Pharmacology & Therapeutics 224 (2021) 107836

Contents lists available at ScienceDirect

Pharmacology & Therapeutics

journal homepage: www.elsevier.com/locate/pharmthera

Signal transduction in primary cilia – analyzing and manipulating GPCR and second messenger signaling

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ARTICLE INFO

Available online 18 March 2021

Keywords:
Second messengers
cAMP
Ca^{2+}
Cilia
GPCR

ABSTRACT

The primary cilium projects from the surface of most vertebrate cells, where it senses extracellular signals to regulate diverse cellular processes during tissue development and homeostasis. Dysfunction of primary cilia underlies the pathogenesis of severe diseases, commonly referred to as ciliopathies. Primary cilia contain a unique protein repertoire that is distinct from the cell body and the plasma membrane, enabling the spatially controlled transduction of extracellular cues. G-protein coupled receptors (GPCRs) are key in sensing environmental stimuli that are transmitted *via* second messenger signaling into a cellular response. Here, we will give an overview of the role of GPCR signaling in primary cilia, and how ciliary GPCR signaling can be targeted by pharmacology, chemogenetics, and optogenetics.

Contents

Abbreviations: 5-HT_6, serotonin receptor 6; AC, adenylyl cyclase; ADP, adenosin di-phosphate; ADPKD, autosomal dominant polycystic kidney disease; AKT, protein kinase B; ARL13B, ADP-ribosylation factor-like protein 13; BBS, Bardet-Biedl syndrome; BMP, bone morphogenetic protein; bPAC, photo-activated adenylyl cyclase from Beggiato; Ca^{2+}, calcium; cAMP, cyclic adenosine mono phosphate; DRD1, dopamine receptor D1; DRD5, dopamine receptor D5; DAG, diacylglyerol; DREADD, designer receptor exclusively activated by designer drugs; EP4, prostaglandin E4; EPAC, exchange protein directly activated by cAMP; IFT, intraflagellar transport; IP_3, inositol 1,4,5-trisphosphate; FDA, U.S. Food and drug administration; FFAR4, free fatty acid receptor 4; GCaMP, calcium sensors; GDP, guanosin di-phosphate; GFP, green fluorescent protein; GPS, GPCR proteolysis site; GPCR, G-protein coupled receptor; GRK, G protein-coupled receptor kinases; GTP, guanosin tri-phosphate; Hh, Hedgehog; IMCD-3, inner medullar collecting duct 3; INPP5E, inositol phosphate 5-phosphatase; KISS1R, kisspeptin receptor 1; MC4R, melanocortin 4 receptor; MCHR1, melanin-concentrating hormone receptor 1; NPY, neuropeptide Y; NPYR, neuropeptide Y receptor; PC-1, polycystin 1; PC-2, polycystin 2; PDE, phosphodiesterase; PGE2, prostaglandin E2; PI3K, phospho-inositol (3) kinase; PKA, protein kinase A; PKD, polycystic kidney disease; PKD1-L1, polycystic kidney disease protein 1 like-1; PKD2-L1, polycystic kidney disease protein 2 like-1; PtdIns(4,5)P2, phosphatidyl (4,5) di-phosphate; PtdIns(3,4,5)P3, phosphatidyl (3,4,5) tri-phosphate; RPGR, retinitis pigmentosa GTPase regulator; SMO, Smoothened; sAC, soluble adenylyl cyclase; SSTR3, somatostatin receptor 3; tmAC, transmembrane adenylyl cyclase; TRP, transient receptor potential; V2R, type 2 vasopression receptor; WAT, white adipose tissue; WNT, Wingless-related integration site.

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https://doi.org/10.1016/j.pharmthera.2021.107836

1. Introduction

Perception of the environment and transducing this information into a cellular response is essential for any living organism. To this end, many cells employ cilia that function as cellular antennae. Cilia are microtubule-based membrane protrusions, emanating from the surface of most vertebrate cells. Cilia come in two different flavors: motile cilia/flagella are only found on specialized cells, where they control cell movement or the generation of fluid flow (Mitchison & Valente, 2017), whereas immotile, primary cilia protrude from the surface of almost every mammalian cell. Primary cilia receive stimuli from the environment and locally transduce this information into an intracellular response (Anvarian, Mykytyn, Mukhopadhyay, Pedersen, & Christensen, 2019; Goetz & Anderson, 2010; Nachury & Mick, 2019; Pazour & Witman, 2003). In this review, we will focus on primary cilia.

The ciliary membrane is continuous with the plasma membrane. Nonetheless, primary cilia contain a unique lipid and protein repertoire, separated from the cell body. This unique identity is maintained by specific structural elements. Primary cilia originate from the basal body, a modified mother centriole, which is part of the centrosome. The basal body serves as a nucleation site for the microtubules forming the core structure of all cilia, the axoneme. Along the axoneme, the intraflagellar transport (IFT) machinery transports proteins anterogradely to the ciliary tip or retrogradely to the ciliary base via the molecular motors kinesin-2 and dynein-2, respectively (Rosenbaum & Witman, 2002). Free diffusion of proteins from the cell body into the cilium is prevented at the transition zone at the ciliary base. One central component that mediates the transport across the transition zone is the BBSome, a multi-subunit protein complex that is not required for maintaining cilia integrity, yet, an important determinant of the protein composition of cilia (Fig. 1).

Mutations in genes that encode for ciliary or centrosomal proteins cause cilia dysfunction, which underlies the development of severe diseases commonly referred to as ciliopathies. Ciliopathies have pleiotropic phenotypes and impact several organs. Typical symptoms of ciliopathies, such as polydactyly, left-right asymmetry, or congenital heart defects arise from ciliary dysfunction during embryonic development. In contrast, other phenotypes, such as cystic kidneys or the development of obesity demonstrate that cilia also fulfill fundamental functions in the adult. Non-syndromic ciliopathies present only one clinical feature, e.g., retinal ciliopathies, whereas syndromic ciliopathies often exhibit additional features, affecting the heart, liver, central nervous system, adipose tissue, gonads, or bones (Hildebrandt, Benzing, & Katsanis, 2011; Reiter & Leroux, 2017).

The molecular mechanisms underlying the sensory function of primary cilia are not well understood. It has been shown that primary cilia participate in a variety of different signaling pathways. These include Hedgehog (Hh), WNT, receptor tyrosine kinase, transforming growth factor β (TGF-β), and bone morphogenetic protein (BMP) signaling (Anvarian et al., 2019). A detailed understanding of how these pathways evoke a cilia-specific response is, however, lacking.

In recent years, a picture has emerged that second messengers, in particular cyclic adenosine mono phosphate (cAMP), transduce extracellular information within the primary cilium into an intracellular response (Fig. 1). This concept has been well described for specialized primary cilia, i.e., the sensory olfactory cilia. Here, odorant binding activates G protein-coupled receptors (GPCRs), evoking an intracellular response through the second messenger cAMP (Kaupp, 2010). While the immediate downstream effectors for cAMP in olfactory cilia are well characterized, this information is missing for primary cilia.

Nevertheless, it has been generally accepted that the prototypic primary cilium uses signaling strategies also found in specialized sensory cilia, and it has been proposed that cellular functions are controlled through ciliary GPCRs and second messenger signaling (Fig. 2). Numerous GPCRs have been identified in primary cilia (Hilgendorf, Johnson, & Jackson, 2016; Mykytyn & Askwith, 2017; Schou, Pedersen, & Christensen, 2015). In the following, we will give an overview of GPCR signaling, its role in primary cilia, and how ciliary GPCR signaling can be targeted.

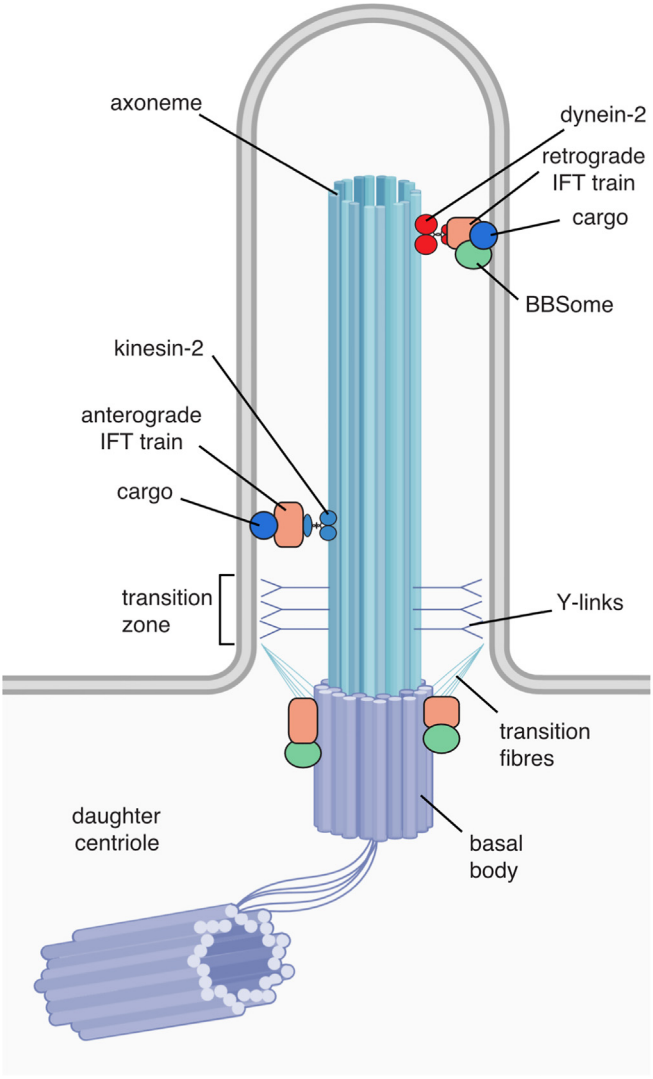

Fig. 1. Primary cilia. Primary cilia consist of a microtubule-based axoneme that originates from the basal body, a specialized mother centriole. Free diffusion of large proteins into and out of the primary cilium is prevented by the transition zone and the transition fibres. Proteins within the cilium are transported in a kinesin-2-dependent manner using anterograde intraflagellar transport (IFT) trains, whereas retrograde IFT trains transport cargos in a dynein-2-dependent manner. The BBSome functions as a cargo adaptor complex for the IFT-dependent removal of proteins from cilia. Figure has been generated using *biorender*.

2. G protein-coupled receptors (GPCRs)

2.1. GPCRs – transduction of extracellular cues

The superfamily of GPCRs comprises the most versatile receptor families in vertebrates. GPCRs sense a variety of different stimuli, ranging from extracellular ligands to photons or membrane tension. GPCRs can be grouped into six different classes: class A (the rhodopsin family), class B1 (the secretin family), class B2 (the adhesion family), class C (the glutamate family), class F (the frizzled–smoothened family), and the taste 2 family. GPCRs without a known natural ligand or stimulus are called orphan GPCRs (Tang, Wang, Li, Luo, & Liu, 2012). Generally, GPCRs consist of seven transmembrane domains with an extracellular

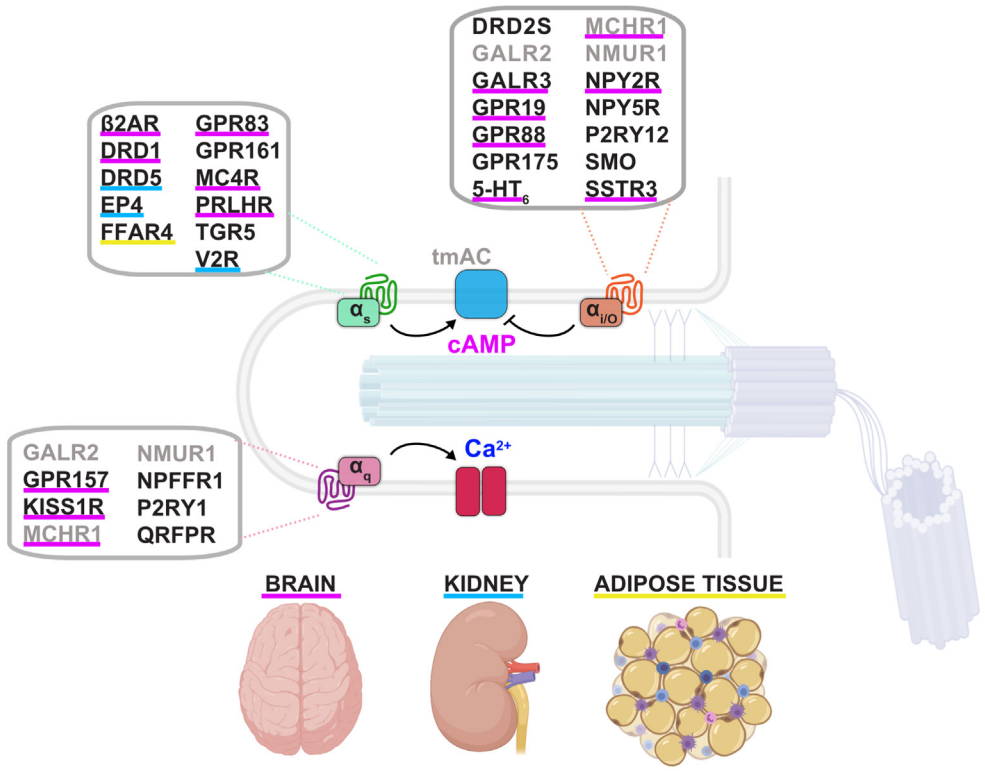

Fig. 2. Ciliary G protein-coupled receptor (GPCR) composition. The listed GPCRs have been identified in primary cilia of different tissues. Colored underlining highlights the tissues, in which the respective GPCRs have been identified (Brain: purple, kidney: blue, adipose tissue: yellow). GPCRs have been grouped according to their respective G proteins. GPCRs in grey as listed twice, as coupling to two different G proteins has been suggested. Figure has been generated using *biorender*.

N and an intracellular C terminus to transduce extracellular into intracellular information by coupling to G proteins. Activation of canonical GPCR signaling at the plasma membrane induces a conformational change upon ligand binding, which activates the G protein by exchanging the bound GDP for GTP (Hilger, Masureel, & Kobilka, 2018). G proteins are heterotrimeric, consisting of α, β, and γ subunits. The α subunit determines G protein function. There are four different G proteins: $G\alpha_s$, $G\alpha_{i/o}$, $G\alpha_{q/11}$, and $G\alpha_{12/13}$. $G\alpha_s$ and $G\alpha_{i/o}$ couple to transmembrane adenylyl cyclases (ACs), either stimulating or inhibiting their activity, which leads to an increase or decrease of intracellular cAMP levels, respectively. $G\alpha_{q/11}$ activates phospholipase C (PLC) in the plasma membrane, which hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP_2) into inositol 1,4,5-trisphosphate (IP_3) and diacylglyerol (DAG). IP_3 receptors conduct calcium upon IP_3 binding, whereas DAG activates protein kinase C (PKC), leading to target protein phosphorylation. $G\alpha_{12/13}$ specifically target Rho guanine exchange factors (RhoGEFs), thereby regulating a wide variety of cellular functions.

2.2. GPCR trafficking and inactivation

Inactivation of GPCRs usually involves GPCR internalization via endocytosis and trafficking to the endo-lysosomal compartment (Eichel & von Zastrow, 2018). The conformational changes induced by activation do not only activate G proteins but also result in GPCR phosphorylation at intracellular domains via G protein-coupled receptor kinases (GRKs). For ciliary GPCRs, there is direct evidence for the phosphorylation via GRK2 (Pal et al., 2016). Phosphorylation at the C terminus recruits β-arrestins, which inhibits further G protein activation and assembles additional factors for clathrin-mediated endocytosis at the plasma membrane. Recruitment of β-arrestin2 to activated ciliary GPCRs has been demonstrated by several groups (Green et al., 2016; Ye, Nager, & Nachury, 2018). However, spatial constraints do not allow the formation of endocytic vesicles within primary cilia, hence, GPCRs need to be removed before they can be endocytosed. Here, the BBSome functions as a cargo adaptor that allows recruitment to IFT trains, which drive activated GPCRs out of cilia in a β-arrestin2-dependent manner (Ye et al., 2018). Recently, BBSome recruitment to activated GPCRs has been shown to involve K63-linked ubiquitin chains on the C-terminal tail that follow β-arrestin2 recruitment in kidney cell primary cilia (Shinde, Nager, & Nachury, 2020). The ubiquitin ligase responsible for ubiquitylation at the C terminus as well as the precise position of ubiquitylation still need to be identified.

While downstream signaling pathways in primary cilia still remain enigmatic for most ciliary GPCRs, the following paragraphs will give an overview of the various GPCRs identified in primary cilia of different cell types and their proposed downstream signaling pathways and cellular functions (Fig. 2).

3. GPCRs and primary cilia

3.1. GPCRs in primary cilia of the neural tube

Embryonic development in vertebrates strictly depends on primary cilia. Cilia are required to detect and interpret Hh morphogens to adapt cellular gene expression and, thereby, determine cell fates in the embryonic neural tube (Ericson, Briscoe, Rashbass, van Heyningen, & Jessell, 1997). Virtually all components involved in Hh signaling are present in primary cilia, including the Hh receptor Patched as well as the GLI transcription factors that regulate target gene expression. How morphogen binding to Patched ultimately results in a concentration-dependent modulation of GLI proteins is a central question in Hh signaling research and sheds light onto the general mechanisms of cilia signaling (Gigante & Caspary, 2020). Hh signaling in the cilium employs two GPCRs with opposing functions. On the one hand,

the oncogenic GPCR Smoothened (SMO) is a central activator of Hh signaling, often mutated in Hh-driven cancers (Pak & Segal, 2016). On the other hand, GPR161 is a constitutively active, orphan GPCR that functions as a negative regulator of the pathway. In unstimulated cells, SMO is absent from cilia, while ciliary GPR161 activates cilia-resident ACs by coupling to $G\alpha_s$ to increase ciliary cAMP. Upon pathway activation, GPR161 is removed from cilia and SMO accumulates, which is supposed to lower ciliary cAMP levels by SMO coupling to $G\alpha_{i/o}$ and inhibition of ciliary ACs. Hence, regulating the localization of these antagonistic GPCRs in cilia appears as a central mechanism to modulate cell fate determination by Hh morphogens during embryonic development (Gigante & Caspary, 2020; Nachury & Mick, 2019).

3.2. GPCRs in primary cilia of neurons in the brain

The major route for neuronal communication is via synapses. Yet, the majority, if not all neurons in the central nervous system possess a primary cilium (Bishop, Berbari, Lewis, & Mykytyn, 2007; Händel et al., 1999), which enables them to detect additional cues from the extracellular environment. Primary cilia dysfunction in the brain, as observed in a number of ciliopathies, results in central nervous system dysfunction, such as mental retardation or hyperphagia (Hildebrandt et al., 2011). A number of GPCRs have been shown to be enriched in neuronal cilia of the brain. Among the first to be identified was the somatostatin receptor 3 (SSTR3) (Händel et al., 1999; Schulz, Handel, Schreff, Schmidt, & Hollt, 2000). SSTR3 specifically localizes to neuronal primary cilia in the cerebellum, amygdala, hypothalamus, and hippocampus. Somatostatin is a common peptide neurotransmitter that affects a number of physiological processes, such as motor activity, sleep, as well as sensory and cognitive processes (Martel, Dutar, Epelbaum, & Viollet, 2012). In turn, mice lacking SSTR3 in the hippocampus display deficits in learning and memory (Einstein et al., 2010). SSTR3 is coupled to $G\alpha_i$ (Fig. 2) and, thereby, to cAMP signaling. A prominent AC isoform in neuronal primary cilia is AC3, which is widely used as a marker for neuronal primary cilia (Bishop et al., 2007). Mice lacking AC3 in the hippocampus also display deficits in learning and memory (Wang et al., 2009). Binding of somatostatin to SSTR3 evokes a decrease in ciliary cAMP levels through inhibiting AC3 activity via $G\alpha_i$ activation. Consistently, in NIH-3T3 fibroblasts, stimulation of ectopically expressed SSTR3-GFP reduced ciliary cAMP levels after stimulation with 100 nM somatostatin (Jiang, Falcone, Curci, & Hofer, 2019). However, experimental evidence for a similar function of endogenous SSTR3 in neuronal primary cilia is still lacking. Furthermore, how a change in ciliary cAMP levels elicits a downstream cellular response in neurons remains unknown.

The $G\alpha_i$-coupled melanin-concentrating hormone receptor 1 (MCHR1) forms heteromers with SSTR3 in neuronal primary cilia (Green, Gu, & Mykytyn, 2012). Similar to SSTR3, the ciliary localization of MCHR1 is BBSome-dependent (Berbari, Lewis, Bishop, Askwith, & Mykytyn, 2008). MCHR1 stimulation has been shown to induce cilia shortening through a $G\alpha_{i/o}$-dependent AKT phosphorylation signaling pathway that also involves actin polymerization (Hamamoto et al., 2016; Tomoshige et al., 2017).

Another GPCR identified in neuronal primary cilia in the brain is the serotonin receptor 6 (5-HT_6 receptor) (Brailov et al., 2000; Hamon et al., 1999). The 5-HT_6 receptor is $G\alpha_s$-coupled (see Fig. 2), predominantly expressed in the striatum, and has been implicated in various cognitive processes (Brodsky et al., 2017). Manipulating 5-HT_6 receptor activity alters primary cilia morphology, such as cilia length in the striatum (Brodsky et al., 2017). In an Alzheimer's disease mouse model, neuronal primary cilia in the hippocampus were elongated, but treatment with 5-HT_6 reduced ciliary and axonal length (Hu, Wang, & Zhang, 2017). Furthermore, altering 5-HT_6 receptor activity also modified axon initial segment morphology (Hu et al., 2017; Wang, Hu, Sun, & Zhang, 2019). Thus, 5-HT_6 receptors seem to control neuronal morphology and function. Similar to the 5-HT_6 receptor, the dopamine receptor D1 (DRD1) is another $G\alpha_s$-coupled GPCR that localizes to neuronal primary cilia with unknown physiological function (Domire et al., 2011; Marley & von Zastrow, 2010). Targeting of DRD1 to cilia has been studied in greater detail: D1R is delivered to cilia from the extra-ciliary plasma membrane via its cytoplasmic C-terminal tail by an unresolved mechanism involving Rab23, the IFT-B complex, and the kinesin KIF17 (Leaf & Von Zastrow, 2015). Furthermore, knocking out *Bbs7* in mice results in accumulation of DRD1 in neuronal primary cilia, indicating that the BBSome also controls the ciliary localization of DRD1 (Zhang et al., 2013).

Neuropeptide Y (NPY) is a prominent neuropeptide regulating food intake and energy consumption (Beck, 2006), whose function has been associated with primary cilia. Interestingly, two $G\alpha_i$-coupled NPY receptors, NPY2R and NPY5R, localize to primary cilia (Guo et al., 2019a; Loktev & Jackson, 2013). *Bbs1* knockout mice that model Bardet-Biedl Syndrome, lack NPY2R in primary cilia of hypothalamic neurons and become obese. Knockout mice fail to respond to NPY2R receptor stimulation with a reduction in food intake (Loktev & Jackson, 2013). Another GPCR associated with feeding behavior is the prolactin-releasing hormone receptor (Omori et al., 2015). It is localized to neuronal primary cilia on the surface of the third ventricle, close to the hypothalamic periventricular nucleus (Omori et al., 2015). However, its precise molecular and physiological function remains to be determined. Apart from the NPY receptors, the melanocortin receptors, in particular MC4R, seem to be important for primary cilia function in the hypothalamus (Siljee et al., 2018). Mutations in *MC4R* account for 3–5% of all severe obesity cases in humans (Vaisse et al., 2000). Strikingly, obesity-associated mutations in *MC4R* impair the ciliary localization of the receptor (Siljee et al., 2018), underlining the role of primary cilia in controlling body weight.

Most ciliary GPCRs that have been associated with the regulation of energy homeostasis, couple to cAMP signaling. The main enzyme synthesizing cAMP in neuronal primary cilia is AC3 (Bishop et al., 2007). Strikingly, mutations in *ADCY3*, the gene encoding AC3, cause monogenic severe obesity (Saeed et al., 2018), and mouse models lacking AC3 become obese (Cao et al., 2016). These findings highlight the importance of cAMP signaling in controlling neuronal and in particular neuronal primary cilia function in the brain.

In contrast, the ciliary functions of a number of additional GPCRs that have been identified in neuronal primary cilia are not known and await further exploration (see Fig. 2). Among them is the $G\alpha_q$-coupled kisspeptin receptor KISS1R, whose ciliary function has been proposed to control gonadotropin releasing hormone-dependent neuronal activity (Koemeter-Cox et al., 2014). The widely distributed β-adrenergic receptor β2AR (Yao et al., 2016) has been reported to localize to primary cilia in hippocampal neurons, where it interacts with PKD2L1, but its function in cilia remains enigmatic. GPR19 (Badgandi, Hwang, Shimada, Loriot, & Mukhopadhyay, 2017), GPR83, the pyroglutamylated RFamide peptide receptor (QERFPR/GPR103), Galanin receptor 3 (GALR3) (Loktev & Jackson, 2013), and GPR88 (Marley, Choy, & von Zastrow, 2013) have been robustly detected in primary cilia in the brain (Omori et al., 2015), but their function has not been elucidated. Based on a sequence homology search for known ciliary targeting signals and expression profiling, additional GPCRs are likely to be localized to primary cilia of the central nervous system, such as the receptors for Neuromedin U receptor (NMUR1), neuropeptide FF (NPFFR1), and the short isoform of the dopamine receptor D2 (DRD2S) (Omori et al., 2015), as well as GALR2 (Loktev & Jackson, 2013) and the purinergic receptor P2RY1 (Badgandi et al., 2017). So far, their ciliary localization has only been observed in heterologous systems, and their localization to neuronal cilia of the brain and the central nervous system awaits final proof.

3.3. GPCRs in primary cilia of the kidney

One of the first symptoms that has been linked to primary cilia dysfunction is the development of cystic kidneys (Pazour et al., 2000). Primary cilia on renal epithelial cells are supposed to function as mechanosensors that sense fluid flow (Verschuren et al., 2020). How they sense flow has been a matter of debate, and the underlying mechanisms still remain elusive (Abdul-Majeed & Nauli, 2011; Nag & Resnick, 2017; Raghavan & Weisz, 2016). Regardless, primary cilia on renal epithelial cells contain GPCRs to sense environmental cues.

The type 2 vasopression receptor (V2R) may not only be localized in the basolateral membrane of renal epithelia cells but has also been detected in primary cilia (Sherpa et al., 2019). V2R regulates sodium and water resorption in the nephron by *i.* controlling aquaporin-2 expression and trafficking to the apical cell membrane of collecting duct principal cells, *ii.* by activating the urea transporters UT-A1 and UT-A3 in the terminal part of the inner medullary collecting duct, *iii.* by stimulating epithelial sodium channel transport in cortical and outer medullary collecting ducts, and *iv.* by increasing the expression and activity of the sodium potassium chloride cotransporter in the thick ascending limb of Henle's loop (Bankir & Bichet, 2012; Juul, Bichet, Nielsen, & Norgaard, 2014; Sussman, Wang, Chebib, & Torres, 2020). Whether one of these V2R-mediated cellular functions depends on cilia, remains to be investigated. V2R is coupled to $G\alpha_s$ and has been suggested to be localized to primary cilia of renal epithelial cells (Sherpa et al., 2019) that also contain AC5 and AC6 (Mick et al., 2015; Raychowdhury et al., 2009). Activation of heterologously expressed V2R by arginin vasopressin (AVP) in inner medullar collecting duct (IMCD-3) cells stimulated an increase in both, cAMP levels in the cilium and cell body (Jiang et al., 2019). Similarly, prostaglandins stimulated the activities of the ciliary localized, $G\alpha_s$-coupled GPCR prostaglandin E4 (EP4) receptor (Jin et al., 2014a), and also increased cAMP levels in the cilium and cell body when EP4 was heterologously expressed (Jiang et al., 2019). However, the activity of the endogenously, but not heterologously expressed GPCRs needs to be investigated to decipher whether activation of a $G\alpha_s$-coupled ciliary GPCR results in a cAMP increase in the cilium or in the cytoplasm.

Stimulation with prostaglandins, i.e., PGE2, induces cyst formation via EP4 in IMCD-3 cells (Elberg, Turman, Pullen, & Elberg, 2012) and via EP2 in human renal epithelial cells, which requires cAMP production (Elberg et al., 2007). Yet, the molecular mechanism as well as the contribution of signaling in the cilium versus cell body are not well understood. Interestingly, cAMP levels are reportedly increased in a number of polycystic kidney disease (PKD) models (Torres & Harris, 2014), underlining the importance of cAMP-dependent signaling in cyst formation. The precise contribution of ciliary cAMP signaling to disease pathology has to be unraveled in futures studies.

Activation of dopamine receptor D5 (DRD5) reduces systemic blood pressure by increasing renal vasodilation and enhancing natriuresis in the kidney. DRD5 is coupled to $G\alpha_s$ and localizes to primary cilia of proximal renal epithelial, but also vascular endothelial cells (Abdul-Majeed & Nauli, 2011; Upadhyay et al., 2014). Activation of DRD5 with fenoldopam increased ciliary Ca^{2+} levels (Atkinson et al., 2015; Jin et al., 2014b) and ciliary length (Upadhyay et al., 2014), which in turn has been proposed to promote the ciliary response to mechanical stimulation by fluid flow (Atkinson et al., 2015). The increase in ciliary Ca^{2+} levels has been attributed to $G_{\beta\gamma}$-dependent opening of L-type Ca^{2+} channels (Atkinson et al., 2015). However, how a ciliary DRD5 signal is processed to evoke a downstream cell type-specific response remains unknown.

One important molecular player in primary cilia of renal epithelial cells is PC-1, a multi-spanning membrane protein frequently mutated in autosomal dominant polycystic kidney disease (ADPKD) (Bergmann, 2017). PC-1 consists of 11 transmembrane domains and contains a unique feature of adhesion GPCRs, the GPCR autoproteolysis inducing (GAIN) domain. The GAIN domain catalyzes the hydrolysis at a GPCR proteolysis site (GPS), which is located N-terminally of the first transmembrane domain. Mutations in the GPS site result in cyst formation (Qian et al., 2002). PC-1 forms a heteromeric complex with the transient receptor potential (TRP)-like ion channel PC-2 via the N-terminal domain of PC-1, which is cleaved off at the GPS and functions as a soluble ligand that activates the channel complex (Ha et al., 2020). Different G proteins shown to interact with PC-1 are supposed to engage in a number of different signaling pathways (Hama & Park, 2016). Thus, the molecular mechanisms underlying PC-1 function in primary cilia of renal epithelial cells still need to be elucidated.

3.4. GPCRs in primary cilia of the white adipose tissue

The white adipose tissue (WAT) is a highly complex and plastic organ that can undergo extensive remodeling in response to changes in the environment, i.e., excessive food intake. The major function of WAT is to maintain systemic energy balance through the storage of free fatty acids and the secretion of adipokines, which not only communicate locally, but also systemically with other organs to regulate food intake, energy expenditure, and a myriad of metabolic processes (Rosen & Spiegelman, 2014). WAT can expand by either increasing the number of adipocytes (hyperplasia) or by storing more fat in large lipid droplets in the existing pool of adipocytes (hypertrophy). The generation of adipocytes, so-called adipogenesis, can be regulated by primary cilia function. Loss of primary cilia in adipocyte precursor cells, which - in contrast to mature adipocytes - possess a cilium, diminishes adipogenesis (Hilgendorf et al., 2019). Recently, the first GPCR in primary cilia of adipocyte precursors, the free fatty acid receptor FFAR4/GPR120, has been identified (Hilgendorf et al., 2019) (Fig. 2). FFAR4 is activated by long-chain free fatty acids and, specifically, by omega-3 fatty acids (Hirasawa et al., 2005; Oh et al., 2010), which promotes adipogenesis via an increase in ciliary cAMP levels. The rise in ciliary cAMP, in turn, activates EPAC signaling, initiating chromatin remodeling, and changing adipocyte gene expression (Hilgendorf et al., 2019). As WAT responds to a number of different stimuli, future research will be required to reveal whether these stimuli are sensed by other GPCRs in the primary cilium to control WAT remodeling under physiological and pathological conditions.

3.5. GPCRs in primary cilia of cholangiocytes

GPCRs known to localize to primary cilia of cholangiocytes for over a decade are the bile acid receptor 1 (TGR5) (Keitel, Ullmer, & Haussinger, 2010) and P2RY12 (Masyuk et al., 2006). As of now, their precise function in primary cilia of cells in the liver remains ill-defined. So far, ciliary localized TGR5 has been shown to lower cellular cAMP levels in response to bile acids in a cholangiocytes cell model (Masyuk et al., 2013).

4. Calcium and cAMP signaling

4.1. Ciliary calcium channels

Ca^{2+} has been proposed to play a central role in primary cilia signaling, as defects in ciliary calcium channels lead to polycystic kidney disease. Mutations in *PKD1* or *PKD2,* which encode PC-1 and PC-2, respectively, are the most common cause of ADPKD (Bergmann, 2017). While the precise functions of PC-1 and the PC-1/PC-2 complex in the pathogenesis of ADPKD are only poorly understood, PC-2 is a cation channel of the transient receptor potential (TRP) family of ion channels. Due to the proposed function of kidney primary cilia in sensing fluid flow, cilia are thought to fulfill a mechanosensory function (Nauli et al., 2003). However, how and where PC-2 channels are activated is a matter of debate. Studies from many different labs using fluorescence-based calcium sensors in primary cilia could not ultimately resolve whether changes in fluorescence in response to fluid flow are due to motion artifacts of deflected cilia, and whether the

observed changes in Ca^{2+} originate in the cilium or the cytoplasm, as there is no Ca^{2+} barrier between the cytoplasm and primary cilia (Delling, DeCaen, Doerner, Febvay, & Clapham, 2013). Interestingly, the Ca^{2+} conductance in cilia of kidney epithelia cells was independent of PC-2, but dependent on the closely related PKD1-L1 and PKD2-L1 (DeCaen, Delling, Vien, & Clapham, 2013; Delling et al., 2013). While this expands the portfolio of tissue-specific ciliary calcium channels, it also questions the role of PC-2-mediated ciliary Ca^{2+} alterations in the pathogenesis of PKD. Furthermore, the significance of PKD1-L1 and PKD2-L1 in the development of cysts also remains unknown.

Apart from the TRPP family members, PC-1 and PC-2, TRPV4 has been identified in primary cilia and TRPC1 at the ciliary base of kidney epithelial cells (Lee et al., 2015; Raychowdhury et al., 2005). Consistently, so far, only TRPV4 but not TRPC1 could be detected by proteomic analyses of renal epithelial cilia (Ishikawa, Thompson, Yates 3rd, & Marshall, 2012; Kohli et al., 2017; Mick et al., 2015). Nonetheless, TRPC1 and TRPV4 have both been shown to form heterodimers with PC-2, generating distinct biophysical and pharmacological properties. A report on TRPC1 being a stretch-activated calcium channel (Maroto et al., 2005) is consistent with a mechanosensory role that may initiate ciliary calcium signaling (Bai et al., 2008). Alternatively, the large extracellular domain of PC-1 contains numerous cell adhesion domains that could regulate ion channel function by interaction of PC-1 with other proteins. Moreover, there is increasing evidence that Ca^{2+} signals in cilia can be chemically induced, e.g., via activation of cilia-localized DRD5 in endothelial cells and fibroblasts, or in response to Hh signals (DeCaen et al., 2013; Jin, Mohieldin, et al., 2014b).

Apart from the TRP family of calcium channels, the presence of other Ca^{2+} channels in primary cilia has not been verified by independent measures, e.g., mass-spectrometric analysis.

4.2. Adenylyl cyclases

Cyclic AMP has been the first second messenger described (Rall & Sutherland, 1958), and has been extensively studied since then. Intracellular cAMP levels are mainly controlled by ACs, which synthesize cAMP, or by phosphodiesterases, which hydrolyze cAMP. Thus, these two enzymatic activities are the main pharmacological targets to change cAMP levels. The most widely used drug to increase cAMP levels is forskolin or its water-soluble analog NKH477, which both target transmembrane ACs (Fig. 3). There are nine different tmAC isoforms in mammals. AC3 is most widely localized to primary cilia and is therefore used as a ciliary marker for certain cell types (Bishop et al., 2007; Raychowdhury et al., 2009). Other isoforms have been identified in primary cilia of different cell types, e.g., AC5 and 6 in renal epithelia cells (Masyuk et al., 2006), and AC1, 3, 5 and 8 have been identified in primary cilia of osteoblasts (Shi et al., 2017). Forskolin has been widely used to study ciliary cAMP signaling, despite the fact that pharmacological stimulation of tmAC activity acts on all isoforms, independent of their localization in the plasma or ciliary membranes.

Besides the tmACs, there is one soluble AC, which is mainly activated by bicarbonate (Chen et al., 2000). The soluble AC plays a prominent role in sperm flagella, where it is the principal source for cAMP and, therefore, essential for sperm motility (Esposito, Jaiswal, Xie, Krajnc-Franken, & Robben, 2004; Hess, Jones, Marquez, Chen, & Ord, 2005; Vacquier, Loza-Huerta, Garcia-Rincon, Darszon, & Beltran, 2014; Xie, Garcia, Carlson, Schuh, & Babcock, 2006). Interestingly, sAC has been reported to localize to primary cilia, in particular in osteoblasts (Shi et al., 2017). However, functional studies, targeting sAC by pharmacology in primary cilia, are missing.

4.3. Phosphodiesterases

Phosphodiesterases control ciliary signaling and function not only by controlling cAMP levels but also by regulating the targeting of proteins to the cilium. A prime example for the latter is the phosphodiesterase 6D (PDE6D), which binds to prenyl groups of cilia-targeted membrane proteins (Humbert et al., 2012). A central target of PDE6D is the inositol polyphosphate-5-phosphatase E (INPP5E), which hydrolyzes the 5-phosphate of PtdIns(3,4,5)P3 and PtdIns(4,5)P2. INPP5E is prenylated and interacts with PDE6D after extraction from a donor membrane (Humbert et al., 2012). The PDE6D/INPP5E complex is transported to the basal body for ciliary targeting. In line with this finding, the retinitis pigmentosa GTPase regulator (RPGR), whose loss causes the most severe form of retinal ciliopathy, the X-linked retinitis pigmentosa (Meindl et al., 1996), interacts with cargo-laden PDE6D to expose a binding site for the small GTPases ARL2/ARL3 (Wätzlich et al., 2013). ARL3 is localized to primary cilia, where it has been proposed to release lipidated cargos from PDE6D (Gotthardt et al., 2015).

The hydrolysis of cAMP by PDEs, in particular the activity of PDE4 isoforms, has been implicated in the development of autosomal polycystic kidney disease (ADPKD) (Omar et al., 2019). In fact, chronically high cAMP levels have been shown to drive cyst formation (Ong, Devuyst, Knebelmann, Walz, & Diseases, E.-E.W.G.f.I.K., 2015; Wallace,

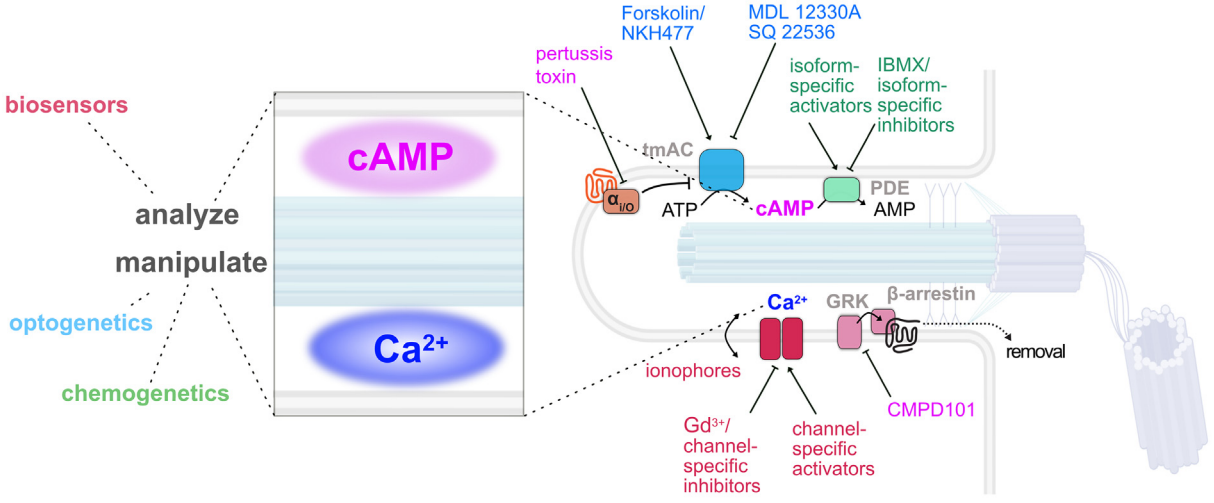

Fig. 3. Manipulation and analysis of GPCR signaling. Pharmacological manipulation applied to ciliary GPCR signaling cascades has been indicated. GPCR activity in cilia can also be modulated by blocking its removal. Furthermore, the downstream second messengers, i.e., cAMP and Ca^{2+}, can be analyzed using genetically-encoded biosensors and manipulated using optogenetics or chemogenetics. Figure has been generated using *biorender*.

2011). Activating specific PDE4 isoforms (long isoforms) reduces cAMP levels and inhibits cyst formation (Omar et al., 2019) (Fig. 3). While PDE6D is the only PDE significantly enriched in proteomic studies of kidney primary cilia (May et al., 2021), it is likely that other PDEs (present both inside and outside cilia) contribute to the hydrolysis of cAMP in cilia.

4.4. The role of Ca^{2+} and cAMP in cilia length control

Manipulation of Ca^{2+} as well as cAMP concentrations have both been shown to modulate ciliary length. The length of the primary cilium has been proposed to be important for its sensory functions, i.e., mechanotransduction (Flaherty, Feng, Peng, Young, & Resnick, 2020). In fact, different disease symptoms, such as obesity or polycystic kidneys have been associated with alterations in ciliary length (Besschetnova et al., 2010; Ritter et al., 2018). Tubulin supply determines axoneme growth or shrinkage and is regulated by IFT (Avasthi & Marshall, 2012). IFT velocities have been reported to be modulated by cAMP-dependent IFT complex phosphorylation and also by prostaglandin-signaling via the ciliary GPCR EP4 (Jin, Ni, et al., 2014a). Moreover, pharmacological manipulation of Ca^{2+} and cAMP concentrations of whole cells as well as optogenetic manipulation of ciliary cAMP levels directly impact cilium length by altering IFT (Besschetnova et al., 2010; Hansen et al., 2020). In addition to cilia length regulation by axoneme dynamics, overexpression of ciliary membrane proteins, such as GPCRs or fusion proteins targeted to cilia, increase cilia length (Hansen et al., 2020; Pal et al., 2016). Hence, the expression levels of ectopically expressed proteins that are utilized to study primary cilia should be carefully assessed to avoid unwanted alteration of the cilia functions under investigation.

5. Pharmacological targeting of ciliary signaling

GPCRs are the largest group of therapeutic drug targets (Sriram & Insel, 2018). The ciliary GPCR, for which most drugs have been developed, is the class F GPCR and oncogene SMO. Moreover, SMO mutations have been associated with a variety of Hh-driven cancers, including the most common type of skin cancer, basal cell carcinoma, and frequent childhood brain cancers, such as medulloblastoma (Skoda et al., 2018). Hence, the identification of specific SMO inhibitors, such as the naturally occurring teratogen alkaloid of the corn lily, cyclopamine (Chen, 2016), has led to the development of therapeutics to fight associated cancers. In fact, the Hh pathway inhibitor Vismodegib was the first SMO inhibitor approved by the FDA for the treatment of basal cell carcinoma (Gould et al., 2014). Moreover, SMO agonists and antagonists are invaluable pharmaceutical reagents that help to unravel the molecular details of Hh signaling and the general principles in cilia signaling.

The pharmacological repertoire for other cilia-specific GPCRs is still limited. However, the therapeutical potential is tremendous, as the collection of known ciliary GPCRs is expanding (Anvarian et al., 2019; Hilgendorf et al., 2016; Mykytyn & Askwith, 2017). As discussed above, ligands that can be sensed by several GPCR isoforms, such as dopamine, neuropeptide Y, somatostatin, or serotonin receptors have cilia-specific receptor isoforms (see Fig. 2). This may allow pharmacological manipulation by receptor-specific drugs, as long as the receptors are exclusively localized to the cilium and other isoforms, which would also be targeted by the drug, are not localized elsewhere in the cell. More cilia-specific pharmaceuticals are emerging; e.g., agonists for the ciliary omega-3 fatty acid receptor FFAR4 promote adipogenesis by activating cAMP production in pre-adipocyte primary cilia (Hilgendorf et al., 2019). As described above, V2R has been reported to localize to renal epithelial cilia (Sherpa et al., 2019). Although the precise molecular mechanism underlying V2R function in renal cilia is unknown, the V2R receptor antagonist Tolvaptan is the first drug that has been approved for the treatment of PKD by the FDA. Vasopressin levels are elevated in PKD patients already at an early phase of the disease (Bankir & Bichet, 2012; Zittema et al., 2012). Thus, blocking V2R was shown to attenuate PKD development and progression (Gattone 2nd, Wang, Harris, & Torres, 2003; Torres et al., 2004), and it also impedes disease progression in ADPKD patients (Edwards et al., 2018). On a cellular level, Tolvaptan reduced vasopressin-induced cell proliferation, chloride secretion, and cyst growth (Reif et al., 2011).

Due to our detailed understanding of GPCR signal transduction and protein trafficking, there is additional pharmacology available to modulate GPCR activity by interfering with downstream signaling via G proteins or by interrupting GPCR trafficking. Hence, commonly used pharmaceuticals to block GPCR signaling on the level of G proteins also inhibit ciliary GPCR signaling. Pertussis toxin catalyzes ADP-ribosylation of $G\alpha_i$ proteins (Fig. 3), and it has been applied to block ciliary GPCR signaling of both SSTR3 and Hh signaling (Ye et al., 2018). Similarly, CMPD101, a GRK2 kinase inhibitor (Thal, Yeow, Schoenau, Huber, & Tesmer, 2011), has been shown to impair Hh signaling by altering SMO trafficking to primary cilia (Bosakova et al., 2020) (Fig. 3). The effect of CMPD101 on Hh signaling in fibroblasts is $G\alpha_s$-dependent, but independent of GPR161 (Pusapati et al., 2018), which has been shown to control ciliary cAMP levels during Hh signaling. These results argue for additional $G\alpha_s$-coupled GPCRs that regulate Hh signaling.

The pharmacological manipulation of calcium channels is subject to intensive investigation and a number of substances, such as Gd^{3+} or more specific agonists and antagonists are available. However, it should be noted that the localization of individual calcium channels is not restricted to primary cilia, such that even subclass-specific reagents have limitations to investigate cilium-specific functions.

6. Approaches to manipulate and image ciliary signaling in live cells

6.1. Imaging cAMP dynamics in primary cilia

Measuring changes in cAMP levels in living cells has been revolutionized by the development of genetically-encoded, fluorescent cAMP biosensors. Since the generation of the first cAMP biosensors, consisting of either protein kinase A subunits (Adams, Harootunian, Buechler, Taylor, & Tsien, 1991) or the cyclic nucleotide-binding domains of EPAC (Zaccolo et al., 2000), various cAMP biosensors have been published (Klausen, Kaiser, Stuven, Hansen, & Wachten, 2019; Willoughby & Cooper, 2008) (Fig. 3). Recent technical advances now allow the direct measurement of changes in ciliary cAMP levels using different cAMP biosensors (intensiometric, ratiometric, different cAMP-binding domains) (Hansen et al., 2020; Jiang et al., 2019; Marley et al., 2013; Moore et al., 2016). Utilizing these imaging approaches, it has been shown that the GPCR GPR88, when localized to primary cilia, regulates the DRD1-dependent cAMP response in cilia (Marley et al., 2013). Furthermore, cAMP biosensor imaging suggested that ciliary cAMP levels are 5-fold higher than in the cytoplasm due to basal activity of the Ca^{2+}-sensitive AC5 and AC6 isoforms in cilia (Moore et al., 2016). Stimulation of Hh signaling reduced ciliary cAMP levels, decreased PKA activity, and changed gene expression in mouse embryonic fibroblasts (Moore et al., 2016). These results have been challenged by others, demonstrating that ciliary cAMP levels are not higher than in the cytoplasm, and that ciliary GPCR activity is promoted by Hh signaling (Jiang et al., 2019). The reason for this observed discrepancy is not known. One explanation could be that the design of the biosensors differs in respect to cAMP affinity and ciliary targeting between studies.

6.2. Imaging Ca^{2+} dynamics in primary cilia

In contrast to cAMP, Ca^{2+} changes in the cell cannot only be measured using genetically encoded biosensors (Fig. 3), but also using Ca^{2+}-sensitive fluorescent dyes. Similar to pharmacological approaches, chemical dyes do not provide a spatial resolution that allows imaging of Ca^{2+} dynamics exclusively in the cilium. Furthermore, despite being

small, some of them, e.g. X-rod-1-AM, seem unable to enter the cilium and detect ciliary Ca^{2+} levels (Su et al., 2013).

The first genetically encoded biosensor in cilia was based on the GCaMP3 biosensor fused to a Ca^{2+}-insensitive mCherry moiety at the C terminus of SMO (Delling et al., 2013). Imaging of the Ca^{2+} dynamics in the cilium vs. cell body using this sensor revealed that cilia constitute a unique Ca^{2+} compartment that can be regulated by PKD1-L1/PKD2-L1 without substantially changing Ca^{2+} levels in the cell body. These findings were supported by electrophysiological recordings, measuring the properties of Ca^{2+} channels in primary cilia (DeCaen et al., 2013).

In parallel, a similar strategy, employing the Ca^{2+} sensor GECO1.0 fused to $5HT_6$ receptor, was used to investigate flow- and chemically-induced ciliary Ca^{2+} dynamics in fibroblasts and kidney epithelial cells (Su et al., 2013). The Ca^{2+}-responsive mechanosensory hypothesis postulates that primary cilia sense mechanical forces, e.g., flow, through opening of Ca^{2+}-permeable ion channels, i.e., PC-2 (Nauli et al., 2003). This hypothesis is based on different experimental set-ups that also include imaging approaches using a cilia-targeted GCaMP3 (Jin, Mohieldin, et al., 2014b). However, in a well-controlled study, a GECO1.2-based ratiometric, ciliary Ca^{2+} sensor, fused to the ciliary ARL13B protein, failed to detect mechanically induced changes in primary cilia Ca^{2+} levels (Delling et al., 2016). As the sensor readily detected Ca^{2+} dynamics in cochlear hair cell bundles, it was concluded that mechanosensation does not involve Ca^{2+} signaling in primary cilia in mice (Delling et al., 2016).

A similar experimental approach has been used in live zebrafish embryos in the left-right organizer, the so-called Kupffer's vesicle. Here, intraciliary Ca^{2+} oscillations were measured, which are required to establish left-right asymmetry (Yuan, Zhao, Brueckner, & Sun, 2015). In the mouse embryo, imaging of ciliary and cytoplasmic Ca^{2+} dynamics using GCaMP6 fused to $5\text{-}HT_6$, revealed Ca^{2+} transients in the crown cells at the node. These Ca^{2+} transients displayed a left-right asymmetry, which was lost in the absence of fluid flow or after knocking out *PKD2*. A detailed analysis suggested that only the ciliary Ca^{2+} transients seem to initiate left-right symmetry breaking in the mouse embryo (Mizuno et al., 2020).

In summary, the role of Ca^{2+} signaling in controlling primary cilia functions is not fully understood and many questions still remain. Tools that allow the manipulation and analysis of second messenger signaling in primary cilia in a spatio-temporally-controlled manner will pave the way to address these questions.

6.3. Optogenetics and chemogenetics to control ciliary cAMP and Ca^{2+} levels

One elegant way to specifically control Ca^{2+} signaling in primary cilia has been applied by a chemogenetic approach using designer receptors exclusively activated by designer drugs (DREADDs) (Guo et al., 2019b) (Fig. 3). The cilia-targeted DREADD hM3Dq, called cilia-hM3Dq, has also been used to demonstrate that ciliary GPCR signaling promotes interneuronal connectivity and inhibitory circuit formation (Guo et al., 2017). Moreover, optogenetics can in principle overcome the limitations of using pharmacology to study ciliary signaling by controlling cellular signaling in a spatial and temporal manner by light. Optogenetics was first employed to study sperm flagella (Jansen et al., 2015; Raju et al., 2019), but has recently also been applied to primary cilia (Guo, Otis, et al., 2019b; Hansen et al., 2020; Prosseda et al., 2020) (Fig. 3). Here, the photo-activated adenylyl cyclase bPAC has been used in primary cilia of different cell types to manipulate cAMP signaling. Photo-activation of bPAC in primary cilia of deep cerebellar neurons revealed a role of ciliary cAMP signaling in controlling axonal growth cone dynamics (Guo, Otis, et al., 2019b).

Besides cAMP and Ca^{2+}, phosphoinositides, in particular phosphoinositide-3 kinase (PI3K)/AKT signaling seems to be important in regulation axonal growth cone dynamics and, thereby, axonal tract development. The involvement of this pathway was demonstrated using a blue light-activated CIBN/CRY2 dimerization optogenetic system to selectively recruit PI3-kinase, phosphatidylinositol 5-phosphatase modules (5-Ptase module of INPP5E), and AKT to the ciliary membrane (Guo, Otis, et al., 2019b). Using a similar approach, it has been demonstrated that primary cilia, more specifically ciliary PI3K/AKT signaling, controls pressure in the eye (Prosseda et al., 2020). Analyzing ciliary signaling using chemo- or optogenetics requires targeting of the engineered proteins to the cilium. This is predominantly achieved by fusing a ciliary targeting sequence or even a whole ciliary protein to respective tools. However, direct fusion to a ciliary targeting sequence may result in loss-of-function of the fusion protein. To overcome this limitation, nanobody-based targeting of optogenetic tools to the primary cilium has been developed (Hansen et al., 2020). This approach revealed a complex interplay between ciliary and cell body cAMP signaling in controlling cilia length in mIMCD-3 cells (Hansen et al., 2020).

7. Summary and perspectives

In summary, tools that allow spatial and temporal manipulation of ciliary signaling have recently been applied to study the role of ciliary cAMP or Ca^{2+} signaling in controlling cellular functions. By driving these approaches forward, recent tool developments help to delineate the sequence of events underlying ciliary signaling and provide powerful means to reveal fundamentally new features of ciliary signaling and function.

Declaration of Competing Interest

The authors declare that they have no conflict of interest.

Acknowledgment

The project was supported by grants from the Deutsche Forschungsgemeinschaft (DFG): SPP1926: grant WA3382/2-1 (to DW), SPP1726: grant WA3382/3-1 (to DW), TRR83/SFB (to DW), FOR2743 (to DW), SFB1454 (to DW), under Germany's Excellence Strategy – EXC2151 – 390873048 (to DW), and SFB894/TP-A22 (to DUM).

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