Abstract

This chapter presents recent works on Myoclonin1/EFHC1 a protein encoded by an epilepsy causing gene of juvenile myoclonic epilepsy (JME), one of the most frequent forms of idiopathic or genetic generalized epilepsies.

Myoclonin1/EFHC1 is a microtubule-associated protein involved in the regulation of cell division. In vitro, EFHC1 loss of function disrupted mitotic spindle organization, impaired M phase progression, induced microtubule bundling and increased apoptosis. EFHC1 impairment in the rat developing neocortex by ex vivo and in utero electroporation caused a marked disruption of radial migration. This effect was a result of cortical progenitors failing to exit the cell cycle. On the other hand, defects in the radial glia scaffold organization and in the locomotion of postmitotic neurons. Mutant analysis of Defhc1 loss- and gain-of-function alleles in vivo in Drosophila revealed a number of neuronal defects, including abnormal synaptic development characterized by extensive satellite bouton formation, increased frequency of spontaneous neurotransmitter release, and aberrations in dendritic arbour morphogenesis.

Thus, Myoclonin1/EFHC1 is a regulator of cell division and neuronal migration during cortical development synaptic bouton and dendritic morphogenesis. Disruption of these properties lead to JME, being now therefore considered as a developmental disease.

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Juvenile myoclonic epilepsy (JME) is the most frequent form of idiopathic/genetic generalized epilepsy. It accounts for 2–12% of all epilepsies. JME symptoms of myoclonias and tonic-clonic convulsions appear in adolescence in an otherwise normal person with normal neurological and cognitive functions (Delgado-Escueta and Bacsal, 1984). Five percent of these patients, five percent of their affected family members and eight percent of a 252 patient...
cohort followed for over 20 years only have seizures when triggered by external factors such as alcohol use, fatigue, menstruation and sleep deprivation.

To understand the disease mechanisms underlying the stages of susceptibility and epileptogenesis in JME, many have tried to define its complex heritability and identify the genes corresponding to the fifteen chromosomal loci so far linked to the disease\(^2\) (see also chapter \ldots in this book).

In 2004, Suzuki et al. identified several heterozygous missense mutations in a gene called \(EFHC1\)\(^3\) in different unrelated families with JME probands. Since then, heterozygous non-sense, deletion frame shifts and novel missense mutations have been identified in various populations of Italy, Austria and Chile\(^4\)\(^-\)\(^7\). Nine percent of sporadic JME cases detected in families consecutively seen in epilepsy clinics of Mexico and Honduras and 3% of clinic patients from Japan carry mutations in \(EFHC1\). This represents the highest number and percentage of mutations found for a juvenile myoclonic epilepsy causing gene of any population group\(^7\). The gene encodes a 75-kDa protein with three DM10 domains of unknown function and a single EF-hand motif, a \(\text{Ca}^{2+}\)-binding domain. The transcript is observed in many cell type of human tissue including brain (in particular in ependymal or periventricular cells) but also best expressed in dividing cells with highest levels in lung and testis\(^3\). It was first proposed that \(EFHC1\) was “pro-apoptotic” when overexpression in hippocampal neurons \textit{in vitro} induced apoptotic cell death. This effect was significantly reduced by any of the five mutations associated with JME. Patch-clamp analysis of BHK (Baby Hamster Kidney) cells transfected with \(\text{Ca}_{2.3}\) VDCC (voltage-dependent calcium channel) and \(EFHC1\) showed significantly increased R-type \(\text{Ca}^{2+}\) currents. So, the pro-apoptotic effect of \(EFHC1\) was assigned to this enhancing effect on \(\text{Ca}^{2+}\) through \(\text{Ca}_{2.3}\) VDCC\(^3\).

In 2005, another research group pointed out that \(EFHC1\) is orthologous to Rib72, an axonemal protein of \textit{Chlamydomonas reinhardtii}. They demonstrated that \(EFHC1\) is abundantly expressed in mouse tissues that have motile cilia or flagella, including the brain, and suggested that it plays a role in the intrinsic properties of these organelles\(^8\).

One of our laboratories previously reported that the subcellular distribution of \(EFHC1\) in different cell lines varied during the cell cycle. In interphase cells, the protein is present in the cytoplasm and nucleus, except nucleoli and is particularly concentrated at the centrosome. During mitosis, \(EFHC1\) is localized at spindle poles of the mitotic spindle and also at the midbody during cytokinesis\(^9\). These results suggest that \(EFHC1\) could play an important role during cell division and in particular during brain development since mRNA expression is higher at embryonic stages as compared to adult\(^10\). More recently, we demonstrated that \(EFHC1\) is a microtubule-associated protein (MAP) playing a key role in neuronal migration\(^11\). In this chapter we review these putative roles of \textit{Myoclonin 1} / \(EFHC1\) or during brain development and during adulthood. We posit an hypothesis that JME is a developmental disease involving neuronal migration and synaptic bouton and dendritic morphogenesis.

**EFHC1/MYOCLONIN1, A PROTEIN OF UNKNOWN FUNCTION**

\(Myoclonin 1\) / \(EFHC1\) gene is located on chromosome 6 (6p11–12) between markers D6S1960 and D6S11024, spans 72 kb and contains 11 exons. This gene encodes a protein of 640 amino acids. A domain search identified three tandemly repeated so called DM10 domains, a motif with unknown function. This protein also contains a single EF-hand, a well known \(\text{Ca}^{2+}\)-binding motif, from which it was named \(EFHC1\) for \textit{EF-hand Containing 1} (Figure 1). This motif is located at the C-terminus between amino acid 578 and 606 and encoded by a nucleotide sequence present in exon 10.
The EF-hand motif

Krestinger, when exploring the crystal structure of paravalbumin, first described the EF-hand domain\textsuperscript{12}. It corresponds to a helix-loop-helix motif, the loop constituted by 12 amino acids, capable of binding Ca\textsuperscript{2+} and linking two perpendicular alpha helix named E and F (Figure 2). The EF-hand denomination comes from the fact that this motif can be represented with a symbolic right hand in which the E helix corresponds to the index and the F helix to a thumb\textsuperscript{13}. The EF-hand proteins belong to the family of Ca\textsuperscript{2+} binding proteins, which include more than 1000 members classified in 66 different sub families. One distinguishes two groups according to their cellular properties: on one hand, the buffer proteins implicated in the transport and regulation of intracellular calcium concentration (calbindin, calretinin for instance) and, on the other hand, the calcium sensors that transfer informations from calcium influx to specific intracellular molecular pathways (calmodulin, troponinC)\textsuperscript{14}. Most of the EF-hand proteins contain however single or multiple pairs of such helix-loop-helix motifs. They are typically present in globular forms and create a cooperative calcium link between the two motifs, resulting in an increased affinity of the protein for the calcium ion\textsuperscript{15}. 

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*Figure 1. Schematic representation of the EFHC1/Myoclonin1 gene, the long and short forms of the EFHC1 protein and the different mutations found co segregated with the JME phenotype*

The transcript undergo alternative splicing in exon 4, resulting in a C-terminally truncated protein, eliminating the EF-hand domain and two DM10 sequences. This last short form therefore only contains 278 amino acids, the first 240 being common with the entire molecule.

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*Myoclonin1/EFHC1 in cell division, neuroblast migration, synapse/dendrite formation in juvenile myoclonic epilepsy*
Figure 2. The EF-hand domain

In A: schematic representation of a EF hand motif constituted by two helices E and F perpendicularly placed and linked by a 
$\text{Ca}^{2+}$ binding 12 amino acids sequence. In B: symbolic representation of the same motif as a right hand in which the E helix 
correspond to the index and the F one to the thumb.

At the loop level, the residues in 1, 3, 5, 7, 9 and 12 position, also named $+X$, $+Y$, $+Z$, $-Y$, $-X$, $-Z$ are responsible for binding calcium, forming a geometrical pentagonal bi pyramidal structure (Figure 3). The residues $+X$, $+Y$ and $+Z$ provide a coordination link, while the residue $-Z$ must provide two of them via the carboxyl groups of its lateral chain. The residue $-Y$ regulate calcium via an oxygen of its principal chain and the residue $-X$ links indirectly the 
calcium by molecule of water. The most conserved amino acids of the loop are the aspartate in $+X$ position and the aspartate or the glutamate in $-Z$ situation. It is important to notice that 
the EF-hand motifs exists in non canonical positions in which the length or the composition 
of the calcium binding loop vary, implicating a calcium binding mechanism with a different 
system of coordination $^{16}$. The EF-hand domain of EFHC1 is probably one of them since $+Y$ and $-Z$ residues do not correspond to the consensus sequence of a canonical domain. Moreover, 
the EFHC1 protein only contains one single EF-hand motif. Therefore, these observations show 
that EFHC1 is a non conventional EF-hand protein.
The DM10 domains

The DM10 domains contain appreciatively 105 residues, and are so far of unknown function. The DM10 domains proteins are found in classes as various as mammals (human, rat, mice, dogs, cows), fishes (Tetraodon, Danio rerio), insects (Anopheles, Drosophila), amphibians (Xenopus tropicalis), protists (Trypanosomia, Giardia Lamblia, Schistosoma japonicum, Chlamydomonas reinhardtii) and nematodes (C.Elegans). In Chlamydomonas and in mammals, these motifs are only found in two types of proteins. The first are type 7 diphosphate nucleosides kinases (NDK7) exhibiting a N-terminal DM10 domain and two catalytic NDK motifs. The NDK catalyse the transfer of the ATP terminal gamma-phosphate on the GDP to form GTP and ADP. These proteins play crucial roles in cellular proliferation, differentiation, development and progression of tumors. The second family consists on proteins with three DM10 domains and one C-terminal EF-hand motif among which one finds EFHC1, its paralog EFHC2, its ortholog Rib72 in Chlamydomonas and EFHC1 and EFHC2 in drosophila. In contrast with the NDK’s, these proteins do not exhibit any enzymatic activities. Their properties remained to be elucidated and have received, more recently, new enlightenments which will be reviewed below.

The phylogenetic analysis of these different proteins shows that each of the 3 DM10 domains are more strongly conserved between the different proteins than among them for the same protein. For instance, the first DM10 domain of EFHC1 is more similar to the first DM10 of Rib72 and EFHC2 as compared with the second or third domains of EFHC1. In Chlamydomonas, the proteins containing DM10 domains are strongly linked to the flagellar axonema. This suggests that these domains could act as flagellar NDK regulators or as specific subunits implicated in axonemal assembly or in the localisation of these proteins in this structure.
EFHC1/MYOCLOMIN1, A WIDELY DISTRIBUTED PROTEIN

So far, the transcript of the Myoclonin 1 has been detected in several structures in various adult and developing tissues or cell models in culture, using either PCR, northern blotting or in situ hybridization. This last approach however raised unconvincing results in determining the presence of the transcript in several tissue, including Brain, although both PCR on brain tissue samples and cells in culture or northern blotting from various tissues unequivocally demonstrated its expression. This could indicate a particularly low level of transcription or a fast turnover of the mRNA encoding the Myoclonin1 protein.

Accordingly, using an anti-myoclonin1 polyclonal antibody, the protein EFHC1 has been, in a first approximation, detected by immunohistochemical analyses in several structures of adult mouse brain including hippocampus, cerebellum, cerebral cortex, thalamus, hypothalamus, amygdala and upper brainstem. In addition signals were observed in soma and dendrites of both in situ and hippocampal primary culture neurons.

Four years later, however, the same authors created Efhc1 knock-out mouse and by using this null-mutant mouse as a negative control they found that the signals obtained by this above mentioned polyclonal antibody remained in the null-mutant and that the signals were largely non-specific. Revisiting the distribution of the protein using a newly generated anti-myoclonin1 monoclonal antibody (yielding more specific myoclonin1 signals in Western blot and immunohistochemical analyses), they 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 were, therefore, inconsistent with their previous report. Using a new monoclonal antibody (6A3-mAb) to mouse myoclonin1 and verifying its specificity in Western blot and immunohistochemical analyses by using Efhc1-null mice as negative controls, this 6A3-mAb specifically recognized myoclonin1 protein at embryonic choroid plexus and postnatal ependymal cells. 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.

We then have revisited the distribution and cellular localization of EFHC1 in adult mouse brain studied by immunohistochemistry using a previously well validated anti-EFHC1 antibody. In the adult mouse neocortex, the labeling was present in cells of all cortical layers particularly in cells resembling pyramidal neurons in cortical layer V and in the piriform cortex. Within the hippocampus, neuronal cells in the CA and the dentate gyrus (DG) regions were stained. Expression was not restricted to the pyramidal cells layer but was also observed in cells located in the stratum oriens, radiatum and lacunosum moleculare. At higher magnification, the protein appeared evenly distributed in the cytoplasm. A labeling was also clearly observed in Purkinje cells of the cerebellum and, in particular, the first order apical dendrites. In all these experimental conditions, the immunostaining disappeared or was considerably attenuated after preabsorption of the antibody with purified immunizing peptide.

EFHC1 immunofluorescence was in particular clearly observed in cerebellar Purkinje cells with a labeling of the soma, the nucleus and the dendritic arborisations extending throughout the molecular layer. A faint signal was also present in the molecular and granule cell layers. The last one was identified by co-labeling using anti-NeuN antibody.

Double immunofluorescence was performed in order to specify the nature of EFHC1-expressing cells in the adult mouse cortex. Counting the number of double labeled cells indicates that the majority (78%) of EFHC1 positive cells express NeuN and so correspond to...
neurons. There were also 20% of EFHC1 positive cells that express GFAP and thus correspond to astrocytes. The labeling was also present in the proximal portion of astrocytic processes. Another way to illustrate the eventual expression of this gene by neural structures is the use of nerve cell lines in culture. We extensively used this approach and showed clear synthesis of EFHC1 by mouse neuroblastoma cells (N2A cells), as already illustrated in the past with HEK-293, HeLa and COS cell lines. Furthermore, in order to establish how early, in culture the EFHC1 gene was expressed, we investigated its presence in neural stem cells (NSC). These NSC were prepared from embryonic mice striata, as previously described. They were allowed to form neurospheres. First, the expression of EFHC1 transcript was confirmed by RT-PCR. Then, double immunofluorescent labeling was performed using anti-nestin, a neural stem cells marker, and anti-EFHC1 antibodies. EFHC1 labeling was present in all cells composing neurospheres. Putting together, these results clearly demonstrate the ability of neuronal cells both in vivo and in vitro to synthesize EFHC1 from the earliest stages of development.

**EFHC1/MYOCLONIN1 CALCIUM SIGNALLING AND APOPTOSIS**

To investigate the functional significance of EFHC1 and its mutants in neurons, Suzuki et al. (2004) transfected mouse hippocampal primary culture neurons with enhanced green fluorescent protein (EGFP)-EFHC1 expression constructs. EFHC1-positive neurons had shorter neurites and fewer branches 16 h after transfection and showed signs of neurodegeneration and cell death, including shrinkage of the cell body and fragmentation of processes 48 h after transfection, whereas control cultures seemed to be healthy. Cells transfected with EFHC1 were TUNEL-positive, indicative of apoptosis. Next, they investigated the effects of EFHC1 mutations on cell survival by counting GFP-positive surviving cells attached to the dishes at various time points, irrespective of cellular morphologies. The cell-death effect of EFHC1 was substantially reduced by any of the five mutations associated with JME and by the double mutation 229C↔A and 662G↔A. In contrast, the three coding polymorphisms that were also present in the control population did not affect cell death considerably. Although the numbers of surviving cells transfected with mutations associated with JME seemed close to that of vector-transfected cells, the cells transfected with mutations associated with JME had unhealthy morphology 48 h after transfection, implying that the mutations did not disrupt EFHC1 function completely. They also analyzed the effects of EFHC1 isoforms on cell survival. Transfection with a construct expressing transcript B resulted in moderate cell death, and coexpression of the wild-type transcript and transcript B had intermediate effects. The cellular functions of the protein encoded by transcript B are not known, but the fact that this isoform excludes the mutation 757G↔T from its open reading frame suggests that this isoform may not have a large role in the pathogenesis of JME.

As already mentioned, the EF-hand superfamily regulates many aspects of cell function, such as Ca$^{2+}$ buffering in the cytosol, cell proliferation and signal transduction. In the central nervous system, calmodulin and the related EF-hand containing neuronal Ca$^{2+}$-sensor proteins have many important roles in neuronal signaling. The basic functional unit consists in a pair of EF-hand motifs, but in the case of human EFHC1 it is not clear if it is present in pairs or not.

Murai et al. (2008) reported the first structural and thermodynamic analyses of human EFHC1C-terminus containing the last DM10 domain and the EF-hand motif. The target protein was expressed mainly in the soluble form and purification protocol including tag removal was successfully established. The final purified protein presented high stability. The secondary structure was measured by CD spectroscopic, showing 34% of a-helices and 17% of b-strands. It was demonstrated that the protein can form dimers in the absence of DTT. Tandem mass spectrometry (MS/MS) analysis with the help of SearchXLinks program suggests that Cys575...
participates in intermolecular S–S bond formation. In addition, DTNB assay showed that reduced EFHC1C has only one accessible free thiol per molecule. ITC data showed that reduced EFHC1C binds to just one divalent ion (Ca$^{2+}$ or Mg$^{2+}$) whereas, in an oxidized (dimeric) state EFHC1C has its ion binding site blocked$^{24}$. To date, it is not clear if EFHC1 is a Ca$^{2+}$-sensor or a Ca$^{2+}$-buffer protein. However, full EFHC1 presents 19% identity and 44% similarity with the Ca$^{2+}$-regulator calmodulin, the classical example of EF-hand protein that suffers conformational changes after ion binding. Additional experiments should be performed in order to better determine the influence of Ca$^{2+}$ or Mg$^{2+}$ binding in EFHC1 dimerization process. Whether dimerization is a physiological conformation remains to be elucidated.

Because EFHC1 contains a Ca$^{2+}$-sensing EF-hand motif and because abnormalities of voltage-dependent Ca$^{2+}$ channels (VDCCs) have been described in human and mouse epilepsies, Suzuki et al. (2004) investigated whether the observed cell death is due to modulations of VDCCs. RT-PCR showed that most of the VDCC subtypes were expressed in mouse primary culture neurons, albeit at varied levels. Treatments of EFHC1-transfected primary culture neurons with several antagonists of VDCC subtypes indicated that SNX-482, antagonist for Ca$_{v}$.2.3, specifically increased the survival rates of EFHC1-positive neurons.

Moreover, patch-clamp analyses of baby hamster kidney (BHK) cells stably expressing Ca$_{v}$.2.3 and transiently transfected with EFHC1 showed that EFHC1 substantially increased the R-type Ca$^{2+}$ current generated by Ca$_{v}$.2.3$^{3}$. Co-transfection with constructs expressing P/Q-type VDCC (Ca$_{v}$.2.1) and EFHC1 did not increase Ca$^{2+}$ currents. The effects of EFHC1 on Ca$_{v}$.2.3 were extensive and unique, even when compared with the effects of the auxiliary subunits of the Ca$^{2+}$ channels. These results suggest that EFHC1 enhances Ca$^{2+}$ influx through Ca$_{v}$.2.3 and stimulates programmed cell death. Mutations associated with JME partly reversed the increase in R-type Ca$^{2+}$ currents by EFHC1. Incomplete reversal of EFHC1-induced Ca$^{2+}$ influx through Ca$_{v}$.2.3 may be responsible for the precarious state of calcium homeostasis sensitive to the triggering effects of sleep deprivation, fatigue and alcohol in individuals with JME. The three coding polymorphisms had weaker or no reversal effects, implying that they could be functionally benign or less malignant. Transfection with the transcript B isoform moderately increased Ca$^{2+}$ current. These results were consistent with those of the cell-death analyses$^{3}$.

**EFHC1/MYOCOLUMN1 IN MITOSIS AND CELL DIVISION**

**EFHC1 co localize with mitotic spindles**

We have shown$^{9}$ that the subcellular localization of EFHC1 varied during the cell cycle. The most prominent finding was the association with the mitotic spindle and the midbody. This was observed in different cell lines (HEK293, COS-7, HeLa, NIH3T3, SKNBE) for the overexpressed EGFP-tagged and the endogenous protein. Those results suggest the involvement of EFHC1 in cell division, and in particular, in the organization of the mitotic spindle and the midbody. However, during mitosis, a fraction of EFHC1 remains localized in the cytoplasm, suggesting that only a portion of the protein is recruited by the mitotic apparatus, as it is the case for other mitotic apparatus associated proteins.

Co-localization of EFHC1 with alpha-tubulin was observed at the mitotic spindle suggesting a possible interaction with microtubules. Such an association was suggested for the *Chlamydomonas* protein Rib72 in flagellar axoneme$^{19,20}$. As mitotic spindle, axoneme and cilia are microtubule-based structures, this further supports the hypothesis of a possible interaction of EFHC1 with microtubules.
In non dividing cells, we consistently observed a strong accumulation of EGFP-tagged and endogenous protein in one (occasionally two) perinuclear spot(s). Using anti-gamma-tubulin antibody, we identified those spots as centrosomes, suggesting that EFHC1 could be a centrosomal component. This is further supported by the proteomic analysis showing the presence of the Chlamydomonas Rib72 in centrioles, a centrosome component. Moreover, the concentration of EFHC1 at spindle poles during mitosis, where centrioles are located, suggests an involvement in the functioning and/or the organization of the mitotic spindle. Molecular components common to centrosome and mitotic spindle have been described.

Deletion analyses of EFHC1 demonstrated that its N-terminal portion (construct N92) is required and sufficient for its association with the mitotic spindle and the midbody. In the light of our observations made with truncated EFHC1 constructs in mitotic cells, the role of DM10 domains remains unclear.

The association of EFHC1 with the mitotic apparatus and the centrosome constituted at this time a clear, new and original finding. Correlation between our observations and previous findings, demonstrating the association of the Chlamydomonas Rib72 with flagellar axoneme and mRib72-1/Efhc1 with motile cilia and flagella, further supported the concept of EFHC1 interaction with microtubule-based structures. Other axonemal proteins have also been found to be associated with the mitotic spindle and/or the centrosome and vice versa.

In other respects, our hypothesis of a potential involvement of EFHC1 in cell division is supported by several arguments. It was shown that the EFHC1 transcript expression in the mouse brain is higher during embryogenesis, when cell division is prominent, than in the adult brain. The EFHC1 promoter was also demonstrated to be a putative target of the E2F4 transcription factor, a member of the E2F transcription factor family that plays a crucial role in cell cycle progression and is therefore important in cellular proliferation. Moreover, EFHC1 transcript expression is modulated during HeLa cell cycle.

**EFHC1 is a microtubule associated protein (MAP)**

To determine more precisely which region of EFHC1 mediates this association, we expressed various enhanced green fluorescent protein (EGFP)-tagged truncated EFHC1 proteins in HEK293 cells and carried out immunocytochemistry with an antibody to α-tubulin. We found that EGFP-hN45, but not EGFP-hN30, colocalized with the mitotic spindle, suggesting that the first 45-amino-acid region contains a motif that is required for the localization of EFHC1 with the mitotic spindle.

This colocalization prompted us to investigate whether EFHC1 interacts with α- and/or γ-tubulin. Using immunoprecipitation procedures on HEK293 cell lysates, we found that EFHC1 and α-tubulin co-precipitated mutually and that EFHC1 did not interact with γ-tubulin. To determine whether EFHC1 interacts directly with α-tubulin, we used in vitro co-sedimentation assays using pure prepolymerized microtubules and different purified GST-tagged truncated EFHC1 proteins. We found that GST-hEFHC1 and GST-hN45, but not GST-hN30 and GST, co-sedimented with microtubules, indicating that there was a direct interaction between EFHC1 and α-tubulin through the first 45-amino-acid region. This association was not affected by the presence of Ca²⁺. We therefore estimated the affinity of EFHC1 for microtubules and found an equilibrium dissociation constant of about 1.5 μM, indicating that EFHC1 binds to microtubules with a relatively high affinity. Finally, we carried out in vitro tubulin polymerization assays with GST-hEFHC1. We did not detect any substantial change in either the polymerization rate or the nucleation phase in the presence of GST-hEFHC1. This indicates that EFHC1 is not required for the polymerization of microtubules in vitro. It is noteworthy that the region involved in the binding to α-tubulin (that is, the first 45 amino acids) showed
no obvious homology with the MTBDs of other known MAPs. Thus, EFHC1 would appear to be a unique MAP, unrelated to conventional previously described MAPs\textsuperscript{11}.

**EFHC1 is required for mitotic spindle organization**

In addition to its association with α-tubulin, we found that overexpression of EGFP-hN92, EGFP-hN60 or EGFP-hN45 in HEK293 cells resulted in severe mitotic spindle defects, including monopolar spindle and chromosome alignment failure during metaphase. We quantified their occurrence in cultures overexpressing different EGFP-tagged EFHC1 proteins and found that the percentage of mitotic defects was significantly higher for EGFP-hN92, EGFP-hN60 and EGFP-hN45 compared with EGFP-hEFHC1 or EGFP.

Notably, truncated forms that did not associate with the mitotic spindle did not promote spindle defects, suggesting that binding to microtubules is necessary for this phenotype. EGFP-hDM101, which contains both the N-terminal region and the first DM10 domain, failed to induce mitotic spindle defects. This suggests that at least the first DM10 domain is required for proper EFHC1 function at the mitotic spindle. Moreover, we observed that monopolar spindles showed an abnormal spherical distribution of chromosomes around a pair of closely and centrally located centrosomes, indicating that the centrosome was duplicated, but that there was an insufficient separation to form a bipolar spindle. In the next experiments, we only used the EGFP-hN45 protein, as it produced, in our opinion, the most important phenotype. Using an antibody to the C terminus of EFHC1, we observed that EGFP-hN45 saturated the microtubule’s EFHC1 binding sites and, as such, acted as a dominant-negative protein.

Moreover, we transfected HEK293 cells with validated shRNAs and observed a significant increase in the number of disorganized mitotic spindles with the presence of monopolar spindles and misaligned metaphase compared with controls. Expression of rEFHC1, an shRNA-resistant form, rescued the mitotic spindle defects, demonstrating the specificity of the shRNA effect.

Finally, we found that the mitotic index was significantly higher in cultures expressing EGFP-hN45 and hEFHC1 shRNA than in control cultures. In contrast with control cells that were distributed across all mitotic phases, EGFP-hN45 and hEFHC1 shRNA–expressing cells were mostly arrested at prometaphase, with a concomitant decrease in the number of cells in anaphase/telophase and cytokinesis, resulting in subsequent M phase delay or arrest. Collectively, these results obtained by independent approaches (RNAi and dominant-negative protein) strongly suggest that EFHC1 is involved in mitotic microtubules organization and M phase progression\textsuperscript{11}.

**EFHC1 impairment induces microtubule bundling and apoptosis**

During interphase, EGFP-hN45 and hEFHC1 shRNA expression markedly induced microtubule bundling around the cell periphery. These bundled microtubules were extremely stable and did not depolymerize in the presence of nocodazole. Microtubule bundling was significantly higher in cultures expressing EGFP-hN45 and hEFHC1 shRNA than in those expressing EGFP-hEFHC1, EGFP or control shRNA. Moreover, using in vitro microtubule bundling assays, we found that GST-hN45, but not GST-hEFHC1 or GST, induced microtubule bundling, indicating that the MTBD of EFHC1 is capable of promoting microtubule bundling formation in vivo and in vitro when expressed alone.

Moreover, in cultures with impaired EFHC1 function, we observed frequent occurrences of nuclear abnormalities such as fragmented and picnotic nuclei, as seen in apoptotic cells. Therefore, we carried out TUNEL assays and found a significant increase of apoptosis in cells overexpressing EGFP-hEFHC1, EGFP-hN45 and hEFHC1 shRNA compared with the EGFP.
control. Finally, to determine the cell cycle phase distribution of apoptotic cells expressing EGFP or EGFP-hN45, we sorted apoptotic EGFP-positive cells by flow cytometry and carried out cell cycle analysis. We observed a significant enhancement of EGFP-hN45 cells in S and G2/M phases, with a concomitant decrease of cells in the G0/G1 phases compared with EGFP controls indicating that mitotic spindle defects induced apoptosis.

**EFHC1/MYOCCLONIN1 AND EARLY NEUROBLAST MIGRATION**

**EFHC1 contributes to radial migration and radial glia integrity in the developing neocortex**

On the basis of previous studies reporting a higher expression of EFHC1 during brain development and on its key role on cell division, we further investigated the role of EFHC1 on cerebral cortex development by modulating the expression of rEFHC1 into ventricular zone (VZ) cells of the rat developing neocortex using both in utero and ex vivo electroporation (figure). In order to inactivate rEFHC1 functions through RNAi-mediated depletion, we validate the effectiveness of rEFHC1 shRNA vector by Western blot on extracts from microdissected ventricular/subventricular zone (VZ/SVZ) after ex vivo electroporation.

**Figure 4. Role of EFHC1 in radial neuronal migration**

Distribution of EGFP-positive cells (in gren) in different cortical regions (ventricular zone/SVZ, intermediate zone and cortical plate, CP) 4 d after ex vivo electroporation of rat brains at E17 with control shRNA (left) and rEFHC1 shRNA (right) showing a significant disruption of neuroblasts migration toward the CP when EFHC1 synthesis is invalidated in the cells. Scale bars represent 200 μm (see ref… for more details).

To determine whether EFHC1 influence radial migration of neocortical neurones in rat neocortex, we examined the position of EGFP+ cells in the VZ/SVZ, intermediate zone (IZ) or cortical plate (CP) 1 and 4 d after ex vivo electroporation at E17 of plasmid encoding Con shRNA, rEFHC1 shRNA, EGFP-hN45, rEFHC1 shRNA and EGFP-hEFHC1 or EGFP-hEFHC1 alone. At day 1, cells were mostly located within the VZ/SVZ with no obvious difference in the position of cells in the five different transfected conditions. By day 4, the expression of EGFP-hN45 and rEFHC1 shRNA induced a dramatic disruption of radial migration compared to Con shRNA (Fig.). In the presence of rEFHC1 shRNA, most of cells were localized in the VZ/SVZ whereas few cells reached the CP. RNAi-mediated alteration of radial migration was rescued by the expression of EGFP-hEFHC1 although expression of EGFP-hEFHC1 alone had no significant effect. When electroporated with EGFP-hN45, cells...
migrated out the VZ/SVZ but failed to enter the CP and thus accumulated in IZ. It is interesting to note that similar results have been observed after in utero electroporation of rEFHC1 shRNA (Fig.) Thus, the impairment of EFHC1 appeared to have a direct effect on radial redistribution to the CP.

Furthermore, immunohistochemistry with anti-BLBP antibody revealed a disruption of radial glial processes extension with concomitant irregular accumulation of cells strongly stained for BLBP in the VZ/SVZ. Together, these results indicate a crucial role for EFHC1 in the integrity of radial fibre network. Finally, expression of rEFHC1 shRNA or EGFP-hN45 induced structural disorganisations of VZ/SVZ cells with many cells presenting an enlarged multipolar morphology and lacked, or only had a very short, leading process, sometimes not radialy oriented. Moreover, we observed the presence of many round-shaped cells with condensed chromosomes, suggesting apoptotic cells. This was supported by the decreased number of EGFP+ cells in the VZ/SVZ of cortices expressing rEFHC1 shRNA and EGFP-hN45. To test this hypothesis, we performed TUNEL assays and showed a significant increased number of apoptosis in brain slices with impaired EFHC1 function.

**EFHC1 is essential for mitosis and cell cycle exit of cortical progenitor cells**

Since cortical progenitor cells division occurs prior to and is tightly coupled to neuronal migration, we decided to study the influence of EFHC1 on mitosis, proliferation and cell cycle exit of cortical progenitors by ex vivo electroporation on E17 rat brains. We analysed the effect of EGFP-hEFHC1, EGFP-hN45 and rEFHC1 shRNA expression on spindle MTs organization of VZ/SVZ cortical progenitors. We observed that both EGFP-hEFHC1 and EGFP-hN45 associated with the mitotic spindle, although EGFP-hN45 and rEFHC1 shRNA induced mitotic spindles defects similar to those observed in vitro (monopolar spindles and mis-aligned chromosome at metaphase. To assess whether these abnormalities influence mitotic progression, we performed immunohistochemistry with anti-phospho-Histone H3 (pH3) antibody, a mitotic marker. This mitotic index (percentage of transfected cells in mitosis) was significantly increased by impairment of EFHC1 function compared to Con shRNA. This accumulation of mitotic cells strongly suggests an M phase arrest/delay of progenitor cells, as we observed in vitro. Moreover, pH3 immunoreactivity showed ectopic localization of mitotic chromosomes associated with EFHC1 defect. Whereas most Con shRNA mitotic chromosomes were normally localized along the ventricular lumen, many EFHC1 deficient mitotic chromosomes were found in the region above the ventricular surface.

Furthermore, we explored whether EFHC1 control the rate of cell cycle exit of cortical progenitors. For this purpose, brains slices were exposed to a short pulse of BrdU (1 h) 24 h after ex vivo electroporation and fixed 24 h later. Triple immunolabelling with antibodies directed against GFP, BrdU and Ki67 allowed us to establish the cell cycle exit index (percentage of transfected cells that were exiting the cell cycle within 24 h. We found that the cell cycle exit index was significantly decreased in brains slices expressing rEFHC1 shRNA.

Finally, to quantify the effect of EFHC1 deficiency on cortical progenitor population, we determine the percentage of transfected cells expressing Sox2, a progenitor marker, using immunohistochemistry. The fraction of VZ/SVZ cells expressing Sox2 was significantly enhanced by EFHC1 inhibition.

All together, these data suggest that the impairment of EFHC1 function in the developing neocortex interfere with both cell division and cell cycle exit of cortical progenitors leading to their accumulation.
EFHC1 is required for locomotion of post-mitotic neurons

Cortical development is a complex process involving strictly regulated sequence of neuronal proliferation, differentiation and migration to specific cell layers. Our results identified an influence of EFHC1 on proliferation and differentiation of cortical progenitors. To investigate the involvement of EFHC1 on radial migration of post-mitotic neurones, we performed focal electroporation of plasmid encoding Con shRNA or rEFHC1 shRNA in the IZ of brain slices at E19 after ex vivo electroporation of RFP plasmid to label VS/SVZ progenitors cells. This method allowed us to differentiate progenitors (EGFP+ /RFP+) from post-mitotic neurones (EGFP+/RFP−). We analysed the cortical scattering of EGFP+/RFP− cells 2 d after electroporation using an arbitrary scale divided into 10 bins (Fig). Compared to Con shRNA, expression of rEFHC1 shRNA impaired cortical redistribution of post-mitotic neurones in the CP as the majority of cells were located in bins corresponding to the upper IZ with concomitant decreased of cells in the CP. In order to confirm these results, we realized ex vivo electroporation at E17 of plasmid encoding EGFP or EGFP-hN45 under the control of NeuroD promoter, allowing the expression of protein only in post-mitotic neurones. Cortical scattering of EGFP+ cells was analysed 4 d after electroporation. Compared to EGFP, only a subset of cells had reached the lower CP in brains expressing EGFP-hN45. These results suggest that EFHC1 is also implicated in the locomotion of post-mitotic neurones11.

EFHC1/MYOCLOVIN1, A KEY CILIAR COMPONENT PLAYING A ROLE IN THE FUNCTION OF EPENDYMA

Myoclonin1 is a homologue of P72/Rib72, a flagellar protein of Chlamydomonas. P72/ Rib72 is thought to have important roles in assembly and function of the axoneme. Indeed, it has been shown that Rib 72 is an essential component of the flagellar axonemal ribbon (a stable structure formed by protofilaments of tubulin), that is implicated in the calcium dependent flagellar motility. On the other hand, Rib72 homologs have been observed in organisms containing motile cilia or flagellas as humans, mice, sea urchin, or drosophila. Moreover, the mouse ortholog of EFHC1 is expressed in many tissues with motil cilia and flagellas as lungs, testis, or ependymal cells. Interestingly, in brain the ependymal cells lining the border of the ventricles are highly ciliated and supports, in adult, a rostral directed CSF flow pulling along new forming neuroblasts. One can postulate that a default of EFHC1 highly expressed in these cilia could lead to abnormal migration of newly formed neuroblast in adult modifying the cytoarchitectony of the gabaergic cortical interneurons as an epileptogenic mechanism.

Suzuki et al 2008 raise intriguing possibilities that “(choroid) plexusopathy” or “ciliopathy” are the pathological basis of epilepsies caused by the EFHC1 mutations. They 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 epithélium (derived from the same layer of cells that forms the ependymal lining of the ventricles). 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.

In 2009, the same authors generated viable Efhc1-deficient mice. Most of the mice were normal in outward appearance and both sexes were found to be fertile. However, the ventricles of the brains were significantly enlarged in the null mutants but not in the heterozygotes. Although the ciliary structure was found intact, the ciliary beating frequency was significantly reduced in null mutants. In adult stages, both the heterozygous and null mutants developed frequent spontaneous myoclonus. Furthermore, the threshold of seizures induced by pentylenetetrazol was significantly reduced in both heterozygous and null mutants. These
observations seem to further suggest that decrease or loss of function of myoclonin 1 may be the molecular basis for epilepsies caused by EFHC1 mutations.

THE MAP MYOCLONIN 1 IS ESSENTIAL FOR THE NORMAL DEVELOPMENT AND FUNCTION OF THE NEUROMUSCULAR JUNCTION SYNAPSE IN DROSOPHILA

Very recently, one of the two Drosophila homologs of EFHC1, CG8959, named Defhc1 has been functionally characterized (Rosetto et al., submitted; personal communication). In agreement with studies in mammalian systems, they found that Defhc1 is capable of binding to microtubules. Transfected Defhc1 distributes both to the cytoplasm and to the nucleus of resting cells while in dividing cells the protein localized to the mitotic spindle. Defhc1 knockout Drosophila displayed normal appearance and behavior. However, morphology of the neuromuscular junction synapse appeared aberrant with an increase in the number of satellite boutons, structures that have been regarded as potential ramifications. Defhc1 is capable of binding to microtubules and overlaps in vivo with axonal microtubules. In the NMJ synapse, disruption of Defhc1 function leads to a decrease in the number of microtubule loops, whose presence correlates with halted bouton division, suggesting that Defhc1 is a negative regulator of this process. In addition, they show that the increase in satellite boutons requires an intact microtubule cytoskeleton and is accompanied by an increase in spontaneous neurotransmitter release. This important work further shows that loss of Defhc1 induces overgrowth of the dendritic arbor and in vivo overexpression of Defhc1 in dendritic neurons substantially reduces dendrite elaboration. These results suggest that, at least in this model, Defhc1 functions as an inhibitor of neurite growth by finely tuning microtubule cytoskeleton dynamics. Corollary to this, these findings also suggest that the susceptibility stage of EFHC1-dependent JME may result from augmented spontaneous neurotransmitter release due to overgrowth of neuronal processes.

EFHC1 OR MYOCLONIN1 DEFECT CAUSES JME AS A DEVELOPMENTAL DISEASE AFFECTING THE PROPERTIES OF THE MICROTUBULES IN THE NEURONO-GLIAL PROCESSES AND CORTICAL SYNAPTIC FUNCTIONS (“MICROTUBULOPATHY”)

The works reviewed above all converge to a clear demonstration: EFHC1 or Myoclonin 1, a gene significantly mutated in JME families, is a new microtubule associated protein (MAP). Microtubules affect multiple facets of neuronal development and function, with prominent roles in the growth and maintenance of axons and dendrites but also the fine tuning of the synaptogenesis. Here we will cite some examples from our previous mentioned data, supporting the above concept.

As discussed, the mitotic index (percentage of transfected cells in mitosis) was significantly increased by impairment of EFHC1 function compared to Con shRNA. This accumulation of mitotic cells in VZ/SVZ strongly suggests an M phase arrest/delay of progenitor cells, as we observed in vitro. The nuclei of the radial glia progenitors (RGP) realize an interkinetic migration along the apico-basal axis in relation with the different phases of the cell cycle. The mitotic cells are located at the border of the ventricular lumen, and the nuclei of the cells in S phase are located in the half basal part of VZ. The microtubules, the centrosome, and certain MAPs contribute to this important process. It has been shown indeed that the proteins that interact with Dynein, LIS1 and Dynactin as well as the centrosomal proteins Cep 120 and TACC are necessary for the movement of the nuclei from its basal position toward the apical centrosome during the G2 phase. Their dysregulation indeed lead to the occurrence of ectopic
cellular divisions. Since EFHC1 appears to be an essential centrosomal MAP it could play a key role in this mechanism. The nuclei of the EFHC1 depleted cells would be then unable to realize their G2 phase baso-apical migration, being forced to divide in basal position and to acquire the basal progenitor phenotype.

Another example concerns the radial glia. These cells play a crucial function during corticogenesis in controlling two important process of the radial migration, i.e.: the somatic translocation and the cellular locomotion. Defaults in these processes lead to migration defects and disturbances in the laminar cortical organisation. The dynamic regulation of the microtubules by certain MAPS like MAP1a, MAP4, MAP7 or the tektins play a clear role in the extension of the radial glia processes. In addition, the deletion of one of these protein, DCLK, disturbs the cyto arhitectony of the radial glia scaffold. Again, we have shown that invalidation of EFHC1 disrupts morphology of the radial glia organisation: the bipolar structure disappeared, the cellular extensions are significantly reduced, and sometimes even the radial organisation is disrupted. This in part can be responsible for the default of migration observed of the post mitotic neuronal progenitors.

The locomotion of these neurons within the VZ is also reduced in cases where EFHC1 is deleted. Migration by locomotion consists of the extension of the anterior process (nucleokinesis) and the retraction of the posterior one. Again, recent studies have shown a key role of LIS1, dynein, NDEL1 and DCX in the coupling of nucleus and centrosome during this phenomenon. As a MAP associated with the centrosome, EFHC1 could be implicated in this mechanism. This hypothesis is reinforced by the fact that movement of the nucleus toward the centrosome during interkinetic nuclear migration, during nucleokinesis and the locomotion needs the support of the microtubules and the same regulatory proteins.

In one word as in hundred, all the phenotypes observed with the functional defaults of EFHC1 in the neocortical structures during early brain development mimic those observed wit other MAPs including LIS1, NDEL1 ad DCLK. Whether or not these molecules are privileged partners of EFHC1 has still to be studied.

Finally, the Drosophila neuromuscular junction (NMJ) synapse has been a useful genetic systems in which to assay the role of the microtubule cytoskeleton and model its role in inherited neurological disease. For instance, mutations in the microtubule-interacting protein Futsch (MAP1b) alters synaptic morphology and VAP-33A regulates synaptic bouton sprouting through interactions with the microtubule cytoskeleton. Atypical Protein Kinase C has been shown to control bouton formation by regulating the synaptic cytoskeleton, and hereditary spastic paraplegia gene spastin and spichthyin regulate synaptic structure and growth through the control of axonal microtubules. Furthermore, the Drosophila fragile X-related gene has been shown to regulate Futsch/MAP1b thereby modulating both synaptic architecture and neurotransmission strength.

Microtubules are essential structural components of dendrites as well, and several genes have been reported to influence dendrite growth and arbor expansion through their action on microtubules. For example, stathmin regulates dendrite arborization by controlling the dendritic microtubule dynamics and knockdown of doublecortin, a microtubule-associated protein, in hippocampal neurons results in reduced branching, length and complexity of the dendrites. Furthermore, Very-KIND, a KIND domain–containing RasGEF, controls dendrite growth through regulation of microtubule-associated protein. Support for an important role of microtubules in dendritic arbor shape has also come from studies in flies demonstrating that in dendritic arborization neurons the Knot transcription factor mediates control of microtubule-based arbor outgrowth by inducing expression of the microtubule-severing protein spastin.

Myoclonin1/EFHC1 in cell division, neuroblast migration, synapse/dendrite formation in juvenile myoclonic epilepsy
Together, these studies indicate that microtubules play key functions in the control of neurite structure and function and that mutations perturbing microtubule dynamics are causatively linked with inherited neurological disorders, that could therefore be called “microtubulopathies”.

CONCLUSIONS

For the first time, a gene responsible for an IGE encodes a MAP utilizing an unusual MTBD and not an ionic channel. Thus, beside the classical theory of channelopathies causing IGE, our work opens new perspectives in the identification of the precise molecular and cellular mechanisms of IGE, including subtle pathologies of early neuronal migration (“microdysgenesis”) and/or synaptogenesis. We hypothesize that mutations of EFHC1 induce subtle neuronal migration or synaptic formation defects that lead to abnormal epileptogenic circuitry during cortical maturation. Eventually, the elucidation of such mechanisms will provide new targets for better pharmacological treatment of epilepsy.

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