

The Effects of Amiloride on Seizure Activity, Cognitive Deficits and Seizure-Induced Neurogenesis in a Novel Rat Model of Febrile Seizures

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Abstract Accumulating data suggest that sodium–hydrogen exchangers (NHEs) play a key role in modulating seizure activity by regulating neuronal pH in the brain. Amiloride, an inhibitor of NHEs, has been demonstrated to be effective in many seizure models, although its efficacy for prolonged febrile seizures (FS) remains unclear. In this study, we investigated whether amiloride could produce neuroprotective effects in a prolonged FS model in which FS were induced in rat pups at postnatal day 10 using a heated air approach. Amiloride was administered by intraperitoneal injection at three different doses (0.65, 1.3 and 2.6 mg/kg). Pretreatment with amiloride significantly delayed the onset of the first episode of limbic seizures, whereas posttreatment with amiloride decreased escape latency in the Morris water maze test compared to post-FS treatment with saline. Amiloride also inhibited seizure-induced aberrant neurogenesis. In conclusion, this study demonstrated the antiseizure activity of amiloride. In particular, posttreatment with amiloride resulted in cognitive improvement; this finding provides crucial evidence of the

neuroprotective function of amiloride and of the therapeutic potential of amiloride in FS.

Keywords Sodium–hydrogen exchangers · Amiloride · Febrile seizures · Neurogenesis

Introduction

Febrile seizures (FS) are the most common type of convulsive events in infants and young children [1, 2], with an incidence of between 2 and 4 % [3–5]. One-third of infants and young children with FS, especially prolonged seizures, experience recurrent FS. Furthermore, approximately 2–8 % of the infants and young children who experience recurrent FS go on to develop temporal lobe epilepsy, which has been associated with memory deficits [6–8]. Several studies have demonstrated that the administration of phenobarbital or valproic acid can prevent FS [9, 10], but the adverse effects of these drugs outweigh the potential benefits [10]. Historically, the use of antiepileptic drugs (AEDs) to treat FS has not proven to be effective [11, 12]. Thus, there is a need to discover safer and more efficacious drugs to prevent or treat FS.

Traditional strategies for AED development have largely focused on augmenting GABAergic transmission, inhibiting excitatory amino acid transmission, or inhibiting ion channel (Na^+ / Ca^+) activity [13]. However, a growing body of evidence points to the involvement of sodium–hydrogen exchangers (NHEs) in the modulation of seizure activity in neuronal cells [14]. NHEs are proteins that are expressed in the plasma membrane of nearly all mammalian cells. By exchanging intracellular H^+ for extracellular Na^+ , the antiporter plays an essential role in a variety of cell functions, including pH regulation, volume

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homeostasis and cell growth [15]. Various investigators have reported the efficacy of NHE activation in enhancing glutamate release by increase in intracellular Na^+ and reversing the $\text{Na}^+/\text{Ca}^{2+}$ exchange function. Furthermore, the inhibition of NHEs contributes to intracellular acidosis, which has been reported to suppress epileptiform activity in rat temporal slices [16].

Amiloride, an NHE inhibitor, has been shown to be effective in suppressing pilocarpine- and electroshock-induced acute seizures [17, 18]. Amiloride has also been reported to modulate seizure generation and propagation in a pentylentetrazole-induced seizure model, which resulted in improvements in cognitive function [19]. However, the effects of amiloride on FS have not been specifically addressed. Thus, the aims of the present study were to investigate the effect of amiloride in a prolonged FS rodent model and to examine the effects of amiloride on memory in FS rats. Because seizure-induced neurogenesis might contribute to learning and memory dysfunction [20], whether amiloride treatment affects seizure-induced neurogenesis was also studied.

Materials and Methods

Animals

Sprague–Dawley rats were bred and maintained in a quiet facility under a controlled temperature (24–25 °C), 50–60 % humidity and a 12-h light schedule. All animals were provided free access to food and water [21]. Hyperthermic seizures were induced on postnatal day 10, and experimental rats were kept with littermate controls in home cages. The rats were weaned on postnatal day 21 and housed 2–3 animals per cage. All experimental procedures were approved by the Fourth Military Medical University Animal Ethics Committee.

Drugs

All drug treatments in this study were conducted with amiloride (Sigma) or a saline control treatment.

Generation of Experimental Prolonged FS

FS were induced on postnatal day 10 using a method adapted from Baram et al. [22]. Pups were placed at the bottom of a large box through which warm dry air (41–48 °C) was circulated using a standard hairdryer that was fitted at the top of the container. The behavioral seizures that are induced by hyperthermia are characterized by sudden movement arrest, body flexion, and the biting of an extremity; these behaviors are occasionally followed by

clonus. Seizures were maintained for a total of 30 min. The pups were then placed on a cool surface and monitored for 20 min before being returned to their home cages.

Arterial Blood pH Analysis

The rats were sacrificed at 0, 5, 10, 15 and 20 min after the induction of FS. A heparin-coated syringe was used to collect whole-blood samples from the left ventricles of rats from all five groups at each time point for arterial blood gas analysis. A blood gas analyzer (ABL800 FLEX, Radiometer Medical A/S, Copenhagen, Denmark) was used to measure pH.

BrdU Labeling

5-Bromodeoxyuridine (BrdU) was used to label newborn cells. All rats received intraperitoneal (*i.p.*) injections of BrdU (50 mg/kg per injection, dissolved in PBS, Sigma-Aldrich) over a 2-h interval on the first day after the generation of experimental prolonged FS in Experiment 4.

Western Blot Analysis

Tissue samples were extracted from the hippocampus and homogenized with 2 % sodium dodecyl sulfate (SDS), 100 mM dithiothreitol, and 10 % glycerol. The tissue samples were stored at 4 °C until needed. The samples were analyzed by electrophoresis in 10 % polyacrylamide SDS gels. After electrophoresis, the proteins were transferred to a polyvinylidene fluoride (PVDF) membrane and blocked by immersion for 2 h in Tris-buffered saline (TBS) containing 5 % dry milk. The membranes were incubated at 4 °C overnight with a goat anti-NHE1 antibody (1:500, Santa Cruz) and a goat anti-NHE4 antibody (1:500, Santa Cruz). After the incubation, the membranes were washed 3 times with TBST (TBS containing 0.2 % Tween-20) and were then incubated for 1–2 h with HRP-conjugated secondary antibodies (1:10,000, Millipore) at room temperature. The membranes were washed with TBST 3 times, and the reactions were visualized using a chemiluminescent reagent (ECL, Menlo Park, CA) and captured on film.

Tissue Preparation

Following behavioral testing, the rats were deeply anesthetized with chloral hydrate (100 mg/kg, *i.p.*) and perfused transcardially with 0.9 % saline followed by 4 % paraformaldehyde (pH 7.4) to fix the brain. Cryoprotection was achieved with 30 % sucrose in 0.01 M PBS. The brains were cut into 30- μm coronal floating sections through the entire dentate gyrus using a cryostat (Leica). The free-floating sections were processed for immunostaining as

described below. For BrdU immunohistochemistry, the sections were first incubated in 2 N HCl at 37 °C for 40 min and then neutralized with 0.1 M borate buffer (pH 8.5) for 10 min.

Immunohistochemistry

Floating sections were incubated with mouse anti-NeuN (1:300, Chemicon), mouse anti-DCX (1:500, Santa Cruz) and rat anti-BrdU antibody (1:300, Abcam) in PBS containing 1 % BSA and 0.3 % Triton-X100 at 4 °C overnight and then incubated with a biotinylated anti-mouse or anti-rat IgG secondary antibody (1:300, Vector) for 2 h at room temperature. NeuN, DCX and BrdU were visualized by incubating the sections with an avidin–biotin–peroxidase complex (1:300, Vector) for 2 h. Peroxidase activity was observed by incubating the sections in 0.1 % 3,3-diaminobenzidine (Sigma) and 0.05 % H₂O₂ in PBS for 5 min at room temperature. The sections were observed under a Leica DMIRB and photographed.

Morris Water Maze

The Morris water maze test was conducted in a large circular black pool (150 cm in diameter) containing water (24 ± 2 °C) that had been colored with a nontoxic black pool dye to contrast with the rat. A 15-cm-diameter black-colored round platform was placed 2 cm below the water surface. All of the rats were placed in the water maze room 1 h before the daily water trials to acclimate them to the environment. The rats were given a maximum of 90 s to find the hidden platform and were allowed to remain on the platform for 15 s. The rats were guided to the platform if they failed to find it within 90 s. The rats were tested with daily sessions of four trials per day for five consecutive days. For each trial, the swimming trajectory and the latency to locate the hidden platform were recorded. During an additional test on the sixth day, the test platform was removed and the number of crossings and the percentage of time spent in the target quadrant were measured.

Study Design and Drug Treatment

This study consisted of four experiments with different specific aims. The total number of rats used in this study was 153. A flow chart of the experimental procedures is shown in Fig. 1.

Experiment 1 NHE1/4 protein levels at days 1, 3 and 7 after FS were measured by western blot analysis in a control group and in a group of rats with hyperthermia-induced seizures. An n = 9 was used for both groups.

Experiment 2 The effects of pretreatment with amiloride on epileptogenesis were observed. In this experiment, each

rat received an *i.p.* injection of either amiloride (0.65, 1.3 or 2.6 mg/kg) or saline 1 h before the induction of FS. The doses were chosen based on a previous study [17]. Each of the four groups (n = 6 per group) were subjected to hyperthermia-induced seizures. The group treatments were as follows: FS + saline, FS + 0.65 mg/kg amiloride, FS + 1.3 mg/kg amiloride and FS + 2.6 mg/kg amiloride.

Experiment 3 The effects of amiloride pretreatment on arterial blood pH were evaluated at 0, 5, 10, 15 and 20 min after the induction of experimental FS. In this experiment, each rat received an *i.p.* injection of amiloride (0.65, 1.3 or 2.6 mg/kg) or saline 1 h before the induction of FS. Additionally, a group of age-matched controls without FS was included. An n = 3 was used for each of the five groups at each of the time points. The group treatments at each time point were as follows: Control, FS + saline, FS + 0.65 mg/kg amiloride, FS + 1.3 mg/kg amiloride and FS + 2.6 mg/kg amiloride.

Experiment 4 The effects of amiloride on cognitive performance were investigated. Animals were randomly divided into 5 groups of 6 rats each. Each rat received an *i.p.* injection of amiloride (0.65, 1.3 or 2.6 mg/kg) or saline 1 h after the termination of seizures. The drug treatments were administered via once-daily *i.p.* injections for 28 days. The grouping method was the same as described above.

Quantification and Statistical Analysis

NeuN⁺, BrdU⁺ and DCX⁺ cells were counted in every sixth section in a mediolateral direction under a light microscope at 200× magnification. All results are presented as the mean ± SEM. Comparisons were performed using analysis of variance (ANOVA), and multiple comparisons were performed using post hoc least significant difference comparisons. *P* values < 0.05 were considered significant.

Results

Dynamic Changes in NHE1/4 Expression in the Hippocampus After FS

The distribution of NHE1/4 in the CNS has been described as widespread, and both isoforms play important roles in epilepsy. Accordingly, we investigated changes in NHE1/4 protein expression in the hippocampus after FS induction using western blot analysis. As shown in Fig. 2a, NHE1/4 protein levels gradually increased after the onset of seizures. There was a large increase in the protein levels of NHE1/4 that persisted from day 1 to day 7 following seizure onset. Statistical analysis revealed that the expression

Fig. 1 Schematic illustration of the experimental protocol used in this study. **a** Schematic illustration of Experiment 2; **b** Schematic illustration of Experiment 4

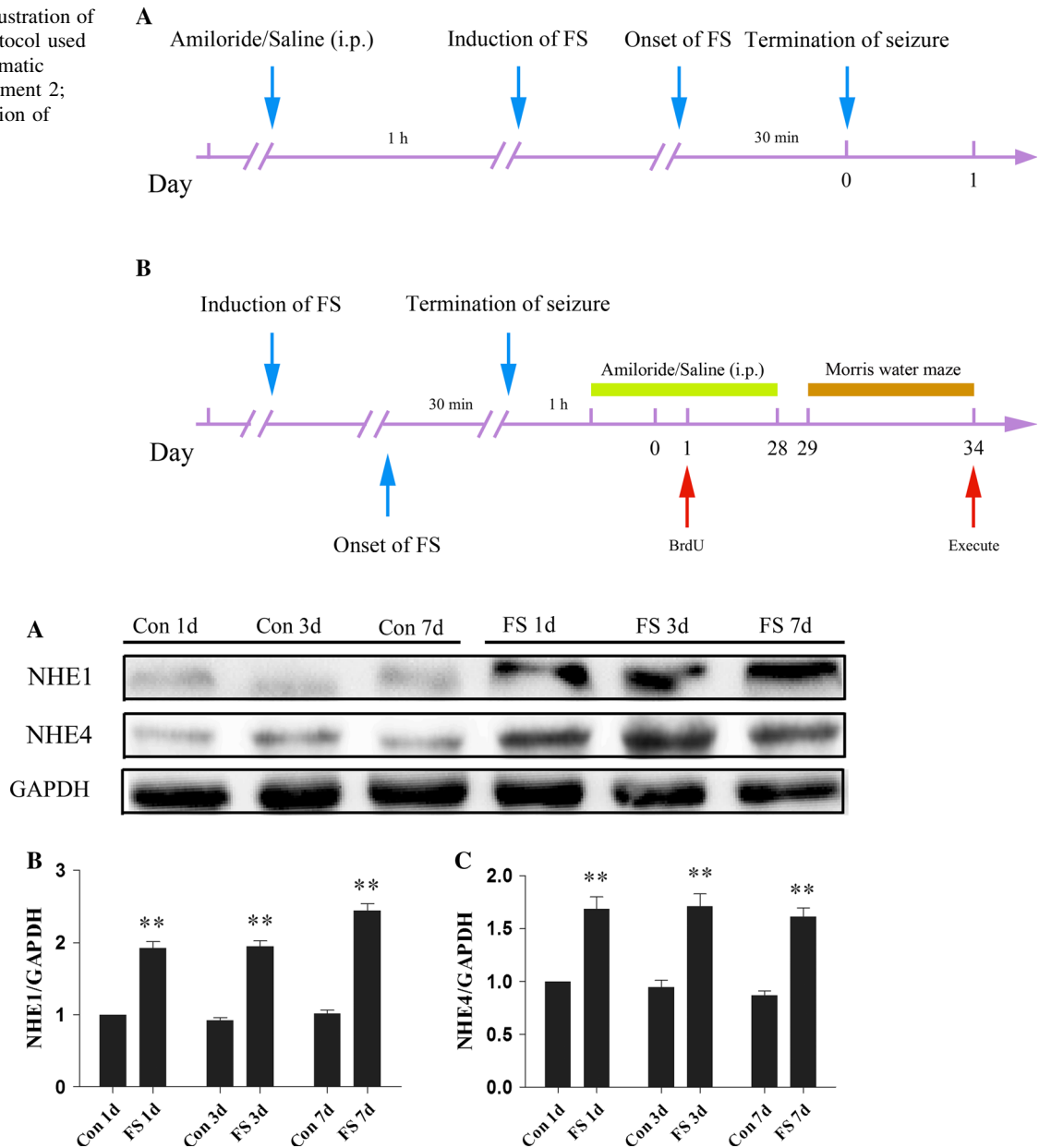


Fig. 2 Dynamic expression of sodium–hydrogen exchanger1/4 (NHE1/4) in the development of a febrile seizure (FS) model. **a** Western blot analysis of NHE1/4 expression levels in the hippocampus of rats at different time points after FS induction; **a**, **b** NHE1 protein levels were increased at 1, 3 and 7 days after FS

compared with the control; **a**, **c** NHE4 protein levels were increased at 1, 3 and 7 days after FS compared with the control. Quantitative analysis of protein expression was performed using Image-Pro Plus 6.0. The data are expressed as the mean \pm SEM, ** $P < 0.01$ compared with the control

of NHE1/4 protein at each post-seizure time point was significantly higher than the protein levels in control animals ($P < 0.01$) (Fig. 2b, c).

Effects of Amiloride Pretreatment on FS

We next examined the effects of amiloride using an animal model of FS. All rats that were exposed to hyperthermic conditions experienced generalized convulsions (GCs);

none of the GCs resulted in mortality. Body temperatures among the groups were similar before hyperthermia. Notably, weighing the rats before and after the procedure indicated little evidence of dehydration ($<3\%$ change in body weight). Amiloride (0.65, 1.3 and 2.6 mg/kg) treatment significantly increased the latency to GCs compared to FS + saline treatment ($P < 0.01$) (Fig. 3). These results indicate that treatment with amiloride before FS effectively suppressed seizures in our model of FS.

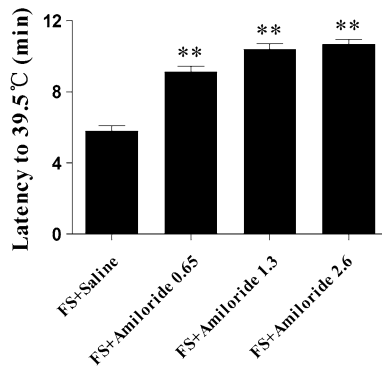


Fig. 3 Effects of amiloride pretreatment on hyperthermia-induced seizures. Amiloride (0.65, 1.3 and 2.6 mg/kg) significantly increased the latency of FS. The data are expressed as the mean ± SEM, ***P* < 0.01 compared with the FS + saline group

Effects of Amiloride Pretreatment on Arterial Blood pH at Different Time Points After Seizure Induction

A prior study reported that experimental FS was precipitated by hyperthermia-induced respiratory alkalosis [23]. Thus, we next tested whether amiloride affected arterial blood pH in our animal model of FS. Arterial blood pH was measured at 0, 5, 10, 15 and 20 min after the induction of experimental FS. Consistent with the previous study, a significant increase in brain pH was observed after the induction of FS in model animals compared to controls (*P* < 0.01, Fig. 4). Additionally, arterial blood pH appeared to gradually increase after the induction of seizures. However, compared to FS + saline treatment, pretreatment with amiloride (0.65, 1.3 or 2.6 mg/kg) did not affect arterial blood pH.

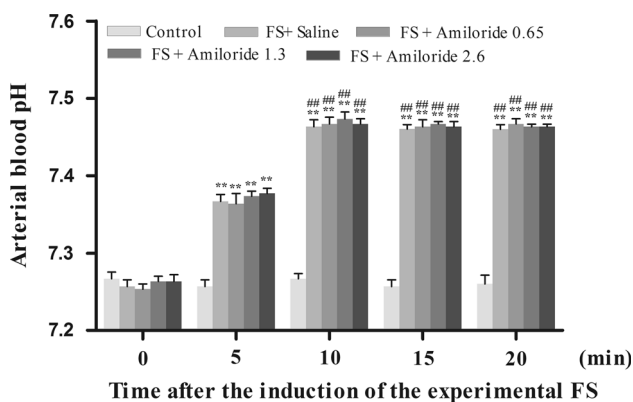


Fig. 4 Effects of amiloride pretreatment on arterial blood pH. Arterial blood pH was measured at 0, 5, 10, 15, and 20 min after the induction of FS. The data are expressed as the mean ± SEM, ***P* < 0.01 compared with the 0 min group after the induction of FS. ###*P* < 0.01 compared with the 5 min group after the induction of FS

Effects of Amiloride Posttreatment on Cognitive Function

In the Morris water maze, rats that experienced hyperthermia-induced seizures exhibited significant learning and memory impairments compared to controls without FS. This conclusion was based on an increased time to locate the platform (escape latency) over the whole training period compared to that of control animals (Fig. 5a). Furthermore, the amiloride (0.65, 1.3 and 2.6 mg/kg)-treated FS animals learned significantly better than FS animals that were injected with only vehicle (*P* < 0.01, Fig. 5a). During the probe trial test (escape platform removed), the number of target area crossings was recorded, and the amiloride (0.65, 1.3 and 2.6 mg/kg)-treated FS rats performed significantly better than the vehicle-treated FS rats (*P* < 0.01, Fig. 5b).

Effects of Amiloride on Hippocampal Damage

Because the hippocampus is critically involved in learning and memory, we examined hippocampal damage by counting the number of neurons in the hippocampal region 5 weeks after FS (Fig. 6). Consistent with previous results [24], we did not find a decrease in the number of neurons in rats that experienced hyperthermia-induced seizures. Further, amiloride posttreatment with FS animals also did not reduce the number of neurons. Thus, it is likely that the cognitive impairments that were observed in the FS animals occurred independent of a decrease in the number of neurons in the hippocampus.

Effects of Amiloride on Seizure-Induced Neurogenesis

Considering the possible effects of seizure-associated aberrant neurogenesis on learning and memory dysfunction, we next examined whether amiloride treatment affected seizure-induced neurogenesis. We employed BrdU labeling of dividing cells and perfused rats 5 weeks after the induction of seizures. FS induced a strong increase in the number of BrdU⁺ cells in the dentate gyrus compared with control treatment (*P* < 0.05), whereas amiloride (0.65, 1.3 and 2.6 mg/kg)-treated FS animals showed a significantly lower number of BrdU⁺ cells compared to vehicle-treated FS rats (*P* < 0.05) (Fig. 7). In addition, the expression of doublecortin (DCX), an immature neuronal marker, in amiloride (0.65, 1.3 and 2.6 mg/kg)-treated FS rats was also inhibited compared to that in vehicle-treated FS rats at 5 weeks after the induction of FS (*P* < 0.05, Fig. 8). These results suggest that amiloride inhibited seizure-induced neurogenesis.

Fig. 5 Effects of amiloride on memory. **a** The latency of rats to find the hidden platform; **b** The number of target area crossings. The data are expressed as the mean \pm SEM, $*P < 0.05$ compared with the FS + saline group

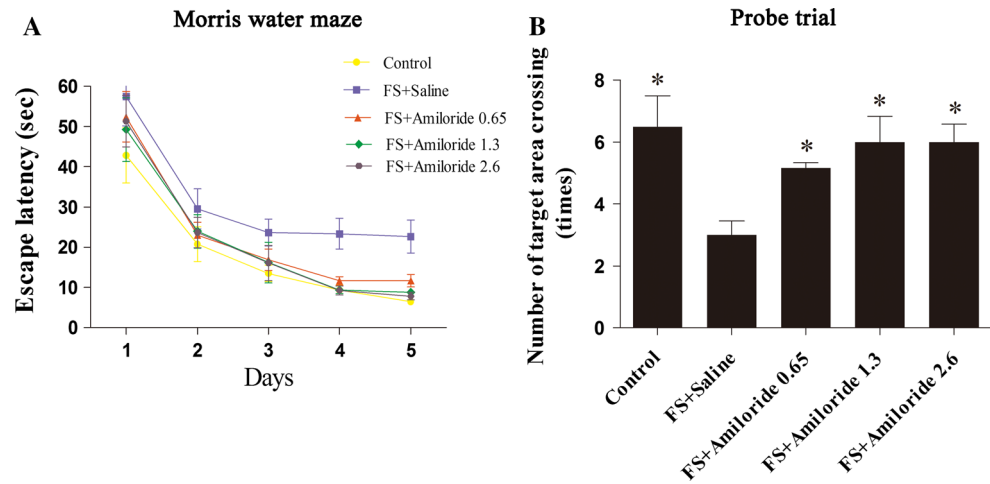
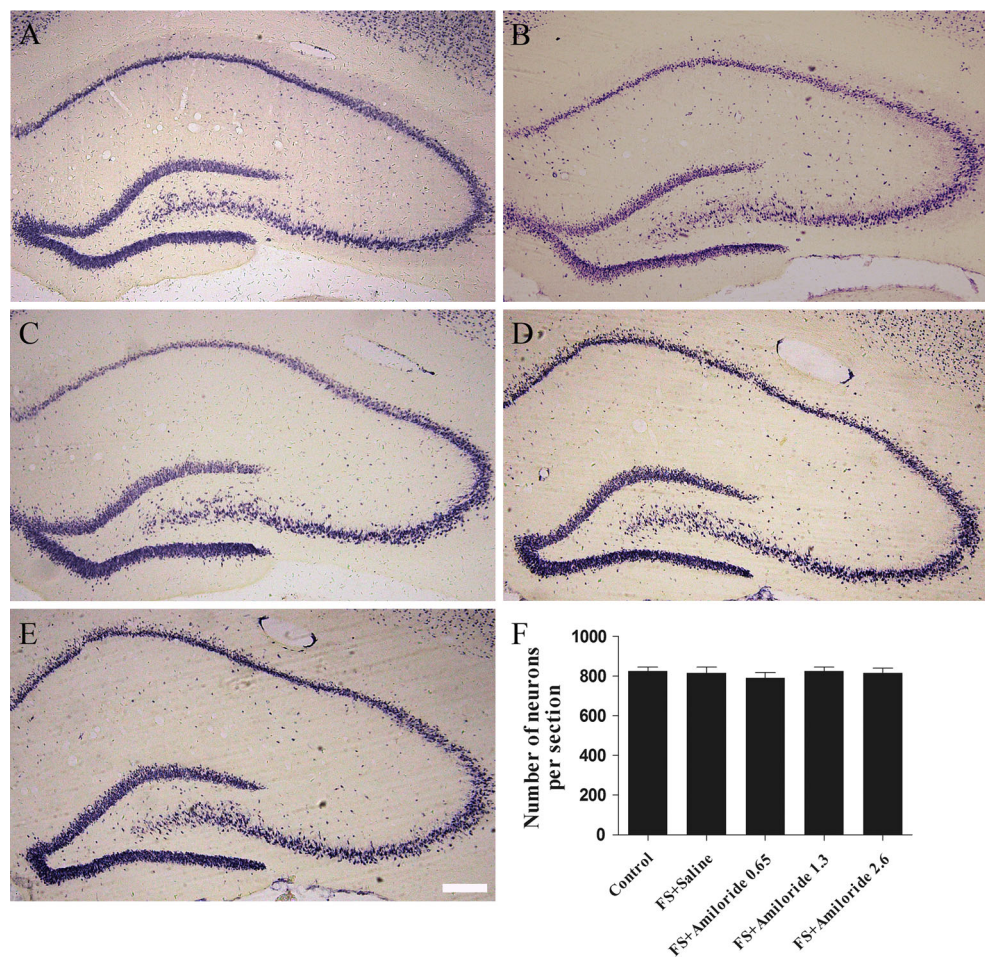


Fig. 6 Representative coronal sections showing the severity of neuronal damage in the hippocampal region of rats with FS. **a** Control; **b** saline-treated FS rats; **c** amiloride (0.65 mg/kg)-treated FS rats; **d** amiloride (1.3 mg/kg)-treated FS rats; **e** amiloride (2.6 mg/kg)-treated FS rats; **f** quantitative analysis of amiloride (0.65, 1.3 and 2.6 mg/kg) treatment on neuronal loss in the hippocampus 5 weeks after FS. The data are expressed as the mean \pm SEM; scale bars 200 μ m

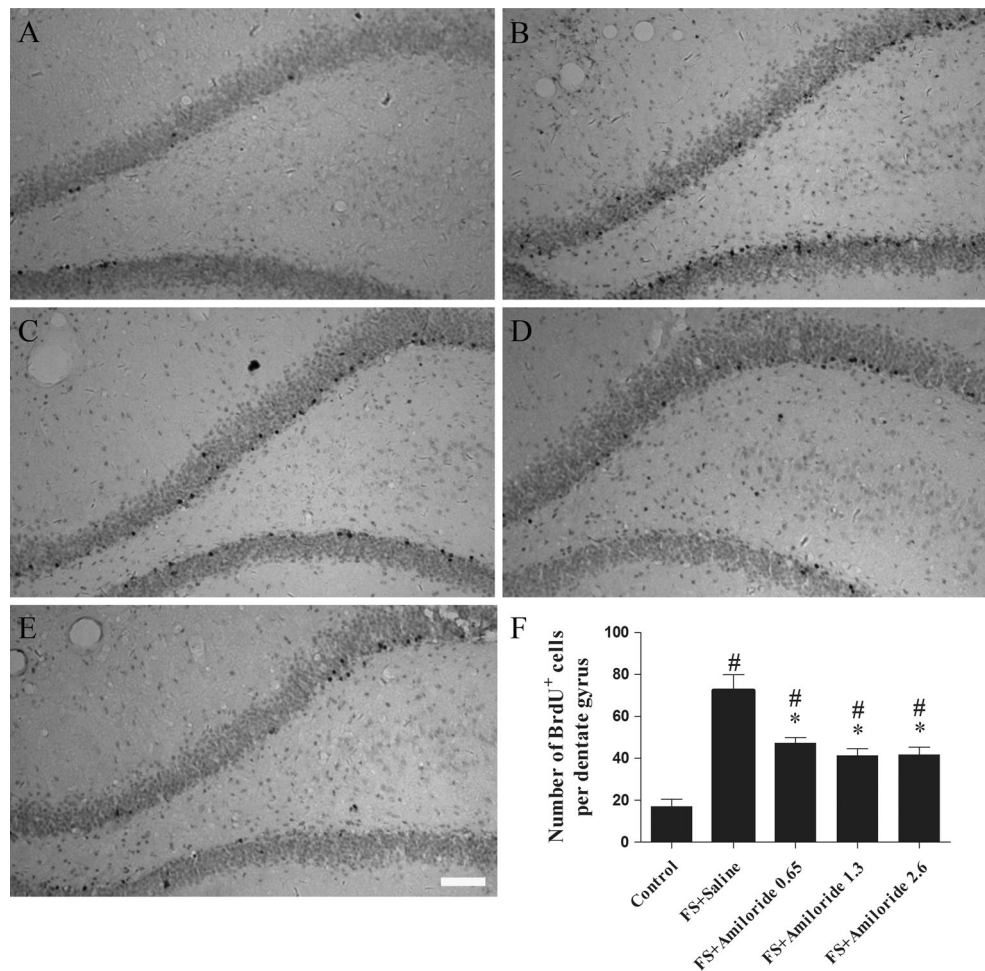


Discussion

FS commonly occur in children under the age of 5 years and can be simple or complex. Complex FS early in life are accompanied by high risk for the later development of epilepsy and cognitive deficits [25]. However, effective

treatments for FS and its associated cognitive impairments are still limited. A previous study showed the protective effect of amiloride against pentylentetrazole-induced seizures. Considering these data, we evaluated the effect of amiloride in an animal model of FS. First, we found that the protein expression of NHE1/4 was significantly

Fig. 7 Amiloride decreased the number of BrdU⁺ cells in the granular cell layer of the dentate gyrus 5 weeks after FS induction. **a** Control; **b** Saline-treated FS rats; **c** Amiloride (0.65 mg/kg)-treated FS rats; **d** Amiloride (1.3 mg/kg)-treated FS rats; **e** Amiloride (2.6 mg/kg)-treated FS rats; **f** Quantitative analysis of amiloride (0.65, 1.3 and 2.6 mg/kg) treatment on neuronal loss in the granular cell layer of the dentate gyrus 5 weeks after FS. Scale bars 50 μ m. The data are expressed as the mean \pm SEM, #*P* < 0.05 compared with the control group. **P* < 0.05 compared with the FS + saline group



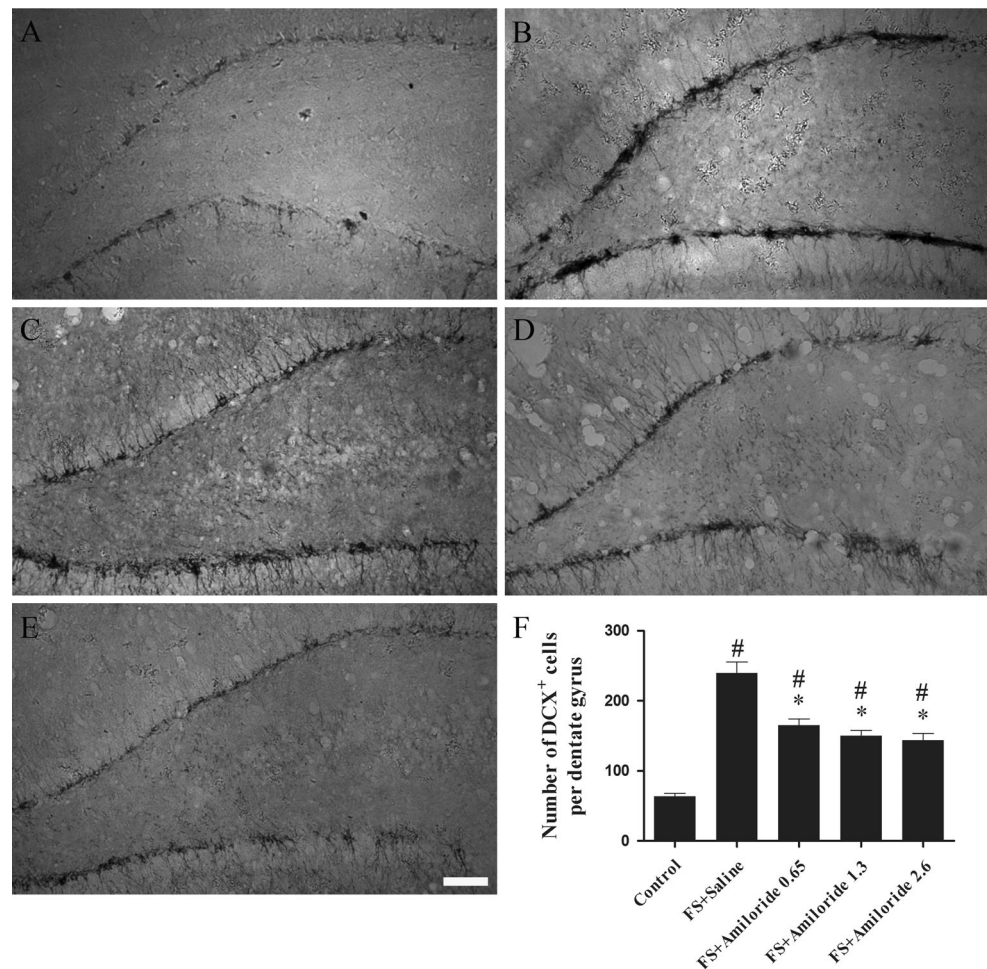
elevated in the hippocampus of immature rats following hyperthermia-induced seizures. We further demonstrated that amiloride delayed the onset of FS. Moreover, amiloride could improve cognitive performance in FS animals and decreased seizure-induced neurogenesis after FS. Altogether, these results provide novel evidence for the neuroprotective potential of amiloride in rodent models of FS.

The mechanisms responsible for the triggering of hyperthermia-induced seizures remain largely unknown. Prior research has indicated a relationship between hyperthermia-induced seizures and brain pH. The authors of this previous study reported that an increase in the body temperature of rat pups led to a pronounced increase in the respiration rate, which was followed by an increase in brain pH that triggered ictal activity. Consistent with that previous study, we found that the onset of seizure activity coincided with an increase in pH. However, FS did not occur immediately following brain alkalosis in amiloride-treated FS animals. Moreover, in our model, amiloride did not decrease the rate of the increase in pH. Furthermore, detailed understanding of the mechanisms that underlie the

process by which hyperthermia-induced respiratory alkalosis mediates ictogenesis is lacking. Thus, it is likely that amiloride suppresses the occurrence of hyperthermia-induced seizures by a method other than the modulation of hyperthermia-induced respiratory alkalosis.

As an inhibitor of NHEs, amiloride has been shown to confer neuroprotective effects in various neuropathological conditions, including brain injury and ischemia [26]. Amiloride also exerted anticonvulsant effects in pilocarpine-induced seizures [17]. In this study, we found that amiloride delayed the onset of FS. Although the mechanism underlying this antiepileptic effect is not yet fully understood, intracellular free protons play an important role in the modulation of neuronal electrical activity [27], and NHEs mediate the electroneutral exchange of intracellular H⁺ with extracellular Na⁺ to regulate pH [28]. To date, nine mammalian NHE isoforms (NHE1-9) have been identified [16]. Amiloride is known to block subunits 1 and 4, which are highly abundant in the hippocampus, a region that is important in epileptic seizures. Bonnet and colleagues reported that inhibition of NHEs resulted in intracellular acidification [14], which was suggested to

Fig. 8 Amiloride decreased the number of doublecortin-positive (DCX⁺) cells in the granular cell layer of the dentate gyrus 5 weeks after FS induction. **a** Control; **b** Saline-treated FS rats; **c** Amiloride (0.65 mg/kg)-treated FS rats; **d** Amiloride (1.3 mg/kg)-treated FS rats; **e** Amiloride (2.6 mg/kg)-treated FS rats; **f** Quantitative analysis of amiloride (0.65, 1.3 and 2.6 mg/kg)-treatment on neuronal loss in the granular cell layer of the dentate gyrus 5 weeks after FS. Scale bars 50 μ m. The data are expressed as the mean \pm SEM, #*P* < 0.05 compared with the control group. **P* < 0.05 compared with the FS + saline group



attenuate NMDA receptor activation and glutamate neurotoxicity. Further, intracellular acidosis has been reported to terminate epileptiform discharges [29]. Using a model of FS, we revealed an increase in the protein expression of NHE1/4 in the hippocampus, indicating the involvement of NHEs in FS epileptogenesis. Thus, the antiseizure activity of amiloride may be attributed to inhibition of NHEs. Moreover, amiloride is known to inhibit acid-sensing ion channels (ASICs), and blockade of ASICs significantly inhibited the increase in neuronal firing and decreased the amplitude and frequency of seizure-like bursting activity; these effects may be partially responsible for the anticonvulsant actions of amiloride [30]. However, determining the exact mechanism by which amiloride delays the onset of FS requires further investigation.

Recently, a growing body of evidence has demonstrated seizure-induced impairments in hippocampus-dependent learning and memory in animal models and patients with complex FS [31–33]. Patients with FS require additional treatment in addition to AED therapy to correct the accompanying neurological deficits. However, no study has identified a drug to treat this neurological problem, even in animal

models. The ideal solution would be to use an AED that provides not only seizure protection but also a positive effect on cognitive impairments. As part of the drug development process, a putative AED should be routinely screened for its neurological effects outside of its antiepileptic effects. The present study demonstrated an improvement in memory that was associated with amiloride treatment, as demonstrated by a decrease in the latency to find a hidden platform in the Morris water maze test. The mechanisms behind this cognitive improvement are not clearly understood, although it has been speculated that seizure-associated aberrant neurogenesis might contribute to learning and memory dysfunction [34]. Lamotrigine and valproate, two of the most commonly used AEDs, have been shown to inhibit seizure-induced neurogenesis in the hippocampus [34, 35] and to protect animals that experienced status epilepticus from seizure-associated cognitive impairments. Here, we report that amiloride significantly decreased the number of aberrant cells in the dentate gyrus after FS, which may be partially related to its cognitive improvements.

Interestingly, our study found increased seizure-induced neurogenesis 5 weeks after experimental FS. A previous

study that examined the effects of hyperthermia-induced neurogenesis in the immature hippocampus at 3, 7 and 28 days post-seizures found no evidence of increased neurogenesis after hyperthermia-induced seizures [36]. However, in that study, the total seizure time was less than 20 min compared to the 30 min used in our study. Indeed, seizure duration is a key factor for the pattern of hippocampal neurogenesis. A previous study reported that more aberrant cells were detected post-seizures following long seizure durations compared to short ones [37]. Moreover, Shi et al. [38] found that neonatal seizures exhibited opposite modulatory effects on neurogenesis over different time windows; early down-regulation was followed by up-regulation. Similarly, increased neurogenesis following experimental FS was observed in our study. The possible mechanisms of the counteractive effect of amiloride on seizure-induced neurogenesis in the hippocampus were not investigated in the current study. However, Akt signaling, which is essential for regulating cell proliferation [39], has been studied in this context, and recent data suggest that amiloride decreases the proliferation of GBM8401 glioblastoma cells through Akt phosphorylation [40]. However, additional experiments are needed to determine the detailed mechanism responsible for the decrease in seizure-induced neurogenesis following amiloride treatment.

Conclusion

In conclusion, our data showed that amiloride pretreatment had anticonvulsant potential in a hyperthermic model of FS, whereas amiloride posttreatment attenuated memory impairment and inhibited aberrant seizure-induced neurogenesis in rats. Thus, amiloride may be a promising drug for FS therapy, although more intensive experimental and clinical investigations are warranted to explore the full potential of amiloride in epilepsy management.

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Compliance with Ethical Standards

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

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