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# Epilepsy protein myoclonin1 interacts with inositol 1,4,5-trisphosphate receptor and reduces calcium ions stored in endoplasmic reticulum

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## Abstract

**Aim:** Mutations in the *EFHC1* gene have been identified in patients with various epilepsies, including juvenile myoclonic epilepsy (JME). Mice with *Efhc1* deficiency also exhibit epileptic phenotypes. The protein myoclonin1, encoded by *EFHC1*, is not expressed in neurons but in cells with motile cilia, including choroid plexus and ependymal cells lining of brain ventricles. However, the molecular mechanisms by which *EFHC1* mutations cause epilepsy remain unclear. Because of the involvement of inositol 1,4,5-trisphosphate receptor type 1 ($IP_3R1$) in epileptic phenotypes and the involvement of myoclonin1 in calcium ions ($Ca^{2+}$) signaling, we investigated possible functional interplay between myoclonin1 and $IP_3R1$.

**Methods:** We performed immunohistochemical staining of brain tissues and co-immunoprecipitation assay of myoclonin1 and $IP_3R1$, and $Ca^{2+}$ imaging analyses using human HeLa.S3, mouse embryonic fibroblasts, or glial cells derived from *Efhc1* homozygous knockout (*Efhc1*$^{-/-}$) and wild-type (WT) littermates.

**Results:** Myoclonin1 was revealed to be well co-expressed with $IP_3R1$ at choroid plexus and ependymal cells, and these two proteins bound to each other. Endoplasmic reticulum (ER) of *Efhc1*-deficient mouse (

*Efhc1*$^{-/-}$) cells showed larger amounts of $Ca^{2+}$ than that of WT mice, and $IP_3$-induced $Ca^{2+}$ release (IICR) from ER was higher in *Efhc1*$^{-/-}$ cells than that of WT. Furthermore, myoclonin1 was revealed to interact with beta subunit of glucosidase II (PRKCSH), also known as a protein kinase C substrate 80K-H, which interacts with $IP_3R1$. Myoclonin1 further binds to $IP_3R2$ and $IP_3R3$.

**Conclusions:** These results indicate that myoclonin1 modulates ER-$Ca^{2+}$ homeostasis through interactions with $IP_3Rs$ and PRKCSH, and suggest that myoclonin1 dysfunctions cause impaired intracellular $Ca^{2+}$ mobilization. Its relevance to the epileptic phenotypes of patients with *EFHC1* mutations is now of interest.

## Keywords

*EFHC1*, myoclonin1, juvenile myoclonic epilepsy, inositol 1, 4, 5-trisphosphate receptor, protein kinase C substrate 80K-H, endoplasmic reticulum-calcium ions store

## Introduction

Human *EFHC1* gene encodes a 640 amino acid protein, myoclonin1, harboring three tandemly repeated DM10 domains and one EF-hand calcium-binding motif at C-terminus [1]. We originally and mistakenly reported that myoclonin1 is expressed in neurons of mouse brain [1], but our subsequent studies using *Efhc1*$^{-/-}$ mouse and a newly-developed mouse monoclonal antibody for myoclonin1 (6A3-mAb) revealed that myoclonin1 is actually not expressed in neurons but dominantly expressed in choroid plexus at embryonic stages, and at motile cilia of ependymal cells, tracheal motile cilia, and sperm flagella at postnatal stages [2–4]. *EFHC1* heterozygous missense mutations in patients with epilepsies, including juvenile myoclonic epilepsy (JME), have been repeatedly reported by us and many other groups [1, 5–15]. A missense mutation in *EFHC1* has also been identified homozygously in patients with intractable epilepsy of infancy [16]. We further reported that *Efhc1*-deficient mice exhibited spontaneous myoclonic seizures, increased seizure susceptibility to chemo-convulsant pentylenetetrazol (PTZ), decreased beating frequency of ependymal cells' cilia as well as that of choroid plexus epithelial one, and enlarged brain ventricles [3, 17].

We have shown that over-expression of myoclonin1 in mouse hippocampal primary culture neurons activates R-type voltage-dependent calcium channel ($Ca_v2.3$), resulting in excessive intracellular calcium ions ($Ca^{2+}$) influx and rapid cell-death, and these effects were compromised by *EFHC1* mutations found in JME patients [1]. We further reported that myoclonin1 interacts with transient receptor potential M2 (TRPM2) channel, which is $Ca^{2+}$-permeable cation channel, and potentiates the channel activity [18]. It is of interest whether myoclonin1 interacts with any other proteins to modulate $Ca^{2+}$ signaling and homeostasis.

Endoplasmic reticulum (ER)-$Ca^{2+}$ homeostasis is maintained by 1,4,5-trisphosphate receptors ($IP_3Rs$) and $IP_3$, which induce release of $Ca^{2+}$ from ER [19]. There are three subtypes of $IP_3Rs$, $IP_3R1$–3, in mammals with distinct regulation and distribution throughout the body. $IP_3R1$ is a predominant subtype in brain, and is involved in diverse functions such as development, axon-guidance, and cognition [20–23]. Because of the involvement of $IP_3R1$ in epileptic phenotypes [24] and the involvement of myoclonin1 in $Ca^{2+}$ signaling, as also suggested by its EF-hand $Ca^{2+}$ binding motif [1], we investigated possible functional interplay between myoclonin1 and $IP_3R1$. Here we report that in mouse brain, $IP_3R1$ is well co-expressed with myoclonin1 at choroid plexus and ependymal cells, and $IP_3R1$ binds to myoclonin1. Myoclonin1-deficiency significantly increased levels of ER-$Ca^{2+}$ store ($[Ca^{2+}]_{ER}$) and $IP_3$-induced $Ca^{2+}$ release (IICR) activity in cells from *Efhc1*$^{-/-}$ mice. In addition, we find that myoclonin1 binds to beta subunit of glucosidase II (PRKCSH), which has been known to interact with $IP_3R1$ [25]. Our findings indicate that myoclonin1 regulates ER-$Ca^{2+}$ homeostasis through interaction with $IP_3R1$.

## Materials and methods

### Mice

*Efhc1*- and *Ip$_3$r1*-deficient mice used for this study have been developed and reported previously [3, 24]. The heterozygous mice were maintained on C57BL/6J background. Heterozygous knockout *Efhc1*$^{+/-}$ or *Ip$_3$r1*$^{+/-}$ mice were interbred to obtain wild-type (WT), heterozygous, and homozygous knockout mice. Food and water were available ad libitum, and cages (of less than 5 animals) were kept at 23°C on a 12-h/12-h light/dark cycle, with the lights off at 20:00. When euthanasia of mice was necessary, cervical dislocation was performed in accordance with institutional guidelines.

### Antibodies

Mouse 6A3-mAb was reported previously [2, 4]. Following antibodies were also used: IP$_3$R1 [KM1112 [26], 18A10 [24], 10A6 [27], H-80 or C-20 (Santa Cruz Biotechnology, USA)], IP$_3$R2 (KM1083), IP$_3$R3 (KM1082 [26] and BD Transduction lab, USA), FLAG (Sigma, USA), Myc (Cell Signaling Technology, USA), GAPDH (Santa Cruz Biotechnology), PRKCSH (Santa Cruz Biotechnology), and DsRed (Invitrogen).

### Immunohistochemistry

Mice were deeply anesthetized with three types of mixed anesthetic agents (0.3 mg/kg medetomidine, 4.0 mg/kg midazolam, and 5.0 mg/kg butorphanol) and perfused intracardially with 0.9% NaCl, followed by 4% paraformaldehyde (TAAB, UK) in phosphate buffered saline (PBS). Preparations of mouse sagittal brain sections (paraffin) from embryonic day 14 (E14), unknown sex, and postnatal day 15 (P15) male mice and immunohistochemical staining were carried out as described previously [2, 3]. The 18A10 antibody for IP$_3$R1 and 6A3-mAb for myoclonin1 were used for staining. Colorimetric and fluorescence images were acquired using the AX80 light-microscope (Olympus, Japan) and TCS SP2 confocal laser scanning microscope (Leica, Germany), respectively.

### Expression constructs

*EFHC1* clone was described previously [1]. We amplified parts of *IP$_3$R1* (GenBank: NM_002222), *IP$_3$R2* (NM_002223), *IP$_3$R3* (NM_002224), and *PRKCSH* (NM_002743) from human adult brain cDNA by PCR and cloned them into pcDNA3-MycN, pcDNA3-FlagN, or pcDNA3-monomeric red fluorescence protein (mRFP) vectors. We introduced mutations by using QuickChange Site-Directed Mutagenesis kit (Agilent Technologies, USA) and confirmed nucleotide changes as well as integrity of full sequences by DNA sequencing.

### Cell culture and transfection

Mouse embryonic fibroblasts (MEFs) were prepared as described previously from E16 unknown sex mouse tail [28]. Once the cells were confluent in poly-L-Lysine coated 60 mm dish (IWAKI, Japan), it was stored at –80°C until needed. To obtain glial cells, culture medium of hippocampal neuron culture prepared from E16 unknown sex mouse brain [1, 2] was replaced with Dulbecco's Modified Eagle Medium (D-MEM) + 10% fetal bovine serum (FBS) + 30 U/mL penicillin and 30 mg/mL streptomycin (P/S) at 2 days in vitro (DIV) and cultured for 18 DIV. During the culture of MEFs or glial cells, visual inspection revealed no differences in cell viability or morphology between cells derived from WT and *Efhc1*$^{-/-}$ mice. The glial cells were harvested and stored in –80°C for subsequent assay. MEFs and HeLa.S3 cells were transfected with expression constructs using Lipofectamine LTX and PLUS reagent (Thermo Fisher Scientific, USA) according to the manufacturer's protocol. Briefly, the cells were exposed to the lipid-DNA complex in serum-free medium (Opti-MEM I; Thermo Fisher Scientific). Subsequently, the cells were incubated in D-MEM supplemented with 10% FBS. Transfected cells were analyzed 24–48 hours post-transfection. The HeLa.S3 cell line was originally obtained from the American Type Culture Collection and cultured under standard conditions. Cells were authenticated by short tandem repeat profiling and were free of mycoplasma contamination.

### Immunoprecipitation and western blot analysis

We washed transfected HeLa.S3 cells twice in PBS and lysed in lysis buffer [10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.5 or 1% NP40 and supplemented with protease inhibitor (Complete, Roche, Switzerland)]. Co-immunoprecipitation (co-IP) studies were carried out as described previously [1]. We probed blot with anti-FLAG, anti-Myc, or anti-DsRed antibodies and developed it with Western Lightning kit (Perkin Elmer, USA). Antibodies, $IP_3R1$ (KM1112, 10A6, H-80 or C-20, Santa Cruz Biotechnology), $IP_3R2$ (KM1083), $IP_3R3$ (KM1082 and BD Transduction lab), PRKCSH (Santa Cruz Biotechnology), myoclonin1 (6A3-mAb), and GAPDH (Santa Cruz Biotechnology) were used for western blot analysis. Wherever necessary, we stripped blots and re-probed with respective antibodies.

### $Ca^{2+}$ imaging

$Ca^{2+}$ imaging analyses were done as described previously [29]. We placed the cells on an inverted microscope IX70 (Olympus) and observed through an objective lens UApoN40XO340 (Olympus), etc. Some of the images were acquired using Olympus IX81-ZDC. The cells were illuminated by a xenon lamp through excitation filters, 340AF15 (Omega, USA) and 380HT15 (Omega), alternately. We used a computer-controlled filter exchanger Lambda 10-2 (Sutter, USA) to switch filters. We used a dichroic mirror and an emission filter 430DCLP (Omega) and 510WB40 (Omega), respectively. To eliminate $Ca^{2+}$ influx, we performed all experiments in absence of extracellular $Ca^{2+}$. We measured IICR with addition of 5 μM histamine [30] for HeLa.S3 cells or 300 nM bradykinin (BK) [31] for MEFs, whereas $[Ca^{2+}]_{ER}$ was measured with addition of 5 μM ionomycin [32]. All measurements shown were representative results from two to four independent experiments (used 3 dishes per condition were used in each experiment).

### Statistical analysis

Data were presented as mean ± standard error of the mean (s.e.m.) and statistical significance of differences between means was tested using unpaired *t*-test. Statistical significance levels were defined as $P < 0.05$.

## Results

### Myoclonin1 and $IP_3R1$ are co-expressed in choroid plexus and ependymal cells in brain

Immunohistochemical analyses revealed that myoclonin1 and $IP_3R1$ were abundantly expressed in choroid plexus at E14 (Figure 1A–C). As reported previously [2], the expression of myoclonin1 in choroid plexus was very transient, appearing at embryonic stages (E14–18) and gradually switching off until P15 (Figure 1D). In contrast, the $IP_3R1$ expression in choroid plexus was observed at E14 (Figure 1B and C) and postnatal period (Figure 1D), suggesting its continuous expression through embryonic and postnatal stages. At P15, myoclonin1 was prominently expressed in motile cilia of ependymal cells but also moderately in cell body (Figure 1D); the somatic expression was more prominent in the 3rd ventricle as reported previously [2]. The $IP_3R1$ expression was mainly observed in the somata and not in the cilia of ependymal cells (Figure 1D). Altogether, these results indicate that myoclonin1 and $IP_3R1$ proteins are co-expressed in embryonic choroid plexus and postnatal ependymal cells.

### Myoclonin1 interacts with all $IP_3R$ subtypes

We next investigated whether myoclonin1 binds to $IP_3Rs$. Because a number of proteins interact with either N- or C-terminal cytosolic regions of $IP_3R1$ and modulate its activity [19], we selected these regions to test their ability to interact with myoclonin1 for co-IP assay. It actually revealed that myoclonin1 binds to the C-terminal region of $IP_3R1$ but not to the N-terminal region or to Endophilin, a randomly selected protein with a molecular weight of 50 kDa used as a negative control (Figure 2A, B and Figure S1). Using a series of C-terminal deletion constructs, we further narrowed down the interacting region to amino acid residues (a.a.) 2565–2625 by co-IP assay (Figure 2A and C). Inversely, co-IP analyses using a series of deletion fragments of myoclonin1 revealed that each of the three DM10 domains of myoclonin1 independently bound to $IP_3R1$ (Figure 2D and E). C-terminal regions of three $IP_3R$ subtypes ($IP_3R1$, $IP_3R2$, $IP_3R3$) are

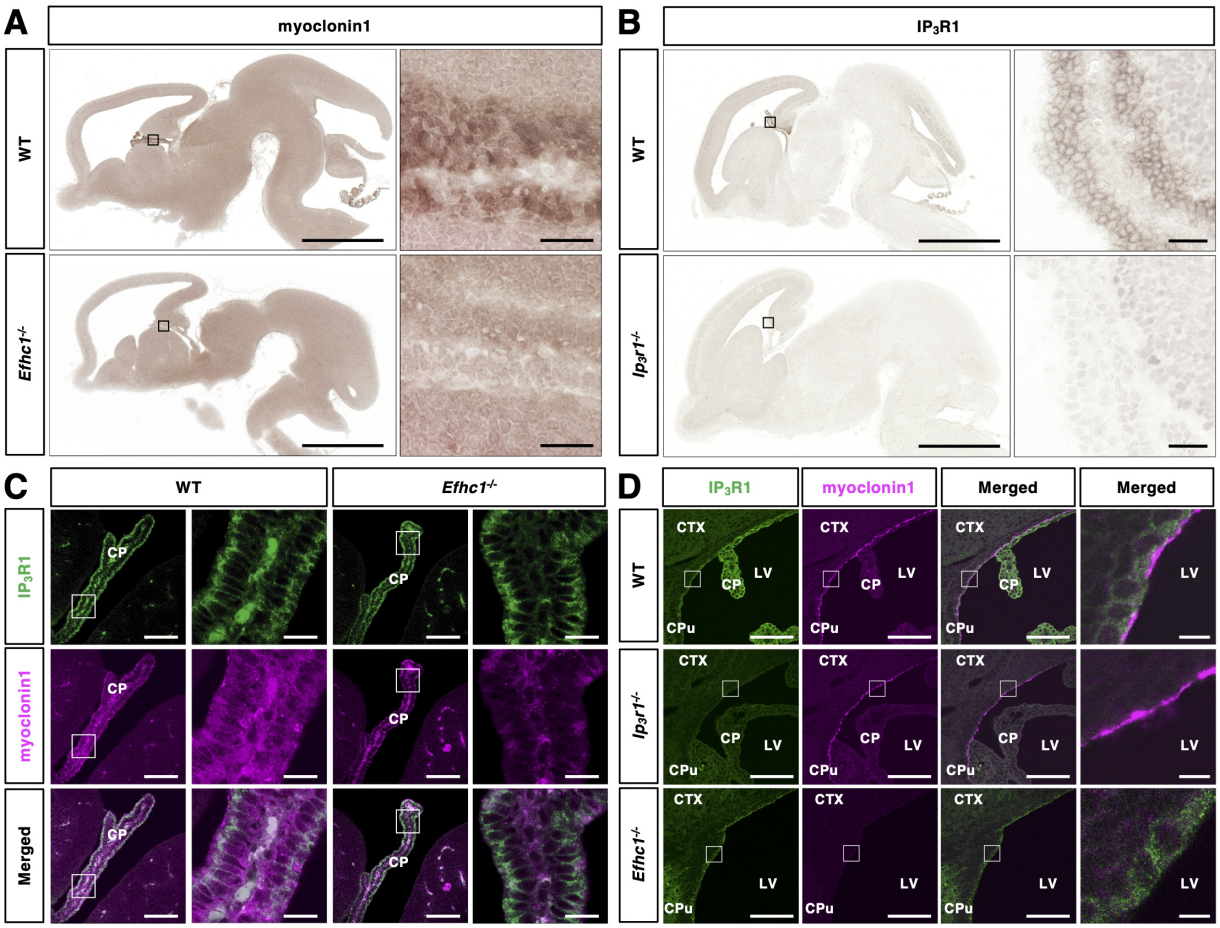


**Figure 1. Co-expressions of myoclonin1 and $IP_3R1$ in embryonic choroid plexus and postnatal ependymal cells.** (**A**–**C**) Colorimetric or fluorescent immunohistochemical analyses showed co-expression of myoclonin1 (brown in **A**, magenta in **C**) and $IP_3R1$ (brown in **B**, green in **C**) in choroid plexus of mouse brain at E14 (sagittal brain sections, *n* = 2 WT, 2 *Efhc1*$^{-/-}$, 2 *Ip*$_3$*r1*$^{-/-}$). (**D**) Co-expressions of $IP_3R1$ (green) and myoclonin1 (magenta) were observed in ependymal cells at P15 (sagittal sections, *n* = 2 WT, 2 *Efhc1*$^{-/-}$, 2 *Ip*$_3$*r1*$^{-/-}$). Boxed areas are enlarged. CP: choroid plexus; CPu: caudate putamen; CTX: cortex; E14: embryonic day 14; $IP_3R1$: 1,4,5-trisphosphate receptor 1; LV: lateral ventricle; P15: postnatal day 15; WT: wild-type. Scale bars = 1 mm (low-magnification images in **A**, **B**), 25 μm (high-magnification images in **A**, **B**), 100 μm (low-magnification images in **C**, **D**), 15 μm (high-magnification images in **C**), and 8 μm (high-magnification images in **D**)

highly conserved (Figure S2A), and a co-IP assay revealed that all C-termini of these $IP_3R$ subtypes similarly bound to myoclonin1 (Figure S2B).

## Myoclonin1 interacts with an $IP_3R$ regulator PRKCSH

PRKCSH is a gene that encodes the PRKCSH, also known as 80K-H, involved in protein folding in the ER. A previous report showed that PRKCSH was identified as an interacting protein of $IP_3Rs$ by yeast two-hybrid screening and regulates its activity [25]. Because binding sites of $IP_3R1$ for PRKCSH and myoclonin1 look overlapping, we investigated interactions among these three proteins. As previously reported, co-IP revealed that a.a. 2555–2594 of $IP_3R1$ bound to PRKCSH (Figure S3). A co-IP of myoclonin1 and a series of PRKCSH deletion constructs, including two clones, a.a. 32–528 and 184–528, which were identified as fragments binding to $IP_3R1$ [25] and additional newly designed one, revealed that a.a. 400–448 of PRKCSH binds to myoclonin1 (Figure 3A–E and Figure S1). Similarly to $IP_3R1$ (Figure 2D and E), the three DM10 domains of myoclonin1 independently bound to PRKCSH (Figure 3F and G).

## Myoclonin1 regulates $[Ca^{2+}]_{ER}$

We subsequently measured levels of $[Ca^{2+}]_{ER}$ in MEFs and glial cells derived from *Efhc1*$^{-/-}$ and WT littermates by applying ionomycin, an ionophore that induces formation of $Ca^{2+}$-permeable pores in cellular membranes, leading to complete emptying of $[Ca^{2+}]_{ER}$ independently of $IP_3Rs$ activation [32]. The assay showed that $[Ca^{2+}]_{ER}$ levels of the *Efhc1*$^{-/-}$ cells were significantly higher compared to WT (Figure 4A and

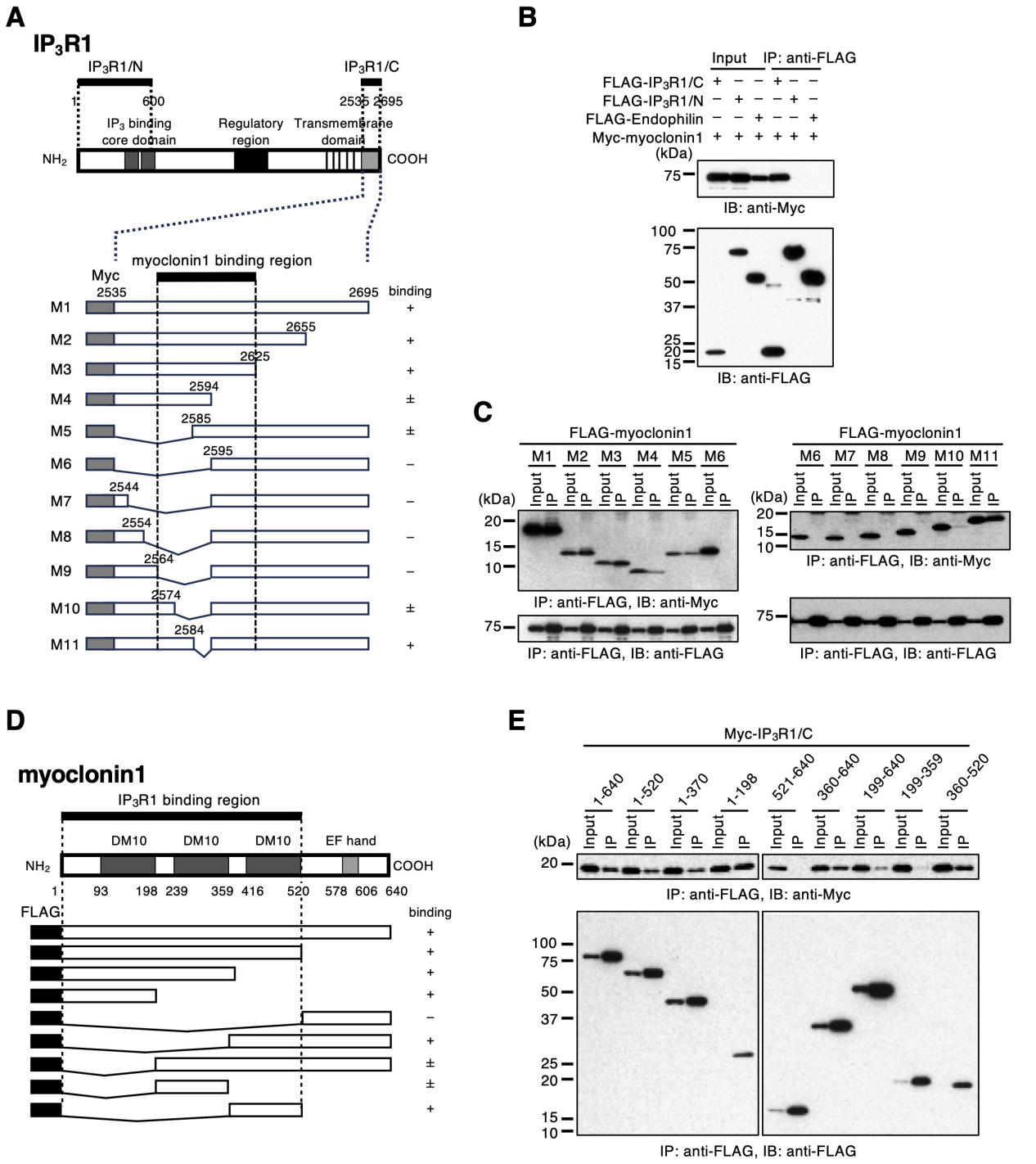


**Figure 2. Myoclonin1 interacts with C-terminus of $IP_3R1$.** (**A**) Schematic diagrams of $IP_3R1$, its deletion constructs, and binding activity. Bold black lines indicate $IP_3R1$ N- (a.a. 1–600) and C- (a.a. 2535–2695) terminus (top). A segment (a.a. 2565–2625; black line, middle) in C-terminal region of $IP_3R1$ contains a binding site for myoclonin1. (**B**) Western blots of co-IP analysis showing that myoclonin1 interacted with $IP_3R1$ C-terminus but not with N-terminus and Endophilin (negative control). (**C**) The interacting region of $IP_3R1$ C-terminus to myoclonin1 was narrowed down to a.a. 2565–2625. (**D**) Schematic diagram of myoclonin1 deletion constructs and binding activity. A bold black line (top) contains a binding site for $IP_3R1$. (**E**) Each of the three DM10 domains of myoclonin1independently bound to $IP_3R1$. The degree of interaction is indicated by +, ±, or – (**A**, **D**). /C: C-terminal; /N: N-terminal; a.a.: amino acid residues; co-IP: co-immunoprecipitation; IB: immunoblot; IP: immunoprecipitation; $IP_3$: 1,4,5-trisphosphate; $IP_3R1$: 1,4,5-trisphosphate receptor 1. Input: 5% of cell lysate

B). We also measured IICR by addition of BK, which stimulates phospholipase C (PLC) metabolism of phosphatidylinositol-4,5-bisphosphate (PIPP) to $IP_3$ [31], and observed a higher IICR level in *Efhc1*$^{-/-}$ MEFs than in WT (Figure 4C). Western blot analyses revealed that myoclonin1 expression was abrogated in the *Efhc1*$^{-/-}$ MEFs, while those of $IP_3Rs$ and PRKCSH remained unchanged (Figure S4). These results indicate that myoclonin1 deficiency enhances $[Ca^{2+}]_{ER}$ and IICR.

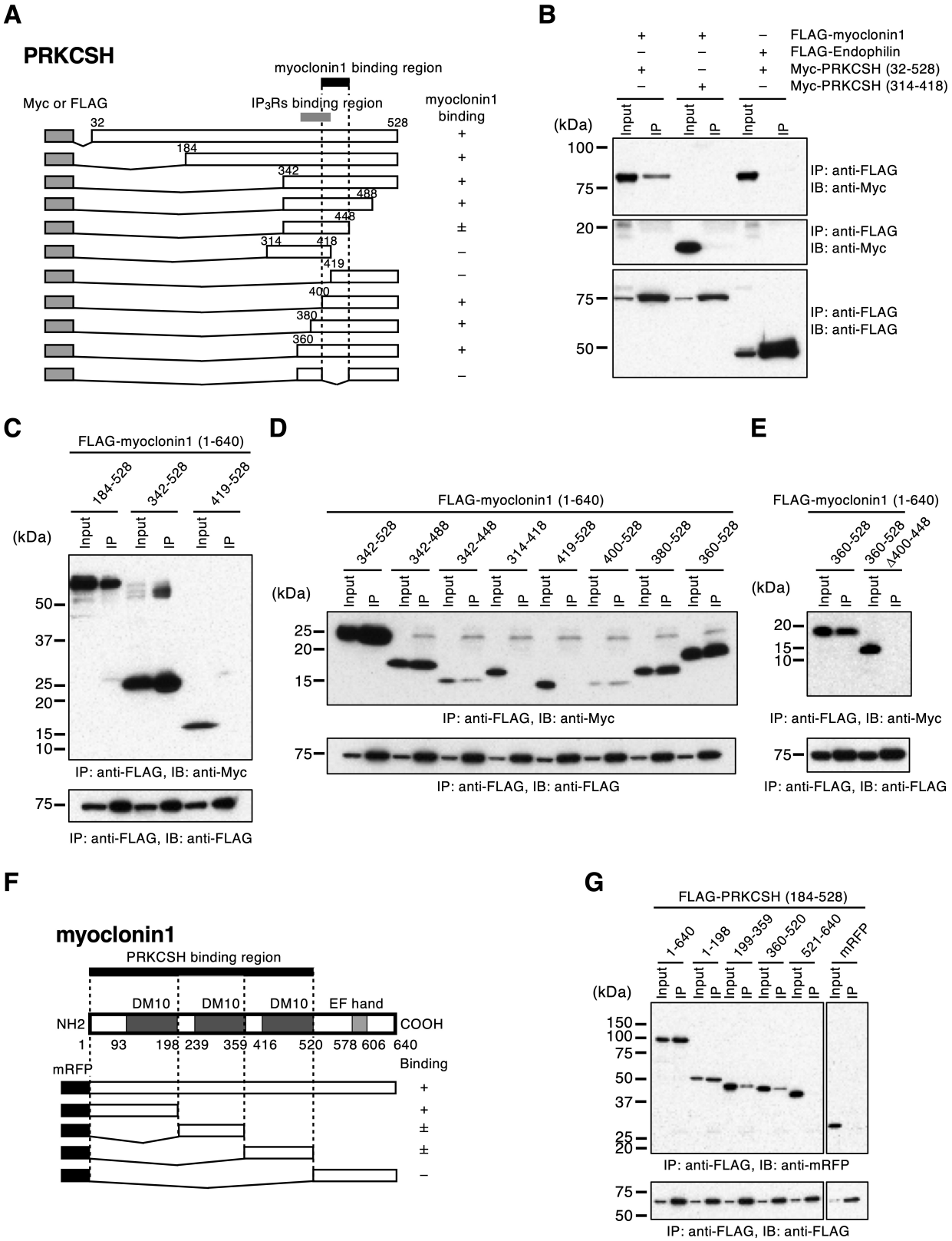


**Figure 3. Myoclonin1 interacts with PRKCSH at its interaction site for $IP_3R1$.** (**A**) Schematic diagram of PRKCSH deletion constructs and binding activity. A short segment (a.a. 400–448; black line) in PRKCSH contains binding site for myoclonin1. Other one (a.a. 365–418; gray line) has been reported as a binding site for $IP_3Rs$ [25]. (**B**–**E**) A region of PRKCSH a.a. 400–448 is critical to bind to myoclonin1. (**F**) Schematic diagram of myoclonin1 deletion constructs and binding activity. A bold black line (top) contains a binding site for PRKCSH. (**G**) PRKCSH is bound to myoclonin1 similarly to $IP_3R1$. The degree of interaction is indicated by +, ±, or – (**A**, **F**). a.a.: amino acid residues; IB: immunoblot; IP: immunoprecipitation; $IP_3R1$: 1,4,5-trisphosphate receptor 1; PRKCSH: beta subunit of glucosidase II. Input: 5% of cell lysate; Δ: internal deletion

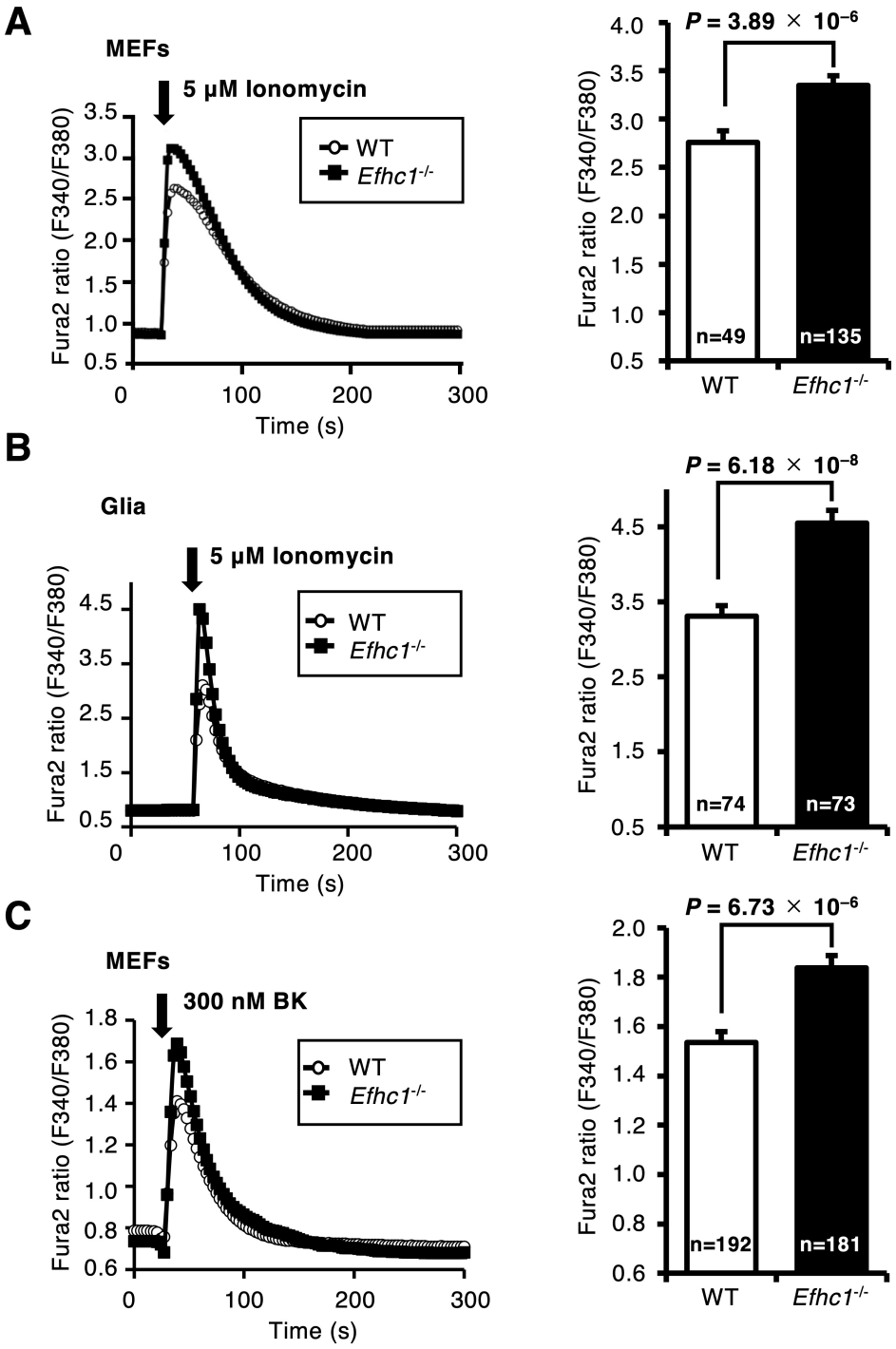


**Figure 4. Myoclonin1 deficiency enhances $[Ca^{2+}]_{ER}$ and IICR.** Ionomycin releasable $[Ca^{2+}]_{ER}$ was significantly higher in *Efhc1*$^{-/-}$ cells than in WT. (**A**) $n$ = 49 WT, 135 *Efhc1*$^{-/-}$ MEFs, unpaired $t$-test, WT vs. *Efhc1*$^{-/-}$, $P = 3.89 \times 10^{-6}$, df = 182. There was a significant difference between the mean values. (**B**) $n$ = 74 WT, 73 *Efhc1*$^{-/-}$ glial cells, unpaired $t$-test, WT vs. *Efhc1*$^{-/-}$, $P = 6.18 \times 10^{-8}$, df = 145. There was a significant difference between the mean values. (**C**) IICR induced by BK was significantly higher in *Efhc1*$^{-/-}$ MEFs than in WT ($n$ = 192 WT, 181 *Efhc1*$^{-/-}$, unpaired $t$-test, WT vs. *Efhc1*$^{-/-}$, $P = 6.73 \times 10^{-6}$, df = 371). There was a significant difference between the mean values. All measurements shown were representative results from two to four independent experiments (used 3 dishes, cells derived from 2 independent animals per genotype, were used in each experiment). Arrows indicate time point of addition of ionomycin or BK. df: degrees of freedom; $n$: total number of cells measured; $P$: $P$-value; IICR: 1,4,5-trisphosphate-induced calcium ions release; WT: wild-type; $[Ca^{2+}]_{ER}$: endoplasmic reticulum-calcium ions store; MEFs: mouse embryonic fibroblasts; BK: bradykinin

We further investigated whether myoclonin1 reduces $[Ca^{2+}]_{ER}$ and IICR. Human HeLa.S3 cells were transfected with mRFP-fused myoclonin1, which did not affect expression of $IP_3$Rs and PRKCSH (Figure S5A). $Ca^{2+}$ imaging revealed that $[Ca^{2+}]_{ER}$ by applying ionomycin and IICR by histamine, which generates $IP_3$ through activation of PLC [30], were significantly decreased by myoclonin1 over-expression (Figure S5B

and S5C). To confirm the effect, myoclonin1 was further re-introduced into *Efhc1*$^{-/-}$ MEFs. $Ca^{2+}$ imaging also revealed that both $[Ca^{2+}]_{ER}$ by ionomycin and IICR by BK were significantly attenuated in mRFP-myoclonin1 expressing MEFs compared to control one (Figure S5D and S5E). Together, these results indicate that myoclonin1 lowers $[Ca^{2+}]_{ER}$ and is involved in maintenance of ER-$Ca^{2+}$ homeostasis.

## Discussion

Here in this study, we showed that myoclonin1 forms a protein complex with $IP_3Rs$ and PRKCSH, and regulates $IP_3$-mediated $Ca^{2+}$ release. Together with our previous observations of the bindings of myoclonin1 with $Ca_v2.3$ [1] and with TRPM2 [18], the results suggest that myoclonin1 is involved in intracellular $Ca^{2+}$ mobilization.

A notable observation is that myoclonin1 and $IP_3R1$ are co-expressed in ciliated cells, such as choroid plexus and ependymal cells in the brain. We also found that myoclonin1 deficiency enhances $[Ca^{2+}]_{ER}$ and IICR in cells from *Efhc1*-deficient mice. It has previously been reported that (1) $Ca^{2+}$ initiates beating of cilia and flagella [33], and (2) elevation of cytosolic $Ca^{2+}$ contributes to increased cilia beating frequency (CBF) of ependymal cells [34, 35]. Moreover, blockage of cilia-mediated cerebrospinal fluid (CSF) inflow has been known to be sufficient to predispose another mouse model to seizures [36]. These may let us assume that reduced CBF by decreased cytosolic $Ca^{2+}$ causes seizures in the *Efhc1*-deficient mice. However, in our previous study of the mice, we showed that reduced CBF was observed only in homozygous *Efhc1*$^{-/-}$ mice but not in the heterozygous ones (*Efhc1*$^{+/-}$), which still showed seizure phenotypes such as frequent spontaneous myoclonus and increased seizure susceptibility to PTZ [3]. These results indicate that there is considerable inconsistency between seizure phenotypes and CBF in heterozygous *Efhc1*$^{+/-}$ mice. Taken together, these observations may suggest that blockage of cilia-mediated CSF inflow and reduction of CBF themselves may not or minimally contribute directly to the seizure phenotypes observed in the *Efhc1*-deficient mice, and an alternative pathway (see below) would be required to explain the molecular basis of epilepsies in the mice.

We have reported that myoclonin1 is dominantly expressed in choroid plexus epithelial cells at embryonic stage [2], and in this study, we found that myoclonin1 and $IP_3R1$ are well co-expressed in those cells as well, and these two proteins bind each other as mentioned above. In addition, we also have reported that CBF of neonatal choroid plexus epithelial cells from *Efhc1*-deficient mice was significantly lower than that of WT mice [17]. Based on these findings, we assume that myoclonin1 possibly plays critical role in choroid plexus epithelial cells. Further, the cells synthesize neurotrophic factors and other signaling molecules, including insulin, that are secreted in response to increased intracellular $Ca^{2+}$ levels [37]. Previous study has shown that $IP_3R1$-deficient mice suffer from epilepsy [24]. In our $Ca^{2+}$ imaging analyses, we observed increased $[Ca^{2+}]_{ER}$ and enhanced IICR through $IP_3R1$ in cells from *Efhc1*-deficient mice. These findings suggested that impaired $IP_3R1$-mediated $Ca^{2+}$ signaling may disrupt the secretion of signaling molecules from choroid plexus cells. Such disruption could lead to alterations in synaptic plasticity (long-term potentiation/long-term depression) and abnormal neural circuit formation, which may also underlie the epileptic phenotypes in these mice.

In resting cells, $[Ca^{2+}]_{ER}$ is tightly regulated by a balance between $Ca^{2+}$ release via $IP_3Rs$, reuptake by SERCA pumps, and the ER translocon [38–40]. In myoclonin1 deficient models, elevated $[Ca^{2+}]_{ER}$ and enhanced IICR suggest that myoclonin1 may modulate ER-$Ca^{2+}$ homeostasis by influencing either $Ca^{2+}$ leak or SERCA activity. Myoclonin1 deficiency could reduce ER-$Ca^{2+}$ leak, leading to excessive $[Ca^{2+}]_{ER}$ accumulation, which may then trigger a compensatory downregulation of SERCA activity to restore $Ca^{2+}$ balance. This mechanism is consistent with previous report that SERCA function is sensitive to luminal $Ca^{2+}$ levels [38]. Thus, myoclonin1 likely fine-tunes ER-$Ca^{2+}$ dynamics through its influence on leak and reuptake mechanisms.

Although we previously reported that myoclonin1 interacts with $Ca_v2.3$ [1] and TRPM2 [18], $Ca_v2.3$ is mainly expressed in neurons and may not be much expressed in choroid plexus and ependymal cells. Therefore, we currently assume that it may not be critically involved in the JME pathology. Meanwhile,

TRPM2 is expressed in ependymal cells and therefore it is highly possible that TRPM2 is also involved in the pathology through myoclonin1-$IP_3R1$ pathway.

In addition to *EFHC1*, we have identified and reported multiple pathogenic mutations of *CILK1* (ciliogenesis associated kinase 1) gene, formerly known as *ICK* (intestinal-cell kinase), in patients affected with JME from a number of families [41]. High level of CILK1 expression was observed in ependymal and choroid plexus cells in postnatal mouse brain [41, 42]. Moreover, pathogenic mutations of several other ciliogenesis associated proteins, CDKL5/STK9 [43, 44], SDCCAG8 [45, 46], PRICKLE1 and PRICKLE2 [47, 48] have also been reported in patients with epilepsies, including JME. These observations support the notion that functional impairments of the cells with motile cilia in brain, such as cell-cell communication, synthesis of neurotrophic factors and signaling molecules, secretion of the molecules, and CSF or ion homeostasis, are likely the basis of epileptic seizure phenotypes in patients with *EFHC1* mutations.

To further explore the link between seizures and altered intracellular $Ca^{2+}$ signaling in non-neural cells such as choroid plexus and ependymal cells, future studies should examine neural activity and $Ca^{2+}$ imaging in acute brain slices and in vivo. Furthermore, testing whether pharmacological modulation of $IP_3Rs$ can restore $Ca^{2+}$ homeostasis and attenuate seizure phenotypes may provide mechanistic insights and identify potential therapeutic targets for JME. Given that JME is characterized by thalamocortical circuit hypersynchrony [49], future investigations at the circuit level would be of great interest to further elucidate how intracellular calcium dysregulation contributes to network-level pathophysiology.

Our results presented here indicate that myoclonin1 participates in controlling intracellular $Ca^{2+}$ mobilization in a manner that involves $IP_3Rs$. This could be molecular basis underlying pathology of epilepsies caused by *EFHC1* mutations and potential candidate for prevention strategies and treatments of epilepsies.

## Abbreviations

$[Ca^{2+}]_{ER}$: endoplasmic reticulum-calcium ions store

6A3-mAb: monoclonal antibody for myoclonin1

a.a.: amino acid residues

BK: bradykinin

$Ca^{2+}$: calcium ions

$Ca_V2.3$: R-type voltage-dependent calcium channel

CBF: cilia beating frequency

*CILK1*: ciliogenesis associated kinase 1

co-IP: co-immunoprecipitation

CSF: cerebrospinal fluid

DIV: days in vitro

D-MEM: Dulbecco's Modified Eagle Medium

E14: embryonic day 14

ER: endoplasmic reticulum

FBS: fetal bovine serum

IICR: 1,4,5-trisphosphate-induced calcium ions release

$IP_3$: 1,4,5-trisphosphate

$IP_3Rs$: 1,4,5-trisphosphate receptors

JME: juvenile myoclonic epilepsy

MEFs: mouse embryonic fibroblasts

mRFP: monomeric red fluorescence protein

P15: postnatal day 15

PBS: phosphate buffered saline

PLC: phospholipase C

PRKCSH: beta subunit of glucosidase II

PTZ: pentylenetetrazol

TRPM2: transient receptor potential M2

WT: wild-type

## Supplementary materials

The supplementary figures for this article are available at: https://www.explorationpub.com/uploads/Article/file/100699_sup_1.pdf.

## Declarations


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### Author contributions

TS: Conceptualization, Investigation, Writing—original draft, Visualization, Funding acquisition. KA: Conceptualization, Investigation, Writing—original draft, Visualization. HM: Conceptualization, Methodology, Investigation, Resources, Writing—review & editing, Visualization. II: Investigation, Writing—review & editing. KM and AM: Resources, Writing—review & editing, Funding acquisition. KY: Conceptualization, Resources, Writing—review & editing, Supervision, Funding acquisition. All authors read and approved the submitted version.

### Conflicts of interest

The authors declare that they have no conflicts of interest.

### Ethical approval

The animal study was approved by the Animal Experiment Committee of RIKEN (W2019-1-006) and by the Institutional Animal Care and Use Committee of the Nagoya City University (NCU; 23-025). All animal breeding and experimental procedures were performed in accordance with the ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines and regulations of the RIKEN and the NCU. This animal study adheres to the Guide for the Care and Use of Laboratory Animals.

### Consent to participate

Not applicable.

### Consent to publication

Not applicable.

### Availability of data and materials

The raw data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.


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## Publisher's note



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