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Original Research

The Expression and Effects of Rev-Erbα in Different Cerebral Regions of Mice With Acute and Chronic Epilepsy

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Abstract

Background: Epileptic seizures exhibit distinct circadian rhythms. Disruption of this rhythm creates a vicious cycle that further exacerbates the seizures. Rev-Erbα is a key circadian regulator that plays a significant role in epilepsy. In this study we aimed to investigate the expression and effects of Rev-Erbα and its agonist SR9009 across different brain regions during acute status epilepticus (SE) and chronic epilepsy (CE). **Methods:** C57BL/6J mice were randomly divided into control, epilepsy (SE and CE), and SR9009+epilepsy (SR9009-E) groups. The distribution and expression of Rev-Erbα in the medial prefrontal cortex (mPFC), hippocampus (HP), and occipital visual cortex (V2M) of mice with SE and CE mice were detected using immunofluorescence and western blotting. Electroencephalography (EEG), the open-field test, and the Y-maze test were applied to evaluate epileptic activity, anxiety, and spatial memory. **Results:** Rev-Erbα expression differed significantly across cerebral regions among the control, epilepsy (SE and CE), and the SR9009-E groups ($p < 0.05$). SR9009-E mice had fewer seizures and less abnormal EEG discharge ($p < 0.05$) and showed better behavioral outcomes, including reduced anxiety-related behaviors and improved spatial memory, compared with epileptic mice ($p < 0.05$). Immunofluorescence showed higher neuronal numbers in the SR9009-E group than in the epilepsy group ($p < 0.05$). **Conclusions:** Rev-Erbα dysregulation is tightly linked to epilepsy progression. Activation of Rev-Erbα exerts antiepileptic and neuroprotective effects and improves epilepsy-related neurobehavioral deficits, thereby offering a novel strategy for epilepsy treatment.

Keywords: NR1D1; epilepsy; neurons; circadian rhythm; behavioral research

1. Introduction

Epilepsy is a common chronic neurological disorder characterized by recurrent and unpredictable seizures [1]. Although antiseizure medications have markedly improved epilepsy treatment, approximately 30% of patients still develop drug-resistant epilepsy [2,3]. Therefore, a precise understanding of epilepsy pathogenesis is an urgent clinical need.

The clinical manifestations and propagation pathways of epileptic seizures depend heavily on the anatomical network in which they originate. Among these networks, the frontal, temporal, and occipital lobes are the most common and functionally representative sites of origin for focal epilepsy [4]. Interestingly, seizures arising from these brain regions all exhibit significant circadian patterns, though their peak phases and triggering mechanisms may differ [5]. For instance, frontal lobe epilepsy often occurs during sleep [6], and temporal lobe epilepsy is closely associated with sleep-wake transitions [7]. In contrast, some occipital lobe epilepsies may be influenced by diurnal variations in visual stimulation [8]. This clinical correlation strongly suggests that the inherent physiological rhythms of different brain regions, along with their sensitivity to circadian signals, may constitute distinct, region-specific windows of epileptic susceptibility. It is noteworthy that extensive clinical and preclinical studies have revealed that epileptic seizures exhibit significant circadian rhythmicity, with many patients experiencing a marked increase in seizure frequency during specific periods [9] (e.g, the sleep-wake transition phase). Clinical data further validate that enforced circadian rhythm disruption exacerbates seizure burden: shift work, a classic real-world model of chronic circadian misalignment, elevates patients' seizure frequency by 1.5–2-fold on average [10]. This phenomenon strongly suggests that the endogenous circadian clock system may be deeply involved in the initiation and modulation of epilepsy.

Reciprocal pathological crosstalk exists between epileptic activity and circadian homeostasis. Recurrent epileptic seizures severely disrupt normal sleep architecture, and altered sleep further synchronizes abnormal neuronal discharges, impairs suprachiasmatic nucleus (SCN) rhythmic output, and disturbs the circadian expression rhythm of local clock genes. This forms a self-perpetuating vicious cycle: "epilepsy, sleep-circadian disruption and increased seizure susceptibility" [11]

The mammalian circadian system relies on cell-autonomous molecular clocks to coordinate physiological adaptation to diurnal environmental fluctuations [12], with the hypothalamic SCN governing global rhythmic output via a conserved cascade of core clock genes [13]. Rev-

Erbα (official gene symbol nuclear receptor subfamily 1 group D member 1 (NR1D1)) is a core negative transcriptional repressor within the canonical circadian feedback loop, which sustains regular 24-hour biological oscillations [14]. Apart from its canonical circadian regulatory function, multiple preclinical studies have uncovered a close association between dysregulated Rev-Erbα signaling and epileptogenesis [15]. Rev-Erbα governs three key pathological processes underlying epilepsy progression: neuronal excitability, central neuroinflammation, and neuronal survival; abnormal downregulation of Rev-Erbα in epileptic lesions exacerbates neuronal injury and facilitates recurrent seizures [16]. Despite these correlative observations, the expression pattern of Rev-Erbα in different brain regions and its temporal dynamics during epilepsy remain unclear. Accordingly, the current study systematically characterizes Rev-Erbα expression across three major seizure-prone brain regions—the medial prefrontal cortex (mPFC), hippocampus (HP), and occipital visual cortex (V2M). We further adopt a pharmacological intervention strategy to observe phenotypic changes in seizure activity, neuronal integrity, and epilepsy-associated behavioral deficits following Rev-Erbα agonist treatment. SR9009 is a potent, highly specific, and blood-brain barrier-permeable small-molecule agonist that selectively activates Rev-Erbα. This compound serves as a convenient pharmacological reagent to elevate Rev-Erbα protein levels in the central nervous system for phenotypic observation [17].

Since epilepsy is tightly associated with circadian rhythm disturbances and Rev-Erbα acts as a critical component governing circadian oscillations, we infer that Rev-Erbα bears vital relevance to epilepsy progression. To test the hypothesis, we first characterize the spatiotemporal expression landscape of Rev-Erbα in pilocarpine-induced acute status epilepticus (SE) and chronic epilepsy (CE) mouse models. Secondly, to observe alterations in the epilepsy of Rev-Erbα, we compare the difference in seizure severity, neuronal injury, and neurobehavioral performance before and after SR9009 administration. This will enrich the understanding of circadian clock molecular alterations during epileptogenesis, and provide new strategies for the treatment of epilepsy.

2. Materials and Methods

2.1 Experimental Animals

Male C57BL/6J mice (6–8 weeks, 21–25 g) were housed individually under standard conditions with free access to food (standard rodent chow) and water. The housing environment maintained a temperature of 21–23 °C and a relative humidity of 50–60%. A 12-hour light/12-hour dark cycle was implemented, with illumination from 7:00 AM to 7:00 PM (Zeitgeber time, ZT). Mice underwent a one-week acclimation period before the experiment. All procedures were conducted in accordance with the principles of minimizing animal suffering and reducing animal use.

2.2 Animal Experimental Design

2.2.1 Experimental Groups

The experiment included four groups:

(1) Control group (n = 40): Mice received intraperitoneal injections of saline instead of pilocarpine.

(2) Epilepsy group: SE (n = 40) and CE (n = 20) models as described above;

(3) SR9009 +epilepsy group (n = 30; 3-day treatment n = 15; 7-day treatment n = 15): the SE and the CE groups received SR9009 (50 mg/kg, i.p.) [18] for either 3 or 7 consecutive days. SR9009 was administered once daily at ZT12 (7:00 PM, the beginning of the dark/active phase), dissolved in 10% DMSO, 40% PEG300, 5% Tween-80, and 45% saline.

(4) SR9009-only control group (n = 15): Control mice received SR9009 (50 mg/kg, i.p.) for 7 consecutive days.

2.2.2 SE and CE Model Establishment

For SE induction, mice were pretreated with scopolamine hydrobromide (1 mg/kg, i.p., S1876, Sigma-Aldrich, St. Louis, MO, USA) 30 minutes prior to pilocarpine administration to minimize peripheral cholinergic side effects. Acute status epilepticus (SE) refers to the acute phase within 24 hours after pilocarpine-induced (P6503, Sigma-Aldrich) (300 mg/kg, i.p.) seizures and was defined as stage 4 or higher seizures persisting for 30 minutes. Chronic epilepsy (CE) refers to the chronic phase 2–4 weeks after SE, when mice develop spontaneous recurrent seizures (SRSs) as confirmed by video monitoring. SE model was considered successful when stage 4 or higher seizures persisted for 30 minutes; seizures were then terminated with diazepam injection (H12020957, Tianjin Jinyao Pharmaceutical Co., Ltd., Tianjin, China) (10 mg/kg, i.p.) [16]. For SE induction, a total of 50 mice were used. Of these, 40 reached stage 4–5 seizures and were included (success rate: 80%). Five mice died during status epilepticus (mortality: 10%), and five mice did not reach stage 4–5 seizures within 60 minutes and were excluded. CE in mice was defined by the observation of recurrent spontaneous seizures (SRSs) during video monitoring. For CE model development, of the 40 mice that successfully experienced SE, 32 developed recurrent spontaneous seizures within 2–4 weeks (80% progression rate), and 20 were randomly selected for CE experiments.

Observation and scoring of mouse behavior using the modified Racine scale [19]:

Stages 1–2: Facial automatisms, tail stiffness, and wet dog shakes.

Stage 3: In addition to the symptoms of stages 1–2, add low-intensity tonic-clonic seizures characterized by unilateral forelimb myoclonus.

Stage 4: In addition to the symptoms of stage 3, add bilateral forelimb clonus and rearing.

Stage 5: Bilateral forelimb and hindlimb clonus with loss of postural control, then falling.

Stage 6: Death.

2.3 Sample Collection and Preparation

Samples were collected at four time points: ZT0, ZT6, ZT12, and ZT18 (spanning 24 hours). Prior experiments have shown that Rev-Erbα was lowest at ZT18 in the mPFC and HP [16,20]. Therefore, ZT18 samples were chosen to assess protein and cell-level changes in three cerebral areas of SR9009-E mice. All mice were divided into two main groups. In the first group, mice were euthanized (In accordance with the American Veterinary Medical Association's (AVMA) standard guidelines on the overuse of inhalation anaesthetics, we employed a secondary confirmation method to verify the cessation of vital signs. Specifically, we performed cervical dislocation following exposure to 5% isoflurane (R510-22-10, RWD) to ensure humane euthanasia), and the brain tissues were rapidly dissected for protein detection. The second group was anesthetized and perfused transcardially with saline, followed by 4% paraformaldehyde (PFA, G1101, Servicebio, Wuhan, Hubei, China) for tissue fixation, and then processed for immunofluorescence, Nissl staining, and haematoxylin and eosin (HE) staining. Brain regions were dissected using a mouse brain matrix (1 mm coronal sections, RWD Life Sciences, Shenzhen, China). The mPFC was dissected from the rest of the cortex based on anatomical landmarks (the mPFC comprises the anterior and medial cingulate cortex regions, located at bregma +1.98 to +1.54 mm). HP and V2M were dissected in the same manner, following the Paxinos & Franklin mouse brain atlas.

2.4 Western Blotting

Western blotting was used to detect Rev-Erbα expression levels in the brain tissue. After decapitation, the brains were removed, and the brain tissues were homogenized on ice in RIPA lysis buffer (G2202, Servicebio) with grinding beads, followed by low-temperature ultrasonic grinding. The homogenate was centrifuged at 3000 rpm for 10 minutes at 4 °C. The supernatant was collected, and protein concentration was determined by the BCA method. After loading buffer was added, protein samples were denatured by heating at 95 °C for 10 minutes. Proteins were separated by SDS-PAGE and transferred onto PVDF membranes (IPVH00010, Millipore, Billerica, MA, USA). Membranes were blocked with 5% BSA (GC305010, Servicebio) for 2 hours at room temperature (RT), incubated with the primary antibody at 4 °C overnight, washed with TBST (G2168, Servicebio), incubated with the HRP-conjugated secondary antibody (G3431, Proteintech, Wuhan, Hubei, China) for 2 hours at RT, washed again, and then visualized using enhanced chemiluminescence (ECL). Band intensities were quantified using image analysis software, with GAPDH as the internal control. Primary antibody: NR1D1 (1:1000, 14506-1-AP, rabbit polyclonal, Proteintech). Secondary antibody: HRP-conjugated goat anti-rabbit IgG (1:5000, SA00001-2, Proteintech). Internal control: GAPDH (1:1000, GB11002, rabbit polyclonal, Servicebio).

2.5 Nissl Staining

Paraffin-embedded sections were deparaffinized and rehydrated through a graded series of xylenes and ethanol. Sections were stained with toluidine blue (G1434, Solarbio, Beijing, China) for 10 minutes, rinsed in distilled water, dehydrated through a graded ethanol series, cleared in xylene, and mounted with neutral resin. Nissl bodies, appearing blue-purple, were observed under a microscope (DM750, Leica, Wetzlar, Germany) to assess neuronal morphology.

2.6 H&E Staining

Deparaffinized and rehydrated sections (as above) were stained with hematoxylin for 3 minutes, rinsed, differentiated in 1% acid alcohol, rinsed again, and blued in PBS. Sections were then counterstained with eosin for 10 minutes, dehydrated through graded ethanol, cleared in xylene, and mounted with neutral resin. Cellular morphology was observed under a microscope.

2.7 Immunofluorescence Staining

After deparaffinization, rehydration, and washing with PBS, antigen retrieval was performed. The tissue sections were blocked with 10% normal goat serum (G1208, Servicebio) for 2 hours at RT, followed by incubation with primary antibodies at 4 °C overnight, washed with PBS (G0002, Servicebio), incubated with fluorescent secondary antibodies for 2 hours at RT in the dark, washed again, and mounted with anti-fade mounting medium containing DAPI. Staining was observed using a fluorescence microscope. Primary antibodies: NR1D1 (1:200, 14506-1-AP, rabbit polyclonal, Proteintech); NeuN (1:500, GB15138, mouse monoclonal, Servicebio); GFAP (1:400, 3670, mouse monoclonal, Cell Signaling Technology, Danvers, MA, USA); IBA1 (1:300, ab178847, mouse polyclonal, Abcam, Cambridge, UK). Secondary antibodies: 594 goat anti-mouse IgG (1:200, SA00013-1, Proteintech); 488 goat anti-rabbit IgG (1:200, SA00013-2, Proteintech).

2.8 Electroencephalogram (EEG) Monitoring

EEG electrodes were implanted 7 days before pilocarpine induction (Day-7) to allow for recovery before seizure induction. Animal EEG signals were acquired and preprocessed using the analysis software integrated with the Medusa Plus electrophysiology system (Bio-Signal, Nanjing, Jiangsu, China). Mice were anesthetized with inhaled isoflurane, induced in an induction chamber with 5% isoflurane (R510-22-10, RWD), and subsequently maintained via a nose cone with 1%–2% isoflurane in oxygen. The mouse was shaved, and the head hair was secured in a stereotaxic apparatus. After disinfection, the scalp was incised to expose the skull, which was leveled between the vertex and the base. Using the vertex as the reference point, four holes were drilled at the following coordinates: –1.0 mm AP, ±2.8 mm ML, and –3.8 mm AP, ±2.8 mm ML relative to bregma. A dental drill was used to create im-

plantation holes, and the electrodes were secured with four screws [21]. After implanting the EEG electrodes into the holes, secure them with dental acrylic resin (2947, Shanghai Yuyan Scientific Instrument Co., Ltd., Shanghai, China). Once the resin has hardened, remove the mouse from the stereotaxic apparatus, place it in an incubator for recovery, and return it to its housing cage. A week post-surgery, connect the cranial electrodes to the EEG recording system to perform EEG recordings. After completing the parameter settings, collect data and proceed with the analysis of the frequency and amplitude of epileptiform spikes. An EEG seizure was defined as rhythmic spike or sharp wave activity lasting ≥10 seconds with amplitude ≥2× baseline and clear evolution in frequency or morphology, accompanied by behavioral arrest or Racine stage ≥3. Recordings were sampled with a 0.5–70 Hz bandpass filter. Analysis was performed manually by two blinded reviewers.

2.9 Behavioral Analysis

Behavioral testing was performed on Day 29 (chronic epilepsy baseline) and Day 32 (3-day SR9009) or Day 38 (7-day SR9009). Behavioral tests were conducted between ZT4 and ZT8 to minimize circadian confounds. Behavioral videos were recorded in real time and automatically analyzed by the Smart 3.0 video tracking system (RWD Life Sciences)

Open-field test (RWD Life Sciences, 63016): The experimental apparatus consists of a square arena (45 × 45 × 40 cm) divided into a central zone and peripheral zones. The arena is cleaned with ethanol before testing. Mice are introduced to the testing room 30 minutes before the test to acclimate. Each mouse is placed in the central zone and allowed to freely explore for 5 minutes [22].

Y-Maze Test (RWD Life Sciences, 63006): The maze consists of three identical arms (30 × 8 × 15 cm). Mice are allowed to acclimate to the test environment for 30 minutes before testing. During testing, place the mice at the center point and allow free exploration for 5 minutes. Record the sequence of arm entries [23].

2.10 Statistical Analysis

The study was conducted blind where applicable. Western blot bands and pathological staining results were quantified using ImageJ (U. S. National Institutes of Health, Bethesda, MD, USA). For comparisons between two groups, a two-tailed Student's *t*-test was used. For comparisons involving multiple groups, one-way ANOVA with Tukey's HSD post-hoc correction was applied. For analyses involving two factors (e.g., time × group, brain region × group), two-way ANOVA with Šidák's multiple comparisons correction was used. For circadian rhythm analyses, the significance of the rhythm was assessed using Cosinor analysis (nonlinear regression of a 24-hour cosine function). For Western blot data involving 3 brain regions × 4 ZT points × 5 groups, multiple comparisons were corrected using the Benjamini-Hochberg false discovery rate (FDR) method at $p = 0.05$. Inter-rater reliability for cell counting was assessed using the intraclass correlation coefficient (ICC >0.9 for all measures). p value of <0.05 was considered statistically significant ($*p < 0.05$, $**p < 0.01$, $***p < 0.001$). Behavioral data were recorded in Excel. Data analysis and graph generation were performed using GraphPad Prism 9.5 (version 9.5, GraphPad Software, San Diego, CA, USA) and ImageJ.

3. Results

3.1 Expression of Rev-Erbα in Different Cerebral Regions of Epileptic Mice

To explore the role of Rev-Erbα in epileptic mice, the expression of Rev-Erbα was examined spatially in the mPFC, hippocampus (HP), and occipital lobe visual cortex (V2M), and temporally at different time points by Western blotting. The original Western blot bands are provided in the **Supplementary Material**.

On Western blots, the expression of Rev-Erbα showed region- and time-dependent alterations in epileptic mice ($p < 0.001$). In the mPFC, both SE and CE groups exhibited significantly lower Rev-Erbα expression than controls only at ZT6 ($p < 0.001$), while no significant differences were observed at ZT0, ZT12, or ZT18. Although statistical significance was not reached at every time point, the overall expression level of Rev-Erbα in epileptic mice tended to be lower than that in controls across most time points (Fig. 1b,g,k). In terms of circadian phasing, the peak in the control group occurred at ZT6, which was delayed to ZT12 in both epileptic models. In the V2M, the control group exhibited the highest expression at ZT12, followed closely by ZT6 (Fig. 1c,h,j). In the HP, significant reductions were detected at ZT0 ($p < 0.01$) and at ZT12 ($p < 0.05$), whereas no significant differences were found at ZT6 or ZT18. As observed in the mPFC, the overall expression in the epileptic groups showed a downward trend compared with the control group (Fig. 1a,f,i). The circadian peak in the control group occurred at ZT12, which shifted to ZT18 in the SE group, while the CE group exhibited a broad peak spanning ZT12–ZT18.

3.2 Expression of Rev-Erbα in Different Cerebral Regions in SR9009+Epilepsy Mice

In both the SE and CE groups, Rev-Erbα expression in the mPFC, HP, and V2M was reduced compared to the control group ($p < 0.001$), with significant downregulation observed in the HP and V2M ($p = 0.021$). After SR9009 administration, Rev-Erbα expression in the three cerebral regions was higher than that in the SE group ($p < 0.001$), especially in the V2M ($p < 0.001$; Fig. 1d,e). After long-term SR9009 treatment, Rev-Erbα expression was further increased across the mPFC, HP, and V2M ($p = 0.020$). Notably, expression levels in the HP and mPFC were significantly higher than those in the CE group ($p = 0.007$). More-

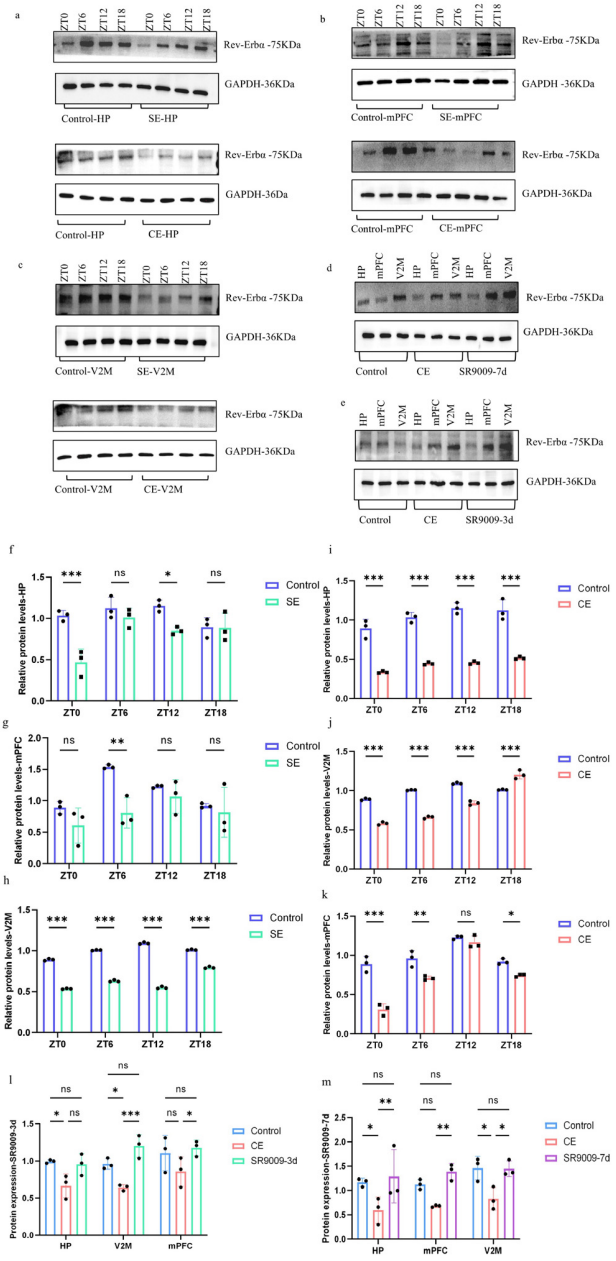

Fig. 1. Expression of Rev-Erbα in epilepsy and Control groups. (a–c) Expression of Rev-Erbα in the mPFC, HP, and V2M brain regions in the SE, CE, and Control groups. (d,e) Expression of Rev-Erbα in the mPFC, HP, and V2M brain regions in the Control, CE, SR9009-3d, and SR9009-7d groups. (f–h) Quantitative statistical graphs of Rev-Erbα expression in the three brain regions in the SE and Control groups. (i–k) Quantitative statistical graphs of Rev-Erbα expression in the three brain regions in the CE and Control groups. (l,m) Quantitative statistical graphs of Rev-Erbα expression in the three brain regions in the Control, acute and chronic epilepsy, and SR9009-7d and SR9009-3d groups. n = 3 valid biological replicates per group after quality screening (5 mice harvested in total), data are shown as mean ± SEM, ns, $p > 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. mPFC, medial prefrontal cortex.

over, the difference in Rev-Erbα expression was not significant between the SR9009-E and control groups in the examined cerebral regions ($p = 0.830$). In the three brain regions, Rev-Erbα expression in the SR9009-E group also differed only minimally between the short-term and the long-term groups ($p = 0.943$; Fig. 1l,m).

Rev-Erbα exhibited a distinct circadian rhythm in the mPFC, HP, and V2M of normal mice. In the pilocarpine-induced SE and CE mice, Rev-Erbα expression was significantly reduced and lost its original rhythmicity in the mPFC, HP, and V2M. These results indicate that the endogenous rhythmic patterns of Rev-Erbα are disrupted in epilepsy.

3.3 Spatial and Temporal Dynamics of Rev-Erbα and Neurons

To determine whether Rev-Erbα affects the nervous system, double-labeling immunofluorescence was performed. The results revealed that Rev-Erbα was widely expressed in astrocytes, microglia, and neurons in the mPFC, HP, and V2M cerebral regions of the control group. Quantitative analysis showed that Rev-Erbα expression was significantly higher in neurons than in astrocytes or microglia. Thus, Rev-Erbα may play a more substantial role in neurons ($p < 0.001$) (Fig. 2a,b). The number of cells co-expressing Rev-Erbα and neurons (NeuN) was then examined, and co-expression was reduced across the different cerebral regions of the epilepsy groups.

Co-expression of Rev-Erbα and NeuN in all three brain regions was reduced in the epilepsy group, with overall levels lower than in the control group across all four time points ($p < 0.001$). These changes showed distinct brain-region specificity and phase specificity. In the SE group, co-expression levels were significantly reduced in the HP and V2M ($p = 0.006$), with a further decline observed in the CE group ($p = 0.001$; Fig. 2e–h). Notably, the most pronounced differences from the control group occurred in the HP at ZT12, where the peak shifted from ZT0 in the control group to ZT12 in CE, and in the V2M at ZT18, suggesting that neuronal clock function in these regions may be selectively impaired at specific time points (Fig. 2e–h).

Overall, Rev-Erbα/NeuN co-expression was reduced in both the SE and CE groups, and its circadian rhythm was altered. However, only minimal differences were observed between the SE and CE groups themselves ($p = 0.984$; Fig. 2c,d,f,h).

3.4 Upregulation of Rev-Erbα Expression Alleviates Neuronal Damage in Epileptic Mice

The effects of Rev-Erbα on neurons in different cerebral regions were further evaluated using immunofluorescence, Nissl staining, and H&E staining.

Nissl staining showed that the number of Nissl bodies in the short-term SR9009-E group was increased relative to the epilepsy group in the mPFC, HP, and V2M. H&E staining showed cellular morphological alterations, disorganized arrangement, and nuclear shrinkage in the mPFC, CA1, and V2M. This trend became more obvious in the long-term group. There was no substantial difference between the SR9009-E and the control group across the three cerebral regions and between the two stages (short-term and long-term). In contrast, by Nissl and H&E staining, the number of damaged cells in the epilepsy group was increased compared to the control group across the different cerebral regions (Fig. 3c,d). Quantitative immunofluorescence analysis showed that Rev-Erbα+NeuN positive cell numbers in three brain regions were higher in the SR9009 group than in the epilepsy group ($p = 0.008$ for SR9009-7d with CE; $p = 0.041$ for SR9009-3d with SE; Fig. 3a,b). However, neuron counts in the SR9009-E group remained lower than in the control group across the different cerebral regions.($p = 0.037$ for SR9009-7d with control; $p = 0.045$ for SR9009-3d with control) (Fig. 3a,b).

3.5 Frequency of Epileptic Seizures and Abnormal Discharges in Mice

By video monitoring, spontaneous epileptic seizures were less frequent in the SR9009-E group (15.17 ± 0.983 seizure episodes/24 h for 3-day treatment and 12.17 ± 2.137 seizure episodes/24 h for 7-day treatment) than in the epilepsy group (21.17 ± 6.211 seizure episodes/24 h). In addition, abnormal spike waves were less frequent in the SR9009-E group than in the epilepsy group on EEG ($p = 0.006$; Fig. 4a–d).

3.6 Anxiety-Like Behavior and Spatial Working Memory in Mice

In the open-field test, mice in the SR9009-E group spent more time in the center zone, less time in the peripheral zone ($p < 0.001$), traveled a greater distance ($p = 0.004$), and produced fewer fecal pellets ($p = 0.113$), compared with the epilepsy group (Fig. 5a–e).

Spatial working memory was assessed by calculating the spontaneous alternation rate of mice in the Y-maze test. In the SE group, the alternation rate was significantly lower than in the control group ($p = 0.024$), and this trend was more pronounced in the CE group ($p < 0.001$). In contrast, the spontaneous alternation rate increased in both the short-term and long-term SR9009-E groups relative to the SE and CE groups, especially in the long-term SR9009-E group, but remained lower than those in the control group ($p = 0.006$; Fig. 5f,g).

4. Discussion

This study first examined the expression of the circadian nuclear receptor Rev-Erbα across different cerebral regions and time points in epileptic mice. It then compared the control, epilepsy, and SR9009-E groups in terms of neuron numbers, seizures, abnormal cerebral discharges, memory ability, and anxiety behavior. The results showed that Rev-Erbα expression varied across the four time points and three cerebral regions. Moreover, as Rev-Erbα expression was upregulated, neuronal damage, seizure frequency, and abnormal discharge were alleviated, and anxiety behavior and spatial memory were improved. These findings indicate that Rev-Erbα plays a beneficial and potentially protective role in epilepsy.

Epileptic seizures exhibit marked circadian rhythmicity [24]. Clinically, most patients with epilepsy are more prone to seizures during sleep, especially during sleep-wake transitions. As a key molecule linking the circadian clock to downstream physiological functions, Rev-Erbα dysfunction may directly influence seizure susceptibility and epileptic networks through multiple pathways

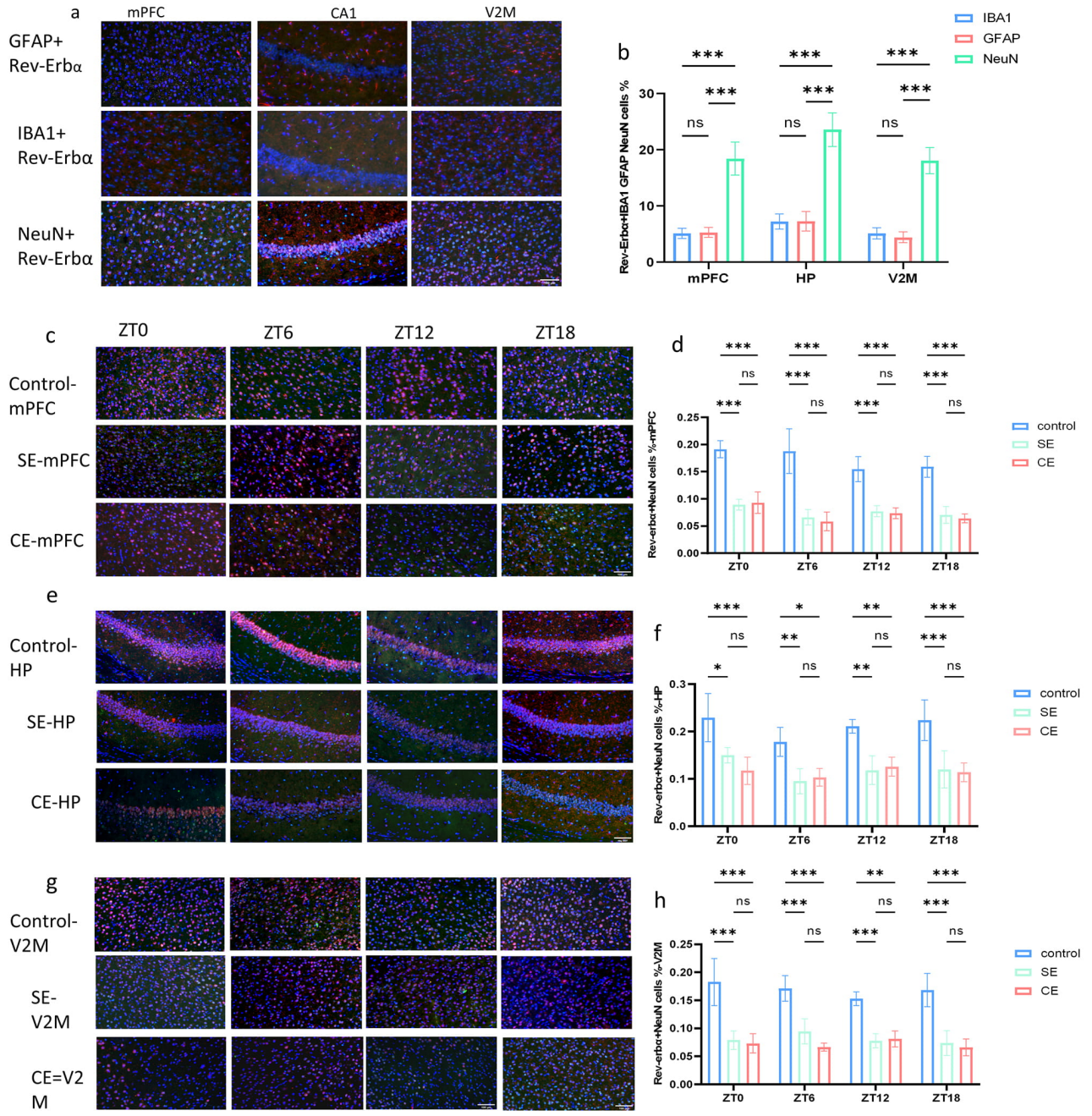

Fig. 2. Spatiotemporal expression of Rev-Erbα and neurons (NeuN). Rev-Erbα (Alexa Fluor 488, Ex/Em = 488/519 nm), NeuN/GFAP/IBA1 (Alexa Fluor 594, Ex/Em = 594/617 nm), DAPI (Ex/Em = 358/461 nm). (a) Co-expression of Rev-Erbα in the Control group with astrocyte marker GFAP, microglial marker IBA1, and neuronal marker NeuN in the mPFC, HP-CA1, and V2M brain regions, respectively. (b) The quantitative statistical graph shows that Rev-Erbα is highly expressed in NeuN-positive neurons. (c,d) Changes in the co-expression of Rev-Erbα and NeuN in the mPFC brain region of the Control, acute epilepsy, and chronic epilepsy groups at four time points. (e,f) Changes in the co-expression of Rev-Erbα and NeuN in the CA1 brain region of the Control, acute epilepsy, and chronic epilepsy groups at four time points. (g,h) Changes in the co-expression of Rev-Erbα and NeuN in the V2M brain region of the Control, acute epilepsy, and chronic epilepsy groups at four time points. n = 3 valid biological replicates per group after quality screening (5 mice harvested in total), data are shown as mean ± SEM, ns, $p > 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, scale bar: 100 μm.

[25,26]. Previous studies have reported that focal seizures often arise in the hippocampus, prefrontal lobe, or occipital lobe; therefore, these regions were selected as the target zones. In normal brain tissue, Rev-Erbα showed distinct circadian rhythms in the mPFC, HP, and V2M regions, with well-defined expression peaks and troughs. In epilep-

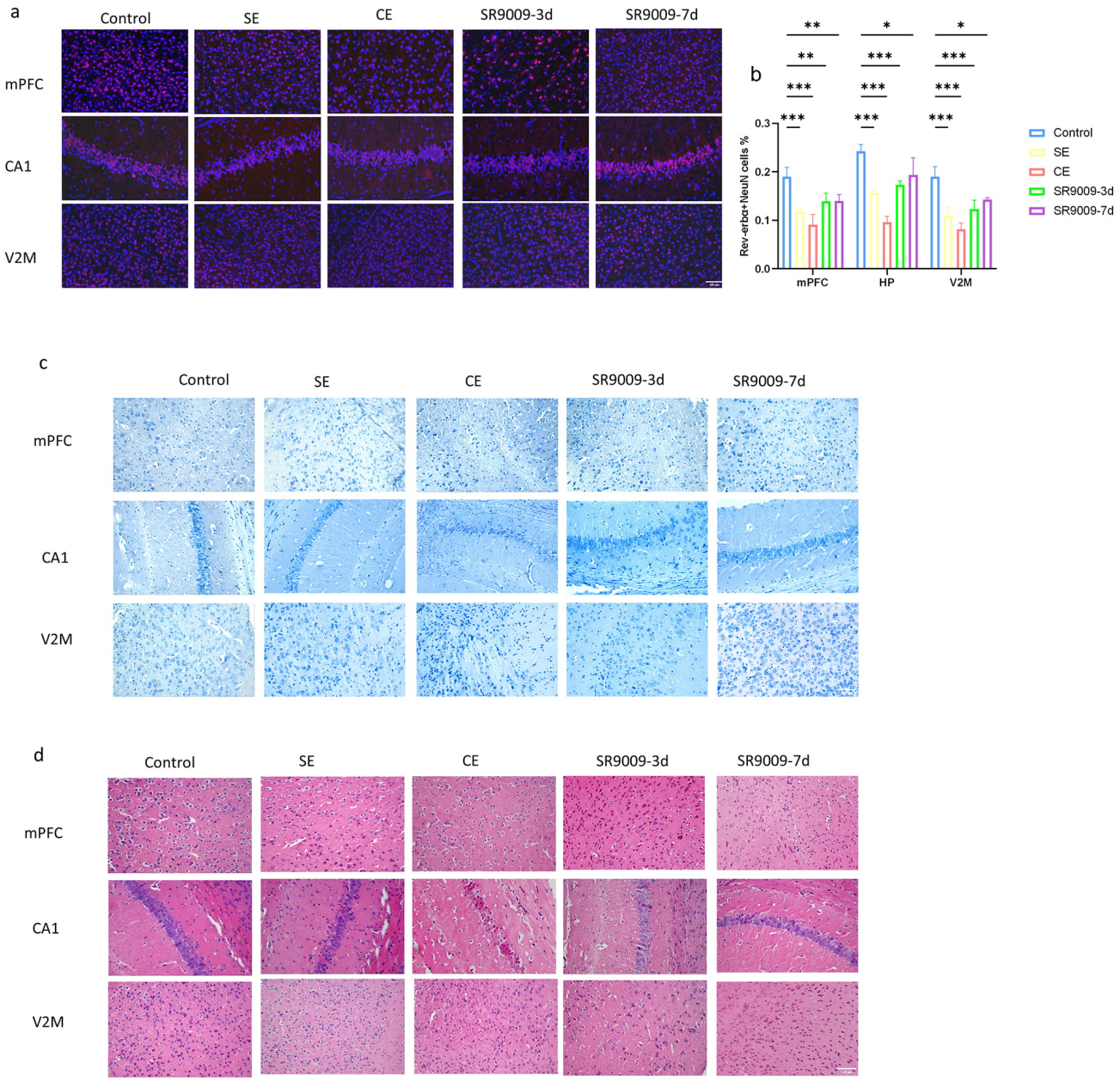

Fig. 3. SR9009 treatment ameliorates neuronal damage. (a) Rev-Erbα+NeuN positivestaining of the mPFC, CA1, and V2M in the Control, acute epilepsy, chronic epilepsy, SR9009-3d, and SR9009-7d groups. Rev-Erbα (Alexa Fluor 488, Ex/Em = 488/519 nm), NeuN (Alexa Fluor 594, Ex/Em = 594/617 nm), DAPI (Ex/Em = 358/461 nm). n =3 valid biological replicates per group after quality screening (5 mice harvested in total); data are shown as mean ± SEM, scale bar: 100 μm. (b) Quantitative analysis of Rev-Erbα+NeuN positive cell expression in three brain regions at ZT18 in the control, epilepsy, SR9009-3d, and SR9009-7d groups. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. (c) Nissl staining of the mPFC, CA1, and V2M in the Control, acute epilepsy, chronic epilepsy, SR9009-3d, and SR9009-7d groups. (d) H&E staining of the mPFC, CA1, and V2M in the Control, acute epilepsy, chronic epilepsy, SR9009-3d, and SR9009-7d groups. scale bar: 100 μm.

tic mice, Rev-Erbα expression was reduced across all three brain regions, consistent with prior research. The results further indicated that these regions retain distinct time-of-day expression rhythms during epilepsy. Compared with the control group, the peak time of Rev-Erbα expression in epileptic mice was delayed (in the mPFC and HP) or advanced (in the V2M). These results suggest that epilepsy disrupts circadian rhythms and alters Rev-Erbα expression across different brain regions. The progressive decline from SE to CE suggests that Rev-Erbα downregulation is not merely an acute response to seizure activity but may contribute to the chronicization process from status epilepticus to established epilepsy.

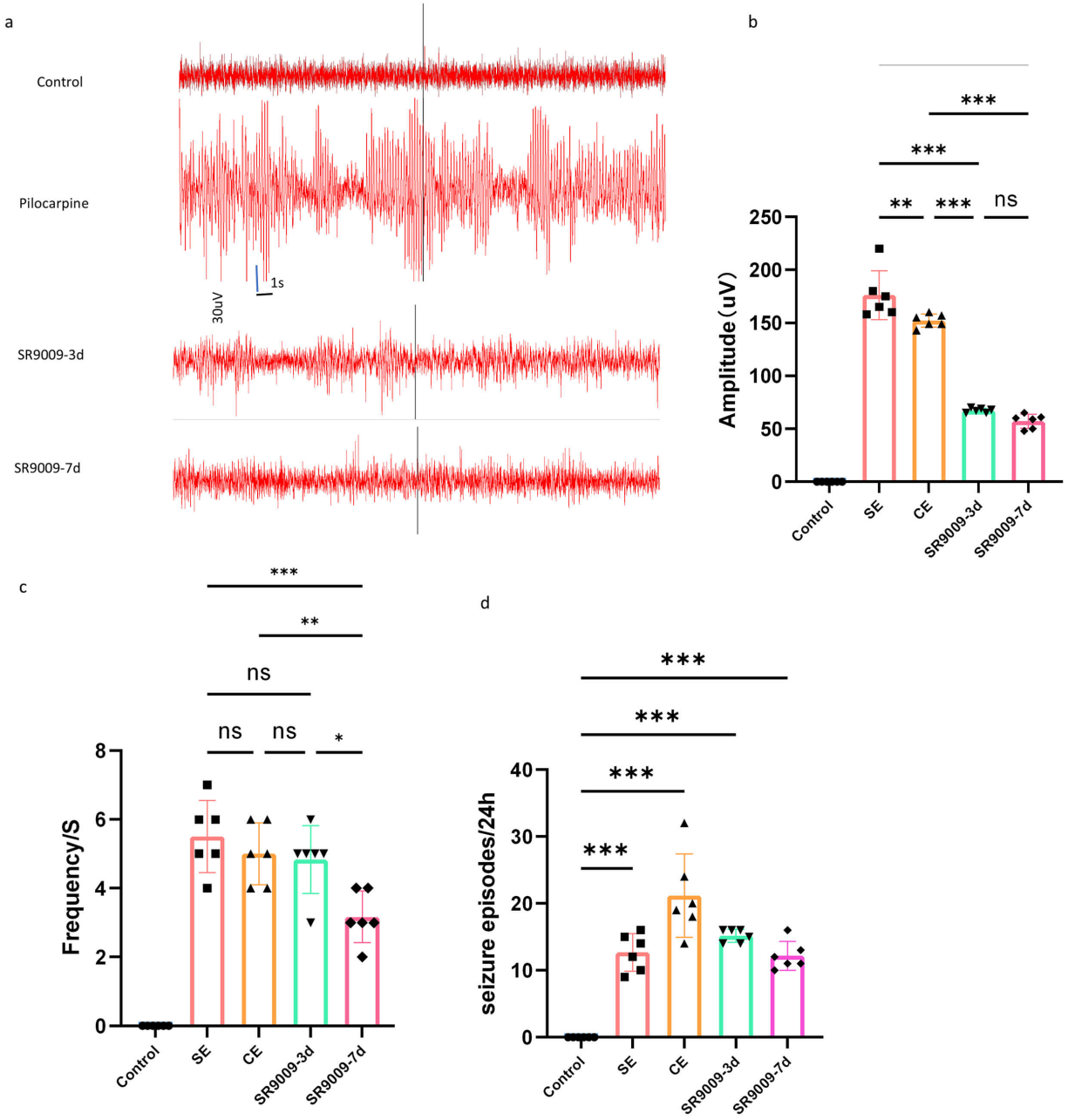

Fig. 4. SR9009 modulates the effects of Rev-Erbα on epileptic seizures. (a) Representative EEG in the Control, Pilocarpine, SR9009-3d, and SR9009-7d groups. (b–d) Seizure frequency and amplitude in the Control, SE, CE, SR9009-3d, and SR9009-7d groups. n = 6 biologically independent mice per experimental group; each mouse completed one full round of EEG, data shown as mean ± SEM, ns, $p > 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Rev-Erbα is widely expressed in both glial cells and neurons [27,28]. Prior studies have shown that activating Rev-Erbα in epilepsy markedly suppresses glial proliferation and inflammatory responses in the hippocampus [16,29]. In our cellular analysis, Rev-Erbα was predominantly expressed in neurons. In the analysis of Rev-Erbα/NeuN co-expression, reduced Rev-Erbα expression was observed across the three brain regions. This reduction persisted across all four time points, with the most pronounced decrease during the chronic phase. This suggests that Rev-Erbα downregulation may be closely associated with epilepsy progression. To further investigate the role of Rev-Erbα in epilepsy, we administered the specific Rev-Erbα agonist SR9009, which can upregulate Rev-Erbα [26]. Based on previous studies and our findings (the lowest expression of Rev-Erbα at ZT18 in the HP and mPFC), ZT18 was chosen as the observation time point.

Western blotting showed that SR9009 treatment at ZT18 significantly upregulated Rev-Erbα expression in all three brain regions, with the most pronounced increases ob-

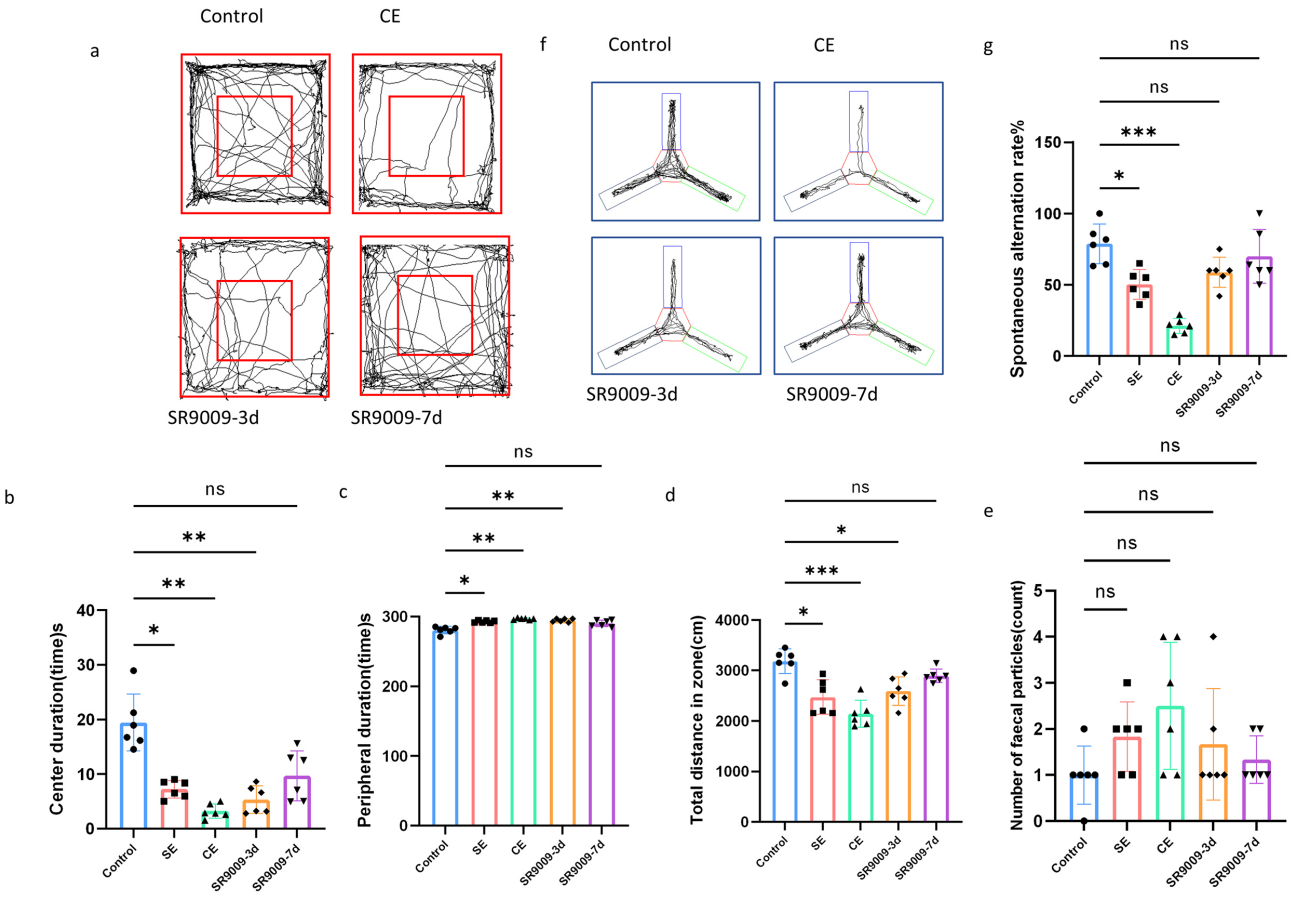

Fig. 5. SR9009 modulates the effects of Rev-Erbα on anxiety-like behavior and spatial working memory. (a,f) Representative trajectory diagram in the epilepsy and SR9009-E groups. (b–e,g) Control, epilepsy, and SR9009-E groups: Anxiety-like behavior and spatial working memory indicators: central zone, peripheral zone, total distance in zone, fecal boli count, spontaneous alternation rate. n = 6 biologically independent mice per experimental group; each mouse completed one full round of behavioral testing, data shown as mean ± SEM, ns, $p > 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

served in the mPFC after short-term treatment and in the V2M after prolonged (7-day) administration. In parallel, we examined NeuN expression in these two brain regions by immunofluorescence and found that neuronal damage was reduced in both areas following SR9009 treatment, indicating that increased Rev-Erbα expression exerts a protective effect. By Nissl and H&E staining, neuronal cell counts increased in the short-term SR9009 group compared with the epilepsy group in the HP. After long-term SR9009 administration, the number of surviving neurons increased significantly, and cellular damage decreased, with the most pronounced effects in the V2M. These findings suggest that Rev-Erbα may play a protective role during the short-term stage and region-specific differences in SR9009 bioavailability or Rev-Erbα turnover, but its protective effects appear in the HP during the long-term stage. Immunofluorescence analysis further showed that NeuN expression increased in the SR9009-E group, compared with the epilepsy group. These findings suggest that Rev-Erbα regulation undergoes specific alterations at different time points and in different cerebral regions, and that Rev-Erbα is highly expressed in neurons. We suggest that in epilepsy, it may exert a protective effect through direct action on neurons. Based on these findings, in different types of focal epilepsy, the timing of medication could be adjusted according to the rhythmic expression of Rev-Erbα, thereby reducing neural damage and alleviating seizures. Further clarification of the role of Rev-Erbα may therefore provide novel strategies for epilepsy drug therapy.

Notably, a subset of epilepsy patients presents with substantial neuropsychiatric comorbidities, with anxiety disorders and cognitive dysfunction reported in 30–50% and 20–50% of cases, respectively [30,31]. Research has reported that the circadian rhythm gene Rev-Erbα in the mPFC is also susceptible to disruption associated with depressive-like behaviors induced by light-dark cycle reversal in mice [20,32]. The connection between the hippocampus and the prefrontal cortex is essential for memory function. Research has shown impairment of learning and spatial memory after epilepsy [33]. Our study suggests that Rev-Erbα is protective in epilepsy. However, how Rev-Erbα influences epileptic seizures and behavioral outcomes

remains unclear. Further investigation showed that following SR9009-induced upregulation of Rev-Erbα, seizure frequency decreased, and EEG showed reduced abnormal discharges with diminished spike amplitudes. The behavioral tests (open field and Y-maze) showed that epileptic mice exhibited increased anxiety-like behavior, reduced locomotor activity, and impaired spatial memory. Together with the elevation of Rev-Erbα expression, epileptic mice showed reduced seizure frequency and abnormal electroencephalographic activity. Anxiety-like behaviors were also alleviated, and spatial memory was significantly improved. These behavioral findings indicate that increased Rev-Erbα expression may help reduce the occurrence of comorbidities in epilepsy.

Limitations

This study has several limitations arising from experimental and technical constraints. First, we did not examine the impact of Rev-Erbα across multiple brain regions using region-specific Rev-Erbα knockouts. Second, the precise molecular pathways by which Rev-Erbα affects epilepsy have not been fully elucidated. Although we show correlations between Rev-Erbα expression and seizure outcomes, we did not investigate downstream transcriptional targets (e.g., Bmal1, Clock, NF-κB) or potential interactions with ion channels. Transcriptomic or proteomic studies are needed to identify Rev-Erbα-regulated pathways in epileptic brain tissue. Third, future studies with larger cohorts and formal power calculations are needed to confirm these findings. Finally, how Rev-Erbα functions within epileptic neural networks requires further in-depth investigation. Future mechanistic studies should focus on identifying Rev-Erbα target genes in neurons and on determining whether its effects are mediated through canonical circadian pathways or non-circadian functions.

5. Conclusions

The persistent loss of Rev-Erbα seen in epileptic mice, coupled with the protective effects observed in this study, highlights the considerable potential of this molecule as a novel therapeutic target, capable of both modulating seizure activity and improving associated neurobehavioral outcomes. As a key circadian regulator, Rev-Erbα is a promising candidate for future epilepsy treatments.

Availability of Data and Materials

The data are available from the corresponding author upon reasonable request.

Author Contributions

ZY contributed to the writing review and editing, conceptualization, project administration; XJ contributed to the writing-original draft, conceptualization, validation, visualization. CM and WW prepared the methodology and formal Analysis. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work

Ethics Approval and Consent to Participate

All animal studies have been approved by the Animal Welfare and Ethics Committee of the Affiliated Hospital of Hebei University of Engineering [IACUC-Hebei-2024-0037]. The study was conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The ARRIVE guidelines were employed for reporting experiments involving live animals, promoting ethical research practices (see **Supplementary Material**).

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Conflicts of Interest

The authors declare no conflicts of interest.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at https://doi.org/10.31083/JIN54266.

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