Mol Neurobiol (2018) 55:3316–3327
DOI 10.1007/s12035-017-0571-x

Adenosine A1 Receptors Play an Important Protective Role Against Cognitive Impairment and Long-Term Potentiation Inhibition in a Pentylenetetrazol Mouse Model of Epilepsy

Qing Zhou [1] · Suiqiang Zhu [1] · Yuchen Guo [1] · Lifei Lian [1] · Qi Hu [1] · Xiaoyan Liu [1] · Feng Xu [1] · Na Zhang [1] · Huicong Kang [1]

Received: 17 February 2017 / Accepted: 21 April 2017 / Published online: 10 May 2017
© Springer Science+Business Media New York 2017

Abstract Epilepsy is a complicated neurological disorder that occurs worldwide and features several kinds of comorbidities in addition to recurrent seizures. One of the most common comorbidities is cognitive impairment, which seriously affects patients' quality of life. Through activating pre- and postsynaptic adenosine A1 receptors (A1Rs), adenosine has demonstrated anticonvulsant and neuroprotective effects in many epileptic animal models. However, whether the neuroprotective effect of A1Rs will protect cognition during epileptogenesis remains unknown. Therefore, by using A1R knockout (KO) mice and establishing a pentylenetetrazole (PTZ)-kindled model of epilepsy, the present study investigated A1Rs' influences on memory and synaptic function. Morris water maze test results indicated that A1R knockout exacerbated the memory impairment induced by PTZ kindling compared with the wild-type group. To further study the synaptic function of epileptic A1Rs KO mice, we recorded long-term potentiation (LTP) in the hippocampal CA3-CA1 pathway, and LTP was highly inhibited in kindled A1R KO mice compared with kindled wild-type mice. To reveal the mechanisms underlying these effects, neuronal loss, cell apoptosis, and relevant synaptic protein levels in hippocampus were assessed. Epileptic A1R KO mice exhibited significant reductions in neuronal cell survival in the CA1 region and a marked increase in the activation of caspase-3 in the hippocampus compared with epileptic wild-type mice. In addition, an obvious decrease in the PSD95 and BDNF expression levels of epileptic A1R KO mice was observed 7 days after complete kindling. In conclusion, these findings indicated that A1Rs play an important protective role against cognitive impairment by reducing neuron loss and increasing BDNF and PSD95 levels. Activation of A1Rs during epileptogenesis might be beneficial to the preservation of epileptic individuals' cognitive functions.

Keywords Adenosine A1 receptors · Epilepsy · Cognitive impairment · Synaptic function · Mechanisms

✉ Huicong Kang
kanghuicong@163.com

[1] Department of Neurology, Tongji Hospital Affiliated to Tongji Medical College, Huazhong University of Science and Technology, 1095 Jiefang Road, Wuhan 430030, People's Republic of China

Introduction

Epilepsy is a chronic neurological disorder that occurs worldwide and is characterized by recurrent seizures. However, individuals with epilepsy are also at high risk of comorbidities [1]. Cognitive impairment is one of the most common comorbidity. Related factors that affect cognitive function in epilepsy patients are centered around the underlying neuropathology, seizure activity, and antiepileptic medication [2–4]. Evidence suggests an independent potential for antiepileptic drugs (AEDs) to preserve or exacerbate cognitive functions [5–9]. Thus, it is meaningful to evaluate the possibility of newly developed anticonvulsant treatment to protect cognitive function in epileptic conditions.

Adenosine-based antiepileptic therapies have emerged as an effective seizure control approach in recent years [10]. The adenosine system mainly exerts an inhibitory effect on neuronal activity modulation in the CNS [11]. Adenosine released immediately after seizure attack is responsible for seizure termination [12]. Its potent anticonvulsant and neuroprotective functions mainly act through pre- and postsynaptic G protein-coupled adenosine A1 receptors (A1Rs) which are highly expressed in the cerebral cortex, cerebellum, and hippocampus. Via presynaptic A1Rs, adenosine inhibits excitatory

amino acid release through Gi/o proteins and voltage-gated Ca^{2+} and K^+ ion channels. Postsynaptically, adenosine stabilizes the membrane potential by modulating the Ca^{2+} and K^+ fluxes [13]. Dynamic changes in adenosine A1Rs have been revealed in a kindling model of epilepsy by our group's previous work [14]. Blockade of A1Rs can promote the progression of seizure activity into status epilepticus [15]. In addition, A1Rs are essential for keeping epileptic foci localized [16].

In addition to the anticonvulsant effect, adenosine A1Rs' neuroprotective effects are also involved in several events associated with other brain disorders such as brain ischemia, Huntington's disease, and Alzheimer's disease [17, 18]. Whether the effects of adenosine A1Rs will protect the cognitive function of epileptic animals remains unknown. In this study, adenosine A1R knockout (KO) mice were kindled with pentylenetetrazole (PTZ), and their cognitive function and hippocampal synaptic plasticity were investigated.

Materials and Methods

Animals

Experiments were performed in mice of the adenosine A1R-deficient strain generated by Professor Jurgen Schnermann, NIDDK/NIH [19]. The animals were kept under conditions of 22 ± 2 °C and 50% humidity in a 12-h dark/light cycle. All experimental procedures and protocols were approved by the Animal Care and Use Committee of Hubei Province, China (Y20081342). International standards of animal welfare were strictly followed.

Heterozygous mice were intercrossed to obtain male homozygous mutant $A1R^{-/-}$ mice and wild-type (WT) $A1R^{+/+}$ mice for use in the experiment. For genotyping, tail DNA was isolated and tested as described previously [19]. The WT and $A1R^{-/-}$ mice were then subdivided into four groups, comprising a WT control group, kindled WT group, A1R KO control group, and kindled A1R KO group.

Establishment of the Epilepsy Model

The kindling procedure was performed using 6-week-old male mice as described previously [20]. Briefly speaking, experiment animals were intraperitoneally injected with PTZ (37.5 mg/kg) (Sigma–Aldrich) once every 48 h, until they were successfully kindled. Vehicle control animals (WT and A1R KO mice) were injected with saline. Animals were observed for 1 h after injection. The seizure stage and frequency of each mouse were recorded. Mice showing three consecutive stage 4 or stage 5 seizures were considered to be successfully kindled. The seizures were rated according to the following criteria [21, 22]: stage 0, no response; stage 1, ear and facial twitching; stage 2, myoclonic jerks without upright position; stage 3, myoclonic jerks, upright position with bilateral forelimb clonus; stage 4, clonic–tonic seizures; stage 5, generalized clonic–tonic seizures, loss of postural control. Seizure frequency was defined as the number of seizures observed over 1 h after PTZ injection, regardless of seizure stage. Animals that were not successfully kindled within 14 injections were excluded from our study.

Morris Water Maze Test

Spatial memory was assessed as previously described [23]. The Morris water maze test consisted of an acquisition training phase (day 1 to day 5) and a removal testing phase (day 6). The test apparatus consisted of a water tank (150 cm in diameter, 50 cm in height), filled with 22 °C water colored white with milk. Orientation was made possible by some fixed geometric figures placed on the top of the curtain around the tank, which were visible to the swimming mice. The tank was equally divided by four points into four quadrants, namely, quadrants 1, 2, 3, and 4. The platform was submerged 1.5 cm below the water surface in the middle of quadrant 3. During the acquisition phase, each mouse underwent four trials per day, with trials spaced 10 min apart. The mice were placed into the tank facing toward the wall from four starting points (randomly selected) four times and allowed to swim freely until they found the platform. Mice were allowed to stay on the platform for 15 s. Mice that failed to find the platform within 60 s were placed on the platform for 15 s. Escape latency, defined as the time taken to find the platform in each trial, was recorded. On day 6, the platform was removed from quadrant 3. Each animal was released into the water from the starting point in the quadrant opposite quadrant 3 and allowed to swim for 60 s in the pool. The escape latency, swimming distance, and swimming speed during the acquisition training phase and the time spent, crossing times in quadrant 3 in the removal testing phase were recorded and analyzed.

Electrophysiology

Seven days after the animals were kindled, three mice from each group were used for electrophysiology experiments. Hippocampal slices were prepared using standard procedures [24]. Briefly, horizontal 300-μm-thick brain slices including the hippocampus were obtained in chilled oxygenated (95% O_2/5% CO_2) artificial cerebrospinal fluid (aCSF) buffer containing (in mM) NaCl 125.0, KCl 3.5, $MgCl·6H_2O$ 1.0, $CaCl_2$ 2, NaH_2PO 1.25, $NaHCO_3$ 25.0, and D-glucose 1.0 (Sigma–Aldrich). Slices were first incubated in aCSF continuously bubbled with 95% O_2/5% CO_2 at 32.5 °C for 30 min and then incubated at room temperature for at least 1 h. After recovery from the acute injury, slices were placed into a submerged recording chamber perfused with 32 °C flowing oxygenated

aCSF. Recordings were made in the CA1 region by stimulating the Schaffer collateral pathway; the stimulating and recording electrodes were placed in the CA1 stratum radiatum, approximately 150 μm apart. The stimuli, whose intensity was sufficient to elicit 40% of the maximal fEPSP (field excitatory postsynaptic potential) response, were given every 20 s for 30 min for the baseline recording. After that, long-term potentiation (LTP) was induced by tetanic burst stimulation: 100 Hz for 1 s, repeated three times at 20-s intervals. After LTP induction, fEPSPs were recorded for at least another 60 min. The fEPSP slope was quantified and analyzed.

Nissl Staining

One day and 7 days after kindling, the brains of mice in all groups were removed and fixed in 4% paraformaldehyde, embedded in paraffin, and sliced into 10-μm-thick sections. Serial sections from the hippocampus were used for Nissl staining and immunohistochemistry. After a dewaxing process to restore water permeability, sections were Nissl stained using toluidine blue solution (G1032, Wuhan Goodbio Technology) for 10 min. Sections were observed with an Olympus BX51 fluorescence microscope (Olympus, Japan). Numbers of surviving intact pyramidal neurons were counted in a high-magnification view (×400; three slices per animal; each group contained three animals) using the counting function of ImageJ.

Immunohistochemistry

After dewaxing and antigen retrieval, the endogenous peroxidase activity of the sections was blocked by 3% H_2O_2 solution. The sections were then blocked in 3% bovine serum albumin (BSA) (Beyotime, China) for 30 min at room temperature and incubated with a 1:500 dilution of rabbit anti-mouse cleaved caspase-3 antibody (GB11009, Wuhan Goodbio Technology) at 4 °C overnight. After being washed three times by PBS, the sections were incubated in goat anti-rabbit secondary antibody (GB23303, Wuhan Goodbio Technology) for 50 min. DAB solution was used for visualization. The cell nuclei were then stained with hematoxylin. The sections were visualized on an Olympus BX51 fluorescence microscope (Olympus, Japan).

Western Blot

One day and 7 days after full kindling, mouse hippocampi were dissected and homogenized in cell lysis buffer (Beyotime, P0013), then incubated on ice for 30 min so that the tissue was completely lysed. Protein concentration was determined using a BCA protein assay kit (Beyotime, China). Protein samples containing equivalent amount of protein were separated by 8 to 12% SDS-PAGE (depending on the molecular size of protein assessed) and transferred to a polyvinylidene fluoride (PVDF) membrane (Immobilon-P, Millipore). Blotting membranes were blocked with 5% BSA (Beyotime, China)-0.05% TWEEN 20 in Tris-buffered saline (TBST) for 1 h at room temperature and then incubated with primary antibodies overnight at 4 °C. After wash-out, the blots were incubated for 1 h with HRP-conjugated goat anti-rabbit secondary antibody (GB23303, Wuhan Goodbio Technology) or HRP-conjugated goat anti-mouse secondary antibody (GB23301, Wuhan Goodbio Technology), and then washed four more times. The immunoreactive bands were visualized using enhanced chemiluminescence (ECL) western blot (WB) detection reagents (Millipore, Billerica, MA, USA). The blots were quantified using ImageJ. The primary antibodies using in this study includes the following: anti-cleaved caspase-3 (1:1000, no. 9664, Cell Signaling Technology), anti-PSD95 (1:1000, ab76115, Abcam), anti-brain-derived neurotrophic factor (BDNF, 1:1000, ab108319, Abcam), anti-NMDAR2B (1:500, ab28373, Abcam), anti-NMDAR2A (1:1000, ab124913, Abcam), anti-NMDAR1 (1:1000, ab109182, Abcam), and anti-glutamate receptor 1 (AMPA subtype) (GluR1, ab109450, Abcam).

ELISA

The level of BDNF was measured using ELISA. One day and 7 days after kindling, the hippocampus was dissected and rinsed with 1× PBS, homogenized in 1× PBS and stored overnight at −20 °C. After two freeze-thaw cycles, the homogenates were centrifuged for 5 min at 5000×g, at 4 °C. The supernatant was removed and assayed. Total protein concentration was determined using BCA protein assay kit (Beyotime China). The level of BDNF was measured with a mouse BDNF ELISA kit (CUSABIO, Wuhan, China) following the manufacturer's protocol.

Results

The Effects of PTZ Kindling on WT and KO Mice

Thirty WT mice and 34 A1R KO mice were used for the PTZ kindling procedure. After 14 times (28 days) PTZ injections, 76.7% (23 in 30) of WT mice and 79.4% (27 in 34) of A1R KO mice were successfully kindled by PTZ. There was no difference in the kindling rate between the two genotypes (chi-squared test, $\chi^2 = 0.843$, $p > 0.25$) (Fig. 1a). The mortality rate was 6.7% (2 in 30) in WT mice and 8.9% (3 in 34) in KO mice; all deaths were caused by severe stage 5 seizure onset. No difference was observed between the two genotypes (chi-squared test, $\chi^2 = 0.099$, $p > 0.75$) (Fig. 1a). A1R knockout caused a significant increase in the mean seizure stage and frequency on days of the first eight PTZ injections (Fig. 1b, c), whereas it decreased the number of

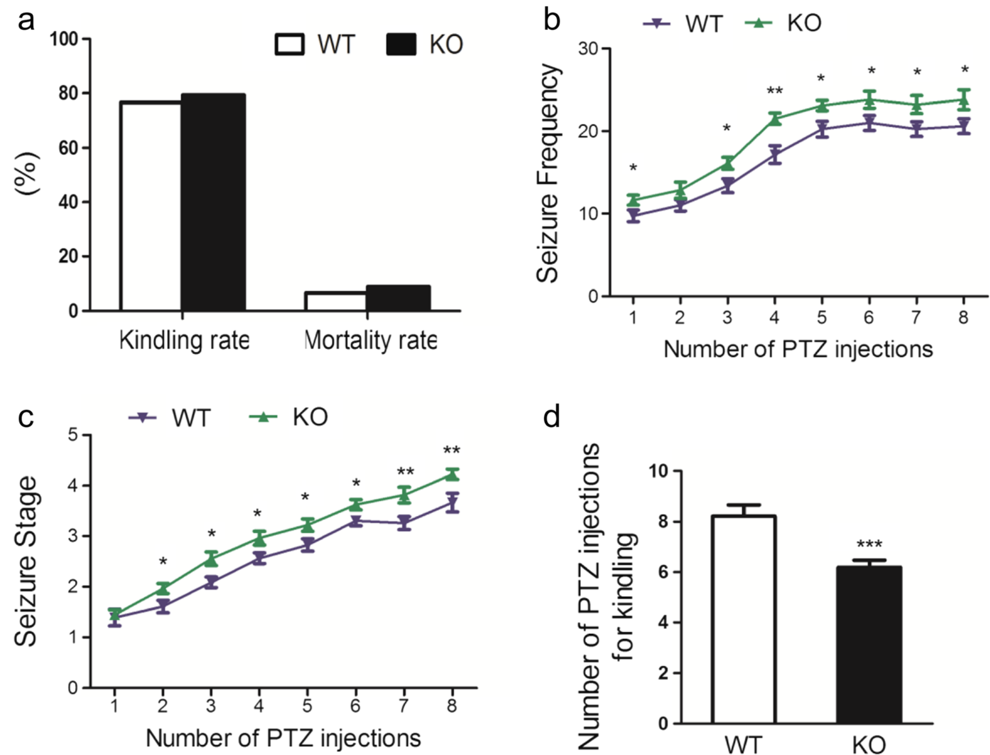

Fig. 1 Effect of PTZ kindling on WT and A1R KO mice. **a** Kindling rate and mortality rate of PTZ kindling procedure in WT and KO mice. No significant differences were observed between the two genotypes (chi-squared test). **b, c** Mean seizure stage and frequency were increased in A1R KO mice compared with WT ones on days of first 8 PTZ injections. **d** A1R KO mice required significantly fewer PTZ injections than WT mice to be successfully kindled (WT $n = 23$, KO $n = 27$, mean ± SEM, ***$p < 0.001$)

PTZ injections for successful kindling compared to WT mice (mean ± SEM, WT 8.217 ± 0.44, $n = 23$; KO 6.185 ± 0.28, $n = 27$, $p < 0.001$) (Fig. 1d).

Learning and Memory Impairment in the Water Maze Test in Mice

A1R knockout exacerbated PTZ kindling-induced memory impairments in the Morris water maze test compared with WT mice (Fig. 2). Between the WT and A1R KO control groups, statistical analysis showed no differences in acquisition, with escape latencies and mean swimming distance decreasing over the acquisition training sessions (two-way ANOVA, latency, $F_{(1,108)} = 1$, $p = 0.3261$; mean distance, $F_{(1,72)} = 0.13$, $p = 0.7273$) (Fig. 2a). During the removal session, the two control groups showed similar crossing times in the platform quadrant (mean ± SEM, WT 6.16 ± 0.38, vs. KO 6.25 ± 0.39, $p = 0.8842$) and spent similar amounts of time in the hidden platform's quadrant (mean ± SEM, WT 21.44 ± 1.56 s, vs. KO 20.10 ± 1.791 s, $p = 0.5746$) (Fig. 2b). On the other hand, compared with control groups, PTZ-kindled mice showed significantly impaired learning and memory functions, indicated by an increase in the escape latency and mean swimming distance during acquisition sessions (two-way ANOVA, latency, $F_{(1,128)} = 15.15$, $p = 0.0005$; mean distance, $F_{(1,76)} = 10.93$, $p = 0.0037$) (Fig. 2a) and a decrease in crossing times (mean ± SEM, control 6.16 ± 0.38, vs. PTZ-kindled 4.57 ± 0.40, $p = 0.0091$) and time spent in the hidden platform's quadrant during the removal testing session (mean ± SEM, control 21.44 ± 1.563 s, vs. PTZ-kindled 16.87 ± 1.503 s, $p = 0.0497$) (Fig. 2b). For the two PTZ-kindled groups, data analysis indicated that A1R knockout aggravated the learning and memory impairment caused by PTZ kindling, as revealed by a significant increase in the escape latency and mean swimming distance during acquisition sessions (two-way ANOVA, latency, $F_{(1,108)} = 9.40$, $p = 0.0049$; mean distance, $F_{(1,84)} = 6.72$, $p = 0.0170$) compared with kindled WT mice (Fig. 2a). Post hoc comparison indicated an increase in the escape latency and mean swimming distance in kindled A1R KO mice compared with WT mice (latency, $p < 0.05$ on day 4, $p < 0.05$ on day 5; mean distance, $p < 0.05$ on day 4, $p < 0.05$ on day 5) (Fig. 2a). During the removal session, kindled A1R KO mice also showed decreasing crossing times (mean ± SEM, WT 4.57 ± 0.40, vs. KO 2.69 ± 0.28, $p = 0.0038$) and time spent in the hidden platform's quadrant compared with kindled WT mice (mean ± SEM, WT 16.85 ± 1.503 s, vs. KO 11.80 ± 1.642 s, $p = 0.0419$) (Fig. 2b).

Inhibition of Hippocampal CA3-CA1 Pathway Long-Term Potentiation After PTZ Kindling

LTP in the hippocampus CA3-CA1 region was depressed in PTZ-kindled mice compared with control mice, with the

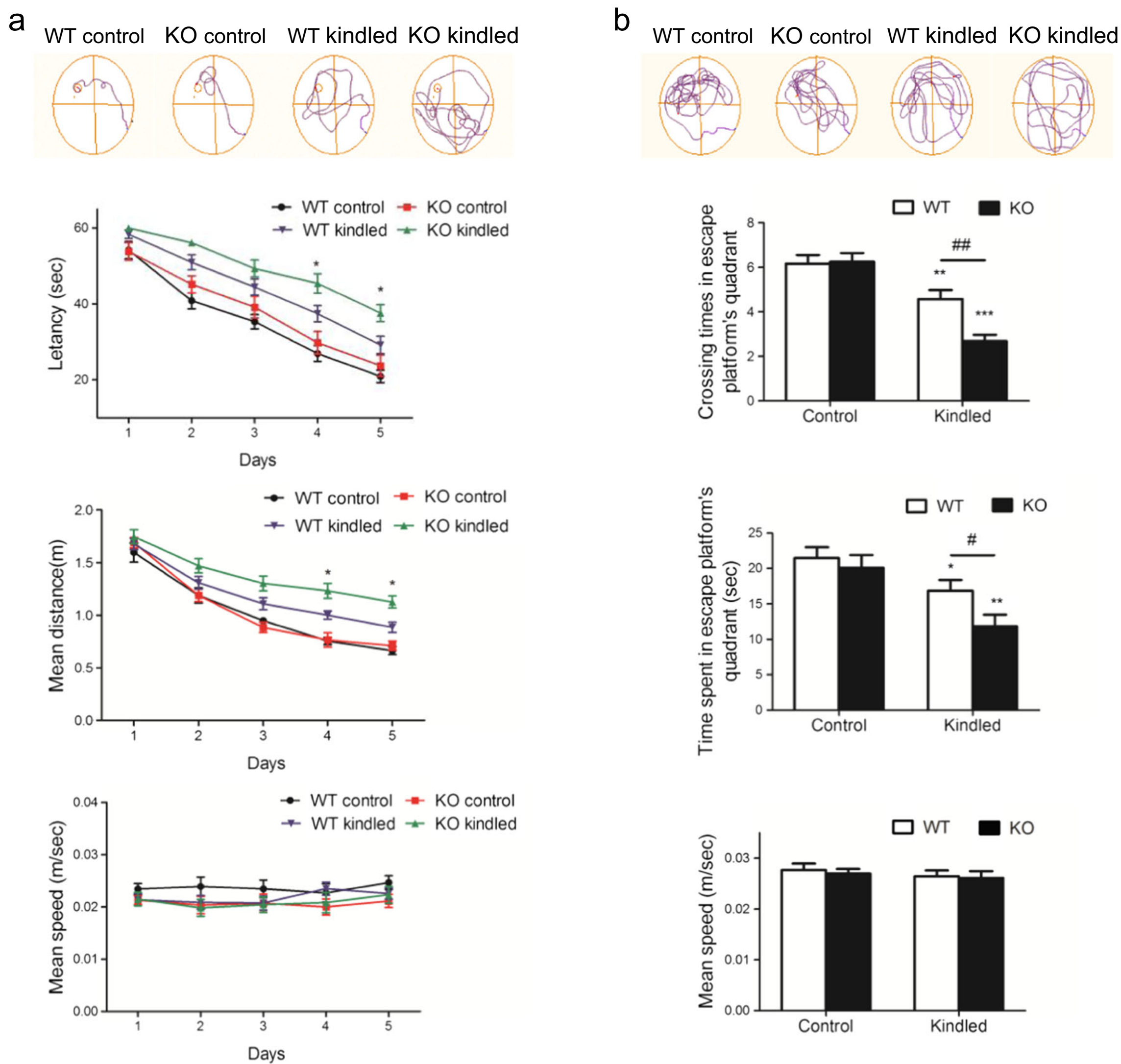

Fig. 2 A1R knockout exacerbated spatial memory function impairment caused by PTZ kindling. **a** During the Morris water maze learning acquisition phase, the latency and mean distance to find the platform significantly increased after PTZ kindling in both WT and KO groups, and A1R KO mice performed the worst. **b** Removal tests were performed on day 6. Crossing times and time spent in the escape platform's quadrant were decreased in PTZ-kindled groups, and kindled A1R KO mice showed the least preference for the target quadrant compared with WT mice. All data are presented as the mean ± standard error of the mean (SEM), $n = 15$ in each group; **a** *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, post hoc comparison between kindled WT and KO mice. **b** *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared to control; #$p < 0.05$, ##$p < 0.01$ compared to kindled WT group

maximum inhibition appearing in the kindled A1R KO mice. Figure 3 shows the time course of the changes in postsynaptic potential slopes and representative averaged field excitatory postsynaptic potentials. There were no significant differences in the increasing rate of fEPSP slopes between control WT and A1R KO mice (fEPSP slope at 50 min, WT 143.5 ± 2.416, $n = 5$, vs. KO 142.9 ± 2.185, $n = 5$, $p = 0.8529$). PTZ-kindled WT mice showed a decreased fEPSP slope growth rate compared to control mice (fEPSP slope at 50 min, kindled WT 131.8 ± 2.396, $n = 4$, vs. WT control 143.5 ± 2.416, $n = 5$, $p = 0.0117$). In addition, A1Rs knockout significantly decreased the rate of increase of the fEPSP slope after PTZ kindling compared to kindled WT animals (fEPSP slope at 50 min, kindled KO 119.4 ± 2.570, $n = 8$, vs. kindled WT 131.8 ± 2.396, $n = 4$, $p = 0.0261$).

Exacerbated Neuron Loss in CA1 Region and Upregulated Active Caspase-3 Expression in Hippocampus of KO Mice After Kindling

Nissl staining was used to assess hippocampal pyramidal neuron loss 7 days after PTZ kindling (Fig. 4). Representative images are shown in Fig. 4a. The number of surviving neurons decreased in the CA1 region 7 days after PTZ kindling compared to the control groups ($n = 3$, $p < 0.01$) (Fig. 4c), and A1R

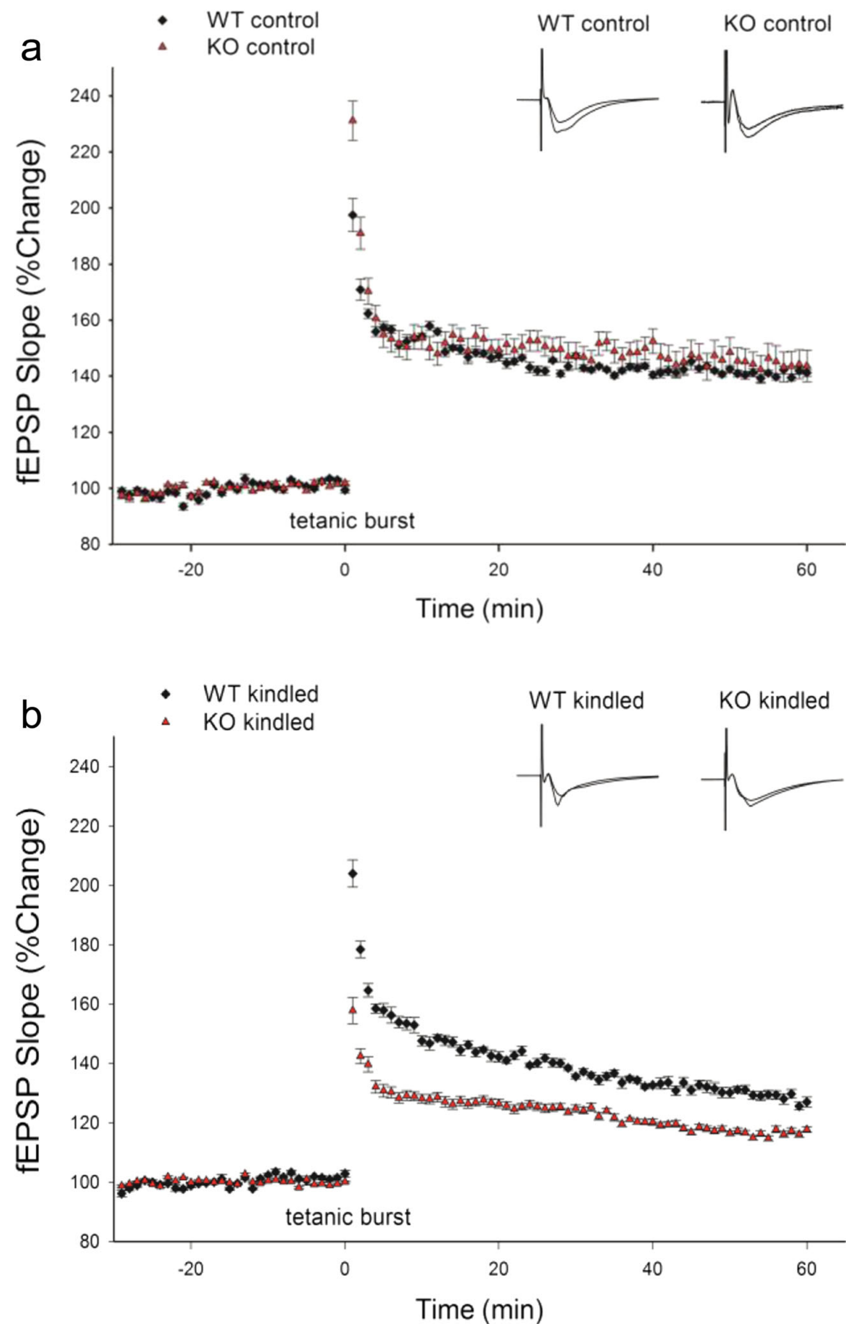

Fig. 3 Long-term potentiation in the Schaffer collateral-CA1 synapses of the hippocampus in WT and A1R KO mice. **a** Theta burst LTP was unchanged in control WT ($n = 5$) and A1R KO ($n = 5$) slices. Both showed significant potentiation of the fEPSP slope. **b** When both genotypes were kindled by PTZ, A1R KO ($n = 8$) slices showed a significantly decreased magnitude of potentiation compared with WT ($n = 4$) slices. fEPSP slope was plotted as percent of baseline. *Each point* represents the mean ± standard error of the mean. Representative averaged field potentials are presented in the *upper right corner*

knockout exacerbated kindling-induced neuron loss in these areas compared to WT mice ($n = 3, p < 0.001$) (Fig. 4c). These results demonstrated that A1Rs had a protective effect against PTZ kindling-induced hippocampal CA1 pyramidal cell loss.

The apoptosis of neurons was detected by immunochemistry staining of active caspase-3. Apoptotic neurons were identified as dark brown active caspase-3 staining cytoplasm merged with blue stained nuclei (Fig. 5). The expression of active caspase-3 increased 7 days after kindling. In addition, compared with the kindled WT group, the kindled A1R KO group showed a significant increase in active caspase-3 expression in CA1, CA3, and dentate gyrus (DG) 7 days after kindling. Western blotting was used to measure the active caspase-3 expression in the hippocampus 1 day and 7 days after PTZ kindling (Fig. 5c). As shown in Fig. 5d, active caspase-3 expression was increased in the A1R KO group compared to the WT group ($n = 3, p < 0.05$) 7 days after kindling. These outcomes indicated that A1R activation can inhibit apoptosis caused by PTZ kindling.

Hippocampal Glutamate Receptors, Postsynaptic Density Protein, and BDNF Change After Kindling

To evaluate the changes in proteins involved in LTP formation and cognition function, western blot analysis was carried out

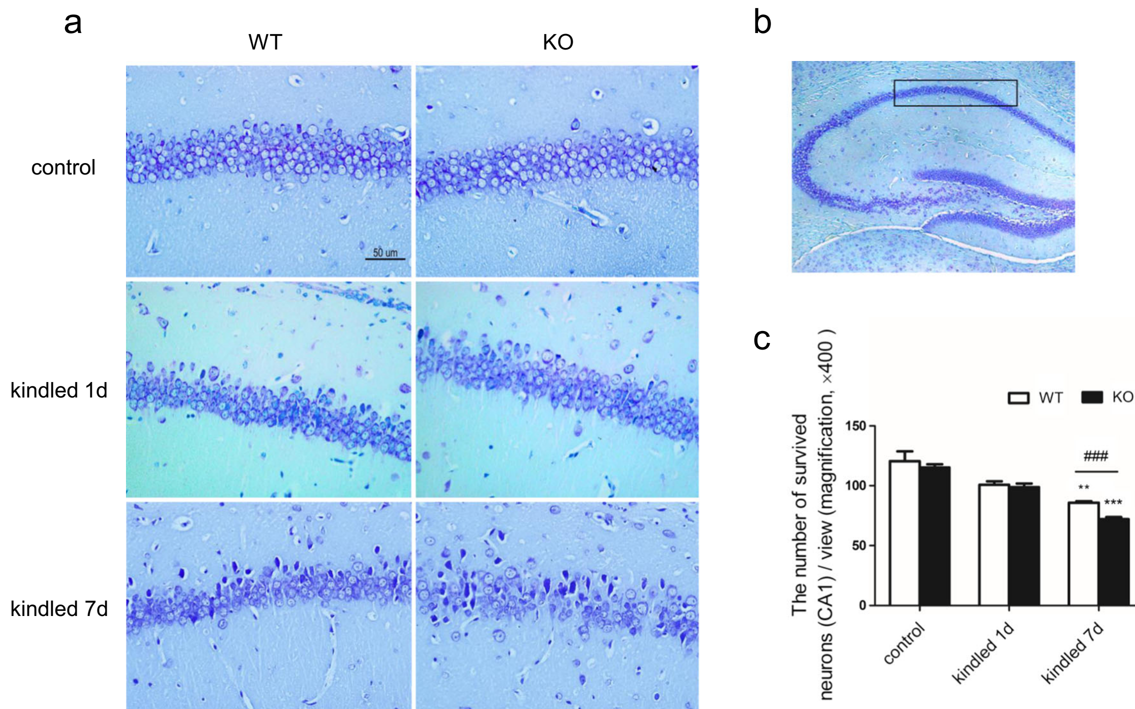

Fig. 4 Effect of A1R knockout on CA1 pyramidal neuron damage 1 day and 7 days after PTZ kindling. **a** Representative Nissl staining of hippocampal CA1 region in control and PTZ-kindled groups. *Scale bars* = 50 μm. **b** Photomicrograph shows the whole hippocampal sample (magnification, ×100) in the coronal plane. CA1 subfield is indicated by the *black frame*. **c** Quantitative values of surviving neurons ($n = 3$). The number of surviving CA1 neurons decreased in PTZ-kindled groups compared with control groups 7 days after kindling. Compared with the kindled WT group, the number of surviving CA1 neurons was significantly reduced in the kindled A1R KO group. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared to control groups; ###$p < 0.001$ compared to PTZ-kindled WT group

to determine the levels of PSD95, BDNF, glutamate NMDA, and AMPA receptors in hippocampi dissected at baseline, 1 day and 7 days after PTZ kindling (Fig. 6). The two genotypes showed no differences in the expression of any of these proteins at baseline.

As for glutamate NMDA receptors, the level of NMDAR2B (GluN2B) was increased 7 days after kindling in both WT and A1R KO groups compared with control groups (7 days after kindling, WT vs. control, $p < 0.01$; A1R KO vs. control, $p < 0.05$), with the kindled WT group increasing more significantly (7 days after kindling, WT vs. A1R KO, $p < 0.05$) (Fig. 6a). The expression of NMDAR2A (GluN2A) was not affected (Fig. 6a). Seven days after kindling, GluN1 and AMPA glutamate receptor GluR1 expressions were obviously decreased in the kindled WT and A1R KO groups (7 days after kindling, WT and A1R KO vs. controls, $p < 0.001$) (Fig. 6b); however, no significant difference was observed between the two genotypes.

Additionally, the expression of PSD95, a postsynaptic density protein that plays key role in synaptic function, was significantly decreased in the A1R KO group compared with the WT group 7 days after kindling (7 days after kindling, A1R KO vs. WT, $p < 0.01$) (Fig. 6c).

The BDNF level was detected by western blot and ELISA. It was increased 1 day after kindling compared with the control groups, and the A1R KO group showed more significant increase compared with the WT group (1 day after kindling, A1R KO vs. WT, $p < 0.001$ for WB; $p < 0.05$ for ELISA) (Fig. 6d, e). However, 7 days after kindling, both WT and A1R KO groups showed a decrease in the expression of BDNF, with the A1R KO group decreasing more significantly compared with the WT group (7 days after kindling, A1R KO vs. WT, $p < 0.01$ for WB; $p < 0.05$ for ELISA) (Fig. 6d, e).

Discussion

The influence of A1Rs on learning and memory is complicated in different physiological or pathological conditions. Under certain pathological conditions, an earlier pharmacological study showed that an A1R-selective agonist prevented scopolamine-induced working memory impairment in the Y-maze test [25]. However, other studies showed that hippocampal A1R activation significantly increased the number of errors in the working memory task [26] and that A1Rs were involved in the chronic morphine treatment-induced impairment of hippocampal CA1 LTP and spatial memory [27]. On the other hand, studies from A1R KO mice indicated that A1Rs may not play such an important role in mediating memory under physiological conditions since A1R KO mice

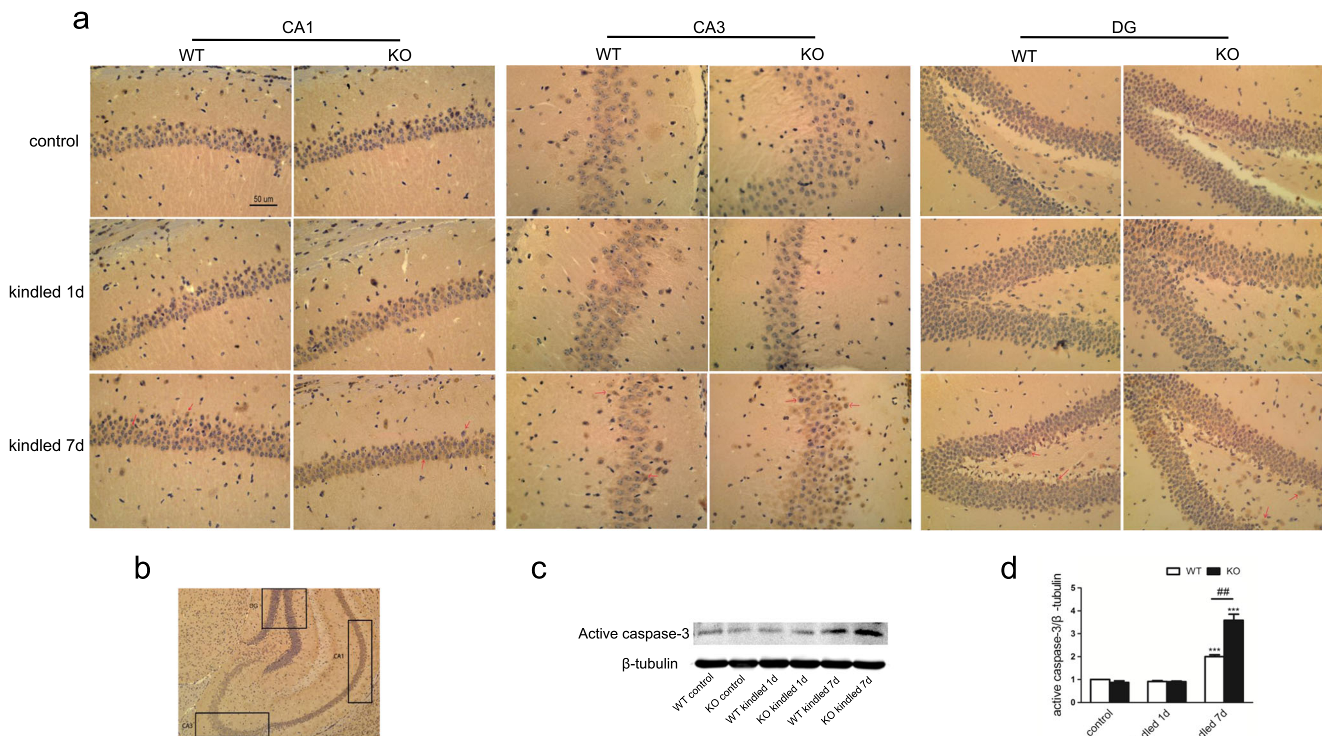

Fig. 5 A1R knockout increased active caspase-3 expression 7 days after PTZ kindling. **a** Representative active caspase-3 immunohistochemistry staining in CA1, CA3, and DG. The active caspase-3 positive neurons are identified as *dark brown* stained cytoplasm merged with *blue* stained nuclei. *Red arrows* indicate the representative active caspase-3 positive neurons. KO mice showed increased active caspase-3 expression 7 days after kindling compared with WT mice. *Scale bars* = 50 μm. **b** Photomicrograph shows the whole hippocampal sample (magnification, ×100) in the coronal plane. CA1, CA3, and DG subfields are indicated by the *black frame*. **c** Western blot analysis of active caspase-3. **d** Relative active caspase-3 protein level in hippocampal tissues (n = 3). ***p < 0.001 compared to control groups; ##p < 0.01 compared with PTZ-kindled WT group

showed no difference from WT mice in spatial reference and working memory in several Morris water maze tasks, and the CA1 LTP was not affected either [28, 29]. However, how A1R will influence learning and memory function under epileptic conditions has not been established. In this study, we conducted the first investigation on the influence of A1Rs on cognitive function in PTZ-kindled epileptic mice.

Spatial memory was assessed by the Morris water maze test. In accordance with previous studies, no differences between genotypes were detected in spatial memory function in the control groups. However, when kindled by PTZ, A1R KO mice displayed worse performance in both the acquisition session and the removal session compared to WT animals. Our result indicated that A1R activation in the epileptic brain may ameliorate cognitive impairment caused by seizures. It should be noted that the different emotional status of kindled A1R KO mice and WT mice might potentially influence their cognitive performance. In our other ongoing study (not yet published), we observed that kindled A1R KO mice showed a more pronounced tendency toward a depressive state in the forced swim test. However, as we observed during the water maze test, the swimming speed displayed no differences between genotypes on any training or testing day, which provides partial evidence against emotional influences on the outcomes of the water maze test.

The phenomenon of LTP is generally viewed as a potential cellular mechanism of learning and memory processes. In accordance with previous studies [29], our study showed that A1R knockout did not influence the LTP elicited by tetanic bursts under normal conditions. This might be because, in the CA3-CA1 pathway, LTP is thought to be mainly caused by postsynaptic mechanisms [30], but the modulatory effects of A1Rs on synaptic transmission are predominantly presynaptic [11, 31], as in the case of mossy fiber-CA3 LTP [32]. However, when the animals were kindled by PTZ, CA3-CA1 LTP was partly inhibited, and the A1R KO mice showed more significant inhibition than WT mice. Given that spatial memory is highly related to LTP at CA3-CA1 synapses [33, 34], it can be assumed that dysfunction of CA3-CA1 synaptic plasticity may contribute to the deterioration of spatial memory in kindled A1R KO mice. Thus, though not critical to synaptic plasticity in the normal brain, A1R activation during the epileptogenic process may help maintain the function of synaptic plasticity in the CA3-CA1 region.

The greater selective neuron loss in the hippocampus and overactivation of the apoptosis pathway after PTZ kindling

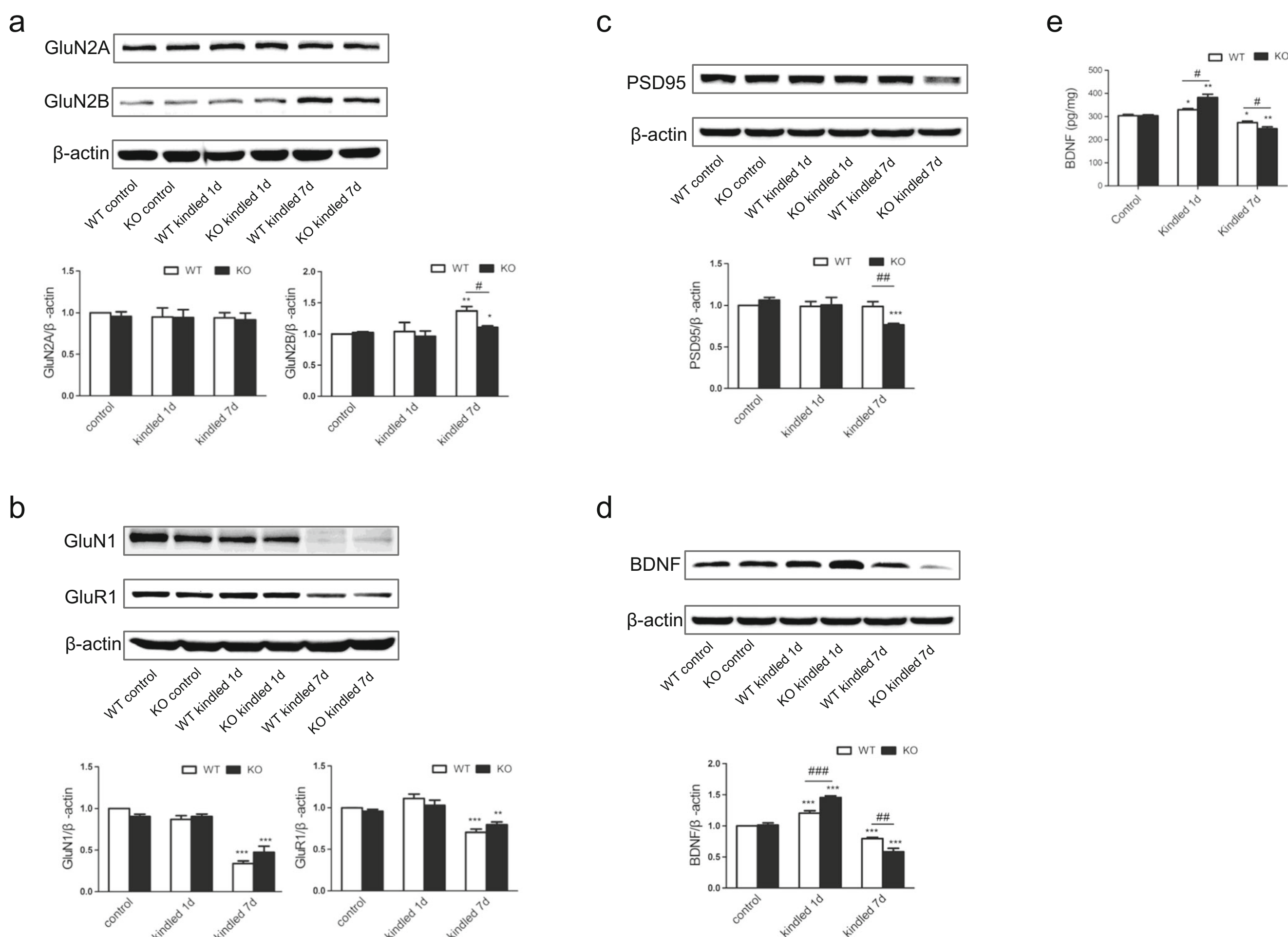

Fig. 6 Effect of A1R KO on synaptic function-related protein expression levels 1 day and 7 days after PTZ kindling ($n = 4$). **a** The expression levels of GluN2A, GluN2B determined by western blot analysis. **b** The expression levels of GluN1 and GluR1 determined by western blot analysis. **c** The expression level of PSD95 determined by western blot analysis. **d** The expression level of BDNF determined by western blot analysis. **e** The concentration of BDNF determined by ELISA in. All data were presented as the mean ± standard error of the mean (SEM). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared to control groups; #$p < 0.05$, ##$p < 0.01$, ###$p < 0.001$ compared with PTZ-kindled WT groups

may account for the more serious cognitive and LTP impairment of A1R KO mice. As shown in the present study, surviving pyramidal neurons in the CA1 region, which are essential for LTP formation, were significantly decreased in kindled A1R KO mice compared to WT animals. In addition, active caspase-3, a feature of apoptotic cells [35, 36], was significantly upregulated in kindled A1R KO mice. Alteration of the glutamatergic system has been demonstrated in the PTZ kindling process [37], and elevated glutamate level was observed in the epileptogenic human hippocampus [38]. Overactivation of NMDARs caused by high extracellular glutamate release during seizures contributes to excessive Ca^{2+} entry into the neurons, which leads to neuronal apoptosis and death [39]. Adenosine can inhibit presynaptic excitatory neurotransmitter release through activation of A1Rs, thus ameliorating cell apoptosis pathway activation and cell loss in the hippocampus.

The correct production and function of hippocampal synaptic transmission-related proteins is the basis for proper cognitive functions. In the present study, PTZ kindling significantly upregulated NMDAR2B expression 7 days later. Previous studies had revealed an increase in hippocampal NMDAR2B expression after PTZ kindling [40], and an increase in the NMDAR2B/NMDR2A ratio may disturb the normal function of synaptic plasticity and negatively modulate early consolidation of hippocampus-dependent memories [41, 42]. However, why the kindled A1R KO mice showed a smaller magnitude of NMDAR2B upregulation compared with WT mice remained unknown. NMDAR1 and GluR1 expression levels were both decreased 7 days after kindling, and no differences were observed between genotypes. The changes in NMDA and AMPA receptors may explain PTZ kindling-induced spatial memory and LTP impairment, but not the differences between kindled WT and A1R KO genotypes.

On the other hand, we observed decreased PSD95 expression in kindled A1R KO mice but not in kindled WT mice.

PSD95 is one of the prominent proteins in the PSD fraction that can bind and cluster glutamate receptors, potassium channels, or other synaptic proteins and link the proteins to cytoplasmic elements [43, 44]. It plays an essential role in synaptic plasticity and the stabilization of synaptic changes during LTP [45]. Thus, the downregulation of PSD95 in kindled A1R mice may partly account for the severe cognitive and LTP impairment. Since PSD95 is a postsynaptic marker, its decreased level in kindled A1R KO mice is probably a consequence of severe neuron loss. However, it remains unknown whether there exist other explanations for the PSD95 protein change.

BDNF is established as a key regulator of synaptic plasticity. Previous evidence indicates that BDNF is one of the major regulators of LTP in the hippocampus [46–49]. BDNF synthesis and secretion occur in an activity-dependent manner. Transient Ca^{2+} influx through NMDA receptors triggers BDNF transcription in cultured hippocampal neurons [50, 51]. Kainic acid-induced seizures and high-frequency stimulation were verified to be able to strongly induce the expression of BDNF mRNA [52, 53]. Thus, in the present study, we observed an increase in BDNF expression 24 h after kindling. As discussed before, A1R blockade leads to excessive glutamate release and Ca^{2+} influx, which can account for the phenomenon that kindled A1R KO mice showed higher BDNF expression 24 h after kindling compared to WT mice. Is more BDNF expression always beneficial? The answer is no. When overexpressed, pro-BDNF is released from neurons [54] and promotes neuronal apoptosis [55–57]. Seven days after kindling, both genotypes showed a decrease in BDNF expression compared to control groups, with A1R KO mice exhibiting the lowest BDNF expression. Since the processes involved in spatial memory are quite sensitive to changes in basal levels of BDNF [58], we can assume that the decrease in hippocampal basal BDNF level in A1R KO mice 7 days after kindling probably contributed to their significant cognitive and LTP impairment.

Summary

In conclusion, the present study revealed that A1R knockout aggravated PTZ kindling-induced memory impairment. The underlying mechanism might be the dysfunction of synaptic transmission, since hippocampal CA3-CA1 LTP was highly inhibited. Dysfunctions of synaptic transmission might be due to severe neuron loss, overactivation of the apoptosis pathway, and abnormal expression of relevant synaptic proteins such as PSD95 and BDNF. Thus, this study indicated that in addition to the anticonvulsant effect, A1R activation during epilepsy development might also help preserve the cognitive functions of epileptic animals, which might be a new approach for the development of adenosine-based antiepileptic therapies.

Acknowledgements This work was supported by the National Natural Science Foundation of China (81201006). We thank Professor Jurgen Schnermann for providing the adenosine A1 receptor knockout mice.

Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflicts of interest.

References

1. Holmes GL (2015) Cognitive impairment in epilepsy: the role of network abnormalities. Epileptic Disord 17(2):101–116. doi:10.1684/epd.2015.0739
2. Lencz T, McCarthy G, Bronen RA, Scott TM, Inserni JA, Sass KJ, Novelly RA, Kim JH et al (1992) Quantitative magnetic resonance imaging in temporal lobe epilepsy: relationship to neuropathology and neuropsychological function. Ann Neurol 31(6):629–637. doi:10.1002/ana.410310610
3. Jokeit H, Kramer G, Ebner A (2005) Do antiepileptic drugs accelerate forgetting? Epilepsy Behav 6(3):430–432. doi:10.1016/j.yebeh.2004.12.012
4. Motamedi GK, Meador KJ (2004) Antiepileptic drugs and memory. Epilepsy Behav 5(4):435–439. doi:10.1016/j.yebeh.2004.03.006
5. Thompson PJ, Corcoran R (1992) Everyday memory failures in people with epilepsy. Epilepsia 33(Suppl 6):S18–S20
6. Meador KJ, Loring DW, Abney OL, Allen ME, Moore EE, Zamrini EY, King DW (1993) Effects of carbamazepine and phenytoin on EEG and memory in healthy adults. Epilepsia 34(1):153–157
7. Meador KJ, Loring DW, Ray PG, Murro AM, King DW, Nichols ME, Deer EM, Goff WT (1999) Differential cognitive effects of carbamazepine and gabapentin. Epilepsia 40(9):1279–1285
8. Martin R, Meador K, Turrentine L, Faught E, Sinclair K, Kuzniecky R, Gilliam F (2001) Comparative cognitive effects of carbamazepine and gabapentin in healthy senior adults. Epilepsia 42(6):764–771
9. Meador KJ, Loring DW, Ray PG, Murro AM, King DW, Perrine KR, Vazquez BR, Kiolbasa T (2001) Differential cognitive and behavioral effects of carbamazepine and lamotrigine. Neurology 56(9):1177–1182
10. Boison D (2005) Adenosine and epilepsy: from therapeutic rationale to new therapeutic strategies. Neuroscientist 11(1):25–36. doi:10.1177/1073858404269112
11. Dunwiddie TV, Masino SA (2001) The role and regulation of adenosine in the central nervous system. Annu Rev Neurosci 24:31–55. doi:10.1146/annurev.neuro.24.1.31
12. During MJ, Spencer DD (1992) Adenosine: a potential mediator of seizure arrest and postictal refractoriness. Ann Neurol 32(5):618–624. doi:10.1002/ana.410320504
13. Fredholm BB, IJzerman AP, Jacobson KA, Klotz KN, Linden J (2001) International Union of Pharmacology. XXV. Nomenclature and classification of adenosine receptors. Pharmacol Rev 53(4):527–552
14. Huicong K, Zheng X, Furong W, Zhouping T, Feng X, Qi H, Xiaoyan L, Xiaojiang H et al (2013) The imbalanced expression of adenosine receptors in an epilepsy model corrected using targeted mesenchymal stem cell transplantation. Mol Neurobiol 48(3):921–930. doi:10.1007/s12035-013-8480-0
15. Avsar E, Empson RM (2004) Adenosine acting via A1 receptors, controls the transition to status epilepticus-like behaviour in an in vitro model of epilepsy. Neuropharmacology 47(3):427–437. doi:10.1016/j.neuropharm.2004.04.015

16. Fedele DE, Li T, Lan JQ, Fredholm BB, Boison D (2006) Adenosine A1 receptors are crucial in keeping an epileptic focus localized. Exp Neurol 200(1):184–190. doi:10.1016/j.expneurol.2006.02.133
17. Gomes CV, Kaster MP, Tome AR, Agostinho PM, Cunha RA (2011) Adenosine receptors and brain diseases: neuroprotection and neurodegeneration. Biochim Biophys Acta 1808(5):1380–1399. doi:10.1016/j.bbamem.2010.12.001
18. Cunha RA (2005) Neuroprotection by adenosine in the brain: From A(1) receptor activation to A (2A) receptor blockade. Purinergic signalling 1(2):111–134. doi:10.1007/s11302-005-0649-1
19. Sun D, Samuelson LC, Yang T, Huang Y, Paliege A, Saunders T, Briggs J, Schnermann J (2001) Mediation of tubuloglomerular feedback by adenosine: evidence from mice lacking adenosine 1 receptors. Proc Natl Acad Sci U S A 98(17):9983–9988. doi:10.1073/pnas.171317998
20. Erdtmann-Vourliotis M, Riechert U, Mayer P, Grecksch G, Hollt V (1998) Pentylenetetrazole (PTZ)-induced c-fos expression in the hippocampus of kindled rats is suppressed by concomitant treatment with naloxone. Brain Res 792(2):299–308
21. Racine RJ (1972) Modification of seizure activity by electrical stimulation. II. Motor seizure. Electroencephalogr Clin Neurophysiol 32(3):281–294
22. Becker A, Grecksch G, Ruthrich HL, Pohle W, Marx B, Matthies H (1992) Kindling and its consequences on learning in rats. Behav Neural Biol 57(1):37–43
23. Morris R (1984) Developments of a water-maze procedure for studying spatial learning in the rat. J Neurosci Methods 11(1):47–60. doi:10.1016/0165-0270(84)90007-4
24. Masino SA, Dunwiddie TV (1999) Temperature-dependent modulation of excitatory transmission in hippocampal slices is mediated by extracellular adenosine. J Neurosci 19(6):1932–1939
25. Hooper N, Fraser C, Stone TW (1996) Effects of purine analogues on spontaneous alternation in mice. Psychopharmacology 123(3):250–257
26. Ohno M, Watanabe S (1996) Working memory failure by stimulation of hippocampal adenosine A1 receptors in rats. Neuroreport 7(18):3013–3016
27. Lu G, Zhou QX, Kang S, Li QL, Zhao LC, Chen JD, Sun JF, Cao J et al (2010) Chronic morphine treatment impaired hippocampal long-term potentiation and spatial memory via accumulation of extracellular adenosine acting on adenosine A1 receptors. J Neurosci 30(14):5058–5070. doi:10.1523/jneurosci.0148-10.2010
28. Gimenez-Llort L, Fernandez-Teruel A, Escorihuela RM, Fredholm BB, Tobena A, Pekny M, Johansson B (2002) Mice lacking the adenosine A1 receptor are anxious and aggressive, but are normal learners with reduced muscle strength and survival rate. Eur J Neurosci 16(3):547–550
29. Gimenez-Llort L, Masino SA, Diao L, Fernandez-Teruel A, Tobena A, Halldner L, Fredholm BB (2005) Mice lacking the adenosine A1 receptor have normal spatial learning and plasticity in the CA1 region of the hippocampus, but they habituate more slowly. Synapse 57(1):8–16. doi:10.1002/syn.20146
30. Malenka RC, Nicoll RA (1999) Long-term potentiation–a decade of progress? Science (New York, NY) 285(5435):1870–1874
31. Fredholm BB, Dunwiddie TV (1988) How does adenosine inhibit transmitter release? Trends Pharmacol Sci 9(4):130–134
32. Moore KA, Nicoll RA, Schmitz D (2003) Adenosine gates synaptic plasticity at hippocampal mossy fiber synapses. Proc Natl Acad Sci U S A 100(24):14397–14402. doi:10.1073/pnas.1835831100
33. Barnes CA, Jung MW, McNaughton BL, Korol DL, Andreasson K, Worley PF (1994) LTP saturation and spatial learning disruption: effects of task variables and saturation levels. J Neurosci 14(10):5793–5806
34. Dragoi G, Harris KD, Buzsaki G (2003) Place representation within hippocampal networks is modified by long-term potentiation. Neuron 39(5):843–853
35. Salvesen GS (2002) Caspases: opening the boxes and interpreting the arrows. Cell Death Differ 9(1):3–5. doi:10.1038/sj.cdd.4400963
36. Ghavami S, Hashemi M, Ande SR, Yeganeh B, Xiao W, Eshraghi M, Bus CJ, Kadkhoda K et al (2009) Apoptosis and cancer: mutations within caspase genes. J Med Genet 46(8):497–510. doi:10.1136/jmg.2009.066944
37. Li ZP, Zhang XY, Lu X, Zhong MK, Ji YH (2004) Dynamic release of amino acid transmitters induced by valproate in PTZ-kindled epileptic rat hippocampus. Neurochem Int 44(4):263–270
38. Cavus I, Kasoff WS, Cassaday MP, Jacob R, Gueorguieva R, Sherwin RS, Krystal JH, Spencer DD et al (2005) Extracellular metabolites in the cortex and hippocampus of epileptic patients. Ann Neurol 57(2):226–235. doi:10.1002/ana.20380
39. Hudspith MJ (1997) Glutamate: a role in normal brain function, anaesthesia, analgesia and CNS injury. Br J Anaesth 78(6):731–747
40. Zhu X, Dong J, Shen K, Bai Y, Zhang Y, Lv X, Chao J, Yao H (2015) NMDA receptor NR2B subunits contribute to PTZ-kindling-induced hippocampal astrocytosis and oxidative stress. Brain Res Bull 114:70–78. doi:10.1016/j.brainresbull.2015.04.002
41. Baez MV, Oberholzer MV, Cercato MC, Snitcofsky M, Aguirre AI, Jerusalinsky DA (2013) NMDA receptor subunits in the adult rat hippocampus undergo similar changes after 5 minutes in an open field and after LTP induction. PLoS One 8(2):e55244. doi:10.1371/journal.pone.0055244
42. Cercato MC, Colettis N, Snitcofsky M, Aguirre AI, Kornisiuk EE, Baez MV, Jerusalinsky DA (2014) Hippocampal NMDA receptors and the previous experience effect on memory. J Physiol Paris 108(4–6):263–269. doi:10.1016/j.jphysparis.2014.08.001
43. Kennedy MB (2000) Signal-processing machines at the postsynaptic density. Science (New York, NY) 290(5492):750–754
44. Ziff EB (1997) Enlightening the postsynaptic density. Neuron 19(6):1163–1174
45. Meyer D, Bonhoeffer T, Scheuss V (2014) Balance and stability of synaptic structures during synaptic plasticity. Neuron 82(2):430–443. doi:10.1016/j.neuron.2014.02.031
46. Bramham CR, Messaoudi E (2005) BDNF function in adult synaptic plasticity: the synaptic consolidation hypothesis. Prog Neurobiol 76(2):99–125. doi:10.1016/j.pneurobio.2005.06.003
47. Edelmann E, Lessmann V, Brigadski T (2014) Pre- and postsynaptic twists in BDNF secretion and action in synaptic plasticity. Neuropharmacology 76 Pt C:610–627. doi:10.1016/j.neuropharm.2013.05.043
48. Lu Y, Christian K, Lu B (2008) BDNF: a key regulator for protein synthesis-dependent LTP and long-term memory? Neurobiol Learn Mem 89(3):312–323. doi:10.1016/j.nlm.2007.08.018
49. Minichiello L (2009) TrkB signalling pathways in LTP and learning. Nat Rev Neurosci 10(12):850–860. doi:10.1038/nrn2738
50. Tao X, Finkbeiner S, Arnold DB, Shaywitz AJ, Greenberg ME (1998) Ca2+ influx regulates BDNF transcription by a CREB family transcription factor-dependent mechanism. Neuron 20(4):709–726
51. Tao X, West AE, Chen WG, Corfas G, Greenberg ME (2002) A calcium-responsive transcription factor, CaRF, that regulates neuronal activity-dependent expression of BDNF. Neuron 33(3):383–395
52. Zafra F, Hengerer B, Leibrock J, Thoenen H, Lindholm D (1990) Activity dependent regulation of BDNF and NGF mRNAs in the rat hippocampus is mediated by non-NMDA glutamate receptors. EMBO J 9(11):3545–3550
53. Patterson SL, Grover LM, Schwartzkroin PA, Bothwell M (1992) Neurotrophin expression in rat hippocampal slices: a stimulus paradigm inducing LTP in CA1 evokes increases in BDNF and NT-3 mRNAs. Neuron 9(6):1081–1088
54. Chen ZY, Patel PD, Sant G, Meng CX, Teng KK, Hempstead BL, Lee FS (2004) Variant brain-derived neurotrophic factor (BDNF) (Met66) alters the intracellular trafficking and activity-dependent secretion of wild-type BDNF in neurosecretory cells and cortical

neurons. J Neurosci 24(18):4401–4411. doi:10.1523/jneurosci.0348-04.2004

55. Teng HK, Teng KK, Lee R, Wright S, Tevar S, Almeida RD, Kermani P, Torkin R et al (2005) ProBDNF induces neuronal apoptosis via activation of a receptor complex of p75NTR and sortilin. J Neurosci 25(22):5455–5463. doi:10.1523/jneurosci.5123-04.2005
56. Bamji SX, Majdan M, Pozniak CD, Belliveau DJ, Aloyz R, Kohn J, Causing CG, Miller FD (1998) The p75 neurotrophin receptor mediates neuronal apoptosis and is essential for naturally occurring sympathetic neuron death. J Cell Biol 140(4):911–923
57. Boyd JG, Gordon T (2002) A dose-dependent facilitation and inhibition of peripheral nerve regeneration by brain-derived neurotrophic factor. Eur J Neurosci 15(4):613–626
58. Leal G, Afonso PM, Salazar IL, Duarte CB (2015) Regulation of hippocampal synaptic plasticity by BDNF. Brain Res 1621:82–101. doi:10.1016/j.brainres.2014.10.019