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Original Article

Visualization of Astrocytic Primary Cilia in the Mouse Brain by Immunofluorescent Analysis Using the Cilia Marker Arl13b

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In vertebrates, almost all somatic cells extend a single immotile cilium, referred to as a primary cilium. Increasing evidence suggests that primary cilia serve as cellular antennae in many types of tissues by sensing chemical or mechanical stimuli in the milieu surrounding the cells. In rodents an antibody to adenylyl cyclase 3 (AC3) has been widely used to label the primary cilia of neurons *in vivo* by immunostaining, whereas the lack of markers for the primary cilia of astrocytes has made it difficult to observe astrocytic primary cilia *in vivo*. Here, we obtained a visualization of astrocytic primary cilia in the mouse brain. In the somatosensory cortex, a large portion of neurons and astrocytes at postnatal day 10 (P10), and of neurons at P56 had AC3-positive primary cilia, whereas only approx. one-half of the astrocytes in the P56 mice carried primary cilia weakly positive for AC3. In contrast, the majority of astrocytes had ADP-ribosylation factor-like protein 13B (Arl13b)-positive primary cilia in the somatosensory cortex and other brain regions of P56 mice. The lengths of astrocytic primary cilia positive for Arl13b varied among the brain regions. Our data indicate that Arl13b is a noteworthy marker of astrocytic primary cilia in the brain.

Key words: primary cilia, astrocyte, ADP-ribosylation factor-like protein 13B

In vertebrates, almost all body cells protrude a single immotile cilium, referred to as a primary cilium. Growing evidence suggests that primary cilia function as a biosensor for extracellular chemical and mechanical stimuli in many types of tissue and that cilia-mediated signaling is required for development and homeostasis [1, 2]. Mutations in human genes that encode cilia-related molecules can lead to several pleiotropic disorders with overlapping symptoms, collectively called ciliopathies, including Bardet-Biedl syndrome (BBS) and Joubert syndrome [1, 2].

Accumulated evidence has revealed that primary cilia play some roles in the central nervous system [3–11]. Several G protein-coupled receptors (GPCRs) and adenylyl cyclase 3 (AC3) have been shown to localize to primary cilia of neurons [12–19], suggesting that extracellular signals can be sensed and amplified by primary cilia of neurons. AC3 is widely used as a marker for neuronal primary cilia throughout many regions of the rodent brain [13]. We have found that neuronal primary cilia in the mouse striatum were lengthened by pharmacological treatments [20, 21].

However, the study of primary cilia of glial cells

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in vivo is constrained by the lack of markers for glial primary cilia. Antibodies to AC3 were shown to label the primary cilia of primary cultured astrocytes [12, 22], and it was demonstrated that astrocytes with AC3-positive primary cilia are rare in the brain sections of 30-day-old mice [13]. Given that the majority of astrocytes are expected to have one primary cilium *in vivo* like other somatic cells, AC3 is inadequate as a marker of the primary cilia of astrocytes.

ADP-ribosylation factor-like protein 13B (Arl13b) is a cilia-localized protein that belongs to the Arf-like small GTPase family [23]. *ARL13B* is one of the causative genes for Joubert syndrome [24]. It was recently revealed that Arl13b-regulated signaling in primary cilia is crucial for the initial formation of a polarized radial glial scaffold in the developing cerebral cortex [25]. This finding led us to investigate whether Arl13b is localized at primary cilia of differentiated astrocytes *in vivo* and to observe astrocytic primary cilia by using Arl13b as a marker in various brain regions of mice in an immunofluorescent analysis, as described below.

Materials and Methods

Animals. Male and female adult C57BL/6J mice were obtained from Charles River Japan (Yokohama, Japan) and used in the present study. All experiments were performed in compliance with the Guidelines for Animal Experiments of the Okayama University Advanced Science Research Center, and were approved by the Animal Care and Use Committee of the Okayama University Advanced Science Research Center. All efforts were made to minimize animal suffering.

Sample preparation. Mice were perfusion-fixed with 4% paraformaldehyde. Brains were quickly removed and immersed in 4% for 24 h followed by cryoprotection with 15% sucrose in 0.1 M phosphate buffer for 48 h at 4℃. Cryoprotected brains were frozen using powdered dry ice and sectioned at a thickness of 20 μm on a cryostat for immunofluorescence staining.

Immunofluorescence staining. Floating brain sections were permeabilized with 0.3% Triton X-100 in phosphate-buffered saline (PBS) with 2% goat serum, 10 mg/mL bovine serum albumin (BSA), and 0.02% sodium azide. The same buffer without Triton X-100 was used for antibody dilution and washes. The following primary antibodies were used: rabbit anti-AC3 (sc-588, Santa Cruz Biotechnology, Santa Cruz, CA, USA, 1 : 500), rabbit anti-Arl13b (a gift from Dr. Tamara Caspary, Emory University, 1 : 5,000), mouse anti-NeuN (MAB377, Millipore, Billerica, MA, USA, 1 : 500), and mouse anti-S100β (S2532, Sigma, St. Louis, MO, USA, 1 : 20,000). The primary and secondary antibody incubations were carried out for 16 h at 4℃ and for 1 h at room temperature, respectively.

The secondary antibodies were Alexa Fluor 594-conjugated goat anti-mouse IgG and Alexa Fluor 488-conjugated goat anti-rabbit IgG (Invitrogen, Carlsbad, CA, USA, 1 : 1,000). Individual AC3- or Arl13b-positive cilia in the brain sections were observed by a BX50 fluorescence microscope with an UPlanFl oil 100X objective lens (Olympus, Tokyo, Japan), and an image of each cilium was captured with a DP50 digital camera (Olympus). Images of at least 50 cilia were captured in the somatosensory cortex of the brain from postnatal day 10 (P10) mice and postnatal day 56 (P56) mice, and in each region of the brain from P56 male and female mice. Cilia length was measured using the NIH ImageJ software.

Statistical analysis. Unpaired *t*-tests were used for the comparisons of the occurrences of AC3-positive cilia and those of Arl13b-positive cilia, and for the lengths of AC3-positive cilia and those of Arl13b-positive cilia in neurons or astrocytes of the somatosensory cortex at P10 and at P56, and for the comparisons between the cilia lengths at P10 and those at P56 in each category. $P < 0.05$ was considered significant.

Results

We first analyzed the localization of AC3 and Arl13b at primary cilia of neurons and astrocytes in the somatosensory cortex of the brain from the P10 and P56 C57BL/6J mice (Fig. 1). Brain sections were immunostained by antibodies to NeuN, a neuron marker, or S100β, an astrocyte marker, with AC3 or Arl13b (Fig. 1A, D). We found that at P10 92% and 89% of the NeuN-labeled neurons had AC3-positive and Arl13b-positive primary cilia, and 89% and 83% of the S100β-labeled astrocytes had AC3-positive and Arl13b-positive primary cilia, respec-

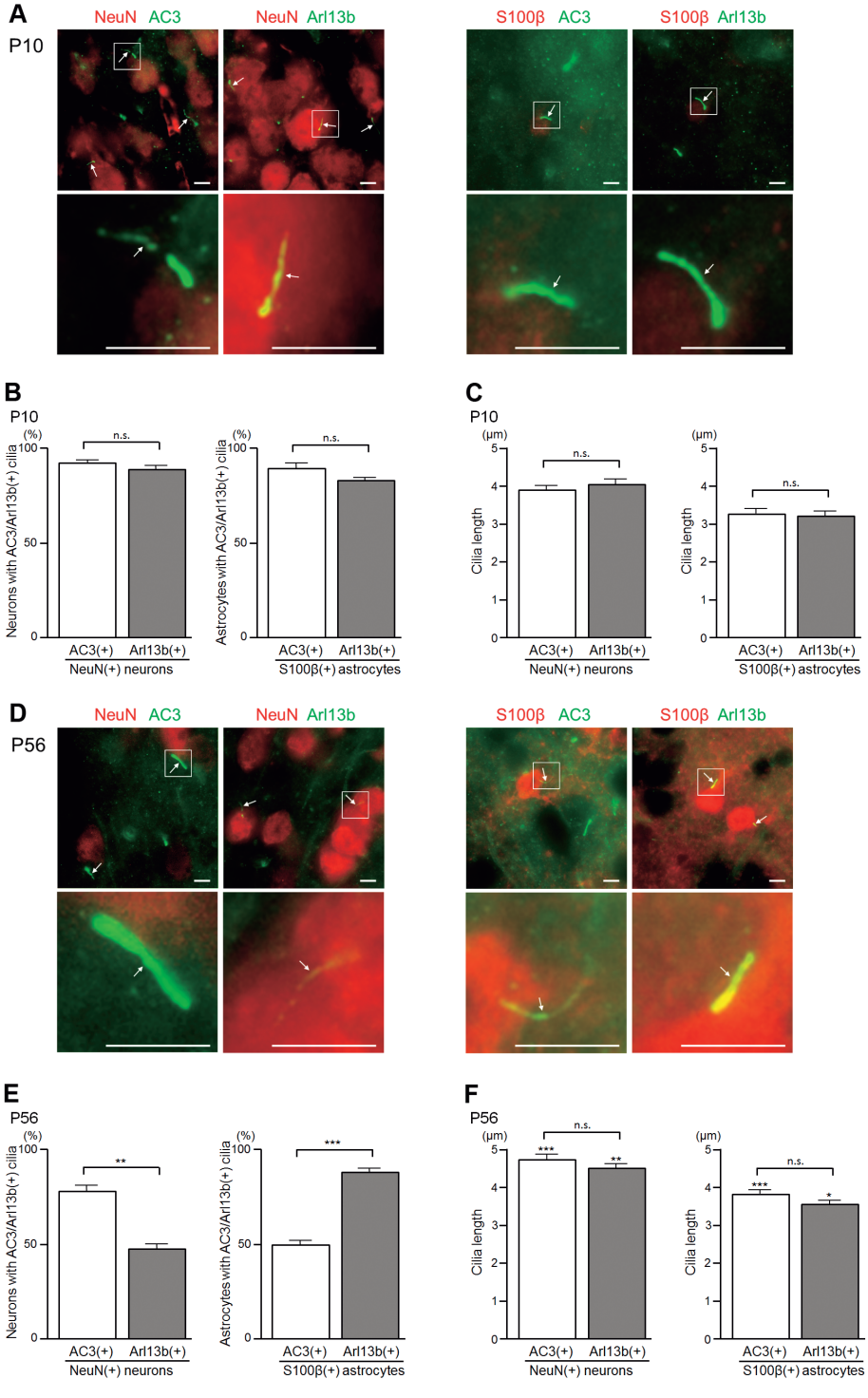

Fig. 1 Validation of AC3 and Arl13b as markers for the primary cilia of astrocytes and neurons. **A**, **D**: Localization of AC3 and Arl13b at the primary cilia of neurons and astrocytes in the somatosensory cortex of C57BL/6J mice at postnatal day 10 (P10) (A) and day 56 (P56) (D). Brain sections were immunostained by antibodies to NeuN (a neuron marker) or S100β (an astrocyte marker) with antibodies to AC3 or Arl13b. The *lower panels* show higher magnification of square region in upper panels. Note that at P56, the Arl13b-immunoreactive cilia signals of neurons and the AC3-immunoreactive cilia signals of astrocytes were faint (D). Arrows indicate primary cilia. Scale bars: 5μm; **B**, In the somatosensory cortex at P10, 92% and 89% of NeuN-labeled neurons had AC3-positive and Arl13b-positive cilia, and 89% and 83% of S100β-labeled astrocytes had AC3-positive and Arl13b-positive cilia, respectively; **E**, In the somatosensory cortex at P56, 78% and 47% of NeuN-labeled neurons had AC3-positive and Arl13b-positive cilia, and 49% and 88% of S100β-labeled astrocytes had AC3-positive and Arl13b-positive cilia, respectively. Data are means $\pm$ S.E.M (n = 3 mice, B, E). The differences between the occurrences of AC3-positive cilia and of Arl13b-positive cilia both in neurons and in astrocytes were significant at P56 (E, $^{**}p < 0.01$, $^{***}p < 0.001$) but not significant (n.s.) at P10 (B). **C**, **F**: Lengths of AC3-positive cilia and Arl13b-positive cilia of NeuN-labeled neurons or S100β-labeled astrocytes in the somatosensory cortex at P10 (C) and at P56 (F). Data are means $\pm$ S.E.M (n > 50 cilia). The differences between the lengths of AC3-positive cilia and of Arl13b-positive cilia were not significant in the neurons or astrocytes at P10 (C) and P56 (F). The cilia length was significantly longer at P56 than that at P10 in each category (C, F, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ in F).

tively (Fig. 1B).

At P56, 78% and 47% of the NeuN-labeled neurons had AC3-positive and Arl13b-positive primary cilia, and 49% and 88% of the S100β-labeled astrocytes had AC3-positive and Arl13b-positive primary cilia, respectively (Fig. 1E). It should be noted that at P56, the Arl13b-immunoreactive cilia signals observed on approx. one-half of the neurons were faint, and at P56 the AC3-immunoreactive cilia signals observed on approx. one-half of the astrocytes were faint (Fig. 1D). This result showed that Arl13b and AC3 are not suitable as markers of neuronal primary cilia and astrocytic primary cilia, respectively.

The cilia length was comparable between the AC3-positive primary cilia and the Arl13b-positive primary cilia, in both the neurons and in astrocytes at P10 (Fig. 1C) and at P56 (Fig. 1F). The primary cilia positive for either AC3 or Arl13b were significantly longer at P56 compared to those at P10, in both the neurons and in astrocytes (Fig. 1C, F). These results revealed that a large fraction of astrocytic primary cilia in the cortex can be visualized by immunofluorescent analysis using an antibody to Arl13b.

We tested the possibility that the majority of primary cilia of differentiated astrocytes in various regions of the adult mouse brain can be visualized by labeling with an Arl13b antibody in immunofluorescence staining. The double-staining of brain sections from the P56 mice with antibodies to S100β and Arl13b (Fig. 2A) revealed that the percentage of S100β-labeled astrocytes with Arl13b-positive cilia in the total S100β-labeled astrocytes ranged from 74% (thalamus) to 95% (amygdala) in the 10 brain regions examined (Fig. 2B). The length of astrocytic primary cilia positive for Arl13b was variable among the 10 brain regions examined within the range from 2.0μm

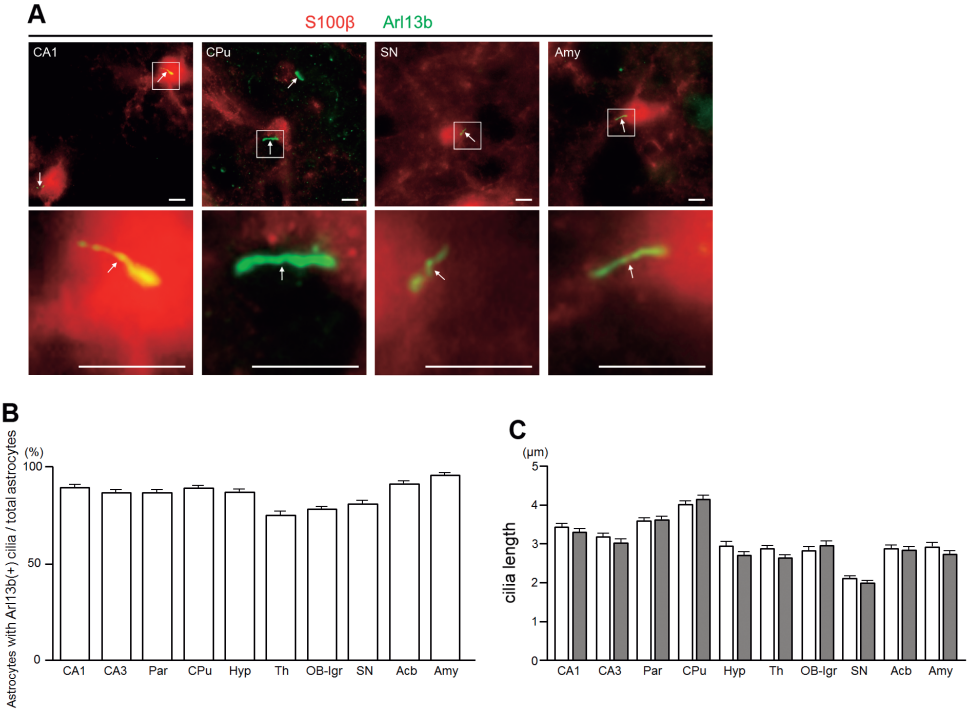

Fig. 2 Visualization of astrocytic primary cilia in the adult mouse brain using Arl13b as a cilia marker. **A**, Primary cilia of S100β-labeled astrocytes were immunostained by Arl13b antibody in various brain regions of the P56 mice. *Lower panels* show higher magnification of the square region in the upper panels. CA1: hippocampal cornet d'Ammon 1, CPu: caudate-putamen, SN: substantia nigra, Amy: amygdala. Arrows indicate primary cilia. Scale bars: 5μm; **B**, The percentage of S100β-labeled astrocytes with Arl13b-positive cilia in the total S100β-labeled astrocytes in various brain regions of the P56 mice. Data are means $\pm$ S.E.M (n = 3 mice). CA3: hippocampal cornet d'Ammon 3, Par: parietal cortex, Hyp: hypothalamus, Th: thalamus, OB-Igr: internal granular layer of olfactory bulb, Acb: nucleus accumbens; **C**, The length of astrocytic primary cilia positive for Arl13b in various brain regions of the P56 mice. Data are means $\pm$ S.E.M (n > 50 cilia). White and gray rectangles indicate data from the male and the female mice, respectively.

(substantia nigra) to 4.1 μm (caudate-putamen) (Fig. 2C). These results reveal that astrocytic primary cilia in various brain regions can be labeled by an Arl13b antibody in immunofluorescence staining.

Discussion

The growing numbers of GPCRs found at the primary cilia of neurons *in vivo* [12–19] suggest that primary cilia function as a biosensor in neurons. AC3 has been used as an established marker for neuronal primary cilia in the validation of ciliary localization of GPCRs. However, little is known about the components that accumulate at the primary cilia of glial cells. Yoshimura *et al.* revealed that the primary cilia of cultured astrocytes express Patched and Smoothened, 2 Hedgehog signaling components, and receive the Hedgehog signal for survival under stressed conditions by using antibodies against AC3 and acetylated tubulin to label the primary cilia of cultured astrocytes [22].

To our knowledge, our present study is the first to comprehensively visualize astrocytic primary cilia i*n vivo*. We observed that both AC3 and Arl13b were frequently localized at neuronal and astrocytic primary cilia in the cortex of the P10 mouse brain (Fig. 1B). A reciprocal expression of AC3 and Arl13b was observed at neuronal primary cilia and astrocytic primary cilia in the cortex of the P56 mouse brain; the majority of neurons had AC3-positive cilia and the majority of astrocytes had Arl13b-positive cilia (Fig. 1E). This heterogeneous expression pattern of AC3 and Arl13b at primary cilia might reflect differentiated cilium functions of neurons and astrocytes in the adult brain.

The lengths of the neuronal cilia and those of the astrocytic cilia were comparable between AC3-immunoreactive cilia and Arl13b-immunoreactive cilia in the cortex of the P10 and P56 mouse brain (Fig. 1C, F), suggesting that both AC3 and Arl13b are expressed in the total tract of each primary cilium. It is of note that the primary cilia of both neurons and astrocytes in the cortex were significantly longer at P56 than at P10 (Fig. 1C, F), indicating the elongation of primary cilia in the nervous system during development.

In the regions of the P56 mouse brain, 74%–95% of the astrocytes had Arl13b-positive primary cilia (Fig. 2B). Regional differences in the length of astrocytic primary cilia in the P56 mouse brain were identified; astrocytic cilia in the caudate-putamen were approximately twice as long as those in the substantia nigra (Fig. 2C).

Further investigations are needed to determine the implications of the Arl13b small GTPase expression in astrocytic cilia. Arl13b-regulated signaling in primary cilia, which participates in the formation of a polarized radial glial scaffold in the developing cortex [25], might have some biological roles in the regulation of the activity of astrocytes in the adult brain. In conclusion, our present data indicate that Arl13b is an outstanding marker of astrocytic primary cilia in the brain and will contribute to analyses of the functions of astrocytic primary cilia.

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References

1. Goetz SC and Anderson KV: The primary cilium: a signalling centre during vertebrate development. Nat Rev Genet (2010) 11: 331–344.
2. Gerdes JM, Davis EE and Katsanis N: The vertebrate primary cilium in development, homeostasis, and disease. Cell (2009) 137: 32–45.
3. Whitfield JF and Chakravarthy BR: The neuronal primary cilium: driver of neurogenesis and memory formation in the hippocampal dentate gyrus? Cell Signal (2009) 21: 1351–1355.
4. Lee JH and Gleeson JG: The role of primary cilia in neuronal function. Neurobiol Dis (2010) 38: 167–172.
5. Lee JE and Gleeson JG: Cilia in the nervous system: linking cilia function and neurodevelopmental disorders. Curr Opin Neurol (2011) 24: 98–105.
6. Green JA and Mykytyn K: Neuronal ciliary signaling in homeostasis and disease. Cell Mol Life Sci (2010) 67: 3287–3297.
7. Han YG and Alvarez-Buylla A: Role of primary cilia in brain development and cancer. Curr Opin Neurobiol (2010) 20: 58–67.
8. Louvi A and Grove EA: Cilia in the CNS: the quiet organelle claims center stage. Neuron (2011) 69: 1046–1060.
9. Einstein EB, Patterson CA, Hon BJ, Regan KA, Reddi J, Melnikoff DE, Mateer MJ, Schulz S, Johnson BN and Tallent MK: Somatostatin signaling in neuronal cilia is critical for object recognition memory. J Neurosci (2010) 30: 4306–4314.
10. Wang Z, Phan T and Storm DR: The type 3 adenylyl cyclase is required for novel object learning and extinction of contextual memory: role of cAMP signaling in primary cilia. J Neurosci (2011) 31: 5557–5561.
11. Davenport JR, Watts AJ, Roper VC, Croyle MJ, van Groen T,

Wyss JM, Nagy TR, Kesterson RA and Yoder BK: Disruption of intraflagellar transport in adult mice leads to obesity and slow-onset cystic kidney disease. Curr Biol (2007) 17: 1586–1594.
12. Berbari NF, Bishop GA, Askwith CC, Lewis JS and Mykytyn K: Hippocampal neurons possess primary cilia in culture. J Neurosci Res (2007) 85: 1095–1100.
13. Bishop GA, Berbari NF, Lewis J and Mykytyn K: Type III adenylyl cyclase localizes to primary cilia throughout the adult mouse brain. J Comp Neurol (2007) 505: 562–571.
14. Hamon M, Doucet E, Lefevre K, Miquel MC, Lanfumey L, Insausti R, Frechilla D, Del Rio J and Verge D: Antibodies and antisense oligonucleotide for probing the distribution and putative functions of central 5-HT6 receptors. Neuropsychopharmacology (1999) 21: 68S–76S.
15. Händel M, Schulz S, Stanarius A, Schreff M, Erdtmann-Vourliotis M, Schmidt H, Wolf G and Hollt V: Selective targeting of somatostatin receptor 3 to neuronal cilia. Neuroscience (1999) 89: 909–926.
16. Berbari NF, Johnson AD, Lewis JS, Askwith CC and Mykytyn K: Identification of ciliary localization sequences within the third intracellular loop of G protein-coupled receptors. Mol Biol Cell (2008) 19: 1540–1547.
17. Soetedjo L, Glover DA and Jin H: Targeting of vasoactive intestinal peptide receptor 2, VPAC2, a secretin family G-protein coupled receptor, to primary cilia. Biol Open (2013) 2: 686–694.
18. Loktev AV and Jackson PK: Neuropeptide Y family receptors traffic via the Bardet-Biedl syndrome pathway to signal in neuronal primary cilia. Cell Rep (2013) 5: 1316–1329.
19. Mukhopadhyay S, Wen X, Ratti N, Loktev A, Rangell L, Scales SJ and Jackson PK: The ciliary G-protein-coupled receptor Gpr161 negatively regulates the Sonic hedgehog pathway via cAMP signaling. Cell (2013) 152: 210–223.
20. Miyoshi K, Kasahara K, Miyazaki I and Asanuma M: Lithium treatment elongates primary cilia in the mouse brain and in cultured cells. Biochem Biophys Res Commun (2009) 388: 757–762.
21. Miyoshi K, Kasahara K, Murakami S, Takeshima M, Kumamoto N, Sato A, Miyazaki I, Matsuzaki S, Sasaoka T, Katayama T and Asanuma M: Lack of dopaminergic inputs elongates the primary cilia of striatal neurons. PLoS One (2014) 9: e97918.
22. Yoshimura K, Kawate T and Takeda S: Signaling through the primary cilium affects glial cell survival under a stressed environment. Glia (2011) 59: 333–344.
23. Caspary T, Larkins CE and Anderson KV: The graded response to Sonic Hedgehog depends on cilia architecture. Dev Cell (2007) 12: 767–778.
24. Cantagrel V, Silhavy JL, Bielas SL, Swistun D, Marsh SE, Bertrand JY, Audollent S, Attie-Bitach T, Holden KR, Dobyns WB, Traver D, Al-Gazali L, Ali BR, Lindner TH, Caspary T, Otto EA, Hildebrandt F, Glass IA, Logan CV, Johnson CA, Bennett C, Brancati F, Group IJSRDS, Valente EM, Woods CG and Gleeson JG: Mutations in the cilia gene ARL13B lead to the classical form of Joubert syndrome. Am J Hum Genet (2008) 83: 170–179.
25. Higginbotham H, Guo J, Yokota Y, Umberger NL, Su CY, Li J, Verma N, Hirt J, Ghukasyan V, Caspary T and Anton ES: Arl13b-regulated cilia activities are essential for polarized radial glial scaffold formation. Nat Neurosci (2013) 16: 1000–1007.