Inhibitory signaling in mammalian olfactory transduction potentially mediated by $G\alpha_o$

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Abstract

Olfactory GPCRs (ORs) in mammalian olfactory receptor neurons (ORNs) mediate excitation through the $G\alpha_s$ family member $G\alpha_{olf}$. Here we tentatively associate a second G protein, $G\alpha_o$, with inhibitory signalling in mammalian olfactory transduction by first showing that odor evoked phosphoinositide 3-kinase (PI3K)-dependent inhibition of signal transduction is absent in the native ORNs of mice carrying a conditional OMP-Cre based knockout of $G\alpha_o$. We then identify an OR from native rat ORNs that are activated by octanol through cyclic nucleotide signaling and inhibited by citral in a PI3K-dependent manner. We show that the OR activates cyclic nucleotide signaling and PI3K signaling in a manner that reflects its functionality in native ORNs. Our findings lay the groundwork to explore the interesting possibility that ORs can interact with two different G proteins in a functionally identified, ligand-dependent manner to mediate opponent signaling in mature mammalian ORNs.

Keywords

Olfaction, olfactory receptor neurons, inhibition, modulation, combinatorial coding

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Introduction

ORs comprise the largest family of mammalian GPCRs (Buck and Axel, 1991). Ligand (odorant) binding to ORs results in the cyclic nucleotide-dependent excitation of ORNs through $G\alpha_{olf}$, a member of the $G\alpha_s$ subfamily (e.g., Belluscio et al., 1998). It has been known for some time that olfactory perception shows ‘mixture suppression’ and ‘mixture synergism’, in which one odorant either reduces or enhances, respectively, the

47 percept of another (e.g., Cain, 1974; Laing et al., 1984), and that at least some of this
48 perceptual modulation can be assigned to the olfactory periphery (e.g., Bell et al., 1987;
49 Laing and Wilcox, 1987). Receptor-driven modulation has since been studied directly
50 (see following paragraph) and was recently shown to be widespread across ORs,
51 indicating that it makes a fundamental contribution to the peripheral olfactory code (Xu
52 et al., 2019). Thus, it is important to understand the processes that modulate cyclic
53 nucleotide-dependent excitation in the dynamic range of activation.

54 Receptor-driven 'mixture suppression', also referred to as inhibition, antagonism,
55 or masking, has received the most attention. Pharmacological, physiological, and
56 computational evidence ascribe odor-evoked inhibition to competitive antagonism (e.g.,
57 Firestein and Shepherd, 1992; Kurahashi et al., 1994; Oka et al., 2004). The implication
58 is that 'mixture suppression' results from a reduction in cyclic nucleotide-dependent
59 excitation due to odorants competing for a common binding site on the OR. Both
60 physiological (Rospars et al, 2008) and computational (Reddy et al., 2017) evidence
61 ascribe odorant-evoked inhibition to non-competitive antagonism in addition to
62 competitive antagonism. Multiple non-competitive processes can result in odorant-
63 evoked inhibition. Some, such as 'odor masking' involving the non-specific action of the
64 antagonist on the cyclic nucleotide gated (CNG) output channel (e.g., Takeuchi et al.,
65 2009), cannot account for the broad ligand specificity of odor-evoked inhibition seen
66 across ORNs (Xu et al., 2019). A non-competitive process linked to odorant-evoked
67 inhibition that is consistent with the ligand specificity seen across ORNs involves
68 phosphoinositide 3-kinase (PI3K)-dependent signaling (Spehr et al., 2002; Ukhanov et
69 al., 2010, 2011, 2013; Yu et al., 2014). Interestingly, the primary product of PI3K-

dependent signaling *in vivo*, PtdIns (3,4,5)P3 (PIP3), competitively competes with cAMP-dependent activation of the CNG channel (Zhainazarov et al., 2004; Brady et al., 2006), potentially confounding a simple mechanistic understanding of receptor-driven 'mixture suppression'. Pharmacological evidence that PI3K-dependent, odorant-evoked inhibition is mediated by a Gβγ subunit implicates a G protein complex in this process (Ukhanov et al., 2011), as does earlier evidence that in heterologous systems at least, the function of an odorant (agonist, antagonist) depends on the G protein used (Shirokova et al., 2005).

Implicating a Gβγ subunit in PI3K-dependent, odorant-evoked inhibition raises the question of the associated Gα protein. While $G\alpha_{olf}$, the most abundant Gα isoform expressed in the cilia of mammalian ORNs, could mediate activation of PI3K signaling through the release of Gβγ, other isoforms occur in cilia-enriched membrane preparations from the olfactory epithelium (OE) (e.g., Schandar et al., 1998; Wekesa and Anholt, 1999; Mayer et al., 2009). These other G proteins may function in processes as diverse as adaptation and cell survival (Watt et al., 2004; Mashukova et al., 2006; Kim et al., 2015a,b), but have also been implicated in signal transduction (e.g., Scholz et al., 2016b). If two different G protein complexes are involved in olfactory signal transduction, it is important to understand whether both are activated by the same OR.

Here, we provide evidence potentially linking PI3K-mediated inhibitory signaling pathway to $G\alpha_o$. We demonstrate that odor-evoked PI3K-dependent inhibitory signaling is no longer detectable in mice carrying an OMP-Cre conditional deletion of $G\alpha_o$. We show that fluorescently-tagged $G\alpha_o$ is trafficked to the cilia of native ORNs using viral-

mediated ectopic expression, and that $G\alpha_o$ expression is reduced in the ORNs of mice carrying the OMP-Cre conditional deletion of $G\alpha_o$ using IHC. We then use single cell RT-PCR to identify an OR expressed by mammalian ORNs that were activated by octanol and inhibited by citral in a PI3K-dependent manner. The functionality of the identified OR (Olr1845) persists in a HEK293T-based pCRE-SEAP assay. Using the same expression system we then implicated $G\alpha_o$ in odor-dependent activation of PI3K by that OR using an ELISA. Collectively, our results are consistent with, although do not prove, that mammalian ORs can interact with at least two different G proteins in a functionally identified, ligand-dependent manner.

Methods

Animals

Experiments were performed on adult female Sprague-Dawley rats, adult CD1 mice, adult M71-SR1-IRES-tauGFP mice, as well as adult C57BL/6 and *cGnαo*$^{-/-}$ mice. All animal procedures were performed in accordance with the University of Florida animal care committee's regulations. Animals were euthanized by inhalation of carbon dioxide and decapitated immediately prior to dissection. All experiments were performed at room temperature (22–25°C) unless otherwise noted. *cGnαo*$^{-/-}$ animal breeding, genotyping, and genomic DNA analyses were performed using published protocols and primers (Chamero et al., 2011; Choi et al., 2014).

In situ hybridization and immunolabeling of cryosections

115 Tissue fixation and cryo-sectioning were performed using published protocols.
116 Briefly, the OE was fixed in 4% paraformaldehyde and then the tissue soaked in 30%
117 sucrose at 4°C before embedding in optimal cutting temperature medium. 12 µM
118 sections were collected under RNase-free conditions and stored at -80°C until use. *In*
119 *situ* hybridization was performed using a modification of published methods (Ishii et al.,
120 2004; Choi et al., 2016a). Briefly, tissue sections were hybridized with digoxigenin-
121 labeled riboprobes for *Gnao* and *OMP* detection. After washing to remove unbound
122 probe, the sections were then incubated with anti-digoigenin-HRP antibody (Roche) and
123 labeling was detected with NBT/BCIP (Sigma). The sections were cover-slipped with
124 Fluormount with DAPI (Southern Biotechnology) and visualized with a 10x and an oil
125 immersion 60x lens on an Olympus BX41 microscope.
126 Immunostaining was performed using modifications of published protocols (e.g.,
127 Choi et al., 2016). Briefly, antigen retrieval was performed by incubating the slides with
128 10 mM sodium citrate buffer (pH 6.0) at 60°C for 30 min. After blocking with 10%
129 (vol/vol) normal goat serum, 1% BSA, and 0.1% Triton X-100, sections were incubated
130 with primary antibodies overnight at 4°C. The antibodies included $G\alpha_o$ (rabbit, 1:200;
131 Santa Cruz Biotechnology) and OMP (goat, 1:500; Wako). The slides were washed
132 with PBS containing 0.1% Triton X-100 and then with secondary antibodies conjugated
133 with Alexa Fluor 488 (Invitrogen). Slides were coverslipped with Fluoromount DAPI
134 (Southern Biotech) and labeling was visualized with 10x and oil immersion 60x lenses.
135
136 ***Calcium imaging***

Acutely dissociated rat or mouse ORNs were imaged using standard published approaches. Briefly, olfactory epithelia were dissected in ice-cold modified artificial cerebrospinal fluid (ACSF) saturated with 95% O_2 and 5% CO_2 that contained (in mM): 120 NaCl, 25 $NaHCO_3$, 5 KCl, 1.25 Na_2HPO_4, 1 $MgSO_4$, 1 $CaCl_2$, 10 glucose. The tissue was transferred in low-Ca^{2+} (0.6 µM free Ca^{2+} buffered with 5 mM EGTA) ACSF supplemented with 0.5 mg/ml papain (Sigma-Aldrich) and, in some cases, 10 units/ml TurboDNAse (Promega). After incubation for 20 min at 37°C in 5% CO_2, the tissue was gently washed with normal oxygenated ACSF several times, minced with a razor blade and triturated with a large bore fire polished glass pipette. The resulting suspension was filtered through a 40 µm cell strainer (BD BioSciences). An aliquot of the suspension was mixed with 10 µM Fluo-3 or Fluo-4 containing 0.04% Pluronic F127 and placed on a glass coverslip coated with concanavalin A (Sigma-Aldrich) in a recording chamber (RC22, Warner Instruments). The volume of the chamber was 200 µL, allowing for complete exchange of the solution during application of odorant and/or inhibitors. In some experiments cells were placed and imaged in 35mm tissue culture dishes with cover glass bottom (FluoroDish, WPI) treated with concanavalin A. Odors were applied using a multi-channel rapid solution changer (RSC-160, Bio-Logic). The cells were illuminated at 500 nm and the emitted light was collected at 530 nm by a 12-bit cooled CCD camera (ORCA-R2, Hamamatsu). Both the illumination and image acquisition were controlled by Imaging Workbench 6.0 software (INDEC BioSystems). Each cell was assigned a region of interest (ROI) and changes in fluorescence intensity within each ROI were analyzed. Continuous traces of multiple responses were compensated for slow drift of the baseline fluorescence when necessary. All recordings were

160 performed at room temperature (22-25°C). Single odorants were of highest purity
161 obtained from Sigma-Aldrich and were prepared fresh as used from 0.5M DMSO stocks
162 kept at -20°C. The complex odorant Henkel-100 was dissolved 1:1 in anhydrous
163 DMSO as a working stock solution.
164
165 ***Viral expression of fluorescently tagged $G\alpha_o$***
166 GFP and mCherry were inserted into the coding sequence of mouse $G\alpha_o$ using
167 site directed mutagenesis to create EcoRI cut sites within the $G\alpha_o$ coding sequence
168 followed by restriction enzyme digestion and T4 ligation. GFP and mCherry were
169 amplified by PCR with primers designed to allow in frame insertion as previously
170 described (Hynes et al., 2004). All constructs were fully sequenced prior to use.
171 $G\alpha_o$:GFP adenovirus (AdV) and $G\alpha_o$:mCherry adeno-associated virus (AAV2/5) were
172 produced using previously described methods (e.g., Zolotukhin et al., 2002; McIntyre et
173 al., 2015). For expression using AV in native tissue, recombinant GFP-fused cDNA was
174 cloned into the vector p-ENTR by TOPO cloning methods. The inserts were then
175 recombined into the adenoviral vector pAD/V5/-dest using LR Recombinase II (Life
176 Technologies, Carlsbad CA). Viral plasmids were digested with PacI and transfected
177 into HEK293 cells. Following an initial amplification, a crude viral lysate was produced,
178 and used to infect confluent 60-mm dishes of HEK293 cells for amplification according
179 to the ViraPower protocol (Life Technologies). AdV was isolated with the Virapur
180 Adenovirus mini purification Virakit (Virapur, San Diego, CA), dialyzed in 2.5% glycerol,
181 25 mM NaCl and 20 mM Tris-HCl, pH 8.0, and stored at −80°C until use. For ectopic
182 expression in native tissue using AAV, the $G\alpha_o$:mCherry fusion was cloned into the

pTR-UF50-BC plasmid vector and virus was propagated in HEK293 cells using the pXYZ5 helper plasmid. For viral transduction of ORNs, mice were anesthetized with a Ketamine/Xylazine mixture and 10-15 µL of purified viral solution was delivered intranasally as a single injection per nostril. Animals were used for experiments at 10 days post-infection. The entire turbinate and septum were dissected and kept on ice in a petri dish filled with oxygenated ACSF. For imaging a small piece of the OE was mounted on the stage of the microscope in a perfusion chamber with the apical surface facing down. High resolution *en face* imaging of freshly dissected OE was performed on an inverted confocal microscope Leica SP5. Images were processed using ImageJ (NIH http://imagej.nih.gov/ij/) and assembled in CorelDraw13 (Corel).

Single Cell RT-PCR

Rat ORNs functionally characterized by calcium imaging were collected with a sterile glass micropipette directly into RT buffer for lysis. Cells were immediately frozen at stored at -80°C. Single cell RT-PCR was performed using a modified approach based on previously described methodology (Touhara et al., 1999). Briefly RT was performed using a Verso RT kit (Thermo Fisher) with an anchored oligo dT primer for 60 minutes at 42°C. RT was followed by PCR detection of OMP and beta actin to exclude cells that were not ORNs and samples contaminated with genomic DNA. PCR with degenerate primers designed to amplify OR genes was performed as follows. The first round of amplification of OR genes was performed in a solution containing 0.4 µM each of the published degenerate primer and an adapter primer targeting the oligo d(T)18-anchor used for the RT, 0.2 mM dNTP, and PrimeSTAR HS Taq (Clontech) and the second

206 amplification used a nested set of primers targeting ORs. Each PCR consisted of 5 min
207 at 95°C followed by 40 cycles at 95°C for 1 min, an annealing temperature dependent
208 on primers for 3 min, and 72°C for 2 min. The PCR products were subsequently cloned
209 into pGEM-T Easy (Promega) followed by sequencing (McLab) of multiple clones for
210 each PCR product.
211
212 ***OR expression constructs***
213 Rat ORs identified by single cell RT-PCR were amplified from genomic rat DNA
214 and mOR261-1 was amplified from genomic mouse DNA. The ORs were cloned into a
215 pME18S-based Lucy-Rho vector (denoted here as pLucy-Rho-OR) (Shepard et al.,
216 2013) for mammalian expression. All constructs were sequenced prior to use.
217
218 ***Culture and transfection of HEK293T cells***
219 HEK293T cells (ATCC) were grown in Dulbecco's modified Eagle medium
220 (DMEM) supplemented with 10% fetal bovine serum, penicillin (100 U/ml), and
221 streptomycin (100 mg/ml) in 5% CO_2 at 37°C. Before transfection, the cells were
222 seeded into 35 mm tissue culture treated dishes and incubated for 24 hours. For pCRE-
223 SEAP and PI3K assays, cells were transfected at 70% confluency using X-treme-GENE
224 HP (Roche) at a ratio of 3:1 with plasmid DNA following the manufacturer's instructions.
225
226 ***pCRE-SEAP assay***
227 cAMP production was measured as previously described (Durocher et al., 2000).
228 HEK293T cells were transfected with the expression vectors pcDNA3.1 Ric-8b (50 ng;

generously provided by Dr. Bettina Malnic, Universidade de São Paulo, Brazil), pcDNA3.1(+) Gαolf (50 ng; Missouri S&T cDNA Resource Center), pcDNA3.1(+) RTP1s (100 ng; subcloned from construct purchased from Thermo Fisher) and pLucy-Rho-OR (1.5 µg). For control experiments cells were transfected as above, however, the pLucy-Rho-OR construct was omitted. Cells were also transfected with 1.5 µg of a pCRE-SEAP, where the expression of the secreted alkaline phosphatase (SEAP) is under regulation of the cAMP responsive elements, (pCRE-SEAP) or a pTAL-SEAP, where the cAMP responsive elements are not present (Clontech; Durocher et al., 2000). Cells were also transfected with 50 ng pcDNA5/TO/LACZ (Invitrogen) to assess transfection efficiency. At 24 hr post-transfection the cells were re-seeded for SEAP analysis and odorants were added at the indicated dilutions at 48 hours post-transfection. Cells and supernatants were collected 20 hr later and centrifuged for 5 min at 5000 g. The supernatants were incubated for 30 min at 65°C and then frozen until analysis. SEAP activity was measured by mixing 100 µl of supernatant with an equal amount of BluePhos substrate (KPL). Samples were monitored for color development at 630 nm in a microwell plate reader. Mean SEAP activity was determined after subtracting the response of cells that were not expressing an OR and is reported in OD630 arbitrary units +/- SEM. Each experiment was repeated in triplicate with three replicates each. The data were analyzed using GraphPad Prism.

PI3K assay

HEK293T cells were transfected with 1.5 µg of pME18s Lucy-Rho-OR, 100 ng of pcDNA3.1(+) RTP1s and 100 ng of the indicated G protein construct, as well as with 0.5

μg of pBTK-PH-YFP (generous gift from Dr. Tamas Balla; (Balla et al., 2009). pcDNA3.1(+)-based constructs for $G\alpha_o$, $G\alpha_{olf}$, and $G\alpha_{oG203T}$ were obtained from the Missouri S&T cDNA Resource Center. At 24 hours post-transfection, cells were split into 35 mm dishes for analysis. After 24 hours, cells were incubated for 1 hour in 0.5% fetal bovine serum in DMEM including phosphatase inhibitors (Boston Bioproducts). For PI3K activation, cells were treated with odorant or DMSO (odorant carrier) for 30 sec and then immediately lysed with ice cold 5% TCA. Cells were scraped from dishes and the lysates were stored immediately at -80°C until analysis. For analysis, lipids were extracted following a chloroform:methanol protocol and used immediately in a PIP3 ELISA following the manufacturer's instructions (Echelon Biosciences). Each experiment was performed in triplicate. Mean PIP3 production was determined by subtracting the response to DMSO and is presented as ΔPIP3 (pM). The data were analyzed using GraphPad Prism.

Results

$G\alpha_o$ is required for PI3K-dependent inhibitory signal transduction in mouse ORNs

Given varied lines of evidence that $G\alpha_o$ is expressed in mammalian ORNs (Mayer et al., 2009; Keydar et al., 2013; Heron et al., 2013; Nickell et al., 2012; Omura and Mombaerts, 2014; Saraiva et al., 2015; Scholz et al., 2016a, Choi et al.,2016; Wang et al., 2017; Zhang et al., 2016) and that $G\alpha_o$ can physically interact with mammalian ORs (Scholz et al., 2016b), we looked for functional evidence to implicate $G\alpha_o$ in PI3K-dependent inhibitory signal transduction in mature mouse ORNs. Mice carrying a global deletion of the *Gnao* gene display a variety of defects that include behavioral issues and

motor control deficiencies likely resulting from impaired neurogenesis and olfactory system development (Jiang et al., 1998; Choi et al., 2016). Therefore, to test the impact of deletion of *Gnao1* on olfactory signal transduction without possible confounds resulting from widespread issues with olfactory system development, we used a conditional Cre-based knockout (KO) model in which inactivation of *Gnao1* through deletion of exons 5 and 6 is restricted to OMP-positive cells, referred to as c*Gnαo1*$^{-/-}$ mice (Chamero et al., 2011; Oboti et al., 2014). Given that OMP expression is restricted to mature ORNs, the OE should develop normally and the impact of Gαo deletion on signaling should be restricted to these cells.

We asked whether depletion of the $G\alpha_o$ protein altered the odor-evoked activity of ORNs by monitoring the responses of acutely dissociated ORNs from C57BI6J and c*Gnαo1*$^{-/-}$ mice to a complex odor mixture (H100) in the presence and absence of the PI3K blocker LY294002 (10 µM) (Fig 1A, 1st and 2nd columns, showing type results for 24 ORNs). We predicted that the response evoked by H100 will reflect excitation evoked by one or more components of the mixture that is tempered by inhibition evoked by one or more other components, and that pharmacologically blocking PI3K will result in an increase in the net response magnitude in instances where PI3K-based inhibitory signaling occurs. All cells were also tested with a higher concentration of H100 than the test concentration (Fig 1A, 3rd column). Only those cells showing a 10% or greater response to the higher concentration of H100, indicating their response was not saturated at the test concentration, were subsequently analyzed. The responsiveness of all the ORNs to an IBMX/ forskolin mixture (Fig 1A, 4th column) confirmed the functional integrity of the isolated ORNs.

298 Our data confirmed the results of previous studies suggesting that ORNs of
299 c*Gnαo1*$^{-/-}$ mice maintain their odor responsiveness (Chamero et al., 2011; Oboti et al.,
300 2014). H100 (1:100,000 dilution) evoked a mean response amplitude from ORNs
301 isolated from c*Gnαo1*$^{-/-}$ mice of 0.25 ± 0.03 (n = 79 ORNs from 4 mice) (Fig. 1C, 1st bar).
302 The mean response amplitude of the ORNs from c*Gnαo1*$^{-/-}$ mice was significantly larger
303 than that observed in the ORNs from WT mice (0.18 ± 0.02, n = 78 ORNs from 5 mice)
304 (Fig. 1C, 2nd bar) (P = 0.04, 1st bar vs 2nd bar), consistent with the inhibitory PI3K
305 signaling pathway not being activated in ORNs from c*Gnαo1*$^{-/-}$ mice.
306 On incubating the ORNs from both the c*Gnαo1*$^{-/-}$ and B6 mice with the PI3K
307 blocker LY294002 (10 μM) prior to treatment with H100, no enhancement of the
308 response was observed in the ORNs from c*Gnαo1*$^{-/-}$ mice, evoking a normalized mean
309 response amplitude of 0.24 ± 0.03 (n = 79 ORNs from 4 mice) (Fig 1C, 3rd bar) (P =
310 0.07, 3rd bar vs 1st bar). The lack of change from baseline recordings would be
311 consistent with the inhibitory PI3K signaling pathway not being activated in ORNs from
312 c*Gnαo1*$^{-/-}$ mice. In contrast, the response of 39 of 78 (50%) ORNs from WT mice was
313 significantly enhanced by PI3K blockade, evoking a normalized mean response
314 amplitude of 0.39 ± 0.04 (n = 78 ORNs from 5 mice) (Fig 1C, 4th bar) (P = 0.<001, 4th
315 bar vs 2nd bar). The significantly smaller mean response of the ORNs from c*Gnαo1*$^{-/-}$
316 mice (P = 0.002, 3rd bar vs 4th bar) than that observed in the ORNs from WT mice would
317 be consistent with the inhibitory PI3K signaling pathway not being activated in ORNs
318 from c*Gnαo1*$^{-/-}$ mice. Presumably odor stimulation should not have activated PI3K
319 signaling in either group of cells treated with LY294002. Thus, finding that blockade of
320 PI3K in WT ORNs resulted in a normalized mean response that was significantly larger

than that of ORNs from the c*Gnαo1*$^{-/-}$ mice (Fig. 1C, 4th bar vs 3rd bar) could potentially indicate $G\alpha_o$-independent activation of PI3K. However, that is not likely since blockade of PI3K had no effect on the response of ORNs from the c*Gnαo1*$^{-/-}$ mice (Fig. 1C, 3rd vs 1st bars), suggesting the magnitude of the response in WT ORNs post-blockade reflects the dynamics of action of the drug when PI3K is activated. Collectively, these findings are consistent with the hypothesis that $G\alpha_o$ is functionally upstream of PI3K in the context of inhibitory transduction in mature ORNs.

$G\alpha_o$ expression in the OE in mice carrying an OMP-Cre based deletion of Gnao1

The loss of PI3K-based inhibition of excitatory signaling in odorant sensitive ORNs in the c*Gnαo1*$^{-/-}$ mice implies that $G\alpha_o$ localizes to the olfactory cilia where transduction occurs and that it is reduced in the c*Gnαo1*$^{-/-}$ mice. A previous study validated reduced Gnao1 gene expression in the c*Gnαo1*$^{-/-}$ mice, but did not find reduced Gnao1 gene expression the total OE (Chamero et al., 2011). Here we show deletion of exons 5 and 6 occurs in the OE at the genomic level using PCR with primers spanning this region (Fig 2A; Choi et al., 2016). The recombined *Gnao1* gene is present as a smaller fragment amplified from DNA isolated from the OE and VNO, but is at low to undetectable levels in the olfactory bulb where there is no OMP expression. Recombination is absent in B6 mice. *In situ* hybridization targets *Gnao1* expression to the mature ORN (OMP-expressing) layer of the OE (Fig 2B; Heron et al., 2013; Saraiva et al., 2015; Choi et al., 2016). Since *Gnao1* mRNA is abundant in ORNs (Heron et al., 2013; Omura and Mombaerts, 2014; Saraiva et al., 2015; Wang et al., 2017), at least some of the recombination potentially could be ascribed to mature ORNs. Immuno-

labeling cryosections of the OE localized expression of the $G\alpha_o$ protein to the axon bundles mature (OMP-expressing) ORNs but was unable to localize expression of the $G\alpha_o$ protein to the distal compartments and/or cilia (data not shown).

We instead determined whether ectopically expressed $G\alpha_o$ can be trafficked to the cilia (McEwen et al., 2008; McIntyre et al., 2015). Mice were intra-nasally injected with adeno-associated virus carrying fluorescently tagged $G\alpha_o$ (Hynes et al., 2004) (AAV $G\alpha_o$:mcherry). *En face* imaging revealed $G\alpha_o$:mCherry in the cilia of transduced ORNs (Fig 3A). In ORNs transduced with adenovirus carrying $G\alpha_o$:GFP, GFP expression co-localized with the ciliary protein Arl13b (Fig. 3C), indicating that these results are not dependent on the identity of the fluorescent tag inserted into $G\alpha_o$ or on the viral vector used for infection. Given that the OE is composed of multiple types of chemosensory cells (e.g., Munger, 2009), we then asked whether $G\alpha_o$ could localize to the cilia of ORNs expressing ORs known to couple to $G\alpha_{olf}$. Using mice expressing tauGFP under the control of the SR1 OR gene, we found AAV expressed $G\alpha_o$:mCherry localized GFP^+ to the cila (Fig. 3B), suggesting it is not excluded from canonical ORNs

$G\alpha_o$ enhances odorant-evoked coupling of a mammalian OR isolated from native ORNs responsive to an identified opponent odorant pair in HEK293T cells

PI3K dependent inhibitory signaling has been demonstrated in both rats and mice (e.g., Brunert et al., 2010; Ukhanov et al., 2010). Several opponent (excitatory/inhibitory) odorant pairs have been identified for rat ORNs (e.g., Ukhanov et al., 2010; 2011), and here use one of those pairs to assess whether a single

366 mammalian OR can activate both PI3K signaling through $G\alpha_o$ and ACIII signaling
367 through $G\alpha_{olf}$.

368 We first measured the calcium signal in acutely dissociated rat ORNs evoked by
369 octanol (OOL, 50 μM) both alone and in combination with citral (CIT, 100 μM). In a
370 subset of OOL-responsive cells, co-application of CIT reduced the peak Ca^{2+} response
371 by 5-fold on average (Fig, 4A). Pre-incubation of the cells with the PI3Kβ and -γ isoform
372 specific blockers TGX221 and AS252424 (200 nM each) rescued the Ca^{2+} response
373 (Fig, 4A), indicating that the antagonism was not the result of direct competition of the
374 odorants for the binding site, but rather activation of the opponent inhibitory PI3K
375 signaling pathway. Individual ORNs with this response profile were collected (Fig, 4B)
376 for single cell RT-PCR using degenerate primers based on conserved regions of
377 mammalian OR sequences (Touhara et al., 1999). Prior to OR amplification, the
378 samples were tested for olfactory marker protein (OMP) expression to ensure that they
379 were mature ORNs (Barber et al., 2000) and with β-actin primers to avoid testing those
380 with detectable genomic DNA contamination (Chan et al., 1997). From a total of ten
381 functionally delimited rat ORNs that met these requirements, we recovered three rat
382 ORs (Olr1845, two ORNs; Olr1479, two ORNs; Olr1231, one ORN; no OR amplified,
383 five ORNs) and cloned the full length sequences for heterologous expression under the
384 control of a CMV promoter with Lucy and Rho tags to enhance their surface expression
385 (Shepard et al., 2013).

386 We then tested the function of the receptors in a pCRE-SEAP assay by co-
387 expressing them with $G\alpha_{olf}$, RTP1s and Ric8b in HEK293T cells along with a cAMP
388 reporter gene (Durocher et al., 2000; Zhuang and Matsunami, 2007). The cAMP

reporter plasmid pCRE-SEAP expresses secreted alkaline phosphatase (SEAP) in response to cAMP binding to cAMP response elements (CRE). Odorants were added at 48 hours post transfection and SEAP activity was measured 20 hours later. All results represent at least three independent replicate experiments. We focused on Olr1845, which responded consistently to OOL in a dose-dependent manner (Fig 5A). The other receptors did not respond consistently and will require further optimization to determine whether they show similar ligand profiles. Olr1845 did not produce measurable responses to other single odorants tested (250 µM) including vanillin, eugenol, and isovalaric acid (data not shown). Olr1845 did not respond to 75 µM CIT alone, but 75 µM CIT suppressed the response to OOL in a graded manner (Fig 5B). Control experiments in which cells were transfected with all of the signaling co-factors, except, Olr1845, and tested in parallel did not show changes in SEAP activity when stimulated with OOL alone or in combination with CIT (Fig. 5B, inset). We then tested the mouse OR OR261-1 (Olfr447), known to respond to OOL (Saito et al., 2009), and confirmed its response to OOL in a dose-dependent manner (Fig 5C). In contrast to Olr1845, OR261-1 responded to 75 µM CIT alone (Fig 5D), which was not affected by increasing concentrations of OOL. This result indicates that not all receptors that respond to OOL respond to CIT, or a mix of CIT and OOL, in the same manner. The experimental results with Olr1845 also serve as a positive control, allowing us to assign the effects seen with Olr1845 to that receptor and not one inherent in the heterologous cell.

To determine whether $G\alpha_o$ enhances the odorant-evoked coupling of Olr1845 to PI3K, we used an ELISA specific for PIP3, the primary product of PI3K activation *in vivo* (Ukhanov et al., 2010). We first co-expressed Olr1845 with RTP1s in HEK293T cells,

412 relying on the endogenous G proteins and associated chaperones. At 48 hours post-
413 transfection, a 30 sec treatment of the cells with CIT or OOL (500 µM), increased the
414 level of PIP3 by 49.52 ± 3.71 pmol and 25.78 ± 7.02 pmol, respectively, (n = at least 3
415 independent replicates) above that in response to carrier (DMSO) treatment alone. This
416 indicates that Orl1845 can activate the PI3K pathway in the heterologous system and
417 that CIT is a stronger PIP3-dependent agonist. The response to CIT is significantly
418 higher than that to OOL (Fig. 6, 1st pair of bars, P=0.04).
419 We then independently co-expressed three different Gα subunits together with
420 Olr1845, each with at least three independent replicates. $G\alpha_o$ overexpression
421 significantly enhanced the increase in PI3K activation in response to CIT, resulting in a
422 PIP3 level of 64.16 ± 1.44 pmol (Fig. 6, 1st bars in the 1st and 2nd pair of bars, P= 0.02).
423 This suggests that $G\alpha_o$ plays a role in mediating PI3K activation in the heterologous
424 system. Again, the response to CIT was significantly higher than that to OOL (Fig. 6,
425 2nd pair of bars, P=0.001). To test whether the increase in PI3K signaling resulted from
426 the functional activity of $G\alpha_o$, we co-expressed a $G\alpha_o$ gene carrying a G203T mutation
427 that is predicted to decrease the ability of the protein to turn over GDP and GTP (Slepak
428 et al., 1993) and attenuate its ability to activate downstream signaling. Unlike native
429 $G\alpha_o$, the mutated G protein subunit resulted in a lower level of PI3K activation by CIT
430 (10.54 ± 3.46 pmol) in comparison to cells expressing only endogenous G proteins,
431 actually significantly decreasing it, and no change in the level of PI3K activation by OOL
432 (25.29 ± 7.62 pmol), indicating that the activity of $G\alpha_o$ is required for this process (Fig. 6,
433 3rd pair of bars vs 1st pair of bars, P = 0.002; 0.97, respectively). $G\alpha_{olf}$ co-expression,
434 which enhances the cAMP response of ORs (Zhuang and Matsunami, 2007), also

435 resulted in lower levels of PI3K activation by both CIT and OOL (21.60 ± 4.17 pmol and
436 3.75 ± 1.24 pmol, respectively) in comparison to cells expressing only endogenous G
437 proteins, actually significantly decreasing them (Fig. 6, 4th pair of bars vs 1st pair of bars,
438 P = 0.007; 0.04, respectively). This latter effect potentially reflects sequestration of the
439 necessary Gβγ subunits from endogenous G proteins (Hippe et al., 2013).
440

441 **Discussion**

442 Our findings potentially associate a second G protein, $G\alpha_o$, with inhibitory
443 signalling in mammalian olfactory transduction by first showing that odor evoked
444 phosphoinositide 3-kinase (PI3K)-dependent inhibition of signal transduction is absent
445 in the native ORNs of mice carrying a conditional OMP-Cre based knockout of $G\alpha_o$.
446 Finding that conditional $G\alpha_o$ deletion eliminates odor evoked, PI3K-dependent inhibition
447 in dissociated ORNs argues that $G\alpha_o$ is functionally upstream of PI3K. We then identify
448 an OR from native rat ORNs that are activated by octanol through cyclic nucleotide
449 signaling and inhibited by citral in a PI3K-dependent manner. We show that the OR
450 activates cyclic nucleotide signaling and PI3K signaling in a manner that reflects its
451 functionality in native ORNs. Collectively these findings raise the interesting possibility
452 that a mammalian OR can interact with two different G proteins in a functionally
453 identified, ligand-dependent manner.

454 We argue that functional data obtained in acutely dissociated ORNs implicate
455 $G\alpha_o$ in mediating inhibitory olfactory signal transduction. Acutely dissociating ORNs
456 destroys the normal polarity of the cells, exposing the entire cell to odors and allowing
457 that $G\alpha_o$-dependent activation of PI3K is not related to transduction per se. Two

findings counter this possibility. First, odorant sensitivity is predominately, if not entirely, localized to the cilia/dendritic knob in dissociated vertebrate (salamander) ORNs (Lowe and Gold, 1991) and the dissociated mammalian ORNs typically retain at least part of their ciliary complement in our hands. Second, the pharmacological effect of blocking PI3K-dependent inhibition seen here in the dissociated ORNs occurs with the same dynamics in ORNs in the intact OE where the normal polarity of the cells targets signaling to the cilia/dendritic knob, and where patch-clamping dendritic knobs shows that PI3K-dependent inhibition acts with msec resolution, setting the peak frequency and the latency of the train of action potentials evoked by an odor mixture (Ukhanov et al., 2010). Thus, we have no reason to assume the functional data are biased by using acutely dissociated ORNs. However, given we did not use littermate controls the possibility remains that the functional data reflect strain differences, which will require further experimentation to resolve.

Implicating $G\alpha_o$ in the activation of PI3K is consistent with evidence that PI3K signaling is sensitive to pertussis toxin, which is indicative of $G\alpha_{i/o}$-dependency in other systems (e.g. Orr et al., 2002; Banquet et al., 2011; Hadi et al., 2013), as well as with evidence that $G\alpha_o$ can signal through interactions of its associated βγ subunits with downstream effectors (Wettschureck, 2005; Steiner et al., 2006; Bondar and Lazar, 2014). Published evidence shows that class 1B PI3Kγ is expressed in mouse ORNs, that PI3Kγ-deficient mice show almost a complete lack of odorant-induced PI3K activity in their OE, and that the ORNs of PI3Kγ-deficient mice show reduced sensitivity to PI3K mediated inhibition (Brunert et al., 2010). The γ catalytic subunit is thought to exclusively associate with the regulatory subunits that mediate binding to the Gβγ

subunit of heterotrimeric G proteins (e.g., Rameh and Cantley, 1999), which in our case would be $G\alpha_o$ activated by the OR.

$G\alpha_o$ also mediates PLC signaling in mature ORNs (Schandar et al., 1998), as well as extracellular-signal-regulated kinase (ERK) signaling by a heterologously expressed OR (Bush et al., 2007) that is associated with cell survival and apoptosis. Since both the PLC and ERK pathways have been associated with PI3K-dependent signaling networks in other systems, temporally distinct waves of $G\alpha_o$ activated PI3K (e.g., Jones et al., 1999; Goncharova et al., 2002) in ORNs could potentially mediate transduction as well as slower activation of cell survival and/or apoptotic pathways. There has been a published report for a $G\alpha_o$-mediated alternate, cyclic nucleotide-independent, PI3K-independent signaling pathway in mammalian ORNs that targets a downstream Cl^- conductance and presumably leads to an excitatory efflux of Cl^- (Scholz et al., 2016b). The functional significance of this pathway is unclear, but as the authors suggest, this pathway may be developmentally important since it appears to be associated with immature ORNs. Further work will be required to relate this finding to the $G\alpha_o$-mediated inhibitory signaling in mature ORNs proposed herein.

We show that excitation and PI3K-dependent inhibition can be mediated by the same OR when expressed heterologously, and that the antagonism is not the result of direct competition for a common binding site. As in the native ORNs from which the rat OR Olr1845 was cloned, OOL acts as an excitatory ligand and CIT as an inhibitory ligand for Olr1845 in a cAMP assay when heterologously expressed. This does not occur when the cells were transfected with the co-factors in the absence of the OR, nor when the cells were transfected with a different OR responsive to the same two ligands.

We also show that CIT is a stronger activator of PI3K than is OOL in a PIP3 assay when Olr1854 is heterologously expressed. Heterologous readouts of OR activation are slow in comparison to transduction, but similar assays reflect the ligand specificity of other ORs tested *in vivo* (Tsuboi et al., 2011), supporting our argument that Olr1845 appears to be capable of directing the pattern of activation elicited by an opponent pair of ligands through two different signaling pathways. This finding for a mammalian OR is consistent with the ability of single insect olfactory receptors to similarly direct the pattern of activation of an ORN in studies using the 'empty neuron' approach (Hallem et al., 2004). Whether all ligands in the molecular receptive range of a given OR can activate PI3K, only to different extents, with the stronger PI3K-dependent agonists being the effective inhibitory ligands for the OR in question, as potentially suggested by Fig. 6, remains for future research. We focused on Olr1845, which allows that our finding could be idiosyncratic for Olr1845 or the OOL/CIT odorant pair, but evidence that $G\alpha_o$ can interact with other mouse ORs (Scholz et al., 2016b), as well as evidence that PI3K-dependent inhibition can be activated by a wide range of conventional odorants in native rat ORNs (Ukhanov et al., 2013), including other opponent odorant pairs (Ukhanov et al., 2011), argues for the generality of this finding across at least a subset of ORs.

Assuming the OR and $G\alpha_o$ interact, the assumption would be they interact in the transduction (ciliary) compartment. As noted, both immature and mature ORNs express $G\alpha_o$ (Mayer et al., 2009; Keydar et al., 2013; Heron et al., 2013; Nickell et al., 2012; Omura and Mombaerts, 2014; Saraiva et al., 2015; Scholz et al., 2016a, Choi et al.,2016; Wang et al., 2017; Zhang et al., 2016). It remains to be determined, however,

whether the protein routinely localizes to the ciliary compartment, notwithstanding limited evidence for positive IHC staining for $G\alpha_o$ in the distal compartments of OMP^+ ORNs in *Gnao1*$^{+/+}$ mice (Choi et al., 2016) and *Olfr73*-positive ORNs (Scholz et al., 2016a). The fact that we could show there appears to be no barrier excluding $G\alpha_o$ from the ciliary compartment is consistent with ciliary expression since cilia are known to largely exclude non-resident proteins (McEwen et al., 2008; McIntyre et al., 2015), although this demonstration leaves open the question of constitutive expression of $G\alpha_o$ in the ciliary compartment. While $G\alpha_o$ is expressed in sustentacular cells (SUSs) (unpublished observations), the possibility that it interacts with signaling in ORNs via ephaptic coupling (Su, et al., 2012) is not consistent with our physiological results obtained in acutely dissociated ORNs. Nor is it consistent with the absence of any evidence that mammalian ORNs and SUSs are grouped in stereotyped functional combinations that would be required to explain the observed ligand specificity.

GPCRs are increasingly appreciated to sequentially activate multiple G proteins such that the outcome of activation does not depend solely on the receptor identity but rather is influenced by extracellular factors such as the range of ligands present, as well as by intracellular factors including the abundance and localization of the G proteins present (e.g., Mashuo et al., 2015 and reviewed in Lohse and Hofmann, 2015; Latorraca et al., 2016). Such 'functional selectivity' (e.g., Luttrell, 2014; Smrcka, 2015) is a key characteristic of allosteric modulation in GPCRs (Christopoulis, 2014). Given that OR-ligand interaction is thought to be 'fast and loose' (Bhandawat, 2005) and growing evidence for loose allosteric coupling of the agonist binding site and the G protein coupling interface in GPCRs (e.g., Lohse and Hofmann, 2015; Manglik et al.,

2015; Wingler et al., 2019), a given OR could interact with both G protein isoforms without implying concurrent activation by a given odorant or the need for simultaneous coupling of the OR to both $G\alpha_{olf}$ and $G\alpha_{o}$. Brief activation of the OR by a PI3K-dependent inhibitory ligand, for instance, could release pre-bound $G\alpha_{olf}$ while resulting in a more favorable structure for binding to $G\alpha_{o}$. The fact that not all G proteins work *in vivo* by having the heterotrimers physically dissociate (e.g., Digby et al., 2006) could provide specificity for signals mediated by the βγ dimer, as in the present context, and avoid confound in the origin of the βγ dimer. However, the idea that ligand-bound GPCRs interact with and activate G proteins (e.g., Audet et al., 2012) is being replaced by emerging evidence that the GPCR and G protein are preassembled into protein complexes in which the G protein influences ligand affinity (e.g., DeVree et al., 2016; Venkatakrishnan et al., 2016). Thus, a subset of the OR expressed by a given cell could be primed for activation of $G\alpha_{o}$, and in turn PI3K inhibitory signaling. Understanding how functional selectivity in ORs could play out at the molecular level awaits further understanding of GPCR signaling in general.

In summary, our findings lay the groundwork to explore the interesting possibility that ORs can interact with two different G proteins in a functionally identified, ligand-dependent manner to mediate opponent signaling in mature mammalian ORNs. Going forward, the primary challenge will be to understand the expression pattern of $G\alpha_{o}$, and potentially other G proteins, in addition to $G\alpha_{olf}$ in the transduction compartment. If $G\alpha_{o}$ is present in cilia, but perhaps at lower levels or not in all neurons, this may be better approached through genetic methods to label and visualize the endogenous protein. The possibility that ORs can interact with multiple G proteins in a functionally identified,

ligand-dependent manner in the context of transduction would be a paradigm-shift in our understanding of how the olfactory periphery sets the combinatorial pattern considered to be the basis of odor recognition and discrimination.

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Author Contributions

EAC, KU, YUB, JCM, and JRM designed the experiments. EAC, KU, YUB carried out the experiments and analyzed the data. EAC, BWA, and JCM prepared the manuscript. All authors discussed and contributed to writing the manuscript.

Conflict of Interest

The authors declare no competing financial interests.

596

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Legends

Figure 1. $G\alpha_o$ is required for PI3K-dependent inhibitory signal transduction in mouse ORNs. A. Representative odor-evoked calcium responses of 23 ORNs acutely dissociated from the main olfactory organ of wild type (W) (left panel) and 23 ORNs from knockout (K/O, *cGnαo1*$^{-/-}$) (right panel) mice, before (column 1) and after (column

2) incubation with the PI3K inhibitor LY294002 (LY). Stimulus: Henkel 100 (H100, 1/100,000 dilution). Column 3, responses to a tenfold higher concentration of H100 (1/10,000). Column 4, responses to a mixture of IBMX (50µM) and Forskolin (50µM). The fluorescence intensity traces were normalized to the maximum fluorescent intensity generated in response to IBMX/Forskolin, and then color coded from blue (minimum fluorescent intensity to yellow (maximum fluorescent intensity). Stimulus pulse duration was 5s for columns 1, 2, and 3, and 10s for column 4. B. Representative time/intensity plots of the calcium responses of W type ORN # 21 in A (top row) and K/O type ORN # 6 in A (bottom row). C. Bar graph comparing the average amplitude of the odor-evoked calcium responses of 79 ORNs from K/O mice and 78 ORNs from W mice before and after incubation with LY. Statistical comparisons based on the Mann-Whitney Rank Sum Test.

Figure 2. OMP-Cre mediated Gnao1 deletion in the olfactory epithelia *of cGnαo1*$^{-/-}$ knockout mice. A. Genomic DNA extracted from the OE, VNO, and OB was examined by PCR for recombination of floxed alleles. C57BL/6 WT (WT) mice were used for comparison. In the KO mice, both the WT and OMP-Cre (Cre) alleles are detected. The recombined Gnao1 (ΔGαo) allele is detected in the OE and VNO of the KO mice as indicated by the smaller fragment in the lower panel. B. Comparison of Gnao1 (Gαo) and OMP expression in the OE of B6 mice by *in situ* hybridization of cryosection from B6 mice. OMP expression is restricted to the mature ORNs.

851 **Figure 3. Ectopically expressed $G\alpha_o$ can enter the cilia of mammalian ORNs.** *En*
852 *face* imaging of ORNs expressing $G\alpha_o$ internally tagged with mCherry or GFP. A. Three
853 examples of AAV infected ORNs ectopically expressing $G\alpha_o$:mCherry. B. $G\alpha_o$:mCherry
854 can be co-localized to SR1-GFP+ ORNs. mCherry expression is found throughout
855 infected ORNs including in the dendritic knobs and cilia. Scale bars represent 10 μM.
856 C. AV infected ORNs of C57BL/6 mice ectopically expressing $G\alpha_o$:GFP and
857 Arl13b:mCherry. $G\alpha_o$ expression overlaps with that of Arl13b indicating that ciliary
858 localization of the ectopically expressed protein does not depend on the vector or tag.
859
860 **Figure 4. Identification of ORNs responsive to the antagonistic odorant pair**
861 **octanol and citral for single cell RT-PCR**. A. Fluo-3 calcium imaging of dissociated
862 rat ORNs was used to identify single cells for RT-PCR. Representative recording of the
863 somatic Ca2+ response from one of ten rat ORNs activated by octanol (OOL; 50 μM) in
864 which citral (CIT; 100 μM) inhibited the response and pretreatment with PI3K inhibitors
865 TGX221 and AS252424 (TGX/AS; 200 nM each) partially relieved the antagonism. B.
866 Image of an ORN identified by calcium imaging prior to collection for single cell RT-
867 PCR.
868
869 **Figure 5. Gαolf and ACIII activation by rat Olr1845 in response to octanol and**
870 **citral.** A. Line graph showing that rat Olr1845 responds in a dose dependent manner to
871 OOL in a pCRE-SEAP assay. Response to OOL is denoted by open circles. B. Bar
872 graph showing that the cAMP response of rat Olr1845 to OOL at the concentrations
873 indicated (dark bars) was reduced in a graded manner when the odorant was presented

in a binary mixture with CIT at the concentration indicated (light bars). Inset: Bar graph showing the response of cells not expressing an OR tested in the same experimental paradigm. C. Line graph showing that a different mouse OR (mOR261-1) also responds in a dose dependent manner to OOL in a pCRE-SEAP assay (open circles). D. Bar graph showing that in contrast to B, the cAMP response of mouse OR261-1 to OOL at the concentrations indicated. (dark bars) was actually enhanced, i.e., shows additivity, when the odorant was presented in binary mixture with CIT at the concentration indicated (light bars). Data are presented as SEAP activity (OD630) -/+ SEM representing at least three independent replicate experiments. Response of cells to DMSO has been subtracted in all cases.

Figure 6. PI3K activation by rat Olr1845 in response to octanol and citral. Bar graph showing the elevation of endogeneous PIP3 in HEK293T cells transfected with rat Olr1845 in response to citral (CIT; 500 µM) and octanol (OOL; 500 µM). PI3K activity was measured by a PIP3 ELISA at 48 hours post-transfection in response to a 30 sec odorant exposure. The PIP3 level in DMSO-treated control cells is subtracted in all instances. The receptor was either expressed alone (endogeneous, 1st pair of bars), with $G\alpha_o$ (2nd pair of bars), with $G\alpha_{oG203T}$ (3rd pair of bars), or with $G\alpha_{olf}$ (4th pair of bars) Data are presented as change in pmol PIP3 ± SEM, representing at least three independent replicates. Probabilities for the various comparisons listed in the text are indicated by the horizontal lines. Statistical comparison based on the Student's t test.