Baker et al., 1988; Vickers et al., 2001; Dolzer et al., 2003; Theodoni et al., 2011). Thus, insects might still respond behaviorally to much higher frequencies in natural odor plumes.

The other neuronal response dynamics that is common to all OSNs expressing diverse receptor type is that the response of OSNs depends on the integration of odorant amount information within a certain period of time. i.e., none of the OR expressing OSNs gave response when stimulated at 10 ms regardless of concentration, but start to respond when stimulated for 20 ms. By doing so, the system can compensate or regain its sensitivity without losing speed of odor detection using longer integration time (Chapter I). Such a phenomenon is possible when transduction happens before spiking (Nagel and Wilson, 2011). However, the system should not keep on integrating as it has to tradeoff being slow (Chapter I). A similar strategy is utilized by other organisms including insects with different integration time window (Firestein et al., 1991; Firestein et al, 1993; Takeuchi and Kurahashi, 2002; Abraham et al., 2004; Wright et al., 2009).

Our data also shows that OSNs expressing the same receptor display different response dynamics to inhibitory vs. excitatory ligands. We show in Chapter (I) that the response duration is longer for inhibitory odors than for excitatory, as found by (Su et al., 2011). We also demonstrate that OSNs cannot resolve pulsed inhibitory stimuli as quickly as excitatory. It is hypothesized that inhibition and excitation utilize a different signal transduction mechanism (Schuckel et al., 2009), however, this is not yet proven. Although the ecological significance of inhibitory odors is not known, we show that differential odor processing take places as early as at the OSN level (Su et al., 2011; Hillier and Vickers, 2011, Chapter I). In summary, our results from Chapter I indicate that the receptor expressed in the OSN confers differences in sensitivity to low concentrations and intermittent stimuli that could impact the behavioral response to odors.

Intracellular signaling modulates OSN kinetics

Modulation of sensitivity to relevant sensory signals, such as through sensitization and adaptation as a function of exposure, is essential for organisms to adapt to a changing environment. Brief exposure to female pheromones has been shown to increase male moth behavioral and physiological sensitivity and response speed (Anderson, et al. 2003, 2007; Anton et al., 2010). Brief exposure to female-emitted sex pheromones also increases male response in rodents (Fewell et al., 2002). Such plasticity is also found in other sensory modalities, such as *Drosophila* visual feature discrimination (Peng et al., 2007) and *Aplysia* sensitization to aversive electrical shocks (Frost et al., 1985). In Chapter IV, we show that OR-expressing OSNs are sensitized to sub-threshold odor pulses for several seconds after being stimulated with a single 500 ms sub-threshold odor pulse. However, OSNs expressing IRs are not sensitized regardless of inter-stimulus interval or concentration (Chapter III). We subsequently show that intracellular signaling in OR-expressing neurons modulates this phenomenon. Specifically, inhibition of cAMP production or mutation of Orco phosphorylation sites impair the sensitization of OSNs. PKC and cAMP have been implicated in long-term potentiation observed in the hippocampus (Akers et al., 1986; Lovinger et al., 1987; Malinow et al., 1988; Kandel, 2001). Similarly, Ca^{2+} dependent phosphorylation and calcium itself were shown to play a role in short-term facilitation (Willian et al., 2013). Likewise, we show that when *Drosophila* flies are presented with a single brief 50 ms short pulse, they exhibit increased behavioral response to a subsequent pulse. Our

study thus shows that intracellular signaling allows for modulation of odor sensitivity at the periphery itself.

Modulation of OrX sensitivity is regulated by PKC

Insect ORs form an OrX-Orco complex, and during activation by the odorant, two kinds of interactions occur, homomeric, i.e. OrX vs OrX and heteromeric interaction, i.e. between OR and Orco (Neuhaus et al., 2005; Benton et al., 2006; Tsitoura et al., 2010; German et al., 2012). There is common consensus that the insect OrX-Orco complex functions as a ligand gated ion channel. Orco has also been shown to affect the OrX-Orco ion conductivity (Wicher et al., 2008; Nichols et al., 2011; Pask et al., 2011; Nakagawa et al., 2012). PKC activation is often critical in mediating the phosphorylation of receptors and/or channels that ultimately alter ion conductivity (Farley and Auerbach, 1986; Madison et al., 1986; Newton, 2001). We thus hypothesized that phosphorylation of the Orco channel by PKC could modulate the sensitivity of OSNs. We show that modification of all PKC phosphorylation sites on Orco abolishes Orco sensitivity to cAMP under heterologous expression (Chapter, III). We furthermore show that phosphorylation by PKC is required for OSN sensitivity to brief odorant stimulations (Chapter, V), and sensitization to repeated stimulation (Chapter, IV), and also modulates response kinetics such as dose response dynamics and latency. These results suggest that PKC signaling is an important regulator in the OSN response. Similarly, mutation of two PKC enzymes reduces the sensitivity of OSNs to brief pulses (Chapter V). Furthermore we show that inhibition of PKC signaling significantly reduces the behavioral response to brief odor pulses. To our knowledge, this is the first report showing a clear phenotype for the involvement of intracellular signaling in odor detection both at the physiological and behavioral levels. We thus conclude that OrX signaling is regulated by cAMP and PKC and maximum OSN sensitivity is controlled by multiple signaling pathways (Chapter, IV and V).

In our pharmacological investigations in Chapters II-IV, we show that inhibition of cAMP reduces the magnitude of the odor response, while activation of cAMP via forskolin, or cholera toxin increases it. These results suggest that cAMP is involved in OrX signaling. We also show that pharmacological inhibition of part of the $G_{\alpha}q$ signaling cascade including PLC and PKC

reduces the odor response (Chapter, III and IV). Finally, from our immunofluorescence we show that PKC is expressed in the dendrite of OSNs, and odorant stimulation activates PKC in the antenna. These results indicate that a complex cellular signaling cascade involving several molecular players takes place in *Drosophila* olfaction. For instance, ligand-receptor binding could activate $G_{\alpha}q$ proteins (Fadool et al., 1995) that subsequently activate PLC leading to the formation of IP_3, that activates cation channels (Fadool and Ache 1992; Stengl, 1994; Lischka et al., 1999; Kain et al., 2008), while DAG activates protein kinase C (Huang, 1989) that phosphorylate receptor protein such as OrX-Orco (Chapter III-V). Activation of $G_{\alpha}s$ could result in production of cAMP (Boekhoff et al., 1990; Breer et al., 1990; Wicher et al., 2008; Deng et al., 2011) that activates Orco (Sargsyan et al., 2011).

Although it is not clear how cAMP directly opens OrX-Orco as the complex does not have known cAMP binding motifs, it is important for maximum OSNs sensitivity (Wicher et al., 2008; Deng et al., 2011, and Chapter IV). cAMP could also activate cyclic nucleotide-gated ion channels that are expressed in the *Drosophila* olfactory system (Baumann et al., 1994), or activate cAMP dependent PKA that could phosphorylate the OrX-Orco complex. *Drosophila* olfactory sensory organs are also enriched with PKA protein (Tunstall et al., 2012). Interestingly, single odorant stimulation can activate more than one G-protein signaling cascade in insects. Zufall and Hatt (1991) have shown that pheromone stimulation resulted in activation of both PKC and cGMP. Odorant stimulation also activates multiple secondary messengers in lobsters (Boekhoff et al., 1994) and mammals e.g in rat (Ronnett et al., 1993; Benbernou et al., 2011). The fast activation of intracellular signaling molecules such as cAMP and PLC (within 50 ms, chapters IV-V) implies that these two independent signaling pathways could function in parallel in insects (Boekhoff et al., 1990; Breer et al., 1990). Interestingly, injection of cAMP under inhibition of PLC or PKC did not improve the OSN response, implying that the two cascades work together to modulate sensitivity (Chapter, IV). A number of studies show that different parallel transduction mechanisms work together, rather than independently for signal amplification (Hille, 2001; Nakagawa et al., 2005; Cheng et al., 2007; Milligan, 2007; Tombola et al., 2008; Nakagawa et al., 2012). In vertebrate OSNs, olfactory transduction shows high cooperativity for signal amplification (Firestein et al., 1993; Kurahashi and Menini, 1997). For insects, different intracellular signaling molecules, cAMP and PLC (Chapter IV), as well as different PKC subtypes (Chapter V), expand the sensitivity, speed and response range of OSNs.

In contrary, stimulation of IR-OSNs did not increase the activity of PKC, and mutation of PKC53E and PKC δ did not change the response dynamics of these OSNs. This shows that IR-OSN signaling is independent of PKC. We also demonstrate that this difference in signal transduction between OR and IR-expressing OSNs is independent of neuronal environment (Chapter, V), and therefore is an intrinsic property of the receptor. This difference between the ancient IRs and recent ORs suggests that ORs may have evolved to optimize sensitivity and speed of odor reception in the insect olfactory system.

Future prospects

In my dissertation I have shown the importance of combined metabotropic and ionotropic signaling for insect olfaction. However, the complexity of the OrX signaling pathway is still formidable. We still know little about the full molecular mechanisms that contribute to OSN signaling. In future studies, it will be interesting to investigate if ligand gated signaling alone is enough to elicit both physiological and behavioral responses. Furthermore, it is unknown whether each subunit of the OrX-Orco complex forms an independent ion channel pore(s) or if they work together as one unit. This dissertation has shown that intracellular signaling molecules are activated due to odorant stimulation of the OrX-Orco complex, determining how insect ORs activate G proteins in olfactory signaling will be important to understand the OrX signaling cascade. In addition, although we show the effect of PKC activation on the OSN response, identification of possible phosphorylation sites on OrX-Orco is essential for full understanding of OSN signaling and regulation, particularly with respect to ion conductivity (Nichols et al., 2011, Pask et al., 2011). Mechanisms for signal termination, which is important for pulse resolution and plume following, are also not known.

General conclusion

We obtained various lines of evidence that OrX-Orco signaling is dependent on intracellular signaling. We conclude that the insect olfactory system has achieved its sensitivity, speed and broad dynamic range through integration of diverse and parallel signaling pathways that could include metabotropic and ionotropic signaling. Thus, integration of multiple signaling cascades can provide a powerful means to establish an efficient and fast sensory system. One possible driving force for the evolution of this unique signaling mechanism may be the evolution of flight that makes olfaction more challenging, thus such signaling mechanism enable insects to follow plume.

Final Summary

Insects are ecologically and economically important organisms. They are pollinators, pests of agricultural products, and vectors for many deadly human and animal diseases. Insects are also highly dependent on olfaction to locate hosts, oviposition sites, and dangers such as toxins or predators. Olfactory organs are the main sensory channel through which insects obtain information regarding the external world. Detailed investigations on olfactory system function are thus a key to implementing olfaction-based pest and vector control or exploitation of beneficial insects. How chemical signals are converted into electric signals by the insect olfactory periphery is still under investigation, particularly regarding how insects have evolved such inordinate sensitivity and fast physiological and behavioral responses to odors. There is a broad consensus that insect odorant receptors function as ligand-gated ion channels. However, ligand gated signaling is generally less sensitive and requires higher concentrations to elicit a response, which does not explain the remarkably high sensitivity observed in insects. The main objective of this thesis was to investigate the molecular mechanism through which high sensitivity, speed and broad dynamic range is achieved by the insect olfactory periphery. To address our questions we employed biochemical, molecular, physiological and behavioral approaches. Insect sensory neurons house three different types of chemoreceptors: odorant receptors (OR), ionotropic receptors (IR), and gustatory receptors (GR), which evolved at different time points in evolution. Using a comparative approach, we were able to show that

neurons expressing these different receptors differ not only in ligand selectivity, but also in sensitivity. IR-expressing neurons, the oldest and ancestral chemoreceptors, were less sensitive and exhibited greater response latency as opposed to the more recently derived OR-expressing neurons. We further showed that this is an innate property of the receptor expressed in the neuron rather than a function of the neuronal perireceptor environment. To investigate the molecular mechanism of this difference in sensitivity in OR-expressing OSNs, we assessed the involvement of intracellular signaling molecules. When intracellular signaling was inhibited in OR-expressing OSNs they became less sensitive and showed delayed response, similar to IR-expressing neurons. Conversely, we did not find a similar effect in IR-expressing OSNs, indicating that intracellular signaling transduction varies between these two types of chemoreceptors. To investigate the impact of intracellular signaling on the behavioral response to odors, we generated different mutants where either PKC genes or co-receptor protein Orco PKC phosphorylation sites were mutated. Mutant flies did not respond to brief pulses of odor and also exhibited greater response latency to odors when tested in a free flight behavioral bioassay. Our results both at the physiological and behavioral levels suggest that modulation of the OSN response by intracellular signaling is indispensable for maximum chemosensory sensitivity and response to brief, intermittent stimulation. The amount of odor emitted from hosts is miniscule and contact with filaments within the odor plume is brief in space and time, especially for insects tracking odor in flight. Therefore, the recent evolution of ORs may have occurred in response to the need for efficient and fast tracking of dynamic odor landscape while in flight.

Zusammenfassung

Insekten sind ökologisch und ökonomisch bedeutsame Organismen. Sie bestäuben Blütenpflanzen, sind aber auch Schädlinge in der Landwirtschaft und Überträger tödlicher Krankheiten. Der Geruchssinn spielt für Insekten eine herausragende Rolle, z.B. für die Nahrungssuche, die Suche nach Eiablageplätzen, aber auch für die Warnung vor Gefahren, wie Giften und Jägern. Ein detailliertes Verständnis der Duftverarbeitung ist der Schlüssel für die Kontrolle von Schadinsekten und für den Schutz nützlicher Insekten. Die Mechanismen, wie in den Duftrezeptoren der Insekten chemische in elektrische Signale umgewandelt werden, sind Gegenstand aktueller Forschung. Besonders interessant ist, wie Insekten eine außerordentliche Sensitivität für bestimmte Geruchsstoffe entwickelt haben, die es ihnen gestattet, schnell und empfindlich auf Duftreize zu reagieren. Es besteht weitgehende Einigkeit, dass die Geruchsrezeptoren bei Insekten Liganden-gesteuerte Ionenkanäle darstellen. Allerdings ist bekannt, dass diese Kanäle schnell, aber weniger sensitiv sind, dieser Wirkmechanismus also die hohe Sensitivität der Duftrezeptoren nicht erklären kann. Das Hauptziel dieser Arbeit ist zu klären, über welchen molekularen Mechanismus die Duftwahrnehmung sensitiv, schnell und in einem breiten Arbeitsbereich funktionieren kann. Zur Beantwortung dieser Frage wurden molekularbiologische, biochemische, elektrophysiologische Methoden eingesetzt und Verhaltensexperimente durchgeführt. Olfaktorische Rezeptorneuronen exprimieren bei Insekten drei Chemorezeptortypen: Odorantrezeptoren (ORs), ionotrope Rezeptoren (IRs) und gustatorische Rezeptoren (GRs). Es konnte gezeigt werden, dass diese Typen nicht nur verschiedene Liganden binden, sondern diese auch mit unterschiedlicher Sensitivität detektieren. Im Gegensatz zu den evolutionär jungen ORs waren die ursprünglicheren IRs weniger sensitiv und antworteten mit zeitlicher Latenz. Diese Unterschiede waren in den intrinsischen Eigenschaften der Rezeptoren begründet und nicht in der Komposition der Rezeptorumgebung. Zur Beantwortung der Frage nach der hohen Sensitivität von ORs wurde die Beteiligung intrazellulärer Signalsysteme an der Duftverarbeitung untersucht. Bei Inhibition solcher Signalkaskaden reduzierte sich die Sensitivität der ORs und ihre Duftantwort erfolgte verzögert, für IRs hatte dies keine Auswirkung. Die OR-Sensitivität wurde durch cAMP erhöht, welche jedoch eine basale Phosphorylierung des Ko-Rezeptors Orco durch die Proteinkinase C (PKC)

zur Voraussetzung hatte. Zur Überprüfung der Verhaltensrelevanz der Regulation der OR-Sensitivität wurden Fliegen mit Mutationen in den Orco-Phosphorylierungsstellen bzw. in Subtypen der PKC hergestellt. Diese Mutanten reagierten nicht auf kurze Duftpulse. Des Weiteren wiesen sie in einem Freiflug-Assay einen verzögerten Flugeinsatz auf. Diese Ergebnisse belegen von der sensorischen Ebene bis hin zum Verhalten, dass die Modulation der OR-Funktion durch intrazelluläre Signale notwendig für eine sensitive Detektion kurzer Duftpulse ist. Möglicherweise hat die Notwendigkeit für fliegende Insekten, schwache, intermittierende Duftpulse sicher zu verarbeiten, die Evolution von ORs, deren Empfindlichkeit regulierbar ist, beigetragen.

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Declaration of independent assignment

I declare in accordance with the conferral of the degree of doctor from the School of Biology and Pharmacy of Friedrich-Schiller University Jena that the submitted thesis was written only with the assistance and literature cited in the text.

People who assisted in experiments, data analysis and writing of the manuscripts are listed as co-authors of the respective manuscripts. I was not assisted by a consultant for doctorate theses.

The thesis has not been previously submitted whether to the Friedrich-Schiller University, Jena or to any other university.

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