ORIGINAL RESEARCH

Inhibition of NMDA Receptors Downregulates Astrocytic AQP4 to Suppress Seizures

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Abstract

Aquaporin 4 (AQP4), a water-specific channel protein locating on the astrocyte membrane, has been found to be antagonist, agonist and undergone closely related to epilepsy. Our previous study showed that inhibition of an *N*-methyl-ᴅ-aspartate receptor (NMDAR) subunit NR2A can suppress epileptic seizures, suggesting that AQP4 is potentially involved in NR2A-mediated epilepsy treatment. In this study, we aimed to explore the relevance of AQP4 in NR2A-mediated seizures treatment in pentylenetetrazol (PTZ)-induced rat models. We performed electroencephalogram (EEG) recording and examined AQP4 expression at mRNA and protein levels, and the downstream molecules of AQP4 as well. It showed that AQP4 expression was increased after the induction of seizures. Lateral ventricle pretreatment of NR2A inhibitor could mitigate the PTZ-induced seizures severity and counterbalance the increase of AQP4 expression. In contrast, NR2A activator that resulted in seizures aggravation could further augment the seizure-related elevations of AQP4 expression. Pharmacological inhibition of AQP4 alone could also suppress the PTZ-induced seizure activities, with decreased expressions of NF-κB p65, interleukin (IL)-1, IL-6, and tumor necrosis factor (TNF)-α in the brain. The results indicated that increased expression of AQP4 might be an important mechanism involved in NR2A of NMDAR-mediated treatment for epileptic seizures, enlightening a potentially new target for seizures treatment.

Keywords Seizures · NMDAR · NR2A · Aquaporin 4 · Inflammation · NF-κB.

Introduction

Epilepsy, a common neurological disorder with an estimated 65 million people affected all over the world, is characterized by repeated spontaneous seizures and abnormal synchronized discharges in neurons (Devinsky et al. 2018). Seizure generation is determined by multiple factors, including *N*-methyl-ᴅ-aspartate receptors (NMDAR), one of the most widely studied receptors of excitatory neurotransmitter glutamate. NMDAR plays a crucial role in the pathogenesis of seizures. The activation of NMDAR mediates neuronal hyper-excitability and excitotoxicity (Lemke et al. 2013). NMDAR non-selective antagonist MK-801 inhibits seizures activities and reduces cell damage caused by status epilepticus (Lemke et al. 2013; Menezes and Da 2017). However, adverse effects of MK-801 made it inappropriate for clinical applications (Xie and Huang 2018; Zhou et al. 2018). More efforts are needed to optimize the NMDAR strategy for epilepsy treatment.

NMDAR is widely distributed throughout the central nervous system and consists of four subunits, NR2A, NR2B, NR2C, and NR2D. The expression pattern of different subunits of NMDA receptors in the brain and their roles in physiology and pathology are different (Kohr 2006; Paoletti and Neyton 2007). Activation of NR2A promoted the expression of brain-derived neurotrophic factor and NR2A antagonist NVP-AAM 077 (PEAQX) prolonged the latent period of seizure onset (Chen et al. 2007). Our previous studies also suggested that PEAQX treatment in rats prolonged the seizure

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latency and seizure duration (Deng et al. 2014). However, the detailed mechanism underlying this regulation remains obscure. Therefore, this study will further explore how the inhibition of NR2A exerts its anti-seizure effects.

NMDA receptors were activated after traumatic brain injury, triggering massive Ca^{2+} influx, intracellular calcium overload (Rozov and Burnashev 2016) and excitotoxicity (Mei et al. 2018). Meanwhile, it was found that the expression of aquaporin 4 (AQP4), a water-specific channel protein locating on the astrocyte membrane, was significantly increased after traumatic brain injury. Interestingly, the elevation of AQP4 subsided if pretreated with NMDAR inhibitor MK-801 (Chen et al. 2018). AQP4 is a water-specific channel protein, which is abundantly expressed in the central nervous system. AQP4 plays an important role in maintaining the balance between water and ion metabolism in the brain and is involved in the neural signal transduction, astrocyte migration, and neuroimmunological functions (Medici et al. 2011; Verkman et al. 2017). Changes in AQP4 expression were found in different neurological diseases, such as Parkinson's disease (Michel et al. 2016), Alzheimer's disease (Burfeind et al. 2017), brain injury, and particularly epilepsy (Das et al. 2012). In epileptic animal models, the upregulation of AQP4 was found in the pathophysiological process of eclamptic seizure-induced cell death (Han et al. 2018), while pharmacological inhibition of AQP4 relieved seizure-induced brain edema and protected neurons from damage (Hubbard et al. 2017). Our previous studies also indicated that acetazolamide (AZA), an AQP4-specific antagonist had anti-epileptic effects (Chen et al. 2015; Yu et al. 2015, 2016). However, it is not clear whether NR2A signal pathway and seizure activities have potential links with the regulation of AQP4 expression.

Localizing in astrocytes, AQP4 is known to act as a neuroimmunological inducer. It is well accepted that neuroinflammation and seizures may interact as both cause and effect. A considerable amount of literature provided evidence that inflammation contributes to epileptic seizures (Borges et al. 2003; Choi et al. 2009; Mercado-Gomez et al. 2018). Nuclear factor-κB (NF-κB), a central mediator of inflammatory processes, targets several pro-inflammatory genes, including tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1), interleukin-6 (IL-6) (Ridder and Schwaninger 2009). Several studies reported that the expression levels of NF-κB p65, IL-1β, IL-6, and TNF-α in the brains of patients and rats with seizures were higher than that of the healthy control group (Patterson et al. 2015; Shi et al. 2018). In AQP4 knockout mice, the production of NF-κB p65, IL-1β, IL-6, and TNF-α in the brain was decreased (Dai et al. 2018; Sun et al. 2018). Therefore, we hypothesized that AQP4 might act as a homeostatic regulator in the CNS, involved in NR2A-mediated seizures activities and excitotoxicity-induced neuroinflammation.

Methods and Materials

Animals

Adult healthy Sprague–Dawley (SD) male rats, weighing 280 ± 30 g, were housed in the Experimental Animal Center of Guangzhou University of Traditional Chinese Medicine under standard temperature and humidity control environment with free access to food and water. All animal experiments in this study were carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The animal use protocol was reviewed and approved by the Experimental Animal Care and Ethics Committee of Experimental Animal Center of Guangzhou Medical University.

Establishment of PTZ-Induced Acute Seizure Model

The seizure model was established by i.p. injection of pentylenetetrazol (PTZ) according to a previous study (Kouis et al. 2014). In brief, rats were first injected with 40 mg/kg PTZ, and their behavior was monitored. If rats did not show typical seizure behavior, they were injected with half of the first dose (20 mg/kg) after 10 min and subsequently 10 mg/kg PTZ every 10 min until seizure onset. Rats in the control group were injected with the same amount of physiological saline. According to the Racine scale (Racine 1972), the severity of seizures was divided into the following categories: Grade 0: No seizure; Grade I: rhythmic mouth and facial twitching; Grade II: rhythmic nodding or tail flicking; Grade III: single limb twitch; Grade IV: bilateral anterior limb rigidity or twitching with standing; Grade V: comprehensive tonic–clonic with fall. The successful criterion of seizure model was that animals exhibited grade IV to V seizures at least 3 times within 30 min (The animal grouping and treatments were shown in Table 1).

Lateral Ventricle Injection

Rats were weighed and anesthetized by i.p. injection of 2% pentobarbital sodium (30 mg/kg). The head was fixed on a stereotaxic apparatus; the hair was shaved, and the head skin was cut longitudinally, and the anterior fontanelle was marked. According to the mapping of "The Rat Brain in Stereotaxic Coordinates" by George Paxions et al. (Paxinos and Watson 1996) (0.9 mm post-anterior bregma, 1.6 mm beside the sagittal suture and 4.0 mm below the subdural surface), drugs were injected into the lateral ventricle by a microinjector. The microinjector's needle remained for 10 min and was withdrawn slowly. The skin of the head was sutured and disinfected with vitality iodine.

Table 1 Animal grouping and treatments

Group	Lateral ventricular injection		Intraperitoneal injection
Control	10 μl normal saline	30 min later	4 ml/kg Normal saline
SE	10 μl normal saline		40 mg/kg PTZ
PEAQX	10 μl 4 nM PEAQX		4 ml/kg Normal saline
PEAQX+SE	10 μl 4 nM PEAQX		40 mg/kg PTZ
GLYX-13	10 μl normal saline		3 mg/kg GLYX-13
GLYX-13+SE	10 μl normal saline		3 mg/kg GLYX-13+40 mg/kg PTZ
GLYX-13+AZA+SE	10 μl normal saline		3 mg/kg GLYX-13+50 mg/kg AZA+40 mg/kg PTZ

Electroencephalogram (EEG) Recording

After lateral ventricle injection, the hippocampus (5.6 mm posterior to bregma, 4.5 mm lateral, 2.6 mm ventral to the dura mater) was located. The skull was drilled, and a stainless steel bipolar copper core electrode was inserted into the subdural 3.0 mm. EEG in each rat was recorded by the BL-420E biological function experimental system simultaneously.

Detection of AQP4 by Immunohistochemistry

Brains were dissociated after transcardial perfusion and fixed in 4% paraformaldehyde for 72 h. Frozen tissues were cut into 30-μm-thick sections and soaked in 3% H_2O_2 (10% methanol: 0.3% H_2O_2) for 30 min. Sections were incubated in normal goat serum blocking solution for 1 h, and subsequently in rabbit anti-AQP4 primary antibody (1:80, Cell Signaling Technology, USA) for overnight at 4 ℃, and in goat anti-mouse IgG (1:200, BIOSS, Beijing, China) for 1 h at room temperature. Sections were washed thoroughly with PBS. Finally, sections were developed in the DAB color solution for 5–10 min, followed by patching, conventional dehydration, transparent, and sealing. The negative control group was incubated with PBS instead of primary antibody. Under the same light intensity and magnification (×40), five fields in hippocampus CA3 subregion were randomly selected for each section. Image J software was used for the semi-quantitative analysis of AQP4 expression.

Western Blot Detection

Rat hippocampus was dissected and homogenized in tissue lysis buffer. The homogenates were centrifuged at 12,000 rpm for 15 min, and the supernatant was collected. The BCA method was used to measure protein concentration. Protein samples were loaded at 50 μg/well, subjected to 12% SDS-PAGE vertical electrophoresis, and then electrotransferred to the PVDF membrane. The membranes were incubated at room temperature for 1 h with 5% skim milk, washed by TBST, then incubated with rabbit anti-AQP4 antibody (1:1000, Cell Signaling Technology, USA) and rabbit NF-κB p65 (1:1000, Cell Signaling Technology, USA) overnight at 4 ℃, washed by TBST, incubated with goat anti-rabbit secondary antibodies (1:5000, Bioss, Beijing, China) for 1 h at room temperature, and washed by TBST thoroughly. Proteins were then exposed to ECL (Bioworld, USA) by the imager (Bio-Rad, USA). Band densities were digitally quantified by Image J software, and internal reference was used for normalization; the expression levels of target proteins were shown as relative ratios.

RT-qPCR

The total RNA of hippocampus was extracted with Trizol (Invitrogen, Shanghai, China) and reversely transcribed into cDNA by ReverTra Ace qPCR RT Master Mix with gDNA Remover (TOYOBO, Japan). The oligo-dT primers were synthesized by Invitrogen (Shanghai, China) and the sequences of the primers used in this study are shown in Table 2. Quantification of gene transcripts was performed with the Quantstudio 5 PCR instrument (Life Technologies,

Table 2 Sequence of primers for RT-qPCR

Gene	Forward sequence	Reverse sequence
β-actin	5′-GGAGATTACTGCCCGGCTCCTA-3′	5′-GGAGATTACTGCCCGGCTCCTA-3′
AQP4	5′-TGCAGCAGAGAGACATCAT-3′	5′-TGGAGACGTCATCTGTGAGC-3′
NF-κB P65	5′-TCAAAGGGGAACGGACATAG-3′	5′-ACCAGGATTCTGTGGCAATC-3′
IL-1β	5′-CTGTACTCGTGGGATGATG-3′	5′-ACAGTG CATCATCGCTGTTC-3′
IL-6	5′-CAGTTGCCTTCT TGGGACT-3′	5′-TCTGACAGTGCATCATCGCT-3′

USA) using the SYBR Green Real-time PCR Master Mix (TOYOBO, Japan). qPCR reaction conditions were 95 ℃ for 60 s, PCR stage including 95 ℃ for 15 s, 60 ℃ for 15 s, 72 ℃ for 45 s,40 cycles. The PCR products were subjected to melting curve analysis. The expression of the target genes was calculated using the difference between the target gene and β-actin RNA cycle threshold (Ct) (Δ Ct). The fold change between the experimental group and the control group (ΔΔ Ct), the expression change fold was $2^{-\Delta\Delta Ct}$. The relative expression (relative quantity, RQ) of the control group was 1, and the experimental group was a fold of changes relative to 1.

Detection of AQP4 and NF- κB p65 by Immunofluorescence

Rat brain sections were permeabilized with 1% Triton X-100 in PBS solution for 10 min at room temperature, washed thoroughly with PBS, blocked with 5% bovine serum albumin (BSA) for 30 min, incubated in rabbit anti-AQP4 antibody (1:200, CST, USA) and mouse anti NF-κB p65 antibody (1:100, CST, USA) overnight at 4 ℃, washed thoroughly with PBS, incubated in dark in goat anti-rabbit Secondary Antibody Alexa Fluor® 594 (1:200, Invitrogen, Shanghai, China) and Goat anti-mouse Secondary Antibody Alexa Fluor® 488 (1:200, Invitrogen, Shanghai, China) for 1 h at room temperature, and washed thoroughly with PBS. Finally, the sections were stained with 1 μg/ ml DAPI (1:500, Invitrogen, Shanghai, China) in the dark for 5–10 min, washed thoroughly with PBS and mounted. Stained sections were examined and imaged under confocal microscopy (Leica, Germany). Image J software was used for semi-quantitative analysis.

ELISA

The standard working solution and samples were added to the microplate at 100 μl per well, coated with film and incubated at 37 ℃ for 90 min. The solution was then discarded, and the samples were dried without washing. The biotinylated antibody/antigen working solution (100 μl) was added to each well immediately, mixed evenly and incubated at 37 ℃ for 1 h. After washing, the enzyme conjugate working solution was added into the plates (100 μl per well), mixed evenly, coated with film, and incubated at 37 ℃ for 30 min. After washing, each well was added with 90 μl substrate solution (TMB), mixed evenly, coated with film, and incubated at 37 ℃ for about 15 min in the dark. The reaction was terminated by adding 50 μl of termination solution per well. The optical density (OD value) of each well was measured immediately at 450 nm wavelength by enzyme labeling instrument.

Statistical Analysis

Data were analyzed using SPSS 16.0 software. The statistical difference between two groups was assessed by unpaired t test. $P<0.05$ was considered statistically significant.

Results

AQP4 Expression in Rat Hippocampus was Increased After PTZ-Induced Seizures
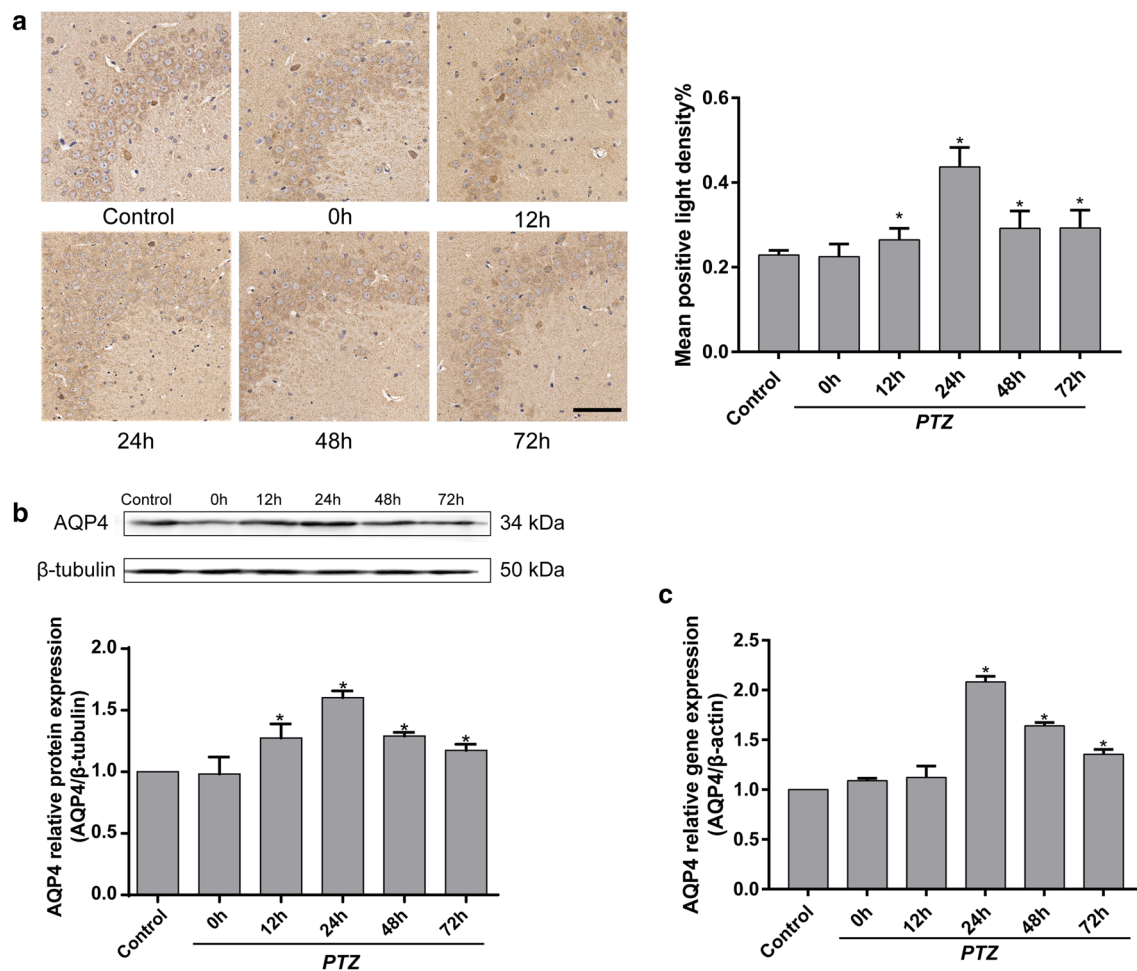
To investigate the dynamic changes of AQP4 expression in rat hippocampus after seizures, the expression of AQP4 was assessed by immunohistochemistry, Western blot, and RT-qPCR at 0 h, 12 h, 24 h, 48 h, and 72 h after seizure induction. The AQP4 expression in the hippocampal CA3 region at different time points after PTZ-induced seizure was assessed by immunohistochemistry. We found that AQP4 expression began to increase at 12 h following seizure induction, and reached the highest level at 24 h. AQP expression levels went down at 48 h and 72 h, but still higher than the control (Fig. 1a). The results obtained from Western blot and RT-qPCR showed that the dynamic changes in AQP4 protein and mRNA were consistent with the immunohistochemistry results.

Effects of NR2A Signaling on PTZ-Induced Seizures

To investigate whether NR2A signal strength might be involved in the PTZ-induced seizure, NR2A antagonist NVP-AAM 077 (PEAQX) or agonist rapastinel (GLYX-13) were administered by lateral ventricle injection before seizure induction. Behavioral observations (Fig. 2a) showed that PEAQX pretreatment significantly prolonged seizure latency and shortened seizure duration, while NR2A activation by GLYX-13 led to increases in seizure sensitivity indicated by latency and duration.

NR2A Signaling Affected AQP4 Expression

To investigate if dynamic regulation of AQP4 expression was involved in NR2A-mediated alteration in seizure sensitivity, we assessed the expression of AQP4 protein and mRNA in the hippocampus of the rats pretreated with NR2A antagonist and agonist and undergone the PTZ-induced seizures. Western blot and RT-qPCR results (Fig. 3a, b) showed that the expression of AQP4 protein and mRNA decreased after PEAQX pretreatment and increased after GLYX-13

Fig. 1 Dynamic changes in AQP4 expression in rat hippocampus after PTZ-induced seizure. **a** (left) Expression of AQP4 assessed by immunohistochemistry in the hippocampal CA3 region at different time points after PTZ-induced seizure. Scale bar: 100 μm. (right) The morphometric analysis of the mean positive immunoreactivity density of the AQP4 staining. *$P < 0.05$ compared to Control. Data are represented as Mean $\pm$ SD ($n = 8$ in each group). **b** Western blot and densitometric analyses of AQP4/β-tubulin in the hippocampus at different time points after PTZ-induced seizure. *$P < 0.05$ compared to Control. **c** Relative gene expression of AQP4 in the hippocampus at different time points after PTZ-induced seizure. *$P < 0.05$ compared to Control. Data are represented as Mean $\pm$ SD ($n = 8$ in each group)

pretreatment, suggesting a tight link between NR2A signaling and AQP4 expression.

Inhibition of AQP4 Signaling Suppressed Seizures

As inhibition of NR2A led to paralleling decreases between AQP4 expression and seizure sensitivity, we next explored the relationship between AQP4 signaling and seizure sensitivity. By analyzing seizure activities and EEG wave amplitude, we found that pro-seizure effects of NR2A antagonist GLYX-13 were counteracted by AQP4 inhibitor AZA, indicated by seizure latency and duration (Fig. 2). The epileptic wave amplitude in EEG recording (Fig. 2b, c) showed similar changes that were consistent with the behavioral observation. Administration of AZA alone could have a significant beneficial effect on seizure sensitivity (Fig. 4). These results

suggested that the effects of NR2A signaling on seizures might be achieved by modulating AQP4, and AQP4 signaling strength was associated with seizure sensitivity.

AQP4 inhibition decreases seizure-induced cytokines

To investigate if AQP4 inhibition could suppress seizure-related neuroinflammation, we assessed the expression of cytokines, including NF-κB p65, IL-1β, IL-6, and TNF-α. After pretreatment of AZA, we firstly examined the expression of AQP4 and NF-κB p65 in the hippocampal CA3 region at 24 h after seizure induction by PTZ using immunofluorescence, Western blot, and RT-qPCR. The expression levels of IL-1β, IL-6, and TNF-α in the hippocampus were

a

b

c
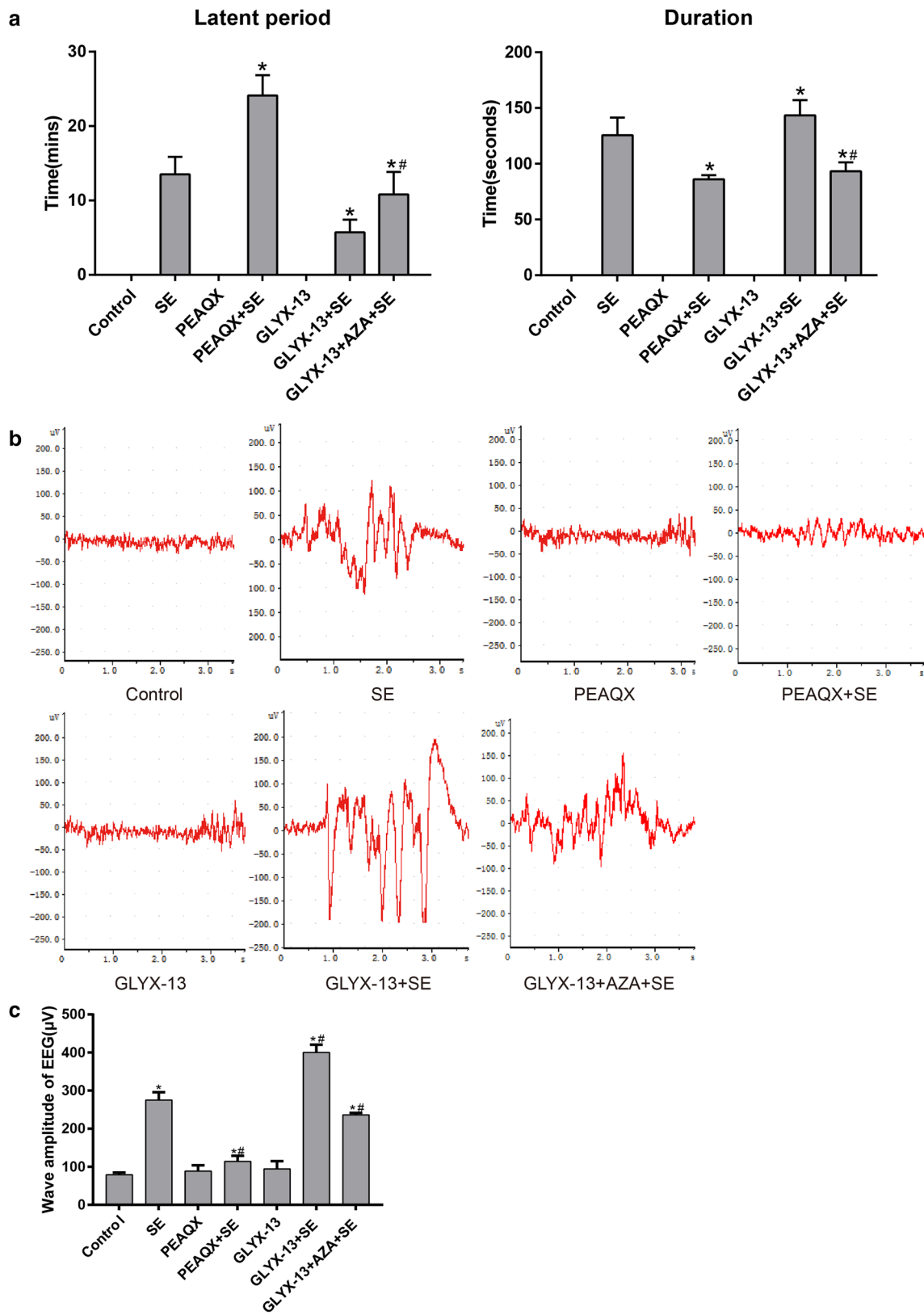
◀**Fig. 2** Effects of NR2A antagonist /agonist pretreatment in the rat model of PTZ-induced seizure. **a** The histograms of the latent period and duration of seizure in each group. *$P < 0.05$ compared with the SE group, #$P < 0.05$ compared with the GLYX-13 + SE group. Data are represented as Mean ± SD ($n = 8$ in each group). **b** Hippocampal EEG recording in each group. **c** The histogram of the brain wave amplitude in each group. *$P < 0.05$ compared with the control group, #$P < 0.05$ compared with the SE group. Data are represented as Mean ± SD ($n = 8$ in each group)

assessed by ELISA and RT-qPCR. The immunofluorescence showed that the expression of NF-κB p65, IL-1β, IL-6, and TNF-α was downregulated by AZA pretreatment, indicating that inhibition of AQP4 signaling could suppress seizure-related neuroinflammation (Fig. 5).

Discussion

In this study, we demonstrated the dynamic changes of AQP4 expression in the hippocampus of rats after seizures and the role of AQP4 in seizure sensitivity and neuroinflammation. The results showed that the expression of AQP4 in the hippocampus of rats increased and the highest expression was observed at 24 h after seizure induction, when neuroinflammation was initiated. NMDAR signaling is correlated to the expression of AQP4 and seizure sensitivity. Inhibition of NR2A led to the concomitant decreases of AQP4 and seizure severity. Additionally, AQP4 inhibitor itself could depress seizures and reduce the production of neuroinflammation cytokines, including NF-κB p65, IL-1β, IL-6, and TNF-α.

AQP4 is the most abundant aquaporin in the mammalian brain and is mainly present in the astrocyte membrane. A large number of studies have shown that the expression of AQP4 is increased in patients with seizures. For example, Medic et al. determined the expression of AQP4 in the patients with seizures by immunohistochemistry and Western blotting, and the results showed that the expression of AQP4 was increased (Medici et al. 2011). In a mesial temporal seizure rat model, expression of AQP4 in the hippocampus was significantly upregulated (Duan and Di 2017). Here, we found that AQP4 expression increased after an epileptic seizure, and reached a peak at 24 h. Based on this result, we performed the following experiments, including the effects of NR2A antagonist and agonist on AQP4 expression at 24 h after seizure induction. The pathogenesis of epilepsy is complicated. One of the currently recognized essential causes is the imbalance of excitatory amino acids and inhibitory amino acids in the central nervous system (Szepetowski 2017). That is, the excitatory glutamic acid is increased, or the inhibitory γ-aminobutyl acid is reduced. The NMDA receptor is a subtype of the glutamate ion-type receptor, which has many allosteric regulatory sites, and different

NMDA receptors correlate with seizure-related excitotoxicity and excitotoxicity-induced neuroinflammation (Luo et al. 2011; Schrattenholz and Soskic 2006). NR2A subunit is the predominant subunit of NMDA receptor in mature neurons (Tovar and Westbrook 1999), while NR2B-containing NMDARs are the predominant subtype at extrasynaptic sites (Liu et al. 2004). Thus, it is not surprising that NR2B inhibition did not result in the expression changes of AQP4 and affected seizure activities. Several studies have shown that downregulating NR2A inhibits epileptic seizures (Wang et al. 2017a, b; Yang et al. 2018), which is consistent with our results. However, there is no research reporting the relationship between NR2A and AQP4. Li X et al. studied the inhibitory effect of magnesium sulfate on eclampsia and found that the pretreatment of magnesium sulfate into the eclampsia-like seizure rat model significantly reduced convulsive seizures and decreased the expression of AQP4 (Li et al. 2017). Verkman's study found that the threshold of seizures in AQP4 knockout mice was significantly increased, and the incubation period of seizures was prolonged (Verkman et al. 2017). Previous studies showed that inflammatory cytokines upregulate AQP4 expression (Ito et al. 2006; Ohnishi et al. 2014; Wang et al. 2018). In this study, we inhibited the expression of AQP4 with AZA and found that AZA can downregulate the expression of NF-κB p65, reduce the levels of inflammatory factors IL-1β, IL-6, and TNF-α, and depress seizures. Verkman et al. suggested that AQP4 as a potential drug target in neurological disorders (Verkman et al. 2017). Wang suggested that inhibiting the upregulation of AQP4 and inflammatory cytokines can protect neurons from brain ischemia (Wang et al. 2017a, b). Except for decreasing the release of inflammation cytokines, AQP4 as a water channel, plays an important role in the formation of brain edema (van Vliet et al. 2007). Downregulating AQP4 can depress the seizures and relieve the edema after seizures to protect neurons vitality (Shin et al. 2014). Thus, AQP4 could be a better approach to anti-epileptic development. In addition, a recent study showed that the AQP4 inhibitor TGN-020 had attenuated LPS-induced lung injury, reduced pro-inflammatory cytokine release, including IL-1α, IL-1β, IL-6, TNF-α, IL-23, and IL-17A (Guo et al. 2019). Our previous study found that inhibition of AQP4 with AZA inhibited seizures, which is consistent with above mentioned findings (Chen et al. 2015; Yu et al. 2015, 2016). Other studies showed that downregulation of AQP4 decreased the levels of NF-κB p65, IL-1β, IL-6, and TNF-α. Consistent with previous studies (Cai et al. 2016; Sun et al. 2017; Yin et al. 2018), in this study, we found that AQP4 inhibitor AZA reduced the production of NF-κB p65, IL-1β, IL-6, and TNF-α and suppressed epileptic seizures. Our results, together with others, indicate
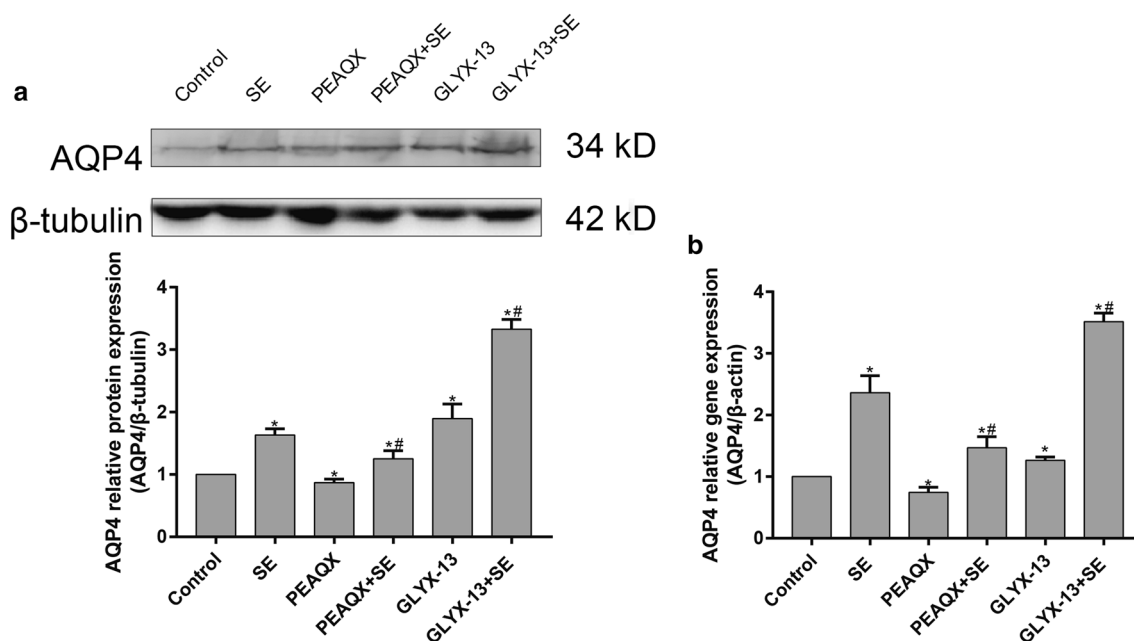
Fig. 3 Expressions of AQP4 with NR2A antagonist/agonist pretreatment. **a** Western blots and densitometric analyses of AQP4/β-tubulin in the hippocampus of rats in each group. *$P < 0.05$ compared with the Control group, #$P < 0.05$ compared with the SE group. Data are represented as Mean ± SD ($n = 8$ in each group). **b** Relative mRNA expression of AQP4 in the hippocampus of rats in each group. *$P < 0.05$ compared with the Control group, #$P < 0.05$ compared with the SE group. Data are represented as Mean ± SD ($n = 8$ in each group)

that inhibition of AQP4 may exert anti-epileptic effects by reducing the production of pro-inflammatory cytokines.

In this study, we presented data to highlight a possible signal link between two membrane channels NR2A and AQP4. These data include (1) upregulation of AQP4 after seizures. The time window of the elevations is associated with the seizure-induced neuroinflammation, indicating AQP4 might be involved in the process. (2) NR2A signal modulation led to changes in seizure activities, demonstrating that NR2A signal is highly involved in the PTZ-induced model. (3) NR2A signal modulation could lead to accordant changes in AQP4 expression, suggesting a tight link between NR2A and AQP4 in the seizure model. (4) Functionl modulation of AQP4 did correlate with seizure sensitivity and seizure-induced neuroinflammation.

NMDAR is commonly considered neuron-specific. However, previous studies showed that NMDA receptors are also expressed in glial cells (Skowronska et al. 2019; Wu et al. 2017). Consistently, it is reported that astrocyte could express inducible functional NMDA receptors without the presence of neurons (Zhou et al. 2010). Even NR2A and AQP4 might locate in different types of cells (e.g., neurons and astrocyte), our findings suggested a possible signaling link between NR2A and AQP4. It is well known that glial cells have a direct role in the regulation of synaptic strength and neuronal excitability. Vice versa, inhibition of NR2A signaling might rapidly induce some changes in the

membrane of glial cells to become "excitable" glia cells, consequently, lowering seizure threshold and precipitating seizures. In our results, we found a link between NR2A and AQP4 changes involved in seizure activities. Their signaling between NR2A and AQP4 might be either coupled on cell membrane or bridged in a way of glia-neuron communications. It still lacks evidence, so far, to know in which way they might interact. For our hypothesis, the activation of NMDA receptor (NR2A) might lead to the release of ATP (Eyo et al. 2014), which might act on P2Y1 receptor in astrocytes and thus regulate AQP4 expression or function.

As discussed above, we only provided evidence for the concurrent involvement of NMDAR and AQP4 in seizures. Considering NR2A subunit is the subunit of predominant NMDA receptor in adult rodents, it is reasonable to speculate that NR2B antagonist may not play an effective role in NMDAR-related seizures directly. The interaction between NR2A and AQP4 might be through direct binding, or mediated by other molecules in astrocytes, or most likely mediated by glia-neuron signaling mechanisms. These detailed underlying mechanisms need further investigations. Previous reports have not reported the relationship between NR2A and AQP4, except that Chen et al. suggested that AQP4 expression increased significantly after traumatic brain injury and was downregulated mediates microglia activationted by the NMDAR inhibitor MK-801 (Chen et al. 2018). Our
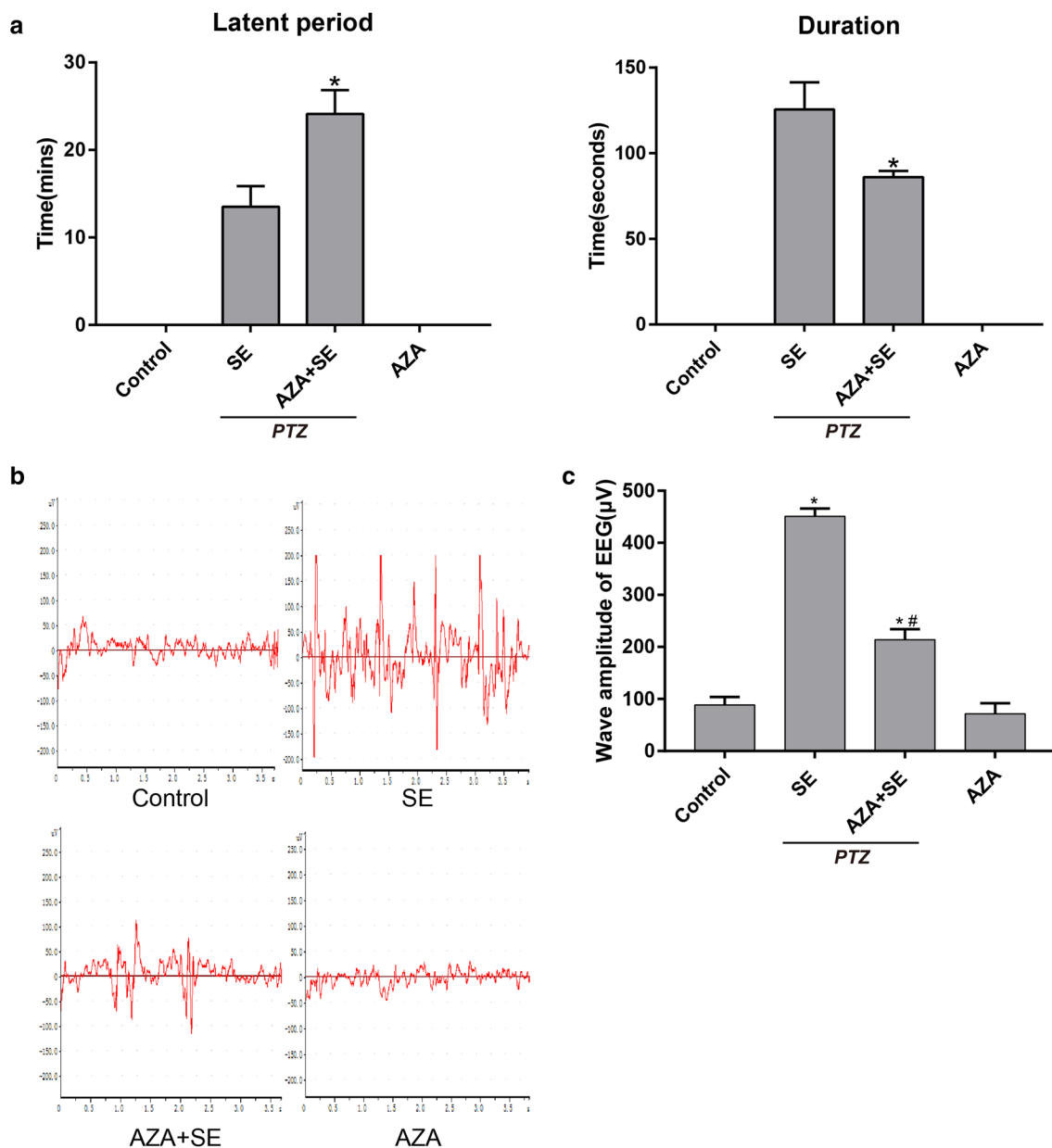
Fig. 4 Effects of AQP4 inhibitor AZA in the rat model of the seizure. **a** Histograms of the latent period and duration of rats in each group. *$P < 0.05$ compared with the SE group. Data are represented as Mean $\pm$ SD ($n = 8$ in each group). **b** Hippocampal EEG recording in each group. **c** The histogram of the brain wave amplitude in each group. *$P < 0.05$ compared with the Control group; #$P < 0.05$ compared with the SE group. Data are represented as Mean $\pm$ SD ($n = 8$ in each group)

findings might potentially link the two epilepsy-related molecules, NR2A and AQP4. We could not exclude a possibility that the changes in AQP4 might be induced by neuronal activities rather than NR2A signaling directly. In fact, we tend to hypothesize that changes in AQP4 are caused by NR2A-mediated neuronal activities. On the other side, the PTZ-induced seizure activities might trigger uncontrolled, pathological activation of NR2A, leading to excitotoxicity and neuroinflammation via AQP4 pathways. The intermediate signals between NR2A and AQP4 remain largely unknown. Nonetheless, the findings might support the potential function of decreased AQP4 expression is involved in NMDAR activation and seizure activities and can be targeted to reduce seizures.
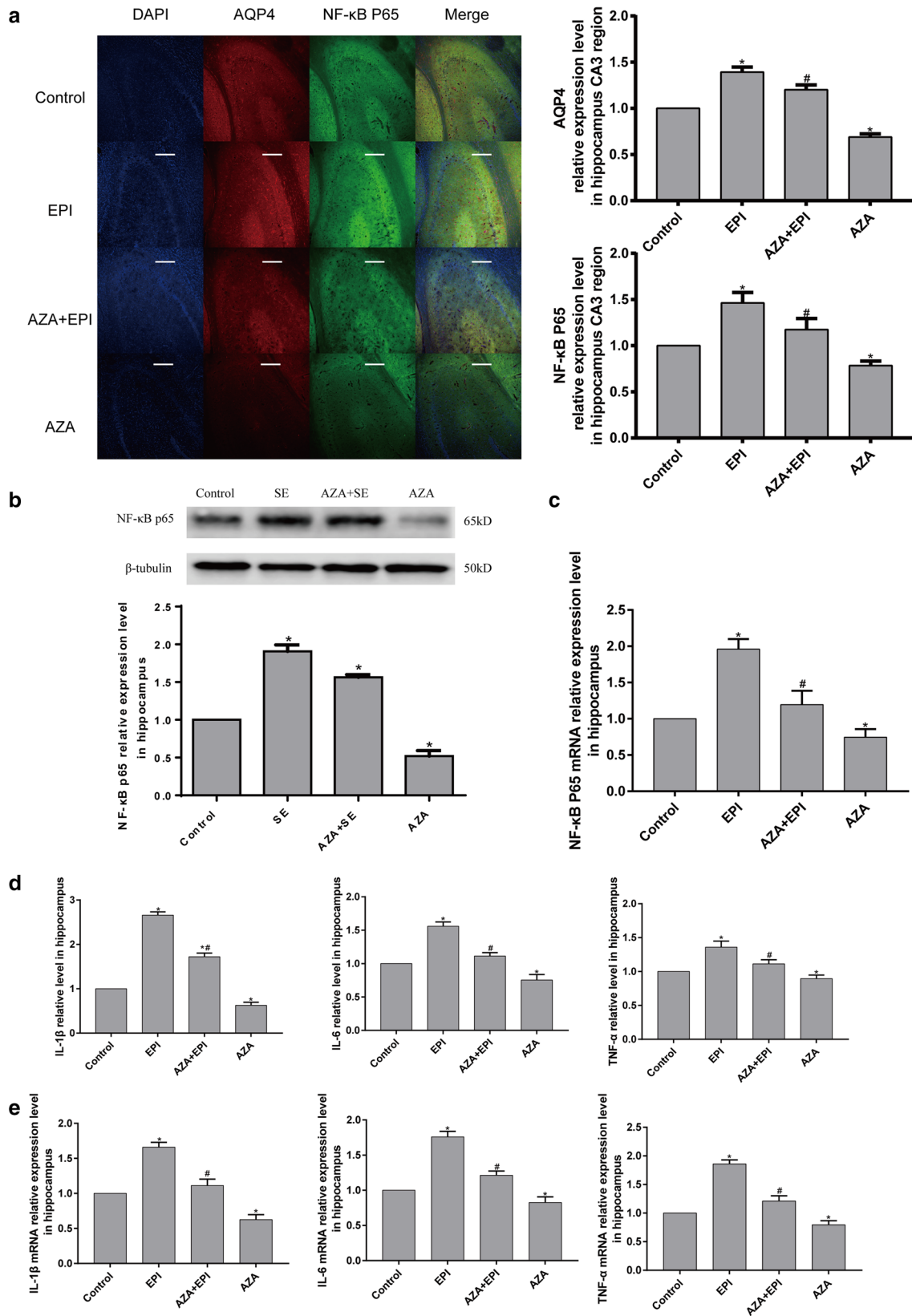
Fig. 5 Effects of AQP4 inhibition on NF-κB p65, IL-1β, IL-6 and TNF-α levels in rat hippocampus. **a** Representative immunofluorescent images (left) and morphometric analysis(right) of AQP4 and NF-κB p65 in the hippocampal CA3 region in each group. Scale bar: 100 μm. $*P < 0.05$ compared with the Control group, $\#P < 0.05$ compared with the SE group. Data are represented as Mean±SD ($n = 8$ in each group). **b** Western blot and densitometric analyses of NF-κB p65/β-tubulin in the hippocampus of each group. $*P < 0.05$ compared with the Control group, $\#P < 0.05$ compared with the SE group. Data are represented as Mean±SD ($n = 8$ in each group). **c** Relative mRNA expression of NF-κB p65 in the hippocampus of each group. $*P < 0.05$ compared with the Control group, $\#P < 0.05$ compared with the SE group. Data are represented as Mean±SD ($n = 8$ in each group). **d** Relative levels of IL-1β, IL-6, and TNF-α in the hippocampus of each group. $*P < 0.05$ compared with the Control group, $\#P < 0.05$ compared with the SE group. Data are represented as Mean±SD ($n = 8$ in each group). **e** Relative mRNA expression of IL-1β, IL-6, and TNF-α in the hippocampus of each group. $*P < 0.05$ Compared with the Control group, $\#P < 0.05$ compared with the SE group. Data are represented as Mean±SD ($n = 8$)

Authors' Contributions All authors contributed to the study conception and design. SL, XZ, and PX designed the study. YH, ZZ, ZL, YL, YH, SD, and XC performed the research. XC analyzed the data. YH wrote the manuscript. XZ and PX revised the manuscript. All authors have read and approved the final manuscript.

Compliance with Ethical Standards

Ethical Approval All procedures performed in studies involving animals were following the ethical standards of the Experimental Animal Care and Ethics Committee of Experimental Animal Center of Guangzhou Medical University (GY2017-038).

Conflict of Interest Statement The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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