Ins and outs of GPCR signaling in primary cilia

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Abstract

Primary cilia are specialized microtubule-based signaling organelles that convey extracellular signals into a cellular response in most vertebrate cell types. The physiological significance of primary cilia is underscored by the fact that defects in assembly or function of these organelles lead to a range of severe diseases and developmental disorders. In most cell types of the human body, signaling by primary cilia involves different G protein-coupled receptors (GPCRs), which transmit specific signals to the cell through G proteins to regulate diverse cellular and physiological events. Here, we provide an overview of GPCR signaling in primary cilia, with main focus on the rhodopsin-like (class A) and the smoothened/frizzled (class F) GPCRs. We describe how such receptors dynamically traffic into and out of the ciliary compartment and how they interact with other classes of ciliary GPCRs, such as class B receptors, to control ciliary function and various physiological and behavioral processes. Finally, we discuss future avenues for developing GPCR-targeted drug strategies for the treatment of ciliopathies.

Keywords ciliopathies; G protein-coupled receptors; intraflagellar transport; neuronal signaling; primary cilia

DOI 10.15252/embr.201540530 | Received 10 April 2015 | Revised 24 June 2015 | Accepted 1 July 2015

EMBO Reports (2015) 16: 1099–1113

See the Glossary for abbreviations used in this article.

Introduction

Signaling through G protein-coupled receptors (GPCRs) regulates a vast array of cellular and physiological processes throughout the eukaryotic kingdom. GPCRs constitute a substantial and highly diverse family of seven transmembrane (7TM) receptors that transmit assorted signals from the extracellular environment to the cell through both G protein-dependent and G protein-independent pathways, which regulate the activity of various cellular signaling networks. GPCRs are encoded by about 800 different genes in humans [1,2]. This large number of GPCRs enables cells to respond to sensory inputs as diverse as odorants, light, lipids, ions, amines, and nucleotides as well as signaling peptides and proteins, such as hormones, morphogens, and neurotransmitters [3]. Hence, GPCRs are well-established targets for almost half of all therapeutic drugs.

Yet, many GPCRs have been denominated “orphan receptors,” because their natural ligands have escaped identification so far [4].

GPCRs are grouped into six classes based on sequence homology and functional similarity. These include the rhodopsin-like receptors (class A), the secretin receptor family (class B), metabotropic glutamate/pheromone (class C), fungal mating pheromone receptors (class D), cyclic AMP (cAMP) receptors (class E) and frizzled/smoothened (class F) [5]. All GPCRs share a common topology consisting of an extracellular N-terminus (e1) followed by 7TM-spanning α -helices (H1–H7) that are separated by three intracellular (i1–i3) and three extracellular loops (e2–e4), respectively, and a C-terminus (i4) projecting into the cytosol (Fig 1A) [6]. Most GPCRs exert their function through pathways involving interaction and activation of heterotrimeric G proteins, although G protein-independent signaling mechanisms occur, for example via receptor-interacting proteins that regulate both agonist-promoted and agonist-inhibited GPCR signaling [7] as well as G protein-interacting protein cross talk with non-GPCR signaling [8,9]. In the absence of an agonist, GPCRs bind to the heterotrimeric G protein complex: a GDP-bound G_α protein ($G_{\alpha s}$, $G_{\alpha q}$, $G_{\alpha i/o}$, and/or $G_{\alpha 12/13}$) and the $G_{\beta\gamma}$ heterodimer (Fig 1A). Once the GPCR encounters its agonist, the receptor transmutes into its active conformation, which allows GTP exchange with GDP on the G_α protein that in turn dissociates from the $G_{\beta\gamma}$ subunits and prompts downstream signaling through secondary messenger pathways [10,11]. Similarly, $G_{\beta\gamma}$ activates a variety of signaling events as outlined in Figure 1A.

In many cases, GPCRs localize to specific subcellular domains for optimization of detection and transduction of both external and cytoplasmic cues to ensure proper regulation of cell-specific functions. As an example, synaptic processes are modulated through the spatiotemporal localization of GPCRs in the highly polarized neuronal membrane environment such as for the type-1 cannabinoid receptor (CB1R), which is a major brain GPCR that predominantly localizes and functions in axons and specific presynaptic nerve terminals [12]. Growing evidence further points to the function of GPCR-mediated signaling from the nuclear membrane to activate intranuclear signaling events that regulate physiological function in cardiac myocytes [13]. The subcellular domains include defined membrane parts at the cell surface, including lipid rafts/caveolae as well as β -arrestin-dependent endocytosis via clathrin-coated pits that serve as signaling platforms to control compartmentalization of GPCR-mediated signaling [14,15]. In this regard, GPCRs may function as scaffolds for the recruitment of GPCR-interacting proteins, which modulate the localization of GPCRs to specific intracellular compartments known as signalosomes [15]. In this review,

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Glossary

5-HT6	5-hydroxytryptamine (serotonin) receptor 6
7TM	Seven transmembrane
ABCC4	ATP-binding cassette sub-family C member 4
ACIII	Adenylate cyclase type III
ARF	ADP-ribosylation factor
ARL	Arf-related protein
ASAP1	Arf-GAP with SH3 domain, ANK repeat and PH domain-containing protein 1
BBS	Bardet–Biedl Syndrome
cAMP	3′-5′-cyclic adenosine monophosphate
CDE	Clathrin-dependent endocytosis
CiPo	Ciliary pocket
CNS	Central nervous system
CTS	Ciliary targeting sequence
DR	Dopamine receptor
DRD2S	Short isoform of dopamine receptor D2
EP4	Prostaglandin E receptor 4
ERK	Extracellular signal-regulated kinase/Mitogen activated protein kinase
EVC	Ellis–van Creveld syndrome protein
FZD	Frizzled
G protein	Guanosine nucleotide-binding protein
GAS	Growth arrest-specific protein
GALR	Galanin receptor
GEF	Guanine nucleotide exchange factor
GFP	Green fluorescent protein
GIPR	Gastric inhibitory polypeptide receptor
GLI	Glioma-associated oncogene homolog
GLI-A	Activator form of GLI
GLI-R	Repressor form of GLI
GNP	Granule neuron progenitor
GnRH	Gonadotropin-releasing hormone
GPCR/GPR	G protein-coupled receptor
GRK	G protein-coupled receptor kinase
GTP	Guanosine triphosphate
HH	Hedgehog
HTR6	5-hydroxytryptamine (serotonin) receptor 6

IFT	Intraflagellar transport
ILK	Integrin-linked kinase
IMCD3	Inner medullary collecting duct cells 3
KIF3A	Kinesin-like protein 3A
KIF7	Kinesin family member 7
KISS1R	Kisspeptin receptor 1
MB	Medulloblastoma
MCHR1	Melanin-concentrating hormone receptor 1
NMUR1	Neuromedin U receptor 1
NPFFR1	Neuropeptide FF receptor 1
NPYR	Neuropeptide Y receptor
PAC1	PACAP receptor 1
PACAP	Pituitary adenylate cyclase-activating polypeptide
PGE2	Prostaglandin E2
PKA	cAMP-dependent protein kinase
PRLHR	Prolactin-releasing hormone receptor
PTC	Patched
QRFP	Pyroglutamylated RF amide peptide receptor
RAB	Ras-related protein in brain
RABIN	RAB-interacting protein
RAN	Ras-related nuclear protein
RPE	Retinal pigment epithelium
SCN	Suprachiasmatic nucleus
SHH	Sonic HH
SMO	Smoothened
SNARE	NSF attachment protein receptor
SSTR3	Somatostatin receptor 3
T2R	Bitter taste receptor 2
TGN	Trans-Golgi network
TGR	G protein-coupled bile acid receptor
TRAPP	Transport protein particle
TUB	Tubby
TULP	Tubby-like protein
TZ	Transition zone
VNO	Vomerionasal organ
VPAC	Vasoactive intestinal peptide receptor
WNT	Wingless/Int

we focus on the cilium as a unique domain for GPCR-mediated signaling.

Seminal work dating back to the early 1980s and 1990s established how specialized non-motile cilia of photoreceptor cells and olfactory receptor neurons mediate sensory signaling by displaying light and odorant stimulated GPCRs in close physical vicinity to their cognate sensory stimuli (reviewed in [16,17]) (Fig 1B and C). Such vital sensory roles of cilia in the visual and olfactory systems naturally led to the question whether non-motile primary cilia, displayed on the surface of most non-dividing cells in our body, could have evolved an analogous disposition for GPCR signaling, that is, could the primary cilium afford functional benefits to GPCR signaling stimulated by a diffusible agonist? Indeed, through the last decade, a number of GPCRs, once thought to be activated by freely diffusible ligands on the plasma membrane, have been shown to exhibit a pronounced functional and subcellular preference for the ciliary membrane compartment. The ciliary GPCRs identified so far belong to three major classes of the GPCR superfamily: A, B, and F. In the following, we present an overview of ciliary GPCR signaling and describe how the dynamic localization and trafficking of these receptors into and out the cilium is regulated, as well as how such receptors cross-talk with other classes

of GPCRs for the spatiotemporal regulation of cellular and physiological processes.

Ciliary structure and assembly

Cilia are microtubule-based, membrane-enclosed projections on the surface of most eukaryotic cells [18]. They generally fall into two classes, defined by their axonemal arrangement of microtubules and capacity to function as motile and/or signaling units. Classical motile (9 + 2) cilia have axonemes with nine outer doublets and two inner singlets of microtubules as well as radial spokes and axonemal dynein arms that promote motility. Non-motile (9 + 0) cilia typically lack the central pair of microtubules and structures associated with motility, and emanate as solitary organelles from the centrosomal mother centriole (basal body) in most non-dividing vertebrate cell types (Fig 2A). They function as mechano-, osmo-, and chemosensory units that control cellular and physiological processes during development and in tissue homeostasis. Ciliary axonemes of both motile and non-motile cilia are assembled onto the basal body by intraflagellar transport (IFT), which is characterized by kinesin-2- and cytoplasmic dynein-2-mediated bidirectional

Figure 1. Overview on GPCR signaling and function of ciliary GPCRs in vision and olfaction.

(A) Examples of sensory inputs for GPCR-mediated signaling pathways through activation of G proteins. (B) List of known ciliary GPCRs in photoreceptor and olfactory receptor neurons. (C) A cartoon of a rod photoreceptor neuron and localization of light-sensitive GPCRs (rhodopsin) in the disk array of the outer segment, which comprises a modified primary cilium. (D) Cartoon of olfactory receptor cells and localization of ciliary GPCRs that detect odorants. Please see text for details and references.

transport of IFT particles, with associated ciliary cargo, from the ciliary base toward the tip and back [19] (Fig 2B). The membrane surrounding the ciliary axoneme is continuous with the plasma membrane of the cell, but is enriched for specific membrane proteins and lipids that confer the cilium with unique sensory properties. Structural and functional barriers comprising a transition zone (TZ) at the ciliary base ensure the selective passage of proteins into and out of the ciliary compartment, and transition fibers basal to the functional barriers connect the ciliary base to the plasma membrane [20–22]. The region between the primary ciliary membrane and the plasma membrane, referred to as the periciliary membrane, is frequently infolded to produce a ciliary pocket (CiPo) that comprises an active site for exocytosis and clathrin-dependent endocytosis (CDE) of ciliary receptors [23,24].

The sensory capacity of primary cilia is maintained through the spatiotemporal localization of specific receptors and downstream

signaling components along the cilium–centrosome axis, including receptor tyrosine kinases (RTK) [25], transforming growth factor beta receptors (TGFβRs) [24], Notch receptors [26], receptors for extracellular matrix (ECM) proteins [27], and ion channels [28] as well as class A, B, and F GPCRs, which are the focus of this review. The medical significance of primary cilia is becoming increasingly evident, since defects in assembly, structure, and sensory function of these organelles are associated with a plethora of diseases and syndromic disorders (ciliopathies), including nephronophthisis and polycystic kidney disease as well as Bardet–Biedl (BBS), Alström (AS), Joubert (JS), and Meckel–Gruber (MKS) syndromes [29], manifested by congenital heart disease, craniofacial and skeletal patterning defects, neurodevelopmental disorders, and cognitive impairment as well as obesity [30–32].

Sorting and targeting of receptors to the cilium rely on multiple pathways. They include the polarized trafficking of vesicles from

Figure 2.

the trans-Golgi network (TGN) and recycling endosomes directly to the ciliary pocket followed by selective conveyance of the proteins across the ciliary barriers. Alternatively, receptors may move through a lateral transport pathway from the plasma membrane to the ciliary membrane [33–35] (Fig 2B), a scenario recently proposed for the dopamine receptor, D1R, as discussed below. Targeting of receptors from the TGN to the cilium is thought to be guided by discrete ciliary targeting sequences (CTSs; Table 1) that interact with specific trafficking modules, which regulate the budding,

transport, docking, and fusion of post-Golgi carriers or recycling endosomes at the ciliary base (for recent reviews, see [21,33]). This process has been particularly well studied in the outer segment of vertebrate rod and cone photoreceptors in the retina, which are modified cilia with vision class A GPCRs (rhodopsin and photopsins) that absorb photons to activate the G protein transducin, causing hyperpolarization of the cell thus inhibiting synaptic release [36]. In rod cells, rhodopsin localizes to flattened disks of discrete self-contained vesicles within the compartment of the outer

Figure 2. Primary cilia are sensory organelles that coordinate GPCR signaling during development and in tissue homeostasis.

(A) Electron micrographs (EMs) of the primary cilium: (i) transmission EM of a longitudinal section of a neuronal primary cilium emerging from the centrosomal mother centriole, which functions as a basal body (BB). The region between the ciliary membrane and plasma membrane, referred to as the periciliary membrane, is often infolded to produce a ciliary pocket (CiPo) that comprises an active site for exocytosis and clathrin-dependent endocytosis of ciliary receptors (courtesy of Joseph Gleeson). (ii) Scanning EM of a fibroblast primary cilium with a ciliary pocket; asterisks mark microvilli (courtesy of Peter Satir). (iii) Transmission EM of a cross section of a fibroblast primary cilium at the ciliary transition zone showing the 9 + 0 microtubules arrangement of the axoneme (with permission [144]). (B) Cartoon illustrating IFT and trafficking processes at the primary cilium to control ciliary assembly and targeting of GPCRs to the ciliary membrane. Receptor transport from the TGN to the ciliary pocket is mediated by ARF4, IFT20, and TCXEX-1 together with a complex consisting of FIP3, ASAP1, and RAB11/RAB8/Rabin8 subcomplex. The docking of vesicles near the periciliary membrane switches to IFT in a process involving the IFT-B components IFT57 and IFT20 as well as ARL6 and the RAB8-binding protein, Rabaptin5. Please see text for further details. (C) List of GPCRs known to localize to the primary cilium. (D) Immunofluorescence micrographs with examples of GPCRs localizing along the axis of the primary cilium in various cell types: (i) localization of melanin-concentrating hormone receptor 1 (MCHR1) in cultured mouse hypothalamic neurons (courtesy of Nicholas Berbari and Kirk Mykityn); (ii) localization of somatostatin receptor 3 (SSTR3) in neuronal primary cilia of the mouse hindbrain (with permission [145]); (iii) localization of dopamine 5 receptor (DR5) to primary cilia in porcine kidney proximal tubule (LLC-PK1) cells (with permission [146]); (iv) localization of kisspeptin receptor 1 (KISSR1) to primary cilia the medial hypothalamus in adult *Cilia^{GFP}* mice (with permission [77]); and (v) localization of smoothened (SMO) to primary cilia in SMO agonist (SAG)-stimulated human embryonic stem cells (hESC) (with permission [147]). Keys: β -tubulin III (neuronal marker), ACIII (adenylate cyclase III, neuronal primary cilium marker), Ac-tub (acetylated α -tubulin, primary cilium marker), Glu-tub (glutamylated α -tubulin, primary cilium marker), and DAPI/DRAQ5 (stains DNA, nuclear marker). Please see text for references and further details

Table 1. Examples of proposed ciliary targeting sequences (CTSs) for various GPCRs in mammalian cells.

GPCR	Examples on proposed ciliary targeting sequences (CTSs)		
	VXPX motif in C-tail	AXXXQ motif in i3	Other motifs in i3
Rhodopsin [40,149]	[SQVAPA]	Not necessary	
SSTR3 [71]		[APSCQ]	
HTR6 [71]		[ATACQ]	
MCHR1 [71,150]	Not necessary	[APASQ]	
NPY2R, GPR88 [80]		Not present	[R/K][I/L]W

We note that some studies have suggested the presence of phenylalanine–arginine (FR) CTSs in the C-terminus of several ciliary GPCRs, but structural evidence suggests that these FR motifs are inaccessible for the ciliary targeting machinery and rather mediate proper protein folding (discussed in [148]).

segment, whereas photopsins localize to disks, which are contiguous with the outer segment plasma membrane of the cone cells, although mammalian cones may contain disks, which are separated from the plasma membrane [37]. For example, the C-terminal CTS of rhodopsin (see below) was shown to directly bind to the small GTPase ARF4, which mediates budding of rhodopsin carrier vesicles at the TGN followed by their translocation to photoreceptor connecting cilia by a complex mechanism involving the RAB11/ARF effector FIP3, the ARF GTPase-activating protein ASAP1 (Arf-GAP with SH3 domain, ANK repeat, and PH domain-containing protein 1), and the RAB11/RAB8/Rabin8 complex [38–41]. These post-Golgi carriers are likely transported by the cytoplasmic dynein-1 motor to the ciliary base via direct interaction between rhodopsin’s C-terminal tail and the dynein light chain Tctex-1 [42]. Additional regulators of ciliary trafficking include the transport protein particle (TRAPP)II complex and TRAPPC8, which are required for the recruitment of Rabin8 to the centrosome [43,44], as well as the IFT-B complex protein IFT20 [45,46].

Additional studies have implicated Bardet–Biedl syndrome (BBS) proteins in ciliary membrane biogenesis/homeostasis, for example, by promoting ciliary trafficking of specific GPCRs [47–49]. The i3-CTSs of these receptors (see Table 1 and below) appear to interact directly with components of the BBSome [48,49], a stable complex of seven BBS proteins that cooperates and interacts with

the RAB11/RAB8/Rabin8 complex to promote cilia membrane biogenesis [50,51]. At the ciliary base, the BBSome helps the vesicle–motor complexes to dock near the periciliary membrane in order to switch the vesicle receptor trafficking to IFT, a process involving the IFT-B components IFT57 and IFT20 as well as ARL6 and the RAB8-binding protein, Rabaptin5 [52]. This leads to stimulation of the GEF activity of Rabin8 toward RAB8, which then interacts with the exocyst complex to mediate receptor fusion with the membrane. RAB8 itself is targeted to cilia via the transition zone-associated complex of RPGR and CEP290, and likely BBS4 [52]. Of note, the BBSome also interacts with the IFT machinery to regulate ciliary export of signaling proteins [53]. As a testament to the importance of the BBSome in proficient targeting of GPCRs to cilia, knockout mice defective in the ciliary protein Tubby or BBSome components display aberrant ciliary localization and signaling of the melanin-concentrating hormone receptor 1 (MCHR1) and somatostatin receptor 3 (SSTR3), which causes blindness and obesity [47,54,55]. For a recent review on the mechanisms in sorting, targeting, and trafficking of rhodopsin to the outer segment, and how mistrafficking is associated with degeneration of photoreceptors, please see [56].

In addition to the GPCRs mentioned above, a growing number of GPCRs are specifically targeted to cilia, including both motile and non-motile cilia. For example, in the airway epithelium, motile 9 + 2 cilia harbor bitter taste class A GPCRs (T2Rs) that signal through the G protein Gustducin when encountering a bitter substance. This causes airways to relax and protects the respiratory system from noxious compounds [57]. Similarly, olfactory transduction is regulated through the combination of hundreds of class A GPCRs that rely on activation of the G protein $G_{\alpha olf}$ in cilia of the main olfactory epithelium (Fig 1B and C). This activates an adenylate cyclase that leads to cAMP production and depolarization of the cell through the opening of several ion channels [58] (Fig 3A). GPCR signaling is also well documented in invertebrate sensilla, such as in chemosensory cilia on dendritic endings of sensory neurons in *Caenorhabditis elegans* (*C. elegans*). These cilia play a unique role in regulating behavioral responses, including social feeding and dauer formation as well as avoidance and mating responses [59]. Systematic characterization of these cilia has contributed significantly to the understanding of ciliary targeting of GPCRs. As an example, a recent study identified novel *cis*- and *trans*-acting mechanisms, that is, amino acid motifs and trafficking pathways, required for ciliary

Figure 3.

Figure 3. Examples of ciliary GPCR signaling.

(A) GPCR signaling in olfactory cilia relies on the cAMP-dependent opening of ion channels, leading to an influx of Na^+ and Ca^{2+} ions into the ciliary compartment, which in turn activates chloride channels, causing efflux of Cl^- , which results in a further depolarization of the cell. Abbreviations: Olf: ligands for olfactory receptors. (B) Outline of trafficking and signaling processes associated with MCHR1 and SSTR3 signaling in neuronal primary cilia. The BBSome and TULP3, IFT-A, and KIF3A control the localization of the receptors to the ciliary base and further into the ciliary membrane. Abbreviations: M: melanin-concentrating hormone; S: somatostatin. (C) Outline of signaling processes associated with D1R and GPR88 signaling in primary cilia. D1R is activated by catecholamines both in the cilium and at the plasma membrane, but receptor activation is specifically inhibited within the cilium by GPR88. (D) Outline of trafficking and signaling processes associated with HH signaling in primary cilia. Please see text for references and further details.

localization of GPCRs [60]. The components shown to be required for localization of GFP-tagged GPCRs to sensilla in *C. elegans* include a number of vesicular and adaptor proteins such as the bbs-1, bbs-8, rab-8, arl-3, rl-13, odr-4, unc-101, and daf-25 as well as TZ and IFT subunits. These GPCRs use different CTSs for ciliary targeting within a given cell type, and CTSs within individual GPCRs mediate ciliary localization via diverse trafficking mechanisms across cell types [60]. In most amphibia, reptiles, and non-primate mammals, the vomeronasal organ (VNO) at the nasal septum also bears GPCRs that in mice are associated with a extensive array of instinctive behaviors, such as aggression, predator avoidance, and sexual attraction [61]. However, VNO cells are generally microvillar rather than ciliary. Similarly, gustatory hair cells in the taste buds of the tongue use microvilli as cellular extensions for sweet, umami, and bitter tasting through the activation of a series of class A and C GPCRs [62].

Recently, signaling molecules were shown to be released into the extracellular environment from the ciliary membrane by the shedding of ectosomes [63–66]. This adds an additional layer of complexity to the trafficking mechanisms of receptors to and from the cilium, although it is currently unknown whether receptors in extracellular vesicles fuse with the ciliary membrane to control signaling processes within the ciliary compartment.

Rhodopsin-like (class A) GPCRs in neuronal primary cilia

A small, but growing number of rhodopsin-like (class A) GPCRs have been demonstrated to localize to primary cilia. In neuronal cells, ciliary GPCRs act as extra synaptic or “unwired” receptors believed to regulate neuronal function by sensing neuromodulators in the local environment. The first class A GPCRs found to be enriched in neuronal primary cilia were SSTR3 [67,68] and serotonin receptor 6 (5-HT6 or HTR6) [69,70], which were detected by immunofluorescence confocal microscopy of cilia on, for example, neurons from the coronal rat brain section, island of Calleja and the olfactory tubercle. Employing C-terminal chimeras and sequence analysis, the discrete GPCR-specific AxxxQ CTS was deduced in the third intracellular loop (i3) of SSTR3 and HTR6 (Table 1), leading to the identification of a third ciliary class A GPCR, MCHR1. As with SSTR3 and HTR6, this CTS is sufficient to localize MCHR1 to cilia in neurons [71].

Interestingly, while most neurons in the brain possess a primary cilium [72], it has been demonstrated that only a subset of ciliated neurons display MCHR1 and HTR6 in the ciliary membrane [47,67,69,70]. MCHR1, which is critical for proficient feeding behavior, was shown to concentrate in neuronal cilia in the hypothalamus in mice [73]. The hypothalamus, a brain region controlling appetite behavior and energy metabolism, relies on ciliary signaling to sense satiety signals from the surroundings. Disruption of cilia by

conditional depletion of *Kif3A* or *Tg737/Ift88* specifically on pro-opiomelanocortin (POMC)-expressing neurons in the hypothalamus causes hyperphagia-induced obesity in mice [74], thus raising the possibility that MCHR1 in neuronal cilia might regulate energy homeostasis. In line with this, as discussed above, the ciliary localization of SSTR3 and MCHR1 was demonstrated to rely on the BBSome in mouse brain sections and in cultured hippocampal neurons [47,49,50]. Mouse models of BBS support a role for the BBSome in targeting GPCRs to cilia, as neurons from mice lacking either the BBS2 or BBS4 protein retain structurally normal primary cilia but fail to accumulate MCHR1 and SSTR3 in the ciliary membrane [47]. These mouse models of BBS provided some of the first mechanistic clues linking BBS phenotypes, for example, obesity, to a defective molecular mis-targeting of GPCRs to the neuronal cilium. Members of the Tubby family, namely Tubby and Tubby-like protein (TULP3), have been demonstrated to mediate IFT complex A-dependent trafficking of ectopically expressed, GFP-tagged, SSTR3 and MCHR1 to cilia [75]. In the neurons of Tubby-deficient (*Tub*) mutant mice, SSTR3 and MCHR1 as well as HTR6 are diminished or excluded from the neuronal primary cilia. As with the *Bbs2* and *Bbs4* mutant mice, *Tub* mice are obese [76], lending further credence to the linkage between GPCR targeting to cilia and energy homeostasis. However, Tubby is not essential for all GPCR trafficking to cilia since another receptor, the odorant receptor mOR28, remains correctly localized to the distal cilia of olfactory epithelial cells of *Tub* mutant mice [54], thus emphasizing the specificity of action exerted by Tubby.

More recently, the kisspeptin receptor (KISS1R) was identified as a novel ciliary class A GPCR in gonadotropin-releasing hormone (GnRH) neurons in the mouse hypothalamus, and it was suggested that primary cilia are required for normal KISS1R signaling in these neurons [77]. KISS1R regulates the onset of puberty and adult reproductive function [77,78], but conditional ablation of primary cilia in GnRH neurons in transgenic mice did not affect their sexual maturation, so further analysis will be needed to understand the function of ciliary KISS1R signaling in the brain [77]. Additional subtypes of rhodopsin-like GPCRs, including dopamine D1, D2, and D5 [79] and neuropeptide Y NPY2R and NPY5R [80] receptors, as discussed below, were found to be specifically localized to primary cilia in different cell types. These findings have been highly instructive for understanding the complex machinery of how GPCRs are targeted to neuronal primary cilia of the hypothalamus to control energy balance, and how defects in neuronal cilia may be linked to neuropsychiatric disorders.

Finally, among the many developmental pathways that have been shown to involve cilia, a recent study utilizing zebrafish genetics and cultured human epithelial cells has added yet another component to the realm of cilia-related GPCR signal transduction systems, namely the prostaglandin signaling pathway. Specifically,

cilium formation and elongation was shown to require the class A GPCR EP4 together with its cognate ligand prostaglandin E2 (PGE2). This signaling requires a signaling cascade of cyclooxygenase-1/2 (COX-1/2), which synthesizes PGE2, and ATP-binding cassette transporter ABCC4 (encoded by the zebrafish leakytail/Lkt locus) that conveys export of PGE2 out of the cell, and subsequent binding of PGE2 to the EP4 receptor displayed on the cilia membranes [81]. The ABCC4-mediated PGE2 signaling at the cilia thereby activates G proteins and cAMP signaling to promote ciliogenesis through an increase in the anterograde velocity of IFT.

Ciliary class F GPCRs of the Hedgehog (HH) and Wingless/Int (WNT) signaling pathways

Studies conducted over the past several years have spawned two major fields of ciliary signaling comprised by the class F GPCRs. This class comprises frizzled (FZD) and smoothened (SMO) receptors, which regulate Wingless/INT (WNT) and Hedgehog (HH) signaling, respectively. Numerous studies have addressed how the primary cilium may coordinate the balance between canonical (β -catenin-dependent) and non-canonical (β -catenin-independent) WNT signaling during development and in tissue homeostasis [82–84]. FZD3, which is a receptor for the WNT5A ligand that promotes non-canonical WNT signaling, has been localized to primary cilia of fibroblasts and kidney epithelial cells [85,86], but the functional coupling of frizzled receptors to the ciliary compartment remains to be elucidated. In contrast, it is well established that one of the foremost tasks of primary cilia is to regulate HH signaling, which relies on the dynamic trafficking of SMO into and out of the cilium to control cellular processes during embryonic development of the axial skeleton, limbs, and the central nervous system (CNS) [87,88]. Further, vertebrate HH signaling is required for the maintenance of tissue homeostasis, cell proliferation, wound repair, angiogenesis [89], anti-inflammatory processes [90] and maintenance of many stem and progenitor cell populations [91,92]. Conversely, loss of negative control of HH signaling contributes to tumor pathogenesis and progression [93–95].

Work from around the mid-2000s established that primary cilia are critical modulators of vertebrate HH signaling (e.g., [96–103]). This generated a novel platform for understanding the mechanisms by which primary cilia coordinate the balanced activation and deactivation of cellular signaling pathways, and how defects in formation or function of primary cilia are associated with developmental disorders and diseases. In the absence of HH ligands, the receptor Patched (PTC) is localized within the ciliary membrane to prevent ciliary entrance of SMO (Fig 3D). In the presence of HH, however, PTC translocates out of the cilium, allowing SMO to enter the cilium and convert GLI (glioma-associated oncogene homolog) transcription factors from their repressor forms (GLI-R) into activator forms (GLI-A) [88]. GLI activation involves the timely inhibition of two major GLI inhibitors, protein kinase A (PKA) and suppressor of fused (SUFU). However, the pathway that transduces signals from SMO to PKA and SUFU remains poorly understood, but likely involves the parallel actions of additional GPCRs as outlined below. Once activated, GLI in complex with SUFU is recruited to the cilium, which leads to the rapid dissociation of the SUFU-GLI complex at the tip of the cilium and translocation of GLI-A to the nucleus to activate target

genes (Fig 3D) [97,104]. This process is regulated by the kinesin-4 family protein, KIF7, which creates a structural compartment at the ciliary tip that induces the dissociation of the SUFU-GLI complex [105].

Two additional protein components, the Ellis-van Creveld syndrome proteins EVC and EVC2, add an extra layer to the regulation of GLI by promoting two critical steps: SUFU/GLI dissociation and GLI ciliary traffic. The mechanism by which EVC/EVC2 regulate HH signaling, although still poorly defined, involves their direct interaction with SMO, which depends on phosphorylation of SMO. It remains to be clarified how EVC/EVC2 transduce HH stimuli downstream of SMO phosphorylation and promote GLI activation by antagonizing SUFU [88] (Fig 3D). More recently, it was demonstrated that disks large homolog 5 (DLG5) protein plays a critical role in the early point of the SMO activity cycle by interacting with SMO at the ciliary base to induce the accumulation of KIF7 and GLI at the ciliary tip for GLI activation [106]. Finally, the IFT complex B protein, IFT25, is required for proper HH signaling by coupling GLI, SMO, and PTC to the IFT machinery that co-regulates the dynamic trafficking of the HH components into and out of the cilium [107].

SMO has many biochemical properties resembling those of the GPCR superfamily, as discussed in Ruiz-Gomez *et al* [4], and recent evidence obtained from different organisms and *in vitro* studies implicate large G protein complexes containing the $G_{\alpha i}$ subunit in SMO signaling [108,109], although this mechanism remains to be linked to the primary cilium. More direct evidence has been obtained for the ciliary G protein, the Gnas-encoded tumor suppressor $G_{\alpha s}$, which recently was found to suppress HH signaling in granule neuron progenitors (GNPs) through activation of the cAMP-dependent pathway to fine-tune the processing and activation of GLI transcription factors, thereby regulating ciliary activities of HH signaling. Gnas expression was shown to determine progenitor cell competency in initiation of medulloblastomas (MBs), and low levels or loss-of-function of $G_{\alpha s}$ was demonstrated to define a subset of aggressive SHH-MB. Hence, $G_{\alpha s}$ inhibits MB formation in part by suppressing SHH signaling [110]. In this model, $G_{\alpha s}$ serves as a point of convergence between SMO and other $G_{\alpha s}$ -coupled GPCR signaling pathways, as discussed below, to adjust SHH signaling and MB formation. Evidence from other sources further substantiates a ciliary GPCR-like function of SMO. β -Arrestins, well known for their function in GPCR protein recycling [111], have long been known for their vital role in endocytosis of SMO and signaling to GLI transcription factors. On top of these functions, β -arrestin1 (arrestin-2) or β -arrestin2 (arrestin-3) depletion abrogates SMO trafficking to the cilium as well as SMO-dependent activation of GLI [112]. Emerging evidence suggests that both G protein-coupled receptor kinases (GRKs) together with β -arrestin2 function in the cilia or at the ciliary base (Fig 3D). In line with the known roles of GRK2 in GPCR and SMO signaling [113], a study linked GRK2 to SMO function in cilia. GRK2, together with the growth arrest-specific protein GAS8/GAS11, a microtubule-associated subunit of the dynein regulatory complex (DRC) [114], was proposed to facilitate SMO signaling [115]. GAS8 ablation compromises signaling and ciliary accumulation of SMO, whereas overexpression of GAS8 augments SMO activity in a GRK2-dependent manner [115]. The SMO signaling pathway differs, however, in some aspects from the canonical mechanisms of GPCR stimulation. Finally, integrin-linked kinase (ILK) was

shown to be required for translocation of SMO to the primary cilium as well as for SHH-dependent GLI activation in the embryonic mouse cerebellum [116], and it was suggested that ciliary SMO accumulation is regulated by the interaction between ILK and β -arrestins at the ciliary base [116,117].

Cross talk between different ciliary GPCR pathways

The unearthing of SSTR3, HTR6, and MCHR1 in the primary cilia paved the way for a new string of studies devoted to the exploration of novel GPCRs in cilia. Specifically, these studies have dealt with the issue of cross talk between converging GPCR pathways functioning concurrently in the primary cilia. What has remained less clear is the ciliary component(s) that regulate the activities of the downstream effectors cAMP and PKA in the GPCR pathways. Specifically, in the case of the HH pathway SMO has been proposed to act as a GPCR that inhibits PKA by inducing the G_i family of heterotrimeric GTPases [4,108,109], but this idea has stirred some controversy [118]. The SMO–GLI link may alternatively represent a non-canonical route by which the HH pathway regulates PKA. Instead, as outlined below, the inhibitory function of PKA on SMO now seems to be the convergence point by which additional ciliary GPCRs can antagonize PKA.

One of the enduring mysteries in the mechanism determining cAMP-regulated PKA activation may have come closer to an answer by recent work identifying the class A orphan GPCR, GPR161, in the pathway of the “canonical” PKA-mediated inhibition of GLI protein function [119]. GPR161 is expressed in the brain and essential for neural tube patterning [120]. Endogenous GPR161 is confined to primary cilia in a wide array of cell types and, unlike SMO, requires TULP3/IFT-A for its ciliary localization [75,121] (Fig 3D). In line with this, a null mutant in *Gpr161* generally phenocopies the *Tulp3*/IFT-A mutants by causing augmented HH signaling and decreased levels of both GLI2/3 proteins and reduced processing of GLI3 into GLI3-R, which is strongly reminiscent of either *Pka* null or *Sufu* mutant mice [122,123]. As ciliary trafficking of SMO is not controlled by TULP3/IFT-A, GPR161 seems a likely candidate for a ciliary receptor responsible for the defects in neural tube patterning found in TULP3-deficient mice. Additionally, constitutive active GPR161 amplifies cellular cAMP levels and knockdown of G_{as} protein, which is likely to be coupled to GPR161, rescues/neutralizes this phenotype [119], indicating that GPR161 might be the long-sought factor for establishing the basal cAMP gradient and activation of PKA in the ciliary HH signaling pathway. The rise in cAMP levels could activate PKA and thus might provide a compelling explanation for the reduced GLI3-R levels in the *Gpr161* mutant. How GPR161 regulates the adenylyl cyclases important for cAMP signaling, however, is still unknown.

A GPCR of the secretin (class B) family, the pituitary adenylyl cyclase-activating polypeptide (PACAP) receptor, PAC1, has been reported to cooperate with GPR161 in the HH signaling pathway, in cells treated with both HH and PACAP agonist, to regulate GLI protein phosphorylation by PKA [124]. This association may fine-tune the transcriptional and physiological function of GPR161 in the HH pathway. The function of PAC1 in the HH cascade may be best reconciled in terms of the proposed “two-brake” model (Fig 3D) of the interplay between the HH pathway and PKA-mediated signaling. In this model, the ciliary HH pathway operates as follows. The

absence of HH induces GPR161-dependent PKA activation, which prevents GLI accumulation in the cilia and in turn converts it into GLI-R. Upon HH exposure, Smo translocates into the cilium, which prompts GPR161 to leave the cilium, and PKA activity then stops. This causes GLI proteins to accumulate at the ciliary tip, effectively converting GLI into GLI-A. In cells complemented with both HH and PACAP, PKA remains inactive, and in its place an alternative pool of PKA called PKA_{GPCR} is activated by PAC1. Only when the PKA_{GPCR} activity is adequately high, it will restrain ciliary translocation of GLI and in turn stimulate GLI-R formation. PAC1-deficient mice display disrupted ventricular ependymal cilia and hydrocephalus-related abnormalities, suggesting that PAC1 is a genuine actor in ciliary signaling [125].

Indirect role of primary cilia in regulating GPCR signaling and cAMP levels

Downstream of ciliary GPCR signaling, so far only one effector has been reproducibly shown to transduce the GPCR activation into the cell. This is the adenylyl cyclase type III (ACIII), a component of the cAMP-dependent, G protein-coupled signaling cascade whose activation increases production of cAMP, which is a prerequisite for the ensuing array of cellular processes [72,126]. ACIII is highly enriched in neuronal cilia in the hippocampus and cortex of brain [72], highlighting the important function of the primary cilium as a focal point in which GPCRs act alone or perhaps together with additional GPCRs as oligomeric/heteromeric assemblies. While the basal cAMP levels appear to be controlled by GPR161 and G_{as} , both of which are confined to the cilia, favoring that they are functional in this compartment, recent evidence points to an indirect role of cilia in the basal cAMP regulation. The dopamine receptor is a catecholamine receptor that signals by G proteins. Three isoforms of the dopamine receptor, D1R, D2R, and D5R, have previously been demonstrated to localize to primary cilia [79,127,128]. In case of D1R, disruption of the BBSome augments D1R accumulation in cilia [48,129] and ectopic expression of D1R appears to markedly increase ciliary length [127]. However, disrupting cilia in cells expressing D1R does not perturb the graded overall D1R-mediated cAMP response upon agonist addition [128]. Recently, D1R was shown to be delivered to cilia directly from the extraciliary plasma membrane. The cytoplasmic tail of D1R appears to target the receptor to the ciliary membrane, a process that is mediated by the joint forces of the IFT-B complex as well as the ciliary kinesin, KIF17, in conjunction with the small GTP-binding protein, RAB23. RAB23 is a potent ciliary mediator and may play broader roles in GPCR targeting to cilia, as overexpression of RAB23 alone can force strong ciliary localization of a non-ciliary GPCRs [35].

In a model cell system expressing two GPCRs, D1R and GPR88, both of which normally co-express in the brain and accumulate in cilia, GPR88 appears specifically to inhibit the graded D1R-mediated cAMP signaling response after catecholamine addition. A control GPCR, in this case the β 2AR receptor, not found to localize to cilia, exhibited normal cAMP activity when co-expressed with GPR88. However, cilia depletion in this same model system, and accompanying GPR88 plasma membrane dispersal, resulted in ablated β 2AR response whereas D1R stimulation was unaffected after catecholamine exposures, suggesting that the selective inhibitory

function of GPR88 on ciliary D1R occurs only when GPR88 is trapped inside the ciliary compartment [128]. Hence, the primary cilium seems to function as a plasma membrane niche that selectively excludes or “insulates” the GPCR-dependent cAMP activities of one GPCR pathway from affecting signaling by other GPCRs. Such a discrete function of the primary cilia to “insulate” specific receptors to restrain or perhaps fine-tune their activities is supported by other recent studies. A similar finding was reported for the G protein-coupled bile acid receptor 5 (TGR5), which is enriched in the cilia of cholangiocytes [130,131]. The selective association of TGR5 on the plasma membrane or ciliary membrane was found to define the cholangiocyte functional response to bile acid (BA) signaling. In non-ciliated cholangiocytes, TGR5 ligands increased cAMP levels and cell proliferation but inhibited the ERK growth signaling pathway. Conversely, in ciliated cholangiocytes, treatment with TGR5 ligand resulted in the opposite response; that is, cAMP levels and cell proliferation decreased, whereas ERK signaling was activated.

Systematic approaches to identify new ciliary GPCRs

A number of recent GPCR screens may enable a leap forward in understanding the extent and diversity of GPCRs functioning in primary cilia. One of the first systematic screens of ciliary GPCRs was performed in *Chlamydomonas reinhardtii* to indirectly identify receptors affecting cell viability or flagellar length, motility, or severing. Out of 1,280 small-molecule compounds tested, 142 were found to induce shortened flagella, 133 resulted in absent flagella, and 126 activated the flagellar deflagellation pathway, that is, induced loss of flagella rather than resorption. Among these compounds, inhibitors of serotonin, melatonin, opioid, histamine, and catecholamines/norepinephrine/epinephrine receptors scored multiple times [127], raising the possibility that many new GPCRs might be identified in cilia, although caution must be exercised when drawing such conclusions based on ciliary length measurements. However, compounds targeting dopamine receptors that were found to change ciliary length in *Chlamydomonas* were supported by the expression of dopamine D1 receptors in cultured mammalian fibroblasts [127], thus suggesting a role for the dopamine responsive system in ciliary length regulation.

Both the dopamine receptor, here the short isoform of dopamine receptor D2 (DRD2S), and the NPY2R were identified in cilia in another recent screen [132], adding further credence to the role of these receptors pathways in cilia. This screen analyzed the subcellular localization of 138 non-odorant human or mouse GPCRs and found, besides DRD2S and NPY receptors, the prolactin-releasing hormone receptor (PRLHR), neuropeptide FF receptor 1 (NPFFR1), and neuromedin U receptor 1 (NMUR1) enrichment in cilia.

Another study, intended to reckon the full catalog of GPCRs operating in neuronal cilia, specifically hypothalamic GPCRs functioning in energy homeostasis, screened 42 GPCRs and found a total of seven candidate GPCRs (when fused to GFP) competent to localize to cilia in retinal pigment epithelium (RPE) cells [80]. These include PGR15L, the NPY family receptors 2 and 5 (NPY2R and NPY5R), the orphan receptor GPR83, the galanin receptors 2 and 3 (GAL2R and GAL3R), and the pyroglutamylated RF amide peptide receptor (QRFP). Among the seven GPCRs, the neuropeptide Y receptor (NPY2R) showed likely implications in anorexia in BBS and tubby

mice. In BBS mice, the cognate ligand specific for NPY2R, neuropeptide PYY3-36, failed to induce the predicted anorexigenic effect. PYY3-36 stimulation in cell lines derived from these mice resulted in reduced cAMP levels, suggesting that the primary cilium enhances ligand-dependent NPY2R signaling. Domain swapping between a non-ciliary GPCR (NPY1R) and the ciliary NPY2R as well as mutation analyses revealed a [R/K][I/L]W CTS in the third intracellular loop (i3) region of both NPY2R and GPR83 (Table 1), which is required and sufficient for ciliary localization, whereas a second motif [RRQK] in the same region seemed not to be sufficient for ciliary targeting. Interestingly, a [AXXXS] CTS is absent in NPY2R and GPR83, indicating the presence of diverse mechanisms by which GPCRs are targeted to the primary cilium by *trans*-acting factors, such as BBS and Tubby family proteins [80].

In addition to the GPCRs known to function in the ciliary compartment, other GPCRs have been found to regulate HH signaling, although it is less clear whether such functions are restricted to cilia. A mouse TM3 Leydig cell-based screen for GPCR inhibitors, employing a pathway-dependent luciferase reporter gene assay, recently identified a novel GPCR within the HH pathway. A cyclohexyl-methyl aminopyrimidine chemotype compound (CMAP) that inhibited GLI-activated signaling in a SMO-independent manner was identified. The GPCR was identified as the orphan receptor GPR39, which under serum starvation is activated by CMAPs to stimulate Gq-coupled, Gi-coupled, and β -arrestin-mediated signaling pathways [133]. Thus, GPR39 is a novel modulator of HH signaling capable of intercepting the pathway downstream of SMO.

Future work and drug development for GPCR-associated ciliopathies

The unearthing of primary cilia as subcellular platforms for GPCR signaling has set the stage for future avenues of cilia research. The GPCR transduction pathways that transmit signaling cues through primary cilia are expanding, as are the effector molecules that shuttle through cilia during signaling. This has enabled a leap forward in the understanding how GPCR signaling fine-tunes tissue sculpturing and tissue homeostasis and how developmental and behavioral disorders are related to defects in assembly and function of primary cilia. Evidently, many questions remain, as exemplified by the elusive role of ciliary KISS1R in the onset of puberty and adult reproductive function [77], but learning the roles of converging GPCR pathways in embryonic and later adult development, explicitly in the context of its localization and removal from the ciliary compartment as well as in cross talk with other GPCRs, promises to offer key insights into the role of primary cilia as critical pharmacological targets in the treatment of ciliopathy patients.

Principally, GPCRs may be modulated in any of the sequential molecular steps that transmit the external stimuli into the cell. As discussed, a distinctive feature of this family is their dynamic association with heterotrimeric G protein that changes its conformation when activated. Depending on the nature of protein G binding to its receptor, a cascade of different secondary messengers convey the signal into the cell (Fig 1A). Through desensitization, inactivation, and internalization, the signal is subsequently turned off through a controlled process. Indeed, aberrant regulation of ciliary GPCR signaling is associated with a range of neurological disorders,

Sidebar A: Open questions in the field of GPCR signaling in primary cilia

- Annotate the full repertoire of human ciliary GPCRs—as opposed to non-ciliary GPCRs. Are there differences in cilia localization between tissues?
- Develop better biochemical and cell physiological tools to test the hypothesis that cilia function as not only amplifiers (antenna), but also as “insulators” that ensure proper distance between specific signaling proteins
- Understand structurally, at the tertiary level, what makes ciliary GPCRs so special. Amino acid motifs are evolutionary divergent and are often poorly suited as predictors.
- How many diseases have deficient GPCRs in cilia as root cause?
- Signaling in the primary cilium involves the interaction between different GPCRs that either leads to activation or downregulation of specified signaling pathways. The modes of interaction are unknown and should be resolved in order to understand the spatiotemporal regulation of ciliary signaling
- Why are CTSs for ciliary GPCRs so diverse and what roles do post-translational modifications play in their targeting to the cilium?

highlighting the importance of primary cilia in the brain and the potential routes for treatment with new and existing drugs against cognitive deficits and psychiatric illnesses [32,134,135]. Historically, GPCRs have been demonstrated to be potent targets of a large group of drugs, among other things the treatment of asthma, heart failure or of renal diabetes insipidus [136]. Hence, similar strategies may prove advantageous in the treatment of neurological disorders. More specifically, a range of selective small-molecule GPCR antagonist candidates capable of traversing the blood–brain barrier have yielded promising effects in terms of potency and selectivity [137]. High-throughput screening campaigns have successfully identified potent GPCR drug candidates [138], and most recently, such strategies have identified agonists against, for example, GPR88 [139] and antagonists to a paralogue of the class B GPCR, vasoactive intestinal peptide receptor 2 (VPAC2) [140], which localizes to the primary cilium [141], raising hopes that selective manipulation of converging GPCR pathways specifically in the ciliary compartment is feasible. In case of the design of functionally discriminatory antagonists, efforts are in progress to understand and utilize the GPCR binding site with molecular modeling [142,143].

In summary, the recognition of GPCR signaling in primary cilia has paved the way for future explorations of the many unanswered questions that remain. The advance of novel tools that can accurately monitor and manipulate ciliary signaling events will be important for the development of novel drug strategies in the treatment of ciliopathy patients.

Acknowledgements

We thank Joseph Gleeson for permission to use his transmission EM image of a longitudinal section of a neuronal primary cilium (Fig 2Ai), and Nicholas Berbari for his immunomicrograph of localization of MCHR1 to the primary cilium in hypothalamic neurons (Fig 2Di). This work was supported by the University of Copenhagen Excellence Programme for Interdisciplinary Research and the Danish Council for Independent Research (1331-00254).

Conflict of interest

The authors declare that they have no conflict of interest.

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