Acta Epileptologica

RESEARCH

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miRNA-let-7i modulates status epilepticus *via* the TLR4 pathway

Shu Ou[1,2], Xi Liu[2], Tao Xu[2], Xinyuan Yu[2], Teng Wang[2], Yangmei Chen[2] and Haiyan Luo[2*]

Abstract

Background: Status epilepticus (SE) is a neurological emergency associated with high mortality and morbidity. Many SE episodes cannot be quickly and effectively terminated with current medications. miRNA-Let-7i, a member of the miRNA-Let-7 family, has been found to be associated with a variety of brain pathophysiological and neurological diseases. However, its role in SE remains elusive and requires further clarification.

Methods: The expression of miRNA-Let-7i was detected in temporal lobe epilepsy (TLE) patients and SE model rats using the real-time quantitative polymerase chain reaction (RT-qPCR) method. Behavioral assays were performed in pilocarpine-induced SE model, and a whole-cell current clamp technique was employed to examine neuronal excitability. Neuronal apoptosis was evaluated by Nissl staining and terminal deoxynucleotidyl transferase-mediated dUTP end-labeling (TUNEL) assays.

Results: The expression of miRNA-Let-7i was significantly reduced in the cortex and hippocampus of SE rats. The miRNA-Let-7i agomir and antagomir effectively regulated the levels of miRNA-Let-7i. In particular, the agomir significantly reduced the degree of SE and prolonged the latent period of SE, whereas the antagomir increased the degree of seizures and shortened the latent period. In addition, the agomir significantly decreased the frequency of action potentials, while the antagomir significantly increased it. Nissl staining and TUNEL assays demonstrated that the agomir increased the survival and decreased the apoptosis, while the antagomir had the opposite effects. In addition, a Toll-like receptor 4 (TLR4) inhibitor rescued the effects of antagomir on SE behavior and expression of IL-6 and TNF-α. Similar results on miRNA-Let-7i expression and effects of TLR4 inhibition were found in brain tissues of TLE patients.

Conclusions: The miRNA-Let-7i—TLR4 regulatory pathway is involved in SE, which provides insights into the pathogenesis of SE.

Keywords: miRNA-let-7i, Status epilepticus, TLR4, Apoptosis

Background

Status epilepticus (SE) represents the most severe form of epileptic disorders or even the initial manifestation of various injuries, such as traumatic brain injury and stroke [1, 2]. The goal of SE treatment is to promptly terminate seizure activity, which can reduce the associated mortality and morbidity [3]. Currently, there are some pharmacologic and non-pharmacologic treatments for SE [3], but many super-refractory cases of SE do not respond well to the treatments [4], largely due to the illusive mechanisms underlying SE.

MicroRNAs (miRNAs) are a class of small non-coding RNAs which are essentially important for post-transcriptional control of gene expression while not directly involved in protein coding [5]. Previous research has shown that miRNAs are involved in a wide array of biological processes of the central nervous system [6, 7]. In epilepsy, miRNAs can modulate the axonal and dendritic structures, as well as the repertoire of

*Correspondence: haiyanL_cqmu@163.com
[2] Department of Neurology, The Second Affiliated Hospital of Chongqing Medical University, Chongqing 400010, China
Full list of author information is available at the end of the article

neurotransmitter receptors, ion channels, and transporter sites [8]. Recently, a meta-analysis study has revealed a total of 378 differentially expressed miRNAs (274 up-regulated and 198 down-regulated) in post-SE models in at least one study [9].

miRNA-Let-7i, a member of the miRNA-Let-7 family, has been associated with various brain pathophysiological conditions, including inflammation, neuronal regeneration and apoptosis, and neurotransmitter disorders [7, 10–12]. Expression of miRNA-Let-7i has been shown to decrease in the brain tissues of animal models of Alzheimer's disease [10]. In another report, miRNA-Let-7i was revealed to be a negative regulator of BDNF and Pgrmc1 and can subsequently disrupt the release and protective effects of progesterone-induced BDNF against stroke [13]. In this study, we aimed to investigate if miRNA-Let-7i is also involved in SE.

Methods

Human subjects

Patients with intractable temporal lobe epilepsy (TLE) were recruited from Xinqiao Hospital of the Third Military University, Chongqing, China. They had typical clinical manifestations and electroencephalogram features, and were diagnosed with intractable TLE according to the 1981 International Classification of Epilepsy Seizures from the International League Against Epilepsy (ILAE). Before surgery, all patients underwent careful presurgical evaluations, which included a detailed history review, neurological examination, neuropsychological testing, interictal and ictal electroencephalograms, and neuroradiological assessment. The temporal neocortex of the control group was obtained from individuals who underwent craniocerebral surgery due to severe traumatic brain injury. The control patients had no history of epilepsy. Pathological assessments of the brain tissue from the control group were normal.

All patients gave written informed consent to participate in this study. This study protocol complied fully with the Declaration of Helsinki and was approved by the Ethics Committee of the Chongqing Medical University.

Animal experiments

Adult male Sprague–Dawley rats weighing 200–300 g were obtained from Chongqing Medical University, China, and housed at controlled room temperature (23 ± 1 °C) and light conditions (12 h light-dark cycles), with *ad libitum* access to water and food. All animal protocols were approved by the Commission of Chongqing Medical University for Ethics in Animal Experiments. All efforts were made during experiments to reduce the suffering of animals.

The agomir or antagomir (Guangzhou RiboBio Co., Ltd.) was injected (agomir, 2 nM in 4 µl; antagomir, 4 nM in 4 µl) bilaterally in the dorsal hippocampus (injection site: medial/lateral, ±2.5 mm; anterior/posterior, −3.2 mm; dorsal/ventral, −2.6 mm), at a speed of ~ 0.5 µl/min. DDH_2O (4 µl) was injected as a control.

Three days after intrahippocampal injection, the lithium pilocarpine-induced SE model was established as described elsewhere [14, 15]. In brief, the rats were intraperitoneally injected with lithium chloride (127 mg/kg; Sigma-Aldrich, St. Louis, MO), followed 20 h later by pilocarpine (50 mg/kg, i.p., Sigma-Aldrich) to induce the onset of SE. The seizure activity was rated according to the Racine's scale [16]: grade 0, no response; grade 1, staring and reduced locomotion; grade 2, activation of extensors and rigidity; grade 3, repetitive head and limb movements; grade 4, sustained rearing with clonus; and grade 5, generalized tonic-clonic seizures (GTCS) with loss of posture and death. Seizures were observed for 60 min. Then the animals were administered with diazepam (10 mg/kg, i.p.) to terminate seizures.

Western blotting analysis

The surgically resected temporal neocortex cortex tissues from patients and rat brain tissues were homogenized, respectively. Proteins were extracted using a protein extraction kit (Keygen Biotech, Nanjing, China) following the manufacturer's instructions. A BCA protein assay kit (Beyotime, China) was used to determine the protein concentration. Then the proteins were separated by SDS–PAGE (5% spacer gel, 60 V for 40 min; 12% and 10% separating gel, 120 V for 80 min) and electrotransferred onto polyvinylidene fluoride membranes at 250 mA for 60 min. Subsequently, the membranes were incubated at 4 °C overnight with the following primary antibodies: rabbit anti-Toll-like receptor 4 (TLR4) (1:1000, Proteintech, China), rabbit anti-IL-6 (1:1000, Proteintech, China), rabbit anti-TNG-α (1:1000, Proteintech, China), or rabbit anti-β-actin (1:3000, Proteintech, China). After 3 washes with Tween-20/Tris-buffered saline for 10 min, the membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (1:3000, Proteintech, China) for 1 h at 37 °C. Finally, the protein bands were visualized in a dark room with an enhanced chemiluminescence substrate (Pierce, Rockford, IL) and a digital scanning microscope (Bio-Rad Laboratories). The protein bands were analyzed with a Fusion FX5 image analysis system (Vilber Lourmat, France).

Real-time quantitative polymerase chain reaction (RT-qPCR)

In brief, total RNA was extracted using TRIzol reagent (Takara, Kusatsu, Shiga, Japan) according to the

manufacturer's instructions. Then RNA purity was determined by measuring the OD260/OD280 ratio using the expected values between 1.8 and 2.0. cDNA was generated using an Applied Biosystems Veriti1-Well Thermal Cycler (Thermo, Waltham, MA). The reverse transcription reaction was performed using PrimeScriptRT Reagent Kit with gDNA Eraser (Takara, Kusatsu, Shiga, Japan) and the miRNA-Let-7i primers at 37 °C for 15 min and 85 °C for 5 s. The RT-qPCR assay was performed in an Eppendorf Realplex Real-Time System (Eppendorf Mastercycle1 ep realplex, Germany) using SYBR1 Premix ExTaqTM II (Takara, Kusatsu, Shiga, Japan). The comparative CT method was used to calculate the relative expression of gene expression. The miRNA-Let-7i primers were as follows: hsa: 5'GGCTGAGGTAGTAGTTTG TGCTGTT3', rno: 5'GGCTGAGGTAGTAGTTTGTGC TGTT 3' (Takara, Kusatsu, Shiga, Japan).

Electrophysiology

The hippocampus was obtained 72 h after intrahippocampal injection of miRNA-Let-7i agomir, antagomir, or vehicle, and cut into coronal sections at 350 μm thickness in an ice-cold sterile slice solution (in mM: 2.5 KCl, 6 $MgCl_2.H_2O$, 1 $CaCl_2$, 1.25 $NaH_2PO_4.2H_2O$, 26 $NaHCO_3$, 220 sucrose, and 10 glucose). Afterward, the slices were perfused with artificial cerebral spinal fluid (ACSF) at pH 7.4, and saturated with a mixture of 5% CO_2 and 95% O_2 at 35 °C for 1 h.

To determine the cell excitability, action potentials (APs) were recorded from pyramidal neurons in the CA1 region using the whole-cell clamp technique. The internal solution contained (in mM) 4 $MgCl_2$, 60 K_2SO_4, 40 HEPES, 60 NMG, 12 phosphocreatine, 0.5 BAPTA, 0.2 Na_3GTP, and 2 Na_2ATP (265–275 mOsm; pH 7.3–7.4). To stimulate seizure activity, a magnesium-free ACSF was applied for 1 h, which was then replaced with normal ACSF. Signals were analyzed with a MultiClamp 700B amplifier (Axon, Union City, CA, USA), digitized at 10 kHz and filtered at 2 kHz. Signals were acquired with the pClamp 9.2 software (Molecular Devices, Sunnyvale, CA).

Nissl staining and TUNEL assay

TUNEL assay was performed to measure the apoptotic neurons using a commercial kit (Roche, Germany). Only TUNEL-positive neurons with nuclear condensate were counted. Nissl staining was performed to detect surviving neurons. Briefly, frozen slices were stained with 0.5% cresyl violet at 50 °C for 5 min. Only neurons with intact morphology and violet nuclei were counted.

Five randomly selected non-overlapping fields in the CA1 and CA3 regions were used for cell counting. The following formula were used for calculation: survival index (%) = (number of surviving neurons/total number of neurons) × 100%, apoptosis index (%) = (apoptotic neurons/total neurons) × 100%.

Immunofluorescence staining

Tissue sections were fixed in 4% polyformaldehyde for 1 min, washed 3 times with PBS, permeabilized with 0.4% Triton X-100 for 15 min at 37 °C, and incubated with 10% goat serum (Zhongshan Golden Bridge) for 30 min at 37 °C. Thereafter, the sections were counterstained with 4′,6 diamidino-2-phenylindole (DAPI) (Sigma-Aldrich) for 5 min to visualize the nuclei and subsequently washed with PBS. Finally, fluorescence was determined by a laser scanning confocal microscope equipped with a FluoView FVX confocal scan head (Leica Microsystems Heidelberg GmbH, Germany).

Statistical analyses

Data are presented as mean ± SD, and were analyzed with Student's *t*-test. $P < 0.05$ was considered as statistically significant. All statistical analyses were implemented using SPSS software, version 13.0.

Results

miRNA-let-7i expression in brain tissues of pilocarpine-induced SE rats

Results of RT-qPCR showed that compared with the control group, the expression level of miRNA-Let-7i was significantly reduced in both the cortex and hippocampus of the pilocarpine-induced SE rats (Fig. 1), which suggested that the miRNA-Let-7i expression change in SE may be a concomitant phenomenon or essentially involved in SE. To verify this possibility, we subsequently explored the effect of the miRNA-Let-7i intervention on SE activity.

Effects of agomir and antagomir on miRNA-let-7i expression

The expression of miRNA-Let-7i was evaluated at 3 days, 1 week, 2 weeks, and 4 weeks after hippocampal administration of agomir or antagomir. We observed that the expression of miRNA-Let-7i in the hippocampus was up-regulated on day 3 after administration of agomir, while this alteration was not seen in control rats. In addition, the increase of miRNA-Let-7i lasted for 4 weeks but it was lower than that on day 3. On the other hand, administration of the antagomir had opposite effects (Fig. 2a, b). We further evaluated the site of expression of agomir and antagomir by immunofluorescence staining. We found that on day 3 after injection, agomir- and antagomir-bearing GFP was localized in the CA1 and CA3 regions (Fig. 2c).

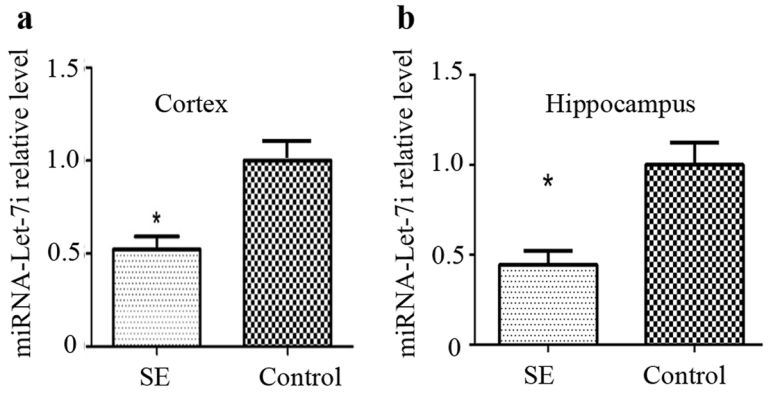

Fig. 1 RT-qPCR analysis of miRNA-Let-7i expression in the pilocarpine-induced SE model rats. Compared with the control group, miRNA-Let-7i expression in the SE group was reduced in both the cortex and the hippocampus (*$P < 0.05$ *versus* control group; $n = 4$ per group)

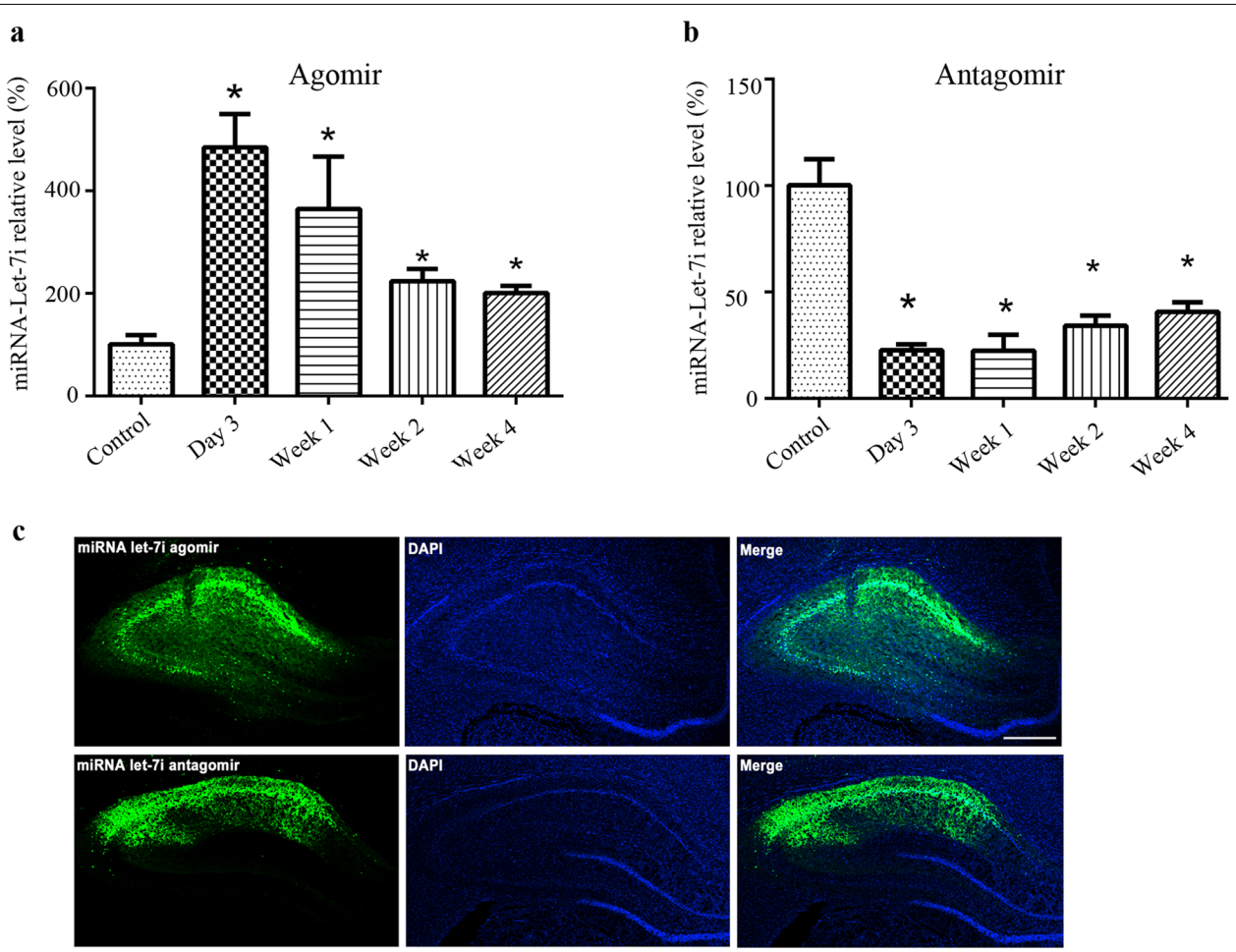

Fig. 2 Effects of the agomir and antagomir on miRNA-Let-7i expression. **a** Relative expression of miRNA-Let-7i in the control group and the agomir-treated group on day 3, week 1, week 2 and week 4 after pilocarpine-induced SE. **b** Relative expression of miRNA-Let-7i in the control group and the antagomir-treated group at day 3, week 1, week 2 and week 4 after pilocarpine-induced SE (*$P < 0.05$ *versus* control group; $n = 4$ per group). **c** Representative images showing GFP expression in the CA1 and CA3 at 3 days after agomir and antagomir injection, and cell nuclei were counterstained with DAPI. Scale bar, 500 μm

miRNA-let-7i regulates pilocarpine-induced SE and neuronal excitability

Based on the changes of miRNA-Let-7i expression after agomir/antagomir administration, we conducted the pilocarpine-induced SE experiment on day 3 following administration. We found that the agomir significantly reduced the degree of seizures, whereas the antagomir increased it (Fig. 3a). The latency from pilocarpine administration to the onset of SE, which reflects the susceptibility to SE induction, was prolonged in the agomir group, while being shortened in the antagomir group (Fig. 3b). The results suggested that the miRNA-Let-7i agomir inhibited SE whereas the antagomir promoted SE, implying that miRNA-Let-7i is involved in SE.

Furthermore, we characterized the function of miRNA-Let-7i in neuronal excitability using the whole-cell patch-clamp technique. Compared with the vehicle group, the agomir decreased the frequency of AP firing, while the antagomir substantially increased it (Fig. 3d). These results indicated that miRNA-Let-7i regulates neuronal excitability.

miRNA-let-7i regulates neuronal apoptosis

Numerous pathological changes, such as neuronal apoptosis, glial hyperplasia, mossy fiber spouting, and synaptic remodeling in neural tissue can occur after SE. To examine the effect of miRNA-Let-7i expression alteration on neuronal apoptosis, rats were intrahippocampally injected with agomir or antagomir one day

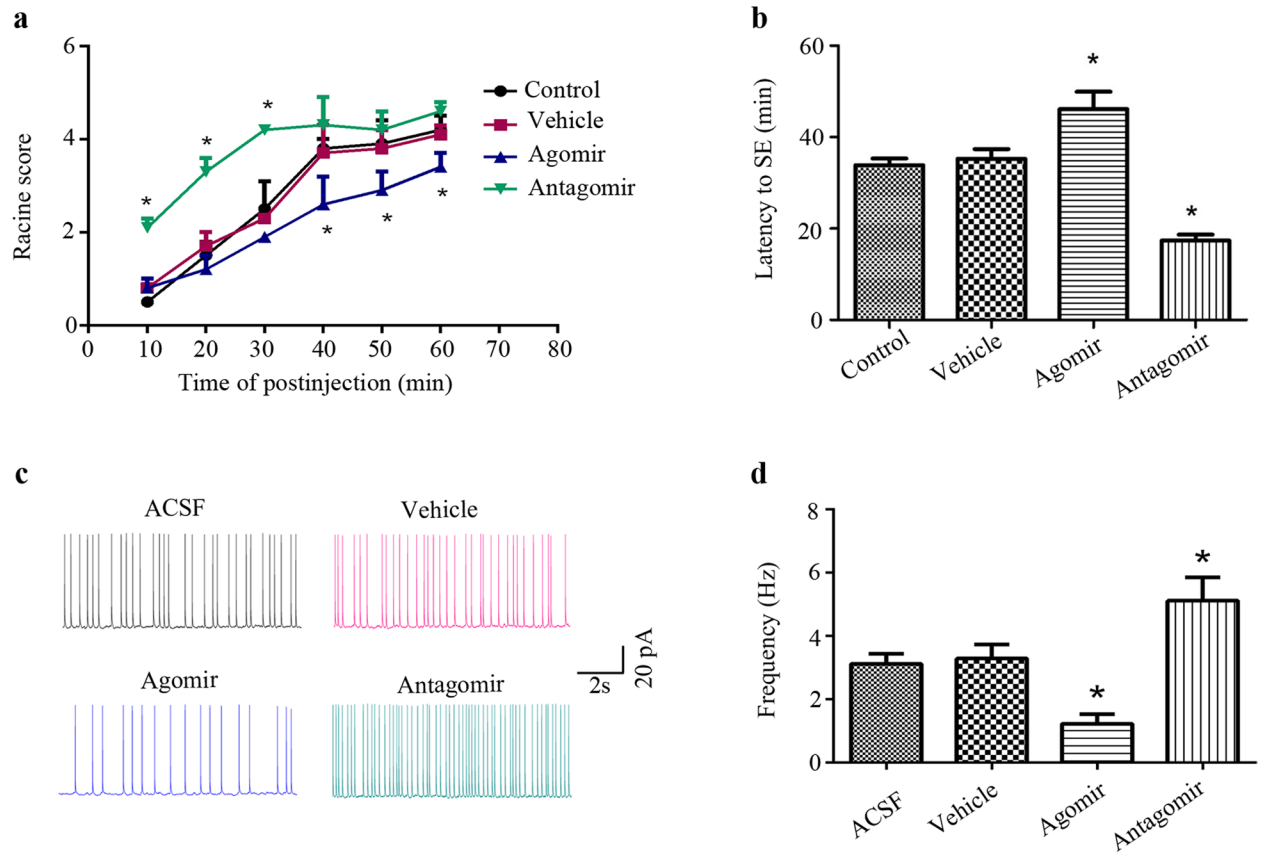

Fig. 3 Effects of the agomir and the antagomir on the pilocarpine-induced SE ($n = 8$ in each group; $^*P < 0.05$). **a** Compared with the vehicle group, the SE Racine scale was significantly decreased in the agomir group at 40 min, 50 min and 60 min ($^*P < 0.05$), and it was significantly increased in the antagomir group at 10 min, 20 min, and 30 min ($^*P < 0.05$). **b** The latency of the agomir group (46.13 ± 3.80 min) was significantly longer than that of the SE group ($P < 0.05$), and the latency of the antagomir group was significantly shorter than that of the SE group (17.38 ± 1.30 min) ($P < 0.05$). There was no significant difference in the latency between the SE group (33.88 ± 1.43 min) and the vehicle treatment group (35.25 ± 2.10 min) ($P > 0.05$). **c** Representative images of action potentials of pyramidal neurons in the CA1 region in Mg^{2+}-free ACSF in the presence of vehicle, the agomir and the antagomir. No difference was observed between the ACSF and vehicle groups. **d** Compared with the vehicle group, the agomir significantly decreased AP firing frequency, while the antagomir significantly increased it. Data are presented as the mean ± SD, $^*P < 0.05$ *versus* vehicle group; $n = 5$ per group

after SE induction. Three days later, neuronal apoptosis and survival were assessed using TUNEL assay and Nissl staining, respectively, in the CA1 and CA3 regions of the hippocampus.

In the CA1 region, the neuronal survival in the agomir group exhibited a trend of increase compared with the vehicle group, but with no significant difference. Conversely, the neuronal survival in the antagomir group was significantly lower than that of the vehicle group (Fig. 4a, b). Additionally, in the CA3 region, the neuronal survival index was significantly increased in the agomir group and decreased in the antagomir group, compared with the vehicle group (Fig. 4a, c). In both the CA1 and CA3 regions, the neuronal apoptosis was significantly lower in the agomir group and higher in the antagomir group, compared with the vehicle group (Fig. 4d-f).

TLR4 is involved in the effect of miRNA-let-7i on SE

Previously, it has been shown that TLR4 may be a downstream target of miRNA-Let-7i. Besides, multiple lines of evidence have highlighted that antagonism of TLR4 can reduce both the incidence and the severity of seizures. Therefore, we next tested if miRNA-Let-7i is involved in SE *via* the TLR4 signaling pathway.

First, we determined the expression levels of TLR4 in the rat hippocampus at 3 days, 1 week, 2 weeks, and 4 weeks after intrahippocampal injection of agomir or antagomir. Compared with the control group, the protein level of TLR4 in the hippocampus was significantly decreased at 4 weeks after agomir administration, while it was considerably increased at the same time point after antagomir administration. Notably, these alterations peaked on day 3 after administration (Fig. 5a). We also found that the protein level of TLR4 and miRNA-Let-7i expression

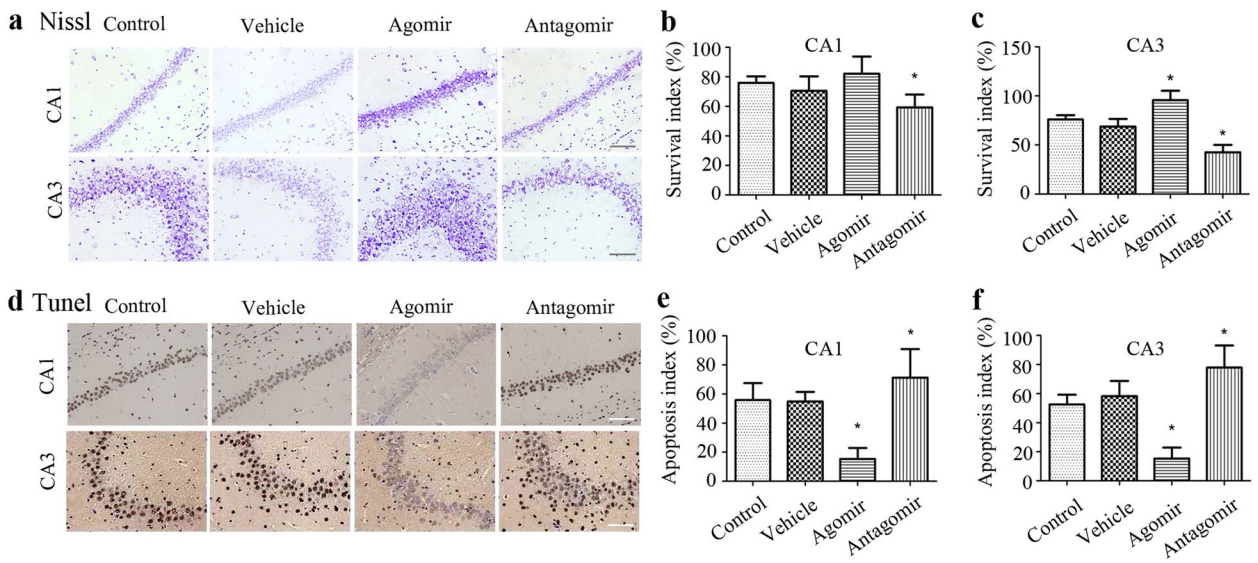

Fig. 4 Effects of the agomir and the antagomir on the survival and apoptosis of neurons in the CA1 and CA3 regions. **a** Representative images of Nissl staining. Scale bar: 100 μm. **b**, **c** Survival index (%) in the CA1 (**b**) and CA3 (**c**) regions of the different groups (*$P < 0.05$ *versus* vehicle group; $n = 3$ per group). **d** Representative images of TUNEL staining in the rat hippocampus. Scale bars: 100 μm. **e**, **f** Apoptosis index (%) in the CA1 (**e**) and CA3 (**f**) regions in the different groups (*$P < 0.05$ *versus* vehicle group; $n = 3$ per group). Mean ± SD

(See figure on next page.)

Fig. 5 **a** Representative Western blot images of TLR4 in the control group and the agomir treatment group at day 3, week 1, week 2 and week 4 after pilocarpine-induced SE. **b** Quantification of immunoblot intensity in (**a**) (*$P < 0.05$ *versus* control group; $n = 4$ per group). **c** Representative Western blot images of TLR4 in the control group and the antagomir treatment group at day 3, week 1, week 2 and week 4 after pilocarpine-induced SE. **d** Quantification of immunoblot intensity in (**c**) (*$P < 0.05$ versus control group; $n = 4$ per group). **e** Behavior analysis of pilocarpine-induced SE showed that the degree of seizures in the antagomir group at various time points (10 min, 20 min, 30 min) was significantly higher than that of the vehicle group, while this increase was reversed in the antagomir + TAK-242 group. **f** The latency to SE in the antagomir group, antagomir+TAK-242 group and vehicle group (*$P < 0.05$ versus vehicle group; $n = 8$ per group). **g** Representative Western blots of TLR4 in the different groups. **h** Quantification results showed that the antagomir increased the expression of both IL-6 and TNF-α, whereas this increase was reversed in the antagomir+TAK-242 group (*$P < 0.05$ versus vehicle group; $n = 8$ per group)

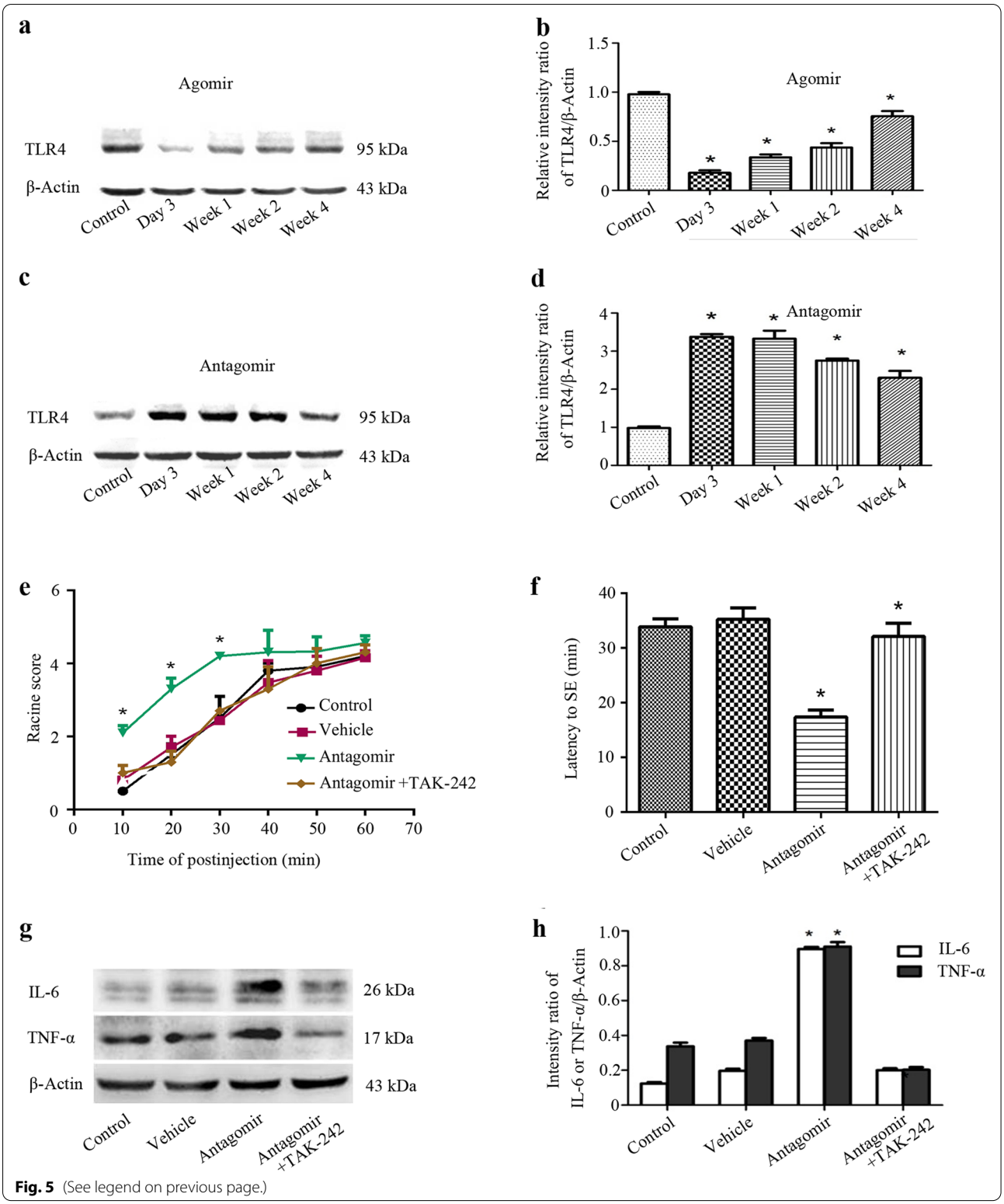

Fig. 5 (See legend on previous page.)

exhibited corresponding changes after miRNA-Let-7i agomir and antagomir administration (Fig. 5a-d).

To test if the change in TLR4 protein level was an accompanying phenomenon or a result of the altered miRNA-Let-7i expression, we systematically administered a selective inhibitor of TLR4 (TKA-242 (0.5 mg/kg)), through the caudal vein, twice on the third day after antagomir injection as previously reported. On the fourth day, pilocarpine was administered to induce SE. Compared with the antagomir + vehicle group, the degree of seizures was inhibited in the antagomir + TKA-242 group, which was comparable with that of the control group (Fig. 5e). Moreover, the reduction in the latency from pilocarpine administration to the onset of SE in the antagomir + vehicle group was rescued by TKA-242 (Fig. 5f).

As IL-6 and TNF-α are essential downstream inflammatory factors of the TLR4 signaling pathway, we also evaluated the protein levels of IL-6 and TNF-α in the hippocampus of rats after the behavioral study. The results showed that the antagomir significantly increased their protein levels, which were rescued by the TLR4 inhibitor (Fig. 5g-h).

Altered expression of miRNA-let-7i and TLR4 in the brain tissues of TLE patients

Based on the animal experiments, we identified that miRNA-Let-7i regulates SE *via* the TLR4 signaling pathway. Since animal models differ greatly from the humans, we subsequently probed whether the expression of the TLR4 protein was altered in tissues collected from epileptic patients. Specifically, we assessed the expression of miRNA-Let-7i and TLR4 protein level in the temporal neocortices from patients with pharmacoresistant TLE ($n = 10$), and in the temporal neocortices from patients who had suffered from head trauma, as the control group ($n = 10$). There were no significant differences in the sex or age between the groups ($P > 0.05$, Table 1). Compared with the control group, the expression of miRNA-Let-7i was significantly lower in the TLE patients (Fig. 6A), whereas the protein level of TLR4 was significantly higher in the TLE patients compared to the control group (Fig. 6B, C). Notably, these changes were consistent with those seen in the rat model of SE.

Discussion

In this study, we found that the expression of miRNA-Let-7i was decreased in brain tissues of both SE rats and TLE patients. Behavioral testing showed that miRNA-Let-7i agomir and antagomir affected the degree and

Table 1 Clinical characteristics of the TLE and control patients

Participant No.	Sex	Age (years)	Disease course (years)	AEDs before surgery	Resection tissue	Pathology
T1	M	57	12	TPM, PHT, CBZ	TNl	G
T2	M	26	14	VPA, CBZ, TPM	TNr	G, NL
T3	F	44	16	CBZ, VPA, TPM, PHT	TNl	G, NL
T4	M	45	24	VPA, CBZ, LTG, TPM	TNl	G, NL
T5	M	36	15	VPA, PB, CBZ	TNl	G, NL
T6	F	18	10	PB, CBZ, VPA, PHT	TNr	G, NL
T7	M	32	10	CBZ, VPA, OXC	TNl	G, NL
78	M	29	4	CBZ, CZP, VPA	TNl	G, NL
T9	F	27	7	CBZ, CZP, LTG	TNr	G, NL
T10	F	31	8	CBZ, PHT, LGT, CZP,	TNr	G, NL
C1	M	39	0	NO	TNr	N
C2	M	58	0	NO	TNl	N
C3	F	41	0	NO	TNl	N
C4	M	29	0	NO	TNl	N
C5	M	44	0	NO	TNr	N
C6	M	23	0	NO	TNl	N
C7	F	22	0	NO	TNl	N
C8	F	49	0	NO	TNr	N
C9	M	17	0	NO	TNr	N
C10	M	49	0	NO	TNr	N

T TLE, *C* Controls, *F* female, *M* male, *AEDs* anti-epileptic drugs, *PB* Phenobarbital, *CBZ* carbamazepine, *VPA* valproic acid, *PHT* phenytoin, *CZP* clonazepam, *OXC* oxcarbazepine, *LEV* levetiracetam, *LTG* lamotrigine, *TPM* topiramate, *TN* temporal neocortex, *l* left, *r* right, *NL* neuronal loss, *G* gliosis, *N* normal

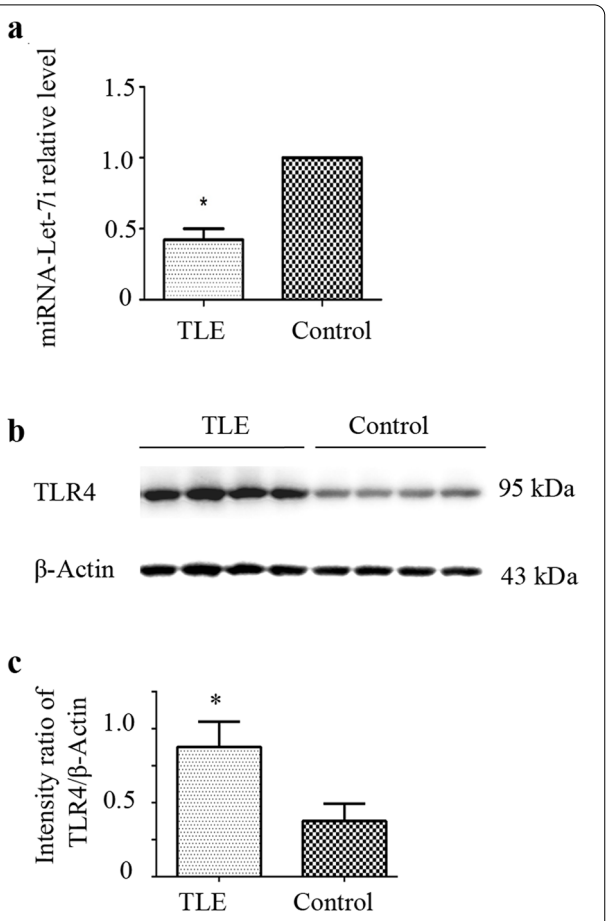

Fig. 6 Evaluation of miRNA-Let-7i expression and TLR4 protein level in TLE patients. **a** The expression of miRNA-Let-7i was significantly decreased in the brain tissues of TLE patients compared with that in the control patients. **b** Representative Western blots of TLR4 in the brain tissues of TLE patients and controls. **c** Quantification of (**b**) showed that the protein level of TLR4 was significantly increased in TLE patients compared with controls (*$P < 0.05$ *versus* control group; $n = 10$ in TLE group and $n = 10$ in control group)

the latency of SE. On the other hand, whole-cell current clamp recordings showed that the frequency of AP firing was also changed after agomir or antagomir administration. Results of Nissl staining and TUNEL assay revealed that the agomir increased the survival and reduced the apoptosis of neurons. Moreover, the TLR4 inhibitor, TKA-242, rescued the effects of antagomir on SE behavior and protein levels of IL-6 and TNF-α. In summary, these results suggest that miRNA-Let-7i modulates SE *via* the TLR4 signaling pathway.

Let-7 is a highly conserved miRNA among animal species and functions as a potent tumor suppressor that is crucial for normal development. In mammals, the level of let-7 increases especially during embryogenesis and brain development [17]. In most organisms, studies have shown that dysregulation of let-7 can result in the onset of many diseases, including neurodegenerative diseases, such as Parkinson's disease, amyotrophic lateral sclerosis, and Alzheimer's disease [18]. A recent study has shown that miRNA-let-7i is a negative regulator of BDNF and Pgrmc1 in glia, while inhibiting let-7i enhances the progesterone-induced neuroprotection and facilitates functional recovery following stroke [13]. Nevertheless, the effect of miRNA-Let-7i on epilepsy remains unclear. Here, we found that the expression of miRNA-Let-7i was significantly decreased in both brain tissues obtained from the pilocarpine-induced acute SE rat model and from patients with refractory epilepsy, compared to their respective control group.

We also evaluated the pilocarpine-induced acute SE behavior in rats. It has been reported that pilocarpine can excite the M-type cholinergic receptors to activate the glutamate excitability pathway, thus inducing acute SE in animals. Further, numerous reports have emphasized that this model well simulates the characteristics and pathological changes of human SE [19, 20]. In this study, we did not use an agomir or antagomir scramble as control but instead used the agomir/antagomir solvent ddH_2O as a control. We found that the up-regulation of miRNA-Let-7i by agomir prolonged the latency to epileptic seizures and reduced the severity of seizures, while inhibition of the expression of miRNA-Let-7i by the antagomir shortened the latency to SE. These findings indicate that miRNA-Let-7i is involved in SE, which provides insights into the pathogenesis of SE and suggests that up-regulating miRNA-Let-7i may be a novel strategy for treating SE. However, this result is inconsistent with another study showing that inhibition of Let-7i had neuroprotection effects in a mouse model of ischemia [13]. Further studies should be performed to address the mechanisms of the opposite effects.

According to the literature, neuronal apoptosis is the basic pathology of SE [21]. Previous studies have demonstrated that knockdown of miRNA-Let-7i can inhibit nerve regeneration [12] and participates in neuronal differentiation, apoptosis and regeneration, which suggests its potential neuroprotective effect [22]. In this study, three days after agomir or antagomir injection into the hippocampus, SE was induced by pilocarpine. Neuronal apoptosis was assessed 3 days after SE using Nissl and TUNEL staining. Our results showed that the apoptosis of neurons in the hippocampal CA1 and CA3 regions in rats after agomir intervention was substantially lower than that in the control group.

TLR4 is a cell-based pattern recognition receptor that mediates binding of exogenous ligands such as lipopolysaccharide and facilitates inflammatory

reactions [23, 24]. Inflammatory cytokines such as IL-6, TNF-α and IFN-γ can be activated by NF-κB in the TLR4-mediated MyD88 adapter-like protein pathway. In addition, inhibition of the TLR4 signaling pathway can reduce the immune-inflammatory response and prevent SE [24–26]. In particular, TAK-242 is a specific inhibitor of TLR4 that affects its normal function [27]. Here, we used TAK-242 to block the function of TLR4, and showed that it rescued the antagomir-induced aggravation of SE. Additionally, TLR4 and its downstream inflammatory factors IL-6 and TNF-α are closely associated with the pathogenesis of SE based on previous studies [28, 29]. It is also important to note that blocking TLR4 can alleviate inflammation and improve SE. In this study, the expression levels of TNF-α and IL-6 in the antagomir group were significantly increased compared with the control group. Furthermore, IL-6 and TNF-α protein levels were considerably rescued by TAK-242 in the antagomir group. Overall, these results suggest that TAK-242 could reduce the severity of SE.

Conclusions

In summary, in this study, we found that the miRNA–Let-7i-TLR4 regulatory pathway in the hippocampus is involved in the pathogenesis of SE, which provides useful and novel insights into the mechanisms and the prevention of SE.

Abbreviations

SE: Status epilepticus; TLR4: Toll-like receptor 4; TLE: Temporal lobe epilepsy; miRNA: microRNA; AP: Action potential; TUNEL: Terminal deoxynucleotidyl transferase-mediated dUTP end-labeling; ILAE: International League Against Epilepsy; GTCS: Generalized tonic-clonic seizures; RT-qPCR: Real-time quantitative polymerase chain reaction; ACSF: Artificial cerebral spinal fluid.

Acknowledgements

The authors would like to thank all the patients and their families for their participation in this study.

Authors' contributions

Ou shu, Yangmei Chen and Haiyan Luo contributed to the experimental design. Shu Ou, Xi Liu, Tao Xu, Xinyuan Yu, Teng Wang and Ying Liu performed experiments. Shu Ou drafted the article. Haiyan Luo revised the manuscript critically. All authors read and approved the final manuscript.

Funding

This work was supported by the Natural Science Foundation Project of Chongqing (cstc2020jcyj-msxmX0611) and the Key Disciplines Program of the Fengjie People's Hospital (2020ZDXY001).

Availability of data and materials

All data generated or analyzed in this study are included in this manuscript and its additional files.

Declarations

Ethics approval and consent to participate

The experiments were conducted in accordance with the Declaration of Helsinki and approved by the ethical committee on human research at the Second Affiliated Hospital of Chongqing Medical University in 2015 (No. 35). Written informed consents were obtained from family members of all patients. For the animal experiment, all procedures used on mice were approved by the Commission of Chongqing Medical University for Ethics of Experiments on Animals and were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Consent for publication

All the patients or their guardian agreed for the publication of this study.

Competing interests

The authors declare that they had no competing interests.

Author details

[1]Department of Neurology, The Fengjie People's Hospital, Chongqing 404600, China. [2]Department of Neurology, The Second Affiliated Hospital of Chongqing Medical University, Chongqing 400010, China.

Received: 2 November 2020 Accepted: 14 March 2022
Published online: 06 July 2022

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