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RESEARCH ARTICLE

Intracellular regulation of the insect chemoreceptor complex impacts odour localization in flying insects

Merid N. Getahun‡, Michael Thoma, Sofia Lavista-Llanos, Ian Keesey, Richard A. Fandino, Markus Knaden, Dieter Wicher¶, Shannon B. Olsson*,§ and Bill S. Hansson§

ABSTRACT

Flying insects are well known for airborne odour tracking and have evolved diverse chemoreceptors. While ionotropic receptors (IRs) are found across protostomes, insect odorant receptors (ORs) have only been identified in winged insects. We therefore hypothesized that the unique signal transduction of ORs offers an advantage for odour localization in flight. Using *Drosophila*, we found expression and increased activity of the intracellular signalling protein PKC in antennal sensilla following odour stimulation. Odour stimulation also enhanced phosphorylation of the OR co-receptor Orco *in vitro*, while site-directed mutation of Orco or mutations in PKC subtypes reduced the sensitivity and dynamic range of OR-expressing neurons *in vivo*, but not IR-expressing neurons. We ultimately show that these mutations reduce competence for odour localization of flies in flight. We conclude that intracellular regulation of OR sensitivity is necessary for efficient odour localization, which suggests a mechanistic advantage for the evolution of the OR complex in flying insects.

KEY WORDS: *Drosophila*, Signal transduction, Orco phosphorylation, Receptors, Flight

INTRODUCTION

Our planet's astonishing number of insect species has been attributed in large part to their adaptation to terrestriality, and then, in the pterygote (winged) insects, to the evolution of flight (Bradley et al., 2009). The evolution of flight has also led to important adaptations to the nervous system (Edwards, 1997). Because of insects' tiny nervous systems, adaptations produce what Roeder referred to as a 'parsimony of nerve cells' (Roeder, 1998), where each cell is individually adapted so that the sensory system can process the largest amount of information with the fewest number of cells. As such, each nerve cell must be optimized to the sensitivity and speed required for flight.

The high-speed performance required for flight also imposes immense constraints on insect sensory systems, as evidenced by visual (Borst and Haag, 2002) and mechanosensory (Sane et al., 2007) adaptations for flight control. Given the well-known importance of chemical cues to the survival of many insects (Hansson and Stensmyr, 2011), it is also likely that the insect olfactory system has undergone adaptations to increase the sensitivity and speed of odour detection in flight. Natural odours are structurally, quantitatively and spatiotemporally complex (Bruce et al., 2005; Riffell et al., 2014). Once emitted from their source, they are dispersed and diluted by the ambient motion of air to form a shifting and filamentous plume (Murlis et al., 1992; Vickers et al., 2001; Koehl, 2006). Nevertheless, flying insects are known for their ability to track wind-borne odour plumes to their source over tens of metres (Carde and Willis, 2008; Van Breugel and Dickinson, 2014). Roeder's 'parsimony' therefore suggests that insect olfactory sensory neurons (OSNs) should be optimized for the sensitivity and speed required for this airborne odour tracking. On the receptor level, an optimal resolution is achieved by special spatial arrangements allowing high cooperativity of signalling elements (Wicher, 2012).

The dendritic membranes of insect OSNs contain diverse families of chemosensory receptors that transform chemical signals from the outside world into electrochemical signals, including: odorant receptors (ORs), gustatory receptors (GRs) and ionotropic receptors (IRs) (Stocker, 1994; Vosshall and Stocker, 2007; Benton et al., 2009). While ORs and GRs are composed of 7-transmembrane proteins analogous to metabotropic receptors, IRs are related to ionotropic glutamate receptors (Benton et al., 2009). ORs also form heterodimers of an odorant-specific OR protein OrX and a ubiquitous co-receptor Orco (Vosshall and Hansson, 2011), and both subunits contribute to ion channel activity (Nichols et al., 2011; Pask et al., 2011; Nakagawa et al., 2012).

These diverse insect chemoreceptor families also appeared at unique points in evolutionary time. While IRs are common to all protostomes (Croset et al., 2010), a recent study by Missbach et al. (2014) indicates the heterodimer OR complex is unique to pterygote insect orders. The co-receptor Orco is highly conserved in insects and Orco homologues have been identified in moths, flies, beetles, social insects and true bugs (Missbach et al., 2014; Krieger et al., 2003; Pitts et al., 2004; Smadja et al., 2009; Yang et al., 2012). Silbering and Benton (2010) also note that the independent evolution of two different chemosensory ion channel families in insects 'argues against the emergence of the observed dichotomy by "chance", and rather points towards specific mechanistic advantages'. Missbach et al. (2014) also suggest that olfactory receptors evolved at the same point in time that vascular plants spread and insects evolved flight. This line of evidence raises a hypothesis that the evolution of the unique heteromeric OR complex found in pterygotes might have conferred a specific mechanistic advantage to insects during flight.

We have previously shown that OSNs expressing the more recently evolved insect ORs were more sensitive to brief odour pulses than cells expressing the more ancient IRs (Getahun et al., 2012). Similarly, olfactory sensory neurons of diverse insect groups

Max Planck Institute for Chemical Ecology, Department Evolutionary Neuroethology, Hans-Knöll-Strasse 8, Jena D-07745, Germany.
*Present address: Naturalist-Inspired Chemical Ecology (NICE), National Centre for Biological Sciences, Tata Institute of Fundamental Research, Bangalore, India.
‡Present address: International Centre of Insect Physiology and Ecology, icipe, Nairobi, Kenya.
§These authors share senior authorship

¶Author for correspondence (dwicher@ice.mpg.de)

D.W., 0000-0001-6443-3399

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can track stimuli of very short duration (Szyszka et al., 2014). However, little is known about the molecular basis for this sensitivity. Studies have shown that the OrX-Orco complex is subject to metabotropic signalling (Stengl, 1993, 2010; Kain et al., 2008; Wicher et al., 2008; Deng et al., 2011; Sargsyan et al., 2011; Getahun et al., 2013; Ignatious Raja et al., 2014; Carraher et al., 2015) and also that OrX-Orco activation enhances secondary messenger production (Wicher, et al., 2008; Miazzi et al., 2016). Neither OrX nor Orco has any obvious homology to known cyclic nucleotide binding domains, and it is not clear how these two proteins interact. One possibility is that cyclic nucleotides act indirectly via kinases that phosphorylate the OrX-Orco complex. Protein phosphorylation by kinases is a frequently observed post-translational modification to adjust protein function including enzymes, ion channels and receptors (Newton, 2001; Azevedo et al., 2015; Antz et al., 1999; Barros et al., 2012; Kanelis et al., 2010). Insect olfactory systems express diverse calcium- and cyclic nucleotide-activated kinases (Schaeffer et al., 1989; Chintapalli et al., 2007; Tunstall et al., 2012), yet any role in signal transduction is unknown. Members of the protein kinase C family (PKCs), in particular, are network genes found to play a role in insect signal transduction, neural connectivity and natural variation in olfactory behaviour (Swarup et al., 2013; Arya et al., 2015). Here, we propose that PKC-mediated intracellular signalling in the recently evolved ORs offers a mechanistic advantage to detect and respond to the brief and intermittent odour information received while tracking plume information in flight. We show that the sensitivity of OSNs expressing the OrX-Orco complex is regulated by intracellular PKC phosphorylation of Orco, while the response of OSNs expressing IRs is not affected by intracellular modifications to PKC. We further show that this signalling mechanism affects insects' ability to exhibit efficient odour localization in several behavioural assays, including a flight tunnel. Our combined results suggest that the unique intracellular modulation of the ionotropic response in ORs increases sensitivity to brief and intermittent odour stimuli encountered during aerial plume tracking, and provide a hypothesis for a mechanistic advantage to the evolution of the OR complex in the pterygote insect orders.

MATERIALS AND METHODS

Drosophila stocks

All experiments were performed on adult, 2–6 day old, wild-type Canton-S *D. melanogaster* male and female flies. Stocks were maintained on conventional cornmeal agar medium under a 12 h light:12 h dark cycle at 25°C and 70% relative humidity.

The Orco mutant (mut) flies were as described in Getahun et al. (2013); endogenous Orco (Orco wt) was replaced with a mutated version of Orco (Orco mut), in which phosphorylation was prevented as a result of S/T to N exchanges in all five PKC sites (Sargsyan et al., 2011), in all Orco cells using the GAL4/UAS system (Brand and Perrimon, 1993). For this, we rescued an Orco null mutation (Larsson et al., 2004) using Orco-GAL4 and UAS-Orco mut, or UAS-Orco wt as a control. The independent insertions Orco-GAL4/+, UAS-Orco/+ and UAS-Orco mut/+ had no effect on the physiology of OSNs, as previously characterized (Getahun et al., 2013). PKC53E EY14093 (BL20790) mutants were obtained from Bloomington at Indiana University, USA. This mutation was previously characterized and is homozygous viable (Murillo-Maldonado et al., 2011). PKCδ e04408 (BL18258) mutants were obtained from the Exelixis Inc. *Drosophila* stock centre; this mutation was characterized in our laboratory. PKCδ 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 the PKCδ gene was confirmed by PCR (primers: 5′-GTACCTGAATGGCGGTGATC-3′ forward and 5′-CAAACGACCACCAATCCACA-3′ reverse). We also used the RNA interference (RNAi) technique to reduce the expression of PKCδ and PKC53E specifically in the OSNs. Lines BL28355 for PKCδ RNAi, BL27491 for PKC53E RNAi and BL34716 for PKC53E RNAi-II were obtained from Bloomington. These lines were crossed with the Orco GAL4 driver line to induce targeted gene silencing in OSNs. To obtain a stable line for behavioural experiments and to increase expression of RNAi, both fly lines were made homozygous. Parental control lines were UAS-ds RNAi (PKCδ)/+, UAS-ds RNAi (PKC53E)/+ and Orco Gal4/+.

Odour stimuli

All odours were obtained from Sigma Aldrich at the 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-butanedione (>97%), pentyl acetate (>99%) and phenyl acetaldehyde (>90%) were diluted in mineral oil (BioChemika Ultra, Fluka); 1,4-diaminobutane (>98%), propionic acid (>99.5%) and butyric acid (>99%) were dissolved in water. For frequency stimulation, we used a custom-built multicomponent stimulus system similar to Olsson et al. (2011) and Getahun et al. (2012). Note that for PKC mutant experiments, Or22a OSNs were stimulated with ethyl hexanoate, a key ligand for Or22a (Hallem and Carlson, 2006), as the response to ethyl butyrate was highly reduced in PKC53E and PKCδ mutants.

Biochemistry

PKC activity analyses were performed according to Ziegelberger et al. (1990) and Maida et al. (2000). Briefly, olfactory antennae of *Drosophila* were cut under a binocular light microscope using fine forceps and the isolated antennae were immediately stimulated with a headspace mixture of odours (ethyl hexanoate, ethyl butyrate, ethyl acetate, methyl acetate, 2,3-butanedione, 2-heptanone, 1-hexanol, pentyl acetate, ethyl-3-hydroxybutyrate). All odours were diluted in mineral oil to 10^{-5} dilution v/v with 100 µl of each mixed in a single vial and presented using a custom-designed stimulus device similar to Olsson et al. (2011). The antennae were stimulated with two 500 ms pulses of the odour mixture and then immediately frozen in liquid nitrogen. For each replicate, approximately 200 antennae were used, and stored at −80°C until analysis. The antennae were crushed under liquid nitrogen and homogenized in 20 mmol l^{-1} Tris-HCl (pH 7.2) in the presence of a protease inhibitor cocktail (Sigma-Aldrich, Germany) and 2.5% octylglucoside at 4°C for 20 min. The homogenate was centrifuged at 10,000 rpm for 15 min at 4°C and the supernatant used for ELISA. PKC kinase kits (ADI-EKS-420A) were obtained from Enzo Life Sciences (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 colour 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.

Western blots

To investigate whether Orco is phosphorylated by PKC, HEK293 cells expressing Orco wt and Orco mut were incubated with 100 µmol l^{-1} 1-oleoyl-2-acetyl-*sn*-glycerol (OAG), a specific PKC

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activator (Alexis, Lörrach, Germany) for ∼3 min in the medium while control cells were not stimulated. Afterwards, cells were washed with ice-cold PBS and for each sample a cell pellet culture density of 5×10^7 was collected by centrifugation. We used ab65400 plasma membrane protein extraction kit (abcam, Cambridge, UK) for the extraction of membrane proteins (Mukunda et al., 2014a). The pellet of each sample was re-suspended with homogenizing buffer in the presence of freshly prepared protease inhibitor and phosphostop cocktail (Roche) to maintain the phosphorylated state. The samples were homogenized in a glass homogenizer on ice. The plasma membrane protein extracts were separated in parallel by 8.5% SDS-PAGE followed by electronic transfer to PVDF membranes (BioRad, Germany). The membrane was then blocked in 5% non-fat dry milk in TBS-T for 2 h at room temperature. The membrane was subsequently incubated with primary polyclonal anti-Orco antibody rabbit (1:5000; kindly provided by Jürgen Krieger, Martin-Luther-University Halle-Wittenberg, Germany) in 2.5% non-fat dry milk in TBS-T overnight at 4°C. The membrane was further washed with TBS-T 3 times for 5 min each and incubated with HRP-conjugated anti-rabbit IgG secondary antibody (1:10,000) for 1 h at room temperature. After washing the membrane in TBS-T 3 times for 5 min each, the proteins were detected using ECL western blotting detection kit (SignalFire™ Elite, Danvers, MA, USA). Relative densitometric analysis of bands was performed using Image J package (http://imagej.nih.gov/ij/).

Once Orco was detected at the right protein size (Carraher et al., 2013; Mukunda et al., 2014a), the same membranes were stripped and reused to test the phosphorylation state of the Orco proteins. As we do not have an antibody that detects phosphorylated Orco, we quantified phosphoserine and phosphothreonine on Orco. For this particular experiment, the membrane was stripped with freshly prepared stripping buffer (1.5 g glycine, 0.1 g SDS, 1 ml Tween, pH 2.2) for 7 min at room temperature by gentle agitation. After mild stripping, the membrane was washed with TBS-T 3 times for 10 min each and subsequently blocked with 5% w/v bovine serum albumin in TBS-T for 1 h at 4°C with agitation. Subsequently, the membrane was incubated with primary anti-phosphoserine antibody ab125277 (1:500 in TBS-T; mouse, abcam) or anti-phosphothreonine antibody (1:250 in TBS-T; mouse, LifeSpan BioSciences, USA) overnight at 4°C in a 50 ml Falcon tube with rotation. The membrane was then rinsed in TBS-T 3 times for 10 min each at room temperature followed by incubation with secondary HRP-conjugated anti-mouse IgG antibody (1:10,000; BioRad Germany). Phosphoserine and phosphothreonine proteins were detected using an enhanced chemiluminescence Elite ECL reagent (Thermo Scientific Pierce, Germany). The specificity of phosphoserine and phosphothreonine antibodies was tested by pre-incubating with phosphoserine and phosphothreonine (www.abcam.com). The relative densitometry of bands was performed using the Image J package (http://imagej.nih.gov/ij/). Relative densities of phosphoserine and phosphothreonine in stimulated cells are given, normalized to unstimulated cells, while the Orco band was used as a loading control.

Immunohistochemistry

Frozen antennal sections were fixed for 10 min in 4% paraformaldehyde, washed 3 times in phosphate buffer (1×PBS) and permeabilized for 30 min in 0.02% Triton-X 100-PBS (PT). After blocking for 1 h with 5% normal goat serum (NGS, Invitrogen) in PT, the antennal sections were immunolabelled with primary antibodies against *Drosophila* PKC (goat, 1:500 dn-16 Sc-15726, lot K 1102, Santa Cruz Biotechnology, Germany) and Orco (rabbit, 1:1000, provided by L. Vosshall, The Rockefeller University and Howard Hughes Medical Institute, USA) and incubated at 4°C overnight. For control samples, *Drosophila* PKC antibodies were pre-incubated with blocking peptide (sc-15726 P, Santa Cruz Biotechnology) at 1:5 ratio antibody:peptide for 2 h at room temperature. Samples were washed 3 times in PT for 10 min and blocked over 30 min with 5% NGS before incubation with the secondary antibodies conjugated to Alexa Fluor 488 (anti-rabbit) and Alexa Fluor 568 (anti-goat) (1:200, Invitrogen). Samples were washed 3 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).

HEK293 cells transfected with GFP-Orco N-terminal fusion protein (Wicher et al., 2008) were grown in 35 mm dishes, similar to Mukunda et al. (2014b), at a density of $\sim1\times10^4$ to 1×10^5 per well. Immunocytochemistry was used to check for phosphorylated serine Orco via PKC activation. Cells were incubated with 30 µl of 100 µmol l^{-1} OAG (a PKC activator) per well diluted in PBS for roughly 3 min, while control (unstimulated) cells were incubated with PBS only. After 3 min, the PBS was aspirated and cells were immediately fixed in 4% formaldehyde in PBS for 5 min. After washing, cells were blocked for 1 h and incubated with anti-phosphoserine mouse antibody (1:500; ab125277, abcam) at 4°C overnight. The next morning, after washing, cells were incubated with Alexa Fluor 546 Cy3 (anti-mouse) for 2 h at room temperature. As a control, non-transfected HEK293 cells were treated in a similar way. We did not check phosphorylated threonine as anti-phosphothreonine mouse antibody was ineffective for immunocytochemistry. Samples were viewed and images acquired using a LSM 880 Zeiss confocal microscope.

Electrophysiology

Both male and female flies, 4–6 days old, were used; each fly was mounted in a cut pipette tip with wax (Hallem et al., 2004; Yao et al., 2005; De Bruyne et al., 1999; 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 1000× magnification with an Olympus BX-51 microscope (Olympus Corporation, Tokyo, Japan). OSN action potentials were amplified, recorded and analysed 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.

Behavioural assays

For all behavioural experiments, ethyl acetate, a known attractant odour (Steck et al., 2012), was used. Flywalk experiments were performed and raw data was pre-processed as described elsewhere (Steck et al., 2012). Briefly, 15 individual 4–6 day old 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 Orco wt (positive control) and Orco mut (experimental) flies were tested to avoid technical artifacts. Flies were continuously exposed to a humidified airflow (∼20°C, ∼75% relative humidity) of 0.3 l min^{-1} (20 cm s^{-1} in the glass tubes). Repeated odour pulses (inter-stimulus interval, 90 s) were released from a multicomponent stimulus device (Olsson et al., 2011) loaded with 100 µl of odour dilutions in mineral oil. Responses were calculated as the mean distance flies covered

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within 4 s after encounter with the odour pulse. In the free-flight trap assay, 30, 4–6 day old female flies, starved for 24 h were released in a 50×50×50 cm mesh cage (Faucher et al., 2013). From preliminary tests, fly catches using either vinegar alone or the single odour were low when tested in the free-flight assay. For this reason, 100 µl of vinegar was used as a background for 100 µl ethyl acetate at 10^{-2} dilution (v/v), and number of trapped flies was counted after 1, 12 and 24 h. Wind-tunnel experiments were performed as described previously (Becher et al., 2010). Flies were tested in a 30×30×100 cm wind tunnel at 25°C and approximately 55% relative humidity with a wind speed of 0.25 m s^{-1} produced by a fan (Fischbach GmbH, Neunkirchen, Germany). We performed preliminary experiments where flies were given two choices: control (mineral oil) or odour. In these experiments, neither wild-type nor mutant flies landed on the control (N=32 for Orco wt and Orco mut; Movie 1). We thus omitted the control trap for subsequent experiments to quantitatively assess behaviour under no-choice conditions. Five populations were tested: Orco wt (positive control; Canton S, N=109), Orco mut (experiment; N=107), PKCδ RNAi (experiment; N=87), PKC53E RNAi (experiment; N=72) and Orco−/− (negative control; N=52). Approximately five, 4–6 day old female flies starved for roughly 24 h were placed in separate vials. A lure containing 100 µl of vinegar and 100 µl of 10^{-2} dilution (v/v) ethyl acetate similar to the trap assay was loaded on separate cotton roll dispensers placed in a plastic vial open at one end. The vial was suspended horizontally approximately 15 cm from the top of the cage and with the open end facing downwind. A paper funnel was used to cover the open end, creating a point odour source. Fly vials from different populations were randomized each day of testing. For testing, all flies from a single vial were released and scored for the following behaviours: (1) upwind-oriented flight (defined by a stereotypical surge and cast anemotaxis within the plume); (2) source approach within 10 cm of the front of the source (i.e. within the plume); and (3) landing (Budick and Dickinson, 2006; Stewart et al., 2010; Van Breugel and Dickinson, 2014). Each trial lasted until all flies exhibited at least one behaviour or a maximum of 10 min had passed.

Data analysis

Co-localized neurons were identified based on spike amplitude. In all cases, f_{max} refers to the maximum spike frequency reached during a given time period (i.e. a 25 ms bin for peristimulus time histogram curves, or the entire stimulus period for concentration–response curves). Sigmoidal concentration–response curves were fitted using Graph Pad Prism 4 with a variable slope parameter (Graph Pad Software Inc., La Jolla, CA, USA). The kinase activity in stimulated homogenate was normalized as a ratio to the unstimulated samples and PKC activity was compared using an independent t-test. Concentration–response curves were compared using independent t-tests between individual dilutions. Behavioural data were analysed using Fisher's exact test and Mann–Whitney test. All statistical calculations were performed using SPSS version 17 (SPSS Inc., Chicago, IL, USA).

RESULTS

Odorant stimulation increases PKC activity in OR-expressing cells and enhances Orco phosphorylation

We propose that OrX-Orco signalling is subject to intracellular modulation indirectly by activating kinases such as PKC. To address the involvement of PKC-based signalling in OSNs during odorant stimulation, we first used immunofluorescence to show that the PKC protein was localized in the sensillum, extending into the sensillum shafts where the OSN dendrites are located (Fig. 1A, co-immunolabelled with anti-Orco; Fig. S1B). The specificity of the antibody was confirmed through pre-incubation of anti-PKC antibody with blocking peptide that abolished PKC labelling (Fig. S1A). We then stimulated the antennae with a mixture of established OR ligands (see Materials and methods). Upon stimulation, PKC activity increased by 3.6-fold compared with unstimulated controls (P=0.001; Fig. 1B). In contrast, odour stimulation in Orco−/− mutants did not affect PKC activity (P=0.75; Fig. 1B). These results indicate that activation of odorant receptors by OR ligands increases PKC activity.

To investigate whether the increased PKC activity observed following odorant stimulation also results in Orco phosphorylation, we performed western blot and immunofluorescence analyses in a heterologous expression system. Treatment of HEK293 cells expressing Orco with the specific PKC activator OAG increased phosphorylation of threonine and serine residues on Orco by 3.4- and 3.6-fold, respectively, compared with untreated cells (Fig. 1C, D); the loading control Orco signal was 1:1 between control and OAG-treated cells (Fig. 1C,D, bottom). To confirm that the change in phosphorylation of threonine and serine residues on Orco is attributed to PKC, we performed the same experiments using HEK293 cells expressing a mutated form of Orco, in which all putative PKC phosphorylation sites are mutated, termed Orco mut (Sargsyan et al., 2011). Mutation of all putative PKC phosphorylation sites in Orco abolished the change in intensity of phosphothreonine (1:1) and phosphoserine (1:0.9) due to OAG treatment compared with unstimulated controls (Fig. 1E,F); the Orco signal between control and OAG-treated cells was 1:1.6 (Fig. 1E,F, bottom). Furthermore, using immunofluorescence analyses, we were able to show the colocalization of Orco and phosphoserine on the plasma membrane in HEK293 cells treated with OAG (Fig. 1G,H; Fig. S1C,D). Together, these results show that PKC activation increases phosphorylation of threonine and serine sites on Orco.

Orco phosphorylation increases OSN sensitivity to brief and intermittent odour stimulation *in vivo*

After assessing PKC-mediated Orco phosphorylation *in vitro*, we investigated its physiological significance *in vivo*. Wild-type *Drosophila* OSNs expressing the olfactory receptor 22a (Or22a OSNs) exhibited a significant response when challenged with a 20 ms odour pulse of ethyl butyrate [10^{-5} dilution (v/v); P=0.02; Fig. 2A]. However, in the absence of Orco phosphorylation by PKC (Orco mut flies), the response of Or22a OSNs to brief odour pulses was significantly reduced (Fig. 2B) and 10× longer stimulations were required to elicit a significant response (P<0.05 up to 200 ms duration; Fig. 2B,C). The response to brief ethyl butyrate [10^{-5} dilution (v/v)] repeated stimulations was also abolished in Orco mut OSNs (Fig. 2D), while the response was recovered in control flies in which an Orco wt rescued Orco−/− in all OSNs (P>0.05; Fig. 2C,D). This indicates that the capacity of OR-expressing OSNs to detect brief and intermittent odour stimuli is reduced in the absence of PKC-mediated Orco phosphorylation. Interestingly, the response to brief stimulations of Orco mut flies could also be recovered by increasing the stimulus concentration by 100× (data not shown), further indicating that the effects observed are a result of reduced sensitivity in the absence of PKC-mediated phosphorylation of Orco. The concentration–response curves of Orco mut cells were also shifted to higher concentrations, especially for brief stimulations (Fig. 2E,F; Table S1; note the higher EC50 values), while there was no change in concentration–response in

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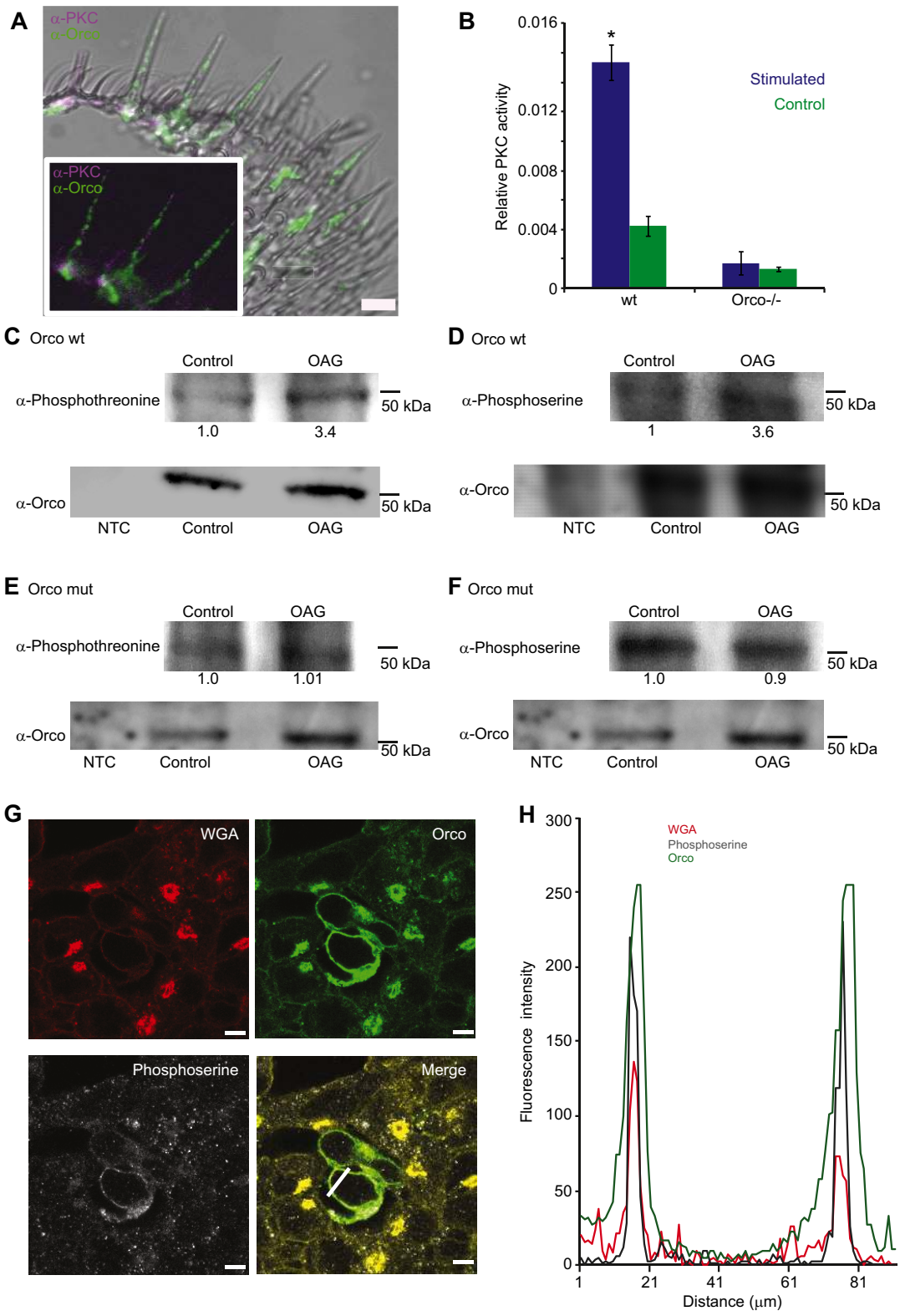

Fig. 1. PKC activity and Orco phosphorylation. (A) Protein kinase C (PKC) expression in adult antennal sections detected with *Drosophila* anti-PKC antibody (magenta, α-PKC) is localized in the dendrites of olfactory sensory neurons (OSNs; inset) co-labelled with anti-Orco antibody (green, α-Orco). Scale bar, 10 µm. (B) Left, relative PKC activity in the wild-type *Drosophila* antenna due to odorant stimulation versus unstimulated control (N=3; P=0.001, independent t-test, error bars show s.e.m.); right, PKC activity upon stimulation of the Orco−/− null mutant fly antenna. (C–F) Western blots showing phosphothreonine (C and E; top) and phosphoserine (D and F; top) residues on wild-type (Orco wt; C and D, bottom) and mutant (Orco mut; E and F; same blot, bottom) Orco proteins extracted from control or OAG-treated HEK293 cells (NTC, non-transfected cells). In all cases, staining demonstrates that Orco protein is expressed. (G) Confocal micrographs of HEK293 cells transfected with GFP-Orco (green), labelled with α-phosphoserine (grey); wheatgerm agglutinin (WGA) was used to label plasma membrane (red). Scale bar, 2 µm. (H) Fluorescence intensity along the paths indicated in the merged image in G. The co-localization of phosphoserine staining and Orco in the plasma membrane is suggested.

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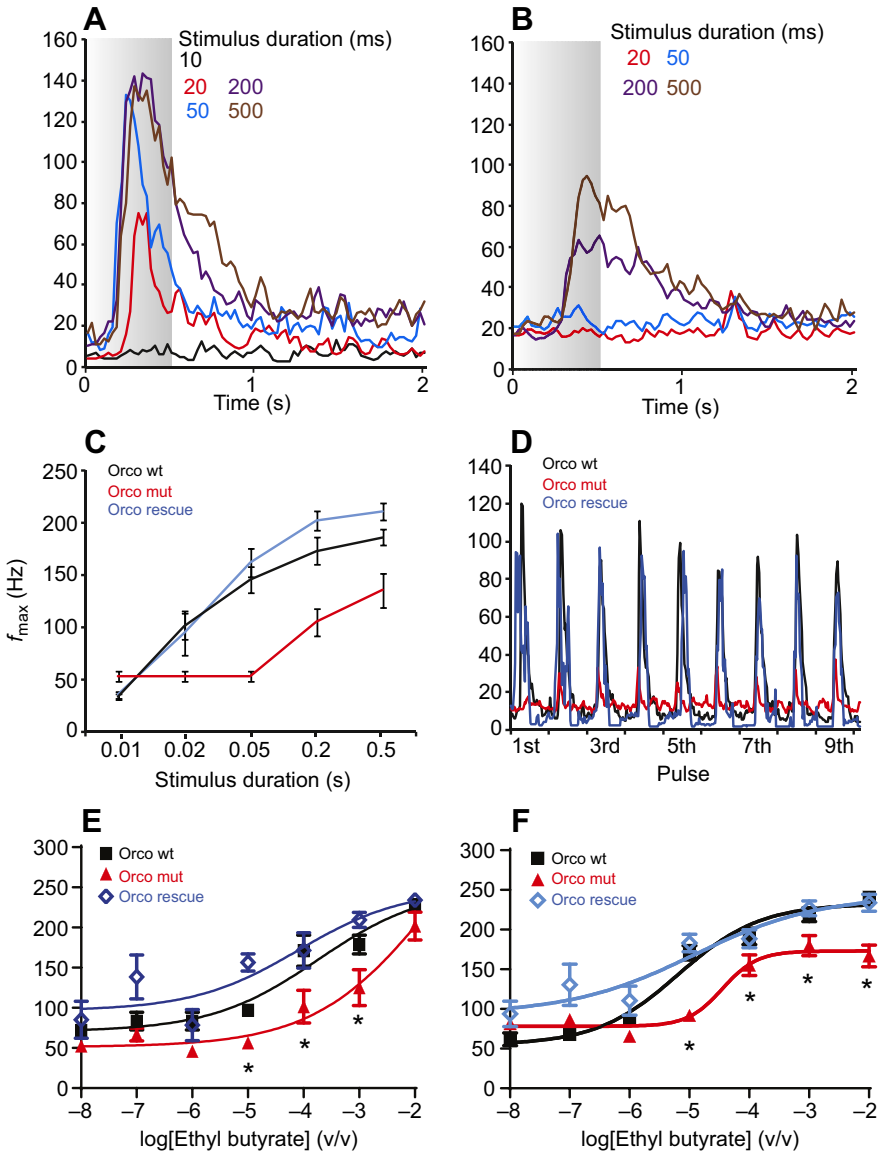

Fig. 2. Odorant-induced responses of Or22a OSNs in Orco wt and Orco mut flies. (A) Mean peristimulus time courses of spike frequencies (f_{max}) in 25 ms bins for Or22a Orco wt OSNs responding to ethyl butyrate [10^{-5} dilution (v/v)]) at varying pulse durations (10–500 ms) for 2 s following stimulus onset (grey). Each trace represents the average of *N*=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 (f_{max}) for Or22a Orco wt and Or22a Orco mut OSNs in A and B, and Orco rescue OSNs to the same stimulus regime as in A (*N*=8–9 for Orco rescue flies; error bars indicate s.e.m.). (D) Mean peristimulus time courses of spike frequencies (f_{max}) of Or22a Orco wt, Or22a Orco mut and Orco rescue OSNs responding to repeated 50 ms pulses of ethyl butyrate [10^{-5} dilution (v/v)] at 1 Hz (*N*=12 Orco wt, *N*=22 Orco mut, *N*=6 Orco rescue OSNs). (E,F) Concentration dependence of the maximum frequency (f_{max}) for Or22a neurons responding to ethyl butyrate stimulation in Orco wt, Orco mut and Orco rescue flies (*N*=5–18; error bars indicate s.e.m., **P*<0.05, independent *t*-test): E, 50 ms stimulation; F, 500 ms stimulation.

Orco wt-rescued OSNs compared with wild-type OSNs (Fig. 2E,F; Table S1). The concentration–response curve shift was also observed in other OSNs (Fig. S2 and Table S1). Taken together, these results suggest the importance of Orco phosphorylation by PKC for OSN sensitivity *in vivo*.

PKC increases OSN sensitivity to brief and intermittent odour stimuli

If the observed loss of sensitivity in Orco mut OSNs is due to a lack of PKC phosphorylation, mutation of PKC genes could also mimic the Orco mut in physiological response. To test this, we generated various *Drosophila* flies with mutations in PKC genes rather than in Orco itself. PKC53E and PKCδ are genes that could be involved in OSN signal transduction (Swarup et al., 2013; Arya et al., 2015). Here, we found that null mutations of both conventional PKC53E and novel PKCδ or suppression of PKC53E and PKCδ genes using RNAi shifted the concentration–response of the OSNs to higher odour concentrations compared with wild-type (*P*<0.05; Fig. 3A; Table S1) or parental controls (Fig. 3C). RNAi treatment also abolished OSN responses to brief odour pulses (*P*<0.05; Fig. 3B,D), but the response could be recovered at 100× concentrations in a manner similar to the Orco mut cells. Similar effects were observed in other OSNs (Fig. S3 and Table S1). We used an alternative RNAi approach targeting PKC53E and the sensitivity to brief repeated odour stimuli was significantly reduced in various OSNs, similar to the other mutants (Fig. S3E–G). Combined, these results further suggest that PKC-mediated signalling regulates the ligand sensitivity of OR-expressing OSNs.

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

To analyse whether the effects of PKC activity on the OSN response are due to intracellular or perireceptor events, we also assessed the response of Or35a OSNs (OR-OSNs), co-localized in the ac3 coeloconic sensillum along with Ir75abc OSNs (Yao et al., 2005). First, we examined the activity of IR-expressing OSNs in the Orco−/− mutant. The single sensillum recording trace shows that the Ir75abc OSN in the Orco−/− mutant responded to odour (Fig. 4A, top left) as in the wild-type (Fig. 4A, bottom left), while the co-localized Or35a OSN in the Orco−/− mutant did not respond to its key ligand (Fig. 4A, top right), unlike the wild-type OSN (Fig. 4A, bottom right). We then checked the activity of PKC in IR-

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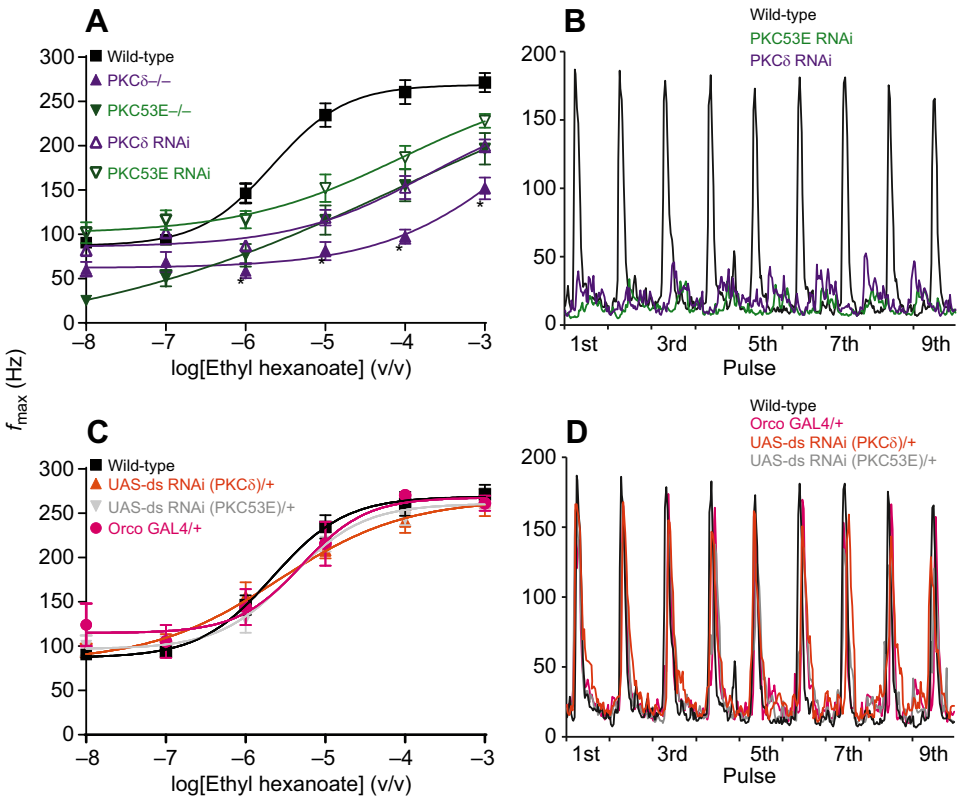

Fig. 3. Odorant-induced response of Or22a OSNs mutant for PKCδ and PKC53E. (A) Concentration–response curves showing maximum frequencies (f_{max}) for Or22a OSNs of different genotypes compared with wild-type, tested with 500 ms pulses of ethyl hexanoate (*N*=8–12; error bars indicate s.e.m.; **P*<0.05, independent *t*-test). (B) Mean peristimulus time histograms of spike frequencies for Or22a OSNs following PKC RNAi treatment compared with wild-type, in response to repeated 50 ms stimulations of ethyl hexanoate [10^{-5} dilution (v/v)] at 1 Hz (*N*=8–11). (C) Concentration–response curves showing maximum frequencies (f_{max}) for wild-type Or22a OSNs and different parental controls tested with 500 ms pulses of ethyl hexanoate (*N*=8–12; error bars indicate s.e.m.). (D) Mean peristimulus time histograms of spike frequencies for wild-type Or22a OSNs and different parental controls responding to repeated 50 ms stimulations of ethyl hexanoate [10^{-5} dilution (v/v)].

OSNs by stimulating the antenna of Orco−/− mutants with a mixture of known IR ligands. As shown in Fig. 4B, the activity of PKC did not change (*P*=0.39), suggesting that, unlike in 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 concentration–response curve (*P*>0.05; Fig. 4C,D; Table S1) unlike OR-OSNs. Finally, we tested the response of Or35a OSNs. As with other OR-OSNs, the concentration–response curve for PKC53E and PKCδ mutants was shifted to higher concentrations, showing a significant difference up to 10^{-3} dilution (v/v) for PKC53E and up to 10^{-2} dilution (v/v) for PKCδ (*P*<0.05; Fig. 4E; Table S1). This result indicates that PKC-mediated signalling in OSNs is independent of the extracellular sensillum environment surrounding the dendrite and is specific to OR-expressing OSNs.

PKC activity and Orco phosphorylation are required for efficient odour tracking

Finally, we investigated the effect of PKC and Orco phosphorylation on the behavioural response of flies using three different behavioural paradigms (flywalk, free-flight and wind-tunnel assays). Orco mut flies responded significantly less than wild-type flies to short pulses of ethyl acetate in high-throughput walking assays (flywalk), where we measured how far the fly moved towards (or in negative values away from) the stimulus within 4 s of odour encounter (Steck et al., 2012; Thoma et al., 2014). Here, we used the same concentration (midpoint of the OSN concentration–response curve) as tested for physiology at 50 ms pulses [10^{-5} dilution (v/v); *P*=0.005; Fig. S4A]. Once we confirmed a preliminary behavioural effect of Orco mut matching the physiology, we then performed free-flight assays to determine a potential role of Orco in flight tracking. In a 24 h free-flight trap assay, more wild-type, parental control and Orco wt rescued flies (Fig. 5A; Fig. S4B) were trapped after 1 h than any Orco mut, PKC53E RNAi, or PKCδ RNAi flies. The response of all mutant flies remained significantly reduced (*P*<0.05) over the entire 24 h period (Fig. 5A).

Lastly, we used wind-tunnel experiments to test our hypothesis that intracellular modulation of Orco affects an insect's ability to exhibit efficient odour localization in flight. When presented with a no-choice single source of ethyl acetate and vinegar at concentrations equivalent to those in the above free-flight assays, nearly 80% of wild-type, Orco wt rescued and parental control flies exhibited upwind anemotactic flight, and 50% reached the source across all trials (Fig. 5B; Fig. S4C; see Materials and methods for behavioural characterization). Orco−/− exhibited some anemotaxis (48%), but could not track the plume to the source. In addition, PKCδ RNAi and PKC53E RNAi flies were significantly different from wild-type flies for all behaviours (Fisher's exact test as in Linn et al., 2005). Orco mut flies were similar to wild-type flies in both upwind flight and approach, but a significantly lower proportion reached the source (34% versus 50%; Fig. 5B). Indeed, several Orco mut flies were observed to hover in front of the source and then fly around it, as if perceiving the odour source as further away than the object in front of them (Movie 1). Unlike the Orco mutants, where only phosphorylation of Orco in the OrX-Orco complex is affected, our PKC mutants are not restricted to Orco – they could also target OrX. The conductivity of OrX-Orco depends on both OrX and Orco (Wicher et al., 2008; Nichols et al., 2011; Pask et al., 2011; Nakagawa et al., 2012); thus, when we mutate PKC53E or PKCδ we could potentially be targeting both OrX and Orco putative PKC-based phosphorylation. While we show PKC subtypes are important for OrX-Orco signal transduction, these enzymes could potentially have additional affects as well. For example, PKC subtypes are expected to have target proteins that affect the electrical activity of an OSN (Wicher

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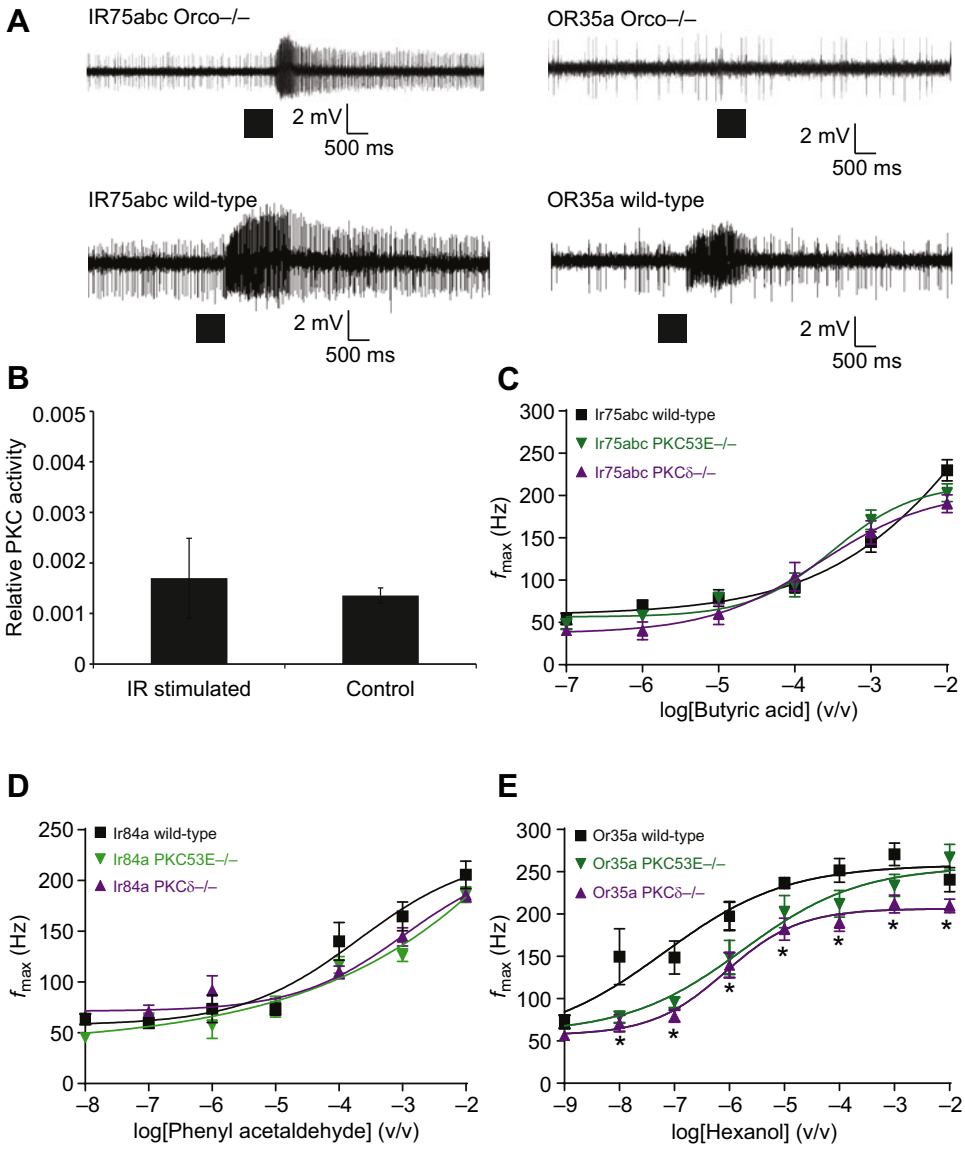

Fig. 4. PKC activity and the ionotropic (IR)-OSN response. (A) Left, 10 s sample trace depicting the response of an Ir75abc OSN to butyric acid [10^{-2} dilution (v/v)] in an Orco–/– mutant fly (top) and a wild-type fly (bottom); right, 10 s sample trace indicating the absence of an Or35a OSN response to hexanol [10^{-5} dilution (v/v)] in an Orco–/– mutant fly (top) and a wild-type fly (bottom). Black squares indicate stimulus presentation (B) Relative PKC ELISA activity due to odorant stimulation of IR-OSNs versus unstimulated control (N=3). (C) Concentration–response curves for Ir75abc wild-type and PKCδ and PKC53E mutant OSNs tested with 500 ms pulses of butyric acid (mean maximum frequency; N=8–13 for wild-type, N=7–10 for PKC53E and PKCδ mutants; error bars indicate s.e.m.). (D) Concentration–response curves as in C for Ir84a wild-type and mutant OSNs responding to phenylacetaldehyde (N=7–12 for wild-type, N=7–11 for PKCδ and PKC53E mutants). (E) Concentration–response curves as in C for Or35a wild-type and mutant OSNs co-localized with Ir75abc OSNs in response to hexanol (N=7–8 for wild-type, N=8–17 for PKCδ and N=7–11 for PKC53E mutants; *P<0.05, independent t-test; error bars show s.e.m.).

et al., 2001). Interestingly, the PKC mutants do not differ from Orco mutants in terms of their source location, which is the major phenotypic difference between Orco mut and wild-type flies (Fig. 5B). These observations suggest that the reduction of peripheral sensitivity in Orco mut flies does not trigger the 'behavioural threshold' (Van Breugel and Dickinson, 2014) necessary for landing behaviour, and the flies overshoot the source. In addition to differences in source localization, Orco mut flies began this oriented flight significantly closer to the source than wild-type flies (52.06±23.065 cm from source for Orco mut flies versus 62.64±22.545 cm for wild-type; P<0.05 t-test). Similar to Orco mut flies, PKCδ and PKC53E mutants also began oriented flight closer to the source in comparison to wild-type flies (46.36 ±26.823 cm for PKCδ, 48.89±27.842 cm for PKC53E; P<0.01 and P<0.05, respectively, t-test). Taken together, these results suggest that disruption of PKC signalling and Orco phosphorylation in OR-expressing cells reduces the success of odour localization in flight. We also tested the behavioural response of different PKC mutant flies to butyric acid (an odorant activating IR-expressing OSNs; Knaden et al., 2012) in the free-flight assay and did not see any difference between the phenotypes, although the response to the acid was low (Fig. 5C); a similar result was obtained when propionic acid was used (data not shown). These results suggest that reduction in PKC activity or Orco phosphorylation at the periphery results in reduced odour localization at the behavioural level.

DISCUSSION

Here, we show a crucial role of Orco phosphorylation and intracellular PKC activity in mediating sensitivity of the OrX-Orco complex to the brief and intermittent odour plume information encountered by flying insects. Specifically, our results indicate that both PKC activity and Orco phosphorylation are important for quick and sensitive odour detection and subsequent odour localization. These phenomena were only observed in Orco-expressing OSNs, suggesting a particular role for this intracellular signalling in this receptor complex. The high performance of insect ORs therefore relies on post-translational regulatory processes.

Our *in vivo* immunofluorescence analyses show that PKC proteins are co-expressed with Orco in the sensillum shaft where the dendritic parts of OSNs are located, and *in vitro* analyses indicate that Orco phosphorylation during odour stimulation is

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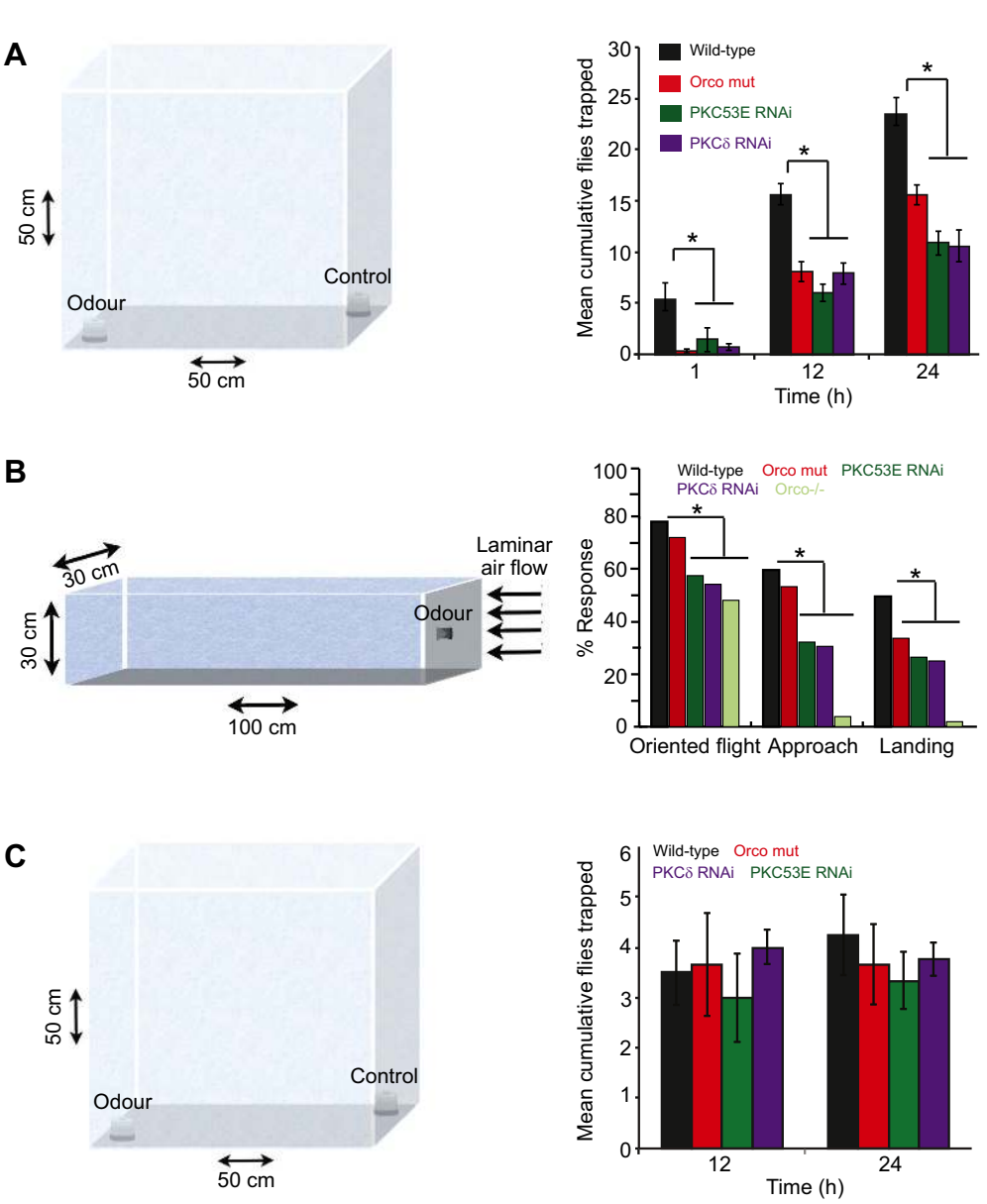

Fig. 5. PKC signalling and olfactory behavioural response. (A) Left, schematic diagram of the free-flight assay; right, mean cumulative wild-type and various mutant flies trapped in free-flight assays at the indicated times (N=6 replicates with 30 flies each; *P<0.05, no flies trapped in mineral oil control, error bars show s.e.m.). (B) Left, schematic diagram of the wind-tunnel setup; right, percentage of mutant flies that flew, approached and landed (*P<0.05). (C) Left, schematic diagram of the free-flight assay; right, mean cumulative wild-type and various mutant flies trapped in free-flight assays at the indicated times with the IR odour butyric acid (N=6 replicates with 30 flies each).

enhanced as a result of PKC activation, but not when putative PKC phosphorylation sites are mutated in Orco mut cells. Single mutations of either PKC53E or PKCδ genes *in vivo* also mimicked the mutations to Orco phosphorylation sites in both electrophysiological and behavioural assays. Orco bears five putative PKC phosphorylation sites, and these different PKCs might act on different sites that are mandatory for proper Orco function (Sargsyan et al., 2011). Reduction or removal of both PKCs had physiological and behavioural effects. Thus, they both appear to be important for normal OR signal transduction.

In contrast to OR-expressing cells, 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. However, the response of OR-OSNs co-localized with these IR-OSNs (Or35a) was reduced, indicating that any effect of PKC modulation is independent of the perireceptor environment where the neurons are expressed, and is specific to OR-expressing cells. Of course, these results cannot exclude modulation of the IR-OSN response by other intracellular signalling molecules, nor do they exclude the role of other intracellular signalling mechanisms in the OR transduction cascade. 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 to be a major factor contributing to the enhanced sensitivity and speed of the odour response in OR-OSNs. Future spectroscopic, imaging and other protein–protein interaction studies are required to test at which site and how these proteins interact. At this time, however, our results can confirm that intracellular signalling through both PKC and Orco phosphorylation affects the sensitivity of insect OSNs to brief and intermittent stimuli.

Odour plumes are composed of brief, punctuate filaments of odour molecules (Carde and Willis, 2008). As such, the reduced response of mutant flies in both no-choice and free-flight assays suggests that disruption of intracellular signalling in OR-expressing cells affects plume tracking. To confirm that these behavioural effects impact flight behaviour, our wind-tunnel results show that disruption of intracellular signalling in OR-expressing cells reduces

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the ability of flies to track odours from long distances and also reduces the success of odour localization. This line of experimentation supports the hypothesis that the evolution of the OrX-Orco complex in flying insects (Missbach et al., 2014) may have occurred to accommodate the high-speed demands of odour-mediated tracking while in flight (Getahun et al., 2012). Obviously, a similar role for intracellular signalling in ORs of other pterygote orders must be demonstrated to give credence to this hypothesis. However, the aforementioned homology of Orco across several insect orders, and the expression of similar intracellular signalling proteins in the dendrite of other insects (Laue et al., 1997; Miura et al., 2005; Maida et al., 2000), suggests that such signalling may be common across the Pterygota.

Our results show that intracellular signalling at the periphery is essential for efficient plume following in flying insects. We stress that it is not the receptor proteins themselves but rather the intracellular modulation of the complex that produces the high sensitivity and capability to follow short and frequently presented stimuli. As such, this does not preclude the presence of Orco in non-flying insects. Indeed, Missbach et al. (2014) note the presence of at least three Orco-like proteins in the apterogote *Thermobia*. However, their functional role remains elusive, given that no OrX proteins have been found in these animals. We therefore propose that the activation of OrX-Orco through intracellular signalling is a unique adaptation in the Pterygota to regulate the sensitivity of OrX-Orco heteromers. Future studies comparing olfactory signalling in pterygote (winged) versus apterygote insects, as well as detailed analyses utilizing high-speed tracking of flying insects and simultaneous measurement of plume structure and physiological response during flight behaviour (Vickers et al., 2001; Bau et al., 2002; Van Breugel and Dickinson, 2014) are necessary to test these hypotheses further.

We therefore propose the hypothesis that the unique signalling mechanism found in OR-expressing OSNs reflects an evolutionary adaptation of the olfactory system to meet the specific challenge of fast and sensitive plume resolution during flight. Insects are the first invertebrates that have developed powered flight and took to 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). It is important to note that while plume following is a well-known feature of many non-flying invertebrates such as blue crabs (Weissburg and Zimmer-Faust, 1994) and nematodes (Kato et al., 2014), the high-speed requirements of flight make sensory detection more challenging. In fact, flying organisms are generally moving more quickly and through faster turbulent flows, which means they encounter the filaments of an odour plume at a much faster rate than walking organisms (Willis et al., 2008; Van Breugel and Dickinson, 2014). This need for speed could be a significant factor driving the evolution of a novel chemoreceptor family capable of increased sensitivity to high-speed odour information.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
M.N.G., S.L.-L., S.B.O., D.W. and B.S.H. conceived and designed the study; M.N.G. performed the experiments and analysed the data; M.N.G., S.B.O. and I.K. performed and analysed the wind-tunnel experiments; M.N.G., S.B.O., D.W. and B.S.H. wrote the manuscript; M.T. and M.K. performed and analysed the Flywalk experiment; R.A.F. helped with western blotting. All authors discussed and agreed on the content of the paper.

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