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Research report

NMDA receptor NR2B subunits contribute to PTZ-kindling-induced hippocampal astrocytosis and oxidative stress

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ABSTRACT

The N-methyl-D-aspartate (NMDA) receptor plays an important role in the pathophysiology of several neurological diseases, including epilepsy. The present study investigated the effect of NMDA receptor NR2B subunits on pentylenetetrazole (PTZ)-kindling-induced pathological and biochemical events in mice. Our results showed that PTZ-kindling up-regulates the expression of NMDA receptor NR2B subunits in the hippocampus and that kindled mice were characterized by significant astrocytosis and neuron loss in the hippocampus. Oxidative stress, including excessive malondialdehyde (MDA) production and decreased enzymatic activities of superoxide dismutase (SOD) and glutathione peroxidase (GSH-PX), were detected in the hippocampus after the mice were fully kindled. Additionally, expression of brain-derived neurotrophic factor (BDNF) in the hippocampus was found to be up-regulated in PTZ-kindled mice. However, selectively blocking NMDA receptor NR2B subunits by ifenprodil significantly suppressed PTZ-kindling-induced hippocampal astrocytosis, oxidative stress and neuron loss. Furthermore, blocking NMDA receptor NR2B subunits also abolished PTZ-kindling-induced BDNF expression. These results indicate that NMDA receptor NR2B subunits contribute to epilepsy-associated pathological and biochemical events, including hippocampal astrocytosis, oxidative stress and neuron loss, and these events might be correlated with up-regulation of BDNF expression.

1. Introduction

Epilepsy is one of the most common neurologic conditions, affecting approximately 1% of the population worldwide. To date, none of the drugs investigated for the treatment of epilepsy have been capable of blocking its development or reversing epileptogenesis (Temkin, 2009). Understanding the molecular and cellular mechanisms involved in the development of epilepsy is becoming increasingly important for the development of appropriate therapeutic targets to modify the epileptic process.

NMDA receptors have been the principal focus of the study of the molecular mechanisms underlying epileptogenesis. Growing bodies of evidence show that NMDA receptors are involved in the pathogenesis of epileptic discharges (Carter et al., 2011; Clasadonte et al., 2013; Di Maio et al., 2011) and seizure-induced selective excitotoxic cell death in the hippocampus (Deshpande et al., 2008; Meldrum, 1993). Among the NMDA receptor subunits, NR2B is the most extensively studied because it is broadly expressed in the cerebral cortex and hippocampus (Cull-Candy et al., 2001; Monyer et al., 1994). NMDA receptor NR2B subunits are believed to play important roles in synaptic plasticity. Although many studies have shown that NMDA receptor NR2B subunits are significant for epileptogenesis or, at least, relevant to it, the plastic cellular changes and the molecular mechanisms involved are still unclear.

Here, we report that NMDA receptor NR2B subunits contribute to PTZ-kindling-induced hippocampal astrocytosis and oxidative stress and that these events might be associated with the up-regulation of BDNF expression.

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).

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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 8:00 A.M. to 8:00 P.M.). Food and tap water were available *ad libitum*. Procedures for generating the PTZ-kindling epilepsy model 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 ifenprodil were purchased from Sigma–Aldrich.

2.3. Kindling procedure

To generate the kindling epilepsy model, mice were intraperitoneally injected with PTZ (35 mg/kg) once every other day for at least nine total injections, and mice showing more than three consecutive stage 4 seizures were considered to be fully kindled. Vehicle control animals were injected with saline. Seizure events during a 30 min period after each PTZ injection were observed. The seizure intensity was scored as follows: Stage 0, no response; Stage 1, ear and facial twitching; Stage 2, convulsive twitching axially throughout 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).

2.4. Brain tissue processing

For immunocytochemistry and Nissl staining, fully kindled mice were killed by intraperitoneal injection of an overdose of urethane 7 days after the last dose of PTZ 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 mice brains were removed and post-fixed overnight in 4% paraformaldehyde and then were cryoprotected by incubation in 30% sucrose in 1× PBS 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 was collected in 1× PBS as free-floating sections and stored at 4 °C until use. For RT-PCR, Western blot and oxidative biomarkers analysis, the hippocampi of the fully kindled mice were dissected 24 h or 7 days after the last dose of PTZ. The dissected hippocampal tissues were then snap-frozen and stored at −80 °C until use.

2.5. Immunocytochemistry

For GFAP immunocytochemistry, free-floating sections were incubated at 4 °C overnight with mouse anti-GFAP primary antibody (1:100; Boster Bioengineering, Wuhan, China). The sections were then rinsed and incubated with fluorochrome-conjugated secondary antibodies (FITC conjugated goat anti-mouse, 1:150, Cwbiotech, Beijing, China). Finally, sections were rinsed and mounted on gelatin-coated slides in DAPI-anti fade mounting medium (SouthernBiotech, Birmingham, AL, USA). One experimenter coded all slides from the experiments before quantitative analysis. The sections were imaged with a laser scanning microscope (Olympus LSM-GB200, Japan). The GFAP immunostaining images were analyzed using Image J software (NIH, Bethesda, MD).

2.6. RT-PCR

Total RNA was extracted from the hippocampus using Trizol reagent according to the manufacturer's instructions (Invitrogen, Carlsbad, USA). The primers for BDNF and GAPDH were as follows: BDNF forward: 5′-ATGACCATCCTTTTCCTTACTATGGT-3′; BDNF reverse: 5′-TCTTCCCCTTTTAATGGTCAGTGTAC-3′; GADPH forward: 5′-ACAGCCGCATCTTCTTGTGC-3′; GAPDH reverse: 5′-GCCTCACCCCATTTGATGTT-3′. The PCR conditions were 30 cycles of denaturation at 98 °C for 10 s, annealing at 55 °C for 30 s, and extension at 72 °C for 60 s. The PCR products were separated by electrophoresis through a 1.5% agarose gel containing 0.5% μg/mL ethidium bromide and imaged using a Gel imaging system (Tanon, Shanghai, China).

2.7. Western blot

The dissected hippocampal tissue of the mice was homogenized in tissue lysis buffer (Beyotime, China). After lysis for 15 min in ice, the samples were centrifuged at 12,000 rpm for 15 min. The protein content of 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) overnight at 4 °C using a Bio-Rad mini-protein-III wet transfer unit (Hercules, CA, USA). Blotting membranes were 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 and were then washed three times. Then, they were incubated with rabbit anti-NR2B (1:2500; Millipore, Temecula, CA, USA), mouse anti-GFAP (1:1000, Boster Bioengineering, Wuhan, China) and rabbit anti-BDNF (1:2000, Abcam, Temecula, CA, USA) antibodies in TBST overnight at 4 °C. After several washes with TBST buffer, the membranes were incubated for 1 h with HRP-linked secondary antibody (1:5,000) (Boster Bioengineering, Wuhan, China) and then washed four more times. 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 Bio-imaging Systems, Jerusalem, Israel). Blots were quantified using Image J software (NIH, Bethesda, MD, USA).

2.8. SOD, CAT, GSH-PX activity and MDA content assays

To detect the enzymatic activities of superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GSH-PX) and malondialdehyde (MDA) content, the dissected hippocampal tissues from the mice were homogenized in tissue lysis buffer (Beyotime, China). After lysis for 15 min in ice, the homogenates were centrifuged at 3000 rpm for 15 min. The protein content of each supernatant fraction was determined using a BCA protein assay, as described in the description of the Western blot procedure. SOD, CAT and GSH-PX enzymatic activities and MDA content in the supernatant were measured using commercially available kits (Jiancheng Bioengineering, Nanjing China). SOD, CAT and GSH-PX enzymatic activities were expressed as units per mg protein, and MDA content was expressed as nmol per mg protein. All assays were conducted according to the manufacturer's instructions.

2.9. Nissl staining and cell counting

The surviving neurons in the hippocampus were visualized by Nissl staining. Briefly, free-floating sections were mounted

onto glass slides in 1× PBS and dried overnight. The mounted sections were rehydrated in distilled water and then immersed in 0.5% cresyl violet in 90 mM acetic acid and 10 mM sodium acetate for 10–30 min until the desired depth of staining was achieved. The sections were rinsed in distilled water, dehydrated in solutions of ascending ethanol concentration (75%, 90%, and 100%), cleared in xylene, and cover-slipped with the mounting medium as described for the immunocytochemistry procedure (SouthernBiotech, Birmingham, AL, USA). The CA1, CA3 and DG hilus subregions of the hippocampus from each animal were captured, and quantitative analysis of cell numbers was performed using Image J software (NIH, Bethesda, MD, USA). Six visual fields (0.6 mm^2) of the hippocampal CA1, CA3 and DG hilus were photographed in each section. The numbers of stained cells in each field were counted at a higher magnification (400×). The data are presented as the number of cells per high-power field.

2.10. Statistical analysis

All data are presented as the means ± S.E.M. Statistical significance was determined using one-way ANOVA or two-way ANOVA followed by Tukey *post hoc* test. Two-tailed Student's unpaired *t* test was used for two-group comparisons. Differences were considered significant when $p < 0.05$.

3. Results

3.1. PTZ-kindling up-regulates hippocampal NMDA receptor NR2B subunits expression

The PTZ kindling model was generated by repeatedly treating the mice with PTZ at a dose of 35 mg/kg every other day for at least 9 injections. Compared to the vehicle control mice, the repeatedly PTZ-treated mice showed progressive increases in seizure severity from almost no observable convulsive behaviors to major tonic–clonic seizures. After the final dose, almost all of the mice became kindled with at least three consecutive stage 4 seizures. To investigate whether NMDA receptor NR2B subunits are involved in the kindling process, we first examined NR2B expression by Western blot in the hippocampus of the kindled mice 6 h and 7 days after the final dose of PTZ. Our Western blot results showed a remarkable enhancement of the NR2B protein level in PTZ-kindled mice compared to vehicle control mice 6 h after kindling (Fig. 1A and B; $F_{(2,12)} = 10.694$, $p = 0.002$), and this up-regulated NR2B protein level was retained for at least 7 days after the mice were fully kindled (Fig. 1A and B; $F_{(2,12)} = 10.694$, $p = 0.009$, One way ANOVA followed by Tukey *post hoc* test). These results suggest that PTZ-kindling up-regulates the expression of NMDA receptor NR2B subunits in the hippocampus.

3.2. NMDA receptor NR2B subunits contribute to PTZ-kindling-induced astrocytosis

Astrocytes constitute the majority of glia populations, and their activation accompanies most brain pathologies. GFAP is an intermediate filament found specifically in astrocytes, and its accumulation indicates astrocyte activation, which is termed astrocytosis. To study the profiles of astrocytosis in the hippocampus of PTZ-kindled mice and to determine whether NMDA receptor NR2B subunits are involved in this event, mice were repeatedly treated with 35 mg/kg PTZ every other day to generate the kindling model, as previously described. After these mice were fully kindled, they were administered an NMDA receptor NR2B subunit antagonist, ifenprodil (5.0 mg/kg) (Ebert et al., 1997; Tsuda et al., 1997) for

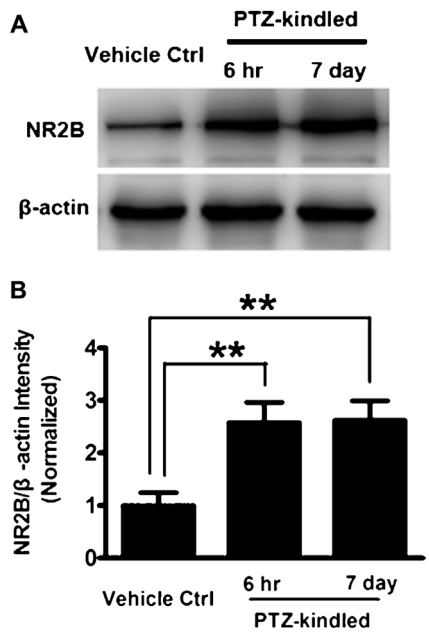

Fig. 1. PTZ kindling up-regulates hippocampal NMDA receptor NR2B subunit expression. Western blot assays of NR2B expression in the hippocampus of vehicle control and fully kindled mice 6 h and 7 days after kindling ($n = 5$). Values are means ± S.E.M. $^{**}p < 0.01$.

seven days at 24 h intervals (Fig. 3A). These mice were then sacrificed 6 h after the last ifenprodil injection for perfusion. Coronary brain sections from vehicle control mice, PTZ-kindled mice, PTZ-kindled ifenprodil-treated mice (PTZ + ifenprodil) and ifenprodil alone-treated mice were immunostained with antibodies against GFAP to assess any astrocytosis in the hippocampus. Our imaging analysis revealed that, in comparison to the vehicle control mice, the GFAP-immunopositive cells in the CA1, CA3, and DG hilus of the hippocampi of PTZ-kindled mice were ramified, forming more spreading branches (Fig. 2B). Two-way ANOVA revealed a significant main effect of PTZ-kindling ($F_{1,16} = 15.374$, $p = 0.001$) and NR2B antagonist ifenprodil treatment ($F_{1,16} = 5.074$, $p = 0.039$), and there was a significant interaction between PTZ-kindling and Ifenprodil treatment ($F_{1,16} = 6.354$, $p = 0.023$). Tukey *post hoc* test revealed that PTZ-kindled mice displayed larger GFAP staining areas compared to the vehicle control mice (Fig. 2C; $p = 0.001$). Blocking NR2B subunits, however, suppressed the PTZ-kindling-induced increase in GFAP staining (Fig. 2C; $p = 0.004$), while ifenprodil alone-treated mice did not show much difference of GFAP staining as compared to vehicle control mice (Fig. 2C; $p = 0.852$). Consistent with the image analysis, our Western blot assays revealed a significant main effect of PTZ-kindling ($F_{1,16} = 18.058$, $p < 0.001$) and ifenprodil treatment ($F_{1,16} = 5.214$, $p = 0.036$), and there was a significant interaction between PTZ-kindling and Ifenprodil treatment ($F_{1,16} = 7.684$, $p = 0.014$). Tukey *post hoc* test revealed that there was a robust increase in GFAP expression in the hippocampi of the PTZ-kindled mice (Fig. 2D; $p < 0.001$) but not in the hippocampi of the PTZ-induced acute seizure mice comparted to vehicle control mice (Fig. 2D; two-tailed Student's unpaired *t* test, $p = 0.606$). Moreover, PTZ-kindling-induced GFAP expression was abolished by NR2B subunit blockade (Fig. 2D; $p = 0.003$). However, blocking NR2B subunit with ifenprodil did not change much about GFAP expression in the normal mice (Fig. 2D; $p = 0.734$). These findings suggest that NMDA receptor NR2B subunits contribute to PTZ-kindling-induced astrocytosis in the hippocampus.

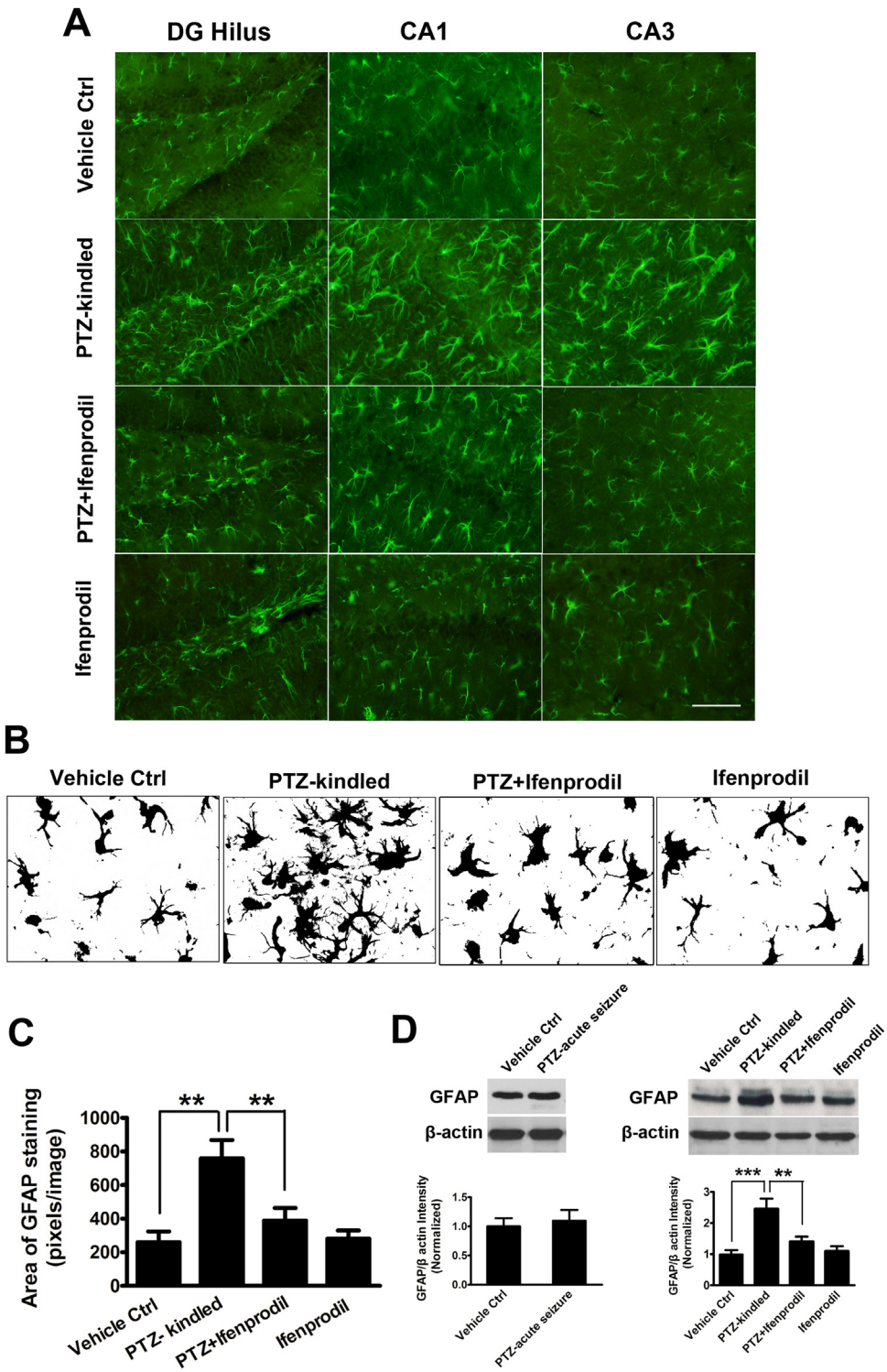

Fig. 2. NMDA receptor NR2B subunits contribute to PTZ-kindling-induced hippocampal astrocytosis. (A) Representative images of GFAP-staining in sub-regions of hippocampal DG hilus, CA1 and CA3 in vehicle control, PTZ-kindled, PTZ-kindled ifenprodil-treated mice (PTZ + ifenprodil), and ifenprodil alone-treated mice, respectively ($n = 4$). (B) Representative converted black and white images used to measure the GFAP-staining by Image J software. (C) Bar graph showed the quantification of areas (pixels) of GFAP staining in the hippocampus of vehicle control, PTZ-kindled, PTZ-kindled ifenprodil-treated mice and ifenprodil alone-treated mice respectively ($n = 6$ images in each group). (D) Western blot assays of GFAP expression in the hippocampus of acute seizure and kindled mice. Bar graphs showed the quantification of hippocampal GFAP protein levels, which were represented as the intensity ratio of GFAP to β-actin in acute seizure and kindled mice ($n = 5$). Values are means ± S.E.M. $^{**}p < 0.01$, $^{***}p < 0.001$, scale bar = 100 μm.

3.3. NMDA receptor NR2B subunits contribute to PTZ-kindling-induced oxidative stress

Neuronal loss and injury have been reported in a variety of neurological conditions for which oxidative stress is a possible mechanism (Beal, 1995). Disruption of the balance between free radical formation and antioxidative defensive systems might cause oxidative stress damage to the brain. To investigate the oxidative stress in the hippocampus of PTZ-kindled mice and to determine whether NMDA receptor NR2B subunits are involved in it,

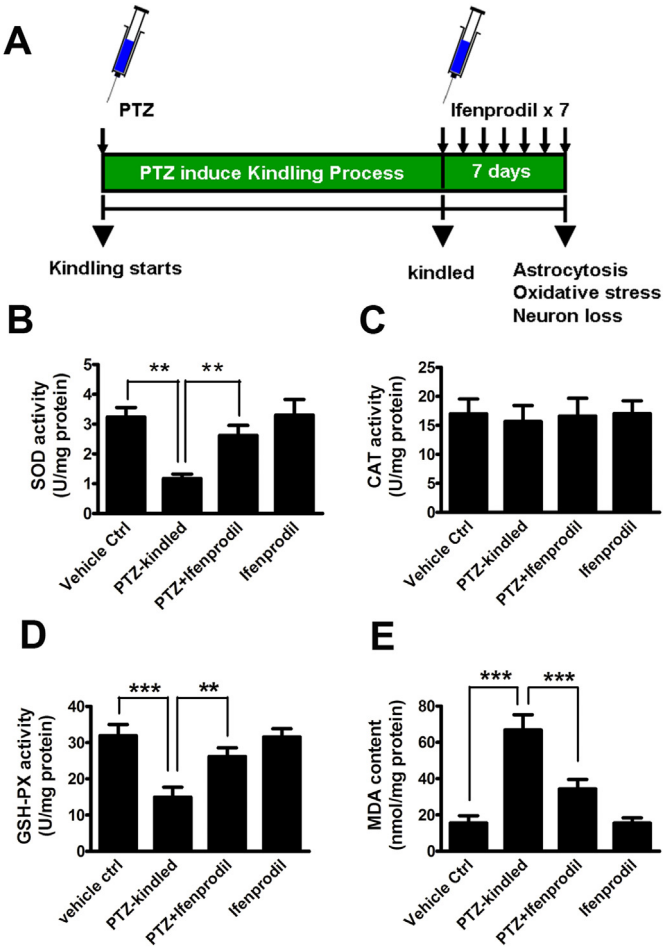

Fig. 3. NMDA receptor NR2B subunits contribute to PTZ-kindling-induced oxidative stress. (A) Schematic representation of experimental design. Mice were repeatedly treated with 35 mg/kg PTZ every other day to induce kindling, immediately after these mice were fully kindled, they were administered ifenprodil at 5.0 mg/kg 7 times at 24 h intervals. These mice were then sacrificed 6 h after the last ifenprodil injection for detection of astrocytosis, neuron loss and oxidative stress. (B-C) Bar graphs represent the enzymatic activities of SOD, CAT and GSH-PX. (E) Bar graph represents the content of MDA ($n = 6$). Values are means ± S.E.M. $^{**}p < 0.01$ and $^{***}p < 0.0001$.

mice were treated with ifenprodil or vehicle immediately after they were kindled. These mice were then sacrificed 7 days after kindling to evaluate the activities of the antioxidant enzymes SOD, CAT, and GSH-PX and the content of the reactive oxygen species (ROS) product MDA (Fig. 3A). Two way ANOVA revealed significant main effects of PTZ-kindling on activities of SOD ($F_{1,20} = 14.722$, $p = 0.001$), GSH-PX ($F_{1,20} = 17.811$, $p < 0.0001$) and content of MDA ($F_{1,20} = 40.387$, $p < 0.001$) and significant main effects of ifenprodil treatment on activity of SOD ($F_{1,20} = 4.485$, $p = 0.047$) and content of MDA ($F_{1,20} = 8.659$, $p = 0.008$). There were significant interactions between PTZ-kindling and Ifenprodil treatment on activity of GSH-PX ($F_{1,20} = 4.798$, $p = 0.041$) and content of MDA ($F_{1,20} = 8.579$, $p = 0.008$). Tukey *post hoc* test revealed that the activities of SOD (Fig. 3B; $p = 0.001$) and GSH-PX (Fig. 3D; $p < 0.001$) were significantly decreased in the PTZ-kindled mice compared with the vehicle control mice, while the activity of CAT was minimally altered (Fig. 3C; $p = 0.9208$). However, blocking NR2B subunits after kindling dramatically rescued the activities of the hippocampal antioxidant enzymes SOD (Fig. 3B; $p = 0.01$) and GSH-PX (Fig. 3D; $p = 0.007$). The content of MDA is an index of lipid peroxidation, and it was significantly increased in PTZ-kindled mice compared to vehicle control mice (Fig. 3E; $p < 0.001$). Blocking NR2B subunits significantly suppressed PTZ-kindling-induced MDA content (Fig. 3E; $p < 0.001$). While blocking NR2B subunits in normal mice has very little effect on the enzymatic activities of SOD ($p = 0.897$), CAT ($p = 0.747$), GSH-PX ($p = 0.053$) and on the content of MDA ($p = 0.993$) (Fig. 3B–E). These data suggest that NMDA receptor NR2B subunits contribute to PTZ-kindling-induced hippocampal oxidative stress damage.

3.4. NMDA receptor NR2B subunits contribute to PTZ-kindling-induced hippocampal neuron loss

It has been demonstrated that seizures lead to permanent changes in brain circuitry. Some of these changes could be associated with seizure progression (Pitkanen and Lukasiuk, 2011). Recent studies have shown that the occurrence of severe seizures leads to cell loss in various regions of the brain, especially the hippocampus (Cardoso et al., 2011). Here, to determine whether NMDA receptor NR2B subunits are involved in seizure-induced cell death, we examined neuron loss by Nissl staining in the hippocampus 7 days after mice were kindled (Fig. 3A). Two way ANOVA revealed significant main effects of PTZ-kindling on number of neurons in CA1 ($F_{1,16} = 9.592$, $p = 0.007$), and DG hilus ($F_{1,16} = 8.155$, $p = 0.011$). Tukey *post hoc* test revealed that extensive neuron loss was detected in the CA1 (Fig. 4A and B; $p = 0.007$) and the hilar region of the DG (Fig. 4A and D; $p = 0.012$) in PTZ-kindled mice compared to vehicle control mice. Blocking NR2B subunits using ifenprodil after kindling partially rescued the neuron loss in the CA1 region (Fig. 4A and B; $p = 0.014$). However, blocking NR2B subunits in normal mice showed very little effect on hippocampal CA1 ($p = 0.151$) and DG hilus ($p = 0.302$) neuron loss (Fig. 4A–D). These results suggest that NMDA receptor NR2B subunits contribute to PTZ-kindling-induced hippocampal neuron loss.

3.5. Selective inhibition of NMDA receptor NR2B subunits suppressed PTZ-kindling-induced BDNF expression in the hippocampus

Seizure activities induce the expression of neurotrophic factors thereby altering neuronal structures and the balance between excitation and inhibition in neural networks, resulting in epileptogenesis (Lukasiuk et al., 2003; Zagulska-Szymczak et al., 2001). BDNF, an important neurotrophic factor, has been implicated in the development of epilepsy (Binder et al., 1999; Tongiorgi et al., 2004). Here, we examined the mRNA and protein levels of BDNF using RT-PCR and Western blot, respectively, in the hippocampus of vehicle control, PTZ-kindled, PTZ-kindled ifenprodil-treated (PTZ + ifenprodil) and ifenprodil alone-treated mice. Two way ANOVA revealed significant main effects of PTZ-kindling on BDNF mRNA level ($F_{1,16} = 66.3$, $p < 0.001$) and protein level ($F_{1,16} = 51.062$, $p < 0.0001$) and significant main effects of ifenprodil treatment on BDNF mRNA level ($F_{1,16} = 54.207$, $p < 0.001$) and protein level ($F_{1,16} = 51.062$, $p < 0.001$). There were significant interactions between PTZ-kindling and Ifenprodil treatment on BDNF mRNA level ($F_{1,16} = 46.208$, $p < 0.001$) and protein level ($F_{1,16} = 29.193$, $p < 0.001$). Tukey *post hoc* test revealed that both BDNF mRNA (Fig. 5A; $p < 0.0001$) and protein (Fig. 5B; $p < 0.0001$) levels were significantly increased in PTZ-kindled mice compared to vehicle control mice, and the PTZ-kindling induced increases in BDNF mRNA (Fig. 5A; $p < 0.0001$) and protein (Fig. 5B; $p < 0.0001$) levels were dramatically reduced by NMDA receptor NR2B subunit blockade. Blocking NR2B subunit in normal mice had very little effect on BDNF mRNA ($p = 0.695$) and protein ($p = 0.651$) levels (Fig. 5A and B). These results indicate that

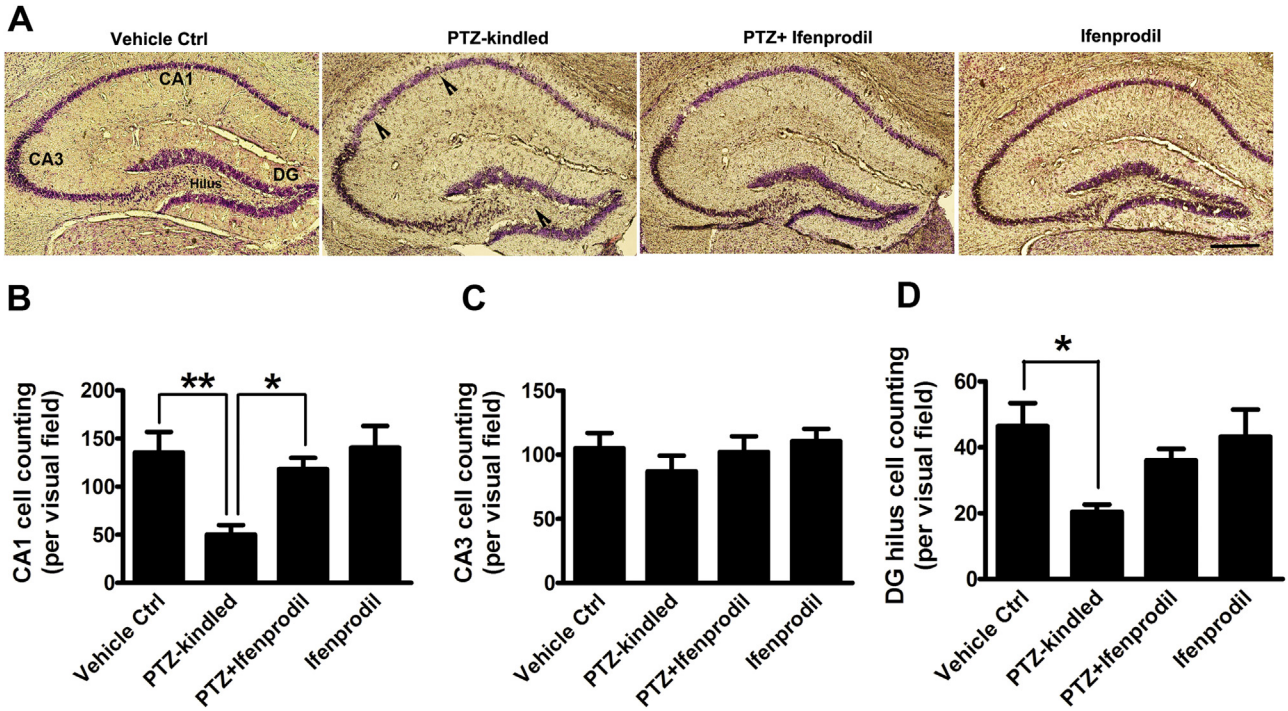

Fig. 4. NMDA receptor NR2B subunits contribute to PTZ-kindling-induced neuron loss in hippocampus. (A) Representative images of Nissl staining of hippocampal neurons in vehicle control, PTZ-kindled, PTZ-kindled ifenprodil-treated (PTZ + ifenprodil) mice and ifenprodil alone-treated mice; arrowheads indicate neuron loss in DG hilus and CA1 region of PTZ-kindled mice. (B–D) Bar graphs represent quantifications of Nissl staining of neurons in hippocampal CA1, CA3 and DG hilus in the above four groups of mice, respectively ($n = 4–6$). Values are means ± S.E.M. $^{*}p < 0.05$ and $^{**}p < 0.01$; Scale bar = 250 μm.

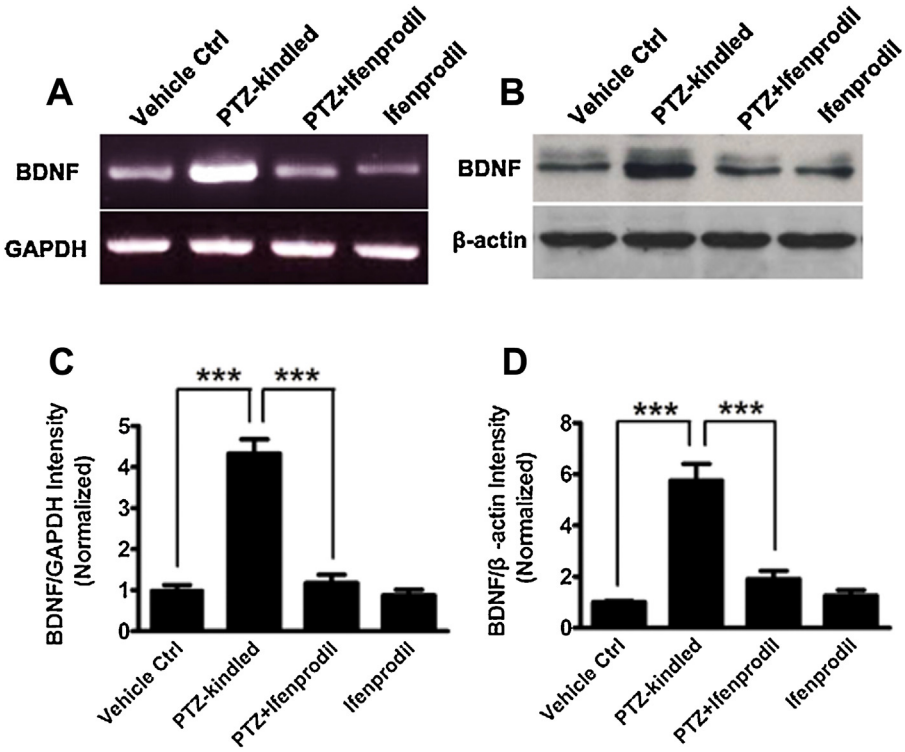

Fig. 5. Selective inhibition of NMDA receptor NR2B subunits suppressed PTZ-kindling-induced BDNF expression in hippocampus. (A) Representative image of BDNF mRNA expression in the hippocampus of vehicle control, PTZ-kindled, PTZ-kindled ifenprodil-treated (PTZ + ifenprodil) mice and ifenprodil alone-treated mice respectively. Bar graph shows the quantification of BDNF mRNA levels, which are presented as the intensity ratio of BDNF to GAPDH ($n = 5$). (B) Western blots were probed with polyclonal rabbit anti-BDNF antibody using protein samples from the hippocampus of vehicle control, PTZ-kindled, PTZ-kindled ifenprodil-treated mice and ifenprodil alone-treated mice, respectively. Bar graph shows the hippocampal BDNF protein levels, which are presented as the intensity ratio of BDNF to β-actin ($n = 5$). Values are means ± S.E.M. $^{***}p < 0.0001$.

PTZ-kindling-induced BDNF expression is dependent on NMDA receptor NR2B subunits.

4. Discussion

It is well established that NMDA receptor activity plays a major role in neuronal excitation in the CNS. Pharmacological and genetic studies of NMDA receptors in *in vivo* and *in vitro* models have suggested that NMDA receptor activation is highly associated with the development of epilepsy. However, the cellular and molecular alterations caused by NMDA receptor activation that underlie the pathogenesis of epilepsy are unknown. Here, we report that NMDA receptor NR2B subunits contribute to PTZ-kindling-induced astrocytosis and oxidative stress in the hippocampus.

NMDA receptors are the major subtype of glutamate receptors, and they normally participate in rapid excitatory synaptic transmission. In mature hippocampal neurons, NR2B subunits are highly distributed both in presynaptic NR1/NR2B receptors that promote glutamate release at excitatory synapses (Berretta and Jones, 1996; Woodhall et al., 2001) and tri-heteromeric NR1/NR2A/NR2B post-synaptic receptors (Rauner and Kohr, 2011). Several lines of evidence suggest that NR2B subunit expression changes during seizures and in tissues chronically affected by epilepsy. NR2B mRNA up-regulation was reported in pyramidal cells of non-sclerotic hippocampi from epileptic patients (Mathern et al., 1998), and NR2B protein levels were found to be increased in the post-synaptic membranes of dysplastic neurons in epileptic foci from patients with focal cortical dysplasia (Colciaghi et al., 2011; Mikuni et al., 1999). A recent study showed that short-term pilocarpine exposure induced NR2B subunit expression in rat primary hippocampal cultures and in rat hippocampi, and this NR2B up-regulation was accompanied by increased expression of NR1 subunits, but not NR2A subunits (Di Maio et al., 2011). Here, we investigated NR2B expression in mice after kindling. Consistent with previous studies, our data showed that PTZ-kindling induced NR2B expression for at least 7 days after the mice were fully kindled. Although increasing evidence has revealed the importance of altered NR2B-containing NMDA receptor expression in human epilepsy (Al-Ghoul et al., 1997; Endele et al., 2010) and in animal models (Al-Ghoul et al., 1997; DeFazio and Hablitz, 2000; Mathern et al., 1999; Ying et al., 2004), the molecular mechanisms involved are still unclear.

Reactive astrocytes are generated in the hippocampus of animal models of epilepsy (Borges et al., 2003; Shapiro et al., 2008) and in the hippocampus of humans with temporal lobe epilepsy (Cohen-Gadol et al., 2004). These reactive changes in astrocytes, termed astrocytosis, generally involve increases in astrocyte size and number (Borges et al., 2003; Shapiro et al., 2008) and are often accompanied with neuronal loss and synaptic rearrangements (Borges et al., 2003; Kron et al., 2010). Reactive astrocytes exhibit an increased expression of GFAP, which is thus used to assess the development of reactive astrocytosis (Pekny and Nilsson, 2005; Wilhelmsson et al., 2004). Emerging evidence has shown that NR2B-containing NMDA receptors are responsible for astrocyte activation (Ahlemeyer et al., 2002; Kato et al., 2006). In agreement with these studies, our data showed that PTZ-kindling significantly increased astrocyte number and size as well as GFAP expression in the hippocampus, and this PTZ-kindling-induced astrocytosis was partially blocked by an NMDA receptor NR2B subunit antagonist. This implies that NR2B subunits are involved in PTZ-kindling-induced astrocytosis. Activated astrocytes release cytokines that induce transcriptional and post-transcriptional signaling in the astrocyte itself and in nearby cells. It has been reported that reactive astrocytes release IL-1β and high-mobility group box 1(HMGB1) proteins, which activate the proinflammatory IL-1 receptor/Toll-like receptor (IL1R/TRL) (Vezzani et al., 2011a), and the activation of IL1R/TLR signaling promotes seizure onset and recurrence in mice. However, blocking this signaling has been shown to reduce seizure activity (Vezzani et al., 2011b).

Increased activation of NR2B subunits caused by high extracellular glutamate released during seizures leads to excessive Ca^{2+} entry into the neurons, which consequently activates Ca^{2+} dependent downstream signaling that includes a variety of proteases and phospholipases. These enzymes mediate proteolysis and oxidative stress, including lipid peroxidation and free-radical generation, leading to neuronal death (Hudspith, 1997). Oxidative events play important roles in a variety of epilepsies (Shin et al., 2011; Waldbaum and Patel, 2010). It remains unclear whether seizure activities induce oxidative stress or whether oxidative injury contributes to epileptogenesis. Impairment of the antioxidative defensive system during seizures has been considered to cause a pathological increase in neuronal damage (Freitas et al., 2005). Superoxide dismutase (SOD) and glutathione peroxidase (GSH-PX) are thought to be two dominant enzymes that act as free radical scavengers to prevent reactive oxygen species (ROS) generation (Niizuma et al., 2010). SOD scavenges the superoxide anion radical (O_2^-) by catalyzing its dismutation to H_2O_2, which is scavenged to water by GSH-PX at the expense of glutathione (Chan, 2001). Malondialdehyde (MDA), an end product of lipid peroxidation, content is an index used to measure the extent of ROS. In the present study, we detected a dramatic decrease in SOD and GSH-PX enzymatic activities and a considerable increase in MDA content indicating that PTZ-kindling significantly induced oxidative stress. Accompanied with this oxidative damage, we observed an obvious neuron loss in the hippocampal CA1 and DG hilus regions. We suspect that this hippocampal neuron loss might be caused by PTZ-kindling-induced oxidative stress. Treatment with the highly specific NR2B antagonist ifenprodil partially suppressed PTZ-kindling-induced oxidative stress as well as neuron loss in the hippocampus. This suggests that NR2B subunits contribute to NMDA receptor-induced oxidation and neuron damage in this PTZ-kindling epilepsy model.

BDNF is a cAMP response element-target gene. Ca^{2+} influx from voltage-dependent calcium channels (Ghosh et al., 1994) or synaptic NMDARs (Hardingham et al., 2002) is sufficient for the initiation of BDNF expression. Previous studies have shown that seizures induce dramatic increases in BDNF mRNA and protein expression in both animal models and humans with epilepsy (Murray et al., 2000; Takahashi et al., 1999; Yan et al., 1997). Intra-hippocampal infusion of BDNF and transgenic over-expression of BDNF increase seizure susceptibility and severity (Croll et al., 1999; Lahteinen et al., 2003; Scharfman et al., 2002; Xu et al., 2004). Here, we detected a significant increase in BDNF mRNA and protein levels in PTZ-kindled mice, and this increase was inhibited by selective inhibition of NMDA receptor NR2B subunits. This suggests that PTZ-kindling-induced up-regulation of BDNF expression is dependent on NMDA receptor NR2B activity.

In summary, we report that NMDA receptor NR2B subunits may contribute to PTZ-kindling-induced astrocytosis, oxidative stress and neuron loss in the hippocampus. These are the major epileptogenic events, and they might be correlated with BDNF expression.

Conflict of interest

The authors declare no competing financial interests.

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