INPP5E controls ciliary localization of phospholipids and odor response in olfactory sensory neurons

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Summary

Cilia of olfactory sensory neurons are distinct in lipid composition from the somatic plasma membrane. Localization of phospholipids shapes the cellular function and further our understanding of the olfactory system in health and disease.

Abstract

The lipid composition of the primary cilia membrane is emerging as a critical regulator of cilia formation, maintenance, and function. Here, we show that conditional deletion of the phosphoinositide 5'-phosphatase gene, *Inpp5e*, causative of Joubert syndrome in terminally developed mouse olfactory sensory neurons (OSNs) led to a dramatic remodeling of ciliary phospholipids that was accompanied by marked elongation of cilia. PI(4,5)P_2_ normally restricted to the proximal segment redistributed to the entire length of cilia in *Inpp5e* knockout mice with a reduction in PI(3,4)P_2_ and elevation of PI(3,4,5)P_3_ in the dendritic knob. The redistribution of phosphoinositides impaired odor adaptation, resulting in less efficient recovery and altered inactivation kinetics of the odor-evoked electrical response and the odor-induced elevation of cytoplasmic Ca^{2+}. Gene replacement by adenoviral expression of *Inpp5e* restored the ciliary localization of PI(4,5)P_2_ and odor response kinetics in OSNs. Our findings support the role of phosphoinositides as a modulator of the odor response and in ciliary biology of native multiciliated OSNs.
Introduction

The olfactory system in animals and humans is optimally tuned to recognize a diverse set of chemical cues and odorants in the environment. In mammals, chemical cues are detected by specialized multi-ciliated olfactory sensory neurons (OSNs) embedded in the olfactory epithelium (OE), transmitting sensory information through action potentials to the olfactory bulb (Firestein, 2001). In most mammalian OSNs, signal transduction is mediated by a canonical cAMP-dependent signaling pathway (Bradley et al., 2005; Kaupp, 2010). Initial binding of an odorant to an olfactory receptor activates a G-protein, Gαolf-coupled cascade that triggers the catalytic activity of adenylyl cyclase 3 (AC3) (Brunet et al., 1996; Jones and Reed, 1989) to generate cAMP. Transiently elevated inside cilia, cAMP opens cyclic nucleotide-gated channels (CNGCs) leading to the influx of calcium ions that in turn activates calcium-dependent chloride channels, (TMEM16B/ANO2) as a secondary amplification cascade (Kaupp and Seifert, 2002; Reisert et al., 2005; Stephan et al., 2009). Importantly, all proteins controlling effective recovery from the transient excitation and overwhelming elevation of intraciliary calcium including cAMP hydrolyzing phosphodiesterase 1C, K+-dependent Na+/Ca2+ exchanger, and the calcium pump, are localized in the ciliary membrane (Cygnar and Zhao, 2009; Mayer et al., 2009; Saidu et al., 2009; Stephan et al., 2012). Despite all the studies that dissected the main components of this cascade, much less is understood of how the transduction is tuned and regulated within the cilia microenvironment to support optimal sensitivity and resolution of the incoming sensory information.

Well known regulators of signaling proteins are the constituents and composition of the cell membranes in which they reside. Emerging evidence indicates that the lipid composition of cilia may differ from the bulk of the plasma membrane (Lechtreck et al., 2013; Zhao et al., 2012). Surprisingly, until recently very little attention was given to the organization of olfactory cilia, in particular, to the lipid membrane ensheathing the axoneme and harboring both polytopic and peripheral olfactory signaling proteins. A gradually building body of evidence suggests some organizational complexity to the olfactory ciliary bilayer. Our previous work demonstrated a differential partitioning of various lipid-anchored GFP probes that bind to the inner leaflet of the olfactory cilia membrane (Williams et al., 2014). This suggested the presence of ciliary membrane domains with distinct lipid compositions. In addition, the cholesterol binding protein caveolin (CAV-1) has been implicated as a scaffold to localize proteins in the odor detection pathway to lipid raft domains (Schreiber et al., 2000). In line with these findings, the olfactory CNGA2 channel was shown not only to have a spatially restricted localization in primary cilium
(PC) but also to be functionally regulated by cholesterol (Brady et al., 2004; Jenkins et al., 2006). Another cholesterol binding protein, Stomatin-like protein 3 (SLP3), was identified in OSNs and localized to the transition zone (TZ) of olfactory cilia (Kobayakawa et al., 2002; Tadenev et al., 2011). Intriguingly, SLP3 co-immunoprecipitated with AC3 and CAV-1 from olfactory cilia isolates (Kobayakawa et al., 2002). Indeed, CAV-1 is not only localized to PC in other cells types but is also implicated in the regulation of cilia length and Sonic-Hedgehog signaling via a polyphosphoinositide (PI) dependent pathway (Maerz et al., 2019; Rangel et al., 2019; Schou et al., 2017).

We now know that PIs are involved in specific aspects of sensory function. For example, elevation of PI(3,4,5)P$_3$ (PIP$_3$) within olfactory cilia can inhibit the CNGCs (Brady et al., 2006; Spehr et al., 2002), whereas odorant stimulation may induce dynamic redistribution of PI(4,5)P$_2$ (PIP$_2$) in the dendritic knob of OSNs (Ukhanov et al., 2016). Recently, PIs were discovered to play a role in ciliogenesis and regulation of ciliary function (Garcia-Gonzalo et al., 2015; Phua et al., 2017). The interplay between the two PIs, PIP$_2$ and PI4P, is crucial to the organization of the cilia transition zone (TZ), and controls protein trafficking and signaling within the PC (Garcia-Gonzalo et al., 2015; Garcia et al., 2018; Phua et al., 2017; Xu et al., 2016). The localization and relative abundance of two PIs, were found in dynamic reciprocity to each other under the tight control of INPP5E, a phosphoinositide 5'-phosphatase that hydrolyzes PIP$_2$ and PIP$_3$ (Bielas et al., 2009; Hasegawa et al., 2016; Kisseleva et al., 2000). Each of the PIs, hydrolyzed by INPP5E, represents a small fraction of all membrane associated lipids, but plays an indispensable role in regulating many aspects of cellular physiology including cell division, vesicle trafficking, and control of transmembrane ionic transport (Balla, 2013; Di Paolo and De Camilli, 2006; Hilgemann et al., 2018; Logothetis et al., 2015). Importantly, mutations in the Inpp5e gene cause its loss-of-function due to mislocalization or impairment in catalytic activity and manifest in a ciliopathy termed Joubert syndrome (JBTS).

To better understand the role of lipids and, specifically PIs in the cell biology of olfactory cilia we sought to investigate the localization and relative abundance of several lipids utilizing a conditional Inpp5e-deficient mutant. A panel of highly selective probes to several important classes of lipids were used in live mouse OSNs in situ. Using this approach and mouse model allowed us for the first time, to analyze the distribution and functional implication of JBTS ciliopathy-related changes to the phospholipid composition of cilia in terminally differentiated mammalian sensory neurons.
Results

Loss of Inpp5e causes PIP\(_2\) redistribution from the knob into the olfactory cilia

INPP5E hydrolyzes with high affinity two phosphoinositide species PI(4,5)P\(_2\) (PIP\(_2\)) and PI(3,4,5)P\(_3\) (PIP\(_3\)) generating PI4P and PI(3,4)P\(_2\), respectively (Kisseleva et al., 2000; Kong et al., 2000). Distribution of PIP\(_2\) in mature OSNs was measured using en face confocal microscopy of intact olfactory epithelium transduced with adenovirus encoding PLC\(\delta\)1-PH (PLCPH) domain tagged with GFP (Fig. 1). In wild-type (WT) littermate control mice, 52.7±10.8 % (n=318, 4 mice) of cells infected with the PLCPH probe had an extremely polarized distribution of PIP\(_2\) with an accumulation in the OSN knob and no ciliary localization (Fig. 1A and C; Fig. S1B). In those cells, PIP\(_2\) was uniformly distributed in the plasma membrane of the knob and adjacent dendrite and extended all the way to the axons (Fig. 1A, right panel; Fig. S1B,C). The total number of cilia (21.8±0.5 cilia per OSN, n=37, 4 mice) and cilia length (29.5±0.5 µm (n=753, 4 mice)) was measured by co-expression of an inert lipid-anchored probe MyrPalm fused to mCherry (MP-mCherry) (Fig. 1A, middle panel). In a fraction of OSNs, however, we detected PIP\(_2\) in a small subset of cilia ranging from 1-5 cilia per neuron, but this allocation was much fewer than the total number of cilia (Fig. 1C; Fig. S1B). The distribution of PIP\(_2\) along the length of a given cilium was highly variable and ranged from a short segment to the full length (Fig. 1A and D). A full-length distribution of PIP\(_2\) was rare and often seen in a single cilium. Overall distribution of PIP\(_2\) and MP-mCherry in the WT cilia result in non-overlapping histograms summarized in Figure 1D.

To get insight in the regulation of phospholipids, in particular PIP\(_2\), in olfactory cilia and OSNs we utilized an olfactory-specific conditional knockout mouse \textit{Inpp5e}^{osnKO}. The mutant was generated by crossing \textit{Inpp5e}^{loxP} founder described previously (Jacoby et al., 2009) with a mouse carrying Cre-recombinase under the promoter of the olfactory marker protein (OMP) expressed exclusively in mature OSNs (Green et al., 2018). Consistent with previous transcriptomic and proteomic data in OSNs (Kuhlmann et al., 2014; Nickell et al., 2012), Western Blot data (representative images and densitometry) of OE extracts show protein expression of a doublet at ~72kD corresponding to INPP5E wildtype and splice variant (Jacoby et al., 2009) that is decreased in the \textit{Inpp5e}^{osnKO} mouse (Fig. S1A). The remaining signal likely reflects the presences of multiple cell types in the OE. The loss of INPP5E in OSNs of \textit{Inpp5e}^{osnKO} mouse severely impacted ciliary PIP\(_2\) distribution resulting in its homogenous
redistribution along the entire axoneme (Fig. 1B). Remarkably, this deficiency affected every cilium (Fig.1C, KO) shifting distribution of PIP$_2$ domain length to a complete overlap with that of the ciliary length marker MP-mCherry (Fig. 1E). Another salient feature of the PIP$_2$ localization in the KO cilia was its abundance within the proximal segment of each cilium, overlapping with the transition zone (Fig. 1B right panel, red arrow). Notably, the mean cilia length was significantly increased from 29.5±0.5 µm in WT littermates to 35.3±0.6 µm in the \textit{Inpp5e}\textsuperscript{osnKO} mice (n=495, 3 mice, unpaired t-test, t=7.363 df=1246, p<0.0001). Cilia length is controlled by an evolutionary conserved process of intraflagellar transport (IFT) (Rosenbaum and Witman, 2002). The loss of INPP5E impacts IFT in primary cilia resulting in the selective accrual of IFT-A particles (Chávez et al., 2015; Garcia-Gonzalo et al., 2015). Surprisingly, we did not find any abnormality in the velocity of IFT-A dependent transport of IFT122 particles or its accumulation inside olfactory cilia of \textit{Inpp5e}\textsuperscript{osnKO} mice (Fig. S2A,B, Movie S1). IFT-B related trafficking of IFT88 was also unaltered with a similar particle velocity to that published previously for the wild-type OSNs (Uytingco et al., 2019; Williams et al., 2014) (Fig. S2C-E, Movie S2).

**Ectopic expression of human \textit{INPP5E} restores the restricted distribution of PIP$_2$ in \textit{Inpp5e}\textsuperscript{osnKO} OSNs**

To address the potential of virally assisted therapy of the JBTS ciliopathy model \textit{in vivo}, we used a rescue adenoviral vector carrying the full-length sequence of human \textit{INPP5E} (NM_019892) fused with GFP on the N-terminal, GFP-INPP5E-FL (Chávez et al., 2015). Ectopically expressed GFP-INPP5E-FL was enriched in the OSN knobs and localized to the full length of cilia in the WT (Fig. S1D) and KO (Fig. 2A). As shown in Figure 2, full-length wild-type INPP5E was necessary for restoration of normal PIP$_2$ distribution in OSNs. Ectopic expression of GFP-INPP5E-FL in \textit{Inpp5e}\textsuperscript{osnKO} OSNs resulted in a significant decrease of PIP$_2$ ciliary domain length as measured with PLCPH-mCherry (Fig. 2B and C). Average length of the PIP$_2$ domain in WT cilia was 4.9±0.27 µm (n=110, 16 cells, 3 mice), in \textit{Inpp5e}\textsuperscript{osnKO} cilia 28.5±1.37 µm (n=54, 5 cells, 3 mice) and in rescued KO cilia 4.2±0.3 µm (n=122, 17 cells, 3 mice), one-way ANOVA, F(DFn, DFd) 86.73 (2,283), p<0.0001) (Fig. 2D). As a negative control we used a catalytically inactive point mutant GFP-INPP5E-D477N (Chávez et al., 2015), which failed to change localization of PIP$_2$ when co-expressed with PLCPH-mCherry in HEK293 cells (Fig. S3). Co-expression of PLCPH-mCherry with GFP-INPP5E-D477N resulted in a significantly larger number of OSNs having a complement of PIP$_2$-decorated cilia, 61.2±0.05 % (D477N, n=61, 3 mice), compared to INPP5E-WT, 17.6±0.09 % (INPP5E-WT, n=83, 3 mice, unpaired t-test,
t=4.536, df=24, p=0.0001) (Fig. 2E-H). Together these data indicate that the catalytic activity of INPP5E is required for restricting the distribution of PIP$_2$ in olfactory cilia.

**Loss of Inpp5e affects multiple phospholipids in OSNs**

One of the main routes of PIP$_2$ synthesis is thought to be by PI5K and PI4K kinase-dependent phosphorylation of PI4P and PI(5)P, respectively (Schramp et al., 2015). PI4P was shown to be highly enriched in PC of several cell types (Chávez et al., 2015; Garcia-Gonzalo et al., 2015) and under the tight control of INPP5E which seems not to use PI5P as a substrate (Conduit et al., 2017; Kisseleva et al., 2000; Madhivanan et al., 2015; Schramp et al., 2015). Adenoviral expression of a probe specific for PI4P, P4M-SidM (Hammond et al., 2014) tagged with mCherry showed low abundance in the olfactory cilia of the control WT mice (Fig. 3A top panel). Conversely, in most OSNs, PI4P was highly enriched in the knob (Fig. 3A). We directly compared levels of PI4P in the knobs of WT and *Inpp5e*$_{osnKO}$ by measuring absolute fluorescence intensity. In the *Inpp5e*$_{osnKO}$ OSNs the mean level of PI4P showed a slight but not significant decrease (Fig. 3D; 179±26 units, WT, n=94, 3 mice and 143±17 units, KO, n=54, 3 mice; t-test, t=0.9777 df=146, p=0.3298). Besides PI2, INPP5E also dephosphorylates PI3 at even higher efficiency than PI2, generating PI(3,4)P$_2$ (Conduit et al., 2012). PI(3,4)P$_2$ was measured using ectopic expression of the Tapp1-PH domain (Fukumoto et al., 2017) and was found mostly restricted to the knobs with a low level in cilia. Its distribution pattern was not changed by the loss of INPP5E (Fig. 3B). However, quantitative analysis of PI(3,4)P$_2$ revealed significant depletion in the knobs of *Inpp5e*$_{osnKO}$ mice (Fig. 3E, 280±11 units, n=830, 3 mice, WT; 174±7, n=858, 3 mice, KO; t-test, t=8.453 df=1686, p<0.0001). Finally, to assay the distribution of PI3, we used GFP-tagged PH domain of Bruxton tyrosine kinase (Btk-GFP), a well characterized highly selective PI3 lipid probe (Balla, 2013). Similar to both PI4P and PI(3,4)P$_2$, PI3 was highly enriched in the OSN knob, with relatively low quantities in cilia (Fig. 3C upper panel). Several other probes selective for PI3 based on the PH domains of ARNO, Akt and Grp1 proteins showed an identical distribution to Btk-GFP in OSNs (data not shown). Intriguingly, quantitative analysis of PI3 in OSN knobs of *Inpp5e*$_{osnKO}$ showed a significant increase by nearly 3-fold (Fig. 3F; 668±64 units, n=60, 3 mice, WT; 1,495±185, n=91, 3 mice, KO; unpaired t-test, t=3.536 df=149, p=0.0005) with very little if any build-up in cilia (Fig. 3C KO bottom panel). An inert membrane lipid anchor probe MP-mCherry did not show any preferred partitioning in the membrane in OSN knobs in the WT and the KO (Fig. 3G, MP-mCherry,
Inpp5e deficiency does not affect overall lipid integrity of the ciliary membrane

We hypothesized that the loss of INPP5E activity resulting in a substantial remodeling of ciliary PIP\textsubscript{2} may impose an additional effect on overall ciliary lipid composition. We first asked if cholesterol which is required for organizing membrane PIP\textsubscript{2}-rich domains may itself be reciprocally affected by its enrichment. The D4H fragment of bacterial toxin perfringolysin-O recognizing cholesterol in inner membrane leaflet, tagged with mCherry (Maekawa and Fairn, 2015) selectively decorated proximal segments of cilia in WT mice (Fig. 4A upper panel, arrowheads). Although D4H-mCherry was enriched in the proximal segment, it did label the full length of cilia albeit not as intensely as the MP-mCherry probe. Consistent with the localization of cholesterol, YFP-Caveolin-1 (a cholesterol binding protein) was also highly restricted to the proximal segment (Fig. S4). Membrane enrichment of PIP\textsubscript{2} in the Inpp5e\textsuperscript{osnKO} however, did not affect overall localization of D4H-mCherry and YFP-Caveolin-1. This suggests only nominal crosstalk between PIs and cholesterol in olfactory cilia (Fig. 4A bottom panel; Fig. S4).

A second phospholipid class particularly enriched in the inner leaflet of the plasma membrane and which regulates the trans-bilayer distribution of cholesterol is phosphatidylserine (Maekawa and Fairn, 2015). In accord with the localization of cholesterol probe D4H-mCherry, ectopically expressed phosphatidylserine sensor Lact-C2-GFP, a fragment of lactadherin, was enriched in knobs and in addition evenly distributed along the entire length of cilia (Fig. 4B). Similar to cholesterol, this pattern was not affected in Inpp5e\textsuperscript{osnKO} mice (Fig. 4B).

We completed this screen by probing lipids relevant to protein trafficking and targeting, sphingomyelin and glycosylphosphatidylinositol (GPI) (Deng et al., 2016; Paladino et al., 2008). Eqt2-SM-GFP, equinatoxin-2 from the sea anemone Actinia equina, is a probe for sphingomyelin which is associated with Golgi-to-membrane vesicle trafficking (Deng et al., 2016). GPI-anchored proteins (e.g. human Folate Receptor-1) are directly targeted to the apical membrane in polarized cells, preferentially partitioning into cholesterol-rich raft domains (Paladino et al., 2008). Eqt2-SM-GFP showed partial enrichment of sphingomyelin in cilia of both WT and Inpp5e\textsuperscript{osnKO} mice (Fig. 4C) whereby GPI-GFP was highly restricted only to the dendritic knobs in OSNs (Fig. 4D).
Loss of Inpp5e impacts ciliary localization of PIP_2 binding proteins

The role of PIP_2 and PIP_3 has been long appreciated as regulators of protein localization and function within structurally defined regions in the plasma membrane (Czech, 2000). Recently, several PIP_2 binding proteins from the Tubby family, implicated in ciliogenesis and ciliary protein trafficking, were shown to be mislocalized in the PC of cells derived from Inpp5e knock-out mice (Mukhopadhyay et al., 2010). Members of the Tubby-like protein family, TULP1 and TULP3 are anchored to the plasma membrane through their C-terminal PIP_2 binding motif (Mukhopadhyay and Jackson, 2011; Santagata et al., 2001). Therefore, TULP1 and TULP3 proteins were used as secondary PIP_2 probe (Hammond and Balla, 2015) and also to test if translocation of proteins with affinity to PIP_2 occurs in Inpp5e^{osnKO} OSNs. Similar to PLCPh distribution, TULP1-GFP and TULP3-GFP were found mostly in the wild-type OSN knobs (Fig. 5A and B upper left panel). However, in the Inpp5e^{osnKO} OSNs, ectopically expressed TULP1-GFP and TULP3-GFP translocated along the full-length axoneme, mimicking the PLCPh redistribution and demonstrating that PIP_2 binding is sufficient for their ciliary entry (Fig. 5A and B bottom panels).

Notably, TULP1 and TULP3 were particularly enriched in the ciliary proximal segment of Inpp5e^{osnKO} OSNs (Fig. 5A and B right bottom panel, arrowheads). Overall, the percentage of knobs showing TULP1-positive cilia was dramatically increased in the OSNs of Inpp5e^{osnKO} mice (Fig. 5A upper right panel, 25.49±0.06%, n=4, 3 mice, WT; 100%, n=6, 3 mice, KO; Mann-Whitney t-test, p=0.0048). The same redistribution of TULP3 was found in cilia of the Inpp5e^{osnKO} mice. (Fig. 5B upper right panel, 30.87±0.12%, n=6, 3 mice, WT; 100%, n=4, 3 mice, KO; Mann-Whitney t-test, p=**0.0095).

Since TULP1 and TULP3 are peripheral membrane proteins, we asked if a different protein with a more complex polytopic structure and known to bind PIP_2 could be translocated into olfactory cilia in Inpp5e^{osnKO} OSNs. Potassium inward rectifier channels (Kir1.x-6.x), particularly Kir2.x members are endogenously expressed in the olfactory system (Prüss et al., 2003) and depend on binding PIP_2 for proper gating (Hansen et al., 2011; Hilgemann et al., 2001; Lee et al., 2016; Logothetis et al., 2015). Indeed, ectopically expressed Kir2.1-mCherry was found highly localized to the knob (Fig. 5C WT left panel) whereas in Inpp5e^{osnKO} mice Kir2.1-mCherry moved into the ciliary membrane (WT: 3.02±0.021%, n=12, 3 mice; KO: 24.34±5.89%, n=10, 3 mice; unpaired t-test, t=3.658, df=20, **p=0.0016). In contrast, other resident proteins expressed in PC (EFHC1, polycystin-2 (PC2)) and not known to bind PIP_2, failed to redistribute
to the full length of cilia in \textit{Inpp5e\textsuperscript{osnKO}} mice (Fig. 5D and E). These data suggest there is specificity to the redistribution of proteins into olfactory cilia following membrane remodeling.

Given the putative role of PIP\textsubscript{2} and Tubby proteins in the localization of GPCRs in primary cilia (Chávez et al., 2015; Mukhopadhyay et al., 2010; Park et al., 2015), we investigated the endogenous localization of an odorant receptor. We assayed endogenous distribution of olfactory receptor M71/72 using \textit{en bloc} immunocytochemical approach. In WT and \textit{Inpp5e\textsuperscript{osnKO}} mice we found very similar homogenous pattern of M71/72 localization along cilia (Fig. S5A,B). Cilia of 20-30 µm in length were observed both in WT and \textit{Inpp5e\textsuperscript{osnKO}} mice which is consistent with our measurements using live \textit{en face} imaging (e.g. Fig.1, MyrPalm-mCherry). This suggests that mechanisms regulating odorant receptor trafficking into OSN cilia differ from GPCRs in PC and raises the question as to the functional consequence of PIP\textsubscript{2} redistribution following loss of INPP5E in OSN cilia.

\textbf{Odor-mediated calcium response is modulated by \textit{Inpp5e}}

Previously PIs were established as modulators of ion channels including olfactory cyclic nucleotide-gated channels (Brady et al., 2006; Hilgemann et al., 2001), and involved, specifically, in the control of the odor response of OSNs (Spehr et al., 2002). We hypothesized that unusually high steady-state accumulation of PIP\textsubscript{2} in cilia as well as elevated PIP\textsubscript{3} in the knob of \textit{Inpp5e\textsuperscript{osnKO}} mouse may result in altered odor-evoked response. We measured odor-evoked calcium transients in the knob of OSNs ectopically expressing GCaMP6f (Fig. 6A-C; Movie S3). A significant decrease of the time constant of termination phase of the GCaMP6f response was observed in \textit{Inpp5e\textsuperscript{osnKO}} compared to the WT OSNs (Fig. 6C-E decay tau, WT: 6.49±0.37 s, n=167, 3 mice; KO: 3.59±0.18 s, n=110, 3 mice, unpaired t-test, t=6.077, df=275, ****p<0.0001). Rise time from 10% to 90% of the GCaMP6f odor response amplitude was also significantly shorter in the KO (Fig. 6F rise time, WT: 1.12±0.07 s, n=46, 3 mice; KO: 0.80±0.08 s, n=30, 3 mice, unpaired t-test, t=2.936, df=74, **p=0.0044).
Odor adaptation is impaired in Inpp5e deficient mouse

Since calcium clearance from cilia and knobs of OSNs is critically involved in shaping the odor response (Stephan et al., 2012), we further analyzed the electrophysiological response to odor in Inpp5e^{osnKO} mice. A short 100-ms pulse of amyl acetate vapor of increasing concentration was applied to the freshly dissected olfactory tissue to build a concentration-response curve (Fig. 7A and B). Overall odor sensitivity was not changed in Inpp5e^{osnKO} mice (two-way ANOVA, F(5, 102) = 0.1858, p = 0.9674) resulting in overlapping dose-response curves (Fig. 7B). However, the kinetics of the response were different in the Inpp5e^{osnKO} mice, reminiscent of the changes observed in a single-cell GCaMP6F response. The EOG evoked by 10^{-2} M amyl acetate reached its maximal magnitude faster (Fig. 7C; 10-90% rise time was 174.5±7.7 ms, n=37, 5 mice, WT; 157.9±10.9 ms, n=40, 7 mice, KO, unpaired Mann-Whitney test, *p=0.0221). Also, the response inactivated faster to the baseline (Fig. 7D; termination phase was fit to a single exponential function yielding time constant of 4.57±0.15 s, n=81, 5 mice, WT; 3.40±0.16 s, n=28, 4 mice, KO; unpaired t-test, t=4.386, df=107, ***p<0.0001). Paired-pulse adaptation paradigm did not reveal any difference between the WT and Inpp5e^{osnKO} mice using a short 100-ms pulse of amyl acetate (Fig. S3). However, we observed a much stronger effect in Inpp5e^{osnKO} mice on adaptation of the EOG response to a repetitive longer 5-s pulse of 10^{-3} M amyl acetate (Fig. 7E and F). Adaptation was measured as the ratio of the peak EOG evoked by the 2\textsuperscript{nd} odor pulse 40 s after the 1\textsuperscript{st} pulse (Fig. 7E and F, black (WT) and green (KO) traces) and recovered slower in the KO (Fig. 7H,I, 2\textsuperscript{nd}/1\textsuperscript{st} peak ratio 0.733±0.026, n=18, 9 mice, WT; 0.514±0.022, n=9, 6 mice, KO; Mann-Whitney test, ***p<0.0001). The effect of the Inpp5e deletion also resulted in a reduced plateau-to-peak ratio (Fig. 7I, ratio of 0.46±0.03 s, n=11, 9 mice, WT; 0.23±0.02, n=13, 6 mice, KO; Mann-Whitney test, p<0.0001). Finally, we analyzed decay kinetics by fitting termination phase of the EOG to a single exponential function yielding time constant of 1,707±124 ms, n=19, 9 mice, WT and 1,311±80 ms, n=20, 6 mice, KO (Fig. 7J, Mann-Whitney test, p=0.0083). We conclude that deficiency in INPP5E through elevated ciliary PIP\textsubscript{2} results in a complex sensory exhaustion at the single cell level.

To assay feasibility of functional rescue, EOG was measured in adult Inpp5e^{osnKO} mice after 10 days of adenoviral ectopic expression of the full-length GFP-INPP5E-FL. A 5-s pulse of 10^{-3} M amyl acetate in the virally treated mice evoked odor response showing prominent recovery of the adaptation and kinetics (Fig. 7F and G). Statistical analysis confirmed a significant change of the EOG parameters (Fig. 7H-J). Ratio between 2\textsuperscript{nd}/1\textsuperscript{st} EOG peak amplitude was increased
PIs, such as PIP$_2$ and PIP$_3$, are implicated in regulation of a vast array of proteins including ion channels and transporters in a tightly regulated spatio-temporal manner (Hilgemann et al., 2018; Hille et al., 2015). A role for PIs, in mammalian olfactory transduction has long been suggested either as second messengers or as constituents of the membrane in which odorant signaling
complexes reside (Spehr et al., 2002; Ukhanov et al., 2016). PIs, which under normal conditions are relatively minor components of the membrane, can directly modulate olfactory signaling proteins like the CNG channel or the olfactory Cl\(^{-}\) channel, TMEM16B (Dibattista et al., 2017; Ta et al., 2017; Zhainazarov et al., 2004). For example, odors may generate a transient change of PIP\(_2\) and PIP\(_3\) directly implicated in inhibiting the output of OSNs (Ukhanov et al., 2010; Ukhanov et al., 2016). Therefore, we hypothesized that redistribution of PIP\(_2\) in OSN cilia of \(\text{Inpp5e}^{\text{osnKO}}\) mice would affect the ability to transduce odor signals. Surprisingly, with disruption of the gradient of PIP\(_2\) and its steady-state enrichment in \(\text{Inpp5e}^{\text{osnKO}}\) OSN cilia, EOG amplitudes were not altered but the response kinetics was accelerated. The more transient EOG response resulted from an acceleration of both the rising phase and termination of the odor response which may lead to an associated sensory exhaustion. Therefore, the exclusion of PIP\(_2\) from the full length of cilia would function to slow odor response kinetics. Indeed, a number of proteins outside the principal signaling components are known to modulate olfactory signaling (Buiakova et al., 1996; Kaneko-Goto et al., 2013; Talaga et al., 2017). To our knowledge the slow-down of odor response kinetics has only been measured with one these protein modulators, cilia- and flagella-associated protein 69 (CFAP69). CFAP69 is an evolutionarily conserved protein localized to OSN cilia and shown to dampen kinetic responses to odors (Talaga et al., 2017). Given the overlapping functional phenotype, it is therefore tempting to speculate that there exists a dynamic reciprocity between CFAP69 or perhaps other orphan house-keeping proteins included in olfactory cilia proteome (Klimmeck et al., 2008; Kuhlmann et al., 2014; Mayer et al., 2009) and ciliary membrane phosphoinositides.

Our data show that redistribution of PIP\(_2\) into the full length of OSN cilia enhanced the rate of calcium extrusion from OSNs following odor stimulation. Therefore, an alternative or complimentary mechanism for the functional effects resulting from loss of INPP5E in OSN cilia, could be derived from the role of PIP\(_2\) as a positive regulator of the Na\(^{+}\)-Ca\(^{2+}\) exchanger as occurs in cardiac cells (He et al., 2000). Calcium extrusion is important for odor response recovery and short-term adaptation in OSNs (Saidu et al., 2009; Stephan et al., 2012; Zufall and Leinders-Zufall, 2000). However, PIP\(_2\) build-up in cilia did not cause any adverse effect on overall odor sensitivity, maximal odor evoked EOG or short-term adaptation (Fig. S5C,D). Instead, it impaired a prolonged form of adaptation induced by 5-s odor pulses. Functionally, our finding parallels earlier evidence of the role of CaMKII kinase that controls a long-form of adaptation (Leinders-Zufall et al., 1999). CaMKII is not known to be directly modulated by PIs,
however, it is certainly possible that indirect modulation of CaMKII occurs with redistribution of PIP$_2$. Such indirect modulation is possible if perturbation of membrane PIP$_2$ can disrupt organization of ciliary membrane microdomains, such as rafts, that may be important for spatio-temporal dynamics of Ca$^{2+}$. Lipid rafts are thought to be crucial in organizing ion channels and other signaling molecules, including CaMKII near the membrane (Hammond, 2016). However, in $\text{Inpp5esnkO}$ mice, we did not measure any ciliary alterations in other membrane lipids nor did we detect an alteration in the ciliary localization of odor signaling proteins. Nevertheless, the exclusion of PIP$_2$ from the full length of OSN cilia contributes to a unique membrane compartment that is optimized for odor detection.

In addition, to a possible direct effect of membrane lipids on ion channels or transporters within the OSN cilium, it is possible that the functional phenotype in OSNs results from perturbation of signaling pathways. Loss of INPP5E not only disrupted cilia localization of PIP$_2$ but also elevated PIP$_3$ in the plasma membrane of the knob and likely in other cellular compartments of OSNs given that INPP5E is localized to Golgi (Kong et al., 2000). Sustained elevation of PIP$_3$ may affect several targets involved in important homeostatic mechanisms, including Akt signaling in close proximity to primary cilia (Hakim et al., 2016). There is evidence for Akt signaling to function in OSNs where it was shown to be activated under prolonged odor exposure promoting neuronal survival (Kim et al., 2015). In other neurons, disruption of Akt signaling can cause an abnormality in axonal growth in the ciliopathy, Joubert syndrome (Guo et al., 2019). While we did not observe any measurable changes in innervation in the bulb (data not shown), it does not exclude the possibility for perturbation of Akt signaling or other pathways to modulate the electrophysiological properties of OSNs. Therefore, the precise mechanism linking alterations in membrane phosphoinositides in OSN cilia to the cellular odor response should be addressed in future studies.

This is the first report to show the localization of lipid species representing broad classes of membrane lipids in olfactory cilia. One of the things that stood out in our analysis of wildtype mice was the stochastic variation in the number of neurons and cilia with PIP$_2$. While PIP$_2$ was largely excluded from the full length of cilia in the majority of neurons, there was a small number of neurons scattered throughout the OE that showed PIP$_2$ in a subset of their cilia (Fig 1A; Fig. S1B). Often these multi-ciliated neurons would have a single cilium in which PIP$_2$ was distributed along the membrane of the full length of the axoneme. This was not observed with the other lipid species we analyzed (Fig. 3,4). The reason for this is unclear but it may reflect the maturation state of the neurons which are renewed on average every several months.
(Mackay-Sim and Kittel, 1991). Alternatively, this may represent a subpopulation of neurons expressing unique odorant receptors or a subclass of neurons expressing noncanonical odor signaling components (e.g. GCD, TRPC2, TAAR receptor neurons) (Munger et al., 2009). Regardless, genetic deletion of INPP5E normalized the distribution of PIP2 and caused a redistribution of the lipid to the full length of cilia in all cells.

Our results on the remodeling of PIP2 in OSN cilia correlate well with previously published studies on the role of INPP5E in PC on different cell types of mammalian, fish and insect origin (Chávez et al., 2015; Garcia-Gonzalo et al., 2015; Park et al., 2015; Xu et al., 2017). However, there are several distinct differences for which olfactory cilia may be unique. One outstanding question is the substrate for INPP5E in WT OSN cilia. In primary cilia, PI4P is enriched in the basal state and is decreased with deletion of INPP5E (Chávez et al., 2015; Garcia-Gonzalo et al., 2015). This suggests the INPP5E uses PIP2 itself as a substrate in PC. In contrast, we did not measure significant levels of PI4P in the OSN cilia and hence no change was measured in Inpp5e^osnKO mice. Importantly, PI4P and PIP2 comprise two nearly independent pools in the membrane and previous studies showed that PI4P may exist in the membrane for a short time as intermediate product channeled from PI4K to PI5K kinases to make PIP2 (Fairn and Grinstein, 2012). Because of this very dynamic process, a steady-state level of PI4P may stay at nearly undetectable low concentration which may be the case in the cilia of mammalian OSNs. Instead, our data suggest that in the knob or at the base of cilia, INPP5E uses PIP3 as a substrate to make PI(3,4)P2. Both lipid species are enriched in the knob and we found that PIP3 levels decreased Inpp5e^osnKO mice. In the PC of medulloblastoma, INPP5E is mostly involved in converting PIP3 into PI(3,4)P2 (Eramo and Mitchell, 2016). The potential for dynamic exchange of lipids from the knob, the proximal segment/transition zone and full-length cilia in OSNs requires further investigation. Nonetheless, we found that steady-state ciliary distribution of PIs other than PIP2 was not significantly changed in Inpp5e^osnKO mice. One explanation is that due to redundancy between multiple 5'-phosphatases, resting levels of PI species level are differentially affected by deletion of INPP5E. This is observed in other systems where a combined knock-down of several isoforms of 5'-phosphatases, SHIP1, SYNJ1/2, OCRL, and INPP5B was required to reveal significant elevation of PIP3 with only a slight decrease of PI(3,4)P2 (Malek et al., 2017). Given that other INPP5E class phosphatases co-exist in OSNs along with INPP5E (Kanageswaran et al., 2015), it would be imperative in future studies to address the growing complexity of the PI pathways in ciliogenesis and ciliary signaling in olfactory system through its development.
There are other aspects of divergence, related to PI pathways, between OSN cilia and a PC. In our study we did not find a major role for the PIP$_2$ gradient in OSN cilia formation or maintenance which is consistent with work in *C. elegans* reporting that increased ciliary PI(4,5)P$_2$ levels are not sufficient to remodel sensory cilia morphology (DiTirro et al., 2019). Other studies, however, have shown that the loss of INPP5E shortened the PC suggesting a complex INPP5E-dependent regulation of ciliogenesis and maintenance (Chávez et al., 2015; Jacoby et al., 2009; Nozaki et al., 2017; Phua et al., 2017). The formation and functioning of cilia as a cellular organelle is maintained by IFT. In PC, there are well defined interactions between the retrograde IFT-A machinery, adaptor proteins and PIP$_2$. For example, the ciliary transport of Gpr161 receptor depends on IFT-122 protein binding to TULP3 which is in turn recruited to the membrane by PIP$_2$ (Boubakri et al., 2016; Mukhopadhyay et al., 2010). This mechanism is also responsible for proper ciliary trafficking of mechanosensitive ion channels NompC and PKD2 in *Drosophila* and *C.elegans*, respectively (Bae et al., 2009; Mukhopadhyay et al., 2010; Park et al., 2015). Further, BBSome core proteins, directly involved in IFT through interaction with kinesin and dynein motors, are able to bind *in vitro* to several PIs with highest affinity to PI(3,4)P$_2$ (Jin et al., 2010). Our previous work demonstrated that the BBSome functions as a *bona fide* constituent of IFT in OSN cilia (Uytingco et al., 2019; Williams et al., 2014). Therefore, it was surprising that we did not find any abnormality in the velocity of IFT-A dependent transport of IFT122 particles or its accumulation inside olfactory cilia of *Inpp5e*$_{osnKO}$ mice. (Fig. S2). IFT-B related trafficking of IFT88 appeared to be also unaltered with a similar particle velocity to that published previously for the wild-type OSNs (Uytingco et al., 2019; Williams et al., 2014). Directly related to this finding, we report no alteration in abundance or ciliary localization of endogenous olfactory receptor M71/72 (Fig. S5). This notion is supported by the lack of any effect of INPP5E deletion on the overall odor sensitivity in the *Inpp5e*$_{osnKO}$ mice. In PC, PIP$_2$ is strongly implicated in the trafficking of GPCRs through a INPP5E/TULP3/IFT-A axis (Garcia-Gonzalo et al., 2015; Maurya et al., 2017; Mukhopadhyay et al., 2017). In addition, Hedgehog signaling is suggested to participate in the ciliary localization of mouse odorant receptors (Maurya et al., 2017). However, our results suggest that the trafficking of odorant receptors into OSN cilia is complex and differs from mechanisms of GPCR localization in PC.
In conclusion, our work provides a novel insight into the organization of membrane lipids in cilia of OSNs in normal and disease-related conditions as well as the functional implications of ciliary membrane lipid perturbation. Ciliopathies associated with altered PIP$_2$ distribution are not limited to INPP5E/JBTS but also occurs in a similar disease of oculo-cerebro-renal syndrome of Lowe (OCRL). Importantly, the ability to rescue ciliary PIP$_2$ distribution and the whole tissue odor response highlight the potential of viral gene therapy treatment of JBTS related phenotypes in the olfactory system and other impacted tissues.

Materials and Methods

Mice

All procedures were approved by the University of Florida Institutional Animal Care and Use Committee, protocol 201908162. Inpp5e$^{flox}$ mouse was made in the lab of Dr. Stephane Schurmans (University of Liege, Belgium). Mice were housed in a standard animal facility room at the University of Florida. To generate an olfactory tissue specific mutant, we generated homozygous Inpp5e$^{flox/flox}$ founders which were crossed with OMP-Cre mice (JAX stock#006668, deposited by Peter Mombaerts). Resulting Inpp5e$^{osnKO}$ mice were genotyped using a standard PCR (Jacoby et al., 2009). Mice of both sexes were used in experiments.

cDNA constructs and adenovirus production

Plasmids containing cDNA fragments were provided as follows (PLCδ1-PH-GFP, Addgene #51407, Btk-GFP Addgene #51463, mCherry-P4M-SidM, Addgene #51471, deposited by Tomas Balla; Tapp1-GFP, a gift from Takeshi Ijuin, Kobe University; D4H-mCherry, a gift from Gregory Fairn, University of Toronto; Lact-C2-GFP, Addgene 22852, deposited by Sergio Grinstein; Eqt2-SM-GFP, a gift from Christopher Burd, Yale University; TULP1 and TULP3, a gift from Saikat Mukhopadhyay, University of Texas Southwestern; Kir2.1, Addgene #32669, deposited by Matthew Nolan; PC2 (PKD2), Addgene #83451, deposited by Thomas Weimbs; Efhc1, a gift from Kazuhiro Yamakawa, RIKEN; IFT122, a gift from Jonathan Eggenschwiler, University of Georgia; GCaMP6f, Addgene 40755, deposited by Douglas Kim). C-terminal catalytic domain of INPP5E was subcloned from PJ-INPP5E (Addgene #38001, deposited by Robin Irvine). Full-length wild-type human INPP5E (NM_019892) was cloned in the lab of
Stephane Schurmans. MyrPalm lipid anchored constructs were described previously (Williams et al., 2014). Catalytically dead INPP5E-D477N was made by a site directed mutagenesis of the wild-type gene using a commercial kit (Q5, cat #E0554S, New England Biolabs). All cDNAs were fused with GFP or mCherry, verified by sequencing and subcloned into the pAd/CMV/V5-DEST™ expression vector using Gateway technology (Invitrogen). Adenoviral vectors were propagated in HEK293 cells using the ViraPower protocol (Invitrogen), isolated with the Virapur Adenovirus mini purification Virakit (Virapur, San Diego, CA) and dialyzed in 2.5% glycerol, 25 mM NaCl and 20 mM Tris-HCl, pH 8.0 (Slide-A-Lyzer Dialysis Cassette, 10,000 MWCO) overnight. Alternatively, purified virus was dialyzed and further concentrated using ultrafiltration device Sartorius Vivaspin-6 (100,000 MWCO).

**Immunodetection of INPP5E**

Freshly dissected olfactory mucosa was homogenized on ice in a lysis buffer (150 mM NaCl, 50 mM Tris, pH 8.0, 1% Triton-X-100 complemented with protease inhibitors) for 20 min. The sample was centrifuged at 10,000 rpm for 10 min at 4°C. The supernatant was then used for a protein concentration assay, using the Bradford detergent-compatible assay according to the manufacturer’s instructions (Bio-Rad, Hercules, CA). Samples were heated for 2 min at 95°C in SDS loading buffer. Thirty micrograms of cell lysis samples were run on a 4–12% Bis-Tris acrylamide gel (Invitrogen). After electrophoretic transfer to nitrocellulose, membranes were incubated with 5% fat-free milk and then with the anti-INPP5E (Proteintech, 17797-1-AP) or anti-Actin antibodies (A5060, Sigma) (diluted 1:500 and 1:1,000, respectively). Bound primary polyclonal antibody was detected with a 1:5,000 dilution of horseradish peroxidase-conjugated goat anti-rabbit IgG (Zymed). The Renaissance Western blot chemiluminescence reagent was used according to the manufacturer’s protocol (Perkin Elmer Life Sciences, Wellesley, MA). Images were captured using the EpiChemi3 Darkroom (UVP, Upland, CA). Intensity of specific bands were measured in NIH ImageJ and presented as a ratio between INPP5E and actin signal.

**Whole mount immunocytochemistry**

Mice were sacrificed by inhalation of carbon dioxide followed by cervical dislocation. Freshly dissected turbinates and septum were drop fixed for 3-4 hours on ice in freshly prepared 4% paraformaldehyde in a phosphate-buffered saline (PBS), pH 7.4 supplemented with 20%
sucrose. Tubes containing the tissue were carefully placed in refrigerator at 4°C and left for the duration of fixation without any movement or agitation. This step was critical for the preservation of cilia known to be extremely sensitive to mechanical damage. The tissue was thoroughly washed in PBS and blocked with PBS containing 3% fetal bovine serum, 2% bovine serum albumin and 0.3% Triton X-100 for 2 h at RT. The tissue was then incubated with primary antibody against mouse M71/72 olfactory receptor (gift from Dr. Gilad Barnea, Brown University) raised in guinea pig, diluted 1:1,000 in the same blocking solution. Finally, the tissue was incubated with secondary anti-guinea pig Alexa 568 (1:1,000) for 2h and placed in antifading mounting agent Vectashield (Vector Labs) on the glass coverslip. Specimen were analyzed in the inverted confocal microscope Nikon TiE-PFS-A1R. Images were post-processed using Nikon Elements software (version 4.30) and NIH ImageJ (Wayne Rasband, NIH http://imagej.nih.gov/ij) and assembled in CorelDraw v.18 (Corel).

En face imaging of adenovirally expressed proteins in live mouse OE

To express genes of interest, 10-20 µl of purified viral construct was intranasally administered to mice ranging between 10-40 days of age. Typically, viral delivery was repeated in three consecutive days. Ten days post infection, mice were anesthetized with CO₂, rapidly decapitated, entire turbinates and septum were dissected and kept on ice in a petri dish filled with freshly oxygenated with carbogen modified artificial cerebrospinal fluid (ACSF) that contained (in mM): 120 NaCl, 25 NaHCO₃, 3 KCl, 1.25 Na₂HPO₄, 1 MgSO₄, 1.8 CaCl₂, 15 glucose, 305 mOsm (adjusted with sucrose), pH 7.4. For imaging, a small piece of the OE was mounted in the perfusion chamber (RC-23, Warner Instruments) with the apical surface facing down and analyzed in Nikon TiE-PFS-A1R confocal microscope equipped with a 60x oil-immersion objective, using preset configuration for acquisition of GFP and mCherry fluorescence. Image acquisition settings were set to avoid pixel saturation and maintained equal when comparing wild-type control and INPP5E-KO tissue.

For total internal reflection fluorescence microscopy (TIRF) en face imaging, virally transduced mice were prepared as above. TIRF imaging was performed on a Nikon Eclipse Ti-E/B inverted microscope equipped with a 100x oil immersion CFI APO TIRF 1.49 NA and an EMCCD camera (iXon X3 DU897, Andor Technology).
Quantification of en face confocal z-stacks and measuring IFT velocity in TIRF time-series.

Confocal z-stacks spanning 5 µm from the uppermost cilia to the dendritic knob of the OSN were flattened using sum intensity projection keeping a 16-bit depth throughout the analysis. Fluorescence was corrected for the background and measured within regions of interest. ImageJ/FIJI was used to generate line-scan kymograms for measuring particle velocities from imported time series. All time-series were corrected for the drift due to any tissue movement using GPU-enabled NanoJ-SRRF plugin (Laine et al., 2019). After trajectories of particle movement were selected per individual identified cilia, the kymograms were extracted using Kymograph plugin. A velocity was calculated using the equation: \( \tan(\alpha \pi/180) \times b/c \) where \( \alpha \) is angle, \( b \) is calibration in µm per pixel and \( c \) is exposure time per frame.

Single cell GCaMP6f calcium imaging of odor evoked response

Calcium imaging was done as described previously (Ukhanov et al., 2016). Mice of 4-6 weeks of age were used for experiments 10-14 days after administration of adenovirus encoding GCaMP6f. Tissue was prepared and mounted the same way as described above. The chamber was transferred to the stage of upright microscope Zeiss Axioskop2F equipped with 40x 0.75NA water-immersion objective lens. Experimental solutions were applied directly to the field of view through a 100 µm diameter needle made of fused silica and connected to the 9-channel Teflon manifold. Each perfusion channel was controlled by the electronic valves (VC-6, Warner Instruments). The calcium response presented as an increase of GCaMP6f fluorescence emanating from the knob and underlying dendrite. The tissue was illuminated using a standard eGFP filter cube BP490nm/535nm (Omega Optical, USA) and the emitted light was collected at 530 nm (BP 530/20 nm, Omega Optical, USA) by a 12-bit cooled CCD camera (ORCA R2, Hamamatsu, Japan). Both the illumination system (Lambda DG-4, Sutter Instruments, USA) and image acquisition were controlled by Imaging Workbench 6 software (INDEC BioSystems). Before processing, fluorescence intensity was corrected for the background. Each knob was assigned a region of interest (ROI) and changes in fluorescence intensity within each ROI were analyzed and expressed as the peak fractional change in fluorescent light intensity (F-Fo)/Fo where Fo is the baseline fluorescence before odorant application. A stock solution of a complex odorant mixture of 42 distinct chemicals (Ukhanov et al., 2013) was made in DMSO as individual 0.5M stock and then mixed to final 1:10,000 dilution with ACSF. ACSF supplemented
with 0.1% DMSO, the odorant carrier, served as the control solution. All 42 odorous chemicals in the stock solution were at the same concentration of 11.9 mM.

Analysis and graphical presentation of calcium imaging data was performed with Imaging Workbench 6 (INDEC), Clampfit 9.2 (Molecular Devices), NIH ImageJ, Microsoft Excel and GraphPad Prism 8.

Electroolfactogram recording

Mice were anesthetized with CO₂, rapidly decapitated, and the head split along the cranial midline. Septal tissue was removed to expose olfactory turbinates. Vapor-phase odors were delivered by a pressurized nitrogen line connected to a sealed 100 ml glass bottle and directly injected into a continuous stream of humidified carbogen flowing over the tissue. Odorants were prepared by diluting pure stock into deionized water and final working concentration calculated as molar (v/v). Responses to odors were recorded with a standard glass micropipette tip-filled with agarose and backfilled with PBS using a Multiclamp 700A amplifier controlled by Multiclamp 700A and Clampex 9.2 software (Molecular Devices). EOG was measured as the maximal peak amplitude from the pre-pulse baseline using Clampfit 9.2 software (Molecular devices).

Statistical Analysis

All statistical tests were done in Prism 8 (GraphPad) following test for normality and by using non-parametric Mann–Whitney test, unpaired t-test or one-way ANOVA, and \( p < 0.05 \) was considered to be statistically significant. All the group statistics are presented as mean ± SEM (standard error of mean).
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Competing interests

The authors declare no competing financial interests.
References


Figure 1. Loss of INPP5E causes redistribution of PIP$_2$ and elongation of cilia in mouse OSNs. (A) PLCPH-GFP, a probe for PIP$_2$, was mostly restricted to the knob of the wild type (WT) OSNs. In a small percentage of OSNs a ciliary segment of varying length up to the full length (black arrows) was also enriched in PIP$_2$. Inert membrane bound lipid probe MP-mCherry was used as a counterstain to label the full length of axoneme opposing highly restricted
localization of PLCPH-GFP resulting in overlapping colors (middle panel, white). PIP$_2$ was evenly distributed in the plasma membrane of the knob as shown in z-stack view (right panel). Yellow lines denote z-stack projection shown at the bottom and right side of the image. (B) Opposite to the WT, in *Inpp5e*$_{osnKO}$ (INPP5E-KO) PLCPH-GFP decorated the entire length of every cilium. Color shifted image was used to accentuate equal distribution of PLCPH-GFP and MP-mCherry labeling (B, middle panel). The PIP$_2$ redistribution is evident also in the z-stack side view showing substantial enrichment at the base of cilia and along the proximal segment (PS, red arrows) whereby PIP$_2$ level in the knob periciliary plasma membrane was not changed (right panel). (C) More than 50% of WT OSNs showed no PIP$_2$ in their cilia. 18% of OSNs had only a single PIP$_2$ positive cilium whereas three other groups of neurons equally represented remaining 30%. Conversely, PIP$_2$ was detected in 100% of OSNs in *Inpp5e*$_{osnKO}$ (KO, green bar). Total of 318 cells in 4 mice were analyzed in the WT group and 36 cells in 3 mice were analyzed in the KO group. (D) Length distribution within the same sets of cells of PLCPH-GFP positive aspects of cilia (PIP$_2$ domain) in WT was substantially shifted to shorter values compared to the full cilia length measured with MP-mCherry, yielding 29.5±0.5 µm (n=753, 4 mice). (E) Distribution of both PLCPH-GFP and MP-mCherry length values showed a complete overlap in *Inpp5e*$_{osnKO}$ OSNs. Average full ciliary length in the KO OSNs, 35.3±0.6 µm (n=495, 3 mice) was significantly longer than in the WT (unpaired t-test, t=7.363 df=1246, ****p<0.0001). Data shown as mean ± SEM.
Figure 2. Virally induced ectopic expression of full-length wild type human INPP5E tagged with GFP (GFP-INPP5E-WT) completely reversed mislocalization of PIP$_2$ in Inpp5e<sup>osnKO</sup> mouse cilia. (A,B) Inpp5e<sup>osnKO</sup> mice were infected at P8-P14 with a triple dose of Ad-GFP-INPP5E-WT and tested 8-10 days later. GFP-INPP5E-WT is enriched in OSN knobs and also localized to cilia. The KO mice were co-infected with PLCPH-mCherry to measure rescue of the PIP$_2$ localization. Several knobs of co-infected OSNs are indicated with arrowheads. (C) Zoomed-in dual-color view of the area marked with a square in (B) shows several knobs of OSNs co-infected with both viruses (arrowheads) resulting in a complete loss of ciliary PIP$_2$ (magenta). (D) Rescue was quantified by measuring length of PIP$_2$ positive ciliary aspect in the WT littermates and KO mice. The KO OSNs were identified within the same preparation by a strong ciliary distribution of PLCPH-mCherry, also lacking any detectable GFP-INPP5E-WT fluorescence. Rescue completely reversed Inpp5e<sup>osnKO</sup> deficiency (PIP2 domain length 4.9±0.27 µm (n=110, 16 cells, 3 mice), WT; 28.5±1.37 µm (n=54, 5 cells, 3 mice), KO; 4.2±0.3 µm (n=122, 17 cells, 3 mice), Rescue, one-way ANOVA, F(DFn, DFd) 86.73 (2,283), ****p<0.0001). (E-G) Inpp5e<sup>osnKO</sup> KO mice in a different group were infected with Ad-PLCPH-mCherry and Ad-GFP-INPP5E-D477N coding for catalytically inactive phosphatase. GFP-INPP5E-D477N mutant was localized to the full cilia length (E). Knobs of co-infected OSNs showing no change in PLCPH ciliary localization are marked with solid arrows. Some knobs had less PLCPH probe localized to cilia (open arrows) reminiscent of the KO phenotype. Expression of GFP-INPP5E-D477N resulted in a significantly smaller number of OSNs having a
complement of PIP2-decorated cilia. This reduction was quantified in (H), 17.6±0.09 % (D477N, n=61, 3 mice), compared to GFP-INPP5E-WT, 61.2±0.05 % (INPP5E-WT, n=83 cells, 3 mice), unpaired t-test, t=4.536, df=24, ***p=0.001). Data shown as mean ± SEM.
Figure 3. Other phosphoinositides in mouse OSNs were almost exclusively restricted to the knobs and changed their level in the INPP5E-dependent manner. (A,D) PI(4)P probe, mCherry-P4M-SidM was not significantly affected by loss of INPP5E showing only insignificant trending decrease in the knobs (179±26 relative units, WT, n=94, 3 mice; 143±17 relative units, KO, n=54, 3 mice; unpaired t-test, t=0.9777 df=146, p=0.3298). (B,E) Tandem PH domain, Tapp1 tagged with GFP was used to specifically label membrane PI(3,4)P$_2$ enriched mostly in the knobs and in cilia only in a small fraction of OSNs. Importantly, overall pattern of PI(3,4)P$_2$ distribution did not change in Inpp5e$^{osnKO}$. Fluorescence intensity, however, measured in OSN knobs showed significant decrease in the KO compared to WT mice (280±11 relative units, n=830, 3 mice, WT; 174±7 relative units, n=858, 3 mice, KO; unpaired t-test, t=8.453 df=1686, p<0.0001). (C,F) PIP$_3$ detected with Btk-PH domain tagged with GFP, was restricted mostly to the knobs with relatively low presence in cilia of the WT and KO. Quantitative analysis of fluorescence showed increase of the intensity in the knobs of the KO (668±64 relative units, n=60, 3 mice, WT; 1,495±185 relative units, n=91, 3 mice, KO; unpaired t-test, t=3.536 df=149, ***p=0.0005). (G) Fluorescence intensity of MP-mCherry used as a negative control, was not
significantly different in the OSN knobs of WT and KO mice (340±31 relative units, n=46, 3 mice, WT; 378±23 relative units, n=70, 3 mice, KO; unpaired t-test, t=1.001 df=114, p=0.3188). Data shown as mean ± SEM.
Figure 4. Distribution of integral membrane lipids was not changed in OSNs and cilia in \textit{Inpp5e^{osnKO}} KO mice. (A) D4H-mCherry, a cholesterol binding probe was enriched in the proximal segment of olfactory cilia equally in the WT and KO OSNs (arrowheads). Cholesterol was detected albeit at a lower level also in the full-length ciliary axoneme. (B) Phosphatydilserine, probed with C2 motif of lactadherin, was uniformly distributed along the cilia and was also enriched in the dendritic knobs of OSNs. (C) Sphingomyelin specific probe, Eqt2-SM-GFP was mostly enriched in the OSN knobs and detected at a low level in cilia. (D) Glycosylated phosphatydilinositol was probed in OSNs with a human folate 1 receptor, GPI-GFP which failed to detect any presence in cilia whereby mostly restricted to the knobs in both the WT and \textit{Inpp5e^{osnKO}} mouse.
Figure 5. Soluble and polytopic proteins with affinity to PIP$_2$ mislocalize in olfactory cilia of Inpp5e$^{osnKO}$. (A) Tubby-like proteins tagged with GFP, TULP1-GFP and TULP3-GFP were preferentially restricted to the knobs in the WT (upper left panel). Build-up of PIP$_2$ in cilia of the KO resulted in complete redistribution of TULP1 (bottom panels). Note that loss of INPP5E activity led to a depletion of TULP1 within knobs revealing proximal segment of cilia decorated with TULP1-GFP (right bottom panel). Quantification of a percentage of the OSN knobs having TULP1-positive cilia per analyzed image showed significant increase in the KO (25.49±0.06%, n=4, 3 mice, WT; 100%, n=6, 3 mice, KO; Mann-Whitney t-test, **p=0.0048). (B) TULP3-GFP, like TULP1, also showed dramatic redistribution between the knob and cilia resulting in a significant increase of percentage of knobs with TULP3-positive cilia (30.87±0.12%, n=6, 3 mice, WT; 100%, n=4, 3 mice, KO; Mann-Whitney t-test, **p=0.0095). (C) Potassium inward rectifier ion channel, Kir2.1-mCherry, a polytopic protein with two membrane-spanning loops and a known affinity to PIP$_2$ also changed its ciliary distribution in Inpp5e$^{osnKO}$ OSNs. Kir2.1-
mCherry moved into the ciliary membrane in a significantly larger fraction of OSNs in the KO (right upper panel, 3.02±0.02%, n=12, 3 mice, WT; 24.34±5.89%, n=10, 3 mice, KO; Mann-Whitney t-test, **p=0.0023). (D, E) As a negative control we used a different ion channel, PC2 (PKD2 or TRPP1) tagged with mCherry (mCherry-PC2) and a microtubule binding protein Efhc1 (GFP-Efhc1) both of which, however, did not change their distribution in the knobs of Inpp5e<sup>osnKO</sup> (upper panels, WT; bottom panels, KO). Data shown as mean ± SEM.
Figure 6. INPP5E is responsible for shaping the odor-evoked intracellular calcium transient in the knob of OSNs. (A,B) Ectopically expressed GCaMP6F was visualized in the en face preparation of mouse OE by wide-field fluorescence microscopy. Bright spots represent numerous OSN knobs. (B) Stimulation micropipette filled with a mixture of 132 different odorants diluted to 1:10,000 in ACSF was positioned as indicated. A single 100-ms pulse at 10psi pressure generated a plume of fluorescein covering an area over the epithelial surface demarcated by a dotted line. (C) Repetitive application of a single odor pulse (arrowheads) evoked nearly identical responses. GCaMP6F fluorescence corrected for background was calculated as (F-Fo)/Fo. (D) Individual traces measured in more than 100 OSNs across several areas and 3 mice per each genotype were averaged to create the graph. Traces were normalized to the peak value before averaging. (E,F) Odor-evoked GCaMP6F response had faster decay in the KO OSNs than the response in the WT control group (WT: 6.49±0.37 s,
n=167, 3 mice; KO: 3.59±0.18 s, n=110, 3 mice, unpaired t-test, t=6.077, df=275, ****p<0.0001). The response in the KO also had faster rising phase (WT: 1.12±0.07 s, n=46, 3 mice; KO: 0.80±0.08 s, n=30, 3 mice, unpaired t-test, t=2.936 df=74, **p=0.0044). To calculate termination phase time constant (decay tau) each individual trace was fit to an exponential function. Rise time 10-90% was defined as time to reach from 10% to 90% of the response peak level. Data shown as mean ± SEM.
Figure 7. A faster single-cell odor response translates in more transient EOG in \textit{Inpp5e}^{osnKO}. (A) Representative EOG traces recorded in response to 100-ms pulse of amyl acetate vapor, driven from the 90-ml head space of bottles containing increasing concentration ranging from $10^{-8}$M to a maximum of 1M (indicated at the individual traces). Odor application is denoted by a black arrowhead. (B) Dose-response relationship showed no significant difference between the WT and KO (WT, n=7, 3 mice; KO, n=11, 4 mice; two-way ANOVA, F(5, 102) = 0.1858, P = 0.9674). (C,D) Rise time of the EOG evoked by a single 100-ms pulse of $10^{-2}$M amyl acetate (rise time 10-90%) was decreased in the KO compared to the WT (WT: 174.5±7.7 ms, n=37, 5 mice; KO: 157.9±10.9 ms, n=40, 7 mice; Mann-Whitney test, p=0.0221), similar to
the time constant (decay tau) of the termination phase (WT: 4.57±0.15 s, n=81, 5 mice; KO: 3.40±0.16 s, n=28, 4 mice; unpaired t-test, t=4.386, df=107, ****p<0.0001). (E) EOG evoked by a longer 5-s pulse of 10^{-3} M amyl acetate applied at the time indicated by a square step (aac, 10^{-3} M) also appeared more transient in Inpp5e^{ΔsenKO} KO (F). Ectopic expression of the full-length wild type INPP5E partially rescued the EOG shape (G). (H-J). The ratio between peak amplitude of second and first EOG response, plateau-to-peak ratio and time constant of termination phase (decay tau) were significantly affected by the loss of INPP5E activity and restored by ectopic expression in OSNs of the wild-type INPP5E. Second/first peak ratio (WT: 0.733±0.026, n=18; KO: 0.514±0.022, n=9; Rescue: 0.582±0.021, n=12; Mann-Whitney t-test, WT vs KO, ****p<0.0001; KO vs Rescue, p=0.0409). Peak/plateau ratio (WT: 0.462±0.028, n=11; KO: 0.230±0.017, n=13; Rescue: 0.336±0.024, n=16; Mann-Whitney t-test, WT vs KO, ****p<0.0001; KO vs Rescue, p=0.0021). Time constant of termination phase (WT: 1.707±0.124 s, n=19; KO: 1.311±0.080 s, n=20; Rescue: 1.991±0.134, n=16; Mann-Whitney t-test, WT vs KO, p=0.0083; KO vs Rescue, ****p<0.0001). Data shown in H-J are based on the experiments performed on 9 WT, 6 KO and 6 rescued mice and presented as mean ± SEM.
Movie 1. Intraflagellar transport of IFT122 particles in olfactory cilia of INPP5E<sup>osnKO</sup> mouse. Live en face TIRF microscopy used to measure ectopically expressed IFT122-GFP particle movement along cilia in multiple OSNs of Inpp5e<sup>osnKO</sup> mouse. Real time rate is 10 frames per second.

Movie 2. Intraflagellar transport of IFT88 particles in olfactory cilia of INPP5E<sup>osnKO</sup> mouse. Live en face TIRF microscopy used to measure ectopically expressed IFT88-GFP particle movement along cilia in multiple OSNs of Inpp5e<sup>osnKO</sup> mouse. Real time rate is 10 frames per second.
Movie 3. Odor application induces transient elevation of GCaMP6f fluorescence in knobs of mouse OSNs. Ectopically expressed GCaMP6f was used to report stimulus-evoked activity in mouse OSNs. Live en face imaging of acutely dissected olfactory epithelium reported change of GCaMP6f fluorescence in the dendritic knobs of Inpp5e<sup>−/−</sup> OSNs. A 100-ms pressure-pulse was applied from the micropipette filled with ACSF and added odor mix. Total of 5 pulses was applied with 60-s intervals in between. Real time rate is 25 frames per second.
Figure S1
Figure S1. Endogenous expression of INPP5E is significantly decreased in the olfactory mucosa of the conditional INPP5EosnKO mice. (A) Double band detected around 70 kD corresponds to the full-length and truncated splice variant of INPP5E (bracket). Intensity of the upper band was calculated as a ratio to the intensity of actin band (shown in relative units, ru). The blot was first probed with anti-INPP5E and then re-probed with anti-actin antibody. Tissue homogenate was prepared from three WT and KO mice (Mann-Whitney unpaired t-test, \( p=0.0144, n=3, \) mean ± SE). (B,C) Distribution of ectopically expressed PI(4,5)P2 probe, PLCPH-GFP in wild-type mouse OSNs. (B) 3-D volume rendering of the z-stack taken from apical surface of the OE down to the initial segment of axons. (C) PLCPH-GFP decorates plasma membrane of OSNs along the full length of olfactory nerve. An image was taken at the position where the nerve enters cribriform plate and olfactory bulb. (D) Ectopically expressed full-length wild-type INPP5E (GFP-INPP5E WT) is localized to the full length of cilia in WT OSNs.
Figure S2
Figure S2. Velocity of intraflagellar transport (IFT) was not affected by the loss of INPP5E.

(A) IFT particles incorporating ectopically expressed IFT122 protein, tagged with GFP (upper panel) was visualized by TIRF microscopy. Kymograph plot was generated by tracks of moving individual IFT particles (bottom panel). (B) Both the anterograde and retrograde transport of IFT122-GFP particles did not change due to the loss of INPP5E (anterograde IFT: 0.417±0.016 µm/s, n=336, WT, 2 mice; 0.414±0.015 µm/s, n=398, KO, 3 mice; unpaired t-test, t=0.1687 df=732, p=0.8661; retrograde IFT: 0.531±0.014 µm/s, n=360, WT, 2 mice; 0.542±0.014 µm/s, n=469, KO, 3 mice; unpaired t-test, t=0.545 df=827, p=0.5859). (C) A snapshot image of \textit{Inpp5e}^{osnKO} OSNs taken during acquisition of a time-series shows multiple single IFT88-GFP particles distributed along the full length of cilia. (E) Kymograms were generated to measure IFT88 particle velocity in anterograde and retrograde directions in \textit{Inpp5e}^{osnKO}, 0.28±0.15 µm/s (n=101) and 0.36±0.02 (n=83), 2 mice, respectively.
Figure S3. Catalytically inactive point-mutant INPP5E-D477N fails hydrolyzing PIP$_2$ in the plasma membrane of HEK293 cells. (A) HEK293 cells were transfected with pAd-PLCPH-mCherry (control), and with added pAd-GFP-INPP5E-WT (INPP5E-WT) and pAd-GFP-INPP5E-D477N (INPP5E-D477N) and allowed for expression for 30 h. (B) Fluorescence of PLCPH-mCherry was measured at the plasma membrane and in the cytoplasm. Ratio of the membrane-delimited and cytoplasm intensity was calculated and plotted in three experimental conditions. Co-expression of GFP-INPP5E-WT (5E-WT) induced translocation of PLCPH probe to cytoplasm due to hydrolysis of PIP$_2$ whereby D477N point-mutant did not. Three groups were compared using a one-way ANOVA and found to be significantly different (F=33.18, (DFn, DFd) = 11.14 (2, 126), ****p<0.0001). Control and D477N groups were not significantly different. Data collected in three independent experiments. Total number of cells used in the experiment, control (n=40), 5E-WT (n=38), and 5E-D477N (n=51).
Figure S4. Localization of caveolin-1 in wild-type and \textit{Inpp5e}osn\textsuperscript{KO} OSNs. (A,B) YFP-Caveolin1 was ectopically expressed in mouse OE and exclusively localized to dendritic knobs and decorated also proximal segment (inset, arrowheads) of olfactory cilia.
Figure S5

Figure S5. Ciliary expression of endogenous mouse olfactory receptor M71/72 and a short-term adaptation were not affected by the loss of INPP5E. (A,B) *En bloc* immunostaining of the OE with antibody against mouse M71/72 OR. Individual cilia often contained numerous particles due to the fixation artifacts. Also visible are fragments of cilia broken during fixation. Otherwise intensity of labeling and overall distribution of M71/72, also shown in enlarged insets, was unchanged in the WT and *Inpp5e*^osnKO^. (C) A brief 100-ms test pulse of amylacetate vapor (AAc -3, 10^-3 M) was applied at time zero followed by a 2nd identical pulse at an indicated time interval. (D) Mean EOG amplitude evoked by the 2nd pulse was plotted at different intervals, showing no difference between WT group, n=12, 5 mice and the KO group, n=12, 4 mice. Slower recovery, however, was detected at 3-s interval. Unpaired Mann-Whitney test, dF=22, **p=0.0027