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Increased expression of the P2X7 receptor in temporal lobe epilepsy: Animal models and clinical evidence

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Abstract. Previous studies have indicated that the adenosine triphosphate-sensitive homomeric P2X7 receptor (P2X7R) plays an important role and exhibits therapeutic potential in a number of brain disorders, including temporal lobe epilepsy (TLE). The aim of the present study was to assess the expression of P2X7R, glutamate (GLU) and glial fibrillary acidic protein (GFAP) in the temporal neocortex and hippocampus of rats with lithium-pilocarpine-induced epilepsy as well as in patients with intractable TLE. The results demonstrated that the levels of P2X7R, GLU and GFAP were significantly upregulated in rats with spontaneous recurrent seizures, whereas they were reduced in rats that were treated with brilliant blue G (BBG), a P2X7R antagonist. To the best of our knowledge, the present study is also the first to demonstrate that P2X7R expression was elevated in patients with intractable TLE. These findings suggest that P2X7R plays a key role in the development of TLE and that BBG treatment may be a promising therapeutic strategy for TLE.

Introduction

Epilepsy is a common cerebral disease characterized by interictal epileptiform discharge, which affects nearly 9 million people in China, among whom ~25% (>2.2 million) are considered to have refractory epilepsy (1). Previous studies have demonstrated that the development of temporal lobe epilepsy (TLE) is associated with both abnormal structure and dysfunction of the brain, such as hippocampal neuron regeneration, mossy fiber sprouting, synaptic reconstruction and astrocyte proliferation (2-4). Developing an effective treatment for TLE remains an important research focus.

The concept of the 'purine receptor' has been used to describe cell membrane adenosine receptors and adenosine triphosphate (ATP) receptors (5). ATP is an energy currency unit that is essential for cell function and organization of biological signaling molecules (6). According to the International Association of Pharmacology classification of receptors and drug names, receptors that are sensitive to extracellular adenosine are referred to as P1 receptors, whereas those that are sensitive to extracellular nucleotides are referred to as P2 receptors. P2 purinergic receptors are subclassified into two types, i.e., P2X and P2Y. P2X is an ATP ligand-gated ion channel. When extracellular ATP binds to the P2X receptor, the P2X channel opens, allowing cations to pass through the cell membrane (7-10).

The P2X7 receptor (P2X7R) is abundantly distributed in the cerebrum (11), including the solitary nucleus, hippocampus, habenular nucleus and substantia nigra pars compacta (12). In addition, it performs numerous physiological functions, such as regulation of neurotransmitter release, stimulation of proinflammatory cytokines and induction of cell damage and apoptosis (13,14). However, whether P2X7R is involved in the development of TLE remains unknown. Therefore, the aim of the present study was to assess the expression of P2X7R in rat and human brain tissue samples from epileptic and non-epileptic subjects using immunohistochemical techniques, in order to elucidate the role of P2X7R in epilepsy, particularly its possible implications in the pathogenesis of TLE.

Materials and methods

Animals. A total of 90 male Sprague-Dawley rats (age, 2 months; weight, 200-250 g) were raised under controlled conditions (temperature 24-25˚C, humidity 50-60% and a 12-h light/dark cycle). The animals had *ad libitum* access to food and water. All procedures were implemented according to the guidelines of the Animal Care Committee of Huazhong University of Science and Technology and the study protocol was approved by the Ethics Committee of Huazhong University of Science and Technology.

Drugs. The following drugs were used in the present study: Pilocarpine hydrochloride (Sigma-Aldrich; Merck KGaA,

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Darmstadt, Germany), lithium chloride (Sigma-Aldrich; Merck KGaA), atropine sulfate (Shanghai Harvest Pharmaceutical Co., Ltd., Shanghai, China), anti-P2RX7 antibody (Abcam, Cambridge, UK), anti-glial fibrillary acidic protein (GFAP) antibody (Abcam), anti-glutamate (GLU) antibody (Sigma-Aldrich; Merck KGaA) and diazepam (Tianjin Jinyao Group Co., Ltd., Tianjin, China). Lithium chloride and pilocarpine hydrochloride were dissolved in physiological saline (0.9% sodium chloride).

Animal model of TLE. First, the rats were administered an intraperitoneal (IP) injection of lithium chloride (127 mg/kg). After 18 h, atropine sulfate (1 mg/kg, IP) was injected, followed by pilocarpine hydrochloride (15 mg/kg, IP) after a further 30 min. The seizure severity was graded according to Racine's standard classification as follows: Stage 1, movement of the mouth and face; stage 2, nodding of the head; stage 3, clonus of the unilateral forelimb; stage 4, clonus and rearing of the bilateral forelimbs; and stage 5, stage 4 seizure with episodes of falling. Pilocarpine hydrochloride injection (15 mg/kg, IP) was repeated every 30 min if there was no seizure, or if seizure activity did not reach stage 4 on the Racine scale. The maximum dose of pilocarpine hydrochloride was 60 mg/kg. When status epilepticus persisted for 1 h, it was terminated by an injection of diazepam (10 mg/kg, IP). The present experiment only used rats graded at stage 4 or 5.

Behavioral observation. A total of 2 weeks after the onset of status epilepticus, all rats underwent uninterrupted video monitoring throughout the daytime to observe whether any spontaneous repeated seizures occurred over 7 weeks. Behavioral parameters, including latency period, frequency of spontaneous recurrent seizure, duration of chronic epilepsy and mortality were analyzed during the entire study period.

Electroencephalogram monitoring. Rats were anesthetized with 10% chloral hydrate (0.3 ml/100 g, IP) to undergo an electroencephalography (EEG). The rats were fixed and two electrodes were inserted underneath the scalp on both sides in the temporal region (0.65 cm in front of the connection of the external ear gate and 0.4 cm beside the center line). The reference electrode was inserted underneath the scalp at the frontal pole midpoint (1.2 cm in front of the external ear gate). EEG waveforms filtered <0.53 Hz and >30 Hz were subjected to analog-to-digital conversion by a dynamic electroencephalography device (Beijing Symtop Instrument Co., Ltd., Beijing, China). When the rats entered the spontaneous recurrent seizure period, they underwent EEG monitoring for 4 h once per week to record EEG waveforms and spike-wave discharges. Some rats with spontaneous recurrent seizures were injected with brilliant blue G (BBG) or saline for 2 weeks and EEG waveforms were recorded 30 min before and 60 min after the injection.

Animal grouping and treatment. This study used a total of 90 male Sprague-Dawley rats, among which 6 were given an IP injection of saline solution to serve as the control group, whereas the remaining 84 were used to establish models of epilepsy. A total of ~21 rats had no epileptic seizures, among which 5 were randomly selected to comprise the no seizure group. The remaining 63 rats were eventually affected by status epilepticus, among which 5 were randomly selected to comprise the acute seizure group. A total of 13 rats died after the onset of status epilepticus, 8 rats died during the latency period, 9 rats were in the spontaneous recurrent seizures group and 28 rats were in a chronic phase of spontaneous recurrent seizures. These 28 rats were randomly divided into two groups: The BBG group (21 rats) and the saline group (7 rats). The BBG group was further randomly divided into 3 subgroups (7 rats each) that were separately administered IP injections of BBG at different doses (50, 100 or 200 mg/kg) for 2 weeks.

Patients with TLE. From June 2017 to June 2018, a total of 25 patients with TLE (15 men and 10 women; age range, 18-55 years) from the Wuhan Union Hospital in China were included in the present study. All the patients had characteristic EEGs and representative clinical features (mean number of seizures, 5.5/month), and were on the highest dose of ≥3 antiepileptic drugs (AEDs), such as valproic acid, oxcarbazepine, ethosuximide, lamotrigine, and clonazepam. All patients underwent presurgical assessment including medical history review, detailed neurological examination and ictal or normal EEG studies; they also underwent magnetic resonance imaging (MRI), video EEG, and sphenoidal electrode EEG prior surgery. All brain MRI scans demonstrated no progressive disease in the central nervous system. All patients underwent surgery to remove the epileptogenic zone in the temporal neocortex. For comparison purposes, from June 2017 to June 2018, 6 patients with head trauma (3 men and 3 women; age range, 12-60 years) whose neocortices were histologically normal and who had no history of epilepsy or use of AEDs were examined. All procedures adhered to the conduct of research involving human subjects established by the National Institutes of Health of China and the Committee on Human Research of Huazhong University of Science and Technology. All subjects provided written informed consent to participate in this study.

Tissue processing. The rats were anesthetized with 10% chloral hydrate (0.3 ml/100 g, IP) and then quickly perfused with 4% paraformaldehyde in PBS. After embedding in paraffin, the brain tissue was sliced into 4-μm sections. Similarly, 4-μm sections were prepared from the 4% paraformaldehyde-fixed and paraffin-embedded human brain tissue samples.

Immunohistochemical staining. First, rat hippocampal sections were incubated with 5% bovine serum albumin (Cell Signaling Technology, Inc., Danvers, MA, USA) in PBS for 30 min at room temperature. Subsequently, the sections were incubated with anti-P2X7 immunoglobulin G (cat. no. ab48871; 1:100), anti-GLU IgG (cat. no. G9282; 1:100), or anti-GFAP IgG (cat. no. ab33922; 1:100) overnight at 4˚C. The sections were washed in PBS for three times for 10 min each time and then incubated with an Alexa® 594-conjugated secondary antibody (cat. no. ab150160; 1:100; Abcam) for 40 min at 4˚C. Then, the sections were visualized with 3,3'-diaminobenzidine for 10 min at room temperature in the dark, rinsed in distilled water and dehydrated in a graded ethanol series. Negative control sections were incubated with PBS instead of the primary

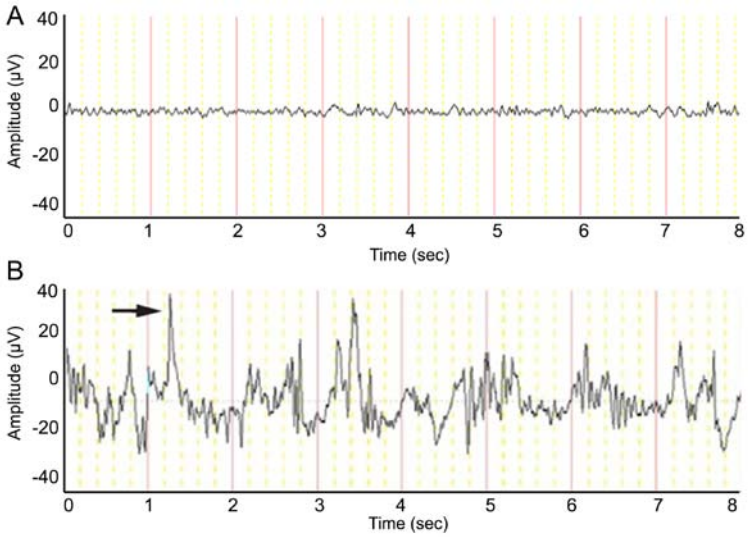

Figure 1. A typical EEG recording. (A) Normal EEG of the control group rats. (B) EEG exhibiting typical spontaneous recurrent seizures in status epilepticus group rats with status epilepticus. The arrow indicates a sharp wave. EEG, electroencephalogram.

antibody. Images were captured via fluorescence microscopy at the same magnification (x200) and grayscale was measured using Image-Pro Plus 6.0 software (Media Cybernetics, Inc., Rockville, MD, USA).

Western blotting. The tissues were harvested, and total cell lysates were prepared using a protein extraction kit (Wuhan Servicebio Technology Co., Ltd., Wuhan, China), following the manufacturer's protocol. Protein concentrations were quantified using a bicinchoninic acid kit (Beyotime Institute of Biotechnology, Haimen, China). Subsequently, 50 μg samples were boiled in gel-loading buffer and separated by 10% SDS-PAGE. The proteins were transferred to polyvinylidene difluoride membranes (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and blocked at 37˚C with 5% skimmed milk powder in Tris-buffered saline for 1 h. Then, the blots were incubated for 6 h at 37˚C with anti-P2RX7 (cat. no. ab48871; 1:1,000; Abcam) and β-actin (cat. no. ab8226; 1:2,000; Abcam) antibodies independently. The membranes were then incubated for 1 h at 37˚C with goat anti-rat horseradish peroxidase-conjugated secondary antibody (cat. no. ab150160; 1:100; Abcam). Chemiluminescent signals were detected using the Enhanced Chemiluminescent Plus kit (KPL, Inc., Gaithersburg, MD, USA) and the signal intensity was measured by densitometry analysis using the Versa-Doc™ Imaging system (version 4.0; Bio-Rad Laboratories, Inc.). β-actin was used as the internal control to normalize the samples.

RNA extraction and polymerase chain reaction (PCR). Hippocampi from rats in each group were treated with TRIzol® (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) reagent to extract total RNA following the manufacturer's recommended protocol. Subsequently, genomic DNA was digested using an RNase-free DNase. A total of 4-6 independent RNA samples were used in duplicate in reverse transcription-quantitative (RT-q) PCR analysis. NanoDrop 2000 (Thermo Fisher Scientific, Inc.) was used to detect RNA concentration. Then, cDNA was transcribed from a total of 500 ng DNase I-treated RNA using a cDNA reverse transcription kit (DP304; Tiangen Biotech Co., Ltd., Beijing, China). Primers used in the study were as follows, P2X7R primers: 5'-GGCGGGGTGACGAAGTTAGTA-3' (forward) and 5'-TTTGTGGGTCCATCCATCCTT-3' (reverse); β-actin primers 5'-TGCTATGTTGCCCTAGACTTCG-3' (forward) and 5'-GTTGGCATAGAGGTCTTTACGG-3' (reverse). The temperature protocol was as follows: 37˚C 15 min and 95˚C 5 min. qPCR analysis was performed on an ABI Prism 7500 sequence detection system (Applied Biosystems; Thermo Fisher Scientific, Inc.) using Thunderbird SYBR qPCR mix (Toyobo Life Science, Osaka, Japan) in 20 μl of reaction mixture. The thermocycling conditions were as follows: Initial denaturation at 95˚C for 5 min, followed by 33 cycles of 95˚C (15 sec) and 60˚C (1 min). Data were analyzed using Sequence Detection Software 1.4 (Applied Biosystems; Thermo Fisher Scientific, Inc.). β-actin was selected as the endogenous reference and data were analyzed using the $2^{-\Delta\Delta Cq}$ method (15).

Statistical analysis. All values were presented as the mean ± standard deviation of three separate experiments and were analyzed by the one-way analysis of variance, which was used to compare multiple groups, followed by a Bonferroni post-hoc test. An unpaired two-tailed Student's t-test was employed to compare the levels of P2X7 receptor and GFAP and GLU in patients with or without intractable epilepsy. Statistical analysis was performed using SPSS software, version 19.0 (IBM Corp., Armonk, NY, USA). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Behavioral analysis. Rats injected with pilocarpine hydrochloride exhibited several behavioral events (data not shown), including stereotypical masticatory movements, hypokinesia, head nodding and wet-dog shakes. This behavior rapidly progressed to generalized limbic seizures recurring every 1-3 min and finally culminating in status epilepticus. A total of 4 rats (4.4%) died when injected with the drug and 63 (73%) eventually developed status epilepticus. Behavioral

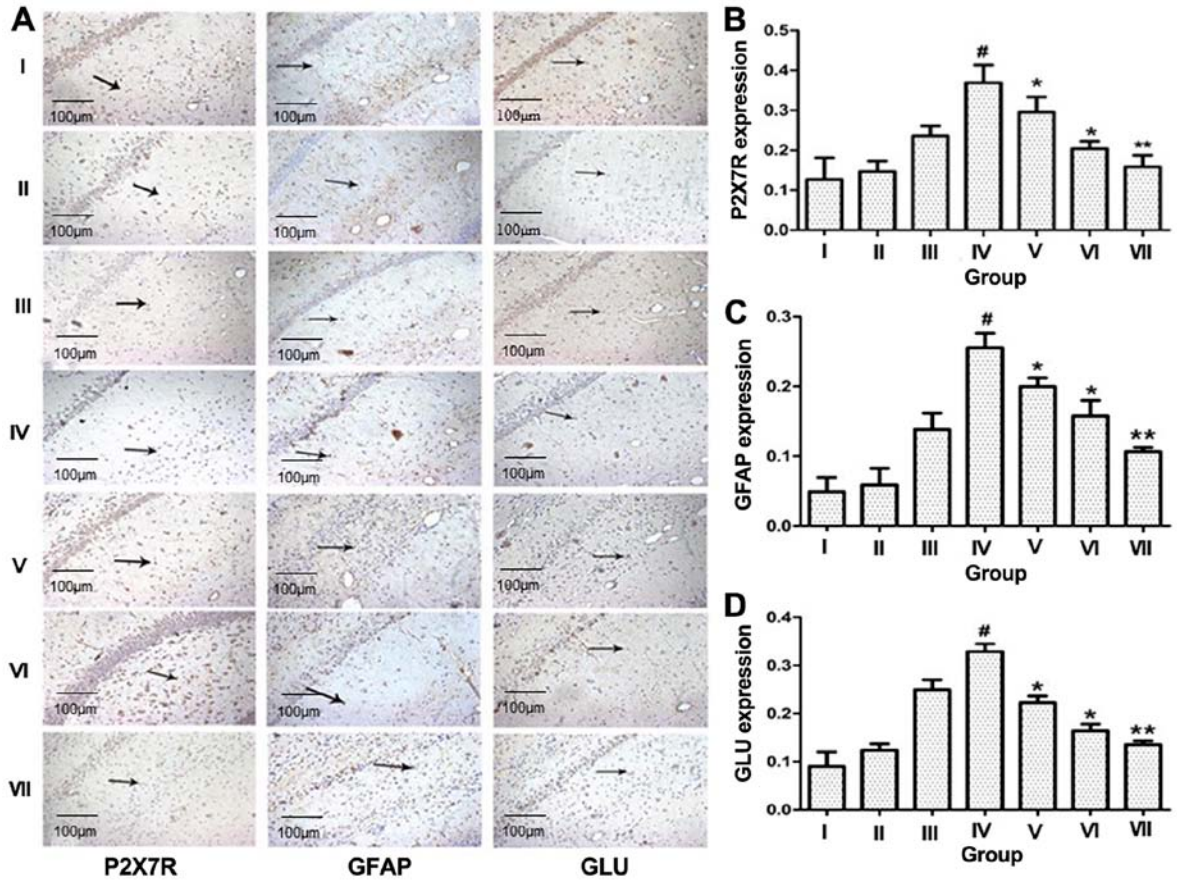

Figure 2. Increased presence of P2X7R, GFAP and GLU in the hippocampal CA3 region of temporal lobe epilepsy rats. (A) Immunohistochemical staining for P2X7R, GFAP and GLU in the CA3 region of the rat hippocampus (x200). Intensity of staining for (B) P2X7R, (C) GFAP and (D) GLU in the CA3 region of the rat hippocampus. One-way analysis of variance was conducted to compare differences among groups. I, control group; II, no seizure group; III, status epilepticus group; IV, spontaneous recurrent seizures group; V, BBG-treated group (50 mg/kg); VI, BBG-treated group (100 mg/kg); VII, BBG-treated group (200 mg/kg). $^{\#}$P<0.01 vs. I; *P<0.05 and **P<0.01 vs. IV. GLU, glutamate; GFAP, glial fibrillary acidic protein; P2X7R, P2X7 receptor; BBG, brilliant blue G.

observations via video surveillance revealed that 28 rats (56%) were in a chronic phase of spontaneous recurrent seizures. Interestingly, one-third reduction in the frequency of spontaneous recurrent seizures was found in the BBG-treated group.

EEG recordings. In the control group, EEG recorded waves of 8-10 Hz and 20-120 μV voltages, followed by low β waves and a single δ wave. In sharp contrast, the 28 rats with status epilepticus exhibited great spike-wave discharges (maximum of 28 Hz and 200 μV). Some spike-wave discharges presented individually, whereas others appeared in a short string (Fig. 1).

P2X7R expression in the rat model

Immunohistochemistry. P2X7R immunoreactivity was found to be high in the cortex, hippocampus, thalamus and amygdala of rats with spontaneous recurrent seizures, but exhibited a significant reduction in the BBG-treated groups 9 [50 mg/kg (V), 100 mg/kg (VI) and 200 mg/kg (VII); Fig. 2], demonstrating a negative correlation with BBG dose. Astroglial activation occurred in the hippocampus of rats with spontaneous recurrent seizures, but this phenomenon was prevented by BBG. Concomitantly, GLU expression was upregulated in rats with spontaneous recurrent seizures compared with the control group, but was also reduced in the BBG-treated groups [50 mg/kg (V), 100 mg/kg (VI) and 200 mg/kg (VII); Fig. 2]. However, there was no significant difference in P2X7R expression between the control group and rats with status epilepticus (I and III, respectively; Fig. 2).

Western blotting and RT-qPCR. Western blotting and RT-qPCR analyses demonstrated that P2X7R expression was increased after spontaneous recurrent seizures within the ipsilateral CA3 subfield of the hippocampus. However, there was no difference between the control group and rats with status epilepticus (Fig. 3). In contrast to rats with spontaneous recurrent seizures, P2X7R expression was significantly reduced in the BBG-treated groups (P<0.05).

P2X7R expression in patients with TLE. Compared with the control group, immunostaining for P2X7R, GFAP and GLU revealed significant upregulation in the temporal lobe of patients with TLE (P<0.05; Fig. 4).

Discussion

To the best of our knowledge, the present study was the first to demonstrate that P2X7R expression is elevated in patients with intractable TLE. This investigation confirmed changes in ATP-gated P2X7R expression in the brain tissue of experimental rats and humans suffering from refractory epilepsy.

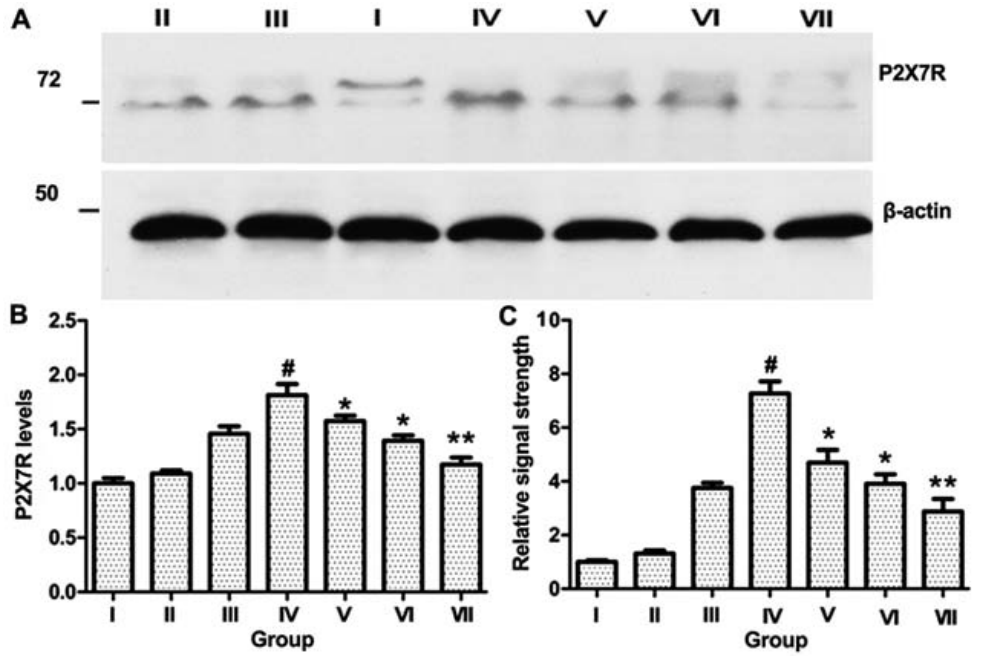

Figure 3. Western blotting and polymerase chain reaction analyses demonstrated that P2X7R expression was increased after spontaneous recurrent seizures within the ipsilateral CA3 subfield of the hippocampus. (A) Western blotting and (B) densitometry analysis of P2X7R protein level in rat hippocampus. The P2X7R protein level was increased in the spontaneous recurrent seizures group. (C) Reverse transcription-quantitative polymerase chain reaction analysis of P2X7R mRNA level in rat hippocampus. The mRNA level exhibited a similar tendency as the protein level. I, control group; II, no seizure group; III, status epilepticus group; IV, spontaneous recurrent seizures group; V, BBG-treated group (50 mg/kg); VI, BBG-treated group (100 mg/kg); VII, BBG-treated group (200 mg/kg). $^{\#}$P<0.01 vs. I; *P<0.05 vs. IV; **P<0.01 vs. IV. P2X7R, P2X7 receptor; BBG, brilliant blue G.

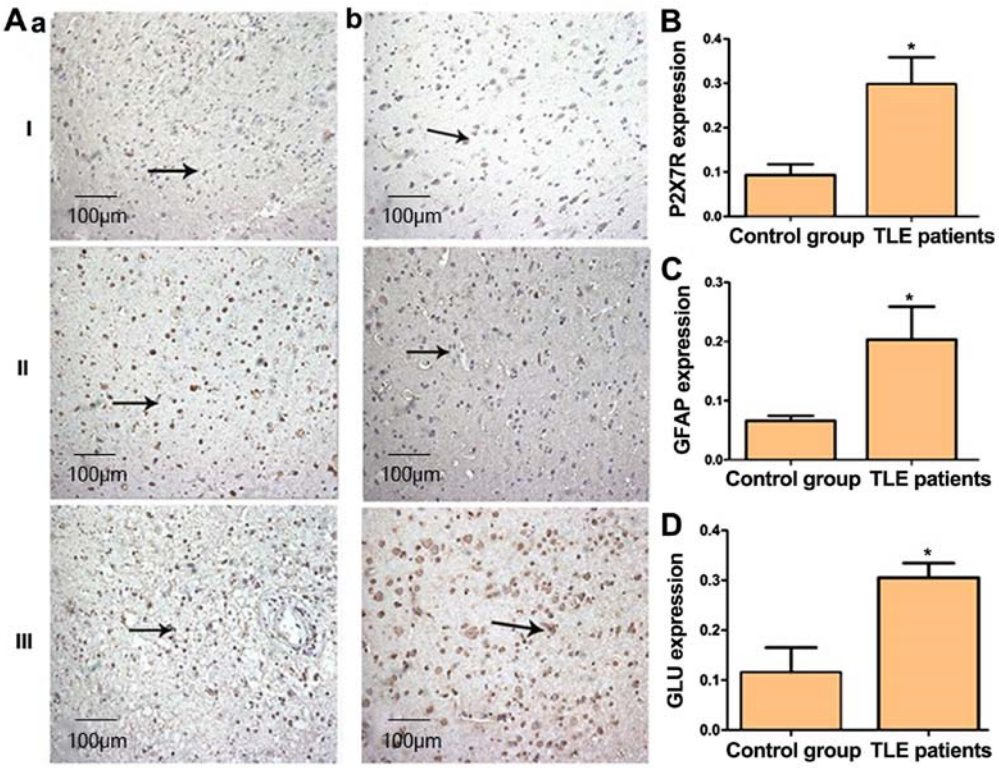

Figure 4. Increased presence of P2X7R, GFAP and GLU in the temporal lobe of TLE patients. (A) Immunohistochemical staining for P2X7R, GFAP and GLU in human brain tissue (magnification, x200). Positive staining is indicated by arrows. (aI-aIII) are control cases and (bI-bIII) are patients with TLE. Intensity of staining for (B) P2X7R, (C) GFAP and (D) GLU in human brain tissue. I, P2X7R; II, GFAP; III, GLU. *P<0.05. GLU, glutamate; GFAP, glial fibrillary acidic protein; P2X7R, P2X7 receptor; TLE, temporal lobe epilepsy.

Furthermore, the expression of GFAP, an astrocytic protein and GLU, the main excitatory neurotransmitter was measured. The results of the present study demonstrated that the levels of these two proteins were increased in the hippocampus of rats

in a chronic phase of spontaneous recurrent seizures as well as in the temporal lobe of patients with TLE. Of note, inhibition of P2X7R using BBG, a P2X7R antagonist, effectively reduced the levels of the two proteins in the rat hippocampus. These results suggest that P2X7R may contribute to the pathogenic mechanism of spontaneous seizures and may represent a novel drug target for seizure prevention.

In humans, epilepsy is a neurological emergency associated with various mechanisms, such as proliferation and repeated activation of astrocytes (16), cerebral cortical developmental disorders (17), immunological dysfunction (18) and disturbance of the glutamatergic system (19). A previous study revealed that TLE is a common type of refractory epilepsy that does not appear to respond to initiation of anticonvulsant therapy (20). In the present study, a pilocarpine-induced seizure model was selected, which highly resembles the morphological and synaptic characteristics of TLE in humans. Rats injected with pilocarpine endure a period of status epilepticus followed by a chronic indefinite period of spontaneous recurrent seizures (21).

P2X7R, a purine receptor, is located in multiple parts of the central nervous system, including the hippocampus, cerebellum, thalamus, striatum and nerve terminals, and performs numerous physiological functions, such as adjustment of neurotransmitter release, stimulation of cytokines and chemotactic factors, as well as induction of cell injury and apoptosis. BBG, a new type of biological stain that can cross the blood-brain barrier, has been demonstrated to block P2X7R with no obvious biological toxicity and has been widely applied in recent P2X7R-related studies (22,23). In addition to the discovery of the P2X7R expression trend in the seizure samples, another main finding was that BBG reduced the levels of both GFAP and GLU in the CA3 region of rat hippocampus in a dose-dependent manner. In addition, BBG-treated rats experienced milder seizures compared with rats that were not treated. The expression of P2X7R was also examined in patients with TLE. Immunohistochemical examination demonstrated that P2X7R expression was increased in patients with TLE compared with in the control group, which was consistent with the results of the present study's animal research, indicating that P2X7R plays a key role in the pathogenesis of intractable epilepsy. Due to ethical restrictions, normal brain tissue samples could not be obtained; therefore, tissue samples from patients with head trauma were used. In addition, although brain tissue samples were only obtained from patients with refractory epilepsy, the results of the present study may provide some insight into epilepsy in general.

The analysis of GFAP and GLU expression suggested that the possible mechanism through which P2X7R contributes to intractable epilepsy may be associated with preventing enhancement of GLU released by astrocytes. Astrocytes play a central role in maintaining homeostasis of the neuronal microenvironment by means of transporting transmitters (24,25). Studies have found that the process of epilepsy is often accompanied by obvious increases in astrocyte number, as well as changes in morphological and biochemical indicators (26,27). In TLE patients, there was a further decline in the number of principal cells in all hippocampal sub-areas analyzed, which was associated with an increase in GFAP immunoreactivity (28,29). As a vital excitatory neurotransmitter, GLU has been a research focus with regard to central nervous system diseases, such as epilepsy (30). Excessive accumulation of extracellular GLU may lead to excitotoxicity of the neurons through different *N*-methyl-D-aspartate receptors and eventually trigger a cascade of intracellular signals that result in neuronal death (31). Earlier evidence has revealed that modulation of GLU by astrocytes plays an important role in the homeostasis of extracellular GLU (32,33), thus participating in the molecular pathogenesis of epilepsy (34,35). The present study demonstrated that P2X7R antagonists may be a potential putative therapy independently or in combination with GLU receptor antagonists.

Further research is required to determine whether BBG affects P2X7R and how this signaling mediates astrogliosis. Moreover, further studies are needed to investigate the effects of P2X7R modulation on TLE.

In conclusion, increased levels of P2X7R were observed in patients and experimental animals with intractable TLE. Therefore, P2X7R may be involved in the pathogenesis of TLE and its suppression may provide a new treatment to promote remission in epilepsy.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

XD designed the study and drafted the manuscript; PS performed the animal experiments, and was a major contributor in writing the manuscript. JH and XL performed the western blot and PCR assay. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All procedures adhered to the conduct of research involving human subjects established by the National Institutes of Health of China and the Committee on Human Research of Huazhong University of Science and Technology. The present study was approved by the Ethics Committee of Huazhong University of Science and Technology. All subjects provided written informed consent to participate in this study.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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