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Sequential expression of Efhc1/myoclonin1 in choroid plexus and ependymal cell cilia

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Abstract

EFHC1 is a gene mutated in patients with idiopathic epilepsies, and encodes the myoclonin1 protein. We here report the distribution of myoclonin1 in mouse. Immunohistochemical analyses revealed that the myoclonin1 first appeared at the roof of hindbrain at embryonic day 10 (E10), and moved on to choroid plexus at E14. At E18, it moved to ventricle walls and disappeared from choroid plexus. From neonatal to adult stages, myoclonin1 was concentrated in the cilia of ependymal cells at ventricle walls. At adult stages, myoclonin1 expression was also observed at tracheal epithelial cilia in lung and at sperm flagella in testis. Specificities of these immunohistochemical signals were verified by using *Efhc1*-deficient mice as negative controls. Results of *Efhc1* mRNA *in situ* hybridization were also consistent with the immunohistochemical observations. Our findings raise "choroid plexusopathy" or "ciliopathy" as intriguing candidate cascades for the molecular pathology of epilepsies caused by the *EFHC1* mutations.

Keywords: EFHC1; Myoclonin1; Epilepsy; Cilia; Ependymal cell; Choroid plexus

The *EFHC1* (EF-hand domain containing 1) gene locates at the chromosomal region 6p12-p11 where genetic linkage analyses mapped the gene for juvenile myoclonic epilepsy (JME) [1,2]. We previously identified five disease-associated missense mutations of *EFHC1* in six Mexican JME families, and proposed *EFHC1* as the gene for JME [3]. Successive studies by us and others found additional *EFHC1* mutations in other ethnic groups including European, Honduran, and Japanese families mainly with JME but also with other and wider range of epileptic phenotypes [4–7].

EFHC1 encodes a protein of 640 amino acids, named as myoclonin1, that harbors three tandemly repeated DM10 domains and one EF-hand motif at the C-terminus. Myoclonin1 over-expression in mouse hippocampal primary culture cells induced cell-death that was significantly lowered by the disease-associated mutations. In patch-clamp analysis, myoclonin1 specifically increased the currents of R-type calcium channel that were reversed by disease-associated mutations [3]. A recent report showed that myoclonin1, or alternatively named mRib72-1, localized in mouse tracheal cilia and sperm flagella but not in immotile primary cilia of NIH3T3 cells [8].

To understand the function of myoclonin1 and its role in the pathogenesis of epilepsy, here in this study we raised a new anti-myoclonin1 monoclonal antibody (mAb) and examined the distribution of myoclonin1 in the mouse brain with the antibody. The specificity of this mAb was verified using lysate or sections of *Efhc1* null-mutant mouse brain. Our results revealed dominant expressions of myoclonin1 in choroid plexus at restricted developmental stages of fetus and in ependymal cell cilia at postnatal stages.

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Materials and methods

Animal experiments. Mice were handled in accordance with the Animal Experiment Committee of RIKEN Brain Science Institute.

A mouse monoclonal antibody against mouse myoclonin1. A synthetic peptide corresponding to amino acid residues 379–397 (QELPPYNGYGLIEDSAQNC) of mouse myoclonin1 was used for the mouse immunization according to the standard protocol [9]. Isotype of this monoclonal antibody (mAb) was confirmed using the mouse monoclonal isotyping kit (Serotec).

Western blot analysis. Preparation of mouse tissue protein samples and Western blot analyses was done as previously described [3]. The following primary Abs were used: anti-myoclonin1 (6A3-mAb), 1:100 dilution or anti-β-tubulin mAb (D-66; Sigma), 1:1000 dilution. A horseradish peroxidase-conjugated anti-mouse IgG Ab (1:5000, Promega) was used for secondary Ab.

Northern blot analysis. Isolation of poly(A)$^+$ RNA (from whole-brain and head of C57BL/6J) and preparation of Northern blot was carried out as previously described [3]. A mouse *Efhc1* cDNA clone containing 321 bp that covers the coding region and 3′-UTR (nts 1670–1990) was amplified by PCR and radioactively labeled with ^{32}P using High Prime DNA labeling kit (Roche Diagnostics). Hybridization and detection were done as previously described [3]. The β-actin probe was used as a control.

Immunohistochemistry. For fixed paraffin tissue sections, mice (C57BL/6J or ICR) were deeply anesthetized with avertin and perfused intracardially with 0.9% NaCl, followed by 4% paraformaldehyde (TAAB) in phosphate-buffered saline (PBS; 10 mM phosphate-buffer, 2.7 mM KCl, and 137 mM NaCl, pH 7.4). The brains were then removed and postfixed for 12 h or upto 3 days at 4 °C in 4% paraformaldehyde in PBS. Paraffin sections (6-μm-thick) were prepared for standard histological protocol [10]. For pre-fixed frozen sections, postfixed brains were cryoprotected with 30% sucrose in 50 mM phosphate-buffer (PB) for 3 days at 4 °C. For fresh frozen sections, mice were deeply anesthetized, and brain was removed. The brains were embedded in Tissue-Tek OCT compound (Sakura), sectioned at 20-μm thickness on a cryostat and mounted on MAS coated glass slides, and used for immunohistochemical staining. Fresh frozen sections were fixed with acetone at 4 °C for 10 min. Immunohistochemical staining was carried out as previously described [10]. Images were acquired under an AX80 microscope (Olympus). The following primary Abs were used: anti-myoclonin1 polyclonal Ab (1:2000 dilution), 6A3-mAb (IgG2a, 1:1000–3000 dilution), and anti-acetylated-α-tubulin mAb (6-11B-1, IgG2b, Sigma, 1:500 dilution). The normal mouse IgG (Santa Cruz Biotechnology) was used as a negative control. For fluorescent immunohistochemistry, the following secondary Abs were used: Alexa Fluor® 488-anti-rabbit IgG, Alexa Fluor® 594-anti-mouse IgG, and Alexa Fluor® 647-anti-mouse IgG2b (Molecular Probes). Images were acquired under a TCS SP2 microscope (Leica).

Immunocytochemistry. Mouse hippocampal cell cultures were prepared basically as previously described [3], except for the absence of 1-β-D arabinofuranosylcytosine in the medium. After wash with PBS containing 0.9 mM $CaCl_2$ and 0.5 mM $MgCl_2$, the cells were fixed with 10% TCA for 15 min on ice. Immunocytochemical staining was carried out as previously described [3]. The following primary and secondary Abs were used: 6A3-mAb (1:1000 dilution), anti-GFAP chicken polyclonal Ab, anti-MAP2 rabbit polyclonal Ab (1:2000, Chemicon), Alexa Fluor® 594-anti-mouse IgG, Alexa Fluor® 488-anti-chicken IgG, and Alexa Fluor® 488-anti-rabbit IgG (1:300 dilution). Images were acquired under a TCS SP2 microscope (Leica).

In situ RNA hybridization. A mouse *Efhc1* cDNA clone used for Northern blot analysis (see above) was used as a template to produce sense and antisense riboprobes using a digoxigenin RNA labeling kit (Roche Diagnostics). Paraffin-embedded brain sections (6-μm thick) of E18 ICR mice were deparaffinized and incubated with 10 μg/ml proteinase K (Invitrogen) in PBS at room temperature for 15 min prior to hybridization. Hybridization and detection reaction were carried out as previously described [10].

Results

The previously described polyclonal antibody against myoclonin1 yielded non-specific signals

We previously reported neuronal expression of myoclonin1 in the immunohistochemical analyses of mouse brain using an anti-myoclonin1 polyclonal antibody [3]. However, recently we created *Efhc1* knock-out mouse (details will be reported elsewhere), and by using this null-mutant mouse as a negative control we found that the signals obtained by the polyclonal antibody remained in the null-mutant and that the signals were largely non-specific (data not shown).

A newly generated anti-myoclonin1 monoclonal antibody yielded specific myoclonin1 signals in Western blot and immunohistochemical analyses

We raised a new mouse monoclonal antibody against mouse myoclonin1 (6A3-mAb) and verified its specificity. The 6A3-mAb recognizes amino acid residues 379–397 of myoclonin1 that locates between the 2nd and 3rd DM10 domains. The isotype of 6A3-mAb revealed to be IgG2a. To check the specificity of 6A3-mAb against endogenous myoclonin1, we performed Western blot analyses using mouse brain lysates from wild type, *Efhc1* heterozygous, and null-mutant mice (Fig. 1A). 6A3-mAb detected a ~75 kDa band in wild type and a less-intense (mostly half amount) band in the heterozygous mouse brain lysates. No signal appeared in the lane of *Efhc1* null-mutant mouse brain lysate, indicating that the 6A3-mAb specifically recognized myoclonin1 protein on the Western blot analysis (Fig. 1A). Western blot analyses containing multiple tissues samples from postnatal 4 days (P4) mouse revealed that myoclonin1 was dominantly expressed in brain and lung (Fig. 1B). In the previous study [3], *Efhc1* mRNA was observed at embryonic 11 days (E11) stage but not at E7. In this study, we found that the expression level of myoclonin1 was largely constant from embryonic to adult stages. Western blot and Northern blot analyses by using embryonic and postnatal stages of mouse brain (E13 days to P12 weeks) and whole head (E11 days) samples revealed that myoclonin1 protein and *Efhc1* transcript were present at 11 days post coitum (dpc), and its expression continued to the postnatal stages (Fig. 1C and D). 6A3-mAb also gave specific signals on immunohistochemical analyses (see below).

Myoclonin1 is expressed sequentially at roof of hindbrain, choroid plexus, and cilia of ependymal cells in mouse brain

To investigate the distribution of myoclonin1, we performed immunohistochemistry of mouse brain sections by using 6A3-mAb. Dominant expression of myoclonin1 was detected in roof of hindbrain at E10 (Fig. 2A), in

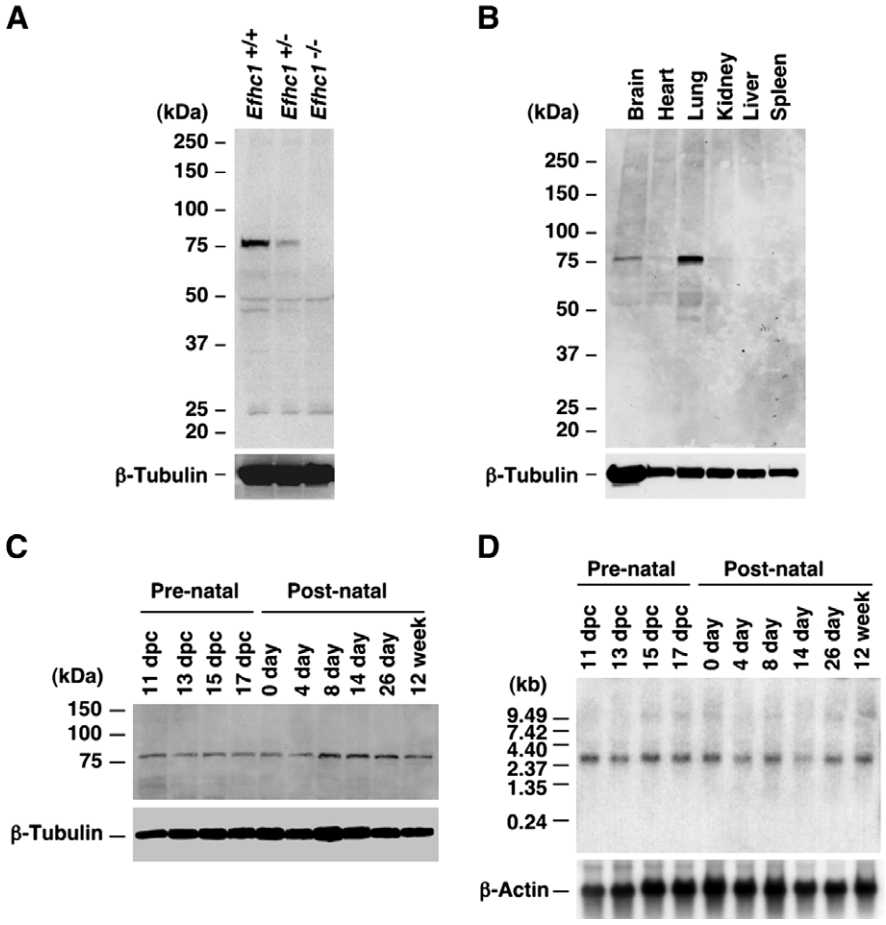

Fig. 1. Western and Northern blot analyses of myoclonin1 and *Efhc1* mRNA. (A) A Western blot of brain lysates from *Efhc1* wild type ($Efhc1^{+/+}$), heterozygote ($Efhc1^{+/-}$), and null-mutant ($Efhc1^{-/-}$) mice, probed with the monoclonal anti-myoclonin1 antibody (6A3-mAb). The myoclonin1 was detected at the expected size (~75 kDa) in the lysates from $Efhc1^{+/+}$, less amount in that of $Efhc1^{+/-}$, but not in that of $Efhc1^{-/-}$. (B) A Western blot of lysates from multiple mouse tissues (brain, heart, lung, kidney liver, spleen of postnatal 4 days). Myoclonin1 expression was dominantly observed in brain and lung. (C) A Western blot containing samples from embryonic (13–17 days postcoitum (dpc)) and postnatal (0 days–12 weeks) mouse brains (whole head was used for 11 dpc). The myoclonin1 was detected in 11 dpc and the expression continued thereafter. The anti-β-tubulin antibody was used as a control and shown in the lower panel (A–C). (D) A Northern blot containing poly $(A)^+$ RNA from embryonic and postnatal stages (the same stages as shown in Fig. 1C). The blot was hybridized with a ^{32}P-labeled probe generated from the *Efhc1* cDNA. The *Efhc1* transcripts were detected at 11 dpc and the expression continued thereafter. The control hybridization with β-actin probe is shown in the lower panel (D).

choroid plexus at E14 (Fig. 2B), and in ependymal cells at E18, P0, and P15 (Fig. 2C–H). At E18, expression of myoclonin1 was further detected in ependymal cells lining the wall of lateral, 3rd and 4th ventricle (Fig. 2I–L). Immunohistochemistry of 1-month-old mouse brain indicated that myoclonin1 was expressed on somata and cilia of ependymal cells lining the wall of lateral and 3rd cerebral ventricles (Fig. 2M–P). Brain sections of the *Efhc1*-null-mutant did not show any signals, and it again approved the specificity of the signals (Fig. 2Q–T). Multiple different conditions for the fixation of brain sections (Pre-fixed frozen or fresh frozen; see Materials and methods.) yielded no differences in the signals. We could not detect any prominent signals in neuronal cells so far. Myoclonin1 signals were also detected at tracheal epithelial cilia in lung and at sperm flagella in testis (data not shown) as previously described [8].

Myoclonin1 partially co-expressed with acetylated-α-tubulin at ependymal cell cilia

Acetylated form of α-tubulin is an important component of axoneme assembly and it may play a prevalent role in the differentiation of microtubule structure and function [11]. Co-localization of myoclonin1 and acetylated-α-tubulin was previously reported in the sperm flagella and tracheal epithelial cilia [8]. We here investigated whether the myoclonin1 co-expressed with acetylated-α-tubulin at the cilia in the brain by double-staining of the P14 mouse brain sections using 6A3-mAb and anti-acetylated-α-tubulin Ab. Expression of myoclonin1 was observed at the cilia of ependymal cells, but not at choroid plexus (Fig. 3A and E). Acetylated-α-tubulin localized at distal cilia and surface of choroid plexus (Fig. 3B and F). Thus, myoclonin1 and acetylated-α-

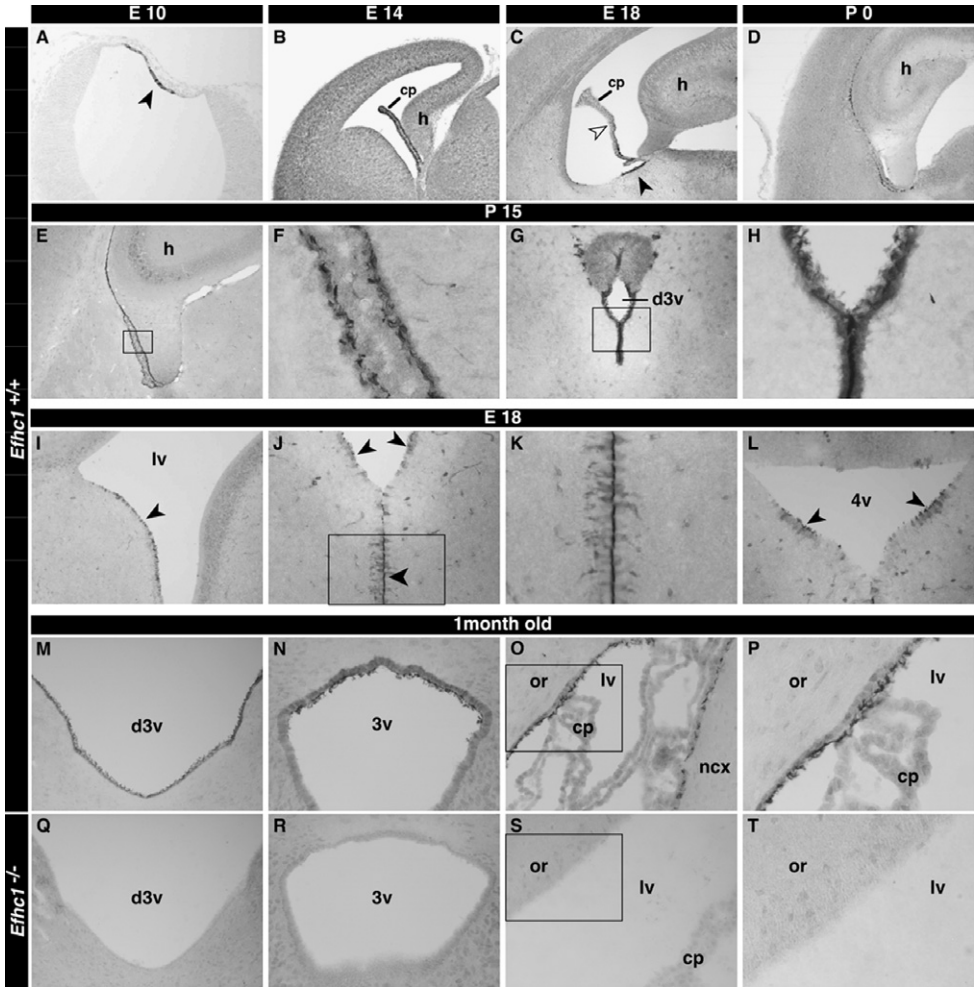

Fig. 2. Distribution of myoclonin1 in mouse brain. Brain sections were stained with the monoclonal anti-myoclonin1 antibody 6A3-mAb and with diaminobenzidine (DAB). Coronal sections from at embryonic day 10 (E10) (A), at E14 (B), at E18 (C), at postnatal day 0 (P0) (D), and at P15 (E–H). The myoclonin1 expression was observed on roof of hindbrain at E10 (A; arrow head), on choroid plexus at E14 (B) and on ependymal cells (C; arrow head) but disappeared from choroid plexus (C; white arrow head) at E18. At the P0 and P15 stages, myoclonin1 expressed highly at cilia and moderately in somata of ependymal cells at the ventricles around hippocampus (D–F) and those lining the wall of dorsal 3rd ventricle (d3v; G,H). (F) and (H) are magnified images of the square regions in (E) and (G), respectively. In the sections from E18 mouse brain, myoclonin1 expression was observed on ependymal cells lining of lateral ventricle (I), dorsal 3rd ventricle (J and K), and 4th ventricle (L). (K) is the magnified image of the square region in (J). (M–P) In 1-month-old *Efhc1*$^{+/+}$ (wild-type) mouse brain, expression of myoclonin1 was observed in somata and cilia of ependymal cells lining the wall of dorsal 3rd ventricle, ventral 3rd ventricle, and lateral ventricles. These observed signals do not appear in *Efhc1*$^{-/-}$ brain sections (Q–T). (P) and (T) are the magnified image of the square regions in (O) and (S) respectively. ncx; neocortex, cp; choroid plexus, h; hippocampus, d3v; dorsal 3rd ventricle, 3v; 3rd ventricle, lv; lateral ventricle, or; oriens layer of hippocampus, 4v; 4th ventricle.

tubulin co-localized at only distal cilia, but not at proximal cilia (Fig. 3D and H).

Myoclonin1 expression at cilia of minor cell species in hippocampal cell culture

We performed immunocytochemistry on culture cells from E16 mouse hippocampus using 6A3-mAb. We did not add 1-β-D arabinofuranosylcytosine in the medium in order to have glial cells as well as neuronal cells in the culture. The strong fluorescence signals for the myoclonin1 protein were observed on cilia of cells, possibly ependymal cells, at after 7 days *in vitro* (div) but not at 4 div. The myoclonin1-positive cells in culture cells at 15 div occupied approximately 10% of the total cell population (16 of 154). Double-staining of the cells (15 div) with 6A3-mAb and the antibody for glial fibrillary acidic protein (GFAP: an astrocyte protein) or one for microtubule associated protein 2 (MAP2: a neuron protein) showed that myoclonin1 signals were observed on a few GFAP-positive cells (Fig. 3I–L), but most of myoclonin1 signals did not overlap with GFAP or MAP2-positive cells (Fig. 3M–T).

Efhc1 mRNA localizes at ependymal cells

To determine the distribution of *Efhc1* mRNA transcripts in mouse brain, we performed *in situ* RNA hybrid-

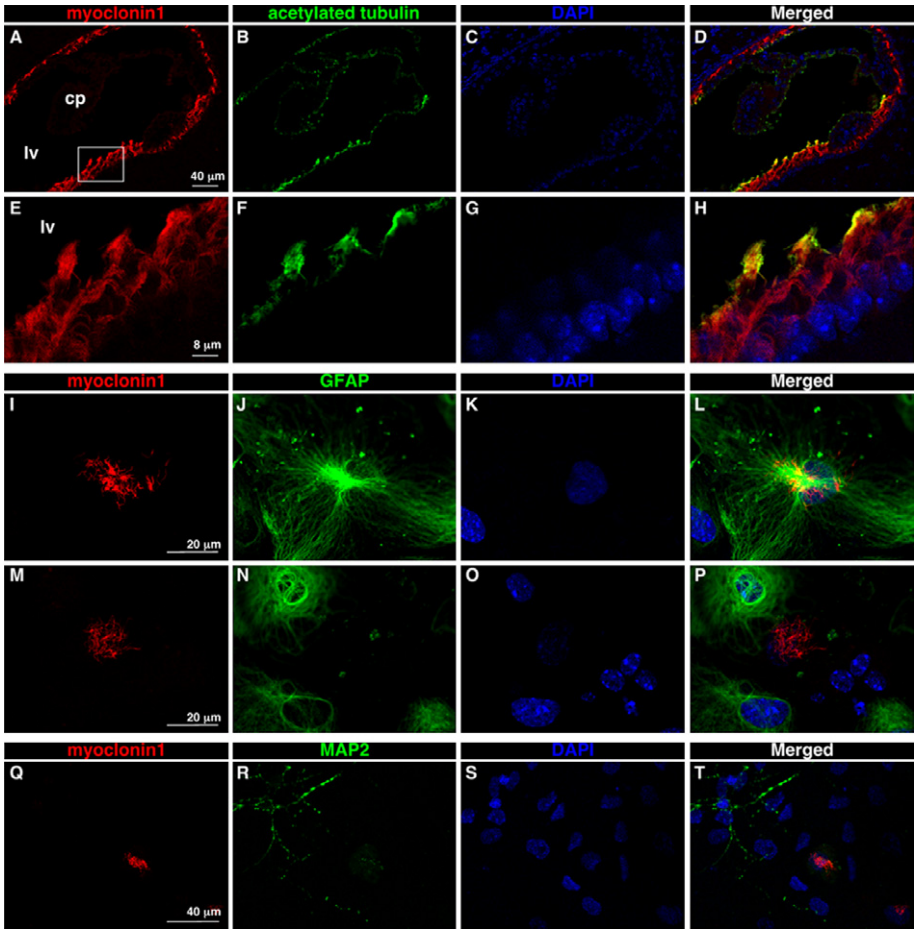

Fig. 3. Myoclonin1 localizes at cilia of ependymal cells. Tissue sections from P14 mouse brain were stained with 6A3-mAb (red), anti-acetylated-α-tubulin antibody (green) and DAPI (blue) (A–H). (D and H) The myoclonin1 expression was observed in cilia of the wall of lateral ventricles (lv) and it partially overlapped with acetylated-α-tubulin. Myoclonin1 signals at proximal cilia of ependymal cells do not overlap with those of acetylated-α-tubulin. (E) is magnified image of the square region in (A). cp; choroid plexus. (I–T) Mouse hippocampal primary cultured cells (15 div) were stained with 6A3-mAb (red) and anti-GFAP antibody (green) or anti-MAP2 antibody (green). (K, O, and S) Nuclear was stained with DAPI (blue). The myoclonin1 expression was observed at the cilia structure (I, M, and Q). The myoclonin1 signals did not overlap with GFAP or MAP2-positive cells (M–T) expect for a minor population of GFAP-positive cells (I–L). (For interpretation of the references in colour in this figure legend, the reader is referred to the web version of this article.)

ization on E18 brain sections using *Efhc1* antisense RNA as a probe. In consistent with the results of immunohistochemistry on E18 mouse brain (Fig. 2I–L), positive signals for *Efhc1* mRNA were detected in ependymal cells surrounding 3rd, 4th ventricles (Fig 4A–G) and central canal (Fig. 4H and I). Hybridization with sense probe produced no staining above background level (data not shown).

Discussion

The present paper described the expression profile of the mouse homologue (orthologue) for the human epilepsy gene, *EFHC1*.

In this study, we showed that myoclonin1 mainly expresses at cilia on ependymal cells surrounding cerebral ventricles in the postnatal mouse brain. No significant signals were observed in other regions and cell types including neurons and astrocytes. These observations are, however, inconsistent with our previous report [3] which described neuronal expression of myoclonin1. The anti-myoclonin1 polyclonal antibody (pAb) used in our previous study recognized over-expressed myoclonin1 protein on Western blot analyses and these observed signals disappeared when incubated with the immunized antigen peptide. Immunohistochemical signals obtained by using this pAb also disappeared with the absorption by the peptide [3]. However, in this study our results with the *Efhc1*-deficient mice revealed that the previous neuronal signals obtained by the pAb are actually non-specific. We therefore raised a new monoclonal antibody (6A3-mAb) to mouse myoclonin1 and verified its specificity in Western blot and immunohistochemical analyses by using *Efhc1*-null mice as negative controls. The 6A3-mAb specifically recognized myoclonin1 protein at embryonic choroid plexus and postnatal ependymal cells. The distribution of *Efhc1* mRNA transcripts at surrounding ventricles in E18 mouse brain was also consistent with those seen in the immunohistochemistry.

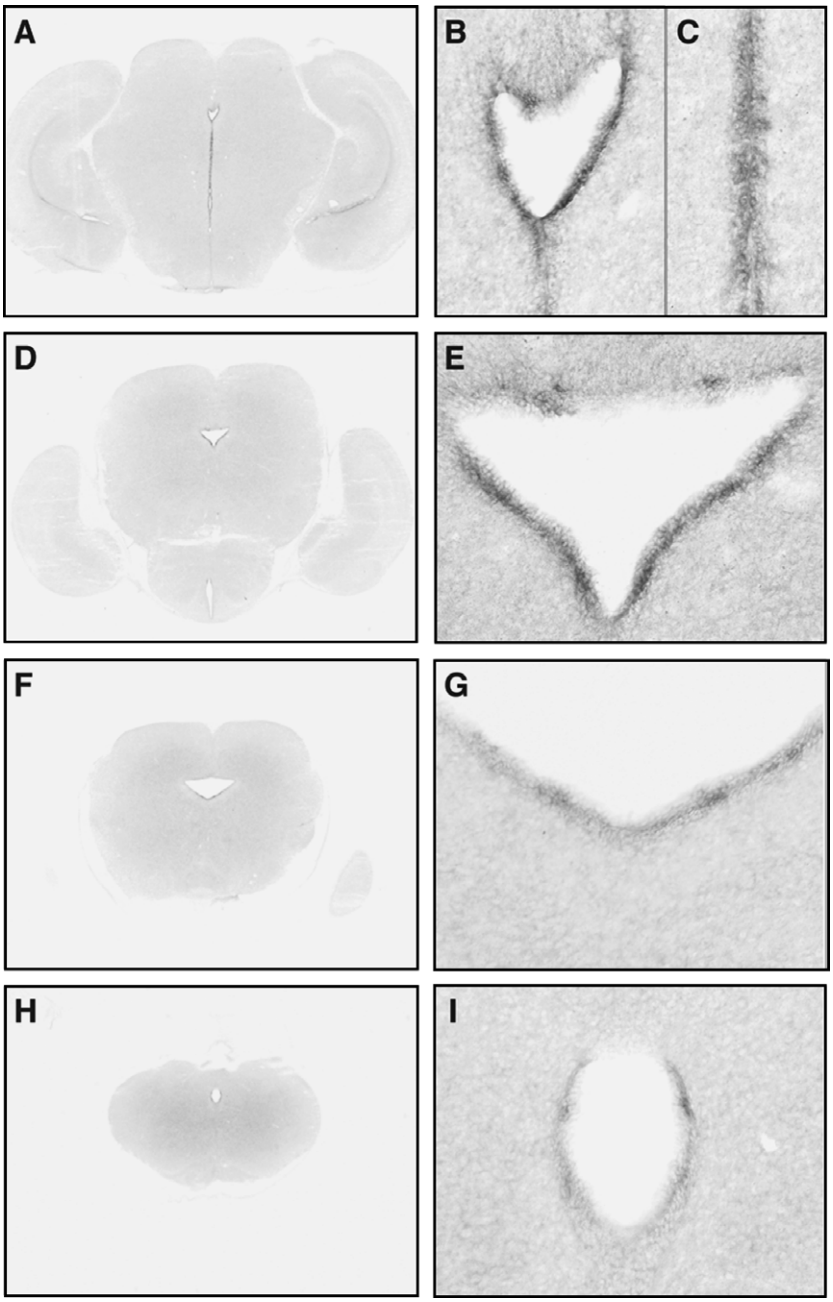

Fig. 4. *In situ* hybridization analysis of *Efhc1* mRNA in mouse brain. The transcripts of *Efhc1* are localized at the wall of ventricles and central canal. (A–I) Serial coronal brain sections of E18 mouse embryos (A, D, F, and H: from cerebrum to spinal cord) were hybridized with digoxigenin-labeled mouse *Efhc1* antisense riboprobes. (B and C) Magnified images around the ventricle region in panel (A). *Efhc1* positive signals were detected in the ependymal cell layer of dorsal 3rd ventricle. (E, G, and I) Magnified images of (D), (F), and (H), respectively. *Efhc1* mRNA expression was observed in a part of the ependymal cell layer in the fourth ventricle wall (E and G) and central canal of spinal cord (I).

Immunocytochemistry also detected the myoclonin1 expression at the cilia structure of cultured cells. In these study, we did not observe myoclonin1 expression in neurons. However, it should be kept in mind that the sensitivities of these analyses may not be high enough to detect moderate or lower levels of myoclonin1 expression in other tissues and cells. In addition, other isoforms of myoclonin1, which the 6A3-mAb cannot recognize, may possibly be expressed in distinct tissues and cells, and therefore further studies are warranted.

Myoclonin1 is a homologue of P72/Rib72, a flagellar protein of *Chlamydomonas* [12,13]. P72/Rib72 is thought to have important roles in assembly and function of the axoneme, and the association between myoclonin1 and tubulin or microtubules was also reported [13,14]. In the previous study [8], most of myoclonin1 positive signals co-localized with acetylated-α-tubulin at the sperm flagellum and tracheal cilia. In this study, we showed that the expressions of myoclonin1 and acetylated-α-tubulin at cilia of ependymal cells in the brain only partially overlaps. The

myoclonin1 signals appeared not only at acetylated-α-tubulin-positive cilia but also at the proximal acetylated-α-tubulin-negative cilia of ependymal cells. Because acetylated-α-tubulin has been assumed to accumulate at stable microtubules [15], our results may suggest that the myoclonin1 is also expressed at proximal cilia of the ependymal cells containing labile microtubules.

Our findings raise intriguing possibilities that "(choroid) plexusopathy" or "ciliopathy" are the pathological basis of epilepsies caused by the *EFHC1* mutations. We found the myoclonin1 expression on the hindbrain at E10 stage and on the choroid plexus at E14 stage. Surface of choroid plexus is comprised of the choroid epithelium (derived from the same layer of cells that forms the ependymal lining of the ventricles) [16]. Since these epithelium cells do not have cilia on cell surface at the embryonic stages, myoclonin1 might have some other roles (e.g. cerebrospinal fluid (CSF) secretion) besides the postnatal ciliary function. CSF maintains homeostasis in the extracellular environment of neuron and glial cells [16]. It also might be possible that the abnormality of CSF secretion is involved in the etiology of the *EFHC1* mutation-dependent epilepsies.

The expression of myoclonin1 at the ependymal cell cilia may also raise a possibility that ciliary malfunction is involved in the development of the epilepsy. The previous reports that myoclonin1 is expressed in sperm flagella and tracheal motile cilia [8], and that the CSF flow, driven by the motile cilia of ependymal cells, establishes a chemical gradient within the subventricular zone, and it is required for the correct migration of neuroblasts in adult mouse brain [17], evoked a proposal that defects of ependymal ciliary motility might have a direct effect on neuronal development and cause JME [18]. Although in this study we actually found the expression of myoclonin1 at ependymal cilia of ventricle walls, it is still necessary to investigate the motility of ependymal cilia and the CSF flow in the *Efhc1* null-mutant mice. Much further studies are required to verify the above hypotheses and to elucidate the pathogenesis of epilepsies caused by the EFHC1 mutations.

Acknowledgments

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