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ORIGINAL PAPER

Characterization of Functional Primary Cilia in Human Induced Pluripotent Stem Cell-Derived Neurons

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Abstract

Recent advances in human induced pluripotent stem cells (hiPSCs) offer new possibilities for biomedical research and clinical applications. Neurons differentiated from hiPSCs may be promising tools to develop novel treatment methods for various neurological diseases. However, the detailed process underlying functional maturation of hiPSC-derived neurons remains poorly understood. Here, we analyze the developmental architecture of hiPSC-derived cortical neurons, iCell GlutaNeurons, focusing on the primary cilium, a single sensory organelle that protrudes from the surface of most growth-arrested vertebrate cells. To characterize the neuronal cilia, cells were cultured for various periods and evaluated immunohistochemically by co-staining with antibodies against ciliary markers Arl13b and MAP2. Primary cilia were detected in neurons within days, and their prevalence and length increased with increasing days in culture. Treatment with the mood stabilizer lithium led to primary cilia length elongation, while treatment with the orexigenic neuropeptide melanin-concentrating hormone caused cilia length shortening in iCell GlutaNeurons. The present findings suggest that iCell GlutaNeurons develop neuronal primary cilia together with the signaling machinery for regulation of cilia length. Our approach to the primary cilium as a cellular antenna can be useful for both assessment of neuronal maturation and validation of pharmaceutical agents in hiPSC-derived neurons.

Keywords Primary cilia · Human induced pluripotent stem cell-derived neuron · iCell GlutaNeuron · G protein-coupled receptor · Melanin-concentrating hormone receptor 1 · Lithium

Introduction

Rodent primary cell cultures have proven to be indispensable for clarifying disease mechanisms and providing insights into gene functions. Nevertheless, it is difficult to persistently translate new findings discovered in rodents into new medicines for humans. In particular, neurodegenerative diseases are hard to study because of the difficulties associated with human neurons. Advances in human induced pluripotent stem cell (iPSC) technology and subsequent advances in neuronal differentiation may partially overcome this problem [1]. At present, several international companies have commercialized high-purity hiPSC-derived neurons or neural progenitor cells. Recently, phenotypes of neurite outgrowth [2, 3], quantification of intraneuronal transport [4], mRNA expression and patch-clamp electrophysiological data of ionotropic glutamate receptors and γ-aminobutyric acid type A receptor [5–7] were reported using commercial hiPSC-derived

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neurons. However, neurobiological analyses focused on other cellular nodes remain poorly understood. Furthermore, a simplified and perceptive assay system using a set of biomarkers is necessary to create a faster model for pharmacological and toxicological assessments.

The primary cilium is a non-motile single cellular organelle that extends from the surface of most growth-arrested vertebrate cells [8, 9]. Cilia are composed of a microtubule backbone (axoneme) that extends from the basal body, a structure derived from the mother centriole. Primary cilia transduce sensory and neuroendocrine signals from the extracellular milieu and play critical roles in the development and homeostasis of many organs, including the neuronal system. Because only selected signaling molecules are allowed to enter the ciliary membrane, the primary cilium is considered a high-level neuronal signaling node in addition to the synapse [10]. Defects in the structure or function of cilia are linked to several ciliopathies, as well as the pathogenesis of various disorders including obesity, polycystic kidney disease, retinal degeneration, and neurological defects [11]. Indeed, disrupted cilia function can impair dendrite outgrowth and synaptic connectivity in neuronal cultures [12, 13]. Meanwhile, G-protein-coupled receptors (GPCRs), which respond to diverse signals and affect numerous cellular processes, are essential for neuronal primary cilia function [14]. To date, a limited set of neuronal cilia-specific GPCRs have been identified in the rodent brain, including somatostatin receptor subtype 3 (SSTR3), serotonin receptor subtype 6 (5-HT6), D1-type dopaminergic receptor, and melanin-concentrating hormone (MCH) receptor 1 (MCHR1) [15–18]. Therefore, primary cilia have been proposed as promising targets for therapeutic interventions, even in neuropsychiatric diseases [19].

Cilia are observed within days in cultured hippocampus, amygdala, nucleus accumbens, and dorsal striatum neurons derived from embryonic day 18 to newborn animals [15–17, 20]. Nevertheless, to the best of our knowledge, there are no studies on the existence and development of neuronal cilia in hiPSCs-derived neurons. Primary cilia in particular cell types have a proper length range at the appropriate time, suggesting the importance of maintaining the optimal cilia length in terminally differentiated neurons [21]. Therefore, as an initial step to explore potential utility of primary cilia, we have shown their presence with the function for cilia length control in hiPSC-derived glutamatergic (excitatory)-neurons by employing commercially available iCell GlutaNeurons along with rat hippocampal slice cultures and telomerase-immortalized human retinal pigment epithelial cell line (hRPE1 cells). Our findings, together with the known functions of primary cilia in vivo, open up the possibility for assessing the therapeutic potential of cilia modulators using various types of hiPSC-derived neurons.

Materials and Methods

Cell and Slice Cultures and Maintenance

We used iCell GlutaNeurons, ≥ 90% pure population of human glutamatergic-enriched cortical neurons according to Cellular Dynamics International (Madison, WI, USA) Product Datasheet. The cells were defrosted according to the protocol provided by the manufacturer. A total of 35×10^4 cells was plated on 12-mm round glass coverslips (Matsunami glass, Osaka, Japan) that had been cleaned with 1 M nitric acid and coated with 0.01% poly-L-lysine (Sigma-Aldrich, St. Louis, MO) in 24-well culture plates. The cells were incubated in BrainPhys Neuronal Medium (STEMCELL Technologies, Vancouver, BC, Canada) containing 2% iCell Neural Supplement B (STEMCELL Technologies), 2% SM1 Neuronal Supplement (STEMCELL Technologies), 1% iCell Nervous System Supplement (STEMCELL Technologies), 1 mM laminin (Sigma-Aldrich), and penicillin G sodium/streptomycin sulfate (PG/SM) (Complete BrainPhys Medium). After 1 day, the medium was changed to Complete BrainPhys Medium containing 50 ng/mL brain-derived neurotrophic factor (iGlu-GM). The medium was subsequently changed every other day. All cultures were maintained at 37 °C in an incubator with 5% CO_2 for 1–20 days in vitro (DIV). The cultured iCell GlutaNeurons were treated with chloride or MCH (Peptide Institute, Osaka, Japan) in iGlu-GM and fixed.

The hRPE1 cells (Catalog Number CRL-4000; ATCC, Manassas, VA) were seeded and grown as described previously [22, 23]. These cells were chosen for its formation of primary cilia under serum-starvation treatment when reaching confluence in culture. Indeed, this cell line is widely utilized for many studies regarding primary cilia assembly and disassembly. The serum-starved hRPE1 cells were 8 h-treated with LiCl, NaCl or KCl (Sigma-Aldrich; 10 and 20 mM) in serum-deprived medium and were subsequently fixed.

For hippocampal slices, Wistar rats (Charles River Japan, Yokohama, Japan) were maintained in a room under a 12 h light:12 h darkness cycle and controlled temperature (23 to 25 °C), with water and food available ad libitum. All experimental protocols were reviewed by Hiroshima University Animal Care Committee and met the Japanese Experimental Animal Research Association standards, as defined in the Guidelines for Animal Experiments (1987). Newborn rats were anesthetized and decapitated for slice culture preparation. We established a rat hippocampal slice culture method for clear detection of primary cilia and ciliary MCHR1 based on a previous study [24]. The brains of 8–12-day-old rats were rapidly removed, and the

hippocampi were dissected and cut into 200-µm sagittal slices using a McIlwain tissue chopper (The Mickle Laboratory Engineering, Surrey, UK). The slices were trimmed for tissue in ice-cold dissection buffer containing 2.5 mM KCl, 0.05 µM $CaCl_2$, 1.7 mM NaH_2PO_4, 11 mM glucose, 20 mM HEPES, 8 mM NaOH, 18 mM NaCl, 0.23 M sucrose, and PG/SM, and placed on 0.4-µm, 30-mm diameter semiporous Millicell Cell Culture inserts (PICM0RG50; Merck Millipore, Germany) in 35-mm petri dishes. Each dish contained 1 ml of starter medium comprising 50% MEM (GIBCO, Grand Island, NY, USA), 25% normal horse serum, 25% Hanks' Balanced Salt Solution, 36 mM glucose, and PG/SM. Six hippocampal slices were placed on each membrane, and maintained at 37 °C in a 5% CO_2 incubator. After 1 day, the medium was changed to Neurobasal Medium (21103-049; Gibco) containing 1.2% B27 Serum-Free Supplement (17504-044; Gibco), 25 mM GlutaMAX I (Gibco), and PG/SM (Slice-GM). One-third of the Slice-GM was changed every 72 h. On day 7 of culture, the slices were treated with chloride or MCH in Slice-GM and fixed.

Immunostaining

iCell GlutaNeurons were fixed with methanol for 10 min at room temperature, washed with Dulbecco's PBS (D-PBS), and blocked with 5% goat serum and 0.1% Triton X-100 in D-PBS (PBSGT) for 45 min at room temperature. The cells were then washed with D-PBS and incubated with primary antibodies in PBSGT overnight at 4 °C. The primary antibodies and their dilutions were: rabbit anti-human Arl13B (17711-1-AP; Proteintech, Chicago, IL, USA; 1:700); mouse anti-rat MAP2 (M4403; Sigma-Aldrich; 1:1000); and mouse anti-human FGFR1OP (B-1; sc-374340; Santa Cruz Biotechnology, Santa Cruz, CA; 1:200). For immunostaining of MCHR1 (goat anti-human MCHR1; C-17, sc-5534; Santa Cruz Biotechnology; 1:300) and Arl13B, 5% donkey serum and 0.1% Triton X-100 in D-PBS (PBSDT) were used for antibody dilution and blocking. Subsequently, the cells were washed with D-PBS and incubated with appropriate species-specific secondary antibodies, Alexa Fluor 488-conjugated goat anti-rabbit IgG (Thermo Fisher Scientific, Rockford, IL, USA; 1:300), Alexa Fluor 546-conjugated goat anti-mouse IgG (Thermo Fisher; 1:300), Alexa Fluor 488-conjugated donkey anti-rabbit IgG (Thermo Fisher Scientific; 1:300) and Alexa Fluor 546-conjugated donkey anti-goat IgG (Thermo Fisher Scientific; 1:300), in PBSGT or PBSDT for 90 min at room temperature. The cells were then washed and counterstained with 4′,6-diamidino-2-phenylindole (DAPI; 1 µg/mL) for 10 min at room temperature. Finally, the cells were washed and mounted on glass slides with VECTOR Shield (Vector Laboratories, Burlingame, CA).

Immunostaining of hRPE1 cells was carried out as described previously [22, 23]. The hRPE1 cells seeded on Lab-Tek plates were fixed in 3.7% paraformaldehyde-PBS solution for 15 min. After washing twice with PBS, the cells were permeabilized, transferred into a blocking solution (20% goat serum in PBS) for 30 min, and labeled with the mouse anti-acetylated tubulin (Ac-tub) primary antibody (T7451; Sigma-Aldrich; 1:2000) for 24 h at 4 °C. The bound antibodies were detected using secondary antibody (Alexa Fluor 488-conjugated goat anti-mouse IgG; Life Technologies Co., Carlsbad, CA; 1:300).

Immunohistochemical staining of hippocampal slices was performed in 6-well culture plates. The slices were fixed for 3 h at 4 °C in fresh 4% paraformaldehyde, washed with D-PBS, heated (70 °C for 20 min) in 10% Histo VT One (Nacalai Tesque, Kyoto, Japan) in D-PBS for antigen retrieval, and blocked with D-PBS containing 5% horse serum and 0.1% Triton X-100 for 2 h. The slices were then incubated with rabbit anti-rat adenylate cyclase 3 (AC3, RPCA-ACIII; Encor Biotechnology, Alachua, FL, USA; 1:5000) and goat anti-human MCHR1 (1:300) primary antibodies overnight at 4 °C [18]. The bound antibodies were detected by incubation with Alexa Fluor 488-conjugated donkey anti-rabbit IgG (1:300) or Alexa Fluor 546-conjugated donkey anti-goat IgG (1:300) secondary antibodies for 2 h at room temperature. The slices were counterstained with DAPI for 10 min and mounted with VECTOR Shield.

Microscopic Images and Analysis

High-magnification images were taken with an FV1000 confocal microscope equipped with a × 60 oil-immersion objective (Olympus, Tokyo, Japan). Ciliary marker, which was positive hair-like appendage longer than 1.5 µm (Ac-tub for hRPE1 cells, Arl13b for iCell GlutaNeurons, Ac-AC3 for hippocampal slices), was counted as primary cilium. Evaluating for cilia length change caused by MCH in hippocampal slices and iCell GlutaNeurons, the length of AC3- and MCHR1-double positive cilium and Arl13b- and MCHR1-double positive cilium was counted, respectively. The primary cilia lengths were measured under a BZ-9000 fluorescence microscope (Keyence, Osaka, Japan) using PhotoRuler Ver. 1.1 software (The Genus Inocybe, Hyogo, Japan). Data for at least 50 cilia per treatment were obtained from at least three independent experiments, and the values are presented as mean ± SEM. Differences between groups were examined for statistical significance with Student's *t* test.

Results

In this study, we used iCell GlutaNeurons, which were previously shown to express functional GluN2B-containing NMDA receptors [7]. Because the cells tended to aggregate and become easily detached at around 3 weeks of culture in our hands, all experiments were performed within Day 20 after plating (20 DIV). Under phase-contrast microscopy, iCell GlutaNeurons had spindle-shaped cell bodies with short processes at 2 DIV. To further characterize the neurons at different stages of culture, immunocytochemistry with an antibody against MAP2 (dendrite marker) was performed from 2 DIV to 20 DIV. The complexity or density of MAP2-positive processes increased with days in culture, especially from 8 DIV to 11 DIV (Fig. 1a, red). Neurons at 18 DIV generally had pyramidal-like cell bodies with long projections. This analysis of iCell GlutaNeurons for expression of a neuronal marker confirmed the high purity of the neurons in culture.

To explore the existence of neuronal primary cilia in iCell GlutaNeurons, the cells were double-stained with an anti-MAP2 antibody and an antibody for the ciliary membrane marker Arl13b (Fig. 1a, red and green, respectively). Arl13b, a small GTPase in the Arf/Arl family, is selectively localized in primary cilia and is necessary for their ability to function as a signaling hub. In fact, Arl13b is expressed in primary cilia in the interneurons of the developing cerebral cortex and cultured hippocampal neurons [20, 25]. In iCell GlutaNeurons at 2 DIV, Arl13b-positive puncta were detected in MAP2-positive cells, and their prevalence and length increased with increasing days in culture. In addition, the basal body marker FGFR1OP [26] was observed at the base of Arl13b-positive protrusions (Fig. 1b) and the percentage of Arl13b positive cilia/cells with FGFR1OP positive cilia/cells was 96.1% (Arl13b- and FGFR1OP-double positive cilia/Arl13b-positive cilia = 173/180). This indicates that primary cilia were present in iCell GlutaNeurons. The prevalence of ciliated cells was evaluated by counting the number of cells with immunoreactivity for Arl13b-positive cilia relative to the total number of MAP2-positive cells. As shown in Fig. 1c, the ratio of ciliated cells was only 4.4% at 2 DIV, but markedly increased to 12.4% at 4 DIV and 22.0% at 6 DIV, before gradually increasing to 28.1% at 11 DIV and 29.1% at 18 DIV. The mean cilia length at 2 DIV was short (2.7 µm), but cells with longer cilia subsequently appeared and the mean length reached 4–5 µm at 18 DIV (Fig. 1d, e). No obvious differences were detected between the ratios and cilia lengths at 18 DIV and 20 DIV (data not shown).

A few pharmacologically prescribed drugs can alter the dynamics of ciliary length. Therefore, we examined whether Arl13b-positive primary cilia in iCell GlutaNeurons had the potential to respond to lithium, a first-line antimanic mood stabilizer. It is well known that lithium elongates primary cilia length in many cell types, including serum-starved NIH3T3 cells, rodent striatal neurons, and human olfactory neuronal precursor cells [19, 27, 28]. In this study, treatment of serum-starved hRPE1 cells with 20 mM LiCl for 8 h increased the cilia length by 28.8% compared with untreated control cells (mean ± SEM: 4.62 ± 0.03 µm vs. 5.95 ± 0.04 µm, $P < 0.01$). On the other hand, stimulation of NaCl and KCl did not affect the ciliary length of hRPE1 cells. Our recent immunohistochemical analyses showed that AC3/MCHR1 double-positive cilia were selectively localized in the rat hippocampus [18]. AC3, enriched in neuronal primary cilia, catalyzes the formation of the signaling molecule cyclic AMP in response to GPCR signaling [8, 14]. Ciliary MCHR1 was also discretely observed in cultured slices derived from the rat hippocampus, and 10 mM LiCl treatment significantly increased the neuronal cilia length by 39.3% (mean ± SEM: 6.14 ± 0.10 µm vs. 8.55 ± 0.16 µm, $P < 0.001$) (Fig. 2a, c). Similarly, 20 mM LiCl treatment caused Arl13b-positive cilia elongation by 40.0% in MAP2-positive iCell GlutaNeurons at 11 DIV (mean ± SEM: 4.74 ± 0.13 µm vs. 6.63 ± 0.23 µm, $P < 0.001$) (Fig. 2b, d). Neither 20 mM NaCl nor 20 mM KCl elongated neuronal cilia length in cultured hippocampal slices and iCell GlutaNeurons (data not shown). These results with NaCl and KCl were consistent with another study using mouse fibroblast NIH3T3 cells and primary cultures of rat striatal neurons [16].

As well as pharmacologically prescribed drugs, the orexigenic neuropeptide MCH was shown to induce cilia shortening in rat MCHR1-expressing hRPE1 cells [22, 23]. We observed an equivalent biological effect of MCH in our rat hippocampal slice cultures. Specifically, the length of MCHR1/AC3 double-positive cilia was reduced by 33.0% after exposure to 1 nM MCH for 6 h (mean ± SEM: 5.97 ± 0.19 µm vs. 4.04 ± 0.16 µm, $P < 0.001$) (Fig. 3a, c). Regarding iCell GlutaNeurons, we initially examined the presence of MCHR1-positive neuronal cilia. Arl13b/MCHR1 double-positive signals were first detected at 10 DIV, and then gradually increased until 16 DIV, when the ratio of MCHR1-positive cilia to Arl13b-positive cilia reached 58.8% (total number of cilia counted: Arl13b- and MCHR1-double positive cilia/Arl13b-positive cilia = 30/51). Addition of 1 nM MCH for 4 h to iCell GlutaNeurons at 16 DIV resulted in 14.5% shortening of Arl13b/MCHR1-double positive cilia (mean ± SEM: 5.22 ± 0.16 µm vs. 4.49 ± 0.15 µm, $P < 0.01$) (Fig. 3b, d), while no effect was observed on the length of MCHR1-negative cilia. Overall, we concluded that Arl13b-positive primary cilia in iCell GlutaNeurons had signaling pathways that governed cilium length regulation by LiCl and MCH.

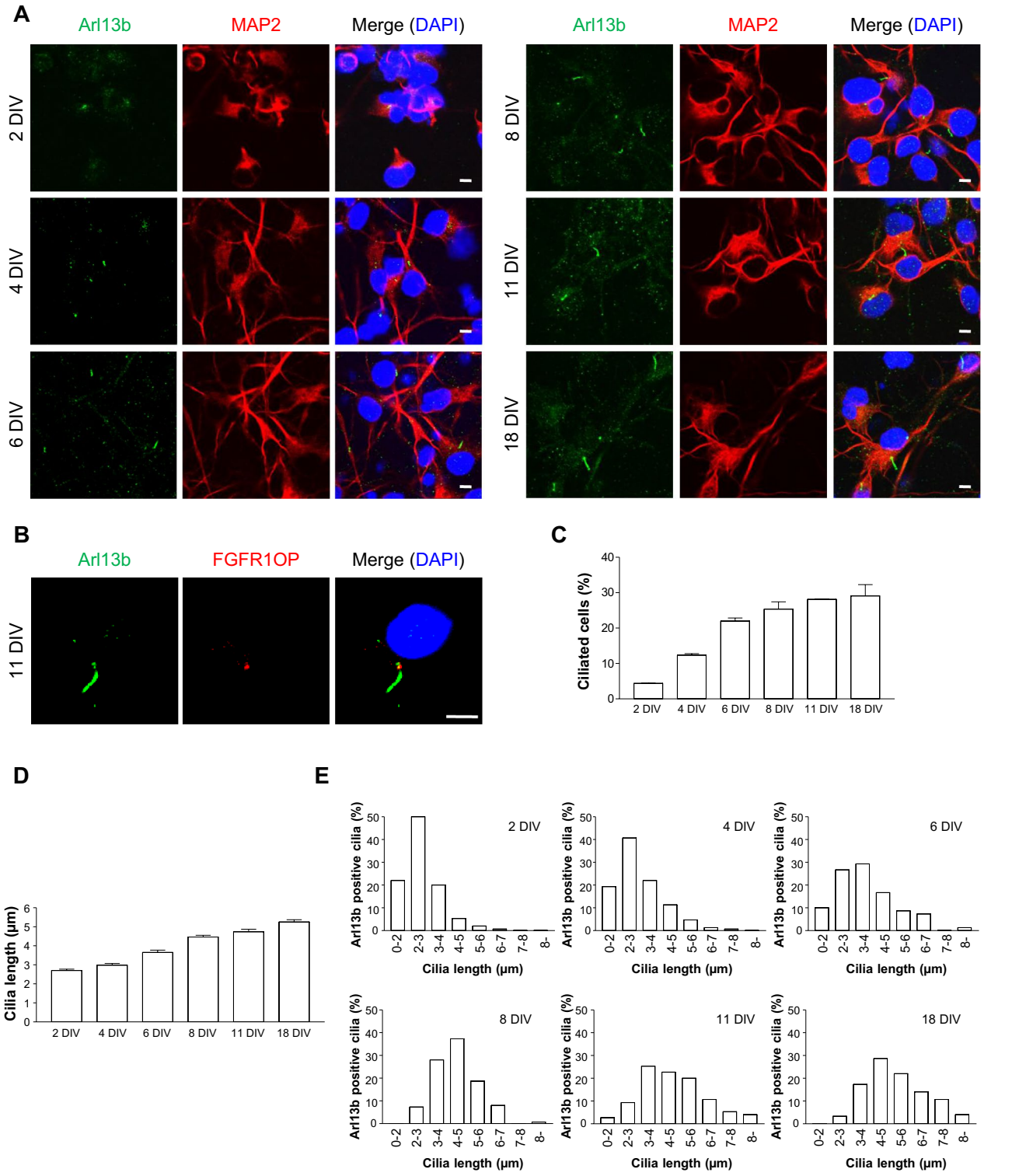

Fig. 1 Characterization of primary cilia in iCell GlutaNeurons. The influence of culture days in vitro (DIV) on the characteristics of primary cilia on iCell GlutaNeurons was examined. **a** Primary cilia and neurons were co-immunostained with antibodies against Arl13B (green) and MAP2 (red). Nuclei were labeled with DAPI (blue). **b** Primary cilia and basal bodies were co-immunolabeled with antibodies against Arl13B (green) and FGFR1OP (red) at 11 DIV. Scale bars, 5 μm. **c** Percentages of ciliated cells (Arl13B-positive) among neurons (MAP2-positive) calculated from at least 200 MAP2-positive cells per DIV group. **d** Mean lengths of Arl13B/MAP2 double-positive cilia measured in randomly selected fields, with data for at least 150 cilia per DIV group. **e** Histograms for the cilia length distributions per DIV group (Color figure online)

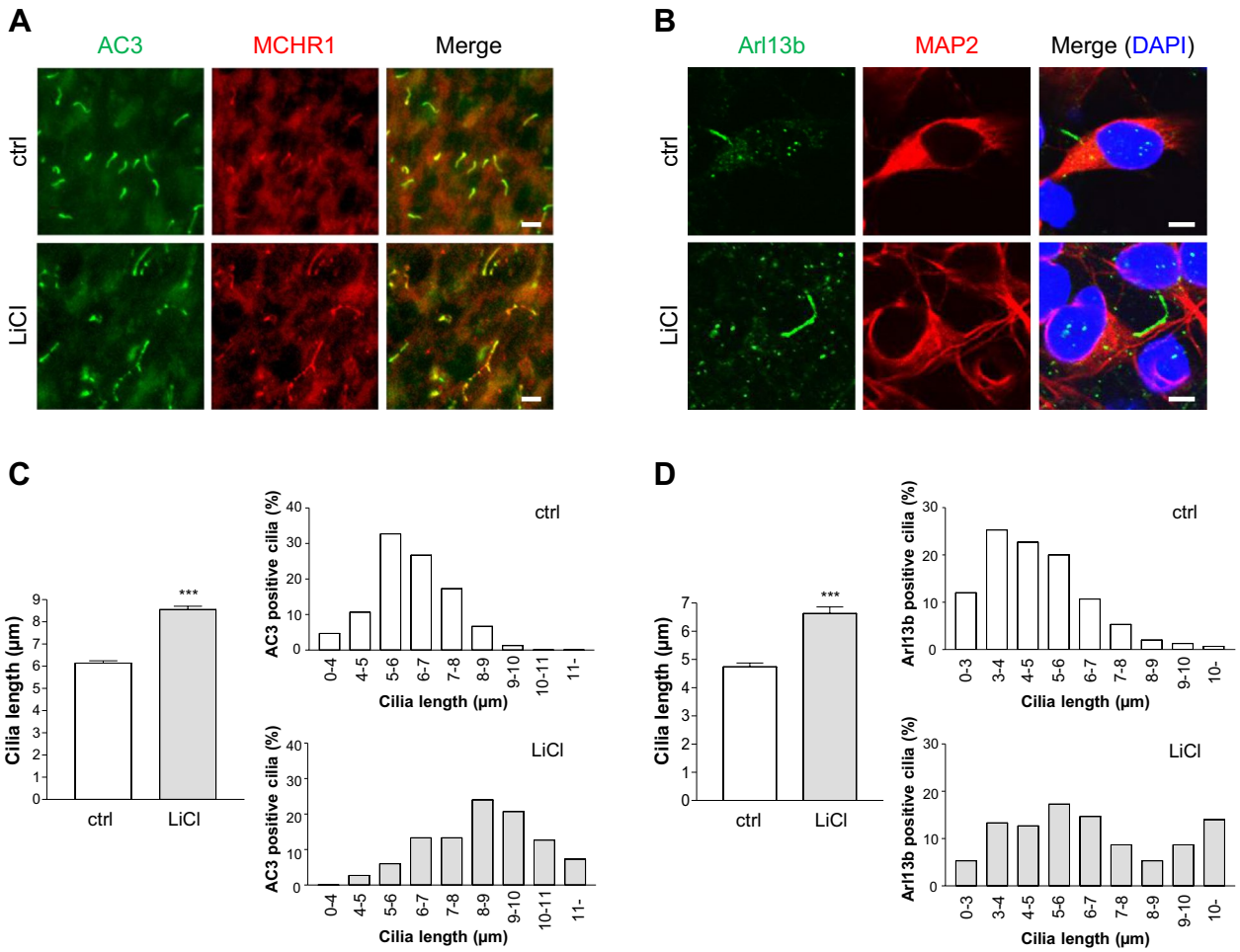

Fig. 2 Primary cilia elongation after treatment of cultured hippocampal slices and iCell GlutaNeurons with LiCl. **a** Rat hippocampal slice cultures were treated with 10 mM LiCl or untreated (ctrl) for 6 h. Primary cilia were co-immunolabeled with antibodies against AC3 (green) and MCHR1 (red). **b** iCell GlutaNeurons were treated with 20 mM LiCl or untreated (ctrl) for 6 h. Primary cilia and neurons were co-immunolabeled with antibodies against Arl13B (green) and MAP2 (red). Nuclei were labeled with DAPI (blue). Scale bars, 5 µm. **c**, **d** Lengths of AC3 single-positive (**c**) or Arl13B/MAP2 double-positive (**d**) cilia. The ciliary lengths were measured in randomly selected fields, with data for 150 cilia per group. Left graph: primary cilia were significantly longer in LiCl stimulated cultures than in control cultures for both hippocampal slices (**c**) and iCell GlutaNeurons (**d**). ***$P < 0.001$. Right graphs: histograms for the cilia length distributions in hippocampal slices (**c**) and iCell GlutaNeurons (**d**) (Color figure online)

Discussion

There is growing evidence for the physiological significance of the primary cilium as a cell antenna [8, 9]. A recent study on ciliopathy patient iPSC-derived cells showed that both the retinal pigment epithelium and lung epithelium required functional primary cilia for complete maturation [29]. In rodent neurons, the synapse is already well recognized as a cellular node, while the neuronal primary cilium is attracting attention as another conserved cellular structure for exploring the complex biological processes underpinning neural development and maturation. However, few studies have described the primary cilia on human iPSC-derived neurons. The present study has revealed for the first time the presence of Arl13b-positive neuronal primary cilia on commercially available iCell GlutaNeurons.

To date, neuronal primary cilia in the brain have been visualized by immunofluorescence staining using commercial antibodies that recognize Arl13b, AC3, and a limited number of GPCRs (SSTR3, 5-HT6, and MCHR1) [10, 14, 30]. For example, an anti-AC3 antibody was utilized for detection of neuronal primary cilia development in the mouse neocortex, revealing that cilia remained undifferentiated from late fetal to early postnatal stages, followed by more or less steady axonemal elongation of the cilia from postnatal days 7–8 [31]. In the present study, immunopositivity for AC3 was found in neuronal primary cilia in rat hippocampal slice cultures, similar to the mouse hippocampus

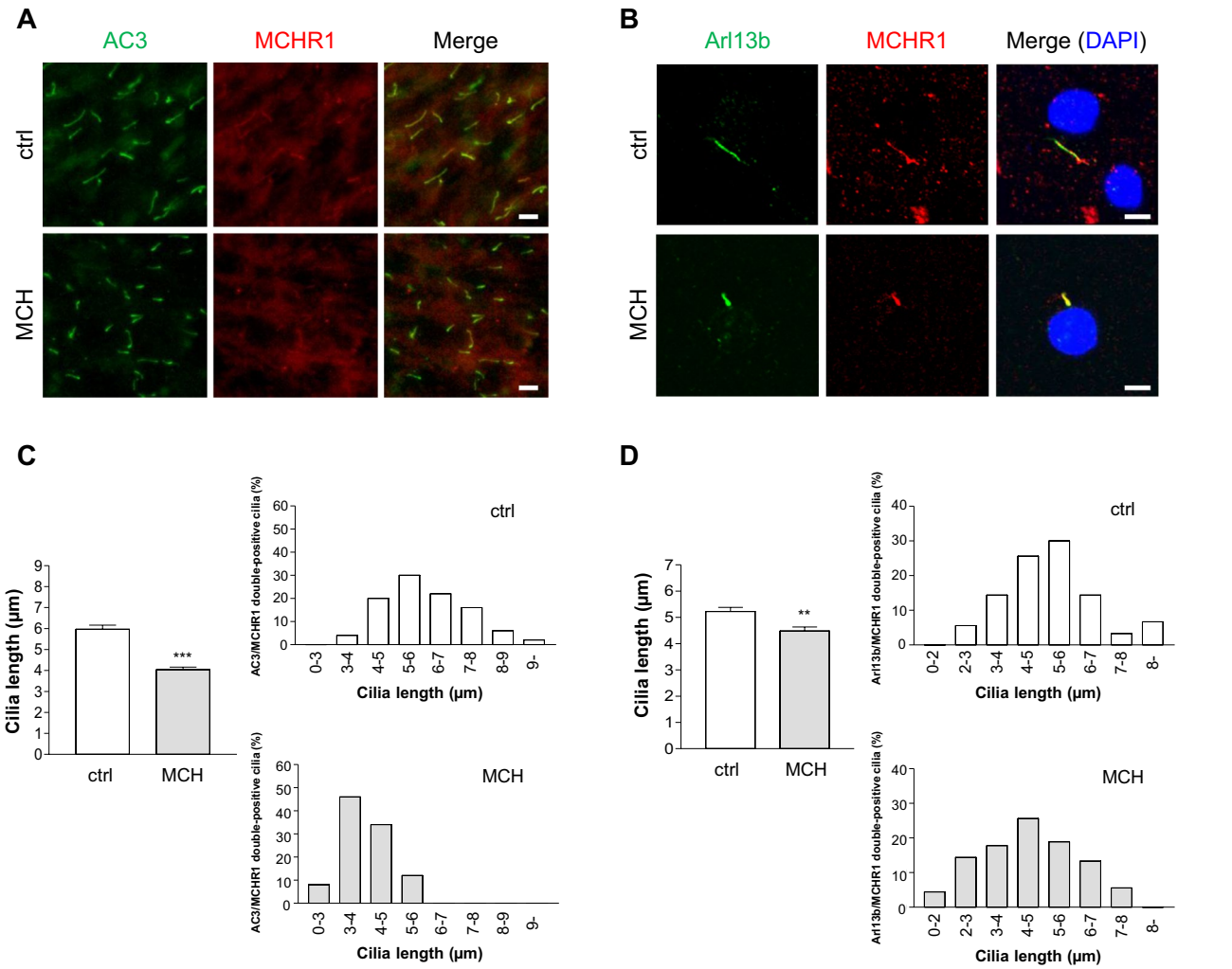

Fig. 3 Primary cilia shortening after treatment of cultured hippocampal slices and iCell GlutaNeurons with MCH. **a** Rat hippocampal slice cultures were treated with 1 nM MCH or untreated (ctrl) for 6 h. **b** iCell GlutaNeurons were treated with 1 nM MCH or untreated (ctrl) for 4 h. Primary cilia were co-labeled with antibodies against AC3 (**a**, green) or Arl13B (**b**, green) and MCHR1 (**a** and **b**, red). Nuclei were labeled with DAPI (blue). Scale bars, 5 μm. **c**, **d** Lengths of primary cilia marker/MCHR1 double-positive cilia. The cilia lengths were measured in randomly selected fields, with data for at least 50 and 90 cilia per group in hippocampal slices (**c**) and iCell GlutaNeurons (**d**), respectively. Left graph: primary cilia were significantly shorter in MCH stimulated cultures than in control cultures for both hippocampal slices (**c**) and iCell GlutaNeurons (**d**). $^{**}P < 0.01$, $^{***}P < 0.001$. Right graphs: histograms for the cilia length distributions in hippocampal slices (**c**) and iCell GlutaNeurons (**d**) (Color figure online)

[18], but not in iCell GlutaNeurons even at DIV 20. According to its data sheet, the anti-AC3 antibody was raised by immunizing a rabbit with the C-terminal 20 amino acids of rat AC3, but the amino acid homology between the rat and human proteins for these residues is only 75%. This may explain the reduced availability of the anti-AC3 antibody for human-derived neurons. Alternatively, it is plausible that the immunodetection method employed in this study was optimal for Arl13b, MCHR1, and FGFR1OP, but not for AC3, in iCell GlutaNeurons. However, it should be noted that no cilia markers are ubiquitously expressed in all neuronal primary cilia, because the expression levels of Arl13b, AC3, and MCHR1 vary among brain regions and developmental stages [15, 18, 25, 31]. In this respect, there remains a need to identify a novel pan-neuronal cilia marker available for rodent brain and human iPSC-derived neurons.

We observed that more than half of Arl13b-positive cilia expressed MCHR1 at 16 DIV in iCell GlutaNeurons. Taking advantage of this finding, we clarified that treatment with MCH led to cilia shortening in three different types of culture, rat MCHR1-expressing hRPE1 cells [22, 23], AC3/MCHR1-expressing rat hippocampal neurons in slice cultures, and Arl13b/MCHR1/MAP2 -expressing iCell GlutaNeurons. The morphological and functional characteristics of both neurons and glial cells are preserved in hippocampal slice cultures [24], but AC3 expression is not associated with

primary cilia on astrocyte in mouse brain [30, 31]. Therefore, the neuronal primary cilia equipped with MCHR1 had the signaling components for cilia length regulation, and this biological effect was conserved between slice culture and neuronal cells, and between rat and human neurons. The MCHR1 system has been implicated in the regulation of energy metabolism, depression, and anxiety in rodents [32–34]. Notably, a significant change in MCHR1-positive cilia length was found in the hippocampus of depression-resistant mice compared with wild-type mice [18]. Children with Bardet–Biedl syndrome, a rare autosomal recessive ciliopathy, showed increased levels of internalization problems including feeling withdrawn, anxiety, and depression [35]. Therefore, ciliary MCHR1 may be related to the pathophysiology of psychiatric disorders through ciliary dynamics in distinct types of human neurons.

Biomarkers for hiPSCs are essential for assessment of cellular differentiation capacity and sustained functionality. In this respect, the present study raises the possibility that examination of primary cilia length and prevalence in iCell GlutaNeurons can serve as a valuable indicator for both neuronal maturation and screening validation for pharmaceutical agents and compound-induced toxicity. Importantly, the effect of lithium on primary cilium elongation was observed in iCell GlutaNeurons, suggesting that this biological phenomenon could be relevant for bipolar disorder patients. Further to the evaluation process, extensive studies focusing on primary cilia in patient hiPSC-derived neurons will reveal novel targeting factors between synapses and primary cilia, which are likely to be involved in neurological disorder-specific pathologies.

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Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

References

1. Takahashi K, Yamanaka S (2006) Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell 126:663–676. https://doi.org/10.1016/j.cell.2006.07.024
2. Wheeler HE, Wing C, Delaney SM, Komatsu M, Dolan ME (2015) Modeling chemotherapeutic neurotoxicity with human induced pluripotent stem cell-derived neuronal cells. PLoS ONE 10:e0118020. https://doi.org/10.1371/journal.pone.0118020
3. Ohara Y, Koganezawa N, Yamazaki H, Roppongi RT, Sato K, Sekino Y, Shirao T (2015) Early-stage development of human induced pluripotent stem cell-derived neurons. J Neurosci Res 93:1804–1813. https://doi.org/10.1002/jnr.23666
4. Nakamura H, Yamashita N, Kanamaru Y, Tachibana T, Sekino Y, Chen S, Gotoh T, Tanaka F, Goshima Y (2015) Quantitative analysis of intraneuronal transport in human iPS neurons. J Pharmacol Sci 128:170–178. https://doi.org/10.1016/j.jphs.2015.06.006
5. Dage JL, Colvin EM, Fouillet A et al (2014) Pharmacological characterisation of ligand- and voltage-gated ion channels expressed in human iPSC-derived forebrain neurons. Psychopharmacology 231:1105–1124. https://doi.org/10.1007/s00213-013-3384-2
6. Berry BJ, Akanda N, Smith AS, Long CJ, Schnepper MT, Guo X, Hickman JJ (2015) Morphological and functional characterization of human induced pluripotent stem cell-derived neurons (iCell Neurons) in defined culture systems. Biotechnol Prog 31:1613–1622. https://doi.org/10.1002/btpr.2160
7. Neagoe I, Liu C, Stumpf A, Lu Y, He D, Francis R, Chen J, Reynen P, Alaoui-Ismaili MH, Fukui H (2018) The GluN2B subunit represents a major functional determinant of NMDA receptors in human induced pluripotent stem cell-derived cortical neurons. Stem Cell Res 28:105–114. https://doi.org/10.1016/j.scr.2018.02.002
8. Louvi A, Grove EA (2011) Cilia in the CNS: the quiet organelle claims center stage. Neuron 69:1046–1060. https://doi.org/10.1016/j.neuron.2011.03.002
9. Ishikawa H, Marshall WF (2011) Ciliogenesis: building the cell's antenna. Nat Rev Mol Cell Biol 12:222–234. https://doi.org/10.1038/nrm3085
10. Wheway G, Nazlamova L, Hancock JT (2018) Signaling through the primary cilium. Front Cell Dev Biol 6:8. https://doi.org/10.3389/fcell.2018.00008
11. Hildebrandt F, Benzing T, Katsanis N (2011) Ciliopathies. New Engl J Med 364:1533–1543. https://doi.org/10.1056/NEJMra1010172
12. Guadiana SM, Semple-Rowland S, Daroszewski D, Madorsky I, Breunig JJ, Mykytyn K, Sarkisian MR (2013) Arborization of dendrites by developing neocortical neurons is dependent on primary cilia and type 3 adenylyl cyclase. J Neurosci 33:2626–2638. https://doi.org/10.1523/JNEUROSCI.2906-12.2013
13. Guo J, Otis JM, Higginbotham H, Monckton C, Cheng J, Asokan A, Mykytyn K, Caspary T, Stuber GD, Anton ES (2017) Primary cilia signaling shapes the development of interneuronal connectivity. Dev Cell 42:286–300. https://doi.org/10.1016/j.devcel.2017.07.010
14. Hilgendorf KI, Johnson CT, Jackson PK (2016) The primary cilium as a cellular receiver: organizing ciliary GPCR signaling. Curr Opin Cell Biol 39:84–92. https://doi.org/10.1016/j.ceb.2016.02.008
15. Berbari NF, Lewis JS, Bishop GA, Askwith CC, Mykytyn K (2008) Bardet–Biedl syndrome proteins are required for the localization of G protein-coupled receptors to primary cilia. Proc Natl Acad Sci USA 105:4242–4246. https://doi.org/10.1073/pnas.0711027105
16. Miyoshi K, Kasahara K, Miyazaki I, Asanuma M (2009) Lithium treatment elongates primary cilia in the mouse brain and in cultured cells. Biochem Biophys Res Commun 388:757–762. https://doi.org/10.1016/j.bbrc.2009.08.099
17. Domire JS, Green JA, Lee KG, Johnson AD, Askwith CC, Mykytyn K (2011) Dopamine receptor 1 localizes to neuronal cilia in a dynamic process that requires the Bardet-Biedl syndrome proteins. Cell Mol Life Sci 68:2951–2960. https://doi.org/10.1007/s00018-010-0603-4
18. Kobayashi Y, Takemoto R, Yamato S, Okada T, Iijima M, Uematsu Y, Chaki S, Saito Y (2018) Depression-resistant phenotype in mice overexpressing regulator of G protein signaling

8 (RGS8). Neuroscience 383:160–169. https://doi.org/10.1016/j.neuroscience.2018.05.005
19. Marley A, von Zastrow M (2012) A simple cell-based assay reveals that diverse neuropsychiatric risk genes converge on primary cilia. PLoS ONE 7:e46647. https://doi.org/10.1371/journal.pone.0046647
20. Hu L, Wang B, Zhang Y (2017) Serotonin 5-HT6 receptors affect cognition in a mouse model of Alzheimer's disease by regulating cilia function. Alzheimers Res Ther 9:76. https://doi.org/10.1186/s13195-017-0304-4
21. Liang Y, Meng D, Zhu B, Pan J (2016) Mechanism of ciliary disassembly. Cell Mol Life Sci 73:1787–1802. https://doi.org/10.1007/s00018-016-2148-7
22. Hamamoto A, Yamato S, Katoh Y, Nakayama K, Yoshimura K, Takeda S, Kobayashi Y, Saito Y (2016) Modulation of primary cilia length by melanin-concentrating hormone receptor 1. Cell Signal 28:572–584. https://doi.org/10.1016/j.cellsig.2016.02.018
23. Tomoshige S, Kobayashi Y, Hosoba K, Hamamoto A, Miyamoto T, Saito Y (2017) Cytoskeleton-related regulation of primary cilia shortening mediated by melanin-concentrating hormone receptor 1. Gen Comp Endocrinol 253:44–52. https://doi.org/10.1016/j.ygcen.2017.08.021
24. Stoppini L, Buchs PA, Muller D (1991) A simple method for organotypic cultures of nervous tissue. J Neurosci Methods 37:173–182. https://doi.org/10.1016/0165-0270(91)90128-M
25. Higginbotham H, Eom TY, Mariani LE, Bachleda A, Hirt J, Gukassyan V, Cusack CL, Lai C, Caspary T, Anton ES (2012) Arl13b in primary cilia regulates the migration and placement of interneurons in the developing cerebral cortex. Dev Cell 23:925–938. https://doi.org/10.1016/j.devcel.2012.09.019
26. Lee JY, Stearns T (2013) FOP is a centriolar satellite protein involved in ciliogenesis. PLoS ONE 8:e58589. https://doi.org/10.1371/journal.pone.0058589
27. Ou Y, Ruan Y, Cheng M, Moser JJ, Rattner JB, van der Hoorn FA (2009) Adenylate cyclase regulates elongation of mammalian primary cilia. Exp Cell Res 315:2802–2817. https://doi.org/10.1016/j.yexcr.2009.06.028
28. Muñoz-Estrada J, Lora-Castellanos A, Meza I, Alarcón Elizalde S, Benítez-King G (2018) Primary cilia formation is diminished in schizophrenia and bipolar disorder: a possible marker for these psychiatric diseases. Schizophr Res 195:412–420. https://doi.org/10.1016/j.schres.2017.08.055
29. May-Simera HL, Wan Q, Jha BS et al (2018) Primary cilium-mediated retinal pigment epithelium maturation is disrupted in ciliopathy patient cells. Cell Rep 22:189–205. https://doi.org/10.1016/j.celrep.2017.12.038
30. Sterpka A, Chen X (2018) Neuronal and astrocytic primary cilia in the mature brain. Pharmacol Res 137:114–121. https://doi.org/10.1016/j.phrs.2018.10.002
31. Arellano JI, Guadiana SM, Breunig JJ, Rakic P, Sarkisian MR (2012) Development and distribution of neuronal cilia in mouse neocortex. J Comp Neurol 520:848–873. https://doi.org/10.1002/cne.22793
32. Chambers J, Ames RS, Bergsma D et al (1999) Melanin-concentrating hormone is the cognate ligand for the orphan G-protein-coupled receptor SLC-1. Nature 400:261–265. https://doi.org/10.1038/22313
33. Saito Y, Nothacker HP, Wang Z, Lin S, Leslie FM, Civelli O (1999) Molecular characterization of the melanin-concentrating-hormone receptor. Nature 400:265–269. https://doi.org/10.1038/22321
34. Macneil DJ (2013) The role of melanin-concentrating hormone and its receptors in energy homeostasis. Front Endocrinol 4:49. https://doi.org/10.3389/fendo.2013.00049
35. Barnett S, Reilly S, Carr L, Ojo I, Beales PL, Charman T (2002) Behavioural phenotype of Bardet-Biedl syndrome. J Med Genet 39:e76. https://doi.org/10.1136/jmg.39.12.e76