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ARTICLE

Inhibition of miR-181a-5p reduces astrocyte and microglia activation and oxidative stress by activating SIRT1 in immature rats with epilepsy

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Abstract

MicroRNAs regulate gene expression at the posttranscriptional level, and this process has been shown to be implicated in the pathological processes of temporal lobe epilepsy. At present, studies about the impact of microRNA-181a (miR-181a) on epilepsy have focused on hippocampal neurons, and the effect of miR-181a on other cells in the hippocampus remains poorly understood. Herein, we explored the role of miR-181a-5p in a lithium-pilocarpine model of epilepticus in immature rats. We found that the hippocampal expression level of miR-181a-5p was increased. Inhibition of miR-181a-5p protected the hippocampus against epilepsy, including hippocampal insults, neuronal apoptosis, astrocyte and microglia activation, neuroinflammation, oxidative stress, mitochondrial function, and cognitive dysfunction. Moreover, miR-181a-5p inhibition exerted a seizure-suppressing effect via SIRT1 upregulation. Overall, our findings reveal the potential role of the miR-181a-5p/SIRT1 pathway in the development of temporal lobe epilepsy, and this pathway may represent a novel target for ameliorating epilepsy and its sequelae.

Introduction

Epilepsy is a common chronic neurological disease that affects 50 million people worldwide (https://www.who.int/mental_health/neurology/epilepsy/en/, accessed Aug 1st, 2019). Epilepsy is characterized by recurring seizures that are caused by neuronal hyperexcitability [1]. The temporal lobe, which is an important site in the development of epilepsy, is the most frequent location of focal onset seizures, making temporal lobe epilepsy (TLE) the most common form of epilepsy [2, 3]. Epilepsy in infants and children is distinct from that in adults due to its clear clinical features, more extensive etiology, and complex developmental process [2, 4]. According to a previous review, ~33–82 out of 100,000 children are diagnosed with epilepsy per year [2]. Although numerous basic and clinical studies promote the development of antiepileptic drugs, more than 30% of patients with epilepsy suffer from drug-resistant epilepsy (DRE) [5]. Moreover, DRE is more likely to lead to severe cognitive delays and poor psychosocial outcomes in children [6]. The mortality of pediatric epilepsy is much higher than that of adult epilepsy [7]. Thus, efforts to further study the pathological mechanism of TLE in children, which would permit the design of selective and interventional therapeutic targets, are urgently needed.

Epilepsy has long been known to be associated with inflammation in previous brain lesions [8]. Evidence from basic and clinical studies suggests that uncontrolled inflammatory responses in the brain play a critical role in the developmental processes of epilepsy [9–11]. During the course of TLE, injury to the temporal lobe causes microglia and astrocyte activation and inflammatory cytokine accumulation, which indicate inflammatory responses in the brain [12, 13]. Sustained inflammation leads to neuronal damage and to recurrent seizures, which exacerbate TLE by inducing synaptic reconstruction and mossy fiber sprouting. In children with intractable epilepsy, a large number of damaged neurons and marked activation of microglia and astrocytes have been observed in the brain [14]. The neuroprotective

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effect of anti-inflammatory drugs in the treatment of epilepsy has been widely studied in both preclinical and clinical epilepsy studies [15, 16]. Anti-inflammatory therapy may be a new strategy to minimize neuronal damage and reduce seizures in childhood epilepsy [17].

It has been suggested that posttranscriptional regulation by microRNAs participates in the pathological processes of TLE, and new therapeutic strategies that target microRNAs have gained substantial interest in epilepsy treatment [18]. miR-181a was previously found to be associated with acute myeloid leukemia [19] and was later discovered to be expressed in the brain and involved in memory formation [20]. Previous preclinical studies have demonstrated the neuroprotective effects of a miR-181a antagomir in cerebral ischemia [21] and traumatic brain injury [22]. Regarding epilepsy, miR-181a has been found to be upregulated in the brains of rats with epilepsy, and a miR-181a antagomir improved learning and memory in rats with pentylenetetrazol-induced epilepsy [23] and attenuated neuronal damage in rats with pilocarpine-induced TLE [24]. Although studies have suggested a functional role of miR-181a in hippocampal neurons during epilepsy development, to date, little attention has been devoted to the effect of miR-181a on other cells in the hippocampus, especially its effect on the activation of astrocytes and microglia in epilepsy.

The involvement of the miR-181a/SIRT1 pathway in various pathological processes has been corroborated by several studies. miR-181a targets the 3′UTR of SIRT1 mRNA and downregulates SIRT1 protein expression at the translational level. Zhou et al. stated that miR-181a downregulates SIRT1 and attenuates hepatic insulin sensitivity. Inhibition of miR-181a increases the protein levels and activity of SIRT1, which may be a novel therapeutic strategy for the treatment of insulin resistance [25]. It has been demonstrated that silencing of the transcription factor ΔNp63 upregulates the expression of miR-181a, resulting in a decrease in SIRT1 and accelerating the senescence of T cells. This study showed that hepatitis C virus-induced T cell senescence is regulated by the ΔNp63-miR-181a-Sirt1 pathway [26]. Moreover, Tan et al. reported that lncRNA ANRIL promotes cell viability and inhibits senescence in vascular smooth muscle cells by downregulating miR-181a and further upregulating SIRT1 [27]. miR-181a mediates oxidative stress-induced proapoptotic factor FoxO1 acetylation and granulosa cell apoptosis by downregulating SIRT1 during follicular development [28]. A study conducted by Huang et al. showed that miR-181a-5p is involved in sepsis by targeting SIRT1 to modulate the inflammatory response and NF-κB pathway activation [29].

SIRT1 (Sirtuin-1), which is a protein deacetylase, is directly implicated in central nervous system (CNS) disorders. SIRT1 deficiency in microglia is related to cognitive decline during neurodegeneration [30]. Wang's study demonstrated that SIRT1 was downregulated in rats with pilocarpine-induced TLE [31]. Furthermore, a SIRT1 activator could inhibit the LPS-induced activation of microglia and astrocytes in the hippocampus and attenuate the cognitive decline of mice, and this effect could be suppressed by a SIRT1 inhibitor [32].

In this study, we hypothesized that the inhibition of miR-181a may protect hippocampal cells against epilepsy by modulating SIRT1. In the present study, we supported this hypothesis using a pilocarpine-induced model of TLE in immature rats. The damage to neurons and the activation of glia, as well as the related molecular mechanisms, were examined.

Materials and methods

Animals and grouping

Male Sprague-Dawley rats (21 days old) were obtained from Changsheng Biotechnology (Liaoning, China). The rats were housed in a standard experimental animal room with controlled temperature (22 ± 1 °C), humidity (50–60%) and a 12 h light/dark cycle, and the rats were allowed free access to water and food. The rats were randomly divided into six groups, with 42 rats in each group: (1) control; (2) Epilepsy; (3) Epilepsy + NC; (4) Epilepsy + anti-181a-5p; (5) Epilepsy + anti-181a-5p + shSIRT1; and (6) Epilepsy + anti-181a-5p+shCon. After seizure assessment, the rats from each group were then randomly divided into two sets. The first set ($n = 36$ per group) was used for histopathological examination and the evaluation of inflammatory responses and oxidative stress 72 h after status epilepticus (SE) induction. The second set ($n = 6$ per group) was used to conduct behavioral studies 42 days after SE induction. All the animal experimental procedures were performed in accordance with the Guidelines for Laboratory Animal Care and Use and approved by the Medical Research Ethics Committee of The First Affiliated Hospital of Zhengzhou University.

Lentivirus transfection

All the rats were anesthetized with pentobarbital (50 mg/kg, i.p.) and fixed on a stereotaxic atlas. The lateral ventricle was located (coordinates from the bregma: anterior-posterior = −0.9 mm, lateral = −1.0 mm, ventral = −4.8 mm), and a puncture needle was implanted into the lateral ventricle. For the rats in the Epilepsy + NC and Epilepsy + anti-181a-5p groups, lentivirus containing negative control or anti-miR-181a-5p (constructed by and purchased from Wanleibio, Shenyang, China) was injected via the needle (4 μL, 0.5 μL/min). The needle was maintained for 10 min

after the injection. For the rats in the Epilepsy + anti-181a-5p + shSIRT1 and Epilepsy + anti-181a-5p + shCon groups, lentivirus containing anti-miR-181a-5p was injected (2 μL, 0.5 μL/min), and then, lentivirus containing SIRT1-targeting shRNA or the control sequence was injected via the needle (2 μL, 0.5 μL/min). The rats in the control and epilepsy groups received the same operation but not the injection. The efficiency of infection was evaluated at day 3 and day 48 (after the behavioral test) after the infection.

Epilepsy induction

Three days after the lentivirus injection, the rats were injected with 125 mg/kg lithium chloride (i.p.) (Aladdin regents, L116330) followed by injection of pilocarpine (30 mg/kg i.p., Aladdin regents, P129614) to induce epilepsy. Scopolamine methyl bromide (1 mg/kg, Aladdin regents, S129958) was administered intraperitoneally 15 min prior pilocarpine injection to reduce the peripheral cholinergic effects. The rats in the control group received equal volumes of vehicle. The seizures were evaluated using the Racine scale [33], and the rats with stage IV or V seizures were used in this study. Oral administration of 10 mg/kg diazepam (Sigma) was performed to terminate the seizures 1.5 h after the onset of SE.

Morris water maze (MWM) test

Morris water maze tests were performed in a cylindrical tank (160 cm in diameter and 50 cm in height) with a black background. The tank contained water (24 cm in depth, 22 ± 2 °C) and was separated into four quadrants numbered in a clockwise manner. A hidden platform (10 cm in diameter) was placed 1 cm below the surface of the water. The rats were trained two times per day for 5 days. On the training days, the rats were placed in the tank facing the wall at four starting points. The time requited for the rats to find the platform was recorded as the escape latency. The rats that failed to find the flatform within 60 s were gently placed on the platform and allowed to remain for 15 s. The latencies of these rats were recorded as 60 s. The probe test was performed on day six. The platform was removed, and the rats were placed in the tank at any of the starting points used on the training days and allowed to swim for 60 s. The times the rat spent swimming across the platform site were recorded.

Pathological examinations

Brain samples were collected, fixed with 4% paraformaldehyde for 24 h at 4 °C and embedded in paraffin. Tissue sections (5 μm thick) from the paraffin blocks of the brain were sliced, dewaxed, and stained for the indicated analyses.

Nissl staining

After being dewaxed, the tissue sections were stained with 0.5% cresyl violet acetate for 10 min, rinsed with distilled water, and then differentiated with 0.25% acetic acid alcohol. The stained slides were dehydrated using alcohol and incubated with xylene. Subsequently, the slices were sealed by neutral balsam and visualized under an Olympus microscope (at ×200 magnification). The Nissl bodies in the hippocampus stained with bluish violet were counted.

Timm's staining

The rats in each group were anesthetized and perfused with GENMED sulfide solution (Reagent A) for 10 min. The brain tissues were placed in 20 mL Reagent A and incubated for 45 min at room temperature. After rinsing with distilled water, the tissues were incubated in 20 mL GENMED fixation solution A (Reagent B) and GENMED fixation solution B (Reagent C) for 16 h. The samples were embedded in paraffin, sliced, deparaffined, and rehydrated. The sections were stained with 200 μL of staining solution (a mixture of 1 mL of GENMED stain solution A (Reagent I) and 25 μL of GENMED stain solution B (Reagent J)). After incubating at room temperature for 80 min, the sections were incubated in 50 mL GENMED cleansing solution (Reagent H). The slides were dehydrated with ethanol, incubated in xylene and sealed with neutral balsam. The sections were photographed with a microscope (at ×400 magnification).

H&E staining

After the sections were dewaxed and rehydrated, they were stained with hematoxylin for 5 min, differentiated with 1% hydrochloric acid alcohol, and stained in a chamber containing eosin for 3 min. Subsequently, the sections were dehydrated by gradient ethanol and permeabilized using xylene. The sections were analyzed under an Olympus microscope (Olympus, Japan) (at ×200 magnification).

Double immunofluorescence staining

After heating at 60 °C for 30 min, the sections were dewaxed and rehydrated. The slices were immersed in antigen retrieval solution and repaired at a sub-boiling temperature for 10 min. The samples were incubated with goat serum to block nonspecific binding. The primary antibodies against SIRT1 (Ms), NeuN (Rb), CD68 (Ms), C3 (Ms), Iba-1 (Rb), and GFAP (Rb) were diluted with PBS (1:100–1:50). The antibody combination (SIRT1-NeuN, Iba-1-CD68, and GFAP-C3) was added to each slide and incubated overnight at 4 °C. The FITC-labeled or Cy3-labeled secondary antibodies (1:200) were then applied for

90 min. The nuclei were stained with DAPI (Beyotime, China) and sealed with anti-fade reagent. The cells from each of the different tissue sections were measured at ×400 magnification using a microscope.

TUNEL-NeuN double immunofluorescence staining

The slides were deparaffinized and rehydrated, and then permeabilized with 0.1% Triton X–100 (diluted in 0.1% citrate buffer). Antigen retrieval was conducted with a citric acid-sodium citrate solution. The slides were labeled with 50 μL TUNEL solution (enzyme solution: label solution = 1: 9) at 37 °C for 60 min. The samples were blocked with goat serum, incubated with the primary antibody against NeuN (1:100) and then incubated with the Cy3-labeled secondary antibody (1:200). The nuclei were stained with DAPI. After washing, the samples were sealed with anti-fade reagent and visualized at ×400 magnification.

Analysis of luciferase activity

To examine whether miR-181a-5p directly binds to SIRT1, pmirGLO (Promega, E133A) vectors containing the wild-type SIRT1 3′UTR or the mutant SIRT1 3′UTR and miR-181a-5p mimics or negative control were cotransfected into 293T cells (Zhong Qiao Xin Zhou Biotechnology Co., Ltd., Shanghai, China) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). The luciferase activity was measured using a dual luciferase reporter assay kit (KeyGen Biotech. Co. Ltd., Nanjing, China).

Measurements of TNF-α and oxidative stress

The content of TNF-α in the hippocampal tissue was assessed using a commercial rat TNF-α ELISA kit (Multi-Sciences Biotech Co. Ltd., Hangzhou, China). The activities of superoxide dismutase (SOD) and catalase (CAT) and the contents of malondialdehyde (MDA) and glutathione (GSH) were measured using commercial kits purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). These parameters were normalized to the protein content, which was determined using a bicinchoninic acid protein assay kit (Solarbio Science & Technology, Co., Ltd., Beijing, China). All the assays were performed following the manufacturer's instructions.

RNA extraction and quantitative real-time PCR (qRT-PCR)

The total RNA was isolated from the hippocampal tissues using the TRIpure Total RNA Extraction Reagent (BioTeke Corporation, Beijing, China) according to the manufacturer's protocol. The concentration of the RNA was determined using a NANO 2000 spectrophotometer (Thermo Scientific, Rockford, IL). The RNA was reverse transcribed using super M-MLV (BioTeke). qRT-PCR was carried out using SYBR Green (Solarbio) and the 2 × Power Taq PCR Master Mix (BioTeke) on an Exicycler 96 (Bioneer, Daejeon, Korea). The primers used were as follows: TNF-α: forward: 5′-GGCTAGAGTACAGTGGCTCG-3′, reverse: 5′-GCAAATCGCAAGGTCAGG-3′, SIRT1: forward: 5′-ATAAATAGGGAACCTCTGCC-3′, reverse: 5′-GCTTTACAGGGTTACAACAA-3′, Serping1: forward: 5′-AGTAAAGAGCAGCCAAGAC-3′, reverse: 5′-CCAGAGCAGGAAGAGGA-3′, S100a10: forward: 5′-CTTACATTTCACAGGTTTGCAG-3′, reverse: 5′-GCCCACTTTTCCATCTCG-3′, PTX3: forward: 5′-AGCCACAGACGTATTAAACAA-3′, reverse: 5′-CCACCCACCACAAACACTA-3′, β-actin: forward: 5′-CCACTGCCGCATCCTCTT-3′, reverse: 5′-GGTCTTTACGGATGTCAACG-3′, miR-181a-5p: RT primer: 5′-GTTGGCTCTGGTGCAGGGTCCGAGGTATTCGCACCAGAGCCAACACTCAC-3′, forward: 5′-AACATTCAACGCTGTCGGTGAGT-3′, reverse: 5′-GGTGCAGGGTCCGAGGTAT-3′, 5 s: RT primer: 5′-GTTGGCTCTGGTGCAGGGTCCGAGGTATTCGCACCAGAGCCAACAAAGCCTAC-3′, forward: 5′-GATCTCGGAAGCTAAGCAGG-3′, reverse: 5′-TGGTGCAGGGTCCGAGGTAT-3′. The fold change in expression relative to the control was obtained using the $2^{-\Delta\Delta Ct}$ method. β-actin and 5 s were used as the internal controls for mRNA and miRNA, respectively.

Measurement of mitochondrial function

The production of ROS and the activities of mitochondrial complexes I, II, III, and IV in the hippocampal tissue were assayed using an ROS assay kit (Nanjing Jiancheng Bioengineering Institute) and complex activity assay kits (Solarbio), respectively, according to the manufacturers' protocols. The mitochondrial membrane potential (MMP) was measured using a MMP assay kit (Beyotime). The hippocampal tissues obtained from the rats were washed with PBS and digested with trypsin for 20 min, and then, the cells were collected. Then, 0.5 mL of a JC-1 staining solution was added to the cells, mixed well, and incubated at 37 °C for 20 min. After centrifugation, the supernatant was removed, and the cells were washed with JC-1 staining buffer (×1) two times and centrifuged. Subsequently, the cells were resuspended in 500 μL JC-1 staining buffer (×1), and the samples were analyzed using a NovoCyte flow cytometer (ACEA Biosciences, San Diego, CA).

Western blot analysis

Hippocampal tissues were lysed using cell lysis buffer for Western blotting and IP (Beyotime, P0013) supplemented

with phenylmethylsulfonyl fluoride (Beyotime). The proteins were boiled in loading buffer, separated in a sodium dodecyl sulfate–polyacrylamide gel (8–15%) and transferred onto polyvinylidene difluoride membranes. The protein-containing membranes were blocked with 5% non-fat milk at room temperature for 1 h and incubated with primary antibodies at 4 °C overnight. Then, the membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit or goat anti-mouse IgG (Beyotime) at 37 °C for 45 min. The protein bands were visualized using an enhanced chemiluminescence reagent (Beyotime), and the gray values were analyzed using Gel-Pro-Analyzer software (Media Cybernetics, Bethesda, MD). β-actin, histone H3, and COXIV (Proteintech, 11242-1-AP) were used as internal controls for the whole protein, nuclear protein, and mitochondrial protein fractions, respectively. The primary antibodies used in this study were as follows: anti-SIRT1 antibody (Novus, NBP-51641), anti-iNOS antibody (Proteintech, 18985-1-AP), anti-Arg1 antibody (Proteintech, 16001-1-AP), anti-HO-1 antibody (Proteintech, 10701-1-AP), anti-NQO1 antibody (Abcam, Ab28947), anti-Nrf2 antibody (Proteintech, 16396-1-AP), and anti-Cyt C antibody (Proteintech, 10993-1-AP).

Statistical analysis

The data are expressed as the mean ± standard deviation (SD). The data were analyzed using SPSS 22.0. The differences were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's test. $P < 0.05$ was considered statistically significant.

Results

Suppression of miR-181a-5p attenuated epilepsy-induced dysfunction of spatial learning and memory

The expression levels of miR-181a-5p and SIRT1 were determined at day 3 and day 48 after in vivo transfection. The results showed that miR-181a-5p was significantly upregulated and SIRT1 was downregulated in the hippocampal tissue of the immature rats with epilepsy. In addition, the shRNA targeting SIRT1 effectively suppressed SIRT1 expression (Fig. 1a–c), which indicated that the transfection was successful and that the effect was sustained throughout the course of the experiment. The results of the luciferase activity analysis confirmed the direct binding of miR-181a-5p and SIRT1 (Fig. 1d). Together, these results suggested that SIRT1 was a direct target of miR-181a-5p in the hippocampus of epileptic rats and that miR-181a-5p negatively regulated the expression of SIRT1.

In the MWM test, the rats in the epilepsy group showed an obvious decline in spatial learning and memory, as evidenced by the significantly prolonged escape latency and the decreased number of platform crossings (Fig. 1e, f). Intracerebroventricular injection of anti-miR-181a-5p markedly improved the memory of the epileptic rats. Although the difference in the escape latency between the anti-miR-181a-5p treatment and epilepsy groups did not reach significance until day 5, the obviously downshifted curve of escape latency and the increased number of platform crossings showed the improvement in learning and memory. However, this effect was inhibited by the shRNA-mediated silencing of SIRT1. These data implied that inhibition of miR-181a-5p improved the cognitive dysfunction in immature rats with epilepsy via SIRT1 activation.

Suppression of miR-181a-5p reduced mossy fiber sprouting and alleviated neuronal loss in the hippocampus

The pathological changes in the hippocampus were examined using Timm's, H&E, and Nissl staining. As shown in Fig. 2a, b, Timm's staining was barely observed in the control rats, while obvious mossy fiber sprouting was observed in the rats with epilepsy. Compared with the control epileptic rats, the anti-miR-181a-5p-treated rats exhibited reduced mossy fiber sprouting, and this phenomenon was reversed by SIRT1 silencing. In addition, H&E staining of the hippocampal tissue sections was performed (Fig. 2c, d). In the SE group, the number of neurons decreased. In addition to the darker cytoplasmic staining and pyknotic nuclei, the irregular cells were in disorderly alignment. In the SE + anti-miR-181a-5p group, the neurons in the hippocampal tissues swelled to a certain extent, the number of neurons was slightly reduced, and the degree of neurodegeneration was markedly reduced. However, these ameliorative effects caused by the inhibition of miR-181a-5p were eliminated by SIRT1-targeted shRNA. In addition, Nissl staining was performed to evaluate neuronal damage (Fig. 2e, f). The results showed significantly reduced Nissl bodies and dissolved neurons in the hippocampus of the immature rats with epilepsy. The anti-miR-181a-5p treatment preserved the neuronal population and normalized the neuronal morphology. However, the protection of neurons provided by anti-miR-181a-5p could be blocked via SIRT1 silencing, as evidenced by the decreased Nissl bodies in the hippocampus of the rats in the SE + anti-miR-181a-5p + shSIRT1 group. All these results indicated that inhibition of miR-181a-5p attenuated the seizure-induced hippocampal insults by activating SIRT1.

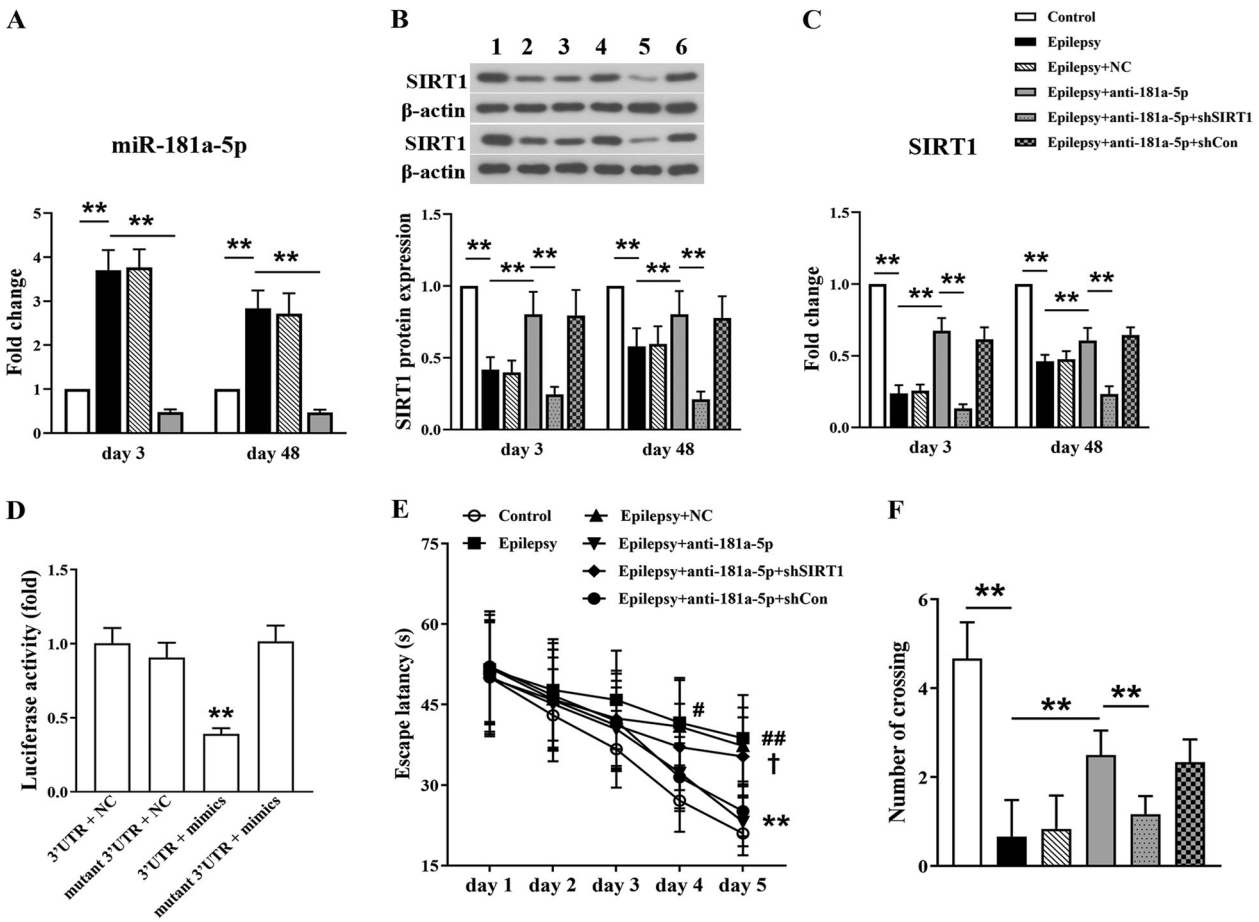

Fig. 1 Suppression of miR-181a-5p attenuated the epilepsy-induced dysfunction of spatial learning and memory. a qRT-PCR measurement of miR-181a-5p in the hippocampal tissues from the control rats and the rats subjected to SE and in vivo transfection at day 3 and day 48 ($n = 6$ per group). **b** The protein level of SIRT1 detected by Western blot in the hippocampal tissues in the control and epileptic rats. **c** The SIRT1 mRNA level detected by qRT-PCR ($n = 6$). **d** The binding of miR-181a-5p to SIRT1 was identified using a luciferase reporter gene assay in 293T cells. The relative luciferase activity was normalized and is shown as the mean ± SD ($n = 3$). **e** Escape latency of the different animal groups during the 5 training days in the MWM test. The data are shown as the mean ± SD based on $n = 6$. **f** The probe test was performed after 5 days of training, and the number of platform crossings was counted ($n = 6$ each group). The statistically significant differences in (**a**–**f**) were analyzed by one-way ANOVA with Tukey's test for multiple comparisons (**$P < 0.01$).

miR-181a-5p suppression enhanced SIRT1 expression in hippocampal neurons and attenuated neuronal apoptosis in immature epileptic rats

To determine which cell type was SIRT1-positive, we performed double immunofluorescence staining for SIRT1 and NeuN (a neuron marker). As shown in Fig. 3a, SIRT1 was colocalized with NeuN in the hippocampus. The expression of SIRT1 was decreased in the NeuN$^+$ neurons of the epileptic rats and was effectively upregulated in the rats pretreated with anti-miR-181a-5p. Neuronal apoptosis was examined using NeuN-TUNEL double staining. The results in Fig. 3b, c showed a significantly increased number of apoptotic neurons in the hippocampus of the epileptic rats. These results also revealed that there were fewer apoptotic neurons in the hippocampal tissues after treatment with anti-miR-181a-5p, and this effect was reversed by SIRT1-targeted shRNA. All the data implied that the miR-181a-5p/SIRT1 pathway was observed in neurons and that miR-181a-5p inhibition reduced neuronal apoptosis through SIRT1 activation in immature epileptic rats.

Suppression of miR-181a-5p inhibited the activation of astrocytes and microglia by activating SIRT1 in the hippocampus of immature epileptic rats

Next, we investigated the effect of the miR-181a-5p/SIRT1 pathway on the activation of astrocytes and microglia in the hippocampus of the epileptic rats. The hippocampal tissues were double-stained with GFAP and C3 (complement component 3, a marker of the reactive A1 phenotype of astrocytes). As shown in Fig. 4a, b, a higher number of C3-labeled GFAP$^+$ cells indicated that the reactive A1 phenotype of astrocytes was significantly

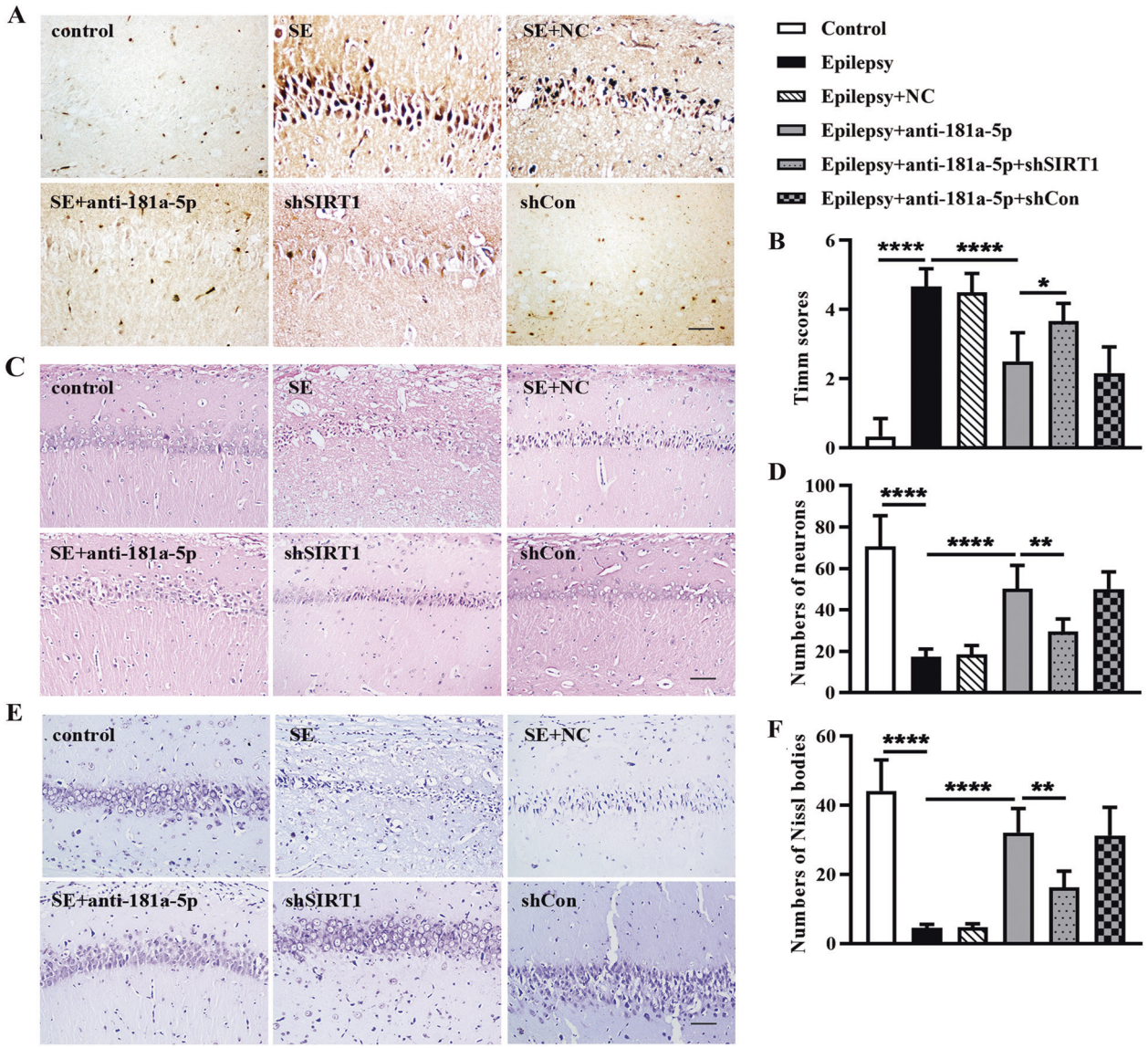

Fig. 2 Suppression of miR-181a-5p reduced mossy fiber sprouting and alleviated neuronal loss in the hippocampus. a Mossy fiber sprouting in the hippocampus of different animal groups observed by Timm's staining. Scale bar, 50 μm. **b** Timm's staining was evaluated and presented as Timm's scores. **c** H&E staining of the hippocampal neurons after SE induction. Scale bar, 100 μm. **d** The numbers of the neurons observed by H&E staining. **e** The images of Nissl staining for Nissl bodies (blue/violet). Scale bar, 100 μm.**f** The numbers of Nissl bodies were counted. The data in panels (**b**, **d** and **f**) are shown as the mean ± SD and were analyzed by one-way ANOVA with Tukey's test for multiple comparisons (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$).

increased in the hippocampus of the epileptic rats. Inhibition of miR-181a-5p reduced the C3 immunoreactivity in the hippocampus. However, the decreased induction of the A1 astrocyte phenotype caused by anti-miR-181a-5p was reversed by SIRT1 knockdown. To further explore the effect of miR-181a-5p on the neurotoxic A1 phenotype and neuroprotective A2 phenotype of astrocytes, specific genes associated with both phenotypes were tested by qRT-PCR. Seizures upregulated A1 astrocyte-related gene (Serping1) expression and downregulated A2 astrocyte-related gene (S100a10 and PTX3) expression. Inhibition of miR-181a-5p restrained the A1 phenotype and promoted the A2 phenotype of astrocytes in the hippocampal tissues of the epileptic rats, and this effect was reversed by SIRT1-targeted shRNA (Fig. 4c). Moreover, the activated microglia were identified by Iba-1-CD68 staining (Fig. 4d, e). The results showed more activated microglia (CD68-labeling of $Iba1^+$ cells) in the hippocampus of the SE rats. Anti-miR-181a-5p pretreatment decreased the ratio of activated microglia ($CD68^+/Iba1^+$ cells), and this ratio was restored by SIRT1 knockdown. Together, these results suggested that inhibition of miR-181a-5p repressed the activation of glial cells due to TLE by activating SIRT1.

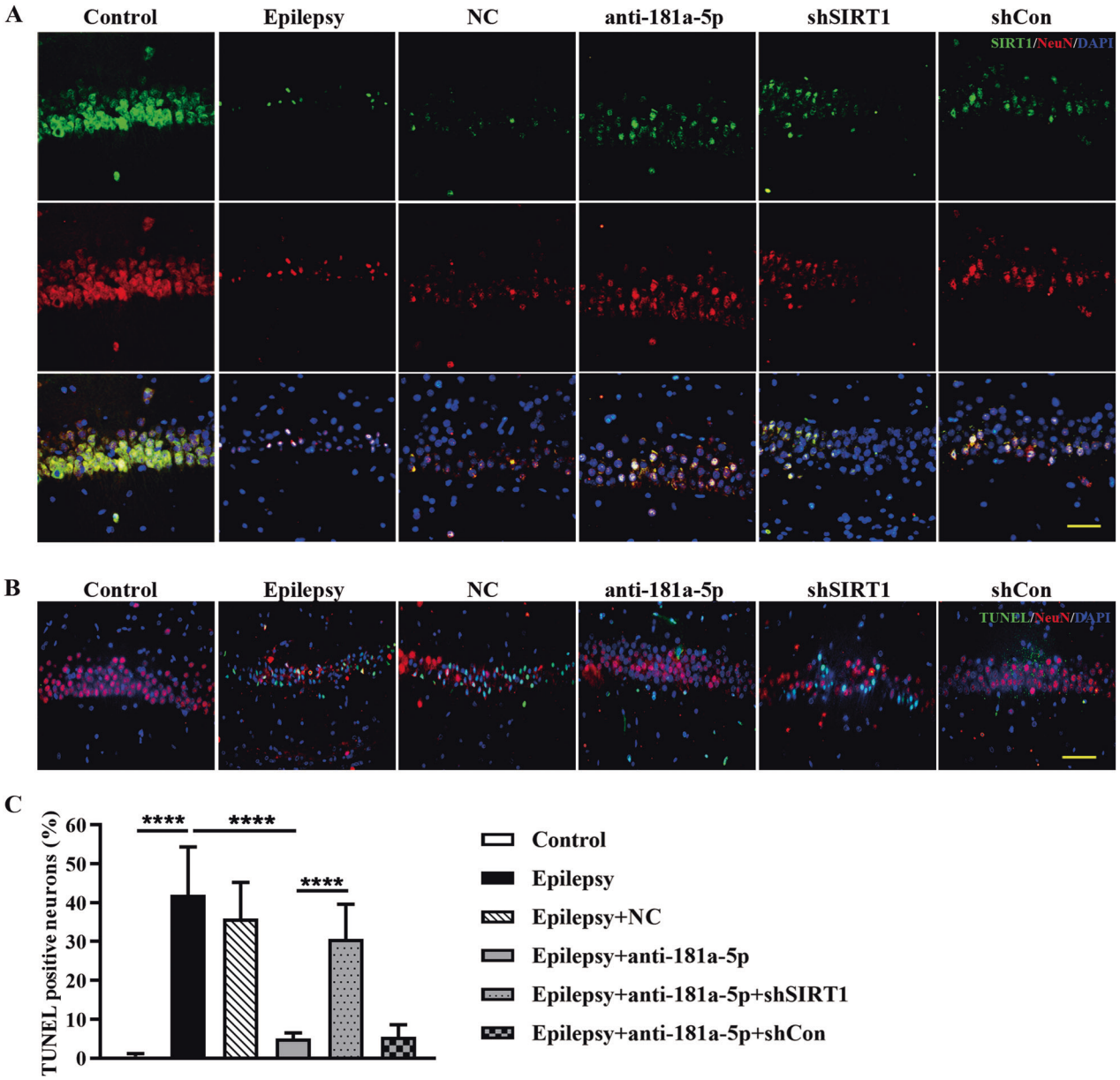

Fig. 3 miR-181a-5p suppression enhanced SIRT1 expression in the hippocampal neurons and attenuated neuronal apoptosis in the immature epileptic rats. a Double immunofluorescence staining of SIRT1 (green) and NeuN (red). Scale bar, 50 μm. **b** Hippocampal neuronal apoptosis visualized by TUNEL-NeuN double immunofluorescence staining in the control and SE rats. TUNEL (green), NeuN (red) and DAPI (blue). Scale bar, 50 μm. **c** The number of TUNEL and NeuN double-positive neurons in the hippocampus. The data were analyzed using one-way ANOVA with Tukey's test for multiple comparisons.

Suppression of miR-181a-5p attenuated neuroinflammation and the degree of oxidative stress in the hippocampus

Considering that activated glial cells release inflammatory factors to mediate inflammatory responses, we further elucidated the effect of the miR-181a-5p/SIRT1 pathway on neuroinflammation. The expression level of the inflammatory cytokine TNF-α was evaluated according to qRT-PCR and ELISA analysis. There was an obvious elevation in the TNF-α levels in the hippocampus of the SE rats. The TNF-α levels in the anti-miR-181a-5p group were significantly reduced compared with those in the SE group. However, SIRT1 silencing reversed the decrease in the TNF-α level caused by miR-181a-5p inhibition (Fig. 5a, b). In addition, neuroinflammation was also assessed using the anti-inflammatory marker Arginase 1 (Arg1) and the pro-inflammatory marker inducible nitric oxide synthase (iNOS). As shown in Fig. 5c, increased expression of iNOS and decreased expression of Arg1 were observed in the SE

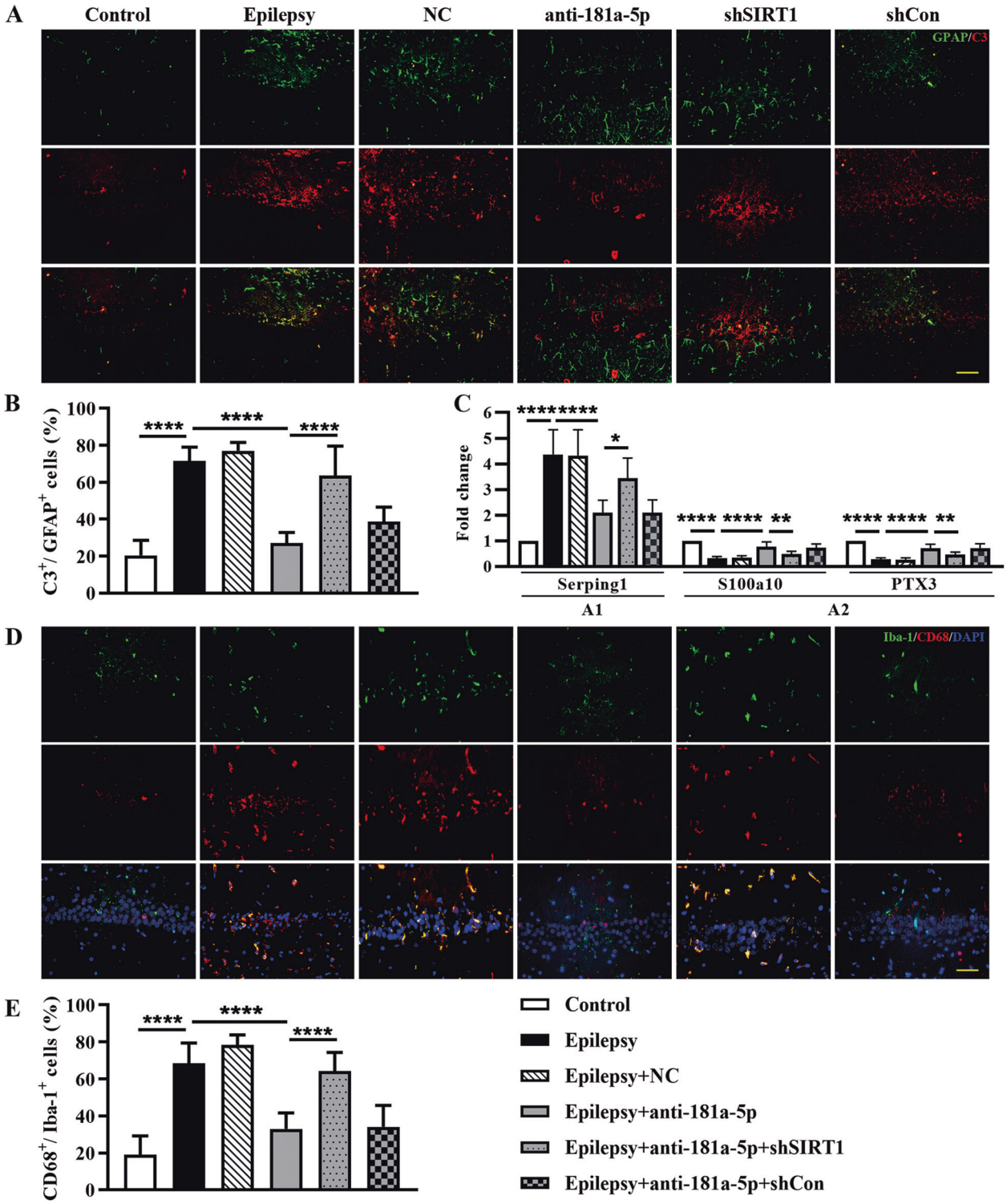

Fig. 4 Suppression of miR-181a-5p inhibited the activation of astrocytes and microglia by activating SIRT1 in the hippocampus of the epileptic immature rats. a GFAP-C3 staining revealed activated astrocytes in response to TLE. Scale bar, 50 µm. **b** The ratio of $C3^+$ cells to $GFAP^+$ cells (the proportion of astrocytes with the reactive A1 phenotype). **c** qRT-PCR of the A1-specific gene Serping1 and A2-specific genes S100a10 and PTX3. **d** Iba-1-CD68 staining showed the activation of microglia in the hippocampus. Scale bar, 50 µm. **e** The ratio of $CD68^+$ cells to Iba-1^+ cells (the proportion of activated microglia). The statistical analyses were based on one-way ANOVA for multiple comparisons.

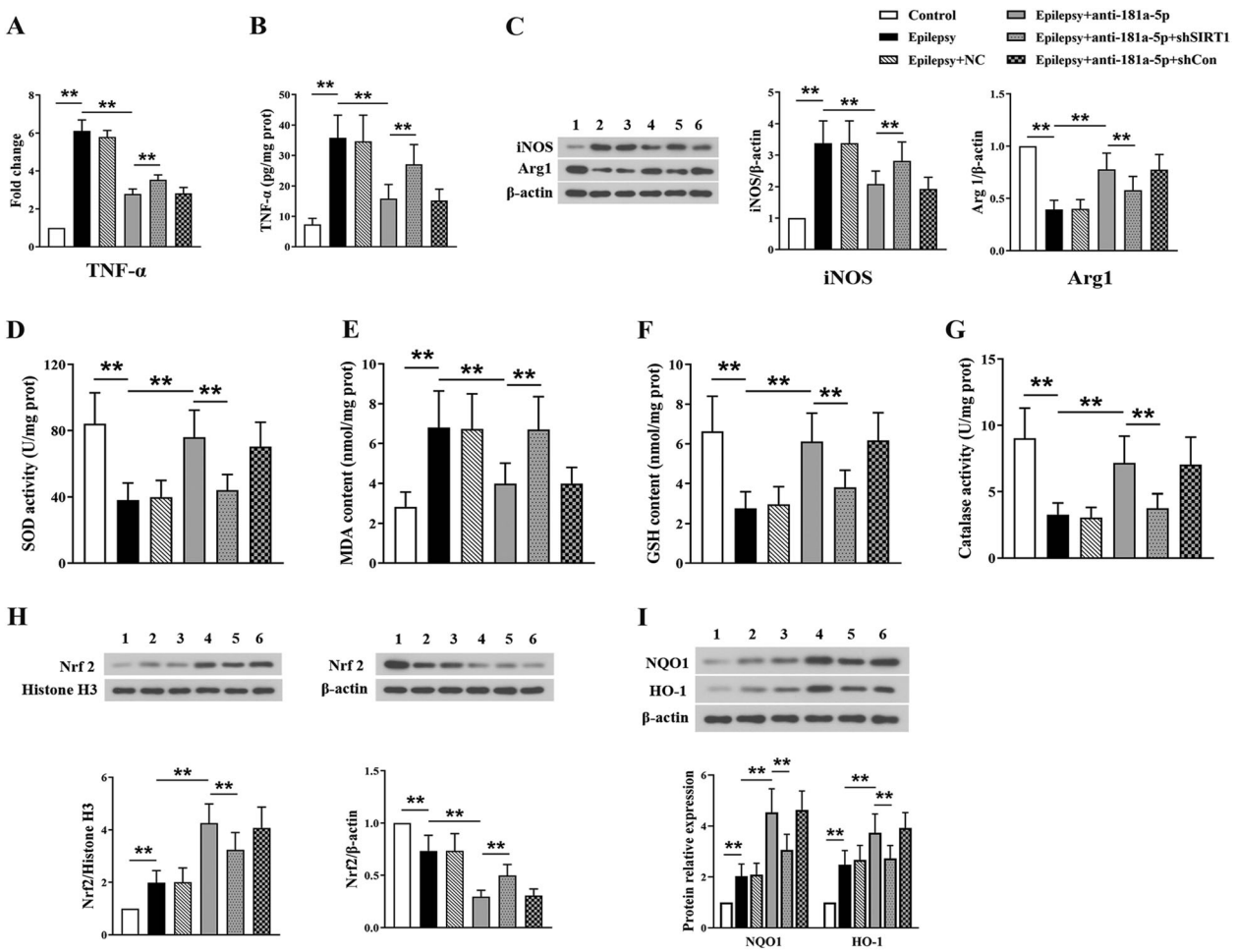

Fig. 5 Suppression of miR-181a-5p attenuated neuroinflammation and the degree of oxidative stress in the hippocampus. a The level of TNF-α in the hippocampus of the control and SE rats was detected by qRT-PCR. **b** ELISA analysis showed the expression of TNF-α in the hippocampal tissues. **c** The protein expression of iNOS and Arg1 determined by Western blot. The oxidative stress markers were analyzed after LiCl-pilocarpine-induced status epilepticus: (**d**) SOD, (**e**) MDA, (**f**) GSH and (**g**) CAT. **h** The expression of Nrf2 in the nucleus and cytoplasm was detected by Western blot. **i** The protein levels of NQO1 and HO-1. Quantitative analysis was performed, and the data are presented as the mean ± SD and were analyzed by on one-way ANOVA with Tukey's test for multiple comparisons ($n = 6$).

group. In contrast, miR-181a-5p inhibition blocked iNOS expression and induced Arg1 expression. However, the levels of these proteins were reversed in the SIRT1 knockdown group. All these data illustrated that the inhibition of miR-181a-5p mitigated neuroinflammation after the induction of SE via the activation of SIRT1.

Seizures not only induce inflammatory responses but also are accompanied by increased oxidative stress. We examined some indexes related to redox balance. The SOD activity, GSH content, and CAT activity in the SE group were decreased. Compared with the SE group, the anti-miR-181a-5p group showed elevated levels of each measured index. SIRT1 knockdown reversed the elevation of these indexes caused by miR-181a-5p inhibition. The MDA levels were prominently increased in the hippocampus of the SE rats. miR-181a-5p inhibition resulted in a decrease in the hippocampal MDA content, and this decrease was effectively reversed by SIRT1-targeted shRNA (Fig. 5d–g). Moreover, some key proteins involved in oxidative stress, including nuclear factor E2-related factor 2 (Nrf2), quinone acceptor oxidoreductase 1 (NQO1), and heme oxygenase 1 (HO-1), were examined. In the hippocampus of the SE rats, the translocation of Nrf2 from the cytoplasm to the nucleus occurred under oxidative stress, and this translocation was followed by the upregulation of NQO1 and HO-1. miR-181a-5p inhibition increased the expression of nuclear Nrf2 and the levels of NQO1 and HO-1. However, SIRT1 knockdown blocked the increase in the protein levels caused by miR-181a-5p inhibition (Fig. 5h, i). Therefore, we concluded that inhibition of miR-181a-5p attenuated oxidative stress during the progression of TLE through the activation of SIRT1.

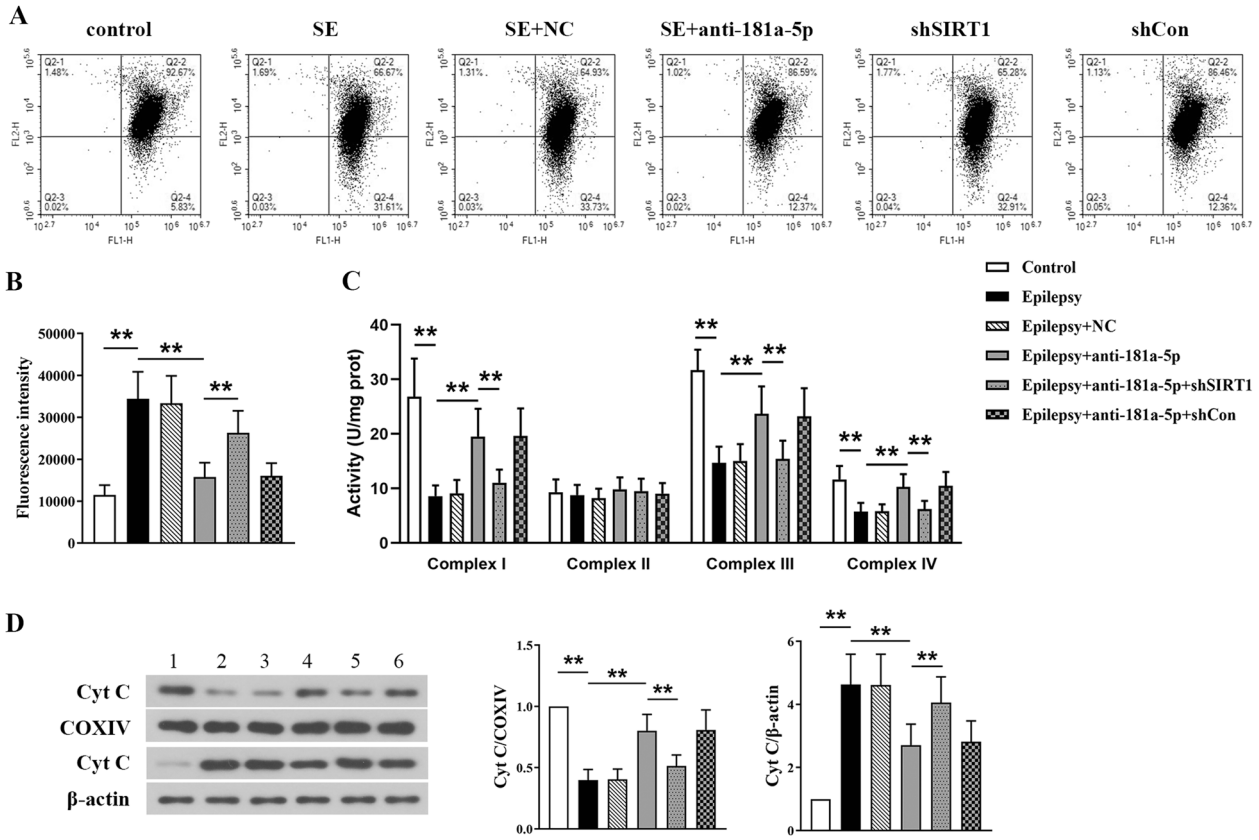

Fig. 6 Suppression of miR-181a-5p improved mitochondrial function in the hippocampus. a The mitochondrial membrane potential of the hippocampal tissues was determined by flow cytometry. **b** ROS production in the different animal groups was determined by fluorescence intensity. **c** The activity of the mitochondrial respiratory chain complexes (complexes I, II, III and IV). **d** The expression of Cyt C in the mitochondria and cytoplasm was determined by Western blot. The data were analyzed by one-way ANOVA with Tukey's test for multiple comparisons (**$P < 0.01$).

miR-181a-5p suppression improved mitochondrial function in the hippocampus

After seizures, the generation of high amounts of free radicals and the consequent oxidative damage produce pathophysiological changes, such as exacerbated membrane damage and altered mitochondrial respiratory chain complex activity. JC-1 fluorescence dye staining was performed to evaluate the MMP. The proportion of JC-1 green fluorescence was increased in the SE group (31.61%), suggesting that the MMP was reduced in the hippocampus of the SE rats. In the anti-miR-181a-5p group, this proportion was reduced to 12.37%, while in the shSIRT1 group, it was elevated to 32.91% (Fig. 6a). Intracellular ROS production was analyzed using the fluorogenic probe DCFH-DA. As shown in Fig. 6b, seizures promoted ROS production in the hippocampus of the SE rats. miR-181a-5p inhibition significantly reduced this SE-induced increase in the ROS levels, while SIRT1 knockdown reversed the decrease in ROS production caused by miR-181a-5p inhibition. We also analyzed the activity of the respiratory chain complexes to assess mitochondrial damage. The results revealed that seizures reduced the activity of complexes I, III, and IV. With miR-181a-5p inhibition, the activity of these complexes was restored, but this recovery was suppressed by SIRT1 knockdown. In addition, we did not observe distinct changes in the activity of complex II (Fig. 6c). To further confirm the results described above, we determined the expression of cytochrome c (Cyt C) in the mitochondria and cytoplasm. In the SE group, Cyt C was released from the mitochondria to the cytoplasm. miR-181a-5p inhibition significantly suppressed the seizure-induced release of Cyt C from the mitochondria to the cytoplasm. However, this suppression of Cyt C release was reversed by shRNA-mediated silencing of SIRT1 (Fig. 6d). Collectively, these data showed that miR-181a-5p inhibition ameliorated mitochondrial dysfunction in immature SE rats by activating SIRT1.

Discussion

An early paper established that the medial temporal lobe (MTL) has an essential role in transferring short-term

(the rearward part of the current space of time) to long-term (an event that has been recollected) memory in association cortices. The MTL is concerned with memory of events and personal experiences (episodic memory), and declarative memory (explicit memory for facts) [34]. Due to the extensive impairment of the mesial temporal lobes, patients demonstrate amnesia and are unable to form and retain new memories [35].

A large number (70%) of children and adolescents suffering from epilepsy exhibit learning difficulties that include intermittent memory, transitory memory disconnections and impaired attention or concentration [36]. The cognitive effects of epilepsy were the major concern stated by children with seizures and their parents [37]. This concern is because memory is one of the specific difficulties in cognitive functioning. Children with epilepsy clearly have memory deficits and are at a disadvantage in learning [38, 39]. Extensive studies have thus focused on the cognitive decline during TLE in children and adolescents. In the current study, we found the upregulation of miR-181a-5p and downregulation of its target gene SIRT1 in a model of pilocarpine-induced TLE in immature rats. Moreover, immature epileptic rats suffered from cognitive deficits, while the inhibition of miR-181a-5p improved the learning and memory disorders via the activation of SIRT1.

A large and growing body of literature has shown the involvement of miRNAs in epilepsy, and the pathology of epilepsy is affected by changes in the expression or functioning of miRNAs. In addition to miR-181a-5p, miR-23a, miR-451, miR-146a, miR-27a-3p, miR-183, and miR-199a-5p were also found to be upregulated in the hippocampus of animal models of epilepsy [31, 40–44]. Notably, miR-199a-5p also targets SIRT1 and has effects on seizures. Knockdown of miR-199a-5p alleviated epileptic damage and protected against loss and apoptosis of neurons, in accordance with the upregulation of SIRT1 and the subsequent deacetylation of p53 [31]. In addition to SIRT1, other human sirtuins have also been implicated in epilepsy. The expression level of sirtuin-2 (SIRT2) is decreased in the hippocampus of patients with mesial TLE [45]. SIRT3 is downregulated in epileptic rats and causes the hyperacetylation of mitochondrial proteins, thereby leading to mitochondrial dysfunction in chronic epilepsy [46]. Kainate-induced seizures result in the increased expression of SIRT5 in the hippocampus of mice [47]. The pathological mechanism of epilepsy is complex and involves many pathways. Our study proposed a role of the miR-181a-5p/SIRT1 pathway in epilepsy and further enriched the underlying mechanism of TLE development, which may provide a novel strategy for the treatment of epilepsy.

Microglia play a critical role in seizure-induced neuroinflammation [48, 49], and they not only produce numerous cytokines and chemokines but also appear to be a target that receives cytokine and chemokine signals in the CNS [50]. In rodent epilepsy models, activated microglia become abundant, which is typically followed by neuronal damage [51, 52]. Prior research in rats with kainic acid-induced epilepsy has also shown that microglial engagement is one of the earliest processes in response to seizures [17]. Similarly, reactive astrocytes produce various cytokines in CNS lesions, and in turn, cytokines can induce astrogliosis [53, 54]. Changes in membrane properties and electrophysiology take place in activated astrocytes, which leads to neuronal hyperexcitability and pro-epileptic changes [55]. It was also confirmed in mice with pilocarpine-induced epilepsy that astrocyte activation is present in the CA3 and CA1 pyramidal cell layers [56]. Consistent with previous studies, we observed the activation of microglia and astrocytes in the hippocampus of immature rats with pilocarpine-induced epilepsy. However, the inhibition of miR-181a-5p abolished microglia and astrocyte activation during the SE-induced inflammatory responses by activating SIRT1. In the hippocampus of epileptic rats, miR-181a-5p inhibition reduced the level of the inflammatory cytokine TNF-α, which might be produced by activated glial cells. Moreover, evidence suggests that NF-κB signaling induced by TNF-α is an important mediator of neuronal injury [57, 58]. Could miR-181a-5p and SIRT1 affect NF-κB signaling via TNF-α during SE-induced neuronal injury? How does the regulation work? These questions deserve more detailed investigations in future studies.

In response to brain injury, glial cells are activated and generate inflammatory mediators that are capable of suppressing and destroying mitochondria. For instance, TNF-α impairs mitochondrial oxidative phosphorylation, reduces ATP production, and promotes mitochondrial reactive oxygen species production. These changes eventually lead to mitochondrial membrane permeabilization, defective mitochondrial dynamics, and cell death. In turn, mitochondrial dysfunction can trigger and increase neuroinflammatory responses [59]. In the current study, we preliminarily revealed that the inhibition of miR-181a-5p suppressed the activation of astrocytes and microglia and improved the dysfunction of mitochondria in the hippocampus of the immature SE rats. Due to the lack of an in vitro model of epilepsy in specific cells, we could not show the amelioration of mitochondrial dysfunction caused by miR-181a-5p inhibition in astrocytes and microglia or neurons. Furthermore, what is the relationship between the reduced astrocyte and microglial activation and the improved mitochondrial dysfunction caused by miR-181a-5p inhibition? This question requires additional work in future studies.

Excessive production of free radicals leads to oxidative stress, which is associated with the pathogenesis of neurodegenerative diseases [60]. It was established that oxidative

stress participates in the occurrence and progression of epilepsy [61]. Antioxidants function as free radical scavengers that can dramatically reduce oxidative stress and inhibit seizures [62]. It has been demonstrated that the activity of antioxidant enzymes (SOD and CAT) was reduced in rats with epilepsy [63, 64]. Our results revealed that the activity of SOD and CAT decreased markedly 72 h after SE, which was consistent with the above findings. However, this observation was different from that of other studies that demonstrated enhanced CAT enzyme activity in rats with pilocarpine-induced SE [65, 66]. A large body of free radicals are generated in rats with the onset of epilepsy, and the antioxidant system is mobilized to scavenge the free radicals. During the transition from the acute period to the recovery period, the antioxidant enzyme activity gradually decreases, which may explain this discrepancy. Notably, the inhibition of miR-181a-5p restored the reduction in SOD and CAT activity through the activation of SIRT1. On the other hand, some oxidative stress markers (MDA and GSH) and key proteins (Nrf2 and NQO1) in the epileptic rats were also measured. Our findings showed that the inhibition of miR-181a-5p provides neuroprotection in immature SE rats partially through mitigating oxidative stress.

In summary, our study showed that miR-181a-5p contributed to the development of epilepsy in immature rats. We also highlighted that the inhibition of miR-181a-5p suppressed the activation of astrocytes and microglia and attenuated oxidative stress by activating SIRT1 in the hippocampus of the immature epileptic rats. Investigations of the underlying mechanisms showed that the inhibition of miR-181a-5p alleviated epilepsy-induced deleterious changes by the upregulation of SIRT1. Taken together, we suggest that miR-181a-5p is a novel seizure regulator that might play a key role in the occurrence and progression of epilepsy, and we establish the miR-181a-5p/SIRT1 pathway as a potential target for preventing and treating TLE.

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Author contributions QL contributed conception and design of the study. HK and JL performed experiments. ZC and JZ analyzed the data. ZZ wrote the draft of the paper. ZL and PT revised the paper. HK, HW, and JW obtained the funding. All authors have read and approved the submitted version.

Compliance with ethical standards

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

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