Neurochemistry International 110 (2017) 25-37

Contents lists available at ScienceDirect

Neurochemistry International

journal homepage: www.elsevier.com/locate/nci

Repeated restraint stress increases seizure susceptibility by activation of hippocampal endoplasmic reticulum stress



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ARTICLE INFO

Article history: Received 15 March 2017 Received in revised form 2 July 2017 Accepted 1 September 2017 Available online 5 September 2017

Keywords: Restraint stress Seizure susceptibility Hippocampus ER stress Oxidative stress

ABSTRACT

A growing body of evidence suggests that stress triggers a variety of pathophysiological responses. Recent studies show that stress produces enduring effects on structure and function of hippocampus, which is one of the most important structures involved in epilepsy. In the present study, we determined the effect of repeated restraint stress exposure on the susceptibility of pentylenetetrazole (PTZ)-induced seizures and the possible mechanisms involved using a rodent model. Our results show that mice subjected to repeated restraint stress exhibited shorter latency to PTZ-induced tonic-clonic seizures and higher seizure severity, suggesting chronic restraint stress increases seizure susceptibility. Following repeated restraint stress, we observed an increased level of endoplasmic reticulum (ER) stress as well as oxidative stress in the hippocampus. Moreover, our results show that chronic restraint stress exposure causes neuron loss in the hippocampus. Inhibition of ER stress with chemical chaperone, tauroursodeoxycholic acid (TUDCA), however, protects against chronic restraint stress-induced neuron loss, suggesting repeated restraint stress-induced neuronal degeneration is dependent on ER stress activation. On the other hand, inhibition of ER stress with TUDCA suppresses restraint stress-induced seizure susceptibility. Taken together, these results indicate that repeated restraint stress increases seizure susceptibility by activation of hippocampal ER stress and ER stress mediated oxidative stress and neurodegeneration. Thus, attenuating ER stress may serve as a potential therapeutic strategy targeted to block stress-induced seizure activities.

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1. Introduction

Prolonged stress exposure induces alterations in hippocampal network and neuronal excitability. Several lines of evidence suggest that chronic stress can affect hippocampal synaptic plasticity (Abush and Akirav, 2013; Joels et al., 2007), dendrite spine density (Radley et al., 2013; Rico et al., 2015) and neurogenesis (Snyder et al., 2011). Hippocampus is often considered as the focus of epileptic seizures, and maladaptive changes of hippocampus induced by stress exposure may increase the vulnerability to

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seizure. In clinic, patients with epilepsy commonly report that stress is a precipitating factor of seizures (Fisher et al., 2000; Maguire and Salpekar, 2013; Nakken et al., 2005). In animal studies, it was demonstrated that repeated stress exposure increases seizure susceptibility (Chadda and Devaud, 2004; Jones et al., 2013). Despite a large amount of experimental evidence support that chronic stress induces seizure susceptibility, it is still unclear how chronic stress contributes to the development of epilepsy or precipitation of seizure.

The endoplasmic reticulum (ER) is responsible for generating and folding secreted and membrane proteins. Disruption of the protein folding or accumulation of misfolded proteins in the ER induces a pathological state known as ER stress. ER stress triggers an adaptive response called the unfolded protein response (UPR), which is a signal transduction pathway responsible for restoring



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protein folding and degradation of misfolded proteins (Lindholm et al., 2006; Xu et al., 2005). However, under chronic ER stress, the UPR will elicit the signals leading to apoptosis (Tabas and Ron, 2011; Urra et al., 2013) and cell death (Walter and Ron, 2011). ER stress is activated and regulated by a variety of factors. Recent studies report that chronic stress can disrupt protein folding process, and consequently leads to ER stress in the central nervous system including cortex, hippocampus and amygdala (Huang et al., 2015; Zhang et al., 2014; Zhao et al., 2013), suggesting a role of ER stress in chronic stress-related neuropathogenesis. ER stress is now implicated in the pathogenesis of several neurological disorders including epilepsy (Bouman et al., 2011; Brennan et al., 2013; Han et al., 2015; Hoozemans et al., 2009; O'Connor et al., 2008; Torres-Peraza et al., 2013; Yamamoto et al., 2006; Zhao et al., 2016). However, the precise molecular mechanism by which ER stress contributes to these neurological diseases remains largely elusive, particularly the mechanisms underlying the effects of ER stress on seizure and epilepsy. Here in this study, we sought to determine whether chronic stress induces seizure susceptibility and to further explore the role of ER stress on chronic stressinduced seizure activity.

2. Materials and methods

2.1. Animals

Male C57/BL6 mice (4 weeks old; weighing 19 ± 2 g at the beginning of the experiments) were obtained from the comparative medicine center of Yangzhou University (Yangzhou, China). The animals were housed in plastic cages and kept in a regulated environment (22 ± 1 °C) with an artificial 12 h light/dark cycle (lighted from 7:00 a.m. to 7:00 p.m.). Food and tap water were available *ad libitum*. Procedures for PTZ induced-seizures and all subsequent experiments were approved by the Animal Care and Use Committee of Medical School of Southeast University. All efforts were made to minimize animal suffering and discomfort and to reduce the number of animals used.

2.2. Drugs

Pentylenetetrazole (PTZ) and tauroursodeoxycholic acid (TUD-CA) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.3. Repeated restraint stress procedure

Animals were maintained under standard laboratory conditions for at least 7 days before starting the stress protocol. For repeated restraint stress, we used a modified version of the restraint protocol described by Mozhui et al., (2010). Briefly, mice were placed in airaccessible cylinders for 2 h daily (14:00 p.m. to 16:00 p.m.) for 3, 7 and 14 consecutive days. Immediately following the final restraint stress, mice were subjected to seizure susceptibility assay or sacrificed to obtain brain tissue for further experiment. To investigate the role of ER stress on oxidative stress and seizure susceptibility, mice were treated with an ER stress inhibitor TUDCA during the 14 days restraint stress procedure. Mice were subjected to seizure susceptibility assay or sacrificed to obtain brain tissue for further experiment as described above.

2.4. PTZ-induced seizures

PTZ was administered intraperitoneally to induce acute seizure at a single dose of 60 mg/kg according to the previous studies (Abdallah, 2010; Mizoguchi et al., 2011; Uma Devi et al., 2006). Mice were then subjected to continuous video monitoring for 30 mins following PTZ administration to observe seizure events. The seizure intensity was scored as follows Stage 0, no response; Stage 1, ear and facial twitching; Stage 2, convulsive twitching axially through the body; Stage 3, myoclonic jerks and rearing; Stage 4, turning over onto the side, wild running, and wild jumping; Stage 5, generalized tonic-clonic seizures; and Stage 6, death (Becker et al., 1995; Mizoguchi et al., 2011; Schroder et al., 1993). Latency to the onset of tonic-clonic seizures, the seizure scores in 5-min blocks during 30 min, and the number of mortality were measured.

2.5. Intracerebroventricular (i.c.v.) injection

For i.c.v. injection, a guide cannula (33-gauge, Plastics One Inc., Roanoke, VA, USA) was implanted in the right lateral ventricle. Briefly, mice were anesthetized and positioned in a stereotaxic instrument with a mouse adapter (David Kopf Instruments, Tujunga, CA, USA). The stereotaxic coordinates for implantation of guide cannula into right lateral ventricle were according to the mouse brain atlas (AP = 0.5 mm relative to bregama; ML = 0.8 mm; DV = -2.5 mm from the skull surface). The guide cannula was then affixed with dental cement and mice were allowed to recover for at least 7 days. After recovery, the patency of the cannula was tested by injection of 250 ng of angiotensinIIin 1.0 µl PBS, since angiotensinII induces drinking response by stimulating preoptic structure (Olivadoti and Opp, 2008; Skott, 2003). Mice with positive drinking response were selected in the subsequent experiment. Injections were performed using a Hamilton syringe attached to the guide cannula. Vehicle (PBS), and vehicle containing TUDCA (1 µg) were injected in control and repeated restraint stressed mice during the 14 days restraint stress procedure every other day for 7 dose (Purkayastha et al., 2011; Young et al., 2012). All injections were 1 µl and the injections were carried out over 60 s and the syringe was left in place for additional 2 min to minimize backflow after each injection. Mice were then subjected to seizure susceptibility assay or sacrificed to obtain brain tissue for further experiment as described above.

2.6. Brain tissue processing

For western blot experiment, the hippocampus was dissected from control and restraint stressed mice. Dissected hippocampal tissues were then snap-frozen and stored at -80 °C until use. For immunocytochemistry experiment, the mice were euthanized by an intraperitoneal injection of an overdose of urethane and were transcardially perfused with 100 mL of saline (0.9% w/v NaCl), followed by 50 mL of 4% paraformaldehyde in 0.05 M sodium phosphate (pH = 7.4, containing 0.8% NaCl). The mouse brains were removed and post-fixed overnight in 4% paraformaldehyde, then were cryoprotected in 30% sucrose in 1xPBS for 72 h. Serial coronal hippocampal sections with a thickness of 25 µm were cut using a cryostat (Leica Microsystems, Wetzlar, Germany) and every sixth section throughout the hippocampus were collected in $1 \times PBS$ as free-floating sections and were stored at 4 °C for future immunocytochemistry studies as described previously (Zhu et al., 2016). For the transmission electron microscopy (TEM) analyses, mice were deeply anesthetized and transcardially perfused with 0.1 M phosphate buffer (PBS, pH = 7.4), followed by 4% PFA and 2% glutaraldehyde. The CA3 subfield of the hippocampus was then removed and processed for electron microscopy.

2.7. Immunocytochemistry and cell counting

The immunocytochemistry studies were performed on freefloating sections as described previously (Zhu et al., 2016). Briefly, the sections were heated (65 °C for 50 min) in antigen unmasking solution (2xSSC/formamide), incubated in 2 M HCl (30 °C for 30 min), rinsed in 0.1 M boric acid (pH 8.5) for 10 min, incubated in 1% H₂O₂ in PBS for 30 min and blocked in PBS containing 3% normal goat serum, 0.3% (w/v) Triton X-100 and 0.1% BSA (room temperature for 1 h), followed by incubation with a mouse anti-GRP78 (1:200; Santa Cruz, Texas, USA), a mouse anti-NeuN (1:200; Abcam, Temecula, CA, USA) and a rabbit anti-CHOP (1:200; Santa Cruz, Texas, USA) and a rabbit anti-CHOP (1:200; Santa Cruz, Texas, USA) antibody at 4 °C overnight. Subsequently, the sections were incubated with fluorescent secondary antibody, a FITC-conjugated goat anti-mouse and a FITC-conjugated goat anti-rabbit antibody (1:200; Cwbiotech, Beijing, China) for GRP78, NeuN and CHOP labeling respectively. Randomly selected immunofluo-rescence images of CHOP, GRP78 and NeuN staining from hippo-campal sections of control and restraint stress mice were analyzed with Image J software (NIH, Bethesda, MD, USA).

2.8. Western blotting

The dissected hippocampal tissues of the mice were homogenized in tissue lysis buffer (Beyotime, China). After being lysed for 15 min on ice, samples were centrifuged at 12,000 rpm for 15 min. The protein content in each supernatant fraction was determined using a BCA protein assay kit (Pierce, Rockford, IL, USA) and samples containing equivalent amounts of protein were applied to 12% acrylamide denaturing gels (SDS-PAGE). After electrophoresis, the proteins were transferred to nitrocellulose membranes (Amersham, Little Chalfont, UK) using a Bio-Rad mini-protein-III wet transfer unit (Hercules, CA, USA) overnight at 4 °C.

The membranes were then incubated with 5% non-fat milk in TBST (10 mmol/l Tris pH = 7.6, 150 mmol/L NaCl, 0.01%Tween-20) for 1 h at room temperature followed by three washes, then were incubated with rabbit anti-HSP70 (1:2 500; Novus, Littleton, CO, USA), rabbit anti-ATF4 (1:2000; Santa Cruz, Texas, USA), rabbit anti-CHOP (1:2000; Santa Cruz, Texas, USA), mouse anti-GRP78 (1:2 500; Santa Cruz, Texas, USA), goat anti-peIF2 α (1:2 500; Abcam, Temecula, CA, USA), mouse anti-NeuN (1:2 500; Abcam, Temecula, CA, USA) and rabbit anti- β -actin in TBST overnight at 4 °C. After several washes with TBST buffer, the membranes were incubated for 1 h with HRP-linked secondary antibody (Boster Bioengineering, Wuhan, China) diluted 1:5,000, followed by four washes. The membranes were then processed with enhanced chemiluminescence (ECL) western blot detection reagents (Millipore, Billerica, MA, USA). Signals were digitally captured using a MicroChemi chemiluminescent image analysis system (DNR Bioimaging Systems, Jerusalem, Israel). Blots were quantified using the Image J software (NIH, Bethesda, MD, USA).

2.9. Measurement of ROS production, enzymatic antioxidant defense, lipid peroxidation and protein oxidation

To detect reactive oxygen species (ROS) production in the brain sections, a cell membrane-permeable superoxide-sensitive fluorescent dye dihydroethidium (DHE) (Sigma-Aldrich, St. Louis, MO, USA) was used as we described previously (Zhu et al., 2016). Briefly, hippocampal sections were incubated with 1 μ M DHE in 0.1 M phosphate buffer (PBS, PH = 7.4) at room temperature for 15 min in the dark. The sections were then washed with PBS three times and mounted on gelatin-coated slides. The fluorescence of DHE was visualized by a confocal laser scanning microscope (Olympus LSM-GB200, Japan) using an excitation wavelength of 520–540 nm. Fluorescence was quantified with the Image J software program (NIH, Bethesda, MD, USA). The enzymatic antioxidant defense was evaluated by detecting copper/zinc superoxide dismutase (CuZn-SOD) and glutathione peroxidase (GSH-Px). The lipid peroxidation was evaluated by detecting the levels of malondialdehyde (MDA)

and 4-hydroxy-2-nonenal (4HNE). Briefly, the dissected hippocampal tissues from mice were homogenized in tissue lysis buffer (Beyotime, China). After being lysed for 15 min on ice, the homogenates were centrifuged at 3000 rpm for 15 min. The protein content in each supernatant fraction was determined using a BCA protein assay as described in the western blotting protocol. The activity of CuZn-SOD and GSH-Px in the supernatant was measured by a commercially available CuZn-SOD and a GSH-Px assay kit (Jiancheng Bioengineering, Nanjing China). The levels of MDA and 4-HNE in the supernatant were measured by a commercially available MDA (Jiancheng Bioengineering, Nanjing China) and a 4-HNE assay kit (Cell Biolabs, San Diego, CA) respectively. The CuZn-SOD and GSH-Px activity was expressed as U per mg protein. The MDA content was expressed as nmol per mg protein while the 4-HNE content was expressed asµg per g protein. The extent of protein oxidation was assayed by measuring the protein carbonyl content using a protein carbonyl content assay kit (Sigma Aldrich). The protein carbonyl content was expressed as nmol per mg protein. All assays were conducted according to the manufacturer's instructions

2.10. Transmission electron microscopy

For the transmission electron microscopy (TEM) analyses, mice were deeply anesthetized and transcardially perfused with 0.1 M phosphate buffer (PBS, pH = 7.4), followed by 4% PFA and 2% glutaraldehyde. The CA3 subfield of the hippocampus was then removed and processed for electron microscopy. Tissue samples were diced and immediately submerged in 2.5% glutaraldehyde (0.1 M sodium cacodylate buffer, pH = 7.2). Each specimen was trimmed and embedded in Spurr's medium. Tissue blocks were post-fixed with osmium, en bloc stained with uranyl acetate, and post-stained with uranyl acetate and lead citrate. Tissue sections were cut to a thickness of 60–70 nm and viewed on 300-mesh coated grids using a transmission electron microscope (JEOL, JEM-1010, Tokyo, Japan). The images were acquired digitally from a randomly selected pool of eight visual fields (800 μ m²) under each condition.

2.11. TUNEL staining

TUNEL staining was performed using the In Situ Cell Apoptosis Detection kit (Boster Bioengineering, Wuhan, China), according to the manufacturer's instructions. Briefly, the sections were rinsed with PBS for 2–5 min. After being treated with 50 μ l 3% H₂O₂ for 10 min at room temperature, the sections were incubated with 20 μ l protein K solutions for 15 min. The sections were then incubated with 50 μ l TUNEL reaction mixture for 1 h at 37 °C in the dark. Further incubation with 50 μ l SABC (1:100) was performed at 37 °C for 30 min. The sections were then rinsed with PBS and stained with DAB substrate for 10 min at room temperature. Images were captured by using a light microscope (Olympus LSM-GB200, Japan). The nuclei of apoptotic cells appear dark brown. Randomly selected TUNEL staining images from hippocampal sections of control and restraint stress mice were analyzed with Image J software (NIH, Bethesda, MD, USA).

2.12. Statistical analysis

All data are presented as the means \pm S.E.M. Statistical significance was determined by using unpaired *t*-test for two groups comparison and by using one-way or two-way ANOVA and repeated-measures ANOVA for multi-group comparisons. Tukey's test was used for *post-hoc* comparisons. Differences were considered to be significant for values of p < 0.05.

3. Results

3.1. Repeated restraint stress increases seizure susceptibility

It is reported that prolonged stress exposure induces alterations in hippocampal network and neuronal excitability. To test whether repeated restraint stress affects seizure susceptibility, we used a non-competitive antagonist of GABAA receptor, pentylenetetrazol (PTZ) to induce seizures in the mice exposed to repeated restraint stress for 3, 7 and 14 days respectively (Fig. 1A). Our data showed that the latency to PTZ-induced tonic-clonic seizures was significantly decreased in 7-day (F_{3.28}=6.294, p=0.008) and 14-day (F_{3.28}=6.294, p=0.005) restraint stressed mice (Fig. 1B). Moreover, seizure severity was significantly increased in 7-day (F_{3.15}=8.240, p=0.006) and 14-day (F_{3.15}=8.240, p=0.006) restraint stressed micecompared to that in vehicle control mice (Fig. 1C). The mortalities in 7 (60%) and 14 days (70%) restraint stressed mice are increased compared to vehicle control mice (40%) when they are induced seizure by PTZ (Fig. 1D). These results indicate that repeated restraint stress decrease the latency and increase the severity of PTZ-induced seizure, suggesting that repeated restraint

stress can increase seizure susceptibility.

3.2. Repeated restraint stress induces hippocampal ER stress

To investigate whether ER stress is activated in the hippocampus of chronic restraint mice, we firstly examined the expression of ER stress-related proteins, including HSP-70, ATF4, CHOP, GRP78, and p-eIF2 α in the mice exposed to repeated restraint stress for 3, 7 and 14 days respectively (Fig. 2A). Our Western blot results revealed that the protein levels of HSP-70, (F_{3,16}=4.511, p=0.011, 14 days restraint stress vs control) ATF4 (F3,16=16.69, p<0.0001, 7 days restraint stress vs control; p<0.0001, 14 days restraint stress vs control; **p**<**0.0001**, 14 days restraint stress vs 3 days restraint stress; p<0.0001, 7 days restraint stress vs 3 days restraint stress), CHOP (F_{3.16}=4.35, p=0.03, 7 days restraint stress vs control; p=0.02, 14 days restraint stress vs control), GRP78 (F_{3,16}=6.28, p=0.006, 14 days restraint stress vs control; p=0.033, 14 days restraint stress vs 3 days restraint stress), and *p*-elF2α (**F**_{3,16}=14.43, *p*=0.013, 7 days restraint stress vs control; p<0.001, 14 days restraint stress vs control; **p**=**0.001**, 14 days restraint stress **vs** 3 days restraint stress) in the hippocampus started to increase in the 3 days restraint



Fig. 1. Repeated restraint stress increases the susceptibility of PTZ-induced seizure. (**A**) Schematic representation of experiment design. Mice were exposed to restraint stress for 3, 7 and 14 consecutive days. Immediately following the final restraint stress, mice were subjected to seizure susceptibility assay. (**B**) The latency to PTZ-induced tonic-clonic seizures was decreased in 7-day and 14-day restraint stressed mice ($\mathbf{n=8}$). (**C**) Seizure severity was increased in 7-day and 14-day restraint stressed mice ($\mathbf{n=6}$). (**D**) The mortality of PTZ-induced tonic-clonic seizures of the control and 3, 7 and 14 days restraint stressed mice ($\mathbf{n=10}$). Values are means \pm S.E.M. **p < 0.01, one-way ANOVA and repeated-measures ANOVA.





Fig. 2. Repeated restraint stress induces expression of hippocampal ER stress-related proteins. (A) Western blot showing the protein levels of ER stress-related protein HSP-70, ATF4, CHOP, GRP78, and *p*-elF2 α in the control and 3, 7 and 14 days repeated restraint stressed mice. (**B**–**F**) Bar graphs showing the quantification of HSP70, ATF4, CHOP, GRP78 and pelF2 α , which were represented as the intensity ratios of these proteins to β -actin (**n**=**5**). Values are means \pm S.E.M. **p* < 0.05, ***p* < 0.01, ****p* < 0.001, one-way ANOVA.

stressed mice and were significantly elevated in the 7 and 14 days restraint stressed mice (Fig. 2B-F). In consistent with the western blot data, our immunofluorescence results showed that hippocampal CHOP (p<0.001) and GRP78 (p<0.001) positive cells are significantly increased in 14 days restraint stressed mice compared to control mice (Fig. 3A–C). To evaluate the rough ER morphology in the hippocampus of repeated restraint stressed mice based on ultrastructural criteria, we performed a transmission electron microscopy (TEM) analysis on the hippocampal CA3 regions of the mice. TEM studies demonstrated that rough ER cisternae were stacked with parallel arrays and there are large amount of ribosomes attached to ER in the hippocampal CA3 neurons of control mice (Fig. 3D). In the 14 days restraint stressed mice, however, the compactness of ER stacks was disrupted, which causes the disorganization of rough ER (Fig. 3D). Moreover, the density of ribosomes attached to ER was decreased (Fig. 3E, p=0.02) and the rough ER length was shortened (Fig. 3F, **p**=**0.006**). Taken together, these data suggest that repeated restraint stress induces hippocampal ER stress.

3.3. TUDCA attenuates repeated restraint stress-induced hippocampal ER stress

Solid evidence suggests that tauroursodeoxycholic acid (TUDCA), a bile salt with ER chaperone-like properties, can reduce ER stress in central nervous system (Keene et al., 2002; Xu et al., 2015). To demonstrate the effect of TUDCA on repeated restraint

stress-induced hippocampal ER stress, we treated mice with TUDCA by intracerebroventricular (i.c.v.) injection (Fig. 4A-B) during 14 days restraint stress procedure and examined the ER stress markers of CHOP and GRP78 by Western blot and immunofluorescence respectively. Our Western blot results revealed that the protein level of CHOP and GRP78 were significantly increased in the hippocampus of repeated restraint stressed mice compared to the control mice. However, TUDCA treatment suppressed the restraint stress-induced increase of CHOP and GRP78 protein levels, while the mice treated with TUDCA alone did not show a significant difference in the CHOP and GRP78 protein level compared to the control mice. A two-way ANOVA revealed a significant main effect of repeated restraint stress on the protein level of CHOP (F_{1,16}=12.68, p=0.003) and GRP78 (F_{1,16}=10.05, p=0.006) and a significant main effect of TUDCA treatment on the protein level of CHOP (F_{1.16}=8.59, p=0.01) and GRP78 (F_{1.16}=7.66, p=0.01), however, there was no significant interaction between restraint stress and TUDCA treatment on the protein level of CHOP ($F_{1.16}=2.61$, **p**=**0.13**) and GRP78 (**F**_{1.16}=**4.06**, **p**=**0.06**). A Tukey post-hoc test revealed that restraint stress significantly increased the levels of CHOP (**p**=**0.003**) and GRP78 (**p**=**0.006**), and TUDCA treatment significantly decreased the restraint stress-induced increase levels of CHOP (p=0.01) and GRP78 (p=0.014), while the mice treated with TUDCA alone did not show a significant difference on the protein levels of CHOP (**p**=**0.13**) and GRP78 (**p**=**0.06**) compared to the control mice (Fig. 4C-E). In consistent with the western blot data, our immunofluorescence results showed that hippocampal



Fig. 3. Repeated restraint stress induces hippocampal ER stress and disrupted ER structure. (**A**) Representative images of ER stress markers CHOP and GRP78 immunostaining in the hippocampal CA3 regions of control and 14 days restraint stressed mice. (**B**–**C**) Bar graphs showing the quantification of CHOP and GRP78-positive cells in the hippocampus of control and 14 days restraint stressed mice (**n**=**5**). (**D**) Representative electron photomicrographs of rough ER morphology in the hippocampal CA3 regions of the control and 14 days restraint stressed mice. (**B**–**C**) Bar graphs showing the quantification of rough ER morphology in the hippocampal CA3 regions of the control and 14 days restraint stressed mice. (**B**–**C**) Bar graphs showing the quantification of rough ER ribosome density and rough ER length in the control and 14 days restraint stressed mice (**n**=**5**). Values are means \pm S.E.M. *p < 0.05, *p < 0.01, unpaired *t*-test. Scale bar = 250 µm in (**A**) and 500 nm in (**D**).

CHOP and GRP78 positive cells are significantly increased in 14 days restraint stressed mice compared to control mice. However, TUDCA treatment suppressed the restraint stress-induced increase of CHOP and GRP78 positive cells, while the mice treated with TUDCA alone did not show a significant difference in the CHOP and GRP78 positive cells compared to the control mice. A two-way ANOVA revealed a significant main effect of repeated restraint stress on the positive cells of CHOP (F_{1,28}=22.89, p<0.001) and GRP78 (F_{1.28}=37.42, p<0.001) and a significant main effect of TUDCA treatment on the positive cells of CHOP ($F_{1,28}$ =8.76, p=0.006) and GRP78 (*F*_{1,28}=18.38, *p*<0.001), and there was a significant interaction between restraint stress and TUDCA treatment on the positive cells of CHOP (F_{1,28}=5.02, p=0.03) and GRP78 (F_{1,28}=23.67, **p**<0.001). A Tukey post-hoc test revealed that restraint stress significantly increased the number of CHOP (p<0.001) and GRP78 (p<0.001) positive cells, and TUDCA treatment significantly suppressed the restraint stress-induced increase number of CHOP (p=0.001) and GRP78 (p<0.001) positive cells, while the mice treated with TUDCA alone did not show a significant difference on the number of CHOP (p=0.615) and GRP78 (p=0.686) positive cells compared to the control mice (Fig. 4F–H). Taken together, these data suggest that TUDCA can reduce repeated restraint stressinduced hippocampal ER stress.

3.4. TUDCA attenuates ER stress-induced oxidative stress in the repeated restraint stressed mice

Growing body of evidence demonstrated that ER stress is closely related to oxidative stress (Dvash et al., 2015; Hasnain et al., 2014). To determine whether oxidative stress is affected by ER stress, we treated mice with ER stress inhibitor TUDCA during 14 days restraint stress procedure and detected the hippocampal oxidative stress level. We firstly examined the hippocampal CA3 ROS accumulation profiles by measuring the DHE-reactive superoxide. Our data show that the repeated restraint stressed mice displayed higher DHE fluorescence intensity compared to the control mice. However, inhibition of ER stress by TUDCA suppressed the restraint stress-induced increase of DHE intensity, while the mice treated with TUDCA alone did not show a significant difference in the DHE intensity compared to the control mice. A two-way ANOVA revealed a significant main effect of repeated restraint stress (*F*_{1.16}=**7.32**, *p*=**0.016**) and TUDCA treatment (*F*_{1,16}=**10.42**, *p*=**0.005**) on the DHE fluorescence intensity and there was a significant interaction between restraint stress and TUDCA treatment on the DHE fluorescence intensity (F_{1.16}=4.80, p=0.044). A Tukey post-hoc test revealed that restraint stress significantly increased the levels of DHE fluorescence (*p*=0.003), and TUDCA treatment significantly





Fig. 4. TUDCA suppressed repeated restraint stress-induced hippocampal ER stress. (A) Chemical structure of TUDCA. (**B**) Schematic diagram of intracerebroventricular (i.c.v.) injection of TUDCA in the lateral ventricle (LV). (**C**) Western blot showing the protein levels of ER stress markers CHOP and GRP78 in the hippocampus of control (Control), 14 days repeated restraint stressed (Stress), 14 days repeated restraint stressed TUDCA treated (Stress + TUDCA) and TUDCA alone treated (TUDCA) mice. (**D**–**E**) Bar graphs showing the quantification of the CHOP and GRP78 protein levels, which were represented as the intensity ratios of CHOP and GRP78 to β -actin(**n**=**5**). (**F**) Representative images of ER stress markers CHOP and GRP78 immunostaining in the hippocampal CA3 regions of Control, Stress, Stress + TUDCA and TUDCA mice respectively. (**G**–**H**) Bar graphs showing the quantification of CHOP and GRP78-positive cells in the hippocampus of Control, Stress, Stress + TUDCA and TUDCA mice (**n**=**8**). Values are means \pm S.E.M. *p < 0.05, **p < 0.01, two way ANOVA. Scale bar = 250 μ m.

suppressed the restraint stress-induced increase of DHE fluorescence levels (**p**=**0.002**), while the mice treated with TUDCA alone did not show a significant difference on the DHE fluorescence (p=0.474) compared to the control mice (Fig. 5A–B). Next, we examined enzymatic antioxidant defense by detecting CuZn-SOD and GSH-Px activity and examined the protein oxidation and lipid peroxidation by detecting protein carbonyl. MDA and 4-HNE. Our data showed that the CuZn-SOD and GSH-Px activities were significantly decreased in repeated restraint stressed mice compared to the control mice. However, pharmacological inhibition of ER stress by TUDCA rescued the CuZn-SOD and GSH-Px activities in the restraint stressed mice, while mice treated with TUDCA alone did not show any significant difference in the CuZn-SOD and GSH-Px activities compared to control mice. A two-way ANOVA revealed a significant main effect of repeated restraint stress on the activity of CuZn-SOD (F_{1.16}=8.66, p=0.01) and GSH-Px (F_{1.16}=2.87, p<0.001) and a significant main effect of TUDCA treatment on the activity of CuZn-SOD ($F_{1,16}=4.45$, p=0.04) and GSH-Px (**F**_{1,16}=**7.913**, *p*=**0.019**), however, there was no significant interaction between restraint stress and TUDCA treatment on the activity of CuZn-SOD (*F*_{1.16}=7.913, *p*=0.013) and GSH-Px (**F**_{1.16}=**4.05**, **p**=**0.06**). A Tukey *post-hoc* test revealed that restraint stress significantly decreased the activity of CuZn-SOD (**p**=**0.003**) and GSH-Px (p<0.001), and TUDCA treatment significantly suppressed the restraint stress-induced decrease activity of CuZn-SOD (**p**=**0.01**) and GSH-Px (**p**=**0.029**), while the mice treated with TUDCA alone did not show a significant difference on the activity of CuZn-SOD (**p**=**0.946**) and GSH-Px (**p**=**0.996**) compared to the control mice (Fig. 5C–D). The protein carbonyl, MDA and 4-HNE content were significantly increased in repeated restraint stressed mice compared to the control mice. However, pharmacological inhibition of ER stress by TUDCA suppressed the restraint stressinduced increase of protein carbonyl, MDA and 4-HNE level, while mice treated with TUDCA alone did not show any significant difference in the protein carbonyl, MDA and 4-HNE content compared to control mice. A two-way ANOVA revealed a significant main effect of repeated restraint stress on the content of protein carbonyl (F_{1.16}=7.913, p=0.013), MDA (F_{1.16}=23.285, p<0.001) and 4-HNE (F_{1,16}=16.314, p<0.001) and a significant main effect of TUDCA treatment on the content of protein carbonyl (*F*_{1,16}=3.276, **p=0.019**), MDA (**F**_{1,16}=**3.514**, **p**=**0.019**) and 4-HNE (**F**_{1,16}=**7.272 p**=**0.012**), and there was a significant interaction between restraint stress and TUDCA treatment on the content of protein carbonyl (*F*_{1,16}=4.031, *p*=0.026), MDA (*F*_{1,16}=2.242, *p*=0.015) but not on the 4-HNE (F_{1.16}=3.162, p=0.094). A Tukey post-hoc test revealed that restraint stress significantly increased the content of protein carbonyl (p=0.013), MDA (p<0.001) and 4-HNE (p=0.001), and TUDCA treatment significantly decreased the restraint stressinduced increase of protein carbonyl (**p**=**0.016**), MDA (**p**=**0.03**) and 4-HNE (**p**=**0.006**), while the mice treated with TUDCA alone did not show a significant difference on the content of protein carbonyl (**p=0.891**), MDA (**p=0.793**) and 4-HNE (**p=0.525**) compared to the control mice (Fig. 5E-G). Taken together, these data suggest chronic restraint stress induces hippocampal oxidative stress and TUDCA attenuates ER stress induced oxidative stress in the repeated restraint stressed mice.

3.5. Inhibition of ER stress by TUDCA reduces repeated restraint stress-induced neuron loss

There is abundant evidence that chronic stress triggers the development and progression of neurodegeneration (Carroll et al., 2011; Sugama et al., 2016; Vyas et al., 2016). To investigate the role of repeated restraint stress on hippocampal neuro-degeneration and the effect of ER stress on repeated restraint stress

mediated neurodegeneration, we used neuronal marker NeuN to label hippocampal neurons (Fig. 6A). Our immunofluorescence results reveal that the number of NeuN positive cells was significantly decreased in the hippocampus of restraint stressed mice, indicating repeated restraint stress induces hippocampal neuron loss. However, inhibition of ER stress by TUDCA rescued the neuron loss in the hippocampus of repeated restraint stressed mice. A two-way ANOVA revealed a significant main effect of repeated restraint stress (F_{1,28}=13.69, p<0.001) and TUDCA treatment (F_{1,28}=4.21, p=0.046) on NeuN positive cells, and there was a significant interaction between restraint stress and TUDCA treatment on NeuN positive cells (*F*_{1.28}=**5.17**, *p*=**0.031**). A Tukey post-hoc test revealed that restraint stress significantly decreased the number of NeuN positive cells (p<0.001), and TUDCA treatment significantly suppressed the restraint stress-induced decrease of NeuN positive cells (**p**=**0.005**), while the mice treated with TUDCA alone did not show a significant difference on the number of NeuN positive cells (p=0.876) compared to the control mice (Fig. 6A–B). Western blot results revealed that protein level of NeuN significantly decreased in the hippocampus of restraint stressed mice, while inhibition of ER stress by TUDCA suppressed the down regulation of NeuN in the repeated restraint stressed mice, which is consistent with the immunostaining data. A two-way ANOVA revealed a significant main effect of repeated restraint stress ($F_{1.16}$ =9.251, p=0.008) and TUDCA treatment (F_{1,16}=9.54, p=0.03) on the protein level of NeuN, and there was a significant interaction between restraint stress and TUDCA treatment on the protein level of NeuN (*F*_{1.16}=6.53, *p*=0.04). A Tukey *post-hoc* test revealed that restraint stress significantly decreased the levels of NeuN (*p***=0.008**), and TUDCA treatment suppressed the restraint stress-induced decrease of NeuN (**p**=**0.025**), while the mice treated with TUDCA alone did not show a significant difference on the protein levels of NeuN (**p**=**0.907**) (Fig. 6C-D). Furthermore, TUNEL staining showed that TUNEL positive cells significantly increased in the hippocampus of restraint stressed mice, while inhibition of ER stress by TUDCA suppressed the increase of TUNEL positive cells in the repeated restraint stressed mice. A two-way ANOVA revealed a significant main effect of repeated restraint stress (F_{1.28}=32.17, p<0.001) and TUDCA treatment ($F_{1,28}$ =9.09, p=0.005) on TUNEL positive cells, and there was a significant interaction between restraint stress and TUDCA treatment on NeuN positive cells (*F*_{1.28}=10.68, *p*=0.003). A Tukey post-hoc test revealed that restraint stress significantly increased the number of TUNEL positive cells (p<0.001), and TUDCA treatment significantly suppressed the restraint stressinduced increase of TUNEL positive cells (p<0.001), while the mice treated with TUDCA alone did not show a significant difference on the number of TUNEL positive cells (p=0.859) compared to the control mice (Fig. 6E–F). Taken together, these results suggest that repeated restraint stress induces hippocampal neurodegeneration, which is dependent on ER stress activation.

3.6. Inhibition of ER stress by TUDCA decreases repeated restraint stressed-induced seizure susceptibility

Activation of ER stress is implicated in the pathogenesis of a range of neurological disorders including epilepsy. To determine whether ER stress is responsible for repeated restraint stress-induced increase of seizure susceptibility, we treated mice with ER stress inhibitor, TUDCA, during the 14 days of restraint stress procedure. Our results show that inhibition of ER stress by TUDCA extended the latency to PTZ-induced tonic-clonic seizures in repeated restraint stressed mice, while TUDCA alone did not affect the latency to PTZ-induced tonic-clonic seizures. A two-way ANOVA revealed a significant main effect of repeated restraint stress (F_{128} =6.64, p=0.016) and TUDCA treatment (F_{128} =1.07,



Fig. 5. TUDCA suppressed repeated restraint stress-induced hippocampal oxidative stress. (A) Representative images of DHE fluorescence in the hippocampal CA3 regions of Control, Stress, Stress + TUDCA and TUDCA mice. (**B**) Bar graph shows the quantification of DHE fluorescence intensity which represents ROS levels in the hippocampus of Control, Stress, Stress + TUDCA and TUDCA mice (n=5). (E-G) Bar graph showing the quantification of the protein carbonyl content and lipid peroxidation products of MDA and 4-HNE in the hippocampus of Control, Stress, Stress + TUDCA and TUDCA mice (n=5). (e=6) Bar graph showing the quantification of the protein carbonyl content and lipid peroxidation products of MDA and 4-HNE in the hippocampus of Control, Stress, Stress + TUDCA and TUDCA mice (n=5). Values are means \pm S.E.M. *p < 0.05, **p < 0.01, two-way ANOVA. Scale bar = 200 μ m.

p=0.037) on the latency to tonic-clonic seizures and there was a significant interaction between restraint stress and TUDCA treatment ($F_{1,28}$ =1.23, **p=0.026**). A Tukey *post-hoc* test revealed that restraint stress significantly increased the latency to tonic-clonic seizures (**p=0.016**), and TUDCA treatment significantly decreased the restraint stress-increased latency to tonic-clonic seizures (**p=0.012**), while the mice treated with TUDCA alone did not show a significant difference (**p=0.957**) compared to the control mice (Fig. 6G). Moreover, inhibition of ER stress by TUDCA suppressed PTZ-induced seizure severity in repeated restraint stressed mice. A

two-way ANOVA revealed a significant main effect of repeated restraint stress ($F_{1,20}$ =**5.90**, p=**0.025**) and TUDCA treatment ($F_{1,28}$ =**2.42**, p=**0.013**) on the seizure severity and there was a significant interaction between restraint stress and TUDCA treatment ($F_{1,28}$ =**2.15**, p=**0.015**). A Tukey *post-hoc* test revealed that restraint stress significantly increased the seizure severity (p=**0.025**), and TUDCA treatment significantly decreased the restraint stressincreased seizure severity (p=**0.045**), while the mice treated with TUDCA alone did not show a significant difference (p=**0.950**) compared to the control mice (Fig. 6H).The mortalities in TUDCA



Fig. 6. TUDCA suppressed repeated restraint stress-induced hippocampal neurodegeneration and seizure susceptibility. (A) Representative images of neuronal marker NeuN staining in the hippocampal CA3 regions of Control, Stress, Stress + TUDCA and TUDCA mice. (B) Bar graph showing the quantification of NeuN positive cells in the hippocampus of Control, Stress, Stress + TUDCA and TUDCA mice (n=8). (C) Western blot showing the protein levels of NeuN in the hippocampus of Control, Stress, Stress + TUDCA and TUDCA mice. (D) Bar graph showing the quantification of protein level of NeuN, which was represented as the intensity ratio of NeuN to β -actin in the Control, Stress, Stress + TUDCA and TUDCA mice (n=5). (E) Representative images of TUNEL staining in the hippocampal CA3 regions of Control, Stress, Stress + TUDCA and TUDCA mice (F) Bar graph showing the

treated restraint stressed mice (50%) are decreased compared to vehicle treated restraint stressed mice (60%) when they are induced seizure by PTZ (Fig. 6I). Taken together, these results suggest that increased seizure susceptibility induced by repeated restraint stress is dependent on ER stress activation.

4. Discussion

In this study, we demonstrate that repeated restraint stress exposure increases the susceptibility of PTZ-induced seizures. Meanwhile, repeated restraint stress triggers hippocampal endoplasmic reticulum (ER) stress, oxidative stress and neuron loss. Inhibition of ER stress, however, suppresses chronic restraint stress-induced oxidative stress and neuron loss. Furthermore, inhibition of ER stress suppresses restraint stress-induced seizure susceptibility. Our results thus imply that repeated restraint stress-induced hippocampal ER stress may contribute to increased seizure susceptibility by triggering oxidative stress and neurodegeneration.

It is well established that adolescence is a transitional period which is characterized by extensive changes in brain structure, neurochemistry and function. Stress exposure during adolescence is found to increases the risk for a variety of neurological disorders (Gomes et al., 2016; Heim and Binder, 2012). Here in this study, we have used the mice of 4 weeks old, which are generally considered as adolescents, to study the effect of chronic restraint stress on seizure susceptibility. Our results show that repeated restraint stress has decreased the latency to PTZ-induced tonic-clonic seizures and increases PTZ-induced seizure severity (Fig. 1B–D). suggesting chronic restraint stress exposure in adolescents increases the seizure susceptibility. It is generally accepted that stress response systems undergo maturational changes, and stress exposure at different developmental stages may have varying or even opposite consequences. A previous study reported that unpredictable chronic stress in juvenile or adult rats have opposite effects (Ricon et al., 2012). Interestingly, some other evidence in animals shows that chronic stress also increases seizure susceptibility in adult. For example, chronic social isolation stress has been shown to decrease the threshold for bicuculline-induced seizure (Chadda and Devaud, 2004) and to increase the seizure susceptibility in response to picrotoxin (Matsumoto et al., 2003). Repeated restraint stress has been shown to enhance vulnerability to amygdala kindling epileptogenesis (Jones et al., 2013). The results of these studies suggest that exposure to stress in both adolescence and adult stage may share a common mechanism to increase the seizure susceptibility. In clinic, the association between stress and seizure susceptibility has been widely accepted. The most common precipitating factor of seizure reported by epilepsy patients is stress (Haut et al. 2003, 2007; Nakken et al., 2005; Sperling et al., 2008).

ER is a central subcellular organelle, which is responsible for synthesizing and folding of secretory and membrane proteins. Accumulating evidence suggests that ER is susceptible to various pathophysiological conditions such as hypoxia, stress and high fat diet, which consequently trigger ER stress (Kim et al., 2008; Zhang et al., 2014). In the present study, we find that chronic exposure of restraint stress leads to induction of hippocampal ER stress as evident by the up-regulation of ER stress markers, including HSP-70, ATF4, CHOP, GRP78, and p-eIF2 α (Fig. 2A–F; Fig. 3A–C). Furthermore, ultrastructure analysis reveals that rough ER

structure was largely disrupted in the repeated restraint stress mice (Fig. 3D–F).

Tauroursodeoxycholic acid (TUDCA) is a hydrophilic bile acid produced endogenously in human bile at small quantities. TUDCA is known as a chemical chaperone of ER and can reduce ER stress (Ozcan et al. 2006, 2009; Xu et al., 2015). Consistently, here in this study, we found that TUDCA specifically suppresses restraint stress-induced ER stress (Fig. 4C–H). There is mounting evidence suggests that there is a link between ER stress and oxidative stress (Cullinan and Diehl, 2006; Dvash et al., 2015; Hasnain et al., 2014; Taura et al., 2013). In the present study, we found that inhibition of ER stress by TUDCA suppresses increased levels of oxidative stress in restraint stressed mice (Fig. 5A–G), indicating ER stress activates oxidative stress in the condition of restraint stress.

It is generally accepted that chronic stress exposure gradually changes the morphology of brain cells and consequently changes the brain activity. An increasing body of evidence suggests that chronic exposure to stress alters the structure and functions of hippocampus (McLaughlin et al., 2007; Sapolsky, 2003; Vyas et al., 2002). A previous study reported that chronic restraint stress significantly induced hippocampal neurodegeneration (Carroll et al., 2011). Consistently, in the present study, we observed a significant loss of neurons (Fig. 6A-B),a down-regulation of neuronal marker (Fig. 6C-D) and an increase of TUNEL-positive cells (Fig. 6E-F) in the hippocampus of the restraint stressed mice, suggesting a significant neurodegeneration in the hippocampus of chronic restraint stressed mice. Although we demonstrated that repeated restraint stress induced neurodegeneration in the hippocampus, the molecular mechanisms involved are still poorly understood. ER stress in the hippocampus is triggered by repeated restraint stress, suggesting the possibility of ER stress involvement in the pathological changes induced by chronic restraint stress. Indeed, here our results show that inhibition of ER stress by TUDCA suppresses the repeated restraint stress-induced neurodegeneration in the hippocampus (Fig. 6A-F), indicating chronic restraint stress-induced increase of neuronal degeneration is dependent on the activation of ER stress. Emerging evidence has suggested that neuronal degeneration plays an important role in pathological plastic changes presumed to underlie the circuit hyperexcitability associated with epilepsy. It is reported that hippocampal mossy cell degeneration causes hippocampal DG cells hyperexcitability (Jinde et al., 2012) and neuronal degeneration may cause spontaneous recurrent seizures (Ben-Ari and Dudek, 2010; Zhang et al., 2002). On the other hand, we find that inhibition of ER stress by TUDCA suppresses the restraint stress-induced increase of seizure susceptibility (Fig. 6G–I), suggesting restraint stress-induced increase of seizure susceptibility is dependent on ER stress activation. Combined with the above evidences, we reason that restraint stress induced increase of oxidative stress and neurodegeneration in hippocampus may contribute to increased excitability of neural circuits of the brain, and could potentially be associated with increased susceptibility to epilepsy.

In conclusion, our data in the present study suggest that repeated restraint stress-induced hippocampal ER stress may contribute to increased seizure susceptibility by initiating a cascade of oxidative stress and neuronal degeneration events. Thus, attenuating ER stress may serve as a potential therapeutic strategy targeted to block stress-induced seizure activities.

quantification of TUNEL positive cells in the hippocampus of Control, Stress, Stress + TUDCA and TUDCA mice (n=8). (G) Bar graph showing the latency to PTZ-induced tonic-clonic seizures of Control, Stress, Stress + TUDCA and TUDCA mice (n=8). (H) Line graph showing the severity of PTZ-induced tonic-clonic seizures of Control, Stress, Stress + TUDCA and TUDCA and TUDCA mice (n=8). (H) Line graph showing the severity of PTZ-induced tonic-clonic seizures of Control, Stress, Stress + TUDCA and TUDCA mice (n=8). (H) Line graph showing the severity of PTZ-induced tonic-clonic seizures of Control, Stress, Stress + TUDCA and TUDCA mice (n=8). (H) Line graph showing the severity of PTZ-induced tonic-clonic seizures of Control, Stress, Stress + TUDCA and TUDCA mice (n=8). (H) Line graph showing the mortality of PTZ-induced tonic-clonic seizures of Control, Stress, Stress + TUDCA and TUDCA mice. Values are means \pm S.E.M. *p < 0.05, **p < 0.01, ***p < 0.01, ***p < 0.01, ***p < 0.01, ***p < 0.01, two-way ANOVA and repeated measures ANOVA. Scale bar = 200 µm.

Conflict of interest disclosure

The authors declare that there are no conflicts of interest.

Acknowledgements

This work was supported by grants from the National Natural Science Foundation of China (81673413 to Xinjian Zhu), Natural Science Foundation of Jiangsu Province (BK20141335 to Xinjian Zhu), the Specialized Research Fund for the Doctoral Program of Higher Education (20130092120043 to Xinjian Zhu).

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