

The microRNA miR-124 suppresses seizure activity and regulates CREB1 activity

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miR-124, a brain-specific microRNA, was originally considered as a key regulator in neuronal differentiation and the development of the nervous system. Here we showed that miR-124 expression was suppressed in patients with epilepsy and rats after drug induced-seizures. Intrahippocampal administration of a miR-124 duplex led to alleviated seizure severity and prolonged onset latency in two rat models (pentylentetrazole- and pilocarpine-induced seizures), while miR-124 inhibitor led to shortened onset latency in pilocarpine-induced seizure rat models. Moreover, the result of local field potentials (LFPs) records further demonstrated miR-124 may have anti-epilepsy function. Inhibition of neuronal firing by miR-124 was associated with the suppression of mEPSC, AMPAR- and NMDAR-mediated currents, which were accompanied by decreased surface expression of NMDAR. In addition, miR-124 injection resulted in decreased activity and expression of cAMP-response element-binding protein1 (CREB1), a key regulator in epileptogenesis. A dual-luciferase reporter assay was used to confirm that miR-124 targeted directly the 3'UTR of CREB1 gene and repressed the CREB1 expression in HEK293T cells. Immunoprecipitation studies confirmed that the CREB1 antibody effectively precipitated CREB1 and NMDAR1 but not GLUR1 from rat brain hippocampus. These results revealed a previously unknown function of miR-124 in neuronal excitability and provided a new insight into molecular mechanisms underlying epilepsy.

Introduction

Epilepsy is a collection of brain disorders that are characterised by recurrent unprovoked seizures and affect 1–2% of the world's population (Ref. 1). Regardless of its underlying causes, a seizure reflects an imbalance between inhibition and excitation that leads to abnormal hypersynchronous electrical activity of neuronal networks (Ref. 2). Recent study has revealed that epilepsy is associated with a cascade of molecular, cellular, and structural alterations (Ref. 3). However, most currently used anti-epileptic drugs (AEDs) fail to prevent or cure the disease, and 20–30% of patients remain refractory to treatment (Refs 4, 5).

MicroRNAs (miRNAs) are endogenous 23-nucleotide RNAs that play an important role in gene regulation. The targets of miRNAs include mRNAs that encode transcription factors, components of the miRNA machinery, and other proteins involved in translational regulation (Refs 6, 7, 8). In vertebrates, distinct miRNAs are expressed in the brain than in any other tissue and play critical roles in multiple neurological diseases including epilepsy (Refs 9, 10, 11). In the nervous system, miRNAs are involved in the control of synaptic function and plasticity (Refs 12, 13, 14).

In pilocarpine-induced seizures, a robust, rapid, and transient increase in the levels of the primary transcript of miR-132 (pri-miR-132) is followed by a subsequent increase in the levels of mature miR-132 (Ref. 15). In vivo microinjection of miR-132 antagonists results in a reduction of seizure-induced neuronal death (Ref. 16). Evidence showed that knockdown or over expression of miRNA may potentially improve functional outcomes in patients with neurological diseases (Refs 10, 17, 18).

MiR-124 is highly abundant in the brain, retina and spinal cord. The expression of miR-124 was first detected in differentiating neurons, suggesting a role for this molecule in neural development (Refs 19, 20, 21). A recent study showed that miR-124 could function at the growth cone or at synapses by modulating synaptic activity and neuronal connectivity (Ref. 22). More importantly, miR-124 is also involved in neurological diseases. For instance, miR-124 is downregulated in brain tumours (Refs 23, 24). In experimental autoimmune encephalomyelitis (EAE), which is an animal model of multiple sclerosis, miR-124 is suppressed in activated microglia (Ref. 25). A potential role of miR-124 in Alzheimer's disease and

TABLE 1.
CLINICAL CHARACTERISTIC OF TLE PATIENTS

Number	Age (Y)	Sex (M/F)	Course (Y)	AEDs before surgery	Resection tissue
1	23	M	4	VPA, TPM, PHT	TNl
2	32	M	6	VPA, CBZ, OXC	TNr
3	24	M	17	PB, PHT, TPM	TNr
4	31	F	12	CBZ, VPA, TPM	TNr
5	45	M	10	TPM, LTG, CBZ	TNl
6	30	M	18	VPA, TPM, GBP	TNl
7	17	F	6	VPA, TPM, CBZ	TNl
8	38	M	11	CBZ, TPM, LTG, PHT	TNr
9	29	F	12	VPA, TPM, OXC	TNl
10	47	F	14	VPA, PB, LTG, PHT	TNr
11	36	F	16	TPM, LTG, CBZ, OXC	TNl
12	22	M	7	VPA, CBZ, LTG	TNl

AEDs, antiepileptic drugs (taken before operation); CBZ, carbamazepine; F, female; GBP, gabapentin; LTG, lamotrigine; l, left; M, male; OXC, oxcarbazepine; PB, phenobarbital; PHT, phenytoin; r, right; TN, temporal neocortex; TPM, topamax; VPA, valproic acid; y, year.

Parkinson's disease has been recently suggested (Ref. 26). These results suggest that miR-124 may serve as a therapeutic target in neurological disorders. However, it remains unclear whether miR-124 is involved in regulation of neuronal excitability and seizures.

In the current study, we have observed that miR-124 is downregulated in epilepsy, as intrahippocampal supplementation with miR-124 inhibited neuronal firing and excitability, as well as susceptibility to epileptic seizures. Furthermore, we have identified that miR-124 regulated the CREB1 gene and CREB1 protein expression. Thus, our findings provide valuable information towards unveiling the mechanisms of human temporal lobe epilepsy (TLE), and a novel target of potentially effective clinical therapies in the future.

Materials and methods

Human subjects

The patients with medically intractable TLE included in our study had typical clinical manifestations and characteristic electroencephalograms. Presurgical assessment consisted of obtaining a detailed history and neurological examination, interictal and ictal electroencephalogram studies, neuropsychological testing, and neuroradiological studies, such as magnetic resonance imaging (MRI).

Temporal neocortex tissues from 12 patients [7 males and 5 females; mean age, 31.17 ± 9.15 years (range, 17–47 years); mean disease course, 11.08 ± 4.64 years (range, 4–18 years)] were chosen at random among 223 specimens from our epilepsy brain tissue bank. All patients were refractory to the maximal dose of at least three AEDs (Table 1). We used temporal neocortex from patients treated for increased intracranial pressure because of head trauma tissue as control. The control subjects had no history of epilepsy or exposure to AEDs, and had no other neurological diseases [three males and three females; mean age, 28.33 ± 13.98 years (range,

15–54 years)] (Table 2). There were no significant differences in age and sex between the TLE and control groups ($P > 0.05$).

Each patient included in this study provided a signed consent form, and our study protocol complied with the guidelines for the performance of research involving human subjects, as established by the National Institutes of Health and the Committee on Human Research at the Chongqing Medical University. The research was conducted in accordance with the Declaration of Helsinki of the World Medical Association.

Animals and treatment

Administration of pilocarpine and intrahippocampal injection. All animal experiments complied with the guide for the Care and Use of Laboratory Animals of the Chongqing Medical University. Lithium-pilocarpine-induced seizures were as described previously (Refs 27, 28). Healthy adult male Sprague–Dawley rats (from the Chongqing Medical University Laboratory Animal Center) weighing 200–300 g were used as experimental subjects.

All rats were housed for 1 week before experiments were performed. The experimental group was divided randomly into the normal control group ($n = 8$) and the experimental groups ($n = 40$). The experimental groups were divided randomly into five subgroups

TABLE 2.
CLINICAL CHARACTERISTICS OF CONTROL GROUP

Number	Age (Y)	Sex (M/F)	Resection tissue
1	24	M	TNl
2	18	F	TNr
3	15	M	TNr
4	27	F	TNr
5	32	F	TNl
6	54	M	TNl

F, female; l, left; M, male; r, right; TN, temporal neocortex; y, year.

TABLE 3.
PRIMERS SEQUENCES USED FOR REAL-TIME PCR

Gene	Forward and reverse primers	Amplified fragment (bp)
GAPDH	5' CATCAAGAAGGTGGTGAAGCA 3' 5' TCAAAGGTGGAGGAGTGGGT 3'	117
CREB1	5' TGCAGACATTAACCATGACCA 3' 5' GTTGCTGGGCACTAGAATCTG 3'	104

according to the period after onset of seizures: 6 h, 1 day, 2 days, 3 days and 7 days. Rats in the experimental group were injected intraperitoneally with lithium chloride (127 mg/kg, i.p.; Sigma–Aldrich, St. Louis, MO, USA) 18 h before the first pilocarpine administration (50 mg/kg, i.p., Sigma–Aldrich, St. Louis, MO, USA). Pilocarpine (10 mg/kg, i.p.) was given repeatedly every 30 min until the rats developed seizures. Roughly 60 min after the onset of the status epilepticus, the animals were injected with diazepam (10 mg/kg, i.p.) to terminate seizures. Seizure activity was rated according to Racine's scale, and only those reached stages 4 or 5 were considered as being successfully kindled (Ref. 29). Control animals were given an intraperitoneal injection of saline.

Intrahippocampal injection was performed as described previously (Refs 30, 31, 32). The CY3-tagged miR-124 mimics (agomir) and FAM-tagged miR-124 inhibitor (antagomir) and the scrambled control miRNA were provided by Guangzhou RiboBio Co., Ltd. (Guangzhou, China) (Ref. 33). miR-124 mimics and inhibitor were RNA duplex and were chemically modified and cholesterol conjugated from a hydroxypropylol-linked cholesterol solid support and 2'-OMe phosphoramidites. Rats were given one treatment of miR-124 mimics (1 nM in a total volume of 5 µl) (RiboBio, Guangzhou, China) or inhibitor (4 nM in a total volume of 5 µl) (RiboBio, Guangzhou, China) into the dorsal hippocampus (injection site: anterior/posterior, –3.3 mm; medial/lateral, ±1.8 mm; dorsal/ventral, –2.6 mm). Mimics-negative control sequences and inhibitor-negative control sequences were injected into the hippocampus as negative control. 72 h after intrahippocampal injection, the rats pre-treated by miR-124 mimics, inhibitor and scrambled controls were treated by pilocarpine administration. One day

post pilocarpine injection, hippocampal tissues were obtained from rats.

To detect the expression of the miR-124 mimics and inhibitor, tissues were sectioned and mounted directly on slides. The intensity of fluorescence was directly detected using a laser scanning confocal microscope (Leica Microsystems Heidelberg GmbH, Germany) equipped with a Fluoview FVX confocal scanning head.

Administration of pentylenetetrazole (PTZ). Healthy adult male Sprague–Dawley rats (from the Chongqing Medical University Laboratory Animal Center) weighing 200–300 g were used as experimental subjects. The experimental group was divided randomly into the normal control group ($n = 8$) and the experimental groups ($n = 16$). The experimental groups were divided randomly into the mimics injection group and the mimics control injection group. Seizures were induced by i.p. injection of PTZ (50 mg/kg). At a dose of PTZ of 50 mg/kg, rats developed seizures and had recovered fully by the time of sacrifice (Ref. 34).

Western blot analysis. Western blot analysis was performed as described previously (Ref. 35). Total and membrane proteins were extracted according to the manufacturers' instructions (Keygen Biotech, Nanjing, China for total proteins; Beyotime Institute of Biotechnology, Shanghai, China for membrane proteins). Protein concentrations were determined using the Enhanced BCA Protein Assay Kit (Beyotime Institute of Biotechnology, Shanghai, China). A total of 50 µg of protein was loaded and then separated by SDS–PAGE (5% spacer gel; 10% separating gel). The primary antibodies used were as follows: rabbit anti-pCREB (1:500, Cell Signaling, Boston, USA),

TABLE 4.
PRIMERS SEQUENCES USED FOR FUSION-PCR

Gene	Forward and reverse primers
CREB-F	5' GCGCTCGAGGTTCCAACACCTGCCTCCA 3'
CREB-R	5' AATGCGCCGCTGGTGGTGGTATGTAAGTG 3'
CREB-mut-F	5' TCCTTGATACGGAATGGGACAGAATTACCCAG 3'
CREB-mut-R	5' CTGTCCCATTCGGTATCAAGGAGCTCAGCACAA 3'
NR1-F	5' CGGGCGATCGCGTCAGCACCGTGGTGTGAG 3'
NR1-R	5' AATGCGCCGCCACAGACCCGAAACTGCGT 3'
NR1-mut-F	5' CCGAGCGCCACGGAACCCCGTGGCGCCCGTGCG 3'
NR1-mut-R	5' GCACGGGTTCCGTGGCGCTCGGGCCCTGGGAG 3'

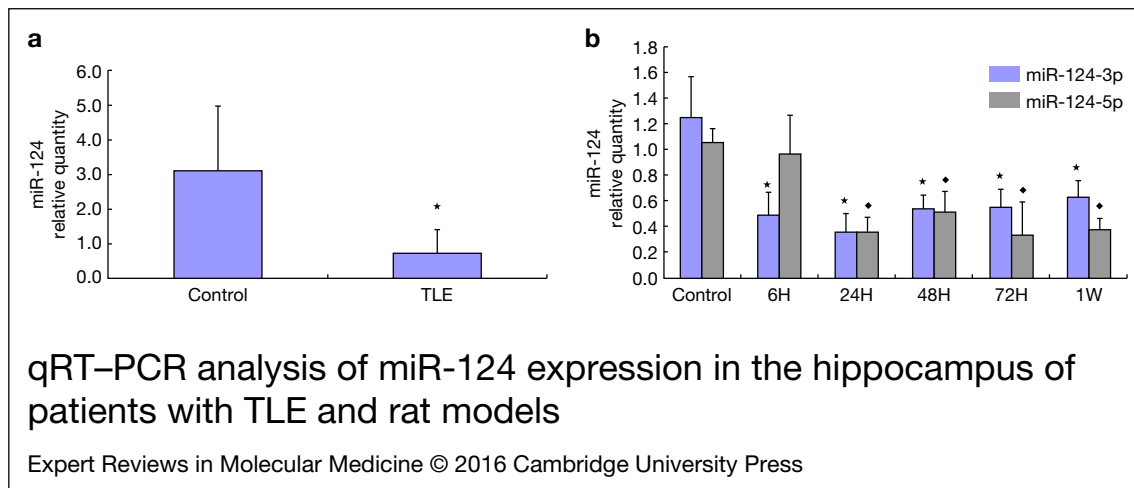


FIGURE 1.

qRT-PCR analysis of miR-124 expression in the hippocampus of patients with TLE and rat models. (a) Relative quantity of miR-124 in the temporal neocortex of controls and patients with TLE. (b) Relative quantity of miR-124-3P and miR-124-5P in the hippocampus of rat models in control conditions and at different time points after seizure. * $P < 0.05$, compared with the control.

rabbit anti-NMDAR1 (1:500, Epitomics, Burlingame, USA), rabbit anti-GLUR1 (1:500, Epitomics, Burlingame, USA) and rabbit anti- β -actin (1:4000, Beijing 4A Biotech Co., Ltd, Beijing, China). After 4 washes with Tween-20/Tris-buffered saline, the membranes were incubated with a horseradish peroxidase (HRP)-conjugated secondary antibody (1:4000, rabbit anti-goat IgG-HRP, Zhongshan Golden Bridge, Inc, Beijing, China). The resulting protein bands were visualised using an enhanced chemiluminescence substrate kit (Beyotime Institute of Biotechnology, Nantong, China) before digital scanning (Bio-Rad Laboratories, California, USA). The resultant pixel density was quantified using the Quantity One software (Bio-Rad Laboratories, California, USA) (Ref. 36).

Immunoprecipitation. Immunoprecipitation was used to survey the binding status between CREB1 and NMDAR1 or GLUR1. Approximately 100 mg of hippocampus tissues was homogenised and added RIPA lysis buffer (Beyotime, Nantong, China). Equal amounts of the proteins were incubated with 2 μ l of Rabbit IgG (1:100, Abcam, Cambridge, UK) as polyclonal-Isotype control or 10 μ l of CREB1 (1:20, Santa Cruz, Dallas, USA) or 4 μ l of GLUR1 (1:50, Abcam, Cambridge, UK) or 4 μ l of NMDAR1 (1:50, Cell Signal, Boston, USA) antibody 8 h at 4°C followed by incubation of 20 μ l of Protein A + G Agarose beads (Beyotime, Nantong, China) overnight at 4°C. The mixture was centrifuged (3000 rpm, 5 min, 4°C) and rinsed three times with RIPA lysis buffer. The mixture was boiled with 1 \times Western blot loading buffer for 5 min. After spinning (3000 rpm, 5 min, 4°C), the supernatants were subjected to Western blot with same set of antibodies as above (Ref. 37).

Quantitative PCR analysis. Total RNA was extracted using the PrimeScript[®] RT reagent Kit according to

the manufacturer's instructions (TaKaRa, Dalian, China). Real-time PCR was performed using a SYBR[®] Premix Ex Taq[™] Kit (TaKaRa, Dalian, China) and the iQ5 Real-Time PCR detection system (Bio-Rad Laboratories, California, USA). The PCR primers used for the amplification of the CREB1 mRNA are listed in Table 3. qRT-PCR was used to analyse the expression of miR-124-3p (miR-124-3p: UAAGGCACG CGGUGAAUGCC) and miR-124-5p (miR-124-5p: CGUGUUCACAGCGGACCUUGAU) and U6 snRNA using the Bulge-Loop[™] miRNA qPCR Primer Set (RiboBio, Guangzhou, China) according to the manufacturer's instructions. The expression level of CREB1 relative to that of GAPDH and the expression level of miR-124 relative to that of U6 were determined (Relative Quantity = $2^{-\Delta\Delta CT}$) (Ref. 38).

Immunohistochemistry. pCREB location and expression were detected by site-specific immunohistochemical staining (Ref. 39). The primary antibody used was a rabbit polyclonal anti-pCREB antibody (1:800, Cell Signal, Boston, USA). Sections were then incubated with a biotinylated goat anti-rabbit secondary antibody (streptavidin-peroxidase kits; Zhongshan Golden Bridge, Inc, Beijing, China). Finally, sections were treated with ABC solution at 37°C for 30 min, washed with PBS, and incubated with DAB (3,3'-diaminobenzidine; Zhongshan Golden Bridge, Inc, Beijing, China) for 3 min. As a negative control, the primary and secondary antibodies were replaced with PBS. An OLYMPUS PM20 automatic microscope (Olympus, Osaka, Japan) was used to collect the images.

Electrophysiology. The rats pre-treated by miR-124 mimics and mimics control were collected 72 h after intrahippocampal injection then followed by pilocarpine administration. One day post pilocarpine injection, hippocampal slices were obtained from rats. Coronal

brain slices (350 μm) were obtained in ice-cold sterile slice solution (containing, in mM: KCl, 2.5; $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$, 1.25; $\text{MgCl}_2 \cdot \text{H}_2\text{O}$, 6; CaCl_2 , 1; NaHCO_3 , 26; sucrose, 220; and glucose, 10). Slices were perfused with artificial cerebral spinal fluid (ACSF) at 35°C for 1 h, and then at room temperature. The ACSF was saturated with a mixture of 95% O_2 and 5% CO_2 at pH 7.4 (Ref. 40).

To measure cell excitability, the whole-cell current-clamp technique was used to record action potentials in CA1 region (5 neurons each group) (Refs 41, 42). The internal solution contained (in mM): 60 K_2SO_4 , 60 NMG, 40 HEPES, 4 MgCl_2 , 0.5 BAPTA, 12 phosphocreatine, 2 Na_2ATP and 0.2 Na_3GTP (pH 7.2–7.3; 265–270 mOsm). To simulate human epilepsy, a magnesium-free solution was applied in ACSF for 1 h. During this time, the neuronal cells underwent sustained seizure activity (Ref. 43). The slice was then bathed with normal ACSF for the remainder of the recordings.

For mEPSC recording, the internal solution was composed of (in mM): 130 Cs-methanesulfonate, 10 HEPES, 10 CsCl, 4 NaCl, 1 MgCl_2 , 1 EGTA, 5 NMG, 5 MgATP , 0.5 Na_2GTP and 12 phosphocreatine (pH 7.2; 275–290 mOsm). Tetrodotoxin (TTX, 1 μM) and Bicuculline (10 μM) were added to block GABA activity. To evaluate NMDAR/AMPA-EPSC in hippocampal neurons, the whole-cell voltage-clamp recording technique was used (Ref. 41). A patch electrode (3–5 $\text{M}\Omega$) was filled with an internal solution containing (in mM): 130 Cs-methanesulfonate, 10 HEPES, 10 CsCl, 4 NaCl, 1 MgCl_2 , 1 EGTA, 5 *N*-methyl-D-glucamine, 12 phosphocreatine, 5 MgATP and 0.5 Na_2GTP (pH 7.2; 275–290 mOsm). Slices were bathed in ACSF containing bicuculline (10 μM) to block GABAA receptors. Evoked currents were generated using a 0.1 Hz pulse (intensity, 50–200 μA ; duration, 400 μs) delivered by a stimulation isolation unit that was controlled by an S48 pulse generator (Astro-Med). A bipolar stimulating electrode (FHC) was positioned $\sim 150 \mu\text{m}$ from the neuron being recorded. The membrane potential of recorded neurons was first held at +40 mV, and the presynaptic stimulation elicited dual component responses (mediated by both AMPARs and NMDARs). After obtaining 30 traces as the baseline, the NMDAR-selective antagonist D-APV (50 μM) was applied; thus, pure AMPAR-mediated EPSCs were obtained. The subtraction of the AMPAR EPSCs from the dual component EPSCs generated the NMDAR EPSCs.

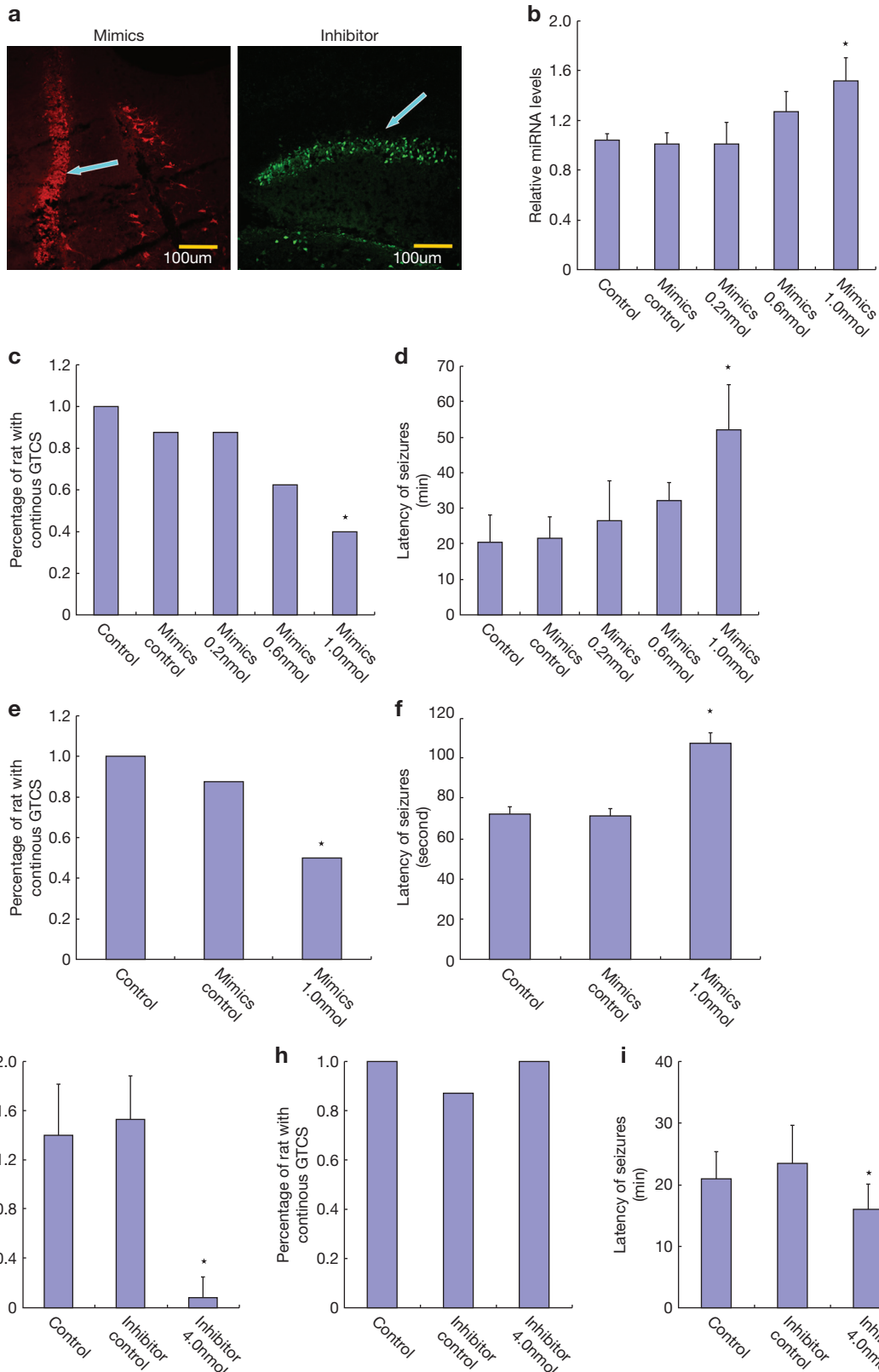
A Multiclamp 700B amplifier (Axon, Sunnyvale, CA, USA) and Digidata 1322A were used to collect and analyse data, which were filtered at 10 kHz and low-pass filtered at 2 kHz, followed by recording using the pClamp 9.2 software (Molecular Devices, Sunnyvale, CA, USA). The Mini Analysis program (Synaptosoft, Leonia, NJ) was used to analyse synaptic activity. Data were collected after currents had been stable for 5–15 min. Results were discarded if the series resistance changed by >15%.

In vivo multichannel electrophysiological recording. Local field potentials (LFPs) analysis was performed as described previously (Refs 44, 45). After anaesthetised by chloral hydrate (350 mg/kg, i.p.), the rats were received one treatment of miR-124 mimics or inhibitor or scrambled controls by intrahippocampal injection (anterior/posterior, -3.3 mm ; medial/lateral, $\pm 1.8 \text{ mm}$; dorsal/ventral, -2.6 mm). Then a recording micro wire array (4 \times 4 array of platinum–iridium alloy wire, each with 25 μm diameter, Plexon, Dallas, TX) was implanted into the right dorsal hippocampus (anterior/posterior, -3.7 mm ; medial/lateral, -2.5 mm ; dorsal/ventral, -2.7 mm). The rats which were treated surgery were recovered for 5 days before the electrophysiological activity was recorded. OmniPlex[®] D neural Data Acquisition System (Plexon, Dallas, TX) was used recording for LFPs. The LFPs signals were digitised at 4 kHz, filtered (0.1–1000 Hz) and preamplified (1000 \times). Neuro Explorer v4.0 (Plexon, Dallas, TX) was used for the LFPs data analysis. Seizures were induced in rats by lithium chloride-pilocarpine and the electrophysiological was continuously recorded (more than 80 min). A typical seizure discharged of electrophysiological was showing as high-frequency (frequency >5 Hz), high-amplitude discharge (amplitude >2 times the baseline) and lasting longer than 5 s (Ref. 44).

Luciferase reporter assay. To confirm the hypothesis that miR-124 targets the 3'UTR region of CREB1, a dual-luciferase reporter assay was used to determine whether miR-124 targeted directly the 3'UTR of CREB1 gene and repressed the CREB1 expression in human-type cells.

A 804-bp segment from the 3'UTR of the CREB1 gene containing the two miR-124 binding sites was produced by PCR with the forward primer 5'-GCGCTCGAGGTTCCAACACCTGCCTCCA-3' and the common reverse primer 5'-AATGCGGCCGCTGGTGGTGGTATGTAAGTG-3', and then cloned into the XhoI/NotI site of pmiR-RB-REPORT[™] (RiboBio, Guangzhou, China). For mutant construct of CREB1 3'UTR, deletion mutagenesis and fusion-PCR were performed. Four fragments, including two mutant miR-124 binding sites and two middle segments, were firstly produced by PCR; the primers are shown in Table 4. The full length of CREB1 promoter, containing the two mutant miR-124 binding sites, was obtained by mixing the two fragments produced from the first-step PCR and then using them as the template in the second PCR reaction with the outermost primers, and then cloned into the XhoI/NotI site of pmiR-RB-REPORT[™] (RiboBio, Guangzhou, China) too. All of the constructs were confirmed by sequencing.

HEK293T cells were obtained from institute of Biochemistry and Cell Biology (Chinese Academy of Sciences, Shanghai, China.). Cells (5×10^4 cells per well) were plated in 24-well plates 24 h before transfection and were maintained in DMEM/F12 (HyClone,



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UT, USA) plus 10% foetal bovine serum (Gibco, NY, USA). The CY3 labelled mimics/negative control were synthesised by RiboBio (RiboBio, Guangzhou, China). HEK293T cells were co-transfected with 100 ng/ml pmiR-RB-REPORTTM (RiboBio, Guangzhou, China), including the 3'UTR of CREB1 [with either wild-type (WT) or mutant-type miR-124 binding sites] and miR mimics or control (RiboBio, Guangzhou, China) at a final concentration of 100 nM using riboFECTTM CP as described by the manufacturer. Luciferase assays were performed with a Dual-Luciferase Reporter Assay System (Promega, Madison, USA) 48 h after transfection. Renilla luciferase activity was normalised to that of firefly luciferase (Ref. 46, 47, 48).

Moreover, to investigate whether NMDAR1 was also a direct target of miR-124, a 946-bp segment from the 3'UTR of the NMDAR1 gene containing the two miR-124 binding sites was produced by PCR and then a Dual-Luciferase Reporter Assay System (Promega, Madison, USA) was performed as described previously.

Statistical analyses. Data were expressed as means \pm standard deviation (SD). All statistical analyses were conducted using the statistical software SPSS 13.0. Data involving more than two groups were assessed by one-way ANOVA, followed by Tukey's HSD post hoc multiple comparison test. Student's *t*-test was used for statistical analysis of differences between the TLE group and the control group in humans. $P < 0.05$ was considered statistically significant.

Results

miR-124 expression is decreased in the temporal neocortex of patients with epilepsy and in a rat model

First we investigated the expression of miR-124 in the temporal neocortex in patients with TLE by qRT-PCR. The results showed the expression of miR-124 was significantly lower in patients with TLE than it was in controls (Fig. 1a). To test whether miR-124 is also downregulated in the hippocampus of rats after pilocarpine-induced seizures, we next determined miR-124 expression in the hippocampus of this rat model; the

results showed that the relative quantity of miR-124-3p was significantly decreased at 6 h after pilocarpine-induced seizures and remained significant low for up to 1 week (Fig. 1b). Similarly, the relative quantity of miR-124-5p was also significantly decreased at 24 h after pilocarpine-induced seizures and remained significant low for up to 1 week compared with control (Fig. 1b).

Effect of miR-124 mimics on seizure activity

After the injection of miR-124 mimics (agomir) as opposed to scrambled miRNA mimics, the miR-124 mimics was observed in the dentate gyrus (DG) and CA3-CA1 region of the rat hippocampus (Fig. 2a). The optimal dose that led to significant expression of miR-124 (40–50% enhancement) was 1.0 nM (Fig. 2b), which did not result in any behavioural abnormalities before seizures was induced.

To test whether miR-124 interferes with the seizure phenotype, we measured the effect of miR-124 mimics on seizure activity. After the injection of pilocarpine, behavioural seizures were detected in each group. Compared with the control group, 1.0 nM mimics led to a significant decrease in the incidence of generalised tonic-clonic seizures (GTCS, Fisher's exact test, $P = 0.038$, Fig. 2c). In addition, 1.0 nM miR-124 mimics significantly delayed seizure onset, as measured by latency. The differences in latency between the 1.0 nM mimics, the control, and the mimics control groups were statistically significant (52.25 ± 12.66 min in 1.0 nM the mimics group, $n = 8$; 20.43 ± 7.62 min in the control group, $n = 8$; and 21.63 ± 6.02 min in the mimics control group, $n = 8$; $P < 0.05$; Fig. 2d).

We then studied whether preinjection of a miR-124 mimics affected PTZ-induced seizures in rat models. After injection of PTZ, behavioural seizures were detected in each group. Compared with the control group, 1.0 nM mimics led to a significant decrease in the incidence of generalised tonic-clonic seizures (Fisher's exact test, $P = 0.021$, Fig. 2e). In addition, 1.0 nM miR-124 mimics delayed seizure onset significantly, as measured by latency. The differences in latency between the 1.0 nM mimics, the control, and

FIGURE 2.

Effect of a miR-124 mimics and inhibitor on rat seizure behavioural activities. (a) Fluorescent image showing positive expression of the miR-124 mimics and miR-124 inhibitor in the DG of the hippocampus. The blue arrow indicates a granule cell; Scale bar, 100 μ m. (b) miR-124 levels in the hippocampus 72 h after intrahippocampal injection of the miR-124 mimics (0.2/0.6/1.0 nM) and mimics control (1.0 nM). $*P < 0.05$, compared with the control, $n = 3$ in each group. (c) Effect of intrahippocampal injection of the miR-124 mimics (0.2/0.6/1.0 nM) and mimics control (1.0 nM) on the percentage of rats with generalised tonic-clonic seizures (GTCS) in pilocarpine-induced seizure rat models. $*P < 0.05$, compared with the control, $n = 8$ in each group. (d) Effect of intrahippocampal miR-124 mimics (0.2/0.6/1.0 nM) and mimics control (1.0 nM) injection on the latency of seizures in pilocarpine-induced rat models. $*P < 0.05$, compared with the control, $n = 8$ in each group. (e) Effect of intrahippocampal miR-124 mimics (1.0 nM) and mimics control (1.0 nM) injection on the percentage of rats with generalised tonic-clonic seizures (GTCS) in PTZ-induced seizure rat models. $*P < 0.05$, compared with the control, $n = 8$ in each group. (f) Effect of intrahippocampal miR-124 mimics (1.0 nM) and mimics control (1.0 nM) injection on the latency of seizures in PTZ-induced seizure rat models. $*P < 0.05$, compared with the control, $n = 8$ in each group. (g) miR-124 levels in the hippocampus 72 h after intrahippocampal injection of the miR-124 inhibitor (4.0 nM) and inhibitor control (4.0 nM). $*P < 0.05$, compared with the control, $n = 5$ in each group. (h) No statistically considerable differences was found between the percentage of rats with GTCS in control, inhibitor control and inhibitor groups. $n = 8$ in each group. (i) Effect of intrahippocampal miR-124 inhibitor (4.0 nM) and inhibitor control (4.0 nM) injection on the latency of seizures in pilocarpine-induced rat models. $*P < 0.05$, compared with the control, $n = 8$ in each group.

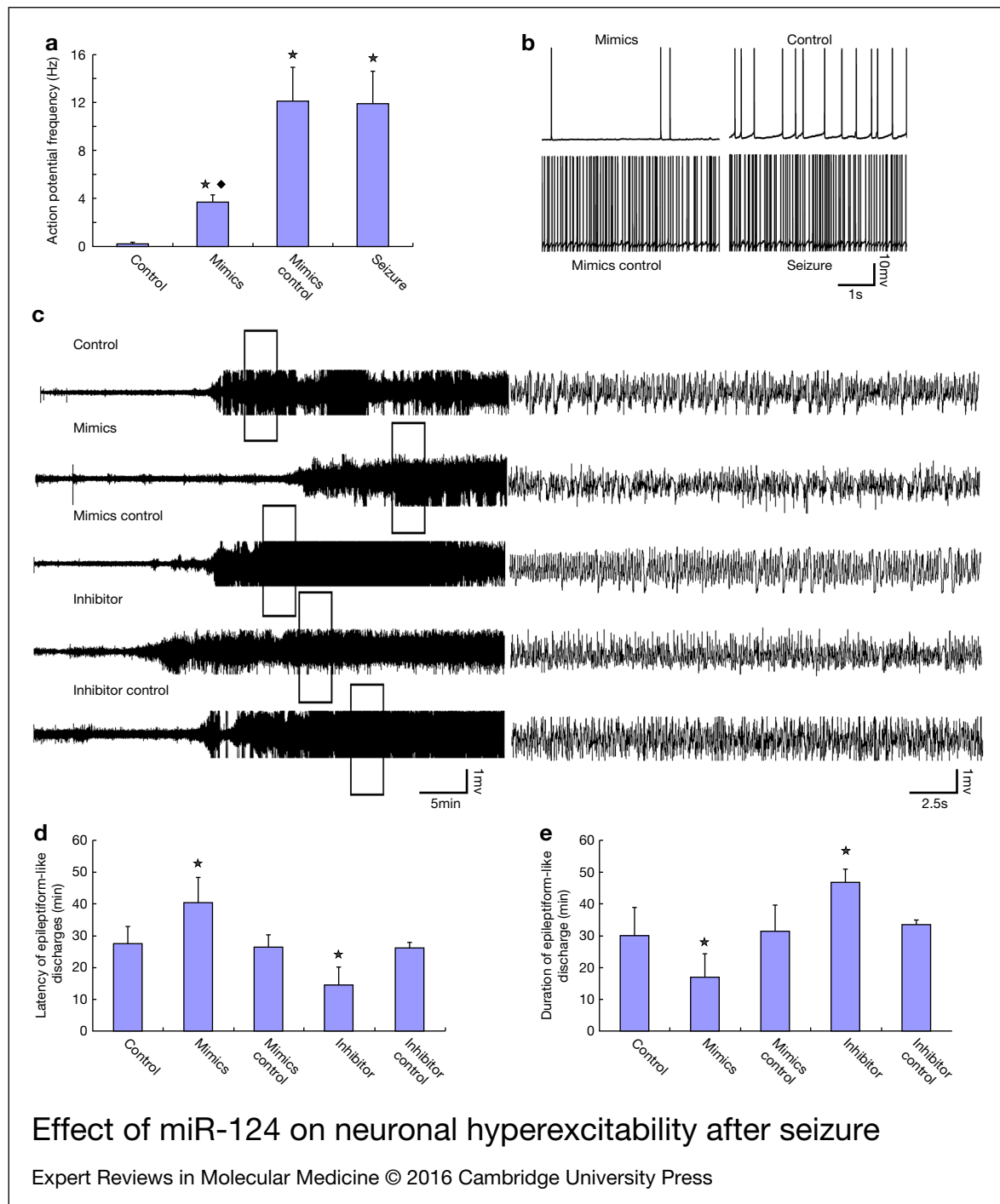


FIGURE 3.

Effect of miR-124 on neuronal hyperexcitability after seizure. (a) Changes of AP frequency. * $P < 0.05$, compared with control, $n = 5$ in each group; $\blacklozenge P < 0.05$, compared with the mimics control, $n = 5$ in each group. (b) Representative traces of action potential (AP) discharges. (c) Typical trace of LFPs on rats treated with miR-124 mimics and inhibitor. (d) The miR-124 mimics significantly prolonged the latency of epileptiform-like discharges and the miR-124 inhibitor significantly shortened the latency of epileptiform-like discharges compared with the controls in a model of pilocarpine induced seizures, $n = 5$, $\star P < 0.05$, compared with the control. (e) The duration of epileptiform-like discharges last shorter on rats treated with miR-124 mimics and longer on rat treated with miR-124 inhibitor in a model of pilocarpine-induced seizures, $n = 5$, $\star P < 0.05$, compared with the control.

the miR-124 mimics control groups were statistically significant (107.25 ± 10.24 s in the 1.0 nM miR-124 mimics group, $n = 8$; 72.38 ± 10.60 s in the control group, $n = 8$; 71.14 ± 9.74 s in the mimics control group, $n = 8$; $P < 0.05$; Fig. 2f).

Effect of miR-124 inhibitor on seizure activity

Similar methods were used for transfection of miRNA inhibitor (antagomir) into hippocampus of rats. After the injection of miR-124 inhibitor as opposed to scrambled miRNA inhibitor, the miR-124 inhibitor

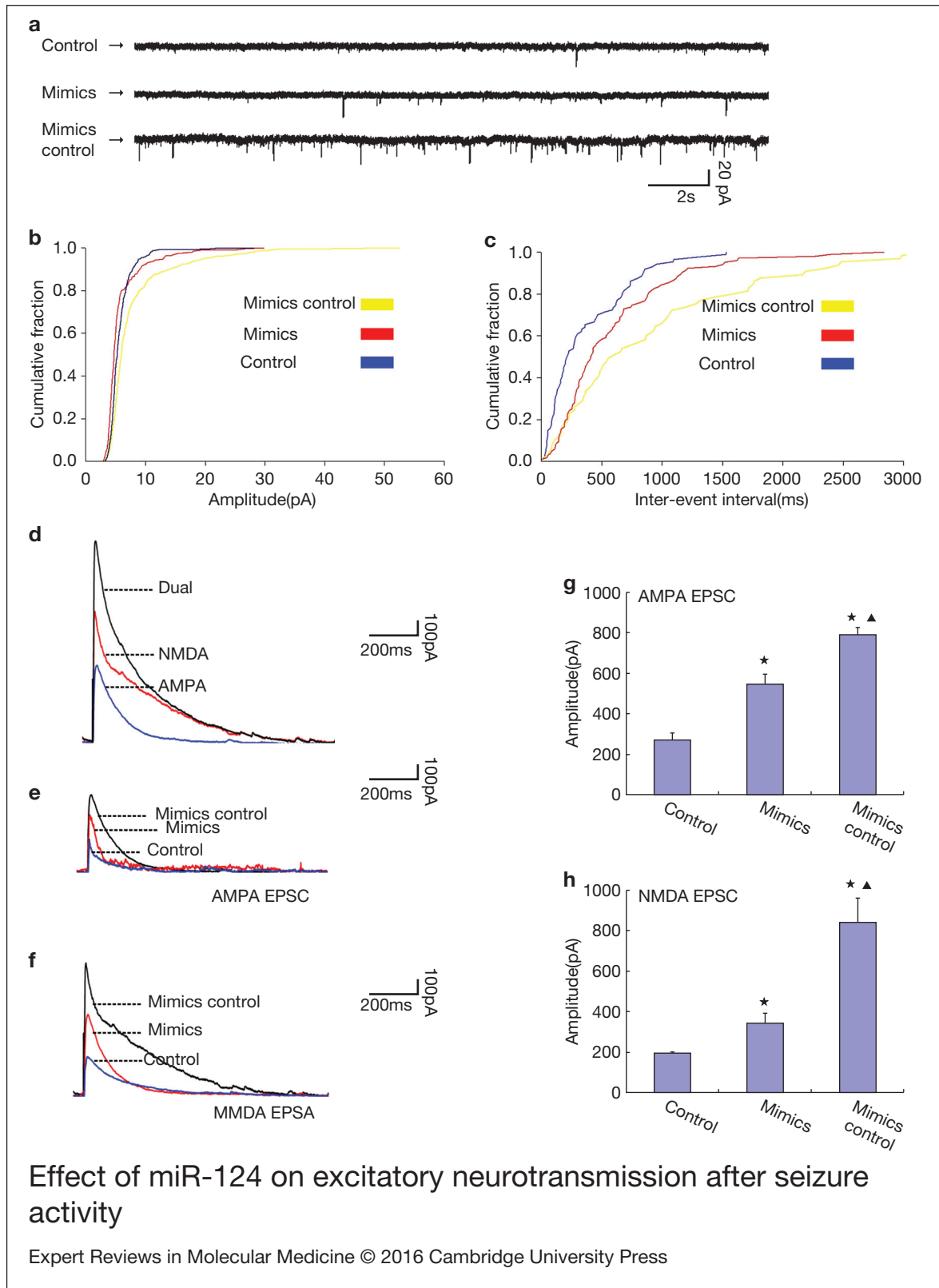
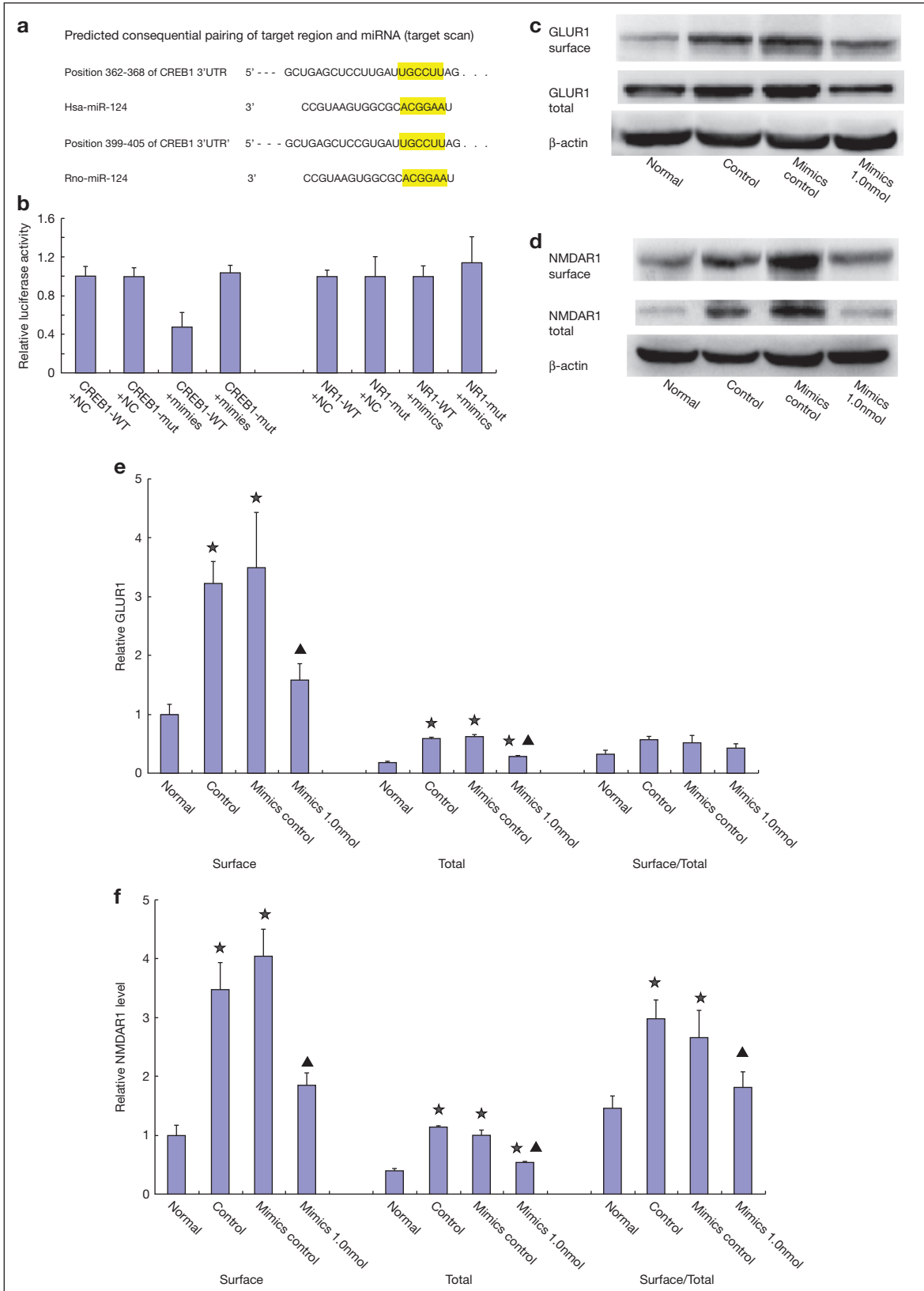


FIGURE 4.

Effect of miR-124 on excitatory neurotransmission after seizure activity. (a) Representative traces of mEPSC recorded in hippocampal pyramidal cells in control (without seizure inducing) group, mimics group and mimics control group. (b, c) Cumulative fractions of amplitude and interevent interval ($n = 5$ in individual groups). (d) Representative traces showing dual components (mediated by AMPARs and NMDARs, as indicated) were recorded from CA1 neurons by holding the membrane potential at +40 mV. (e, f) Sample traces showing AMPAR- (e) and NMDAR- (f) mediated components in control (without seizure inducing) and mimics and mimics control injection in pilocarpine-induced seizure rats, respectively. (g) Summary of AMPAR-mediated currents. (h) Summary of NMDAR-mediated currents. * $P < 0.05$, compared with the control ($n = 5$). ▲ $P < 0.05$, compared with the mimics control, $n = 5$.



Effect of miR-124 on the expression of NMDAR1 and GLUR1 in the hippocampus

was observed in the DG and CA3–CA1 region of the rat hippocampus (Fig. 2a). 4.0 nM dose of miR-124 inhibitor was used which led to significant decrease of miR-124 level ($P < 0.05$, Fig. 2g). To test whether miR-124 inhibitor interferes with the seizure phenotype, we also measured the effect of miR-124 inhibitor on seizure activity. After the injection of pilocarpine, behavioural seizures were detected in each group. No statistically considerable difference was observed in the incidence of generalised tonic–clonic seizures between the 4.0 nM inhibitor, the control and the inhibitor control groups ($P > 0.05$, Fig. 2h). The differences in latency between the 4.0 nM inhibitor, the control and the inhibitor control groups were statistically significant (23.41 ± 6.24 min in the inhibitor control group, $n = 8$; 20.98 ± 4.35 min in the control group, $n = 8$; and 15.98 ± 4.05 min in the inhibitor group, $n = 8$; $P < 0.05$; Fig. 2i).

miR-124 inhibits neuronal hyperexcitability after seizure activity

To test whether the effect of miR-124 on behavioral activity was because of the inhibition of hyperexcitability, we measured action potentials in hippocampal CA1 neurons. As shown in Figure 3, slices from controls (without seizure inducing) exhibited relatively few action potentials. In the brains of rats with seizures induced by pilocarpine, slices with scrambled miRNA mimics control and seizure group showed an outburst of action potentials, whereas slices with 1.0 nM miR-124 mimics exhibited significantly reduced action potential frequencies (Fig. 3a and b; mimics versus control, $P < 0.05$; mimics versus scrambled mimics, $P < 0.05$).

We further used an *in vivo* multichannel electrophysiological recording to record the effect of miR-124 on LFPs. The typical changes of LFPs in rat pre-treated by miR-124 mimics and inhibitor are shown in Figure 3c. We observed that treatment with miR-124 mimics dramatically prolonged the latency of epileptiform-like discharges (Fig. 3d, $P < 0.05$) and shortened the duration of epileptiform-like discharges (Fig. 3e, $P < 0.05$) in a rat model of pilocarpine-induced seizures. Moreover, rats injected with miR-124 inhibitor presented decreased latency of epileptiform-like discharges in a model of pilocarpine (Fig. 3d, $P < 0.05$) while prolonged duration of

epileptiform-like discharges (Fig. 3e, $P < 0.05$). The result of LFPs on pilocarpine-induced seizures model further demonstrated miR-124 may have anti-epilepsy function.

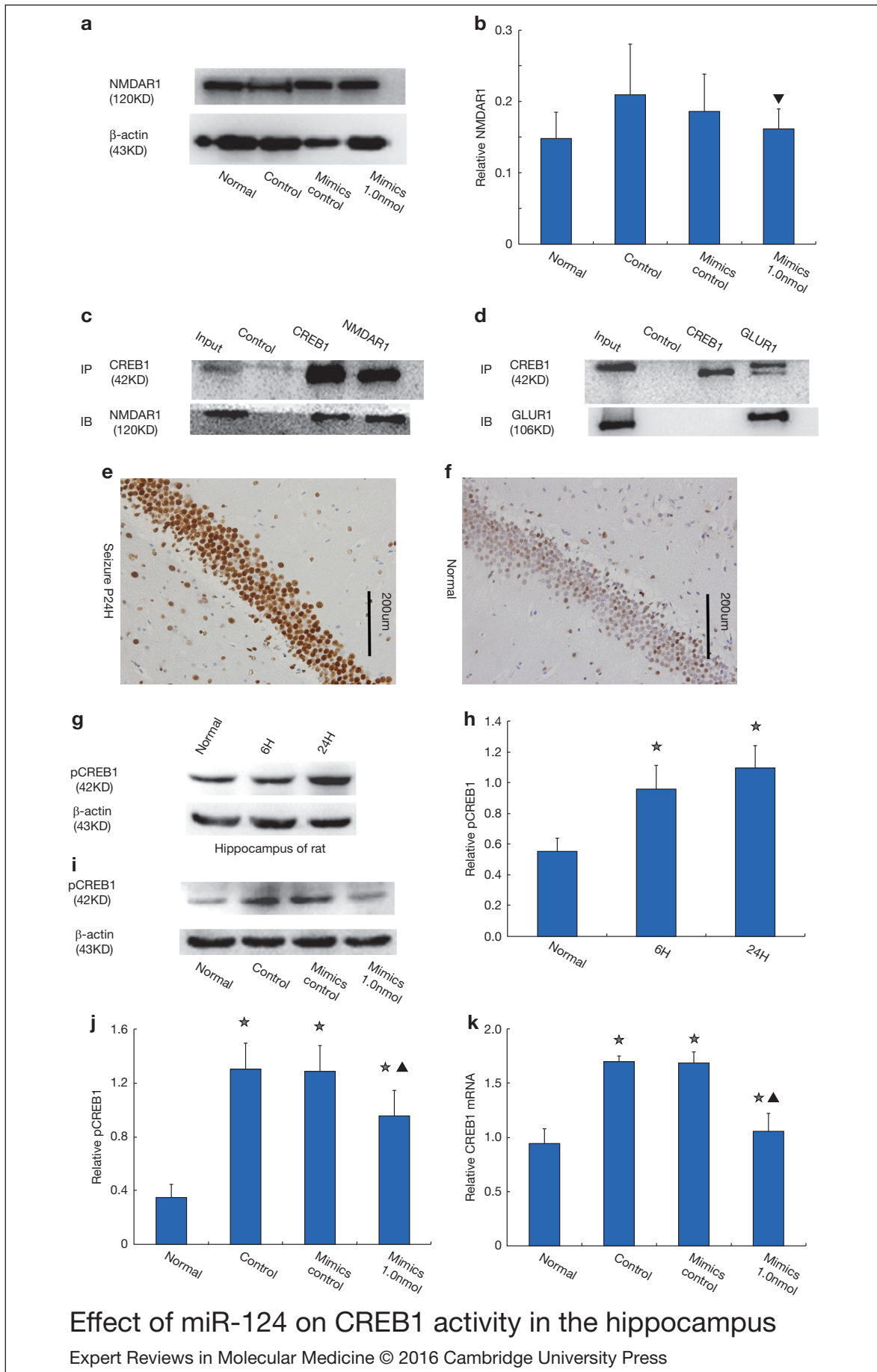
To test if enhanced excitatory neurotransmission contributes to neuronal hyperactivity, mEPSC was recorded in the hippocampal slices. The effective dose of the miR-124 mimics significantly decreased the amplitude and frequency of mEPSC compared with the mimics control and control group ($P < 0.05$; Fig. 4a–c). To identify the AMPA receptor (AMPA) or NMDA receptor (NMDAR) mediates inhibition of excitatory neurotransmission afforded by miR-124, we examined evoked AMPAR and NMDAR currents in the brain slices. In the brain slices of rats with seizures induced by pilocarpine, both AMPAR- and NMDAR-mediated currents were significantly increased compared with those observed in control group animals. However, both AMPAR- and NMDAR-mediated currents were significantly reduced after miR-124 mimics injection (Fig. 4d–h). These results suggest that miR-124 inhibits epileptic hyperactivities through AMPAR- and NMDAR-mediated mechanisms.

miR-124 Interacts directly with the 3'UTR region of CREB1

Using TargetScan (Release 4.2) online searching programs, we have identified CREB1 as the potential target of miR-124. A 100% matched sequence was found at the CREB1 mRNA 3'UTR (Fig. 5a). To confirm the hypothesis that miR-124 targets the 3'UTR region of CREB1, the entire 3'UTR region of CREB1 was cloned into the XhoI/NotI site of pmir-RB-REPORTTM and co-transfected the HEK293T cells with this vector along with either the miR-124 mimics or its negative control. Using the luciferase reporter system, we found that co-transfection of miR-124 mimics along with the 3'UTR of WT CREB1 caused a significant decrease by over 50% in luciferase units compared to controls (Fig. 5b). However, co-transfection of miR-124 mimics not significantly suppressed the luciferase activity of reporter genes containing 3'UTR of NMDAR1 compared with the control after statistics calculation (Fig. 5b). These results demonstrated that miR-124 targeted specifically the 3'UTR region of CREB1.

FIGURE 5.

Effect of miR-124 on the expression of NMDAR1 and GLUR1 in the hippocampus. (a) Diagram of the CREB1-3'-UTR with potential binding-sites for miR-124. (b) Relative luciferase activity of reporters, including WT or mutant CREB1 and NMDAR1 3'-UTR co-transfected with NC or miR-124 mimics. *indicates significant difference at $P < 0.01$, respectively. (c) Sample Western blots showing surface and total levels of GLUR1 subunits in normal (without seizure inducing) and pilocarpine-induced seizure rats without treatment (control), and in rats treated with mimics control and mimics. (d) Sample Western blots showing the surface and total levels of expression of NMDAR1 subunits in normal (without seizure inducing) and pilocarpine-induced seizure rats without treatment (control), and in rats treated with mimics control and mimics. (e) Summary showing the surface expression and the total and surface/total ratios of GLUR1 subunits. * $P < 0.05$, compared with the control. ▲ $P < 0.05$, compared with the mimics control, $n = 3$. (f) Summary showing the surface expression and the total and surface/total ratios of NMDAR1 subunits. ▲ $P < 0.05$, compared with the mimics control, $n = 3$.



miR-124 downregulates AMPAR and NMDAR expression in the hippocampus of rat models

To test whether miR-124 alters AMPAR/NMDAR surface expression, we examined total and surface receptor expression by Western blot analysis. For AMPAR, a significant increase in the expression of both total and surface GLUR1 was present in the hippocampus of rats after pilocarpine-induced seizure activity (Fig. 5c and e). The scrambled mimics (agomir control) did not have an effect on AMPAR expression compared with the group with pilocarpine-induced seizure. The levels of total GLUR1 were significantly suppressed by miR-124, whereas the surface/total ratio of GLUR1 did not change significantly after microinjection of miR-124 mimics (Fig. 5c and e). For NMDAR, seizure activity led to a significant increase in NMDAR1 expression in the hippocampus. However, the surface and total expression of the NMDAR1 protein in the hippocampus of rats with pilocarpine-induced seizure were significantly lower in the miR-124 mimics group than they were in the mimics control group after 72 h of miR-124 mimics injection or mimics control injection (Fig. 5d and f).

Subsequently, we studied whether preinjection of a miR-124 mimics affected the total expression of NMDAR after PTZ-induced seizures in rat models. After the injection of PTZ, seizure activity led to an increase in NMDAR1 expression in the hippocampus, and the miR-124 mimics partially decreased the expression of NMDAR1; however, none of these changes were significant ($P > 0.05$, Fig. 6a and b).

These results indicate that, in the hippocampus of rats, although the total expression of both NMDAR and AMPAR was suppressed by miR-124 24 h after seizures were evoked, only the surface expression of NMDAR was downregulated by miR-124, suggesting that different mechanisms may be involved in the regulation of AMPAR and NMDAR trafficking.

Since there was significant change in AMPAR-mediated EPSCs, but there was no significant change in surface expression of GLUR1, we further examined whether the NMDAR1 and GLUR1 were correlated with acute increase of CREB1. Immunoprecipitation was used to test the interaction of CREB1 with NMDAR1 and GLUR1. Our studies confirmed that the CREB1 antibody effectively precipitated CREB1

and NMDAR1 but not GLUR1 from rat brain hippocampus (Fig. 6c and d).

miR-124 inhibits CREB1 activity

A previous study has demonstrated that miR-124 recognises specific binding sites in the 3'UTR of the CREB1 mRNA in the neurons of *Aplysia*. The miR-124-mediated CREB1 cascade contributes to enhanced synaptic plasticity (Refs 49, 50). In addition, the activation of CREB1 plays a key role in epileptogenesis (Refs 51, 52). Thus, we tested whether miR-124 inhibits neuronal hyperexcitability through CREB1. As shown in Figure 6, in the CA1 area, CA3 subfields, and dentate gyrus, CREB1 was expressed mainly in the nuclei of neurons. Immunostaining of activated CREB (Ser133-phosphorylated CREB, p-CREB) was significantly stronger in the hippocampus of rats with pilocarpine-induced seizure (Fig. 6e) 24 h after the first seizure compared with that observed in control rats (Fig. 6f). Consistently, Western blot analysis showed that p-CREB expression significantly enhanced 6 and 24 h after seizures in rats (Fig. 6g and h). As shown in Figure 6i and j, hippocampal p-CREB was significantly reduced after injection of miR-124 mimics. In addition, miR-124 significantly reduced CREB1 mRNA levels compared with control and scrambled miRNAs (Fig. 6k).

Discussion

The principal finding of this study was that miR-124 levels were suppressed in patients with TLE and in a rat model. Hippocampal injection of miR-124 mimics both alleviated seizure severity and prolonged the latency of seizure. MiR-124 administration inhibited neuronal firing, mEPSC and AMPAR- and NMDAR-mediated currents and NMDAR surface expression. The effects of pre-injection of miR-124 mimics on LFPs also showed shortened duration of epileptiform-like discharges and prolonged latency of epileptiform-like discharges in a model of pilocarpine-induced seizures. In addition, CREB1 activity was significantly down regulated by miR-124 in pilocarpine-induced seizures rats. We also demonstrated that CREB1 is a direct target of miR-124 in human HEK 293T cells. Furthermore, we showed that NMDAR-mediated current changes occur via direct interaction with

FIGURE 6.

Effect of miR-124 on CREB1 activity in the hippocampus. (a) Sample Western blots of NMDAR1 in normal (without seizure inducing) and model rats (PTZ-induced seizure in rat models) without treatment (control), and in animals treated with mimics control and mimics. (b) Summary of the relative NMDAR1 analysed from h. ▼ $P > 0.05$, compared with the normal, $n = 4$. (c, d) Immunoprecipitation was used to survey the binding status between CREB1 and NMDAR1 or GLUR1. (e, f) Immunohistochemistry images show strong (e, 24 h after pilocarpine-induced seizures) and weak (f, control group) immunoreactive staining of pCREB in the dentate gyrus. Scale bar, 200 μm . (g) Sample Western blots of pCREB before (normal) and at different time points of pilocarpine-induced seizure. (h) Summary of pCREB activity showing a significant increase after seizures. * $P < 0.05$, $n = 5$. (i) Sample Western blots of pCREB in normal (without seizure inducing) and seizure rats without treatment (control), and in rats treated with mimics control and mimics. (j) Summary of the relative pCREB analysed from I. * $P < 0.05$, compared with the normal. ▲ $P < 0.05$, compared with the mimics control, $n = 5$. (k) Relative CREB1 mRNA levels in normal (without seizure inducing) and seizure rats without treatment (control) and in animals treated with mimics control and mimics. * $P < 0.05$, compared with the normal. ▲ $P < 0.05$, compared with the mimics control, $n = 5$.

CREB1. Our study revealed a previously unknown function of miR-124 in epilepsy.

The relationship between miRNA and epilepsy has received attention only recently. A genome-wide miRNA profiling study showed that, in human TLE, 165 miRNAs were changed significantly, miR-124 being among those that were downregulated (Ref. 53). Consistently, our study demonstrated that decreased miR-124 expression occurs not only in patients with TLE, but also in animal models for up to 1 week. For the normal human hippocampal tissues as control were not available to study, we did not study the hippocampus in TLE patients. However, discrepancies exist regarding miR-124 changes in animal models. In a mouse model, miR-124 was not included among the miRNAs that were up- or downregulated (Ref. 16). This may be owing to differences in animal species. Using a microarray method, Hu et al. investigated the miRNA expression profile in the same rat model as that used in our study, and found that miR-124a was significantly upregulated in these animals (Ref. 54). However, a quantitative PCR analysis was not performed to confirm this observation.

A limited number of studies have demonstrated that miR-124 may serve as a novel target for therapeutic intervention of neurological diseases. Intrathecal injection of miR-124 reduced persistent and neuropathic pain in a mouse model (Ref. 55). In EAE, peripheral administration of miR-124 leads to deactivation of macrophages and marked suppression of disease (Ref. 25). However, the current treatment for epilepsy is antisymptomatic. None of the antiepileptic drugs (AEDs) have been proven to be curative, and 20–30% of patients are refractory to AEDs (Refs 4, 56). In the present study, hippocampal injection of miR-124 caused a significant reduction in seizure severity in two rat models, indicating that miR-124 exerts a potential role in regulating neuronal excitability and seizure phenotype.

In the present study, miR-124 inhibited neuronal hyperexcitability by suppressing neuronal firing and mEPSC. In addition, NMDAR- and AMPAR-mediated currents and expression were also inhibited by miR-124. Although it is relatively clear that miR-124 promotes embryonic neurogenesis and neuronal differentiation (Ref. 26), the function of miR-124 in the adult central nervous system and mature animals has not been well studied. In *Aplysia*, miR-124 suppresses serotonin-induced synaptic facilitation, thus providing direct evidence that miR-124 is critical in long-term synaptic plasticity in the mature nervous system (Ref. 49). A recent study in sensory neurons of *Caenorhabditis elegans* showed that miR-124 targets to genes that are associated with neurotransmitter release, cell-projection morphogenesis, and translation (Ref. 57), which supports a role for miR-124 in the regulation of synaptic plasticity in our experimental conditions.

It is well documented that CREB1 plays an important role in epilepsy. In patients with medically intractable epilepsy, enhanced CREB1 activation and gene expression occur in the seizure onset zone (Ref. 51). Activation of CREB1 and downstream genes contributes to epilepsy in both humans and rodent models (Ref. 52). Conversely, mice with decreased CREB1 levels exhibit a ~50% reduction in spontaneous seizures, as well as a higher seizure threshold (Ref. 52). In our study, miR-124 administration resulted in CREB1 deactivation and mRNA suppression after epileptiform discharges, suggesting that CREB1 might partly mediate the miR-124 effect on synaptic remodeling. As reported previously, CREB1 enhances NMDAR but not AMPAR-mediated current and surface expression (Refs 58, 59). Consistently, NMDAR but not AMPAR trafficking to the cell surface was reduced, as measured by surface/total protein ratios in our study, suggesting a central role of CREB1 in neuronal excitability and seizure phenotype.

A previous study demonstrated that miR-124 recognised specific binding sites in the 3'UTR of the CREB1 mRNA in the neurons of *Aplysia* (Ref. 49). The miR-124-mediated CREB1 cascade contributed to enhanced synaptic plasticity (Refs 49, 50). In our experiment, injection of miR-124 significantly reduced CREB1 mRNA, suggesting that CREB1 function is associated with miR-124. Using human HEK 293T cells, we have demonstrated that CREB1 is a direct target of miR-124 in human cells. Acute expression of active CREB1 correlates with an increase of both NMDAR surface expression and NMDAR-mediated currents. In this study, the altered activity and expression of CREB1 as a result of miR-124 administration, suggesting CREB1 is a potential mediator in miR-124 regulation of neuronal excitability, while NMDAR1 was a critical substrate of CREB1 which mediated the changes of NMDAR EPSCs.

The present study provided direct evidence that miR-124 is inhibited in patients with epilepsy and in drug-induced seizure rat models, and that the administration of miR-124 alleviates seizure attacks, which may lead to a novel intervention strategy for human epilepsy. However, the detailed mechanisms underlying miR-124 regulation of CREB1 and neuronal excitability remain to be elucidated in the future.

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