FEATURE ARTICLE

Association of Mitochondrial Letm1 with Epileptic Seizures

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Leucine zipper-EF-hand containing transmembrane protein 1 (Letm1) is a mitochondrial protein that is associated with seizure attacks in Wolf-Hirschhorn syndrome. This study aimed to investigate the expression pattern of Letm1 in patients with temporal lobe epilepsy (TLE) and pilocarpine-induced rat model of epilepsy, and to determine if altered Letm1 leads to mitochondrial dysfunction and increased susceptibility to seizures. Using immunohistochemical, immunofluorescent, western blotting, and transmission electron microscopic methods, we have found that Letm1 was significantly decreased in TLE patients, and gradually decreased in experimental rats from 1 to 7 days after onset of seizures. Letm1 knock-down by a lentivirus bearing LV-Letm1-sh resulted in mitochondrial swelling and decreased expression of Letm1 target protein mitochondrial encoded cytochrome B (MT-CYB). Behavioral study revealed that inhibition of Letm1 caused early onset of the first seizure, increased seizure frequency, and duration. However, administration of Letm1 homolog nigericin failed to prevent epilepsy. These results indicate that inhibition of Letm1 and mitochondrial dysfunctions contributes to the development of epileptic seizures. Appropriate Letm1 level may be critical for maintaining normal neuronal functions.

Keywords: lentivirus, Letm1, mitochondria, MT-CYB, temporal lobe epilepsy

Introduction

Epilepsy is characterized by recurrent unprovoked seizures that often require long-term antiepileptic drugs (AEDs) therapy. It affects about 0.5–1% of population worldwide (Hauser et al. 1993). Regardless of active AED therapy, about 20–40% of the newly diagnosed epilepsies are drug resistant (French 2007), and 70–80% of patients with temporal lobe epilepsy (TLE), one of the major focal epileptic forms, finally develop drug-refractory epilepsy (DRE; Schmidt and Loscher 2005). Uncontrolled epileptic seizures, or DRE, could result in neuronal loss, gliosis, and synaptic reorganization (Sutula et al. 1988; Regesta and Tanganelli 1999; Kwan and Brodie 2000) and often lead to cognitive and behavioral problems. However, the mechanisms underlying the pathogenesis of epilepsy are not well understood.

Evidence has shown that mitochondria play a critical role in epilepsy, dysfunction of which not only results from seizures, but may also contribute to epileptogenesis (Kunz 2002; Waldbaum and Patel 2010a, 2010b). Patients with inherited mitochondrial disorders, such as myoclonic epilepsy with ragged red fibers (MERRF) and encephalopathies frequently manifest epilepsies (McFarland et al. 2002). Leucine zipper-EF-hand containing transmembrane protein 1 (Letm1) is a conservative protein localized in the mitochondrial inner membrane. Hemizygous deletion of Letm1 gene is associated with Wolf-Hirschhorn syndrome (WHS), clinical features of which include growth and mental retardation, muscle hypotonia, seizures, and congenital heart defects (McQuibban et al. 2010). Epilepsy is often refractory with frequent status epilepticus (SE; Katagani-Shimono et al. 2005), which occurs usually in the first 2 years of life in patients with WHS (Worthington et al. 2008; Battaglia et al. 2009). Letm1 is deleted in all WHS patients with seizures, but is preserved in those without seizures (Rauch et al. 2001). The study has shown that Letm1 is associated with mitochondrial potassium homeostasis and volume control through its K+/H+ exchanger (KHE) activity (Nowikovsky et al. 2004; Froeschauer et al. 2005; Tamai et al. 2008). Protein synthesis machinery at the inner mitochondrial membrane, including respiratory chain complexes III and IV, is also associated with Letm1 (Lupo et al. 2011). Down-regulation of CG4589, a Letm1 ortholog gene in Drosophila, leads to mitochondrial dysfunction and reduced synaptic neurotransmitter release (McQuibban et al. 2010). These findings suggest that dysfunctional Letm1 and mitochondria play an important role in epilepsy. However, how Letm1 is changed in the epileptic foci, and whether the down-regulation of Letm1 would decrease the epileptic threshold remain unknown.

In this study, we hypothesized that down-regulation of Letm1 may increase the susceptibility to epileptic seizures. We examined Letm1 protein expression in patients with TLE and lithium–pilocarpine-induced seizures in rats. Then, by knocking down Letm1 in the hippocampus of epileptic rats, we investigated changes of mitochondrial morphology and function and behavioral activities of epilepsy. We further studied the effect of Letm1 homolog nigericin on epileptic phenotype.

Materials and Methods

Patient Selection

All patients with TLE in this study had typical clinical manifestations and characteristic electroencephalograms of epilepsy. Samples from 30 patients (16 males and 14 females; mean age 22.4 ± 8.79 years (8–42 years); mean disease course 9.07 ± 6.95 (2–30 years)) who had undergone resection surgery for intractable TLE were randomly chosen from 220 specimens in our epileptic brain tissue bank. Informed written consent form for the use of the tissue in research was obtained before the surgery. All study protocols were compiled with the guidelines for the conduct of research involving human subjects as established by the National Institutes of Health and the committee on human research at Chongqing Medical University. Presurgical assessment included obtaining a detailed history and neurological examination, interictal and ictal electroencephalogram studies, neuropsychological testing, and neuroradiological studies, such as brain X-ray computerized tomography (CT) scan or magnetic resonance imaging (MRI), to localize the epileptic foci for each patient. We did not find any progressive lesions in the central nervous system (CNS) as determined by cranial CT or MRI in the TLE group. All patients had no response to the maximal...
Table 1
Clinical characteristics of TLE and control patients

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<th>Subjects</th>
<th>Gender (M/F)</th>
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<th>Course (year)</th>
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P: patients; C, control; F, female; M, male; AEDs, antiepileptic drugs; VPA, valproate; PB, phenobarbital; CBZ, carbamazepine; TPM, topiramate; PHT, phenytoin; LTG, lamotrigine; OX, oxcarbazepine; C2P, clonazepam; TN, temporal neocortex; l, left; r, right; NL, neuron loss; G, Gliss; N, normal.

doses of 3 or more first-line AEDs, including the traditional and new AEDs. All TLE patients in our study experienced at least one seizure attack within 1 week before the surgery. For comparison, we obtained 9 histologically normal temporal neocortical samples from individuals (5 males and 4 females; mean age 24.67 ± 7.37 years; range 16–36 years) who were treated for increased intracranial pressure due to head trauma without apparent signs of CNS disease or exposure to AEDs. The samples from these patients were taken only for treatment purpose, in which no previous seizures were presented. Table 1 summarized the clinical features of these patients. Statistical analysis showed no significant differences in age and sex between the TLE patients and control groups (P > 0.05).

Pilocarpine-Induced Epilepsy

The experimental procedures of animals were approved by the Commission of Chongqing Medical University for ethics of experiments on animals and were conducted in accordance with international standards as described earlier (Curta et al. 2008). All rats were housed under standard conditions (room temperature: 23 ± 1°C; illumination: 12 h light and dark cycles, with access to food and water ad libitum). Male SD rats weighing 200–220 g from the Experimental Animal Center of Chongqing Medical University were used in this study. Rats in epilepsy groups were given an intraperitoneal injection of lithium chloride (127 mg/kg, i.p., Sigma, USA) 20 h before the administration of pilocarpine. Then, methyl-scopolamine-bromide (1 mg/kg) was given to reduce the peripheral cholinergic adverse reaction. Pilocarpine was given repeatedly (10 mg/kg, i.p.) every 30 min until the rats developed SE. The seizures were scored using Racine’s scale as follows: Stage 0 = arrest, wet dog shakes, and normal behavior; stage 1 = facial twitches (nose, lips, and eyes); stage 2 = chewing and head nodding; stage 3 = forelimb clonus; stage 4 = rearing and falling on forelimbs; stage 5 = imbalance and falling on side or back. Only those reached in stages 4 or 5 were used in our study (Racine 1972). Rats in the control group were intraperitoneally injected with equal volume of saline instead of pilocarpine. Animals in the experimental group were sacrificed on day 0 (control, n = 8), day 1 (n = 8), day 3 (n = 8), and day 7 (n = 8) after seizures were evoked. The seizure activity was observed by 2 observers for 1 h after the first seizure was induced by pilocarpine. All rats were given an intraperitoneal injection of diazepam (10 mg/kg) to stop the continuous seizures 1 h after the onset of the first seizure.

Lentivirus Production and Stereotactic Injection

We amplified the coding sequence of shRNA for Letm1 by reverse transcription polymerase chain reaction and ligated them into the pGC-FU plasmid (Shanghai GeneChem) to produce pGC-FU-Letm1-green fluorescent protein (GFP) (LV-Letm1-sh). A lentiviral vector expressing GFP alone (LV-GFP) was chosen as control. Different shRNAs targeting rat Letm1 gene were screened in primary culture neurons. The LV-Letm1-sh1 (Table 2) that effectively knocked down the expression of Letm1 was chosen. The titer of the lentivirus (LV) was 2 × 109 Tu/mL (made in Shanghai GeneChem Corporation).

Stereotaxic intrahippocampus injection was described earlier (Kanter-Schlifke et al. 2007). Fifty-one male rats were deeply anesthetized with intraperitoneal injections of 3.5% chloral hydrate (1 mL/100 g), and the rats’ head was fixed in a stereotaxia frame (Stoelting Co., Ltd., USA). A volume of 5 µL LV-Letm1-sh (n = 27) and LV-GFP (n = 12) were infused through a glass pipette (0.2 µL/min) bilaterally in the dorsal hippocampus (anterior—posterior −3.3 mm, medial—lateral ±1.8 mm, and dorsal—ventral −2.6 mm). The pipette was left in place for an additional 5 min after injection to prevent backflow. In the control group (n = 12), the LV were replaced by equal volume of saline. The different concentrations (0.5, 1, and 5 µmol/kg, respectively) of nigericin (Shanghai Yingxuan Chempharm Co. Ltd., China), or equal volume of saline (normal saline [NS]), were injected bilaterally in the dorsal hippocampus of 30 rats (n = 3 each group) with (n = 15) or without (n = 15) LV-Letm1-sh treatment a week ago as described above.

Tissue Preparation

Human

All TLE and control samples were collected from patients in the operating room. The anterior 3.5–4.0 cm of the lateral temporal lobe was resected en bloc from the superior temporal gyrus to the collateral fissure. A portion of the excised brain tissue from each studied TLE and control patient was immediately placed in a cryovial and stored in liquid nitrogen until it was later used for western blot. Additional portions of the samples were fixed in 4% buffered formalin for 48 h, then embedded in paraffin, sectioned at 5 µm for immunohistochemical analysis or 10 µm for immunofluorescence analysis, and mounted on polylysine-coated slides.

Rats

Samples were taken on days 1, 3, and 7 (n = 8 each, respectively). Day 1 is calculated from the time of first seizure to the time of sampling. The rats typically showed 2–3 seizure attacks a week. Therefore, the rats exhibited at least 1 seizure attack on days 1 and 3 and...
at least 2 attacks on day 7. At various time points following a seizure, 4 rats from each group were sacrificed by decapitation after an i.p. administration of a lethal dose of chloral hydrate. The resultant brains were dissected to collect both the temporal neocortex and the hippocampus and were placed in liquid nitrogen, and stored at −80°C for western blot analysis. The remaining rats were anesthetized and intracardially perfused with 0.9% saline, followed by 4.0% paraformaldehyde. Following perfusion, both sides of the temporal lobe were excised and immediately fixed in 4% paraformaldehyde. Then, the tissues were embedded in paraffin and sectioned at 5 µm for immunohistochemical analysis and 10 µm for immunofluorescence analysis. In virus-injected rats, the brain tissues used for transmission electron microscopy (n = 9) were prepared as described above. For the observation of distribution of GFP by laser confocal analysis, the rats in LV-GFP (n = 3), LV-Letm1-sh (n = 3), and control groups (n = 3) were sacrificed by decapitation after an i.p. administration of a lethal dose of chloral hydrate. Then, the brain tissues were sectioned at 50 µm at −20°C for laser confocal analysis and mounted on polylysine-coated slides. The tissues used for western blot were prepared as described above.

**Immunohistochemistry**

Ten slides were cut from each sample (30 and 9 samples from TLE patients and controls, respectively, and 8 samples from rats in each group), and 3 slides were randomly selected from each sample for immunohistochemical analysis. All the experiments were carried out in the same condition and repeated 3 times. An avidin-biotin complex (ABC) method was used to detect the expression of Letm1. Tissue sections were deparaffinized in xylene, rehydrated in a graded series of ethanol, and then incubated in H2O2 (0.3%, 15 min). For antigen recovery, sections were incubated with 10 mM sodium citrate buffer (pH 6.0), heated with a microwave oven for 15 min at 92–98°C, and then blocked with normal goat serum (Zhongshan Golden Bridge, Inc., Beijing, China) at 37°C for 30 min. Tissues were then incubated in primary rabbit anti-Letm1 (1:50, Santa Cruz Biotechnology, USA) overnight at 4°C, followed by incubation in secondary goat antirabbit IgG for 30 min at 37°C. Sections were then treated with ABC working solution (Zhongshan Golden Bridge Inc.) for 30 min, washed with phosphate-buffered saline (PBS), and then incubated with 3,3-diaminobenzidine (DAB; Zhongshan Golden Bridge Inc.) for 5 min. Counterstaining was carried out with Harris’s hematoxylin. For negative controls, the primary antibodies were replaced by PBS to exclude false results. The LEICA DM6000B automatic microscope (Leica Microsystems, Heidelberg GmbH, Germany) was used to collect the images. Cell with buffy stain in cytoplasm was considered to be positive. Ten visual fields for each sample were randomly chosen under a light microscope. Image-Pro plus 5.0 software (Media Cybernetics, USA) was used for the quantitative analysis of Letm1 expression. Mean optical density (OD) of each vision field was automatically measured by computer. The final OD values were averaged from 3 independent experiments.

**Double Immunofluorescence Labeling**

Sections were deparaffinized, rehydrated, and antigen recovery as performed in immunohistochemistry. Then, sections were incubated in 10% goat serum (Zhongshan Golden Bridge Inc.) for 30 min at room temperature. Sections were then incubated with a mixture of polyclonal rabbit anti-Letm1 (1:50, Santa Cruz Biotechnology) and a monoclonal mouse antimicrotubule-associated protein 2 (MAP2), or mouse antialgibullary acidic protein (GFAP) at 4°C overnight. Sections were washed and incubated with fluorescein isothiocyanate (FITC)-conjugated goat antirabbit IgG (1:200, Zhongshan Golden Bridge Inc.) and tetramethylrhodamine isothiocyanate (TRITC)-conjugated goat antimouse IgG (1:200, Zhongshan Golden Bridge Inc.) in the darkroom for 60 min at 37°C, then washed with PBS, and mounted in 1:1 glycerol/PBS. For GFP analysis, the tissue was directly mounted on the slides. Intensity of fluorescence was examined by laser scanning confocal microscopy (Leica Microsystems) on an Olympus IX 70 inverted microscope (Olympus) equipped with a Fluoview FVX confocal scan head.

**Western Blotting**

Tissues were homogenized in RIPA lysis buffer (Beyotime Institute of Biotechnology, China), and the supernatant were removed after centrifugation at 4°C (16000g × 10 min). Concentrations of total protein were determined by a bicinchoninic acid protein (BCA) assay (Beyotime Institute of Biotechnology). Total proteins (50 µg per lane) were separated in sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE; 5% spacer gel, 80 V, 30 min; 8% separating gel, 120 V, 90 min) and transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore). Membranes were blocked with 5% skim milk for 1 h at room temperature, and then incubated with primary antibodies (anti-Letm1, rabbit serum) for 2 h at room temperature. Membranes were washed with PBS and then incubated with HRP-linked secondary antibodies (anti-rabbit IgG, 1:2000, Santa Cruz Biotechnology) for 1 h at room temperature. The blotted proteins were visualized using the enhanced chemiluminescence (ECL) reagent (Amersham, UK). The intensity was quantified using a densitometer (Bio-Rad, USA) and ImageJ software (NIH). The OD values of each protein band were calculated, and normalized to β-actin to correct for differences in protein loading. The Student’s t-test was used to compare the mean OD values of Letm1 in both the TLE and control groups (P < 0.05).

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**Figure 1.** Immunofluorescent labeling for Letm1 in the neocortex. (A) Letm1 (green) and MAP2 (red) are coexpressed (merged) in the temporal neocortex of a TLE patient. (B) Letm1 (green) and MAP2 (red) are coexpressed (merged) in the cortex of an epileptic rat at 7 days postseizure. (C) Letm1 and GFAP are not coexpressed in the cortex of an epileptic rat at 7 days postseizure. Arrows show the positive cells (Scale bar = 75 µm).

**Figure 2.** Immunohistochemical images showing Letm1-positive cells in the brain specimens. (A) Weak immunoreactive stainings of Letm1 are shown in the temporal neocortex of a patient with TLE. (B) Relatively strong immunoreactive stainings of Letm1 are shown in the temporal neocortex of a control subject. In the rat model, (C and D) faint immunoreactivity of Letm1 was in the hippocampus (C) and adjacent cortex (D) of a rat at day 7 after seizure. (E and F) Strong immunoreactive staining of Letm1 in the hippocampus (E) and adjacent cortex (F) of a normal rat. The arrows indicate Letm1-positive cells. (Scale bar = 50 µm). (G) Summary of mean OD values of Letm1 in both the TLE and control groups (P < 0.05). (H) Histogram of the mean OD values of Letm1 in the rat cortex (P < 0.05) and hippocampus (P < 0.05), respectively, compared with control.
100 V, 60 min), and then transferred to a polyvinylidene fluoride (PVDF) membrane at 400 mA for 90 min. Then, membranes were incubated at 37°C for 1.5 h in 5% skim milk in order to block the nonspecific binding sites. Membranes were also incubated with polyclonal rabbit anti-letm1 (1:200, Santa Cruz Biotechnology) at 4°C overnight, washed with Tween-20–Tris-buffered saline (TBS) 10 min × 3 times, and incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG antibody (1:5000, Santa Cruz Biotechnology) for 1.5 h at 37°C. A rabbit anti-β-actin antibody (1:5000, Santa Cruz Biotechnology) was used as a loading control. For the detection of MT-CYB, the membranes used for β-actin staining were washed in stripping buffer for 5 min, and then incubated with polyclonal rabbit anti-CYTB (1:200, Santa Cruz Biotechnology) after incubation in 5% skim milk. The resultant OD values were measured by Quantity One 1-D Analysis Software (Bio-Rad Laboratories, Hercules, USA), and letm1 or CYTB over the β-actin ratio was calculated. Experiments were carried out in the same condition and repeated 3 times. The final OD values were averaged from 3 independent experiments.

Transmission Electron Microscope

The rats (n = 3 each group) were decapitated after anesthesia, and the hippocampus were removed and prepared for electron microscopy. Samples were diced and immediately submerged in 4% glutaraldehyde (0.1 M sodium cacodylate buffer, pH 7.2). Each specimen was trimmed and embedded in Spurr's medium. Tissue were postfixed with osmium, en bloc stained with uranyl acetate, and poststained with uranyl acetate and lead citrate. Then, tissue sections were cut into a thickness of 90 nm and observed on 300-mesh coated grids by using a JEOL JEM-2100F (Tokyo, Japan) transmission electron microscope. The evaluation of electron micrographs were carried out by 2 different observers who assigned a single score to each of the areas based on the criteria of Kloner et al. (1978). A grading scale of 0–4 depending on the degree of mitochondrial morphological damage was as follows: (0) normal mitochondria; (1) early swelling as manifested by the separation of cristae and clearing of matrix density; (2) more marked swelling than in grade 1; (3) massive swelling with architectural disruption; and (4) findings in grade 3 plus rupture of inner and outer mitochondrial membranes. We chose 5 samples for each rat randomly. Ten visual fields for each sample were randomly chosen under an electron microscope at ×50 000 magnification. The results of the mean grading scale for each group were obtained by 2 observers.

Statistical Analysis

The values were expressed as means ± standard deviation (SD). Comparisons of >2 groups were performed by an analysis of variance (1-way ANOVA; SPSS17.0), and Student's t-test was used to analyze the differences between 2 groups. The χ² test was used to compare gender differences between TLE patients and controls. A P-value <0.05 was considered as statistically significant.

Results

Localization of Letm1 in Human and Rat Brain Tissues

Letm1 was expressed exclusively in neurons of the temporal neocortex in controls and TLE patients. Ltem1 expression was colocalized with the dendritic marker MAP2 (Fig. 1A, B), but not with astrocytic marker GFAP (Fig. 1C).

Decreased Letm1 Expression in Patients with TLE and a Rat Model

Immunohistochemical study showed that neuronal Letm1 immunoreactivity was stronger in control (Fig. 2B) than in patients with TLE (Fig. 2A), which were significantly different...
LV-Letm1-sh with different degrees and significance. Letm1 expression was decreased in all rats treated with LV injection. In the rat model, the Letm1 immunoreactivity was also significantly decreased in the hippocampus and adjacent cortex (Fig. 2C,E,F,H). Western blotting study showed that Letm1 expression was significantly lower in 26 of the 30 samples in the temporal neocortex of TLE patients than that in controls (0.844 ± 0.079 in control and 0.377 ± 0.042 in patients with TLE, P < 0.05; Fig. 3A,B). There were no significant differences of Letm1 expression in patients with TLE with respect to onset age, frequency, and duration of seizures (Fig. 3E,F,G, P > 0.05). In the rat model, Letm1 levels were gradually decreased from day 1 to 7 after seizures, which was significantly lower than that in control at each time point after seizure (Fig. 3C,D, P < 0.05).

Analysis of Letm1 Expression After Injection of Recombinant Lentivirus
LV bearing GFP was localized in the CA1 of hippocampus and dentate gyrus 3 and 7 days after the LV injection (Fig. 4A,B). The expression of Letm1 was decreased in all rats treated with LV-Letm1-sh with different degrees and significantly decreased 7 days after the LV injection in the LV-Letm1-sh group compared with the control and LV-GFP groups (P < 0.05; Fig. 4C).

Mitochondrial Dysfunction After Down-Regulation of Letm1 by LV-Letm1-sh
The mitochondrial swelling was significantly increased in the LV-Letm1-sh group compared with that in the control and LV-GFP groups (Fig. 5A and Table 3). Similarly, MT-CYB expression was significantly decreased in the LV-Letm1-sh group compared with that in the control and LV-GFP groups as shown by western blotting analysis (Fig. 5B).

Down-Regulation of Letm1 Promotes Acute Seizures in Rat After Administration of Pilocarpine
LV-Letm1-sh knock-down alone did not result in spontaneous seizures. However, in pilocarpine-induced epilepsy, the latency of the first seizures were decreased, the frequency and duration of seizures were increased during the first hour after SE in the LV-Letm1-sh group compared with that in the control and LV-GFP groups (P < 0.05; Fig. 5C). However, no significant difference was found between the control and LV-GFP groups (P > 0.05).

Effect of Nigericin on Seizure Phenotype
A study has shown that nigericin, a homologous protein of Letm1, is an electroneutral ionophore for K⁺/H⁺ exchanger that reduces mitochondrial swelling (Nowikovsky et al. 2004). To test if nigericin may have protective effect on epilepsy, we injected nigericin into the hippocampus and investigated behavioral changes. However, rats treated with nigericin at different concentrations (0.5, 1, and 5 μmol/kg) did not show increased onset latency or decreased seizure frequency or duration, compared with epileptic control (Fig. 5D and Table 4). Similarly, rats treated with nigericin failed to reverse the effect of LV-Letm1-sh facilitation of epilepsy (Fig. 5E and Table 4).

Discussion
The major finding of this study is that Letm1 expression is decreased in both patients with intractable TLE and a rat model of epilepsy. Down-regulation of Letm1 leads to increased mitochondrial swelling and decreased MT-CYB expression, which is associated with enhanced susceptibility to seizures. However, Letm1 homolog nigericin does not show preventive effect on epilepsy.

In our study, the expression of Letm1 is decreased in the neocortex of TLE patients as well as in the hippocampus and adjacent cortex of rat after the onset of seizures. Letm1 is one of the mitochondrial inner-membrane proteins important for normal KHE function (Zotova et al. 2010). Genome-wide RNAi screening has revealed that Letm1 is also a mitochondrial Ca²⁺/H⁺ antiporter (Jiang et al. 2009) and functions as a putative EF-hand Ca²⁺ binding protein in WHS patients (Endele et al. 1999; Schlickum et al. 2004). Evidence has shown that Letm1 gene is deleted in all the WHS patients with seizures, but preserved in those without seizures (Rauch et al. 2001), suggesting that decreased expression of Letm1 might be involved in the development of epilepsy. Although we are not able to equally compare hippocampal Letm1 expression between TLE patients (from the hippocampus) and control (from the neocortex) due to practical and ethical reasons,
animal study do provide direct evidence that seizure activities lead to reduced Letm1 in the hippocampus, damage of which is frequently seen in TLE. Indeed, neuronal loss in the hippocampus is one of the main pathological features of human intractable TLE (Bengzon et al. 2002; Dawodu and Thom 2005).

The present study has demonstrated that mitochondrial swelling occurs after epilepsy in the rat model, and knockdown of Letm1, in turn, increases mitochondrial swelling. Previous studies have shown that mitochondrial swelling and decreased activity of respiratory chain complex results from

Figure 5. Morphological and functional changes of mitochondria and seizure susceptibility after down-regulation of Letm1. (A) Transmission electron microscopic images showing morphological changes of mitochondria before and after LV injection. After seizures are evoked by administration of pilocarpine at day 1, the mitochondria is slight swollen in the neurons of control and LV-GFP groups, but significant mitochondrial swelling are presented in the LV-Letm1-sh group. (B) Upper panel, representative western blotting images of mitochondrially encoded cytochrome b (MT-CYB) expression (42 kDa) in control, LV-GFP, and LV-Letm1-sh groups 1 day after administration of pilocarpine. Lower panel, histogram of the normalized MT-CYB expression in these groups. *P < 0.05, compared with control, #P < 0.05, compared with LV-GFP. (C) Bar-plot summary of seizure susceptibility with or without LV injection. *P < 0.05 compared with control, #P < 0.05 compared with LV-GFP. (D) In the normal rats treated with or without nigericin or normal saline (NS), the latency of the first seizure, frequency of seizures, and the total time spent in seizures during the first hour after SE among each group are without significant difference (P > 0.05). (E) In the rats treated with LV-Letm1-sh 1 week before experiments, the latency of the first seizure, frequency of seizures, and the total time spent in seizures during the first hour after SE among each group are also without significant difference (P > 0.05).

Table 3

<table>
<thead>
<tr>
<th>Group</th>
<th>Grading of scale</th>
<th>Number of mitochondria</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2.58 ± 0.53</td>
<td>734</td>
</tr>
<tr>
<td>LV-GFP</td>
<td>2.61 ± 0.61</td>
<td>765</td>
</tr>
<tr>
<td>LV-Letm1-sh</td>
<td>3.52 ± 0.75*</td>
<td>748</td>
</tr>
</tbody>
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Note: The results are expressed as mean ± SD.

*P < 0.05, LV-Letm1-sh versus control or LV-GFP.
repeated neuronal firings (Chuang et al. 2004), which can trigger neuronal death through both necrotic and apoptotic pathways depending on mitochondrial energy availability (Lorigados et al. 2008; Chuang 2010). On the other hand, impaired mitochondrial ATP production and intracellular calcium deregulation may have impact on membrane potential and excitability associated with epilepsy (Nowikovsky et al. 2009).

Repeated neuronal firing and mitochondrial dysfunction may form a vicious cycle to contribute to neural cell death and the formation of DRE (Kudin et al. 2009; Waldbaum and Patel 2010). Given the key role of Letm1 in mitochondrial ion homeostasis and cell viability (Dimmer et al. 2008; Nowikovsky et al. 2012), it may be predicted that reduced activity of the Letm1 is followed by osmotic matrix swelling, outer membrane rupture, and impaired ATP production (Hasegawa and van der Bliek 2007; Piao et al. 2009). However, how epileptic activity may lead to Letm1 dysfunction is currently unknown.

In our study, knock-down of Letm1 leads to decreased MT-CYB expression and increased susceptibility to seizures. This is consistent with the finding that down-regulation of Letm1 can disturb the mitochondrial protein biosynthesis, including respiratory chain complex of mitochondrial (Frazier et al. 2006). It has been known that Letm1 regulates the biosynthesis of mitochondrially encoded cytochrome b (MT-CYB; Lupo et al. 2011), which is a component of the ubiquinol–cytochrome c reductase complex (complex III or cytochrome b–c1 complex) that is responsible for generating an electrochemical potential coupled to ATP synthesis. The decreased expression of MT-CYB after Letm1 knock-down may be associated with disrupted ATP production, leading to increased excitability and perhaps neuronal death. Therefore, dysfunctional Letm1 together with mitochondrial swelling and disturbed MT-CYB may contribute to deteriorated behavioral phenotype of epilepsy.

However, nigericin, a homologous protein of Letm1 (Nowikovsky et al. 2004, 2009), fails to prevent epileptic seizures, nor does it reverse LV-Letm1-sh facilitation of epilepsy, suggesting that increased Letm1 function is not helpful in reducing seizure severity. Evidence has shown that overexpression of Letm1 could also induce the destruction of mitochondria and apoptosis (Hwang et al. 2010). It is possible that while reduced Letm1 facilitates neuronal firing and seizure, overexpression of Letm1 on the other hand also leads to mitochondrial dysfunction and apoptosis that may contribute to the pathophysiology of epilepsy. Another explanation for the failure of nigericin in preventing epilepsy is that Letm1 target protein MT-CYB involved in membrane integrity, rather than ion exchanger itself plays an important role in the development of epilepsy, which requires further study to clarify.

The LV bearing exogenous shRNA that leads to down-regulation of target genes has been well documented (Blomer et al. 1997; Rubinson et al. 2003). Consistently, significant decrease of Letm1 is shown 7 days after intrahippocampal administration of LV in our study. Although increasing evidence demonstrates that LV reduces the seizure threshold by causing inflammatory reaction (Haase 1986; Auvin et al. 2007; Vezzani et al. 2011), our study does not show spontaneous seizure attacks in animals treated with LV harboring LV-Letm1-sh or scrambled sequence before pilocarpine injection. One possibility is that inhibition of Letm1 is not enough to trigger spontaneous neuronal firings, albeit facilitating existed hyperexcitability. Another possibility is that not all cells in the hippocampus have been transfected with LV bearing LV-Letm1-sh (Fig. 4), which may compromise the effect of Letm1 inhibition.

In conclusion, our study demonstrates that Letm1 is decreased in patients with drug-refractory TLE and experimental model of seizures. Down-regulation of Letm1 increases the susceptibility to seizures, whereas Letm1 homolog nigericin fails to prevent epileptic seizures. It is too early to conclude that Letm1 is a target for antiepileptic therapy.

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References


