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ORIGINAL RESEARCH

FAM134B Attenuates Seizure-Induced Apoptosis and Endoplasmic Reticulum Stress in Hippocampal Neurons by Promoting Autophagy

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Abstract

Autophagy plays a critical role in epileptic neuronal injury, and recent studies have demonstrated that FAM134B plays an important role in regulating autophagy. However, the effect of FAM134B on epileptic neuronal injury remains unclear. In this study, we investigated the role of FAM134B in neuronal apoptosis and endoplasmic reticulum (ER) stress using the hippocampal neuronal culture model of acquired epilepsy (AE) in vitro. We found that in this model, the level of autophagy significantly increased, indicated by an elevated LC3-II/LC3-I ratio. FAM134B overexpression using lentiviral vectors enhanced autophagy, whereas FAM134B downregulation using lentiviral vectors impaired this process. In addition, the ER Ca^{2+} concentration was decreased and the intracellular level of reactive oxygen species was increased in this model. FAM134B overexpression was sufficient to reverse these changes. Moreover, FAM134B overexpression attenuated ER stress as shown by a decrease in the expression of C/-EBP homologous protein and glucose-regulated protein 78, and neuronal apoptosis induced by seizure, while FAM134B downregulation caused the opposite effects. Further, pre-treatment with the selective autophagy inhibitor 3-methyladenine abolished the effects of FAM134B on ER stress and neuronal apoptosis. Altogether, we demonstrate that FAM134B is an important regulator of AE-induced ER stress and neuronal apoptosis by controlling autophagy function.

Keywords FAM134B · Autophagy · Epilepsy · Endoplasmic reticulum stress · Apoptosis

Introduction

Autophagy alterations exist in a variety of neurological diseases, including epilepsy (Giorgi et al. 2015). The Family with sequence similarity 134, member B (FAM134B), which contains a reticulon homology domain (RHD) and LC3-interacting region (LIR), is the firstly identified ER autophagy receptor and exerts an important effect on normal cell homeostasis by regulating autophagy (Islam et al. 2018). FAM134B controls ER turnover by specific binding to microtubule-associated protein 1 light chain 3B (MAP1LC3B), which in turn binds to the phagophore membrane via its C-terminal LC3-interacting region (LIR) (Bhaskara et al. 2019). Recent studies have demonstrated that FAM134B-regulated autophagy is crucial for cell survival, especially under conditions of stress (Khaminets et al. 2015). However, the role of FAM134B-regulated autophagy in epileptic neuronal injury is unknown.

The endoplasmic reticulum (ER) is a vital organelle for neuronal survive, responsible for multiple functions including Ca^{2+} storage, signaling and protein folding, and maturation (Song et al. 2016; Yin et al. 2017). Various cellular stresses such as disturbance of ER Ca^{2+} homeostasis and oxidative stress can contribute to ER stress (Duan et al. 2017; Song et al. 2016). Moderate ER stress promotes the dissociation of glucose-regulated protein 78 (GRP78) to reduce the number of misfolded proteins and restore cell function (Liu et al. 2018). However, exacerbation of ER stress leads to downstream apoptotic signaling molecules activation such as cysteinyl aspartate-specific proteinase 12 (caspase 12) and C/-EBP homologous protein (CHOP) (Liu et al. 2018; Shimodaira et al. 2014). Growing data suggest an interaction between ER stress and autophagy. It has been recognized that ER stress can activate autophagy to reduce cell stress, while excessive autophagy triggers neuronal

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death (Niu et al. 2018; Ko et al. 2015). However, the effect of the FAM134B on seizure-induced ER stress is still elusive.

In this study, we evaluated the effects of FAM134B on neuronal apoptosis and ER stress using lentiviral vector-mediated manipulation of its expression in the hippocampal neuronal culture (HNC) model of acquired epilepsy (AE). In addition, we used the selective autophagy inhibitor 3-methyladenine (3-MA) to gain insights into the mechanisms of FAM134B in this context.

Materials and Methods

Primary Hippocampal Neuronal Cultures

All animal protocols were approved by the animal care and use committee of Zhengzhou University, China, confirming that all animal research and experimental operations are strictly in accordance with international guidelines for animal studies.

Primary hippocampal neurons were generated from healthy newborn Sprague–Dawley rats which were purchased from the Animal Center of Zhengzhou University. Dissociated neurons were plated on poly-L-lysine-coated (0.1 mg/ml) culture plates in 6-well plates at 4×10^5 cells per well and grown in planting medium containing neurobasal-A medium, 2% B27, 2 mM glutamine and 10% fetal bovine serum (Biological Industries, Israel) (Hinze et al. 2017). After 4 h of culture, the planting medium was removed and replaced with maintenance medium containing 2% B27, 2 mM glutamine, and neurobasal-A medium. Half of the maintenance medium was regularly refreshed every 2 days. Purity of hippocampal neurons was measured by immunofluorescence and confirmed to be > 95% by quantifying the proportion of positive cells.

Experimental Design

Hippocampal neurons were plated in 6-well plates at 4×10^5 cells per well. After 4 h of culture, the planting medium was removed and replaced with maintenance medium. The AE model was induced using a conventional method: hippocampal neurons were incubated in a Mg^{2+}-free solution containing 145 mM NaCl, 2 mM $CaCl_2$, 2.5 mM KCl, 0.002 mM glycine, 10 mM HEPES, and 10 mM glucose, with a pH of 7.4 (Blair et al. 2006; Kiese et al. 2017). After 10 days of culture, primary hippocampal neurons were randomly assigned to the following groups: (1) control group, neurons incubated in normal extracellular fluid for 3 h; (2) AE group, neurons cultured in Mg^{2+}-free solution for 3 h and then cultured in maintenance medium; (3) negative control group, neurons incubated with lenti-pGV for 12 h and then cultured in maintenance medium for 72 h before incubation in Mg^{2+}-free solution for 3 h; (4) lenti-FAM134B (upregulation FAM134B) group, neurons incubated with lenti-FAM134B for 12 h and then cultured in maintenance medium for 72 h before incubation in Mg^{2+}-free solution for 3 h; (5) lenti-FAM134B-shRNA (downregulation FAM134B) group, neurons incubated with lenti-FAM134B-shRNA for 12 h and then cultured in maintenance medium for 72 h before incubation in Mg^{2+}-free solution for 3 h; and (6) 3-MA group, neurons incubated with lenti-FAM134B-shRNA for 12 h, cultured in maintenance medium for 72 h and then incubated in the presence of the autophagy inhibitor 3-MA for 24 h before incubation in Mg^{2+}-free solution for 3 h.

MTT Assay

The methyl tetrazolium (MTT) assay was used to assess cell viability. Briefly, hippocampal neurons were seeded in 96-well plates at a density of 2×10^5 cells/ml. After 10 days of culture, the maintenance medium was removed and replaced with 20 µl of 5 mg/ml MTT solution and cultured at 37 °C for 4 h. Then, 150 µl of DMSO was added to dissolve MTT-formazan precipitates prior to incubation for 4–18 h at 37 °C. Absorbance density values were spectrophotometrically measured at 570 nm to calculate the cell viability.

TUNEL Assay

Apoptosis was determined by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay (Roche Diagnostics, Indianapolis, IN) according to the manufacturer's instructions. Briefly, coverslips containing primary neurons were processed for 30 min in 4% paraformaldehyde at 37 °C and then for 5 min in 0.1% Triton X-100 at 37 °C. Afterwards, the coverslips containing primary neurons were incubated for 1 h in TUNEL reaction mixture and then with DAPI in the dark at 37 °C for 5 min. Finally, the coverslips containing primary neurons were mounted with glycerol. In each group, a total of 500 neurons were detected and the positive cell proportion was calculated for statistical analyses.

Determination of Ca^{2+} Concentration in ER

The concentration of ER Ca^{2+} was assessed by using specific fluorescent probes (Mag-Fluo-AM) according to the instructions providing by manufacturer (Jiemei, Shanghai, China). Neurons were plated in 12-well plates at a density of 2×10^5 cells/ml and rinsed with 500 µl reagent A and then cultured in 300 µl reaction mixture in the dark for 60 min at 37 °C. Neurons were then rinsed with reagent A. The concentration of ER Ca^{2+} was quantified using a fluorescence microscope (Olympus, Japan).

Measurement of ROS

The level of intracellular reactive oxygen species (ROS) was determined by using a commercial kit (Nanjing Jiancheng Bioengineering Institute, Jiangsu, China) according to the instructions providing by manufacturer. Neurons were incubated with fluorescent probe 2′, 7′-dichlorofluorescin diacetate (DCFH-DA) in the dark for 30 min at 37 °C. Next, the neurons were resuspended in ice-cold Krebs–Ringer solution. The level of ROS was spectrophotometrically measured at a wavelength of 488 nm.

Immunofluorescence

Immunofluorescence was used to examine whether seizures induce the recruitment of FAM134B into autophagosomes. Coverslips containing primary neurons were fixed for 30 min in 4% paraformaldehyde at 37 °C and then permeabilized for 5 min in 0.1% Triton X-100 at 37 °C. Next, the neurons were blocked for 1 h in 10% goat serum at 37 °C and then incubated with rabbit anti-FAM134B (1:100; Abcam, USA) and mouse anti-LC3B (1:200; CST, USA) at 4 °C overnight, followed by incubation with dye-labeled secondary antibodies at 37 °C for 1 h. The expression of FAM134B and LC3B was observed under a confocal microscope (Zeiss, Germany).

Western Blotting

Hippocampal neurons were scraped on ice in 80 µl of RIPA buffer-containing protease inhibitor. Each sample was added a quarter volume of 5 × loading buffer and boiled at 100 °C for 5 min. These proteins were separated using 12% SDS-PAGE and stained with rabbit anti-β-actin (1:1000; CST, USA), mouse anti-CHOP (1:500; CST, USA), rabbit anti-GRP78 (1:500; Abcam, USA), rabbit anti-caspase12 (1:1000; CST, USA), and mouse anti-LC3B (1:1000; CST, USA) at 4 °C overnight, followed by incubation with secondary antibodies at 37 °C for 1 h. Analysis was performed using Image J software.

Infection with Lentiviral Vectors

Hippocampal neurons were seeded in 6-well plates at a density of 2×10^5 cells/ml. After 5 days of culture, cells were infected with lenti-FAM134B, lenti-FAM134B-shRNA, or lenti-pGV (Jikai, Shanghai, China) at a multiplicity of infection (MOI) of 15. The medium containing lentiviral vectors was removed and replaced with maintenance medium after 12 h. Infection efficiency was observed using a fluorescence microscope (Olympus, Japan) and was verified by Western blot analysis of the expression of FAM134B after 72 h of culture.

Statistical Analysis

All results are expressed as the mean ± standard deviation (SD) and were analyzed using SPSS software version 17.0. Differences between groups were determined by using one-way ANOVA and Newman–Keuls test. A P-value of < 0.05 was considered statistically significant.

Results

FAM134B Overexpression Attenuates AE-Induced Neuronal Apoptosis

The infection efficiency of lentiviral vectors was verified by Western blot analysis of the expression of FAM134B. Relative to the control group, pre-treatment with lenti-FAM134B increased the FAM134B expression and pre-treatment with lenti-FAM134B-shRNA elevated the expression of FAM134B (Fig. 1a). Neuronal viability, as measured by MTT assay, was significantly decreased in the AE group as compared with the control group (Fig. 1b). Overexpression of FAM134B using lenti-FAM134B attenuated AE-induced neuronal death, while downregulation of this protein using lenti-FAM134B-shRNA exacerbated AE-induced neuronal death (Fig. 1b). Statistically, there were no significant differences between the AE group and the negative control group (Fig. 1b).

In addition, the apoptosis rate of cells was assessed by TUNEL assay. Neuronal apoptosis was significantly increased in the AE group (Fig. 2). However, FAM134B overexpression attenuated AE-induced neuronal apoptosis and FAM134B downregulation exacerbated this process (Fig. 2). The difference between the AE group and the negative control group was not statistically significant (Fig. 2).

FAM134B Overexpression Augments Autophagy Induced by AE

The ratio of LC3-II/LC3-I was significantly increased in the AE group as compared to the control group (Fig. 3). Relative to the AE group, FAM134B overexpression enhanced the ratio of LC3-II/LC3-I, whereas its downregulation decreased this ratio (Fig. 3). No differences were found between the AE and negative control groups (Fig. 3).

In order to further assess the effects of FAM134B on autophagy, neurons were co-immunostained for FAM134B and LC3B. We found more extensive colocalization of these proteins in the AE group as compared with controls, and

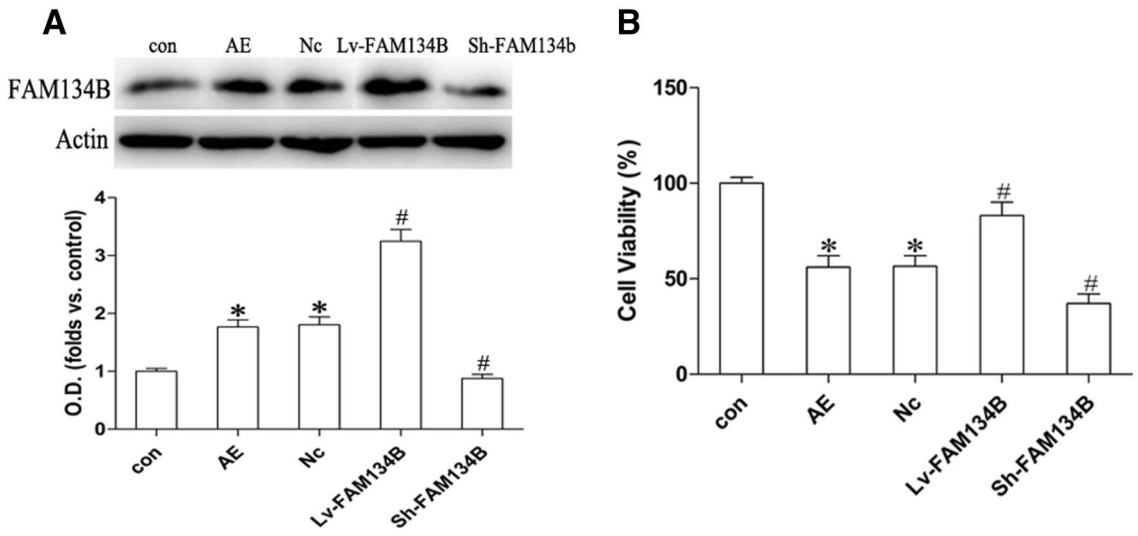

Fig. 1 Effect of FAM134B on hippocampal neuronal damage induced by acquired epilepsy (AE). **a** The infection efficiency of lentiviral vectors was analyzed by Western blot analysis of the expression of FAM134B. Hippocampal neurons were only incubated with lenti-FAM134B and lenti-FAM134B-shRNA for 12 h and then cultured in maintenance medium for 72 h. **b** Cell viability, assessed by MTT assay. Hippocampal neurons were incubated with lenti-FAM134B, lenti-FAM134B-shRNA or lenti-pGV for 12 h and then cultured in maintenance medium for 72 h before exposure to Mg^{2+}-free solution. Data are expressed as mean ± SD of six independent experiments. $^{*}P < 0.05$ compared to the control group, $^{\#}P < 0.05$ compared to the AE group

FAM134B overexpression further increased this elevated colocalization (Fig. 4). On the contrary, downregulation of FAM134B decreased the colocalized areas of FAM134B and LC3B (Fig. 4). No significant differences were found between the AE group and negative control group (Fig. 4). Altogether, these data indicate that the level of autophagy is regulated by FAM134B.

FAM134B Overexpression Attenuates ER Ca^{2+} Release and Intracellular ROS Production Induced by AE

As shown in Fig. 5, compared with the control group, the ER Ca^{2+} concentration was reduced and the intracellular level of ROS was increased in the AE group. This was rescued by FAM134B overexpression, which significantly increased the ER Ca^{2+} concentration and diminished the intracellular ROS level as compared with the AE group (Fig. 5). In contrast, FAM134B downregulation had the opposite effect, exacerbating the defects observed in response to AE. The AE group showed no differences compared with the negative control group (Fig. 5).

FAM134B Overexpression Attenuates AE-Induced ER Stress

We further found that relative to controls, AE caused a significant increase in the GRP78 and CHOP expression (Fig. 6). While FAM134B knockdown further raised these expression levels, its overexpression decreased the GRP78 and CHOP levels compared to that in the AE group (Fig. 6). No significant differences were found between the AE group and negative control group (Fig. 6). These results suggest that ER stress induced by AE is regulated by FAM134B expression.

Inhibition of Autophagy by 3-MA Abolishes the Protective Effects of FAM134B

To further confirm whether FAM134B exerts effects on apoptosis and ER stress through induced autophagy, we took advantage of the selective autophagy inhibitor 3-MA. As shown in Fig. 7, in the AE group, the GRP78 and CHOP levels were increased compared to the control group, and FAM134B overexpression reversed this effect. Interestingly, pre-treatment with 3-MA significantly abolished the effects of FAM134B on neuronal apoptosis and ER stress, indicating that the observed effects were specific to FAM134B (Fig. 7). The AE group and the negative control group showed no statistically significant differences (Fig. 7).

Discussion

Recent studies have implicated that autophagy plays a crucial role in various neurodegenerative diseases and FAM134B plays a functional role in autophagy. (Cai et al. 2019; Niu et al. 2018). However, the effects of FAM134B-regulated

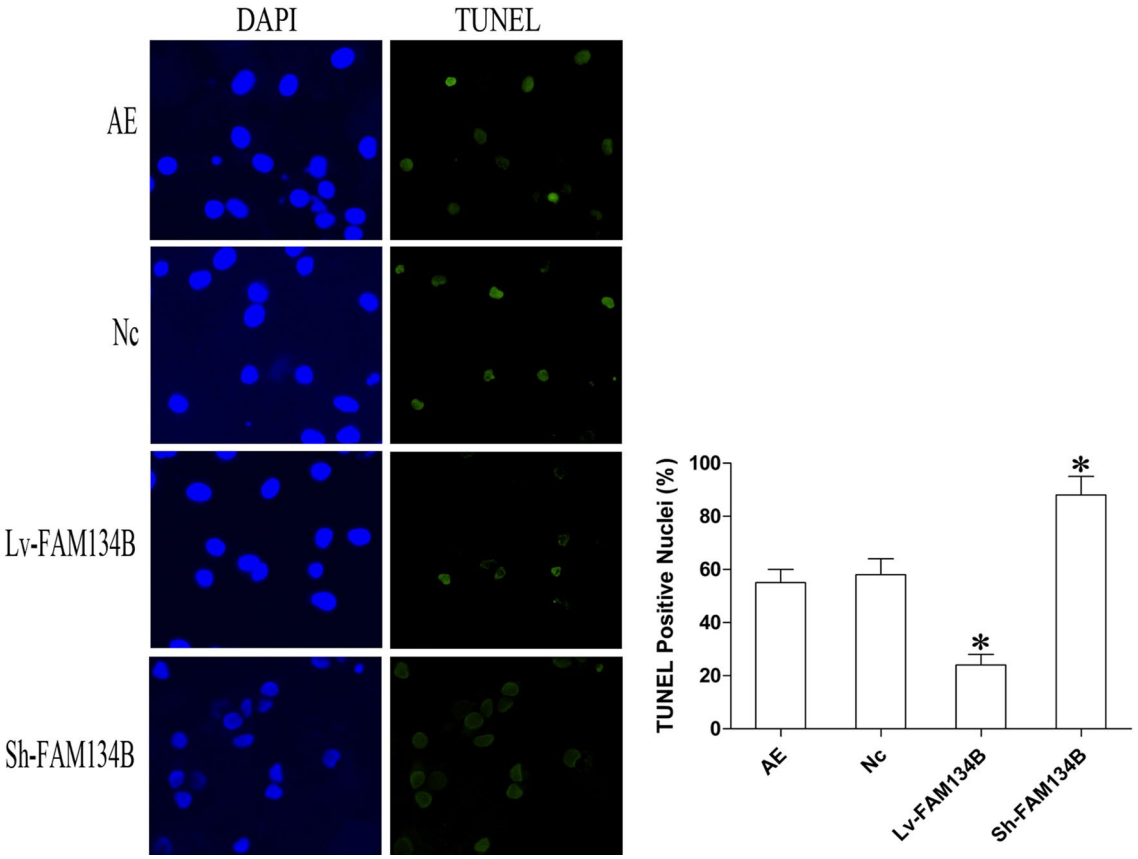

Fig. 2 Effect of FAM134B on hippocampal neuron apoptosis induced by acquired epilepsy (AE). TUNEL assay was used to assess hippocampal neuron apoptosis. Both the AE group and the negative control group exhibit apoptotic neurons (positive cells), whereas in the lenti-FAM134B group, FAM134B overexpression protects neurons against apoptosis induced by AE. In the lenti-FAM134B-shRNA group, FAM134B downregulation exacerbates AE-induced neuronal apoptosis. Data are expressed as mean ± SD of six independent experiments. $^{*}P < 0.05$ compared to the AE group

autophagy on ER stress and neuronal apoptosis induced by AE have not been investigated. In this study, we found that AE can induce ER dysfunction, which is characterized by the depletion of its Ca^{2+} storage and increased intracellular ROS levels. ER dysfunction was also evident through a significant elevation in ER stress and autophagy levels. Importantly, FAM134B overexpression attenuated ER stress and neuronal apoptosis, while FAM134B knockdown led to opposite effects. Moreover, selective inhibition of autophagy abolished the protective effects of FAM134B on ER stress and apoptosis. Altogether, our results indicate that FAM134B exerts neuroprotective effects on seizure-induced ER stress and apoptosis, and that this is likely based on its regulation of autophagy.

Autophagy is an important process for maintaining intracellular homeostasis. Many studies have demonstrated that autophagy modification can alleviate the results of epileptic seizures (Attia et al. 2019). Here, we found that in the HNC model of AE, autophagy increased. In addition, FAM134B overexpression enhanced seizure-induced autophagic

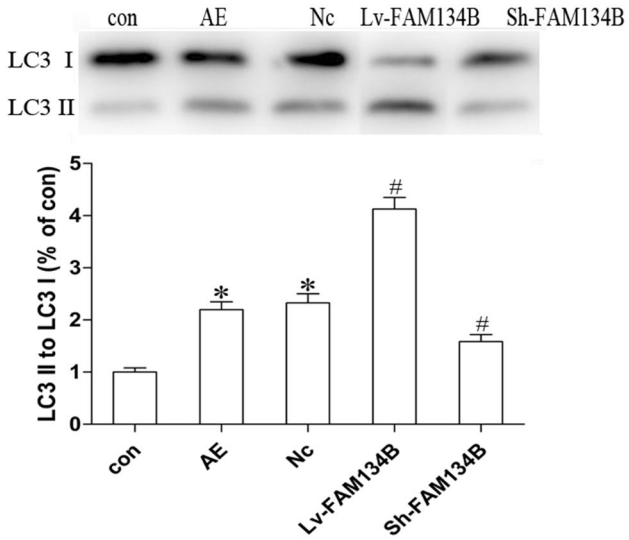

Fig. 3 Effect of FAM134B on LC3-II/LC3-I ratio induced by acquired epilepsy (AE). Western blot and quantitative analysis of LC3-II/LC3-I ratio in hippocampal neurons. Data are expressed as mean ± SD of six independent experiments. $^{*}P < 0.05$ compared to the control group, $^{\#}P < 0.05$ compared to the AE group

activity, as evidenced by increases in the ratio of LC3-II/LC3-I and in the area of colocalization of FAM134B and LCB, whereas FAM134B downregulation resulted in the opposite. These observations indicate that FAM134B plays a crucial role in regulating AE-induced autophagy.

The ER is the most important Ca^{2+} storage organelle. Various cellular stresses, including oxidative stress and the depletion of Ca^{2+} from ER stores, can trigger ER stress responses (Dejeans et al. 2010; You et al. 2016). An increase in the amount of Ca^{2+} released from the ER can boost the generation of ROS, which causes a positive feedback loop leading to further calcium release and stress (Bhandary et al. 2012). Our results demonstrate that the ER Ca^{2+} concentration significantly decreases and the intracellular ROS level increases in the HNC model of AE. These data suggest that seizures can contribute to the disruption of ER homeostasis by causing an increase in Ca^{2+} release from the ER and an elevated oxidative stress level. We observed that FAM134B overexpression significantly attenuated these two processes, while its downregulation further exacerbated them. These results suggest that FAM134B contributes to the recovery of ER function through regulating Ca^{2+} concentration within this organelle and by limiting oxidative stress.

Disruption of ER homeostasis, such as the depletion of Ca^{2+} from the ER, can interfere with protein folding and transport, thus contributing to ER stress (Concannon et al. 2008). Moderate ER stress can improve cell survival through the unfolded protein response (UPR) (Shi et al. 2016), which is mediated by ER transmembrane receptors, including IRE1, PERK, and ATF6. Activation of these receptors upregulates the expression of ER chaperones, such as GRP78 (You et al. 2016). On the other hand, severe and/or prolonged ER stress promotes the activation of pro-apoptotic factors such as CHOP (Lei et al. 2017). In our study, we found that FAM134B overexpression inhibited GRP78 and CHOP expression as well as neuronal apoptosis, while FAM134B knockdown evoked the opposite. These results indicate that FAM134B alleviates neuronal ER stress and apoptosis.

Several studies have implicated that autophagy is closely related to ER stress and that autophagy plays an important role in cell survival and cell death (Ding et al. 2007; Giorgi et al. 2015; Song et al. 2017). Autophagy can inhibit apoptosis by eliminating damaged organelles and misfolded/unfolded proteins, inhibiting caspase activation, and clearing sequestosome 1 (SQSTM1)/p62, thereby protecting cells that are under ER stress. In contrast, dysregulation of autophagy may contribute to several pathological conditions by causing cell death (Ogata et al. 2006; Xu et al. 2017). In this study, we demonstrated that selective inhibition of autophagy using

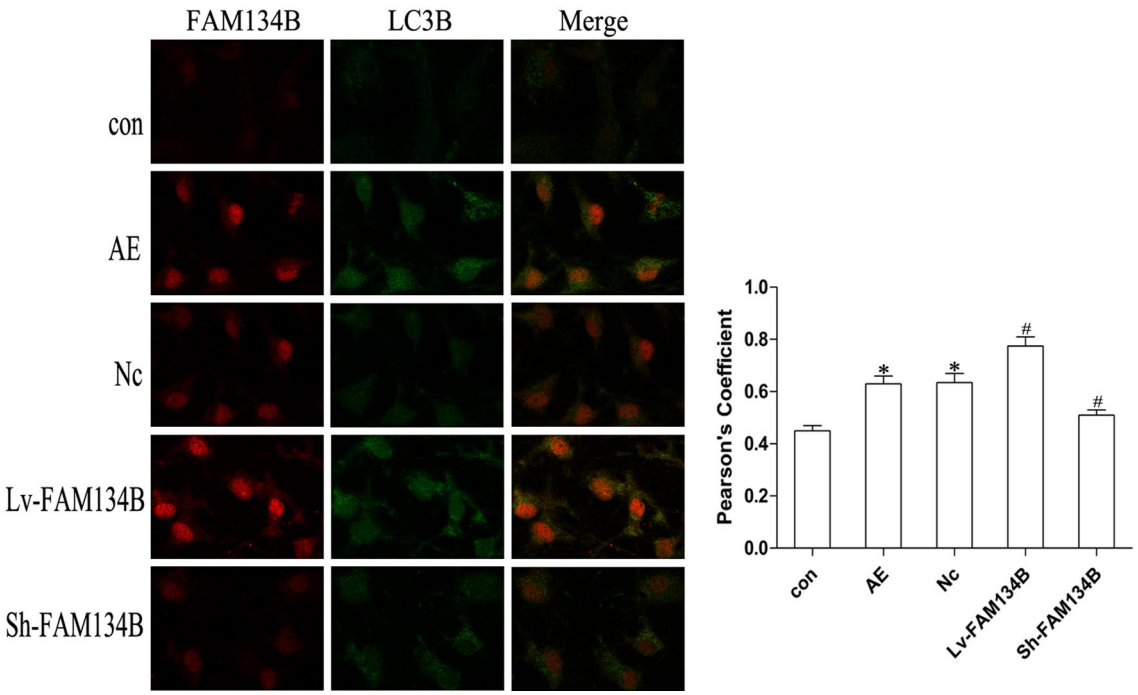

Fig. 4 Effect of FAM134B on co-immunostained for FAM134B and LC3B induced by acquired epilepsy (AE). Representative images of FAM134B and LC3B immunofluorescence in hippocampal neurons. Colocalization of FAM134B with LC3B was determined using the Pearson's coefficient. Data are expressed as mean ± SD of six independent experiments. $^{*}P < 0.05$ compared to the control group, $^{\#}P < 0.05$ compared to the AE group

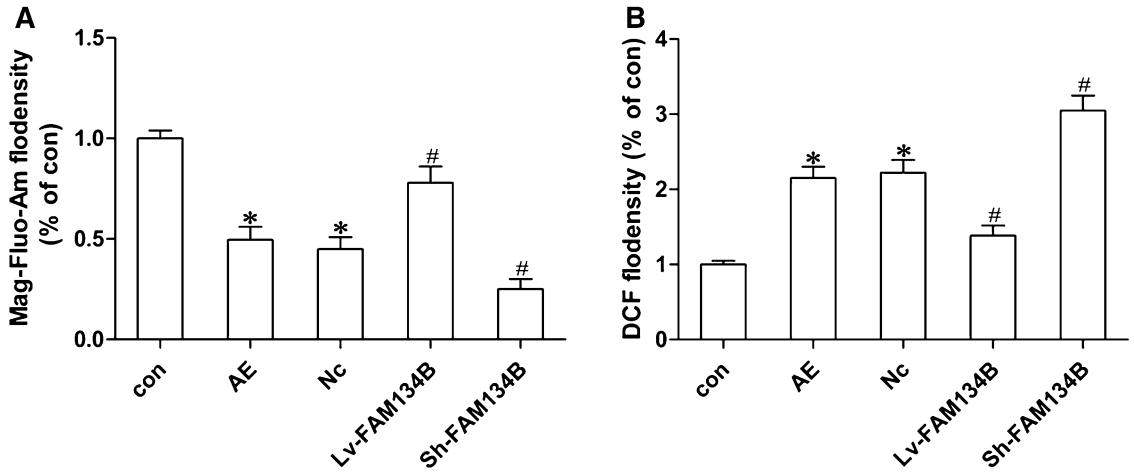

Fig. 5 Effect of FAM134B on changes of endoplasmic reticulum (ER) Ca^{2+} concentration and intracellular reactive oxygen species (ROS) production induced by acquired epilepsy (AE). **a** ER Ca^{2+} concentration in hippocampal neurons. **b** Levels of ROS in hippocampal neurons. Data are expressed as mean ± SD of six independent experiments. $^{*}P < 0.05$ compared to the control group, $^{\#}P < 0.05$ compared to the AE group

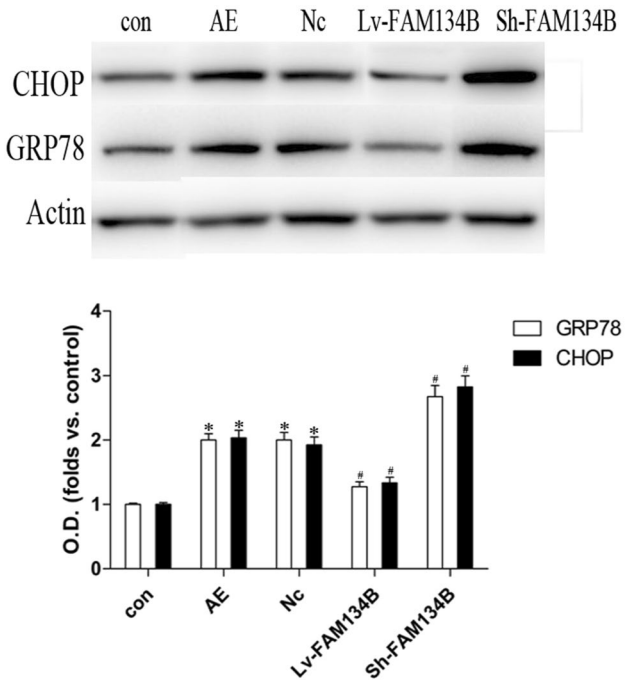

Fig. 6 Effect of FAM134B on glucose-regulated protein 78 (GRP78) and C/-EBP homologous (CHOP) expression induced by acquired epilepsy (AE). Western blot and quantitative analysis of GRP78 and CHOP expression in hippocampal neurons. Data are expressed as mean ± SD of six independent experiments. $^{*}P < 0.05$ compared to the control group, $^{\#}P < 0.05$ compared to the AE group

3-MA significantly abolished the effects of FAM134B on ER stress and neuronal apoptosis. This is consistent with previous studies indicating that autophagy induced by ER stress can reduce cell damage, and that FAM134B inhibition contributes to the misfolding or aggregation of proteins leading to neuronal injury (Ciechomska et al. 2013; Khaminets et al. 2015; Song et al. 2017). Taken together, our results suggest that FAM134B is involved in promoting autophagy function to relieve ER stress and prevent neuronal apoptosis.

In conclusion, our study provides evidence that FAM134B attenuates seizure-induced apoptosis and ER stress in hippocampal neurons, and the underlying mechanism is, at least, in part, based on the regulation of autophagy. Although further studies are needed to unravel the specific mechanisms underlying FAM134B-mediated regulation of autophagy in AE, our results pave the way for the development of novel therapeutic strategies for the treatment of epilepsy.

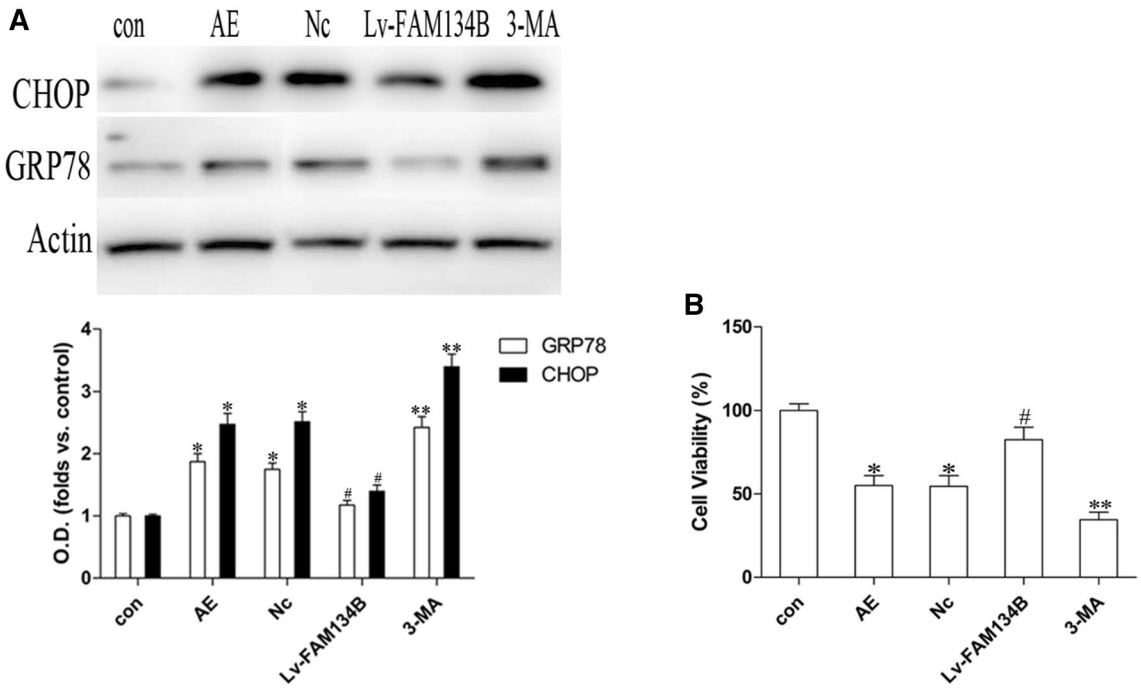

Fig. 7 Selective autophagy inhibitor 3-methyladenine (3-MA) abolishes the protective effects of FAM134B. **a** Western blot and quantitative analysis of GRP78 and CHOP in hippocampal neurons. **b** Cell viability, analyzed using MTT assay. Data are expressed as mean ± SD of six independent experiments. $^{*}P<0.05$ compared to the control group, $^{\#}P<0.05$ compared to the AE group, $^{**}P<0.05$ compared to the lenti-FAM134B group

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Author Contributions NX and CW contributed to conception and design of the study; NX, YL (Yajun Lian), and HZ performed the statistical analysis; YL (Yingjiao Li), YL (Yujuan Li), LD, and XM performed the experiments; NX and YL (Yingjiao Li) wrote the manuscript. All authors contributed to manuscript revision and read and approved the submitted version.

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Compliance with Ethical Standards

Conflict of interest The authors have no conflicts of interest to declare.

Ethical Approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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