NOVEL MECHANISMS OF EPILEPTOGENESIS AND ITS INSPIRED PHARMACEUTICAL TREATMENTS FOR EPILEPSY

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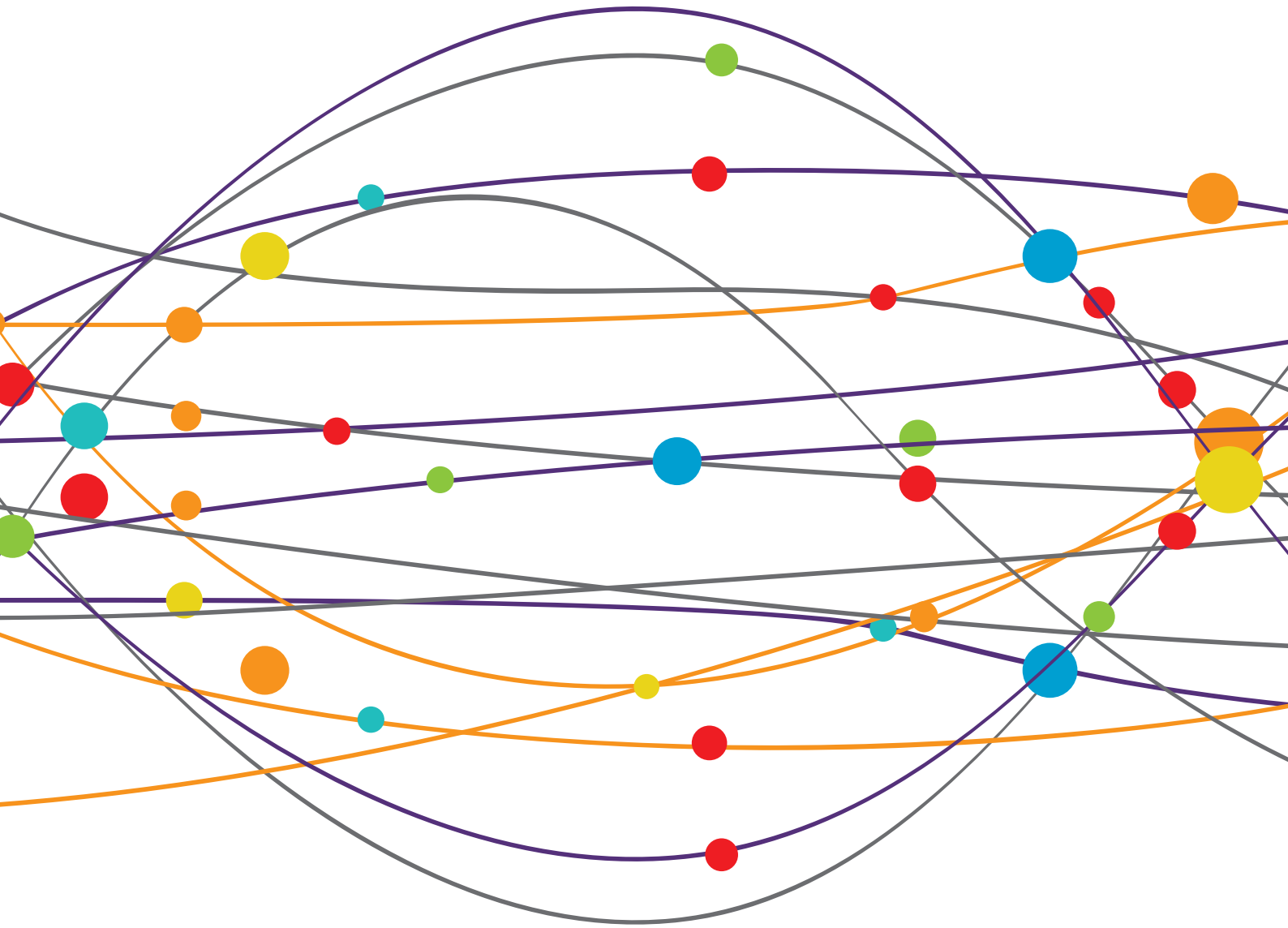

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NOVEL MECHANISMS OF EPILEPTOGENESIS AND ITS INSPIRED PHARMACEUTICAL TREATMENTS FOR EPILEPSY

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EDITORIAL
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Editorial: Novel Mechanisms of Epileptogenesis and Its Inspired Pharmaceutical Treatments for Epilepsy

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Keywords: epilepsy, pathogenesis, pharmacotherapy, precision medicine, treatments

Editorial on the Research Topic

Novel Mechanisms of Epileptogenesis and Its Inspired Pharmaceutical Treatments for Epilepsy

Epilepsy is a common chronic neurological disorder affecting approximately 0.5–1% of the population worldwide (∼50,000,000 people) and it accounts for a variety of neurological disorders characterized by recurrent seizures. More than half of all epilepsies have some genetic basis and single gene defects in ion channels or neurotransmitter receptors are associated with inherited forms of epilepsy (1). In the last decades, epileptogenic mutations have been identified in several ion channel genes, leading to the concept that several epilepsies can be considered channelopathies (2, 3). Functional studies have in some cases provided significant advances in the understanding of the molecular and cellular dysfunctions caused by mutations. However, the relationships between molecular deficits and clinical phenotypes are still unclear. Sun et al. showed that decreased activity of α7 nicotinic acetylcholine receptors (nAChRs) increases the excitability of CA1 pyramidal neurons and reduces the onset time of epilepsy in pilocarpine-induced mouse models. Moreover, the expression of α7 nAChRs is downregulated in human epileptogenic tissues. Overall, their findings confirm that α7 nAChR is an essential regulator of seizure susceptibility. However, the etiology of epilepsy is extremely complex and heterogeneous and both genetic and acquired factors can be responsible for this condition; the cellular mechanisms underlying the epileptogenicity depend on the integrity of the blood-brain barrier, circuit abnormalities, or cellular and molecular defects, leading to epileptogenesis. Nevertheless, there is still a growing need for the identification of accurate biomarkers of epileptogenesis that enable the prediction of epilepsy following a brain insult. Recent technical progress may offer the opportunity for further investigating cortical areas and brain networks involved in cerebral functions and in epileptic discharges. Chen et al. present a comprehensive overview of recent innovations in the role of neuroimaging and EEG in identifying reliable biomarkers of epileptogenesis, whereas Shen et al. provide a review of the mechanisms of secondary epileptogenesis in molecular, cellular, and circuity levels. Zhang et al., instead, used magnetoencephalography (MEG) to evaluate whether the neuromagnetic signals of the brain neurons correlated with the response to therapy in drug-naïve patients and showed that a local frontal epileptic network at 80–250 Hz may increase the risk of drug-resistance in childhood absence epilepsy.

Despite a tremendous increase in the opportunities for non-invasive research on the human brain animal models of epilepsy still allow the investigation of the mechanisms of epileptogenesis and are also useful to study the consequences and co-morbidities of epilepsy and to develop effective treatments. Wang et al. focus their review on the brain-derived neurotrophic factor (BDNF), a

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member of the neurotrophic factor family with an important role in the survival, growth, and differentiation of neurons, and discuss the possibility of BDNF as an underlying target for the treatment of epilepsy, whereas Han et al. highlight the potential role of vascular endothelial growth factor (VEGF) as a critical neurovascular target in modulating epileptogenesis in the animal immature brain after lithium-pilocarpine-induced status epilepticus (SE). Moreover, symptomatic SE is one of the highest risk factors of epileptogenesis as shown by the study by Tong et al. who compared the effect of different drugs in the pilocarpine model of acquired epilepsy.

In humans, SE is a medical emergency associated with acute severe systemic damage and high mortality as confirmed by Li et al. describing a rare nonconvulsive status epilepticus following surgical resection of a pituitary tumor and not taking regular hormone replacement therapy. Nevertheless, the challenge of finding new, more efficacious, and better-tolerated drugs is ongoing. The pipeline for the development of new ASMs with novel mechanisms of action is narrowing with only a few interesting compounds on the immediate horizon. Recent studies prompted the identification of neuroinflammation as a potential target for the treatment of epilepsy, particularly drug-resistant epilepsy, and refractory status epilepticus. In Costagliola et al., a systematic review of the clinical experience with anti-cytokine agents and agents targeting lymphocytes is provided offering promising main therapeutic perspectives in this field. Finally, there is also increased interest in the use of possible alternative treatments. Zhong et al. tested the effect of crocin, the main component of Crocus sativus L., in pilocarpine-induced epileptic mice suggesting a potential anti-epileptic property for this natural compound.

In brief, this issue of Frontiers may serve as a helpful guide for epileptologists, including those beginning their careers and honing their skills, as well as for medical students and residents who want to learn more about the pathophysiology, epidemiology and burden, comorbidities, treatment, and research for the management of epilepsy.

AUTHOR CONTRIBUTIONS

AR: writing of the manuscript. PS: writing of the manuscript and critical revision of the manuscript. Both authors contributed to the article and approved the submitted version.

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Conflict of Interest: AR has received honoraria from Kolfarma s.r.l, Proveca Pharma Ltd, and PTC Therapeutics. PS has served on a scientific advisory board for the Italian Agency of the Drug (AIFA), received honoraria from GW pharma, Kolfarmas.r.l., Proveca Pharma Ltd, and Eisai Inc., and received research support from the Italian Ministry of Health and Fondazione San Paolo.

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BRIEF RESEARCH REPORT
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Nicotinic Acetylcholine Receptor α7 Subunit Is an Essential Regulator of Seizure Susceptibility

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A large body of data has confirmed that α7 nicotinic acetylcholine receptors (nAChRs) play a pivotal role in cognition, memory, and other neuropsychiatric diseases, but their effect on seizure susceptibility in C57BL/6 wild-type mice is not fully understood. Here, we showed that decreased activity of α7 nAChRs could increase the excitability of CA1 pyramidal neurons and shorten the onset time of epilepsy in pilocarpine-induced mouse models. However, compared with the control group, there was no apparent effect of increasing the activity of α7 nAChRs. Moreover, the expression of α7 nAChRs is downregulated in human epileptogenic tissues. Taken together, our findings indicate that α7 nAChR is an essential regulator of seizure susceptibility.

Keywords: cholinergic receptors, α7 nicotinic acetylcholine receptors, epilepsy, seizure susceptibility, CA1 pyramidal neuron

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INTRODUCTION

Nicotinic acetylcholine receptors (nAChRs) are a family of ligand-gated, pentameric ion channels. Understanding the precise roles of nAChRs remains a challenge because they can modulate cholinergic activities both postsynaptically and presynaptically (1). In humans, there are 16 different subunits (α1-7, α9-10, β1-4, δ, ε, γ) of nAChRs, which can be found both in the peripheral and central nervous systems (2–4). Of these, 12 are associated with a wide spectrum of physiological and pharmacological functions (5). The main function of nAChRs is to regulate neuronal plasticity (6) and neuroprotection (7, 8), and the most abundant nAChR subunits are the α4β2 and α7 subunits within the central nervous system. Mutations in the transmembrane regions of the neuronal α4β2 subunit receptors in the neocortex and thalamus can cause autosomal dominant nocturnal frontal lobe epilepsy (9), which is a focal epilepsy in the frontal lobe with attacks typically arising during non-rapid eye movement sleep (10). The α7 subunit is expressed widely in the brain with its highest levels observed in the hippocampus and cortex (11), and its role has been associated with both impairment in cognition and neuropsychiatric phenotypes (12–15).

Our previous studies have indicated that activation of α7 nAChR could decrease seizure susceptibility in Chat-Mecp2$^{-/y}$ mice (16), but the role of α7 nAChR in seizure susceptibility in C57BL/6 wild-type mice and humans remains uncertain. In the present study, we explored the function of α7 nAChRs in seizure susceptibility in wild-type mice. We revealed that decreased α7 nAChR activity in CA1 parvalbumin (PV) neurons in the hippocampus could increase seizure susceptibility in wild-type mice, while the increased activity of α7 nAChR had no significant effect on seizure susceptibility. We also tested the expression of α7 nAChR that was downregulated in tissues of humans, experiencing epilepsy after traumatic brain injury or intracerebral hemorrhage. Our findings suggest that α7 nAChR is an essential regulator of seizure susceptibility.

MATERIALS AND METHODS

All human material was reviewed and approved by the Medical Ethical Committee of Zhejiang Provincial People's Hospital, People's Hospital of Hangzhou Medical College (No. 2019KY255) based on the Code of Ethics of the World Medical Association. All participants provided written informed consent. All animal experimental procedures were examined and approved by the Animal Advisory Committee of Zhejiang Provincial People's Hospital, the People's Hospital of Hangzhou Medical College based on the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Animals and Reagents

We used C57BL/6 wild-type mice to complete the experiment. For acute pilocarpine-induced epilepsy models, C57BL/6 wild-type male mice weighing 25–30 g were used. Before the behavioral test, we kept the mice in a 12/12 h light/dark cycle, and the environmental conditions were controlled. Mice with abnormal body weight and appearance were excluded from the behavioral test. Sexually dimorphic observations were not observed. The primary antibodies used were rabbit polyclonal anti-α7 nAChR (Santa Cruz Biotechnology, USA, Cat#: sc-5544), and rabbit polyclonal anti- glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Cell Signaling Technology, Cat#:5014S). MLA was purchased from Abcam (UK), while pilocarpine and PNU282987 were purchased from Sigma-Aldrich (USA).

Immunohistochemistry

We anesthetized the mice and perfused their brain vessel with 4% paraformaldehyde dissolved in phosphate-buffered saline (PBS). We cut and fixed the whole brains in paraformaldehyde at 4°C overnight and further dehydrated the brains in 30% sucrose in PBS. According to anatomical landmarks, brain sections were cut 35-μm-thick from the regions of interest using a freezing microtome. We treated the brain sections with 10% (v/v) normal donkey serum for immunolabeling in PBS containing 0.3% Triton X-100. Subsequently, we incubated the brain sections with antibodies against anti-α7 nAChR (1:100) at 4°C overnight. We visualized the immunoreactivity with the secondary antibodies of Alexa Fluor 594 donkey anti-mouse, Alexa Fluor 633 donkey anti-goat, and Alexa Fluor 488 donkey anti-rabbit IgG (1:400). We visualized the immunofluorescent images by confocal microscopy (FV1000 Laser scanning confocal microscope, USA).

Human Tissue Preparation and Western Blot Analysis

We dissected 1 g of epilepsy tissue from fresh frozen brain sections that were maintained at −80°C. The tissue was homogenized in a lysis buffer (Beyotime Biotechnology, China) containing 1 mM protease inhibitor phenylmethylsulfonyl fluoride (PMSF) (Beyotime Biotechnology). We collected the supernatants after centrifugation for 10 min at 15 000 rpm. After the protein concentration was measured using Bradford's solution (Beyotime Biotechnology), we boiled the samples in a loading buffer containing equivalent amounts of protein for 5 min (Beyotime Biotechnology). We performed sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred proteins to an Immobilon polyvinylidene fluoride (PVDF) membrane (Millipore) for 100 min at 300 mA. We incubated the membranes with primary antibodies against anti-α7 nAChR (1:100) after blocking with Tris-buffered saline buffer with Tween 20 (TBST) solution (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.1% Tween 20) containing 5% skimmed milk for 1 h at room temperature. After washing with TBST, we incubated the blots with secondary antibodies (1:7,500) for 1 h at room temperature. We detected the signals with enhanced chemiluminescence and developed them on an X-ray film. We digitized the immunoblots on a flatbed scanner and quantified the images using the US National Institutes of Health Image program for densitometric quantification.

Surgeries and Electroencephalogram Measurements

We secured the mice in a stereotactic head frame and made an incision along the midline after the mice were anesthetized with isoflurane. We used 8% H_2O_2 to observe the bregma and the posterior. We placed the electrode over the cranium with three screws (three positions: the first screw (left): A-P: +1.5 mm, lateral: +1.5 mm; the second and the third screws: A-P: −3 mm, lateral: ±3 mm). We placed the cannula (intraventricular drug administration) into the CA1 in the hippocampus (position (right): A-P: −1.94 mm, lateral: ±1.1 mm depth: −2.0 mm). We recorded the electroencephalogram (EEG) results in freely moving mice for 1 week after the surgery. α7 nAChR agonist (PNU282987, a selective α7 nAChR agonist, 1M) and antagonist (methyllycaconitine citrate (MLA), a specific α7 nAChR blocker,100 nM) were injected into the hippocampus in the CA1 by microtubule drug administration. Pilocarpine was injected into the peritoneal cavity following intraperitoneal administration.

We defined the abnormal wave as an amplitude >400 mV. We assessed the pilocarpine-induced seizure stages as follows: onset time was measured from the moment the C57BL/6 mice were injected with pilocarpine until the first epileptic waves were observed. The latency of generalized tonic-clonic (GTC) seizure was measured from the moment the C57BL/6 mice, were injected with pilocarpine until the first GTC seizure epileptic waves were observed. The latency of death was measured from the moment the mice were injected with injection pilocarpine, until the death of the mice.

Electrophysiology

Coronal slices of the hippocampus were performed in approximately 4- weeks-old mice. Slices (300 μm) were prepared with a Vibroslice (Leica VT 1000S) in ice-cold artificial cerebrospinal fluid (ACSF: 125 mM NaCl, 3 mM KCl, 1.25 mM NaH2PO4, 2 mM MgSO4, 2 mM CaCl2, 25 mM NaHCO3 and 10 mM glucose). After recovery for ∼60 min, incubation in ACSF at 33°C was followed by ∼60 min at 22°C, when slices were transferred to the recording chamber and superfused (3 mL min^{-}) with ACSF at 32–33°C. All solutions

were saturated with 95% O_2 and 5% CO_2. The neurons were identified in the brain sections using an upright microscope equipped with a 40× water-immersion lens (Nikon, Eclipse FN1, Japan), and the electrical activity was recorded using whole-cell technology (MultiClamp 700-B Amplifier, Digidata 1440 A analog-to-digital converter, and **PCLAMP** 10.2 software, Axon Instruments Molecular Devices, USA). Pyramidal neurons were recorded in the hippocampal CA1 region. Glass pipettes (3–4.5 MΩ) used for whole-cell recording were filled with internal recording solution: 110 mM K-gluconate, 40 mM KCl, 10 mM HEPES, 2 mM Mg2ATP, 0.5 mM NaGTP, and 0.2 mM EGTA; pH was adjusted to 7.25 with 10 M KOH. We also added DL-2-amino-5-phosphonovaleric acid (DL-AP5; 50 μM, Tocris Bioscience), 6, 7-dinitroquinoxaline-2, 3-dione (DNQX; 20 μM, Tocris Bioscience) and picrotoxin (100 μM, Abcam), to block AMPA-mediated, NMDA-mediated, and GABA-mediated synaptic transmission.

Statistical Analysis

Unless otherwise stated, we expressed the data as mean ± standard error of the mean (SEM); error bars show the SEM (n = number of samples). A two-tailed Student's t-test was used to compare the means from the same group of cells. The differences between more than two groups were tested with two-way analysis of variance. Differences were considered significant at $P < 0.05$.

RESULTS

Expression of α7 nAChR in the CA1 Neurons in the Hippocampus

Mice expressing the channelrhodopsin-2 (ChR2) protein under the control of the choline acetyltransferase (ChAT, a marker for cholinergic neurons) promoter (ChAT–ChR2–EYFP) were used in this study. The hippocampus has been reported to be the main target for the projection of basal forebrain cholinergic neurons (17). To confirm this, we stained the NeuN in hippocampal CA1 neurons in ChAT–ChR2–EYFP mice (**Figure 1A**. left). We found that there are lots of cholinergic neuron fiber projections in this area (**Figure 1A**. middle).

After merging, the results showed that hippocampus CA1 neurons receive dense cholinergic projections (**Figure 1A**. right). Previous studies showed that the main target projection neurons of α7 nAChRs are the PV-positive interneurons, which are the most prominent GABAergic neurons in the hippocampus (16). Therefore, we stained α7 nAChRs in the CA1 neurons (**Figure 1B**). The results showed that α7 nAChRs were concentrated in PV GABAergic neurons, with little expression in non-PV neurons in the CA1 (**Figure 1B**). Taken together, these results indicate that the cholinergic neurons were highly expressed in the hippocampal CA1 PV GABAergic neurons.

Decreased Activity of α7 nAChRs Could Increase the Seizure Susceptibility in C57Bl/6 Wild-Type Mice

To investigate the effects of α7 nAChRs on seizure susceptibility in C57BL/6 wild-type mice, we injected PNU282987 (1 M) and MLA (100 nM) into the hippocampus in the CA1 by microtubule drug administration. Scopolamine methylnitrate (1 mg/kg s.c.; Sigma) was administered 30 min before pilocarpine injection to avoid peripheral cholinergic effects (18). We measured seizure susceptibility by injecting the cholinergic agonist pilocarpine (250 mg/kg i.p.) following intraperitoneal administration, which acts on muscarinic receptors.

To observe the electrographic changes caused by pilocarpine-induced seizures between the vehicle and α7 nAChR agonist and antagonist, we recorded EEGs using bilateral epidural screw electrodes (see **Methods** section) and quantified the electrographic seizures by the latency of onset time, the latency of GTC, and the latency of death. After 10 min baseline recording, we administrated the vehicle and PNU282987 in the CA1 of the hippocampus in C57BL/6 wildtype mice. After recording the EEGs for 15 min, we injected pilocarpine following intraperitoneal administration to induce epilepsy. The results showed that there was no significant difference in the onset time of epilepsy and the latency of GTC (**Figures 2A–C**). Moreover, we found the latency of death was slightly prolonged after administration of PNU282987, but there was no significant difference between the vehicle-treated control group and the C57BL/6 wild-type group (**Figure 2D**). However, the latency of onset time, the latency of GTC, and the latency of death were significantly shortened with MLA treatment (**Figures 2A–D**). The results indicated that a decrease in the activity of α7 nAChRs could increase seizure susceptibility but the increased activity of α7 nAChRs has no effect.

Decreased Activity of α7 nAChRs Could Increase the Excitability of the CA1 Pyramidal Neurons in the Hippocampus in C57BL/6 Wild-Type Mice

To further demonstrate the effect of α7 nAChRs, we recorded the excitability of CA1 pyramidal neurons by checking the number of action potentials (APs) elicited by current injections of various amplitudes (1 s, 0 to +250 pA) in the acute cortical slices. All pyramidal neurons showed high-frequency discharges with increasing currents. We assessed the effect of PNU282987(1 μm), a selective α7 nAChR agonist, on hippocampal slices from C57BL/6 mice. In contrast to the control group, there was no significant change in the excitability of pyramidal neurons in C57BL/6 wide-type mice (**Figures 3A,B**). However, with the bath application of MLA (10 nM), a specific α7 nAChR blocker, the number of APs was significantly higher in the C57BL/6 group compared with that in the control group (**Figures 3C,D**). Taken together, these results revealed that a decrease in the activity of α7 nAChRs could increase the excitability of CA1 pyramidal neurons in the hippocampus.

We believed that the effect of α7 nAChRs on pyramidal neuron excitability could be due to the following two possibilities: the inhibitory input from parvalbumin (PV) interneurons to pyramidal neurons, and the direct modulation of α7 nAChRs on pyramidal neurons (19). To distinguish between the two possibilities, we administered DL-APV, DNQX, and picrotoxin

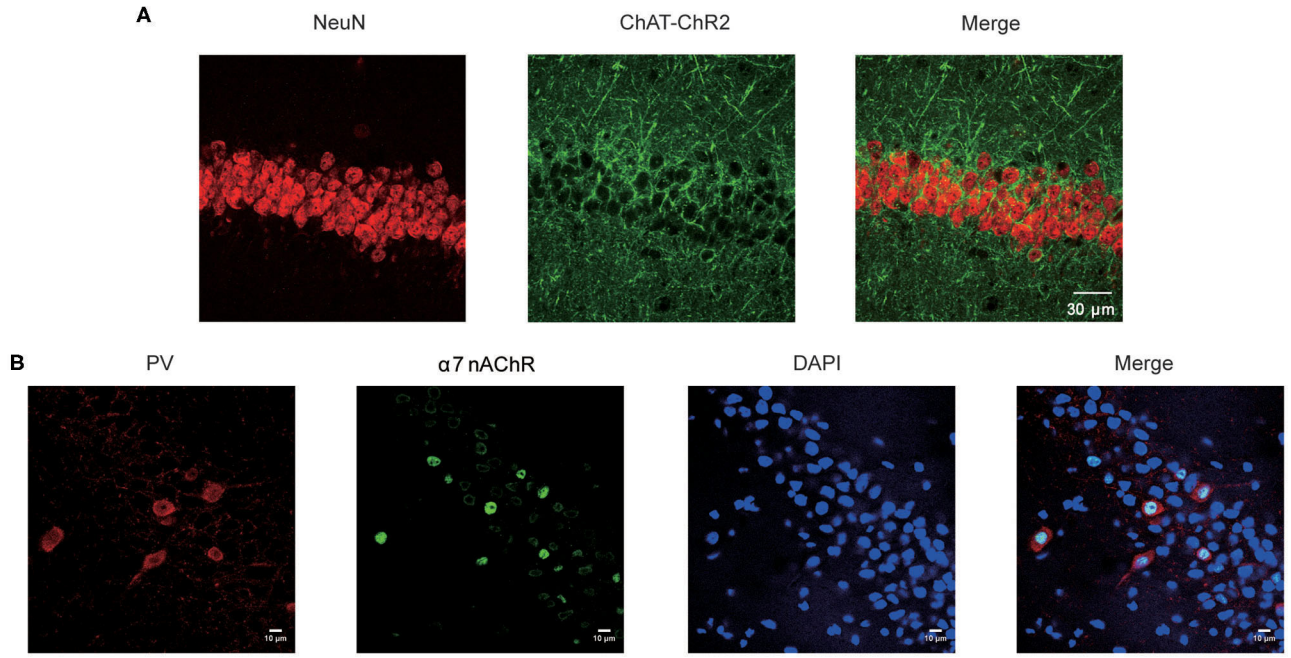

FIGURE 1 | Expression of α7 nAChR in the CA1 neurons in hippocampus. **(A)** Fluorescence images showing CA1 sections stained for the NeuN and the cholinergic neuron projections in the Chat-ChR2-EYFP mice. **(B)** Immunostaining of the hippocampal pyramidal layer with neurons and α7 nAChRs, showing the expression pattern of α7 nAChRs on PV GABAergic neurons and non-PV neurons.

to block NMDA-mediated, AMPA-mediated, and GABAA-mediated synaptic transmission, respectively. The results showed that there was a slight increase but no significant change of pyramidal neuron excitability after the application of MLA (10 nM), suggesting that the excitability of pyramidal neurons is mostly regulated by the inhibitory input from PV neurons (**Figures 3E,F**).

To further study the effect of MLA on the excitability of CA1 pyramidal neurons in epileptic brain slices. We recorded the excitability of CA1 pyramidal neurons in epileptic brain slices before and after administrating MLA. The excitability of pyramidal neurons was increased after epilepsy, and the excitability is slightly higher after adding MLA. However, there was no significant difference in pyramidal neuron excitability after the application of MLA (**Figures 3G,H**). The results indicate that the α7 nAChRs on pyramidal neurons may also play a role in regulating excitability.

Decreased α7 nAChR Expression in Human Epileptogenic Tissues

To further confirm the association between the activity of α7 nAChRs and seizure susceptibility, we assessed the expression of α7 nAChRs in cell membranes by immunoblotting from the epileptogenic foci tissue between subjects with secondary and intractable epilepsy and the those with traumatic brain injury (**Figure 4A**). The results showed that the level of α7 nAChRs in the epilepsy samples was only ∼50% of that in the control group (**Figure 4B**). This observation is also supported by a decrease in α7 nAChR expression in human epileptogenic tissues.

DISCUSSION

This study investigated the association between the activity of α7 nAChRs and seizure susceptibility. We presented evidence that decreased activity of α7 nAChRs could increase seizure susceptibility in C57BL/6 wild-type mice. First, we proved that cholinergic neurons exist, and its subunit receptor-α7 nAChRs mostly expressed in CA1 PV interneurons. Second, we studied seizure susceptibility after the stimulation and inhibition of α7 nAChRs. To address this issue, we used C57BL/6 wild-type mice experiencing pilocarpine-induced seizures as the experimental animals. The results showed that the seizure susceptibility between the PNU282987-treated mice and the control group was not significantly different, but the MLA-treated mice could significantly shorten the seizure susceptibility. Third, the number of APs was significantly increased after MLA treatment; however, there were no significant differences after infusion of PNU282987 in CA1 pyramidal neurons. Furthermore, we found that the excitability of pyramidal neurons was mostly regulated by α7 nAChRs receptors of PV GABAergic neurons. Finally, the study showed that the expression of α7 nAChR protein was reduced in the epileptogenic foci tissue from individuals. In summary, our study identified that decreased α7 nAChRs may be a risk factor for seizure susceptibility.

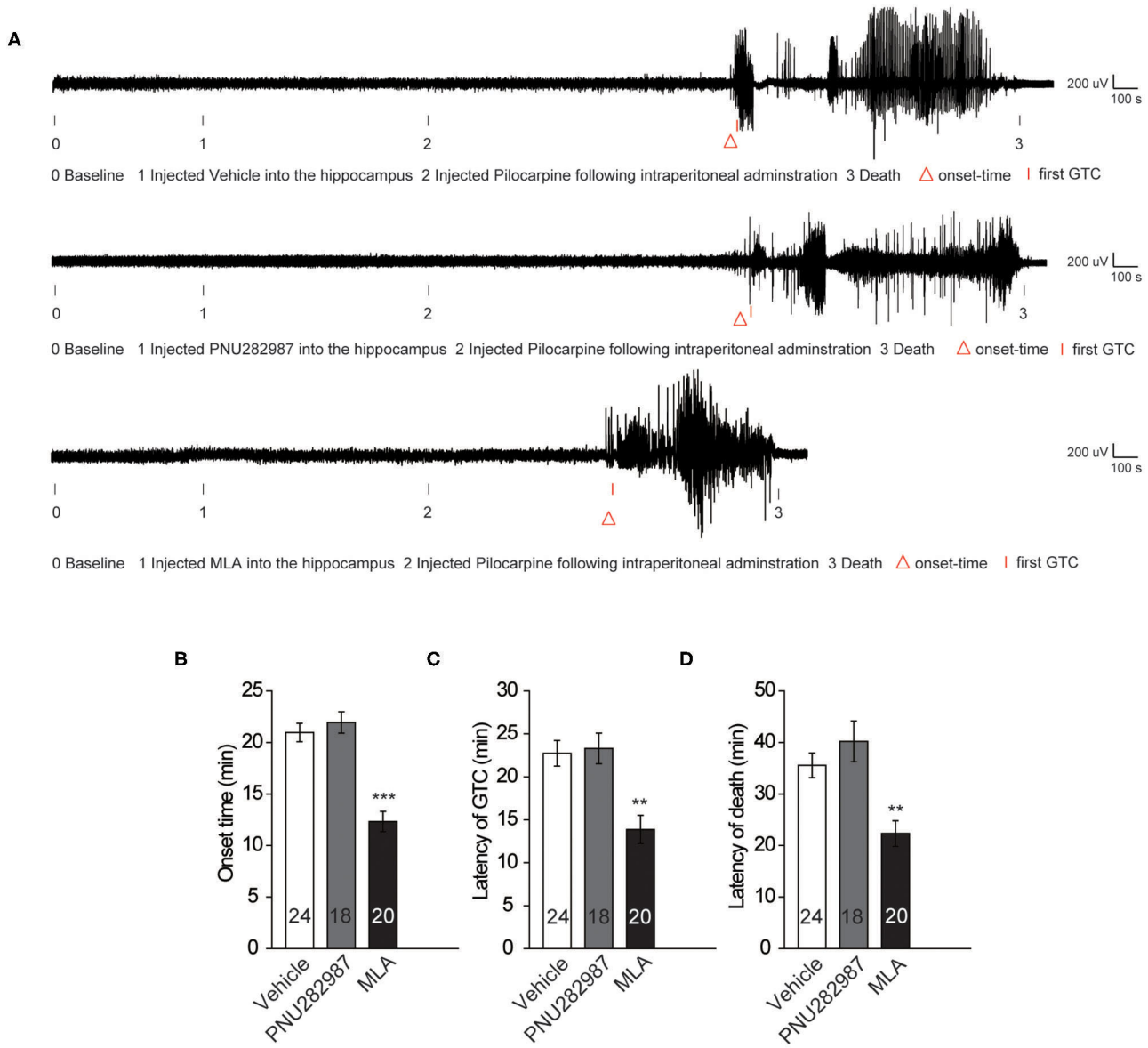

FIGURE 2 | Decreased activity of α7 nAChRs could increase the seizure susceptibility in C57BL/6 wild-type mice. **(A)** Representative compressed electroencephalogram from a cortical lead depicting an electrographic seizure in a C57BL/6 mouse with seizure behaviors identified at the time of occurrence. Background EEG baseline is shown before seizure onset. **(B–D)** Summary histograms of the onset time, latency of GTC, and latency of death, respectively. Two-way ANOVA was used. ***$P < 0.001$; **$P < 0.01$; vs. to vehicle. Error bars are means ± s.e.m.

It has been reported that α7 nAChRs are widely expressed in the hippocampus and thalamus, but they are observed at low levels in the postmortem human brain (20, 21). The α7 nAChRs are concentrated on almost all synapses both presynaptically and postsynaptically in the CA1 region of the hippocampus (22). The initial evidence of α7 nAChR activity in synapses was obtained from rat hippocampal CA1 pyramidal neurons by examining rats' spontaneous activity (23–25). Loss of α7 nAChRs may play a pivotal role in cognitive function damage (26–29). Previous studies have shown that α7 nAChRs have neuroprotective effects (30). The administration of nicotine may attenuate microglial activity and increase the eclampsia-like seizure threshold in the rat hippocampus through the α7 nicotinic receptor (31). This revealed the influence of α7 nAChRs on the threshold in pregnant rats. Previous studies have shown that activation of α7 nAChRs could decrease the seizure susceptibility in Chat-Mecp2$^{-/y}$ mice (16). Therefore, we hypothesized that α7 nAChRs may be associate with epilepsy. In this present study, immunofluorescence results on hippocampal neurons showed that α7 nAChRs are expressed in pyramidal and

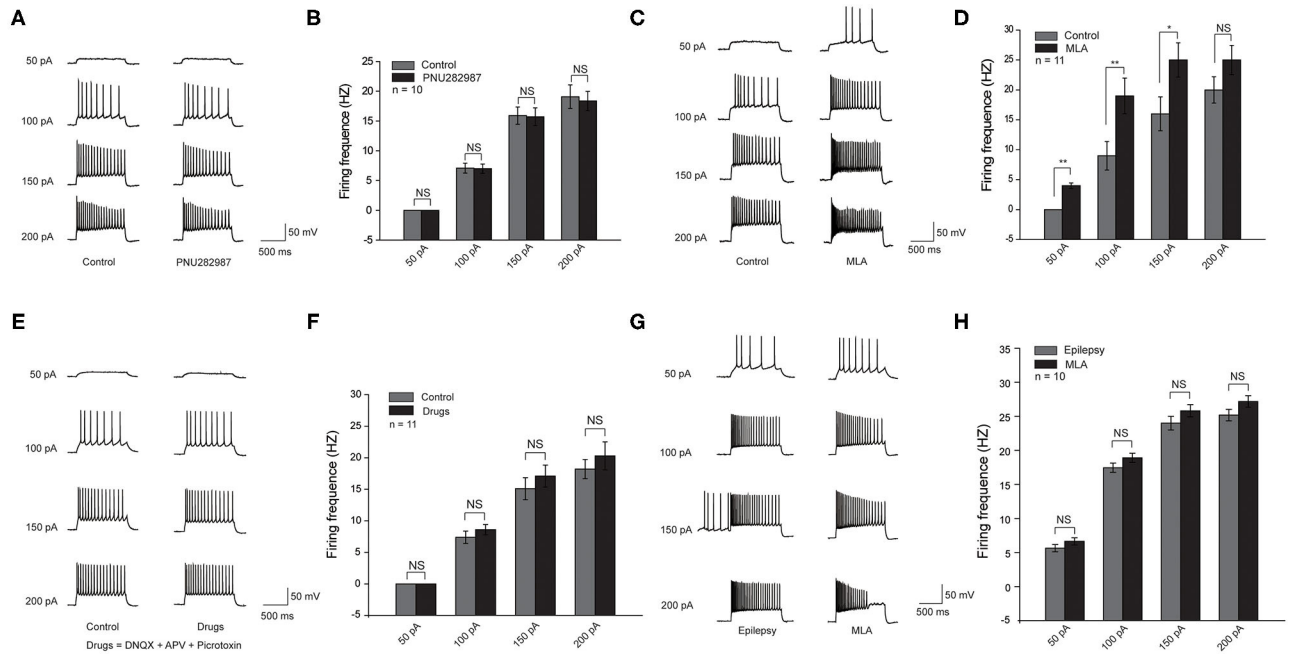

FIGURE 3 | Decreased activity of α7 nAChRs could increase the excitability of the CA1 pyramidal neurons in the hippocampus in C57BL/6 wild-type mice. **(A,C,E,G)** Voltage responses of a representative to in pyramidal neurons to various injection steps (1s) from top to bottom, 50, 100, 150, 200 pA. **(A)** Left: Control group. Right: PNU282987 group. **(B)** Summary histogram showing the number of pyramidal neuron APs. Two-way ANOVA was used. $n = 10$. **(C)** Left: Control group. Right: MLA group. **(D)** Summary histogram showing the number of pyramidal neuron APs. Two-way ANOVA was used. $n = 11$. **(E)** Left: Control group. Right: Drugs group (Drugs = DNQX + APV + Picrotoxin). **(F)** Summary histogram showing the number of pyramidal neuron APs. Two-way ANOVA was used. $n = 11$. **(G)** Left: Epilepsy group. Right: MLA group. **(H)** Summary histogram showing the number of pyramidal neuron APs. All pyramidal neuron membrane potential was kept at −70 mV by injecting a small DC current through the recording pipette. *P*-value was calculated by two-sided *t*-test. $^{**}P < 0.01$; $^{*}P < 0.05$. Error bars are means ± s.e.m.

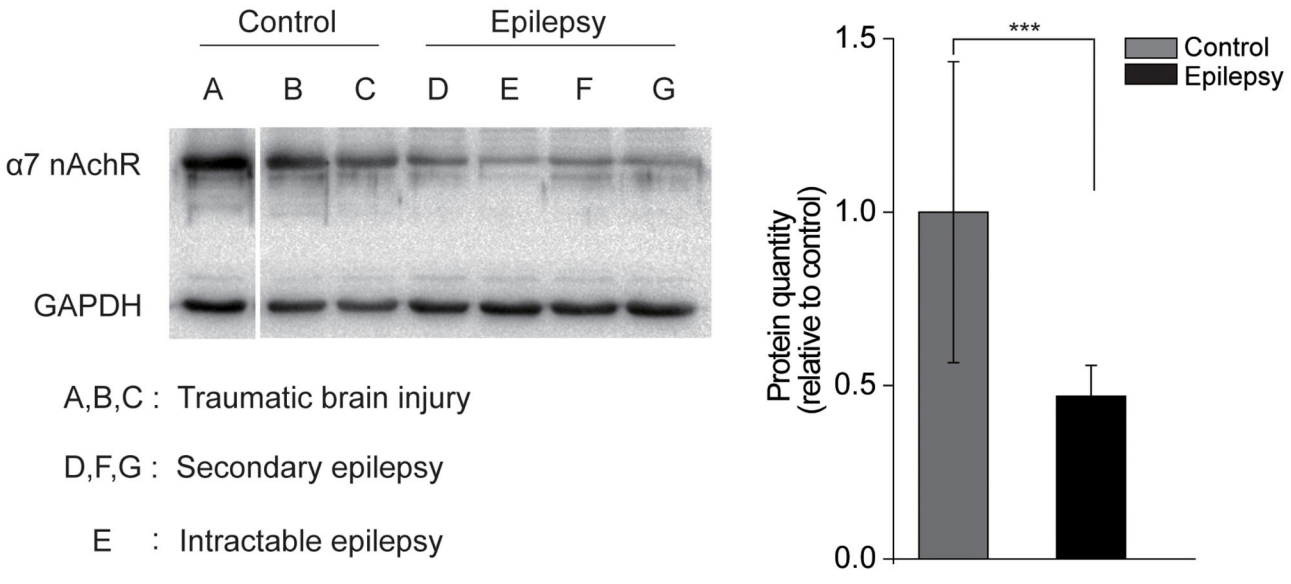

FIGURE 4 | Decreased α7 nAChRs expression in human epileptogenic tissue. **(A)** Immunoblotting with α7 nAChRs in the membrane fraction of cortex from control individuals or individuals with epilepsy. Each lane was loaded with 40 μg of protein with GAPDH as the loading control. **(B)** Quantification of α7 nAChRs proteins in control and epilepsy groups. $n = 3$. Protein levels of epilepsy tissue were normalized to the corresponding ones of non-epilepsy tissue. $^{***}P < 0.001$; Error bars are means ± s.e.m.

PV neurons in the hippocampus. Previous studies showed that PV interneuron axons project to the peribody area of pyramidal neurons and regulate the activity of pyramidal neurons (32). We revealed that treatment with MLA to block α7 nAChRs on CA1 PV interneurons indirectly increased the excitability of these neurons. Further studies are required to examine whether α7 nAChRs in pyramidal neurons affect its excitability. This study only tested the role of α7 nAChRs in the pilocarpine model, and further studies are required to confirm its effects in more epilepsy models.

Decreased excitability of α7 nAChRs on CA1 pyramidal neurons in the hippocampus leads to high seizure sensitivity in mice. α7 nAChR antagonists have been reported to alter the frequency and amplitude of glutamatergic neurons recorded from pyramidal neurons in hippocampal samples obtained from patients with mesial temporal lobe epilepsy with hippocampal sclerosis (33). The results of this study also suggested that the application of α7 receptor antagonists in patients with temporal lobe epilepsy could overexcite pyramidal neurons, a result similar to that of our study. A recent study also demonstrated that the α7 nAChR agonist choline chloride could improve epilepsy, depression, and memory deficits in the PTZ-kindled mouse model (34). This also suggests that α7 nAChRs play a pivotal role in epilepsy. Moreover, in our study, the expression of α7 nAChRs was reduced in human epileptogenic tissues. Our findings suggest that the downregulation of α7 nAChRs contributes to human epilepsy. However, the increased activity of α7 nAChRs on CA1 pyramidal neurons in the hippocampus had no significant effect on seizure sensitivity. Earlier studies also showed that selective allosteric modulators of α7 nAChRs could have potential therapeutic applications in epilepsy (35). Overall, our results suggest that α7 nAChRs are an essential regulator of seizure susceptibility.

DATA AVAILABILITY STATEMENT

The datasets generated for this article are not readily available because this would jeopardize patient privacy. Requests to access the datasets should be directed to sp120@zju.edu.cn.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Medical Ethical Committee of Zhejiang Provincial People's Hospital, People's Hospital of Hangzhou Medical College. The patients/participants provided their written informed consent to participate in this study. The animal study was reviewed and approved by Medical Ethical Committee of Zhejiang Provincial People's Hospital, People's Hospital of Hangzhou Medical College. Written informed consent was obtained from the owners for the participation of their animals in this study.

AUTHOR CONTRIBUTIONS

PS conducted the immunohistochemistry, western blot analysis, electrophysiology experiments, analyzed data, and wrote the manuscript. D-GL conducted the animal behavioral studies, EEG recordings, and collected the data. X-MY supervised all phases of the project and approved the final manuscript. All authors contributed to the article and approved the submitted version.

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Pretreatment Source Location and Functional Connectivity Network Correlated With Therapy Response in Childhood Absence Epilepsy: A Magnetoencephalography Study

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Objective: This study aims to investigate the differences between antiepileptic drug (AED) responders and nonresponders among patients with childhood absence epilepsy (CAE) using magnetoencephalography (MEG) and to additionally evaluate whether the neuromagnetic signals of the brain neurons were correlated with the response to therapy.

Methods: Twenty-four drug-naïve patients were subjected to MEG under six frequency bandwidths during ictal periods. The source location and functional connectivity were analyzed using accumulated source imaging and correlation analysis, respectively. All patients were treated with appropriate AED, at least 1 year after their MEG recordings, their outcome was assessed, and they were consequently divided into responders and nonresponders.

Results: The source location of the nonresponders was mainly in the frontal cortex at a frequency range of 8–12 and 30–80 Hz, especially 8–12 Hz, while the source location of the nonresponders was mostly in the medial frontal cortex, which was chosen as the region of interest. The nonresponders showed strong positive local frontal connections and deficient anterior and posterior connections at 80–250 Hz.

Conclusion: The frontal cortex and especially the medial frontal cortex at α band might be relevant to AED-nonresponsive CAE patients. The local frontal positive epileptic network at 80–250 Hz in our study might further reveal underlying cerebral abnormalities even before treatment in CAE patients, which could cause them to be nonresponsive to AED. One single mechanism cannot explain AED resistance; the nonresponders may represent a subgroup of CAE who is refractory to several antiepileptic drugs.

Keywords: childhood absence epilepsy, antiepileptic drug responders, antiepileptic drug nonresponders, ictal periods, source location, functional connectivity

INTRODUCTION

Childhood absence epilepsy (CAE) is the most common idiopathic, generalized nonconvulsive epilepsy caused by multiple genetic etiologies, representing approximately 10% of pediatric epilepsy. This disease is characterized by brief moments of impaired consciousness and often occurs between the age of 3 and 8, affecting girls more than boys (1, 2). The typical ictal electroencephalography (EEG) shows 3- to 4-Hz generalized synchronous bilateral spike–wave discharges (GSWDs) (3).

CAE is recently considered as a network disorder, and the GSWDs are probably generated through the interconnection between the cortex and thalamic neurons (4). Furthermore, different changes in brain network among CAE patients occur, involving the default mode network (DMN), attention network, and salience network (5–7).

CAE patients are often treated with ethosuximide (ESM), lamotrigine (LTG), and valproic acid (VPA). Currently, ESM has class I evidence for CAE, and it is considered as the first line of treatment to cure it compared to LTG and VPA; however, ESM is unfortunately not available in China (8, 9). Although about two-thirds of patients recover completely, some continue to experience seizures or other psychosocial deficits into adulthood (10–12). Thus, early identification is necessary to plan a replacement therapy, patient counseling, individual support, and early referral. Despite many studies predicting the treatment outcome and prognostic factors in CAE patients (13–16), the reasons why some patients respond well to the treatment while others do not remain poorly understood. Potential causes of intractability include the induction of drug efflux transporters on the blood–brain barrier and the alteration of both the neurotransmitter receptors and target ion channels (17–19).

Magnetoencephalography (MEG) is a non-invasive method to detect the neuromagnetic signals of brain neurons, which is gradually applied in clinical practice (20, 21). MEG can localize epileptic activities and provide a dependable location in the cerebral cortex (22). Compared with functional magnetic resonance imaging (fMRI), MEG can measure at millisecond temporal resolution, which is useful for exploring epileptic neuromagnetic activities (23, 24). Different frequencies provide various temporal windows for processing, and various rhythms are correlated with distinct spatial scales (25). Sources in various frequency bands may uncover distinct corticothalamic networks in CAE patients (26).

Thus, based on the aforementioned background, in this work MEG was firstly used to study the ictal periods of absence epilepsy in untreated CAE patients, and then after the therapy, they were grouped according to their outcome, with the purpose of exploring the differences between antiepileptic drug (AED) responders and nonresponders. MEG was used to analyze the source location and functional connectivity network changes from low to high frequency in 24 drug-naïve CAE patients during ictal periods to investigate whether these alternations were correlated with the response to treatment. This study reveals new potential mechanisms involved in the treatment response of CAE patients, providing an indication for clinical treatment and prognosis.

MATERIALS AND METHODS

Participants

A total of 46 CAE patients were monitored from 2014 to 2020. However, five patients were lost to follow up, 13 were treated with AEDs before the first MEG inspection, and four had no absence seizures during MEG recordings. Thus, 24 CAE patients were finally included in this study. All patients were accompanied by their parent or guardian who provided the informed consent on behalf of the child. The inclusion criteria were the following: (1) diagnosis of CAE by pediatricians according to the 2017 International League Against Seizure Classification (27), (2) an EEG showing 3 to 4 Hz GSWDs with at least 3 s of typical absence seizures, (3) head movement <5 mm during MEG recording, (4) normal neurological and physical condition, and (5) no history of AED treatment. The excluded criteria were the following: (1) presence of metal implants, (2) dramatic head movement and untypical absence seizures during MEG inspection, and (3) history of other neurological, psychiatric, or severe illnesses. During MEG recordings, eight patients were subjected to hyperventilation to evoke absence seizure, whereas the remaining patients had spontaneous absence seizures. A total of 24 patients with typical absence seizures were included in the subsequent analysis after removing inaccurate and incomplete seizures. All participants started the treatment with AEDs after the first MEG recordings as prescribed by the doctors of the Nanjing Children's Hospital in China. Clinical outcomes were evaluated at least 1 year after diagnosis by the parents' reports or EEG scans. The patients were then divided into two groups: one group consisting of AED-responsive patients and another group consisting of AED-nonresponsive patients. An AED-responsive patient was defined as one who had no clinically observed absence seizures and electroclinical discharges during video EEG recording along the follow-up. An AED-nonresponsive patient was defined as one who still had seizures after adequate doses of AEDs or who became seizure-free after the administration of more than two types of AEDs. Patients who became seizure-free after the administration of different types of AEDs due to adverse effects were considered as AED-responsive patients. The criteria were according to previously published papers (28, 29).

MEG

The research data were acquired using the CTF-275-channel whole-head MEG system (VSM Medical Technology Company, Canada) at the magnetic shielding examination laboratory. Before collecting the MEG data, three coils were connected to the bilateral preauricular and nasion area, and the head location procedure was conducted before and after each collection to determine the patient's head locations, matching the MEG sensors prior to inspections. The audiovisual system was applied in monitoring the process. The sampling rate was 6,000 Hz, and the data were collected with noise elimination of third-order gradients. At least six epochs with a period of 2 min were collected for each patient, and then the patients were told to close their eyes and remain still during the scan. Head movement for each collection was restricted to 5 mm. If no ictal data were captured,

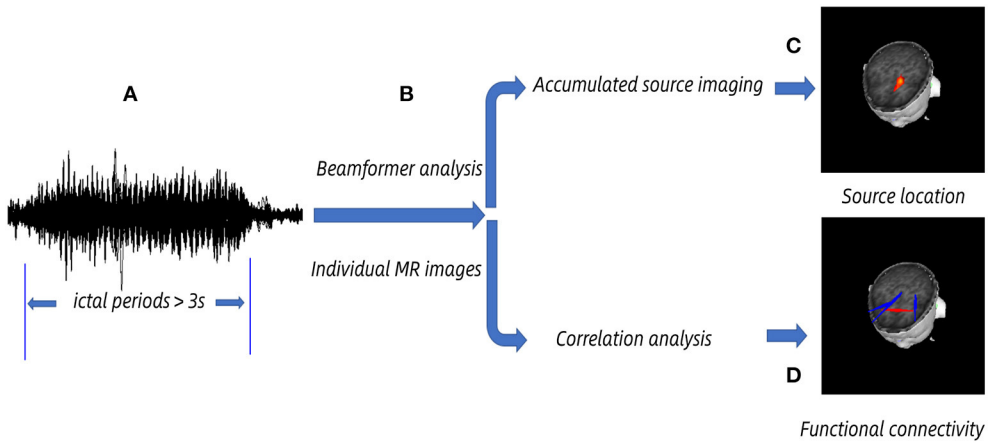

FIGURE 1 | Schematic diagram of magnetoencephalography (MEG) data analysis. MEG data were acquired, and we chose >3 s ictal periods for analysis. **(A)** Ictal periods. **(B)** The beam-former was applied to cast sensor-level signals to the anatomical brain regions according to individual MR images at the source level. **(C)** Accumulated source imaging was generated by spatially overlying the source MEG data and exhibiting the predominant source location. **(D)** Functional connectivity network was obtained by correlation analysis, and the significant network was acquired at a predefined threshold.

another recording was performed after subjecting the patients to hyperventilate to trigger extra absence seizures.

MRI

All patients were subjected to a T1-weighted image with a 3.0-T system magnetic resonance imaging (MRI) examination (Siemens, Germany). The parameters were as follows: the flip angle was 9°, the field of view was 250 × 250 mm, and the matrix was 512 × 512. We acquired 176 sagittal slices of each subject. The MRI marks were placed in the same position as those used in the MEG inspection for a precise co-registration of the MEG and MRI datasets. All the anatomical parts in the MRI could be recognized in the digital MEG images.

MEG Data Analysis and Signal Processing Technology

MEG data were filtered by a 1–4-Hz band pass filter after removing noticeable magnetic artifacts and noise (amplitude >6 pT). The selection of ictal periods was based on 3-Hz GSWDs shown in MEG and clinically observed symptoms. The clinical manifestations were observed by the audiovisual system. The epileptic waveforms were recognized by a specialist neurological physician. The first spike wave and the last slow wave in GSWDs were defined as the beginning and the ending of the seizure separately. An ictal segment of more than 3 s for data processing was chosen (**Figure 1**). The MEG signals of predefined six frequency bands at 1–4, 4–8, 8–12, 12–30, 30–80, and 80–250 Hz were analyzed, and the power-line noise at 50 Hz was avoided.

Source Localization

According to the previously published paper (30), accumulated source imaging (ASI) technique was used to sum the volumes of source activities over a period of time with two-step beamforming methods, which could quantitatively compare the neuromagnetic activity between responders and nonresponders. The ASI was applied in separating correlated sources with multiple source beamformers. The sources were located by ASI using node-beam lead fields. Because each node-beam lead field represented a form of either subspace solution or source-beamformer (30), the entire brain was scanned at a resolution 6 mm (about 17,160 voxels per magnetic source). ASI was obtained using the following formula:

$$Asi\,(r,s) = \sum_{t=1}^{t=n} Q\,(r,t)$$

where Asi stands for the accumulative source strength at location r, s represents time slice, t represents the time point of MEG data, n stands for the entire time points of MEG data, and Q is the source activity at the time point t and source r. Detailed algorithms were described in the previously published paper (30), and this method has been used in several research (31–36).

Functional Connectivity

According to previous studies (30, 35, 36), the ASI algorithms and the correlation analysis were used to elaborate the functional connectivity network between the responders and nonresponders at the source level. The volumetric sources of activities in the whole brain were firstly calculated using individual MR images (31). The signal correlation of virtual sensors from two source pairs was analyzed to estimate the neural networks during the selected ictal period. The correlativity between the virtual sensor signals from the two source pairs was analyzed by calculating the correlation coefficient, and the correlation coefficient was expressed as per the following formula:

$$R\,(xa,xb) = \frac{C\,(xa,xb)}{SxaSxb}$$

where $R(xa, xb)$ represents the correlation between two magnetic sources at position "a" and "b," xa, and xb are the signals from

the paired magnetic sources to calculate the correlation, $C(xa, xb)$ represents the average value of the signals from the paired magnetic sources, and *Sxa* and *Sxb* are the standard deviations of two source signals.

All possible links between every two source pairs were evaluated at the source level to avoid bias, and the neural network distribution was superimposed on the individual MR images (30, 31). Individual MR images were spatially normalized using an MRI template (37, 38), and the neural networks were overlaid on individual MR images. Then, the MEG–MRI data were spatially normalized to the MRI template. The medial frontal cortex (MFC) was specially selected as the region of interest (ROI). The ROI was firstly defined visually and then verified with coordinates through the MRI template. The neural networks between responders and nonresponders were visually recognized in MRI views and displayed in the coronal, axial, and sagittal positions. The midpoint of the right and left preauricular points was the original point ($x = 0$, $y = 0$, and $z = 0$). The blue and red colors indicated the negative and positive connections, respectively. Our MEG processor software could segment and visualize the brain regions in 2D and 3D views.

The threshold was defined as a checkpoint to guarantee the data quality. The t values for the entire magnetic source pairs were used to identify the threshold for the connection and calculated using the following equation:

$$Tp = R\sqrt{\frac{K-2}{1-R^2}}$$

where Tp represents the t value of a correlation, R stands for the correlation of magnetic source pairs, and K represents the number of connected data points. This study specifies the Tp value when $p < 0.05$ was defined as the threshold of functional connectivity network within the two groups.

The algorithms discussed above were analyzed through MEG Processor software (http://sites.google.com/site/braincloudx/). The detailed mathematical algorithms were described in previous studies (30, 31), and this method has been used in several research (35, 36, 39).

Statistical Analyses

The magnetic source location and functional connectivity under six frequency bandwidths were compared between AED-responsive patients and AED-nonresponsive patients using Fisher's test. The clinical data of the patients (age of onset, average age, follow-up time, and duration of absence seizures during MEG recording) between responders and nonresponders were analyzed using two-tailed t-test, and the results were represented as mean ± SD. The types of AED between the two groups were analyzed using Fisher's test. A value of $p < 0.05$ was considered statistically significant. Multiple comparisons were corrected by Bonferroni-corrected methods [i.e., for six frequency bands, $p < 0.0083$ $(0.05/6 = 0.0083)$]. Controlling the false discovery rate could be utilized to solve the problem of type 1 errors (40). Statistical analysis was performed by SPSS 25.0 (IBM SPSS Inc., USA).

RESULTS

Subject Characteristics, Therapy, and Therapeutic Response

The gender ratio among patients was 4:20 (male/female). Thirteen patients were responsive, and 11 were nonresponsive. Among the responders, LTG was administered to nine patients, and VPA was administered to the other four. All the responders became seizure-free. Among the nonresponders, LTG was administered to two patients, VPA was administered to five, and a combination of VPA–LTG was administered to the other four. The seven nonresponders treated with monotherapy still had seizures, whereas the four treated with combination therapy became seizure-free. The average age of the 24 patients was 10.28 ± 2.82 years; the onset age was 6.29 ± 1.33 years, with a mean duration of the seizure of 14.10 ± 4.70 s and a follow-up time of 28.67 ± 22.07 months. The clinical characteristics of the responders and nonresponders were not significantly different (onset age, average age, age at the time of diagnosis, gender, seizure durations, follow-up time, and types of AEDs). The clinical data are shown in **Table 1**.

Source Location

The source location of the nonresponders was mainly in the frontal cortex at 8–12 and 30–80 Hz, especially 8–12 Hz. The source location of the nonresponders was mostly in the medial frontal cortex, which was chosen as the region of interest ($p = 0.005$, $p < 0.0083$; **Figure 2** and **Table 2**). In addition, it is important to note that majority of the areas of MFC were the medial prefrontal cortex (MPFC) and dorsal medial frontal cortex (DMFC) (**Figure 3**), while in the other four frequency bands, the two groups were not statistically different.

Functional Connectivity

Because the source location of the nonresponders was mainly located in the MFC at 8–12 Hz, our hypothesis was that the network involving MFC could be different between the two groups. Thus, MFC was chosen as the ROI. The results were co-registered to each patient's brain MR images using the three fiducial points, and visual inspection was conducted in both 2D and 3D views by two specialist neurological physicians independently. Both excitatory and inhibitory connections were analyzed. The functional connectivity was significantly different between the responders and nonresponders ($p < 0.0001$; **Figure 4**) at 80–250 Hz. At 80–250 Hz, the nonresponders showed strong positive connections in the frontal cortex (excluding other brain areas) compared with the responders. Nevertheless, both the responders and nonresponders showed excitatory connections between the anterior and posterior brain areas in the other five frequency bandwidths, and no significant differences were observed between the two groups in these frequency bands.

DISCUSSION

Our study using MEG revealed that the differences in pretreatment source location and functional connectivity

TABLE 1 | Characteristics of the patients.

Patient	Gender/age (years)	Onset age (years)	Seizure durations (s)	Follow-up time (months)	Initial aed	Aeds added	Seizure-free
1[a]	F/8	6	16.5	12	VPA	N	Y
2[a]	F/9	6	10	23	LTG	N	Y
3[a]	F/8	5	7.1	20	LTG	N	Y
4[a]	F/9	6	7.2	20	LTG	N	Y
5[a]	F/10	8	14.4	20	LTG	N	Y
6[a]	F/13	7	15	68	LTG	N	Y
7[a]	F/11	5	15.5	74	VPA	N	Y
8[a]	F/8	6	5	23	LTG	N	Y
9[a]	F/13	7	14.8	68	LTG	N	Y
10[a]	F/8	7	24	13	LTG	N	Y
11[a]	M/6	5	10.2	14	VPA	N	Y
12[a]	F/7	6	14.7	13	LTG	N	Y
13[a]	M/8	7	18	12	VPA	N	Y
14[b]	M/14	6	20	24	VPA	N	N
15[b]	F/15	8	18.5	25	LTG	VPA	Y
16[b]	F/9	7	9.3	12	LTG	N	N
17[b]	F/10	8	15	26	VPA	N	N
18[b]	F/10	5	11.2	15	VPA	N	N
19[b]	F/11	10	11.3	12	LTG	N	N
20[b]	F/14	4	11.3	67	LTG	VPA	Y
21[b]	M/11	6	18	23	LTG	VPA	Y
22[b]	F/11	5	13	13	VPA	N	N
23[b]	M/7	5	20.5	19	VPA	N	N
24[b]	F/17	6	18	72	LTG	VPA	Y

F, female; M, male; Y, yes; N, no; aeds, antiepileptic drugs; LTG, lamotrigine; VPA, valproic acid.
[a]*mean responder.*
[b]*mean nonresponder.*

network between AED responders and AED nonresponders were frequency dependent, supporting the existence of new mechanisms of AED response in CAE children. However, the clinical characteristics between the responders and nonresponders did not change significantly in our study.

Source Localization

Among the nonresponders, an evident frontal cortex source location at 8–12 and 30–80 Hz, especially the MFC (including MPFC and DMFC) source location at 8–12 Hz was found compared with the responders.

The frontal cortex has numerous high-order functions (41–43), and some studies reported that the orbital/lateral frontal lobe may be of importance for CAE pathophysiology (44, 45). The lower frontal myelin water content in CAE patients reflects an altered neurodevelopment, and the frontal lobe regions are most closely related to the cortical thickness of the entire brain in CAE patients (46, 47). Moreover, to the best of our knowledge and contrary to popular belief, cortical oscillations, instead of sole thalamic rhythmicity, are pivotal in inducing epilepsy and establishing a spike–wave discharge frequency (48), and the frontal cortex is of great importance in initiating and spreading absence epilepsy (44, 49–51).

However, the subgroups of CAE patients related to AED response are insufficiently studied. A previous EEG study revealed that the frontal onset of absence seizures is sometimes harder to control with traditional AED monotherapy and can be considered a special subtype (52). P. W. Carney used EEG–fMRI to classify the diverse individual patterns of frontal cortical activation associated with absence seizures, and they postulated that the involvement of dorsolateral prefrontal cortex in this disease is highly associated to cognitive impairment in CAE, and the group with a primarily negative signal change in the dorsolateral prefrontal cortex has a more benign phenotype compared to the positive group (53).

Furthermore, in our study, the MFC mainly included MPFC and DMFC. MPFC is an important hub of default mode network, which is closely connected to the limbic system (54) and is considered abnormal in CAE patients (5, 55). Thus, it is likely that source locations involving these crucial hubs potentially reveal dysfunctional intrinsic activities and can make CAE patients become more intractable.

The frequency band where the source was located was mainly at 8–12 and 30–80 Hz, both expressing low-frequency brain activities. The α rhythm (8–12 Hz) is an obvious oscillation during wakefulness and relaxation (56). A study has shown that idiopathic, generalized epilepsy patients represent increased

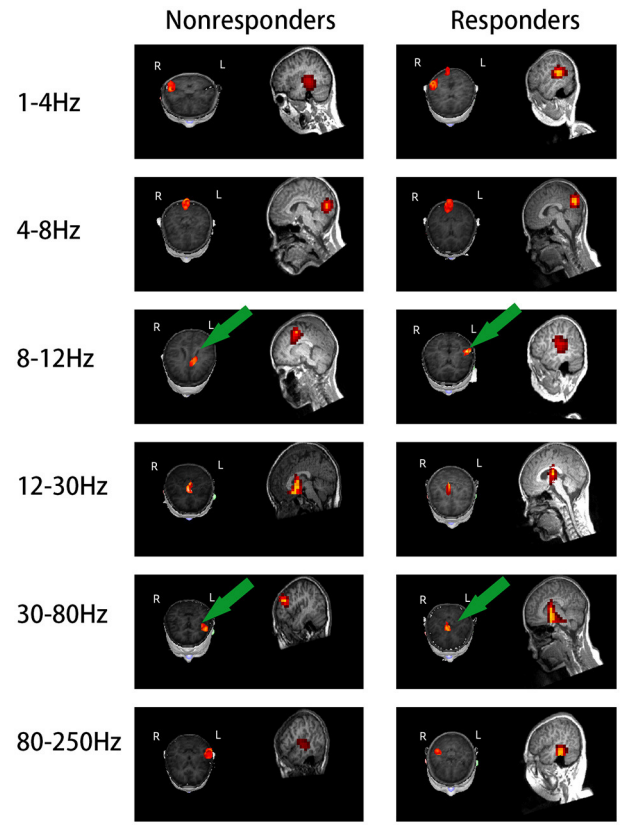

FIGURE 2 | Images of magnetic localization at 1–250 Hz frequency band. Significant differences were labeled with a green arrow. The location in the nonresponders was mainly in the frontal cortex area at 8–12 and 30–80 Hz, especially at 8–12 Hz, as opposite to the responders; the nonresponders showed activation in the medial frontal cortex area.

functional connectivity in low-α frequencies (57). The γ rhythm (30–80 Hz) is important in information processing within cortical networks (58). A research found that the shift in γ power (30–80 Hz) was associated with AED efficacy in two absence epilepsy mouse models (59). Notably, we observed nonresponders located in MFC at 8–12 Hz compared with the responders in our study. A previous study using EEG found that α-power shifted in intractable epilepsy patients, which might reflect dysfunction of a large-scale cortico-thalamic network, including frontal cortex (60). Various frequency bands prefer diverse sorts of connections and different spatial and temporal information integrations. Low-frequency oscillators include numerous groups of neurons in large cerebrum regions as compared with high-frequency oscillators (HFOs) (61). Thus, the source of low-frequency brain activity may not always result in a description of potential epileptogenic zones (33).

Overall, our results made preliminary assumptions that the frontal lobe and especially the medial frontal lobe at α band might be relevant to AED-nonresponsive CAE patients.

Network Pattern

Notably, the source location does not represent the aberrant brain tissue but indicates the area involved in a dynamic network of discharges (62). Epileptic seizures reflect aberrant synchronization, which can only be learnt at the level of neuronal networks (63). Functional connectivity is considered the statistical dependencies among distant neurophysiology events (64). The network perspective assists in understanding the epileptogenesis of epilepsy, which may result in the progression and maintenance of intractable or chronic epilepsy (65).

Therefore, given the importance of MFC that we discovered initially and the advantages of functional connectivity networks, the dynamics of functional connectivity networks involving the MFC were specially studied.

Our results identified a stronger positive functional connectivity in local frontal cortex at 80–250 Hz in nonresponders. Fair DA supposed that, as the brain grows, the connections between the anterior and posterior cortices are strengthened (66). However, the increased connection within the frontal cortex and the decreased connection between the anterior and posterior cortex might not simply be due to immature brain development. A previous study has demonstrated increased functional connectivity within the orbitofrontal cortex in CAE patients (67). Moreover, the network-involved MFC presented decreased connections with other regions. The MFC was a hub of DMN, which was revealed to be abnormal in CAE patients (5). A previous study revealed reduced DMN connectivity in treatment-resistant idiopathic, generalized epilepsy (68). Intractable epilepsy may arise from the start rather than evolve over time, and clinical symptoms are obvious in the early stage of the disease (69). Thus, we speculated that this local frontal connection found in our study might be caused by underlying cerebral abnormalities even before treatment in CAE patients, potentially leading to them to become AED nonresponders, but in our study, given the small sample size of the two groups, we could not definitely extend our results to the entire CAE patient population. Further study is needed in the future.

In our study, the CAE patients were treated with VPA or LTG rather than ESM because ESM is not available in China. The mechanisms used by VPA and LTG to resolve CAE were unclear. Previous studies concluded that T-type calcium channels in nucleus reticularis and cortical neurons are vital in absence seizure expression, which is consistent with the gain-of-function mutation of the Cav3.2 subtype of T-type calcium channels appearing in some CAE patients (70, 71). This evidence might explain the weak and non-selective action of VPA on these channels, which leads to its ineffectiveness (72). In addition, LTG is generally considered as a member of the sodium channel blockers of AEDs, but this mechanism is not explaining its current clinical effect on controlling absence seizures (73, 74). Overexpression of drug efflux transporters in epilepsy, mostly P-glycoprotein, at the blood–brain barrier can be involved in LTG resistance (75, 76). However, the relationship between the pretreatment neural network during ictal periods found in our study and the molecular mechanisms discussed above is unknown. Thus, further studies are needed to investigate the underlying connections between them.

A study speculated that the receptors used by VPA to exert its effect are spread in all parts of the corticoreticular network, potentially explaining the effect of this AED (77). A previous

TABLE 2 | Magnetic location in the brain of the responders and nonresponders.

Frequency band (Hz)	1–4		4–8		8–12		12–30		30–80		80–250	
Group	**N**	**R**	**N**	**R**	**N**	**R**	**N**	**R**	**N**	**R**	**N**	**R**
FC	4	3	4	1	7*	0*	3	2	10*	1*	3	1
(MFC)	3	3	4	1	7*	0*	3	2	5	0	3	0
(LFL)	1	0	0	0	0	0	0	0	5	1	0	1
TC	1	0	1	0	1	2	0	2	0	3	3	6
TPJ	1	2	0	0	0	3	0	1	0	0	0	1
POT	6	5	4	2	2	2	3	2	0	0	0	0
Pc	0	3	1	0	0	1	2	3	1	1	0	0
PCC	0	2	0	0	0	0	2	0	0	1	0	0
PL	1	1	2	1	2	1	1	0	0	0	0	0
MOC	2	4	0	2	1	2	1	2	0	1	0	0
TH	0	3	1	4	1	1	3	3	2	7	1	1
CE	0	0	1	0	0	0	0	0	0	0	1	2
DBA	0	1	0	2	0	2	1	0	3	1	4	3

N, nonresponders; R, responders; FC, frontal cortex; LFL, lateral frontal lobe; MFC, medial frontal cortex; TPJ, temporal–parietal junction; POT, parietal–occipital–temporal junction; pC, precuneus; PCC, posterior cingulate cortex; PL, parietal lobe; MOC, medial occipital cortex; TH, thalamus; CE, cerebellum; DBA, deep brain area.
$^*p < 0.05$.

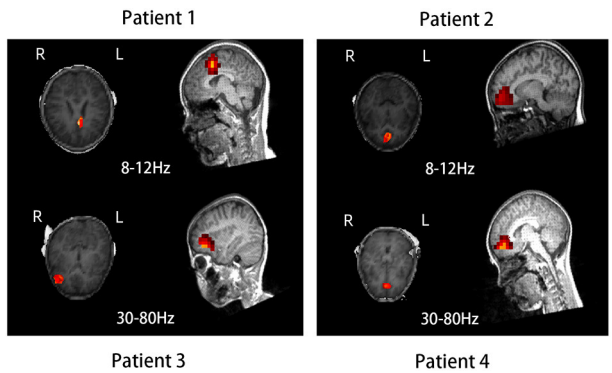

FIGURE 3 | Source location in the dorsal medial frontal cortex (patient 1), medial prefrontal cortex (patients 2 and 4), and lateral frontal cortex (patient 3) in four typical nonresponders at 8–12 and 30–80 Hz.

study evaluating the therapy outcome in CAE demonstrated that the involvement of post-dorsal lateral medial frontal cortex in CAE patients may be the reason of the initial LTG therapy failure, and VPA effectiveness can be associated with the complexity of the neural network (78). Furthermore, a study using a combined EEG–fMRI and MEG analysis revealed that CAE nonresponders have a pretreatment increased connectivity in the frontal cortex, potentially leading to the absence of the effect of ESM on the thalamus in comparison with the effect of ESM treatment in responders (29).

Whereas, in our study changes in the local medial frontal cortex network were discovered in nonresponders, independently on the type of AED the children received, our results potentially suggest that the pretreatment network differences between responders and nonresponders were not specific to one single AED but represented a subgroup of CAE who was refractory to any medical treatment, an aspect that is in agreement with the previous study discussed above (29).

The significant changes of functional connectivity networks were at 80–250 Hz. HFOs are usually divided into ripples (80–250 Hz) and fast ripples (250–500 Hz), which are appropriate for detecting adjacent and strongly connected neural connections and may offer accurate spatial information about cortical malfunction (61). It has been uncovered that ictal HFOs are produced by the disorganization of neural firing, which are highly located in epileptogenic zones (79). Tenney indicated that ictal HFOs prominently appeared in the frontal area in CAE patients (26), and a previous study of our group reported that the ictal HFOs reflected the severity of absence seizures (33). HFOs might also serve for monitoring AED response (80).

To sum up, our results display the abnormal dynamic networks involving the MFC in nonresponders at 80–250 Hz, which might further reveal underlying cerebral abnormalities even before treatment in CAE patients, leading to nonresponsive AEDs.

Nevertheless, the response ratio of the treatment in CAE patients varied from 60 to 95%, which was depending on different factors, including the studied population, the measurement of the outcomes, the length of the follow-up, pretreatment EEG semiology, genetic abnormalities, and different AED mechanisms (15, 16, 81–83). It is probably an understatement to assume that AED resistance was caused by a single mechanism because it could be due to multiple factors coexisting in the same patient.

LIMITATIONS

Our research has certain limitations. Firstly, the clinical data were provided by the parents, potentially resulting in inexact information. Secondly, given the strict inclusion criteria, our

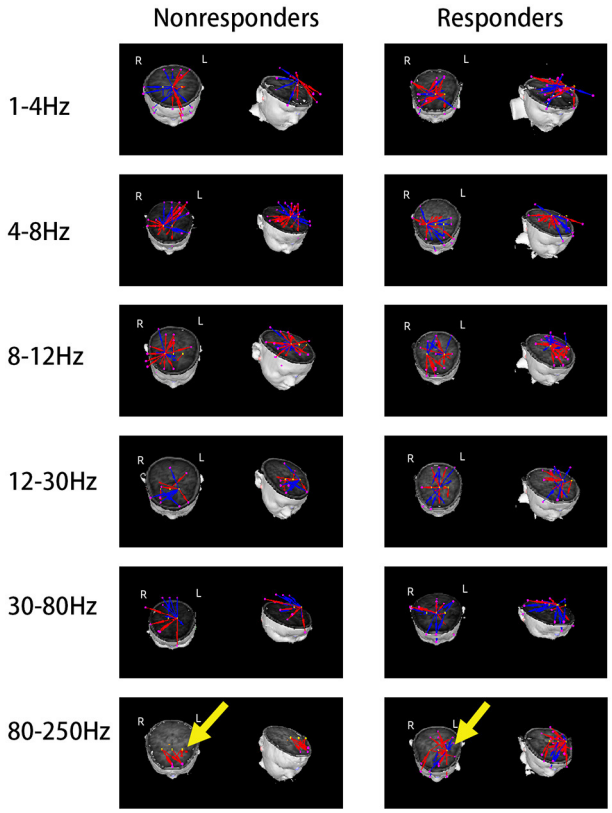

FIGURE 4 | Typical predominant functional connectivity networks at 1–250 Hz. Nonresponders showed a significantly altered functional connectivity network at 80–250 Hz as compared to the responders, represented by local frontal connections and deficient anterior–posterior connections, which were indicated by yellow arrows.

analysis is limited to a relatively small sample size; thus, it is not possible to draw any definitive conclusions, and further studies are needed to confirm our results. Thirdly, the relationship between cortical multi-frequency pretreatment neuromagnetic activities and the underlying molecular mechanisms remained unknown. Finally, the repeatability and reliability of our measurements remain to be proven. Therefore, further research is needed to confirm the underlying mechanisms of AED response in CAE patients.

CONCLUSIONS

Our study demonstrated the pretreatment source location and functional connectivity network associated with the different treatment responses in CAE patients. The frontal cortex and especially the MFC at α band might be relevant to AED-nonresponsive CAE patients. The local frontal positive connection at a high-frequency range in our study might be probably caused by underlying cerebral abnormalities even before treatment in CAE patients, leading to nonresponsive AEDs. It is probably an understatement to assume that one single mechanism was responsible for AED resistance; the nonresponders might represent a subgroup of CAE who were refractory to several antiepileptic drugs. Thus, the specific mechanisms underlying the treatment response need to be further investigated.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by The Affiliated Brain Hospital of Nanjing Medical University. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin. Written informed consent was obtained from the individual(s), and minor(s)' legal guardian/next of kin, for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

KZ analyzed the data and wrote this manuscript. JS and YS contributed to data analysis. KN, PW, and CW recruited patients. QC recorded the MEG data. All authors agreed to publish this manuscript.

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REVIEW
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Update on the Neuroimaging and Electroencephalographic Biomarkers of Epileptogenesis: A Literature Review

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Epilepsy is one of the most common debilitating neurological disorders that lead to severe socio-cognitive dysfunction. While there are currently more than 30 antiseizure medications available for the treatment and prevention of seizures, none address the prevention of epileptogenesis that leading to the development of epilepsy following a potential brain insult. Hence, there is a growing need for the identification of accurate biomarkers of epileptogenesis that enable the prediction of epilepsy following a known brain insult. Although recent studies using various neuroimages and electroencephalography have found promising biomarkers of epileptogenesis, their utility needs to be further validated in larger clinical trials. In this literature review, we searched the Medline, Pubmed, and Embase databases using the following search algorithm: "epileptogenesis" and "biomarker" and "EEG" or "electroencephalography" or "neuroimaging" limited to publications in English. We presented a comprehensive overview of recent innovations in the role of neuroimaging and EEG in identifying reliable biomarkers of epileptogenesis.

Keywords: epilepsy, epileptogenesis, biomarker, neuroimage, EEG

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INTRODUCTION

Epilepsy is one of the most common neurological disorders affecting around 70 million people worldwide. Approximately 2.4 million new cases of epilepsy are diagnosed annually (1). Subsequently, epilepsy is getting increased public health attention as patients with epilepsy have a noticeable reduction in quality of life and employment prospects (2). Although antiseizure medication (ASMs) are considered the first-line treatment of epilepsy, it is still widely recognized that nearly one-third of epileptic patients have drug-resistant epilepsy in which seizures are unable to be controlled with at least two appropriately ASMs (3). One potential reason is that current accessible ASMs merely prevent one from having further spontaneous seizures but do not directly affect or alter the underlying cause contributing to epileptogenesis (4, 5). Through plentiful scientific research on the pathophysiology of epilepsy over the past several decades, there has been an increasing understanding of the pathophysiology of epileptogenesis.

Epileptogenesis refers to the process by which normal brain tissue becomes capable of generating spontaneous recurrent seizures and its progression (6). With the advances in technology, potential promising biomarkers are now available to predict the development of epileptogenesis after

the epileptogenic insult. Furthermore, there are accessible therapeutic biomarkers that predict the treatment prognosis through the identification and exact localization of the epileptogenic lesion and associated network, as well as the severity and progression of epileptogenesis (7, 8). The ideal biomarkers should not just have validity, reliability, and reproducibility; they should also be non-invasive and cost-effective (9). Among all the available biomarkers related to epileptogenesis, neuroimaging and electroencephalogram (EEG) biomarkers are by far the most appealing biomarkers as they are non-invasive and routinely performed as part of epileptic patients' workup protocol.

In this review article, we searched the Medline, Pubmed, and Embase databases using the following search algorithm: "epileptogenesis" and "biomarker" and "EEG" or "electroencephalography" or "neuroimaging" limited to publications in English. The last date of the search was May 31, 2021. We screened the titles, abstracts, and references of all search results to identify potentially relevant studies. We aimed to provide a comprehensive literature review of recent innovations in the role of neuroimaging and EEG as biomarkers of the development of epileptogenesis after the epileptogenic insult.

NEUROIMAGING BIOMARKERS

Several neuroimaging modalities, including structural magnetic resonance imaging (MRI), functional MRI, magnetic resonance spectroscopy (MRS), and positron emission tomography (PET), have already been applied to investigate biomarkers of epileptogenesis and have significantly contributed to our understanding of the pathophysiological mechanisms that underlie the development of epilepsy (**Table 1**).

Structural MRI

MRI is an ideal tool for biomarker studies due to its accessibility and translatability to routine clinic settings. In the lateral fluid percussion injury (LFPI) rat model of traumatic brain injury (TBI), the abnormalities in the surface morphology of the ipsilateral hippocampus at 1-week post-LFPI can predict the occurrence of epilepsy 6 months after TBI (10). Besides, assessing individual MRI parameters in the peri-lesional cortex or the thalamus at 9 days after TBI can also provide high sensitivity and specificity for predicting increased seizure susceptibility at 12 months (11). In the follow-up LFPI study, the presence of diffusion abnormality analyzed by using D_{av} in the perilesional cortex and thalamus at 2 months after the TBI is found to have the highest predictive value for the development of seizure susceptibility at 12 months post-TBI. Similar changes in the MRI structures have been validated at the human level. In the early acute post-TBI phase (within 90 days post-injury), there is evidence showing a positive correlation between hippocampal/temporal structural abnormalities and the onset of seizure activity (11). In addition to the injury severity, the left temporal pole and left frontal cortical thinness are found to be significantly predictive factors for developing seizures after TBI (12). In addition to the cortical and subcortical structures, studies have shown that the distribution and quantification of paravscular spaces (PVSs) can be used as a potential biomarker for the development of epileptogenesis in posttraumatic epilepsy (PTE). Post-TBI epileptic patients are found to have significantly smaller PVSs and asymmetric distribution of PVSs in the suspected epileptogenic hemisphere (13, 14).

Mesial temporal lobe epilepsy (TLE) is reported as a common sequelae of the febrile status epilepticus (FSE) (38). Recently, in the rat model of FSE, reduced T2 relaxation time in the amygdala within 2 h of FSE is observed in the high resolution 11.7T MRI. This finding is shown to have a strong prediction of the later occurrence of TLE following the FSE. It is hypothesized that T2 changes are related to the increased oxygen utilization after FSE termination, which correlates with the activation of the intracellular inflammatory cascades that had been previously implicated in epileptogenesis (15). The result is again validated in the lower resolution 3T MRI (16). These results suggest that the quantitative T2 MRI can be used as a reliable neuroimaging biomarker following FSE for brain injury and structural alterations at the onset of epileptogenesis. Further studies are warranted to validate the reduction of the T2 relaxation time in the amygdala in the development of epileptogenesis in humans before they are ready for the clinical setting. Overall the translational nature of the MRI results has also greatly contributed to clinical advancements as the reported neuro-imaging protocols can be applied safely to epileptic patients.

Diffusion-weighted imaging (DWI) represents the diffusion of water molecules and is particularly useful for detecting acute changes in the brain tissue following status epilepticus (SE) (17). Yokoi and colleagues analyzed the acute DWI data of 22 children with FSE over the period of 9–13 years following FSE and reported that focal epilepsy was significantly more frequent in patients with hippocampal DWI hyperintensity than those without DWI changes (39). Further studies, including a larger cohort, are required to investigate the relationship between DWI changes and subsequent epilepsy in patients with FSE.

Diffusion tensor imaging (DTI) detects the restriction of water diffusion caused by the microstructural organization of tissues. Advancement in post-processing technique allows DTI to detect subtle white matter changes in the early development of epileptogenesis in a structural network-based approach (40). Sierra and colleagues studied and compared the fractional anisotropy (FA) and axial, radial, and mean diffusivities among three subgroups of rats: SE, TBI, and normal controls, at 6–12 months post-injury using 9.4T MRI. FA in the hippocampi was significantly increased in the SE/TBI group compared to the normal control group. Regarding the diffusivities, there was an increase in the $D_{||}$ (associated with axonal damage) after the SE, whereas a decrease in $D_{\perp}$(associated with demyelination) was noted after the TBI in the subfield-specific hippocampi. Thus, the data suggest that the DTI method identifies not only subtle hippocampal changes and progression after epileptogenic brain injuries but also different brain insults based on the different diffusivities (18). Several recent longitudinal studies of the mesial TLE rate models demonstrated that the epileptogenic rats have significant changes in DTI-measured FA in the early

TABLE 1 | Overview of neuroimaging biomarkers of epileptogenesis.

Imaging modality	Animal model	Human epilepsy	References
Structural MRI			
Surface morphology	LFPI model	None	(10)
D_{av}	LFPI model	None	(11)
Cortical thinness	None	PTE	(12)
PVSs	None	PTE	(13, 14)
T2 relaxation time	FSE	None	(15, 16)
DWI	None	Children with FSE	(17)
DTI	LFPI model	None	(18)
	Pilocarpine -induced SE mode	None	(18)
	MSO infused model	–	(19)
Functional MRI			(20)
Global network	KA-induced SE model	None	(21–23)
Local network	KA-induced SE model	None	(21)
MRS			
NAA	Pilocarpine -induced SE model	None	(24, 25)
GABA-A	Pilocarpine -induced SE mode	None	(26)
Myo-inositol	Pilocarpine -induced SE mode	None	(25, 27)
Antioxidant glutathione	Pilocarpine -induced SE mode	None	(25)
PET			
18F-FDG	LFPI model	None	(10)
	KA-induced SE model	None	(23–32)
	Pilocarpine-induced SE model	None	(30)
GABA-A	KA-induced SE model	None	(33–35)
TSPO	KA-induced SE model	None	(36, 37)

LPFI, lateral fluid percussion injury; PTE, posttraumatic epilepsy; PVSs, paravscular spaces; DWI, diffusion-weighted imaging; FSE, febrile status epilepticus; DTI, diffusion tensor imaging; KA, kainic acid; SE, status epilepticus; MSO, methionine sulfoximine; NAA, N -acetyl aspartate; TSPO, 18-kDa translocator protein.

stages. In addition, the FA changes in both gray and white matter progress over time as the animals transitioned from early to late epileptogenesis (19). Although the results suggest that DTI changes can be used as biomarkers of epileptogenesis, no prospective studies of epileptogenesis with DTI have been implemented in humans. Thus, further studies are warranted to implement the finding in the clinical setting.

Functional MRI

Functional MRI detects hemodynamic changes in different parts of the brain by means of blood oxygen level-dependent (BOLD) sequences, an indirect non-invasive measure of neuronal activity (41). Contrary to measuring the structural connectivity with DTI, functional MRI measures functional connectivity between various brain regions. Using the animal intrahippocampal kainic acid (KA) model of mesial TLE, Li et al. compared the fMRI of animals with mesial TLE and animals without epilepsy at 1 week after SE. For global network features, animals with epilepsy showed an overall increase in functional connectivity strength compared to animals without epilepsy. For local network features, animals without epilepsy showed decreased hubness in the hippocampus, whereas animals with epilepsy showed a complete loss of hippocampus hubs with appearance of new hubs in the prefrontal cortex (21). Instead of the hypersynchrony brain network pattern, Christiaen et al. reported a decreased functional connectivity between 1 and 3 weeks post SE after comparing 20 intraperitoneal KA animals and seven healthy control animals (22). The difference might be the result of the different routes of administration of ketamine as intraperitoneal injection resulted in more widespread brain lesions than intrahippocampal injection (21). Furthermore, Bertoglio et al. demonstrated diverging changes in network connectivity in relation to the seizure onset in the KA-induced models of SE. Animals with regular seizure onset (<17 days post-SE) showed a significant hypersynchrony of network connectivity at 4 weeks post-SE, while animals with delayed disease onset (≥17 days post-SE) remained hyposynchronous (23). Although there is a discrepancy across different studies on functional connectivity after TBI or TLE, the current literature suggests that there may be a reorganization of the functional network in early period of epileptogenesis, which may be used as an imaging biomarker in the near future.

MRS

MRS can provide indirect information, such as neuronal health, gliosis, energy metabolism, by analyzing different metabolites in the brain tissue. Several studies have analyzed the changes of N -acetyl aspartate (NAA) and gamma-aminobutyric acid (GABA) in the pilocarpine-induced SE model, a reduced NAA and GABA can be detected in the hippocampus from baseline to the period of epileptic seizures (24–26). However, a decrease in GABA and NAA levels has also been found in patients after TBI without

correlation with epileptic seizures (28). A progressive increase in myo-inositol and antioxidant glutathione before the onset of seizures has been found in pilocarpine-induced SE (25, 27). Furthermore, the level of antioxidant glutathione has shown to be negatively correlated with the frequency of spontaneous seizures (25). Although MRS analyses in seizure-prone brain areas following potential epileptogenic injuries may represent clinically meaningful biomarkers for the early identification of individuals at high risk for developing epilepsy, further human studies are warranted to validate the finding for the clinical setting.

PET

Alteration in the brain metabolic activity has been reported in the early development of the epileptogenesis after the initial epileptic insults. Nuclear imaging modalities such as PET are optimally used to assess functional brain metabolic activity using various radiotracers. Hence, PET has been used in several studies to assess potential mechanisms of epileptogenesis.

In the LFPI model, epileptic rats' ipsilateral hippocampi are reported to have subtle thickening on the surface analysis and 18F-FDG PET hypometabolism at 1 week, 1 month, and 3 months post-injury compared to the non-epileptic group. In addition, all the TBI rats have reported cortical and hippocampal hypometabolism, but the non-epileptic group has a partial recovery of the FDG uptake at 3 months post-injury (10). Thus, an initial reduction in glucose uptake is perhaps the result of both injury itself and early epileptogenesis, but in epileptic rats, there would be no recovery of the initial hypometabolism (29).

Multiple studies using KA and pilocarpine-induced models of SE have shown an initial increase in the glucose uptake during the acute seizures followed by the reduced metabolism at around day 3 of post-SE (24, 30). Furthermore, in the KA models of SE, glucose hypometabolism during early epilepsy correlates with the duration of the latent phase and frequency of spontaneous seizures in the spontaneous recurrent seizure (SRS) model of epilepsy (31, 32).

Several studies used different PET radioligands other than 18F-FDG to investigate the relationship between the density of GABA-A receptors and epileptogenesis in animal models of epilepsy. It is widely observed that GABA-A receptor density is decreased not only in hippocampi but also in several cortical regions in the KA models of SE (33, 34). One study using the focal cortical dysplasia model suggests that the decrease in the GABA-A receptor density may characterize a latent phase of epileptogenesis (35). Further studies are warranted to get more validation, but the promising results suggest that glucose hypometabolism and reduced GABA-A receptors might be the important hallmarks of early epileptogenesis.

Neuroinflammation is another pathological hallmark in one of the major pathophysiologies for epileptogenesis (42). The investigation of inflammation can be performed using a PET scan with radioligands that bind to18-kDa translocator protein (TSPO). TSPO is reported to be highly expressed on the mitochondrial membrane of activated microglia and reactive astrocytes. In the SRS model of epilepsy, TSPO levels at 14 days post-SE are predictive of SRS frequency at the onset of epilepsy (36). Subsequently, the same researcher group reported that TSPO upregulation at 14 days post-SE was associated with epileptogenesis, while TSPO overexpression at 14 days post-SE was associated with seizure frequency (37). Although TSPO-PET results are promising, clinical PET data on this topic is very limited due to the cost and availability of specific radioligands.

EEG

EEG, either non-invasive scalp recording or invasive microelectrode recording, is one of the most utilized modalities in the clinical setting and can monitor brain activity with high temporal resolution and relatively high spatial resolution. Several studies have demonstrated specific changes in the EEG as the potential biomarkers for the early development of epileptogenesis (**Table 2**).

High-Frequency Oscillations (HFOs)

HFOs, i.e., ripples (80–250 Hz) and fast ripples (250–600 Hz), have been studied and shown to be promising biomarkers for epileptogenesis markers over the last decade. In the KA-induced SE model, Bragin et al. reported the appearance of HFOs in the ipsilateral hippocampi dentate nuclei in rats that later developed epilepsy. The author also described that the appearance of the HFO timing was found to be associated with the delay in the onset of the first seizure (43). The same group reported different types of HFOs named repetitive HFO and spikes (rHFOSs), in which rhythmic spikes at the frequency of 10–16 Hz with superimposed pathological HFOs (80–300Hz). The appearance of rHFOSs in the injured cortex and around the adjacent injured cortex within 2 weeks from the initial insult in the LFPI model rats was reported to more likely develop spontaneous seizures later in life (20, 44). Although HFOs are typically detected with invasive intracranial EEG, the advancements in scalp EEG monitoring equipment enable one to record HFOs on scalp EEG in human studies (53). In a cohort of children after a first unprovoked seizure, the presence of scalp ripples can predict the development of epilepsy (45). Further studies, including patients with TBI and febrile seizures, can enhance our knowledge of the role of scalp HFOs as biomarkers of epileptogenesis.

Sleep Spindles and Theta Activity

Changes in the duration and frequency of the sleep spindles, one of the non-rapid eye movement stage II EEG features, have also been reported as a possible biomarker for epileptogenesis. In the LPFI model, Andrade et al. showed that shortening of the sleep spindles' duration and reducing of their frequency during slow-wave to rapid eye movement sleep transition can predict the development of epilepsy. Furthermore, receiver operating characteristics (ROC) analysis showed that spindle duration of <2.13 s (86% sensitivity, 80% specificity) and frequency of spindle <9.19 Hz (64% sensitivity, 60% specificity) could be used as biomarkers in differentiating rats with seizures from those without (46). These findings suggest that sleep spindles changes may be the indicators of widespread functional disturbance in the thalamocortical circuits following initial brain insult and could be used as a potential early biomarker for epileptogenesis.

Milkovsky et al. investigated the role of changes in the hippocampal dynamic in five animal models of epileptogenesis

TABLE 2 | Overview of EEG biomarkers of epileptogenesis.

EEG biomarkers	Animal model	Human epilepsy	References
HFO	KA-induced SE model	None	(43)
rHFOSs	LFPI model	None	(20, 44)
Scalp ripples	None	Children after a first unprovoked seizure	(45)
Sleep spindles	LPFI model	None	(46)
Theta band	Multiple rat and mouse models	None	(47)
Epileptiform activity	None	PTE and TSC	(48–51)
Background asymmetry	None	Epilepsy after stroke	(52)

HFO, High-frequency oscillations; rHFOSs, repetitive HFO and spikes; KA, kainic acid; SE, status epilepticus; LPFI, lateral fluid percussion injury; PTE, posttraumatic epilepsy; TSC, tuberous sclerosis complex.

using the intrahippocampal recording. Among five frequency bands, changes in the dynamic of the theta band on days 2–4 post brain injury showed more than 90% sensitivity and specificity in predicting the animal which would develop epilepsy (47). Overall this finding is intriguing, but given the invasiveness nature of intracranial electrodes, further studies using non-invasive source modeling may help further validating the finding as early epileptogenesis in the human.

Epileptiform Abnormalities and Background Asymmetry

The relation between epileptogenesis and scalp EEG findings such as interictal epileptiform discharges, lateralized periodic discharges, background EEG asymmetry, and electrographic seizures has been reported in humans. Although earlier studies in the 20th century do not give much yield in predicting the occurrence of epilepsy in patients with TBI (54), a recent retrospective study reported that during the acute traumatic phase, the presence of EEG interictal and ictal epileptiform abnormalities, such as sporadic interictal epileptiform discharges, lateralized or generalized periodic discharges, and seizures, are correlated with the development of the PTE at 1 year follow up (48). Punia et al. reported a similar result in adult patients that electrographic seizures or lateralized periodic discharges were related with the development of epilepsy after TBI (49). Two prospective multicenter studies investigated the role of scalp EEG in the infants with tuberous sclerosis complex. Among several EEG abnormalities, the author reported that the epileptiform discharges, not the hypsarrhythmia, showed the high positive predictive value and low negative predictive value in the development of epilepsy (50, 51). Furthermore, in addition to the interictal epileptiform activities, the presence of background asymmetry plays a role in the early development of epilepsy after stroke (52).

CONCLUSIONS AND FUTURE PERSPECTIVES

Here we aimed to provide a comprehensive review of recent innovations in the role of neuroimaging and EEG as biomarkers of epileptogenesis after the epileptogenic insult. Identifying biomarkers of epileptogenesis would greatly facilitate not only diagnosis and treatment but also the early prevention of epilepsy in individuals at risk. Although several studies have identified potentially promising biomarkers for early epileptogenesis, such as changes in the amygdala T2 relaxation time, PVSs, TSPO-PET, global and local network connectivity reorganization, and HFOs, numerous challenges remain to implement the potential neuroimaging biomarkers to the bedside clinical setting. Firstly, the resolution capacity of human neuroimage is significantly lower than animal neuroimage. Thus, the same biomarker which is reported from animal studies may not be directly replicated in human studies. Secondly, each potential biomarker has both disadvantages and advantages, and it is unrealistic to expect that a single biomarker will epitomize the various types of epileptogenesis. Therefore, a combination of EEG and neuroimaging biomarkers might enhance the predictive power of epileptogenicity. Thirdly, the majority of published data are at the animal stage (summarized in **Tables 1**, **2**). Before all these biomarkers can be utilized in the clinical setting, multicenter studies with standardized acquisition parameters and analysis procedures are needed to validate the robustness of biomarkers.

Currently, ongoing multicenter research studies are aiming to find biomarkers and treatments to prevent epileptogenesis. The European Union 7th Framework–funded project Targets and Biomarkers for Antiepileptogenesis (EPITARGET) is a consortium of 18 partners in nine European countries. In addition, the Epilepsy Bioinformatics Study for Antiepileptogenic Therapy (EpiBios4Rx), National Institute of Neurological Disorders and Stroke funded Centers without Walls study, is a collaborative multicenter international study conducted in the United States, Europe, and Australia. To data, biomarkers for epileptogenesis are still in the initial phase of the process. However, there will be more conclusive innovative EEG and neuroimaging early epileptogenic biomarkers and treatments from the multicenter trials to combat epilepsy in the foreseeable future.

AUTHOR CONTRIBUTIONS

GC: contributed to the conception and drafting the manuscript, ZZ: drafting the manuscript. MW and YG: revising the manuscript. BJ: contributed to the conception, drafting the manuscript, and final approval of the version to be published. TA: drafting the manuscript and revising the manuscript. All authors contributed to the article and approved the submitted version.

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CASE REPORT
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Case Report: Hypopituitarism Presenting With Nonconvulsive Status Epilepticus

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Introduction: Hypopituitarism is defined as one or more partial or complete pituitary hormone deficiencies. Nonconvulsive status epilepticus (NCSE) refers to a state of continuous or repetitive seizures without convulsions. In this paper, we review a case of an old female patient with hypopituitarism who presented with NCSE, which is rare in the clinic.

Case Report: This paper describes a 67-year-old female patient with hypopituitarism who presented as NCSE. She had surgical resection of pituitary tumor half a year before the seizures and did not get regular hormone replacement therapy. She presented general convulsive status epilepsy as the initial symptom and got sedation and antiepileptic drug in the emergency room. The seizure was terminated but the patient fell in coma in the following days. The patient had magnetic resonance imaging (MRI) and other inspects, and EEG showed epileptic discharges. Combining these clinical symptoms and examinations, we made the diagnosis of NCSE. Finally, she regained consciousness after the treatment with diazepam.

Conclusion: This case report and literature review investigated the possible mechanism of hypopituitarism presenting with NCSE.

Keywords: nonconvulsive status epilepticus, hypopituitarism, hormone, seizure, case report

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INTRODUCTION

Status epilepticus (SE) refers to a condition caused either by the failure of seizure termination or by the initiation of prolonged seizures. According to the classification of SE proposed by the International League Against Epilepsy (ILAE), NCSE is defined as SE without prominent motor symptoms, including NCSE with coma and NCSE without coma (1). The role of hormones in the pathophysiology of epilepsy has been gradually recognized (2, 3). In this paper, we report a patient with hypopituitarism presenting with NCSE.

CASE REPORT

A 67-year-old woman with diabetes was admitted to the emergency department due to two generalized tonic-clonic seizures and loss of awareness in the interictal period. The patient had never experienced seizures before. Half a year ago she had surgical resection of pituitary tumor and did not get regular hormone replacement treatment. In the days leading up to the seizures, she did not have any signs of discomfort such as fever or headache. In the emergency room

diazepam (5 mg, i.v.) and sodium valproate (50 mg, intravenous pumped at the rate of 160 mg/h) were given immediately as antiepileptic therapy, the seizures were terminated in several minutes and the patient fell in state of sedation shortly after, during which the patient could gradually open her eyes, answer simple questions and no more convulsions were observed. Routine blood tests in the emergency department showed hyperglycaemia (13.0 mmol/L), mild hypernatremia (151.7 mmol/L), and hyperchloremia (111.3 mmol/L). Then she was admitted to the neurology department and rescue measures including oxygen inhalation, antiepileptic therapy with sodium valproate (56 mg/h, 1 mg/kg/h), rehydration, maintaining water, and electrolyte balance were continued. About 3 h later, the patient passed into a lethargic state and she could only open her eyes on hearing our call but could not respond in words (GCS score of 8). The lethargic state continued for several hours and the patient fell in coma then (GCS score of 5), which lasted for the following 3 days. During this period the sodium valproate was pumped for 36 h continuously (56 mg/h for 24 h, 50 mg/h for 12 h, 24 mg/h for 12 h, total dose of 2,232 mg) and no remarkable facial or limb spasms or convulsions were recorded from admission. Tests of thyroid function, adrenocorticotropic hormone (ACTH), growth hormone (GH), cortisol, and sex hormone were conducted and the results supported the diagnosis of hypopituitarism (**Table 1**). MRI of the head was performed and no acute cerebral infarction, cerebral hemorrhage, and inflammatory changes were found (**Figure 1**). A lumbar puncture was performed to the patient on the 2nd day of admission. The cerebrospinal fluid (CSF) showed elevated protein levels (0.60/L, reference range 0.15–0.45 g/L) and increased chloride (137.8 mmol/L, reference range 118–132 mmol/L) and normal glucose levels and CSF pressure (**Table 1**). Autoimmune encephalitis and viral encephalitis tests were both negative, and thus the diagnosis of encephalitis was ruled out. A 24-h electroencephalogram (EEG) revealed sharp waves, spike waves, sharp-slow waves, spike-slow waves in the bilateral frontal region on a continuous slow-wave background with a trend of evolution, and the EEG also recorded paroxysmal electrical activities in this region (**Figure 2**). Combining the symptoms and examinations, the diagnosis of NCSE was considered. On the 3rd day of admission, diazepam (10 mg) was intravenously injected and the patient turned to a state of lethargy gradually and could open her eyes under painful stimulation, confirming the diagnosis of NCSE. Unfortunately, her family refused further treatment and she was discharged on the 3rd day, and she died of respiratory failure induced by epilepsy 5 days after her discharge from our hospital.

DISCUSSION

NCSE refers to a continuous nonconvulsive seizure that lasts for more than 30 min, or multiple nonconvulsive seizures happening during a period of more than 30 min and between which cognitive, motor, and/or sensory disorders is not fully recovered (4). Patients with NCSE can present as a variety of clinical manifestations, among which altered mental status is the most common (5). As the symptoms of NCSE are various, the diagnosis of NCSE relies on the combination of clinical presentation, electroencephalography findings, and clinical and electrographical reactions to antiepileptic treatments (4). The transition from generalized convulsive status epilepticus (GCSE) to NCSE is common: studies found that NCSE was quite common in patients in the ICU, accounting for about 50% (4). In our case, the patient initially presented as GCSE and went into coma which was diagnosed as NCSE finally.

The aetiology and risk factors of NCSE are complex, including acute and chronic pathological conditions (4). The role of endocrine system diseases in the pathogenesis and treatment of epilepsy has been studied continuously.

TABLE 1 | Laboratory data.

Test	Result	Normal range of value	Test	Result	Normal range of value
GH	0.031 ng/ml	(0.126–9.880)	FT3	2.40 pmol/L	(3.28–6.47)
Cortisol (00:00)	18.67 nmol/L	(185–624)	FT4	9.51 pmol/L	(7.64–16.03)
Cortisol (08:00)	17.28 nmol/L	(185–624)	TT3	0.24 nmol/L	(1.01–2.48)
Cortisol (16:00)	25.54 nmol/L	(185–624)	TT4	54.30 nmol/L	(69.97–152.52)
ACTH (00:00)	<1.00 pg/ml	(7.20–63.40)	TSH	0.04 ulU/ml	(0.56–5.91)
ACTH (08:00)	<1.00 pg/ml	(7.20–63.40)	Estradiol	0.02 pg/ml	(10–30)
ACTH (16:00)	<1.00 pg/ml	(7.20–63.40)	Testosterone	<0.1 ng/ml	(0.1–0.75)
Prolactin	28.67 ng/ml	(2.74–19.64)	Progesterone	<0.08 ng/ml	(<1.0 ng/ml)
LH	0.30 mlU/ml	(10.87–58.64)	FSH	10.87 mlU/ml	(16.74–113.5)
CSF protein	0.60 g/L	(0.15–0.45)	CSF chloride	137.8 mmol/L	(118–132)
CSF glucose	3.82 mmol/L	(2.5–4.2)	CSF white blood cell	0.002×10^9/L	(0–8)
CSF red blood cell	0.002×10^{12}/L	0			

Tests of hormone levels support the diagnosis of hypopituitarism.

GH, growth hormone; ACTH, adrenocorticotropic hormone; LH, luteinising hormone; FSH, folliclestimulating hormone; TSH, thyroid-stimulating hormone; FT3, free triiodothyronine; FT4, free thyroxine; TT3, total triiodothyronine; TT4, total thyroxine.

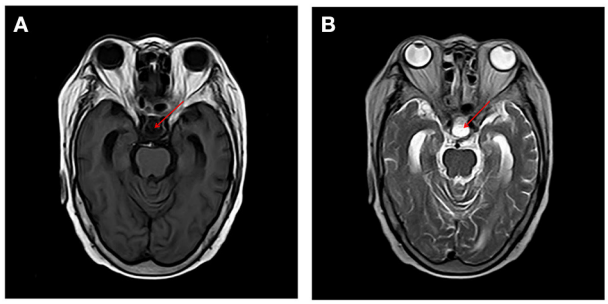

FIGURE 1 | MRI of the brain revealed change after removal of pituitary tumor and no acute cerebral infarction, cerebral hemorrhage, and inflammatory changes were found on T1 and T2 sequences [T1, T1-Weighted Magnetic Resonance Imaging; T2, T2-Weighted Magnetic Resonance Imaging **(A)** T1, **(B)** T2].

Hypopituitarism is defined as deficiency of one or more pituitary hormones produced in the anterior lobe and posterior lobe, including GH, the gonadotropins follicle stimulating hormone (FSH) and luteinizing hormone (LH), ACTH, thyroid stimulating hormone (TSH), prolactin, oxytocin, and antidiuretic hormone (ADH) (6).

Metabolic encephalopathy can also present with disorders of consciousness and it can be differentiated from NCSE by etiology, laboratory examination, and EEG. While increasing slowing of the waking background frequency was observed in hyperglycemia and hypoglycemia, hyponatremia may initially produce posterior slowing followed by more diffuse delta activity (7), hypopituitarism and hypoadrenalism may cause diffuse theta and delta activity. Furthermore, diffuse suppression with scant activity may be found in patients with hypothyroidism (8).

The relation between hormones and epilepsy is complicated. Concerning sex hormones, while androgens, progesterone and its metabolites have been found to have anticonvulsant effects, proconvulsant effects of estrogens were well-described (9). Studies have found that changes in hormone levels correspond to changes in seizures' frequency, which was considered as the effect of hormones on brain excitability (9), and this association was well-documented in catamenial epilepsy, in which the distribution of seizure numbers varied across the days of the menstrual cycle (10). Our patient showed remarkably low level of sex hormones including estradiol, FSH, LH, progesterone, and testosterone, making the evaluation of the role of any single hormone in the seizures complicated. Studies have found increased serum prolactin in about 67% of cases after complex-partial seizures or generalized tonic-clonic seizures (11, 12). Our patient had a relatively high level of prolactin, which was consistent with the previous reports.

The use of sex steroid hormones or their analogs for the treatment of epilepsy has been studied (13). It has been shown that supplementation of progesterone was effective in the management of seizure in patients with anovulatory cycles (14). Indeed, studies have confirmed that hormones including corticosteroids and ACTH were effective in the management of pediatric epilepsies including West syndrome, the Landau–Kleffner syndrome, other epilepsies, and epilepsy syndromes (15, 16).

It has been reported increasingly that stress plays a role in precipitating seizures (17, 18). Preclinical models of epilepsy showed that neuronal excitability and seizure susceptibility were both influenced by stress hormones (19). The hypothalamic-pituitary-adrenal axis is the main neuroendocrine system activated by stress (20). Studies on animal models revealed that neuronal excitability and seizure threshold were affected by stress hormones including corticotrophic hormone and corticosterone (21). It was reported that excitability of neurons was increased under the influence of corticotropin-releasing hormone (CRH) both *in vivo* and *in vitro*, and thus seizure activity was induced (22). Given the negative feedback effect of the hypothalamus-pituitary-adrenal axis, our patient should theoretically have high levels of CRH which may induce seizures.

Under basal conditions, stress hormones can also affect disease activity in seizures (23). In most of the studies, it was shown that increases in cortisol levels were a promoter during epileptogenesis (24, 25). But Ostrowska's team had the opposite conclusion and they found that patients with epilepsy had lower cortisol levels than those without epilepsy (26). Pritchard et al. (27) found that before generalized tonic-clonic seizures and complex partial seizures, the cortisol levels increased, while before simple partial seizures and secondary generalized seizures, the results were opposite (28). More importantly, patients with epilepsy often showed increased cortisol levels (29, 30). While the mechanisms of interaction between stress and seizures are not entirely clear, both of the two would have impact on cortisol levels.

Zhang and Liu (28) conducted a study analysing ACTH and cortisol levels during sleep seizures had found that during and after an epileptic seizure, the ACTH and cortisol levels were higher than those before a seizure. The researchers believed that epileptic seizures may be induced by decrease in ACTH and cortisol levels. A possible explanation may be that at night the decrease in ACTH levels triggers the release of CRH through negative feedback regulation, and the release of CRH increase epilepsy susceptibility and lower epilepsy threshold (21, 31).

Although the mechanisms of epileptogenesis still remain to be unclear, it is well-proved that mitochondrial dysfunction (32, 33) and oxidative stress (34) play important roles in this process. Molecular evidences have revealed that thyroid hormones are involved in normal mitochondrial biogenesis and function (35) and decreased activity of thyroid hormones is related to mitochondrial dysfunction (36). As to oxidative stress, both hyperthyroidism and hypothyroidism can affect antioxidant/oxidant balance (37). The impact of thyroid hormones in different aspects of epilepsy has been demonstrated (37). Thyroid function tests showed obvious hypothyroidism in our patient, which may also play an important role in the occurrence of epilepsy.

Treatments of NCSE should be based on expert opinions as well as individual aetiology including critical illness, subtherapeutic AED levels, etc. Recommendations for first-line

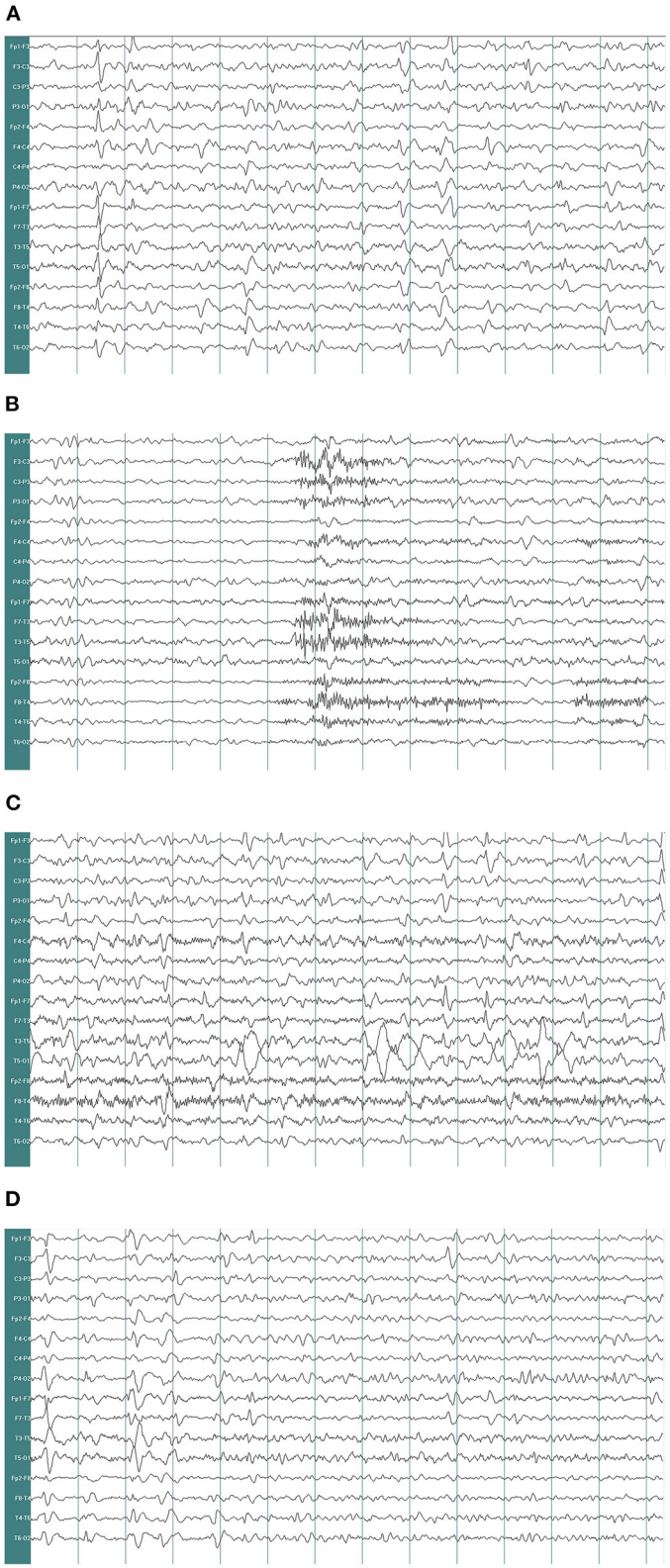

FIGURE 2 | Electroencephalogram recorded. EEG demonstrates δ and θ waves in each lead as background, mingled with sharp waves, spike waves, sharp-slow waves **(A)**. EEG shows a large number of sharp waves, spike waves, sharp-slow waves, spike-slow waves in the bilateral frontal pole, frontal and anterior temporal areas, paroxysmal or continuous, sometimes accompanied by evolution trend **(B–D)** (paper speed: 30 mm/s, high-frequency filter (HF): 30 Hz, low-frequency filter (LF): 0.53 Hz, notchfilers: 50 Hz, sensitivity:7 μv/mm. EEG A provided EEG background and the EEG B showed epileptic discharges).

treatment of NCSE are usually benzodiazepines (4). In our patient, recovery of impaired consciousness after the use of diazepam confirmed the diagnosis of NCSE. Regrettably, the patient did not receive systemic therapy and passed away finally.

CONCLUSION

In summary, our case, an old woman with hypopituitarism presenting with NCSE, led us to further explore the complex relationship between epilepsy and hormones, which contributes to the accurate diagnosis and treatment.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The study involving a human participant was reviewed and approved by the Ethics committee of Jincheng People's Hospital, Jincheng, China. The patient provided her written informed consent to participate in this study. Written informed consent was obtained from the individual for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

LJ put forward research ideas. LX took the responsibility of communicating with the patient's family and obtaining the authorization in this paper. HL was responsible for drafting articles. FY revised the article. HC and WL were responsible for literature searches and final proofreading. All authors contributed to the article and approved the submitted version.

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The Role of Brain-Derived Neurotrophic Factor in Epileptogenesis: an Update

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Epilepsy, which is characterized by spontaneous recurrent seizures, is one of the most common and serious chronic neurological diseases in the world. 30% patients failed to control seizures with multiple anti-seizure epileptic drugs, leading to serious outcomes. The pathogenesis of epilepsy is very complex and remains unclear. Brain-derived neurotrophic factor (BDNF), as a member of the neurotrophic factor family, is considered to play an important role in the survival, growth and differentiation of neurons during the development of the central nervous system. Recent years, a series of studies have reported that BDNF can maintain the function of the nervous system and promotes the regeneration of neurons after injury, which is believed to be closely related to epileptogenesis. However, two controversial views (BDNF inhibits or promotes epileptogenesis) still exist. Thus, this mini-review focuses on updating the new evidence of the role of BDNF in epileptogenesis and discussing the possibility of BDNF as an underlying target for the treatment of epilepsy.

Keywords: epilepsy, brain-derived neurotrophic factor, tyrosine kinase receptor B, treatment target, epileptogenesis

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INTRODUCTION

Epilepsy, which currently afflicts approximately 0.5–1% of population in the world (Fisher et al., 2014; Chen L. et al., 2020), is a chronic progressive central nervous system (CNS) disorder with a sudden abnormal discharge of neurons in the brain. Epilepsy is characterized by spontaneous recurrent seizures (SRS), which can lead to a range of serious consequences including temporary brain dysfunction, accidental injury, neurological damage and cognitive decline (Thijs et al., 2019). The clinical treatment of epilepsy is mostly dependent on anti-seizure drugs (ASDs). However, about 30% patients still failed to control seizures after regular treatment with multiple ASDs, who may gradually develop into intractable epilepsy patients (Wang and Chen, 2019). So there is an urgent need for seeking new therapeutic drugs or strategies. Since the pathogenesis of epilepsy is not yet clarified clearly, more and more researchers focus on the mechanism research of epilepsy and expect to find new therapeutic targets *via* further understanding the underlying mechanisms of several complex neural circuits (Wang et al., 2017; Xu et al., 2019) and associated molecules (Wang and Chen, 2019; Xu et al., 2021).

Several important pathological processes, such as neuronal damage, regeneration, and abnormal neural circuits formation in the epileptogenesis, have been reported (Chen B. et al., 2020; Chen LY. et al., 2020). Accumulated studies have focused on some key molecules that may be involved in these pathological processes, including brain-derived neurotrophic factor (BDNF). BDNF is an important

member of the neurotrophic factor family. It has a major role in promoting neuronal survival, growth and differentiation during the development of the CNS (Binder, 2004; Sharma et al., 2020); moreover, it can maintain the survival and function of the nervous system and promotes neuro-regeneration after injury (Song et al., 2008; Sun and Alkon, 2019). It was found that BDNF mainly exerts its effects through binding to at least two membrane receptors: tyrosine kinase receptor B (TrkB) and low affinity neurotrophic factor receptor (LNGFR, also known as p75) (Carito et al., 2014). TrkB is a high-affinity receptor for BDNF, while p75 is a low-affinity receptor, which is preferentially binded by BDNF precursor (proBDNF). A significant number of studies have shown an association between the BDNF-p75 pathway and psychiatric symptoms such as anxiety, depression and irritability (Lin and Huang, 2020), while very few studies have shown an effect of this pathway on epileptogenesis (Grabenstatter et al., 2014). The BDNF-TrkB pathway triggers a series of cascade reactions in downstream pathways, which is considered to play a main role in BDNF function (Naumenko et al., 2015). The signaling pathways downstream of the BDNF-TrkB include the following three: phospholipase Cγ, phosphatidylinositol 3-kinase and extracellular signal-regulated kinase cascade pathways, which are cross-linked to form a complex signaling network. All of them ultimately lead to the phosphorylation and activation of the cyclic adenosine monophosphate-response element-binding protein (CREB), which mediates the transcription of genes related to neuronal differentiation and survival (Cunha et al., 2010).

Basic and clinical studies have successively found that BDNF in the brain has a broad effect. It can alleviate neurological damage caused by diseases, such as Alzheimer's disease, Parkinson disease, spinal cord injury, stroke, and other neurological disorders (de Boer et al., 2017; Tanila, 2017; Rosa et al., 2019; Vidal-Martinez et al., 2019), and is strongly associated with the development of cognitive function, learning, memory, and depression (Hing et al., 2018). For instance, BDNF couldregulate synaptic plasticity and long-term potentiation (LTP) in the hippocampus and other brain regions to affect learning and memory (Leal et al., 2014). Hippocampal LTP is damaged in mice lacking BDNF in their neurons, and BDNF can enhance synaptic plasticity and LTP in the hippocampus and visual cortex (Mattson, 2008). Due to synaptic plasticity and LTP being closely related with temporal lobe epilepsy (TLE) and long-term memory (Tramoni-Negre et al., 2017), it is speculated that BDNF may be involved in TLE and the long-term memory deficits after TLE (Hattiangady et al., 2020).

In recent years, BDNF and its downstream pathways have also been found to play a role in the epileptogenesis (Walczak et al., 2013), but its exact role and potential mechanisms have not been completely illuminated. Based on the available studies, two opposing hypotheses have been proposed, that is BDNF may facilitate or inhibit the process of epileptogenesis (McNamara and Scharfman, 2012; Lin et al., 2020).

Therefore, to further understand the role of BDNF in the epileptogenesis, we aim to review the two views by summarizing the relevant literature in recent years, which may provide certain references for further elucidating the formation process of epilepsy and finding new targets for epilepsy treatment.

BDNF EXPRESSION INHIBITS EPILEPTOGENESIS

The hypothesis suggested that the expression of BDNF can suppress epileptogenesis by a combined receptor-ligand pathway; and the TrkB binding to BDNF may be the most closely associated with epilepsy between its two corresponding receptor-ligand pathways (McNamara and Scharfman, 2012; Iughetti et al., 2018).

Evidences From Preclinical Trials

In cell experiments, BDNF and proBDNF were found to have a strong regulation in neuronal injury and repair as well as plasticity of synapses, both of which are derived from a protein expressed in the CNS: pre-BDNF precursors (Pre-proBDNF). BDNF and proBDNF mainly bind to TrkB and p75, respectively (Walczak et al., 2013). Their binding activates a series of signal cascade responses that may suppress epilepsy by decreasing pyramidal neuron excitability (Gibon et al., 2015). It is well known that different processes of epileptogenesis eventually pass through two necessary pathways: increased excitability of excitatory synapses and diminished inhibition of GABAergic neurons (Pitkanen and Lukasiuk, 2009). So the results suggested that the proBDNF has another pathway except for transforming into BDNF, which also can suppress epilepsy. In an animal experiment, BDNF was also found to attenuate the decrease in the electrical potential of GABAergic neurons in the brain tissue of epilepsy model of rats (Cifelli et al., 2013). It is hypothesized that BDNF may inhibit epilepsy by promoting phosphorylation of certain subunits of GABAergic neurons to attenuate the disease-induced decrease in GABAergic neuronal excitability (Cifelli et al., 2013). In a recent study, loss of BDNF-TrkB signaling in the cortistatin-expressing interneurons resulted in behavioral hyperactivity and SRS in mice (Maynard et al., 2020). Meanwhile, BDNF could alleviate the inflammatory response associated with epileptogenesis and thus reduce the disruption of the blood-brain barrier, as well as reduce the number of SRS in an epilepsy model of rats (Bovolenta et al., 2010). These results also suggested that BDNF has an inhibitory effect on epileptogenesis. In addition, a study in epileptic pregnant rats and their offsprings reported that the treatment of phenytoin increased the level of BDNF in the offspring born by epileptic rats and exhibited neuroprotection effects. It is suggested that BDNF reduces the CNS damage caused by epilepsy through its neurotrophic effect and then reveals the inhibitory effect on the epileptogenesis (Soysal et al., 2016). Notably, the effects of drugs and hormones themselves on epileptogenesis could not be ignored in this study, so this study should be considered as a side or indirect evidence.

In the study of drugs, Chiu et al. investigated the protective effect of dexmedetomidine on brain injury in a rat model of kainic acid (KA) epilepsy and found that dexmedetomidine ameliorated KA-induced neuronal apoptosis in rats and increased the

expression of BDNF and TrkB meanwhile (Chiu et al., 2019). Also by exploring the effect of pantoprazole on pentylenetetrazol-induced seizures in rats, it is found that pantoprazole treatment delayed seizures, protected memory, and likewise increased the level of BDNF in the brain (Taskiran et al., 2021). From the above two studies, it is hypothesized that BDNF expression may inhibit the formation and recurrence of epilepsy and improve the prognosis through the protective effect of neurons. In addition, it has been found that CREB is an important nuclear transcription factor that regulates signaling pathways in the brain and is closely related to the expression of its downstream BDNF proteins, forming the CREB/BDNF pathway (Jiang et al., 2021). A recent study showed that hesperidin can promote the activation of the CREB-BDNF pathway and maintain physiological levels of BDNF to inhibit pentylenetetrazol-induced convulsions in zebrafish (Sharma et al., 2020). Thus, these drug studies suggested that BDNF expression may inhibit epileptogenesis from a side as well.

In general, many cellular and animal studies have shown a consistent relationship between decreased levels of BDNF and increased incidence of epilepsy, or that recovery of BDNF expression may alleviate epilepsy in experimental epilepsy. Notably, we noted that many studies have found the importance of maintaining normal levels of BDNF in suppressing epileptogenesis, but more direct evidences are still needed to clarify whether increasing BDNF to above physiological levels can significantly suppress epileptogenesis. Furthermore, although these results have suggested that BDNF expression may play a role in inhibiting the epileptogenesis, the precise mechanisms remain unsolved. Therefore, it is worthy of further researching the exact molecular mechanism of epileptogenesis inhibited by BDNF and its corresponding pathways, which may mainly involve in the binding of BDNF and proBDNF to TrkB and p75 followed by a series of signaling cascade reactions, the regulatory role of BDNF on GABA, and the CREB/BDNF pathway and so on.

Evidences From Clinical Studies

Only a few studies reported the relationship between serum levels of BDNF and epilepsy in patients. A clinical study in patients with TLE showed that BDNF serum level in patients with TLE was significantly lower than that in the healthy controlsand the BDNF serum levels correlated with epilepsy duration (Chen et al., 2016). Recently, Poniatowski et al.divided the subject patients (n = 143) into a generalized tonic-clonic seizure group (n = 50) and chronic epileptic patients (n = 93) to determine their serum BDNF levels after seizures separately compared to the control group (n = 48). They found a significant decrease in serum BDNF levels in patients after generalized tonic clonic seizures, at 1 and 72 h after seizures in this group (Poniatowski et al., 2021). These data suggest a direct relationship between the decrease in BDNF levels and the occurrence of epilepsy. However, it is not clear whether the decrease in BDNF levels causes the epileptogenesis or isonly a concomitant phenomenon of epilepsy. Hence, further clinical studies on this issue are warranted. In addition, BDNF is rarely reported worldwide as an epilepsy-related screening index, while BDNF-related genes are now found to be strongly associated with the development of epilepsy in patients. It has been found (Shen et al., 2016) that polymorphisms in BDNF-related genes (Val66Met) will lead to abnormal BDNF secretion and functional alterations, which in turn will affect the progressive course of the disease in patients with TLE. Other clinical studies have found that several miRNAs are involved in the BDNF-TrkB pathway and certain miRNAs can increase the expression of BDNF and activate its downstream signaling pathways, which have an inhibitory effect on the development of epilepsy (Wang et al., 2016). For example, it has been shown that the expression of CREB, a key molecule in the downstream signaling pathway of BDNF, and its corresponding genes increased at the original foci in patients with intractable epilepsy, while miR-124 may retard the process of epileptogenesis by reducing the expression of mRNA corresponding to CREB1 to diminish the effect of CREB1 on its downstream receptor NMDAR (Wang et al., 2016). The close relationship among miRNAs, the BDNF-TrkB pathway, and epilepsy suggests that miRNAs acting on the BDNF-TrkB pathway may be a promising antiepileptic treatment strategy.

BDNF EXPRESSION PROMOTES EPILEPTOGENESIS

The other hypothesis suggests that abnormal activity or increased levels of BDNF play a positive role in inducing epilepsy and promoting epileptogenesis (McNamara and Scharfman, 2012; Iughetti et al., 2018).

Evidences From Preclinical Trials

It has been reported that the deletion of TrkB in mice caused a significant reduction in behavioural evidence for epileptogenesis (He et al., 2004; Kotloski and McNamara, 2010), and BDNF and TrkB expression increased in the amygdala of rats with epilepsy and depression (Liu et al., 2013; Briz et al., 2015). Ghadiri et al. found that BDNF levels were reduced in the hippocampus of rats given 32 mg/kg progesterone in an epilepsy model, while the number of apoptotic cells in the ipsilateral hippocampus and the number of damaged neurons was significantly reduced. Thus, the data showed an antiepileptic effect, which suggested that BDNF may promote neuronal cell damage and apoptosis to induce epilepsy formation (Ghadiri et al., 2019). In addition, decreased percentage of BDNF alleles in KA-induced epilepsy model of rats causes stronger mossy fiber sprouting to lead to the formation of abnormal excitatory circuits in the brain, which are thought to be associated with the epileptogenesis (Skupien-Jaroszek et al., 2021). This finding is consistent with some previous studies that BDNF takes part in sprouting events in epilepsy (Danzer et al., 2002; Scharfman et al., 2002), which suggests BDNF may play a positive role for epileptogenesis *via* promoting the mossy fiber sprouting and the formation of abnormal circuits. In addition, BDNF induces ryanodine receptor channel-mediated Ca^{2+} release and reactive oxygen species (ROS) production (Yang et al., 2020), while oxidative damage to mitochondria leads to disruption of mitochondrial function and cell death signalling, allowing excessive ROS

production and ultimately triggering epilepsy (Quan et al., 2020). Therefore, it is speculated that BDNF may induce epilepsy by causing an increase in ROS production. Overall, BDNF may contribute to the development of epilepsy by activating its downstream pathways to modulate a variety of factors, (e.g., neuronal cell damage and apoptosis, mossy fiber sprouting, increase in ROS, etc.).

TrkB is a main downstream molecule of BDNF. Reduced TrkB receptors in the amygdala and hippocampus can hinder the process of epileptogenesis in TrkB receptor-related knockout mice (Kotloski and McNamara, 2010). The mechanism is believed that increased TrkB receptor activity inhibits Cl^- efflux by reducing KCC2 expression, which inhibits GABAergic neuron function and induces epilepsy (Liu et al., 2014). Similarly, transient inhibition of the TrkB receptor by a TrkB inhibitor can effectively suppress TLE in mice (Liu et al., 2013). REST/NRSF, an upstream gene of BDNF, acts to prevent seizures by inhibiting the activation of the seizure-associated BDNF-TrkB pathway, suggesting a possible role of the BDNF-TrkB pathway in promoting epileptogenesis (Chmielewska et al., 2020). In addition, BDNF overexpression in astrocytes in a lithium-pilocarpine mouse model worsened their phenotype, while neuroprotective effects were exhibited after lithium-pilocarpine treatment in the mice with hippocampal neurons or astrocyte-specific genes deficient of TrkB and significant retention of spatial learning ability was observed in the mice with astrocyte-specific gene deletion of TrkB (Fernandez-Garcia et al., 2020). The data further suggests that the damaging effects of the BDNF-TrkB pathway on nerves may be associated with epileptogenesis.

In summary, these cellular and animal studies have shown that increased BDNF levels seem to be correlated with the incidence of epilepsy and increased BDNF may be companied with the neuronal cell damage. However, the related molecular mechanisms still require to be further investigated, including whether increased BDNF levels have a direct damaging effect on neuronal cells and whether high levels of BDNF can reverse their protective effect on neurons at low levels.

Evidences From Clinical Studies

Although BDNF has a nutritional and supportive effect on neurons under physiological conditions, overexpression of BDNF seems to play an opposite role. Excessive BDNF significantly increases excitability of neurons and increases susceptibility to epilepsy, thus producing damage to neurons and possibly promoting epileptogenesis (Scharfman et al., 2002). BDNF was found to increase excitatory synaptic transmission and decrease the inhibitory effect of inhibitory neurotransmitters on activated synapses, thereby enhancing the transmission of epileptiform discharges in neural networks (Doherty et al., 2019). The clinical study also found that the majority of epileptic patients had higher levels of serum BDNF than healthy individuals, and the expression of BDNF and TrkB receptors were positively correlated. It is suggested that BDNF may play an important role in the occurrence and development of TLE through TrkB receptors (Iughetti et al., 2018). In addition, BDNF and its conjugated receptor (TrkB) is increased in both animal models and human epilepsy patients, especially in the temporal and hippocampal regions which provides a surgical strategy for epilepsy surgery. The distribution of BDNF showed the levels of BDNF and TrkB receptors in the cerebrospinal fluid of patients with TLE are higher than that in peripheral blood. This phenomenon was also seen in some drug trials and studies. Investigating 80 epilepsy patients, who were already using new ASDs, and 13 healthy subjects, a study found that BDNF levels were higher in patients with focal epilepsy and new ASDs levels were negatively correlated with BDNF levels in serum and positively correlated with total quality of life scores in patients with multiple new ASDs (Demir et al., 2020). Therefore, the serum or cerebrospinal fluid test of BDNF may be a convenient and reliable test for prognostic assessment of TLE and medications.

Excessive transcriptional expression of BDNF has also been investigated in epileptic patient (Martinez-Levy et al., 2018). The use of BDNF gene polymorphism to replace the corresponding amino acid (Val66Met replaced by methionine) has a good therapeutic effect on epilepsy, while inhibiting the secretion of activity-dependent BDNF can prevent seizures (Egan et al., 2003; Chen et al., 2004). Some epidemiological studies have also found that BDNF Met66 allele carriers are relatively less prone to epilepsy in patients with Rett syndrome (Nectoux et al., 2008), suggesting that altering the molecular structure of BDNF may have an inhibitory effect on epilepsy formation and BDNF may have an important role in promoting epilepsy formation. A recent investigation from Hong Kong and Malaysia for BDNF genotyping showed a significant correlation between BDNF and risk of symptomatic epilepsy in Malaysian Indians (Sha'ari et al., 2016), suggesting that BDNF polymorphisms may increase the incidence of epilepsy in Malaysian Indians. Thus, all these studies support the idea that high expression of BDNF in certain brain regions may cause epilepsy.

In the above clinical study, BDNF levels were indeed higher in the serum or the cerebrospinal fluid of epileptic patients than that in normal subjects. However, due to technical and ethical limitations, we are generally unable to measure the changes of BDNF in epileptic patients during the epileptogenesis. Therefore, we have not been able to determine whether this elevation of BDNF induces neuronal excitation to promote epilepsy, or it is simply a stressful increase of the body to repair neurons after the onset of epilepsy. More researches are needed to further elucidate this issue in the future.

Outlook

Despite accumulated evidences have indicated BDNF is closely associated with epileptogenesis in recent years, the exact role of BDNF in the epileptogenesis is still controversial, which may be concerned with the expression level of BDNF, the brain region of expression, its upstream and downstream molecules and other factors. It is speculated that there exist two possible hypotheses needed to be further verified. One is the expression level of BDNF in the brain needs to be maintained in certain range; that means, too high or too low may be unbeneficial for preventing from epileptogenesis. The other is that the influence of BDNF expression on epileptogenesis in different neural circuits, brain

area, or subgroup of neurons may also be diverse. In addition, its upstream and downstream molecules may be involved in epileptogenesis. However, the role of several molecules such as p75 on epileptogenesis is still unclear, which is worthy to be further studied and clarified. Therefore, more researches and more direct evidences are needed to further elucidate these issues in the future; especially the elucidation of the role of BDNF in different neural circuits, which may have important significance for further understanding of epileptogenesis. Furthermore, many proteins and molecules within the BDNF-related signaling pathway are expected to be molecular targets for clinical epilepsy detection and treatment, which may be exploited to participate in the clinical treatment, risk assessment and prognosis of epilepsy. Hence, BDNF-related translational research is also a research direction to be focused on in the future.

SUMMARY

BDNF is closely related to epilepsy, since its upstream related genes and downstream receptors are found to be involved in the epileptogenesis. Various proteins and molecules in the whole pathway cross each other to form a complex signal transduction network; meanwhile, it is regulated by various exogenous substances. It may play a key role in the excitatory/inhibitory balance of neurons, which also has important significance of the occurrence and development of epilepsy. Currently, despite BDNF showed its effect in several preclinical researches, clinical studies using BDNF as a therapeutic agent have not been encouraging. Thus, BDNF is considered as a potential therapeutic target but not a drug and the modulation of BDNF and its upstream or downstream molecules by other agents may have certain clinical feasibility.

AUTHOR CONTRIBUTIONS

XW and ZH searched and filtered literatures. XW, ZH, and KZ wrote and corrected the mini-review.

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Secondary Epileptogenesis: Common to See, but Possible to Treat?

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Secondary epileptogenesis is a common phenomenon in epilepsy, characterized by epileptiform discharges from the regions outside the primary focus. It is one of the major reasons for pharmacoresistance and surgical failure. Compared with primary epileptogenesis, the mechanism of secondary epileptogenesis is usually more complex and diverse. In this review, we aim to summarize the characteristics of secondary epileptogenesis from both clinical and laboratory studies in a historical view. Mechanisms of secondary epileptogenesis in molecular, cellular, and circuity levels are further presented. Potential treatments targeting the process are discussed as well. At last, we highlight the importance of circuitry studies, which would further illustrate precise treatments of secondary epileptogenesis in the future.

Keywords: secondary epileptogenesis, mechanisms, pharmacotherapy, neuromodulation, neural circuits

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INTRODUCTION

Epilepsy is one of the most common neurological diseases, affecting nearly 70 million people with ∼1% incidence worldwide (1, 2). Patients with epilepsy suffer from unpredictable seizures. Seizures are characterized by synchronous neural firing originating from the seizure focus. However, more than one epileptic focus may emerge in some patients as the disease progresses, defined as secondary epileptogenesis (3). Frank Morrell initially put forward the term secondary epileptogenesis to describe an independent epileptic focus localized in the homotopic area of the primary focus in the contralateral hemisphere (4). The existence of a secondary focus may lead to further pharmacoresistance and failure of surgical intervention. Although the phenomenon of secondary epileptogenesis has been recognized for long, its mechanism is still "tales from the mist." Hypotheses include excitatory actions of glutamate, depolarized GABA transmission, and long-term alteration of synaptic plasticity. Here, we first summarized the characteristics of secondary epileptogenesis by reviewing both clinical and experimental studies, followed by different aspects of mechanisms. Then available and possible treatments for interfering secondary epileptogenesis in the recent decades were presented. Given the significant advances of experimental approaches, including optogenetics, neuroimaging, and electrophysiology, we suggest understanding secondary epileptogenesis in a circuitry view and proposing open questions for future direction to improve the management of this common and intractable clinical situation.

SECONDARY EPILEPTOGENESIS

Early Findings From Animal Experiment

For many years, it has been known that epileptic discharge in one hemisphere may be related to synchronous discharges in the symmetric region of the other hemisphere (5). However, the precise definition of secondary epileptogenesis was not introduced until the 1960s. Morrell confirmed that after forming the primary epileptic focus, an independent epileptic focus could develop in the symmetric regions (which was also defined as the mirror focus) (4). In another early study, researchers proposed that the direct callosal junction between the primary and the mirror focus is indispensable (6). They questioned whether transecting the corpus callosum in the early stage can prevent secondary epileptogenesis. To address this issue, experiments were firstly performed on cats and rabbits. Ethyl chloride spray was delivered to a small section of the pial surface to produce a relatively small epileptogenic lesion. Recording electrodes were implanted into the sprayed area as well as the symmetric region to record electrical activities. After a few hours, the spike activities in the lesion could be observed. However, several days later, the paroxysmal independent discharges could be recorded by the contralateral electrode. And the callosal transection could prevent this process. Thus, it could be concluded that the callosal pathway is probably the critical route for epileptic propagation to the mirror focus in secondary epileptogenesis (7). In 1975, secondary epileptogenesis was further confirmed in the hippocampal kindled cats by Sato. In that study, epileptic electroencephalographic (EEG) activities were found in different regions apart from the mirror focus (8).

As for rodents, in 1962, Dow et al. firstly reported the development of secondary epileptogenesis on rats. Ten to twenty days after the cobalt application, which would produce a chronic discharging focus at the right cerebral cortex, the epileptic activities were observed at the contralateral hemisphere (9). Another study from Levin et al. confirmed that the contralateral focus, which would show recurrent spontaneous seizures, was not rare in an ethyl chloride freezing induced epilepsy model (10). To analyze the secondary epileptogenesis in rats in detail, Engel applied cobalt powder into the posterior brain of the rats to induce spontaneous epileptic seizures. They found that an independent secondary epileptic focus was easy to develop in the cobalt-induced epileptic model. Furthermore, the secondary epileptic focus was usually more excitable than the primary ones in the latter stage (11). A further histological study found that the RNA level was decreased, and ganglioside sialic acid amount was increased in the secondary focus (12). In addition to the chemical convulsant, kainite acid (KA) induced secondary focus by intra-hippocampal and intra-amygdala microinjection into the mice brain (13). Similar results about secondary epileptogenesis were found on other animal species, regardless of the diverse approaches to induce epileptic seizures. As Engel et al. proposed, the subsequent application of pentylenetetrazol could easily induce epileptic discharges in the contralateral secondary focus at dry ice treated rabbit's cortex (14).

It has to be admitted that using chemical convulsants to induce epileptic seizures has a major limitation. The uncertainty of drug diffusion may further influence other extensive regions beyond the injection site. Thus, further validation of secondary epileptogenesis with a more specific primary focus is necessary. Repetitive electrical stimulation in a routine region was commonly used to investigate the progressive increase of behavioral and EEG seizures (15). Secondary epileptogenesis caused by electrical stimulation has been reported in multiple animal species, including frog, caiman, opossum, and monkey (16, 17). All the animals developed projected or evoked epileptic discharge in the homotopic area. Among them, the squirrel monkeys and rhesus monkeys had the fully independent mirror focus in which the epileptic discharges at the contralateral cortex have no correlation in timeline with that of the primary focus. Furthermore, the time courses of secondary epileptogenesis in lower mammals (e.g., rats, cats, and rabbits) seem to be shorter than those of non-human primates (18).

Besides *in vivo* studies, *in vitro,* ictal models are also beneficial for studying secondary epileptogenesis. In 1997, Khalilov et al. firstly established a well-designed three-chamber *in vitro* model by placing the intact hippocampal structures of neonatal male *Wistar* rats in different compartments of a chamber. Those well-isolated chambers separated by latex membranes could be perfused with different solutions separately (19, 20). Using this *in vitro* model, researchers could study the generation of synchronized neuronal activities and investigate the propagation of local epileptic excitability to distant areas. In their study, the contralateral hippocampus developed an independent epileptic focus after KA treatment on the primary focus despite the application of tetrodotoxin on the commissural fibers to block the neural connections reversibly (21). This *in vitro* preparation of intact hippocampi demonstrated the existence of secondary epileptogenesis in the isolated chamber and could be a promising model to study its mechanisms.

Substantial findings obtained from different models have confirmed the existence of secondary epileptogenesis in different epileptic models (**Table 1**). The most common localization of the secondary focus is the homotopic area contralateral to the primary focus, and the time of formation may be related to the intrinsic epileptic characteristics of the primary focus. However, due to the limitations of experimental techniques, seizures of these early laboratory experiments were mainly triggered by convulsants or electrical stimulations and were characterized by relatively extensive lesion area and vague primary focus (mainly localized in the cortex). Given that only a certain amount of patients had one specific epileptic focus, we suggest that the chronic spontaneous epileptic models may be more appropriate to study the mechanisms and discover potential therapeutic targets for secondary epileptogenesis.

Clinical Evidence of Secondary Epileptogenesis

Interestingly, the secondary epileptogenesis phenomenon was first presented in animal experiments, and clinicians took many years to validate secondary epileptogenesis in patients. The secondary focus, which can generate separate epileptic seizures in patients, was first reported in 1984. Morrell reviewed patients

TABLE 1 | Secondary epileptogenesis in animal models.

Years	Authors (Ref.)	Species	Epileptogenic factors	Primary focus	Secondary focus
1947	Pacella et al. (5)	Monkey	Hydrous oxides of aluminum	Motor cortex	Contralateral motor cortex
1959	Morrell (4)	Rabbit	Ethyl chloride spray	Right cortex	Left cortex
1960	Morrell (7)	Cat	Ethyl chloride spray	Right cortex	Left cortex
1962	Dow et al. (9)	Rat	Cobalt powder	Right frontal lobe	Left frontal lobe
1967	Levin and McCrimmon (10)	Rat	Ethyl chloride spray	Right somatosensory cortex	Left somatosensory cortex
1968	Engel (11)	Rat	Cobalt powder	Left posterior portion	Right posterior portion
1968	Wilder et al. (16)	Frog, cayman, opossum, monkey	Freeze lesion, penicillin	Left cortex	Right cortex
1970	Engel and Morrell (14)	Rabbit	Slivers of dry ice	Right cortex	Left cortex
1972	Westmoreland et al. (12)	Rat	Cobalt powder	Right somatosensory area	Left somatosensory area
1975	Morrell et al. (17)	Frog	Electrical stimulation	Right hippocampal cortex	Left hippocampal cortex
1975	Sato (8)	Cat	Electrical stimulation	Left hippocampus	Right hippocampus
1978	Schwarcz et al. (22)	Rat	Kainic acid	Hippocampus	Contralateral hippocampus
1980	Ben-Ari et al. (6)	Rat	Kainic acid	Amygdala, caudate-putamen, globus pallidus, bed nucleus of the stria terminalis and septum	Contralateral homotopic area
1983	Jibiki et al. (23)	Rabbit	Electrical stimulation	Right visual cortex	Left visual cortex
1991	Kirkby et al. (24)	Rat	Electrical stimulation	Right hippocampus	Left hippocampus
1993	Beldhuis et al. (25)	Rat	Electrical stimulation	Amygdala	Contralateral amygdala
1993	Hiyoshi et al. (26)	Cat	Electrical stimulation	Right amygdala	Left mygdala
1994	Szente and Boda (27)	Rat	4-aminopyridine	Cortex	Contralateral cortex
1996	Federico and MacVicar (28)	Guinea pig	Electrical stimulation	Lateral entorhinal cortex (*in vitro*)	Contralateral lateral entorhinal cortex (*in vitro*)
1997	Forti et al. (29)	Guinea pig	Bicuculline	Right anterior piriform cortex (*in vitro*)	Left anterior piriform cortex (*in vitro*)
1997	Kudo et al. (30)	Cat	Electrical stimulation	Right motor cortical	Left motor cortical
1997	Mihaly et al. (31)	Rat	4-aminopyridine	Right frontal neocortex	Left frontal neocortex
2000	Barna et al. (32)	Rat	4-aminopyridine	Right somatosensory cortex	Left somatosensory cortex
2003	Gajda et al. (33)	Rat	4-aminopyridine	Somatosensory cortex	Contralateral somatosensory cortex
2003	Khalilov et al. (21)	Rat	Kainic acid	Right hippocampus (*in vitro*)	Left hippocampus (*in vitro*)
2005	Arabadzisz et al. (34)	Mouse	Kainic acid	Right hippocampus	Left hippocampus
2005	Gajda et al. (35)	Rat	4-AP	Right cortex	Left cortex
2008	Mouri et al. (36)	Mouse	Kainic acid	Amygdala	Contralateral amygdala
2009,2011	Nardou et al. (37–39)	Rat	Kainic acid	Right hippocampus (*in vitro*)	Left hippocampus (*in vitro*)
2013	Sobayo and Mogul (40)	Rat	Kainic acid	Hippocampus	Contralateral hippocampus
2017	Ito et al. (41)	Rat	Hyperthermia	Right hippocampus	Left hippocampus
2017	Kuang et al. (42)	Rat	Electrical stimulation	Right amygdala	Left amygdala

with benign brain tumors in whom the lesion stayed relatively stable over time. However, EEG recordings confirmed that 34% of those patients could develop an independent secondary epileptogenic focus remote from the tumor sites (13, 43).

The phenomenon of secondary epileptogenesis could be further identified in patients with progressive epilepsy. In those patients, determination of the epileptogenic zone has become a considerable challenge. In a study in 1970, EEG parameters, including the distribution of the beta rhythms and the behavior of the bilateral spike, were analyzed after repetitive injections of thiopental sodium in a total of 82 patients, aiming to guide the epilepsy surgery. The simply routine EEG criteria appeared unreliable for those patients who had widespread epileptic lesions (44). Similarly, Gollwitzer et al. reviewed video-EEG recordings from 100 patients with temporal lobe epilepsy (TLE) and found that the bilateral independent interictal epileptiform activities could be detected in 64% of patients. Their findings suggested that seizure foci were localized in both hemispheres (45). Schmidt et al. reported the phenomenon of seizure recurrence after discontinuing anti-seizure drugs (ASDs) in six patients who had undergone epilepsy surgery (most are temporal lobe surgery). This phenomenon could be attributed to the formation of secondary epileptic foci (46). Another evidence of secondary epileptogenesis was that some patients could develop different

TABLE 2 | Secondary epileptogenesis in patients.

Years	Authors	Sample size	Conclusion
1952	Tukel and Jasper (49)	31 patients with parasagittal epileptogenic lesions	Epileptogenic foci in the cortex near the corpus callosum can cause widespread discharges at both hemisphere.
1961	Falconer and Kennedy (43)	7 patients with small focal lesions (glial hamartomas, angiomas, or other neoplasms)	The EEG disclosed there were bilateral, independent spike discharges in both temporal regions.
1961	Rovit et al. (50)	20 patients	Unilateral carotid amobarbital injection at primary epileptogenic lesions can inhibit bilateral discharges.
1970	Lombroso and Erba (44)	82 patients presenting variety of seizures	Patients with widespread brain involvement in seizure activity are unappropriate for surgery.
1985	Morrell (13)	47 patients with cerebral tumor seen as epilepsy	34% of patients had bilateral, independent epileptiform discharge in their EEGs, and more than one seizure type.
1994	Gilmore et al. (51)	22 patients with complex partial seizures and had temporal lobe neoplasms	The mirror focus is not a contraindication to operation even when the preponderance of interictal discharge is contralateral to the tumor.
1997	Eliashiv et al. (52)	60 patients who had standard en bloc anterior temporal lobe resection	The seizure recurrence at sites distant to the lesion may relevant to years of uncontrolled seizures.
1998	Morris et al. (53)	38 patients with intractable epilepsy and ganglioglioma	Despite years of medically resistant seizures, patients with ganglioglioma can still have good surgical outcomes.
2006	Kimiwada et al. (54)	14 children with partial epilepsy involving the temporal lobe	Radiographic results show the recruitment of hippocampal and thalamic in epileptic network.
2008	Surges et al. (55)	14 patients with tonic–clonic seizures of extrahippocampal onset	Repeated extrahippocampal seizures can result in persistent modifications in hippocampal excitability.
2010	Bortolato et al. (56)	One patient with bilateral foci in frontal lobe	The density of $GABA_A$/benzodiazepine receptor binding in the mirror focus had a significant increase.
2014	Kim et al. (48)	One patient with intractable occipital lobe epilepsy	Occipital lobe epilepsy can also have the mirror focus.
2017	Gollwitzer et al. (45)	100 patients diagnosed with temporal lobe epilepsy	Bilateral independent interictal epileptiform activities could be detected in the progress of TLE.

types of seizures later in the disease course, with an epileptic focus distinct from the primary site. For instance, Morrell reported a patient who developed a new seizure type (automatism followed by head and eye turning to the left) distinct from the habitual seizures with epigastric sensations followed by lip-smacking. (47). These seizures were distinct from the primary ones as a consequence of the formation of other epileptic foci. As mentioned above, the phenomenon of secondary epileptogenesis was reported more often in patients with temporal or frontal lobe epilepsy and rarer in occipital lobe epilepsy. However, Kim et al. reported an exception in occipital lobe epilepsy. The patient had relapses of seizures 10 months after resecting the defined seizure focus located at the left occipital lobe. Further validation confirmed the formation of a secondary focus located in the homotopic area of the right occipital lobe (48).

Clinical evidence on secondary epileptogenesis is still lacking, limited to case reports or series with small sample size. It could occur in different types of epilepsy (**Table 2**). Systematic and comprehensive prospective cohort studies are still needed to assess the prevalence and incidence of secondary epileptogenesis in different types of epilepsy and identify factors related to this phenomenon.

Dilemmas in Treating Secondary Epileptogenesis

Over the last 30 years, about 30 ASDs have been approved and used to help control epileptic seizures (57). In many conditions, the seizure frequency could be reduced after taking ASDs. However, there are still a certain proportion of patients who would become pharmacoresistant. Compared with patients who had only one seizure focus, those with a secondary focus are more susceptible to pharmacoresistance (58). For pharmacoresistant epilepsy, uncontrolled seizures increase the risk of sudden unexpected death in epilepsy and seriously affect the quality of patients' daily life. Resection of the epileptogenic zones turns to be the optimal option. However, a secondary focus could restrict the surgery's efficiency because the presence of a secondary focus can generate epileptic discharges independently, even when the primary focus is resected.

Some researchers propose that the presence of secondary epileptogenic focus might not account for surgical failure in patients with epilepsy. Take the tumor patients, for instance. Resection of the tumor itself was mostly sufficient for seizure control, even if the mirror focus was spared in the surgery (59). Meanwhile, Goldensohn insisted that EEG evidence of multifocal discharge should not be considered when making decisions regarding epilepsy surgery because follow-up research showed that patients with bilateral foci still have a good prognosis after resection of the unilateral seizure focus (60). A study with 22 patients with temporal lobe neoplasms demonstrated that the mirror focus is not a contraindication for epilepsy surgery. Resection of the primary focus resulted in the disappearance of the secondary focus (51). In contrast, another study argued that the seizure relapses after resection were usually due to secondary foci in homotopic regions contralateral to the primary focus

(48). Different phases of secondary epileptogenesis (dependent, intermediate, and independent stages) proposed by Morrell may explain these conflicting reports (13, 47). During the dependent stage, the discharges that originated from the secondary focus are always time-locked to that of the primary focus, which means epileptic discharges at the secondary focus may only be propagated from the primary focus. In the intermediate stage, the discharges of the secondary focus can be different in time phases from that of the primary focus. However, surgical resection of the primary focus can lead to the vanishment of the secondary focus, which means the maintenance of the seizures in the secondary focus needs the existence of the primary focus. Eventually, the secondary focus becomes permanent in the last independent stage. In this stage, the seizure may originate from the secondary focus after the resection of the primary focus. Nevertheless, it should be noted that if the resection of a seizure focus leads to a certain period of seizure-free at first, but followed by a relapse of seizures, other epileptogenic factors such as abnormal stem cells attributed by developmental malformations or tumors may be also taken into account besides secondary epileptogenesis (61).

Thus, although contradictions exist, a conclusion could be drawn that secondary epileptogenesis is one of the major causes of the less favorable surgical outcome. However, the outcome of epilepsy surgery cannot be solely determined on the presence of the secondary focus but should take the different phases into account.

Possible Mechanisms of Secondary Epileptogenesis

Unlike those neurological diseases with clear pathogenesis, multiple epileptogenic factors include tumor, trauma, neuroinflammation, genetic predisposition, etc., have been shown to result in abnormal excitation in the brain and consequently provoked seizures (1). Moreover, uncontrolled repetitive seizures further aggravate epileptic conditions like secondary epileptogenesis. Given that the genesis of secondary epileptic focus largely depends on the seizure propagation from the primary focus, mechanisms of excitability spreading such as neurotransmission and synaptic plasticity are closely related to secondary epileptogenesis. In the following sections, we discuss the possible mechanisms of secondary epileptogenesis systematically.

MOLECULAR MECHANISMS

Involvement of Excitotary Neurotransmission

It is widely accepted that the actions of excitatory neurotransmitters play a vital role in the process of seizure propagation. The glutamate-mediated amplified excitatory activity could lead to the recruitment of excitatory neurons and initiation of the hyperactivity, then the spread of the hyperactivity would cause the seizures (62). Pathological excitatory neurotransmission mediated by glutamate receptors has long been regarded as a major factor in clinical and experimental epilepsy etiology. Ionic glutamate receptors include N-methyl-D-aspartic acid (NMDA) receptor, KA receptor, and α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid (AMPA) receptor, which conjugate with ion channels to mediate fast signal transduction. Khalilov et al. preliminarily revealed the correlation between ionic glutamate receptors and secondary epileptogenesis in the three-chamber model. They reported that the application of NMDA receptor antagonist on the contralateral hippocampus could prevent the formation of the secondary focus but not the propagation of seizure activity (21), which means NMDA receptors were involved in secondary epileptogenesis caused by the long-lasting synaptic excitatory effects originating from the primary focus. Given that the NMDA receptors were composed of two GluN1 obligatory subunits and two regulatory subunits (63), just as Acutain et al. reported, the decreased expression of GluN2A would further lead to increased seizure susceptibility (64). Thus it can be speculated that changes of the NMDA subunits may also underly secondary epileptogenesis. These findings suggest that the activation of the NMDA receptor is necessary for forming the secondary focus.

Similar to the NMDA receptor, other studies verified the role of the AMPA receptor in secondary epileptogenesis. Barna et al. proposed that intracerebral injection of the AMPA receptor antagonist GYKI-52466 into both the primary and mirror focus led to anticonvulsant effects in anesthetized rats treated by 4-AP (32). Also, the role of the AMPA receptors in secondary epileptogenesis of a KA treated rat model was examined. Interestingly, the application of the AMPA receptor antagonist CNQX led to a priority of seizure generation in the ipsilateral hippocampus, while in the selective KA receptor antagonist and the control group, epileptic discharge mainly originated from the contralateral side (40). Another study further verified this by showing that the AMPA receptor antagonist could reversibly suppress the seizure activity originating from the secondary focus (28). Besides, although barbiturate anesthetics have been widely accepted as a GABAergic function enhancer (65), it is also reported that phenobarbital could modulate the expression of AMPA-type glutamate receptor channels (66). Based on this, Nardou et al. compared the effects of diazepam, unrelated to the AMPA receptors and phenobarbital, on the formation of the mirror focus. They reported that phenobarbital but not diazepam could reduce the occurrence of epileptic spikes in the mirror focus with the presence of GABA and NMDA receptor antagonists (37), which laterally provide evidence for the involvement of the AMPA receptors in secondary epileptogenesis.

In brief, epileptic discharges resulted from neuronal hyperexcitability. The repetitive abnormal epileptic discharges in the primary focus can lead to an increased glutamatergic driving force acting on both excitatory NMDA and AMPA receptors of the possible secondary epileptic focus. This process will decrease the threshold of seizure generation and thus contribute to secondary epileptogenesis.

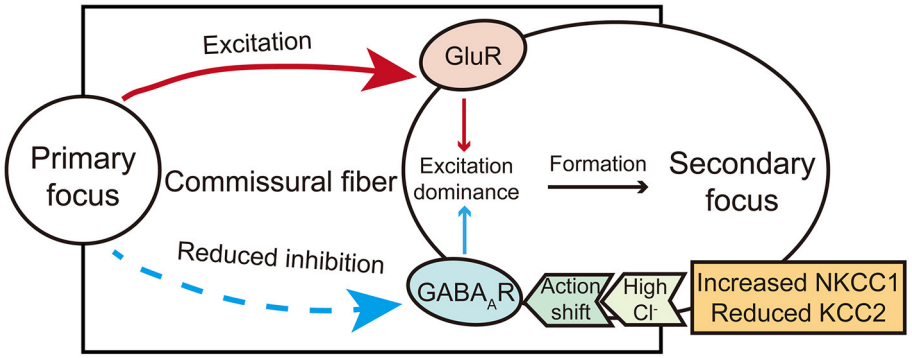

FIGURE 1 | A brief diagram illustrates the action of glutamate and GABA receptors in secondary epileptogenesis. The shift action of GABA from inhibitory to excitatory mediated by chloride co-transporters and excitation actions carried by glutamate commissural fibers co-mediate the formation of the secondary focus.

The Role of Inhibitory Neurotransmission

Besides excitatory glutamatergic synaptic transmission, inhibitory GABA synaptic transmission also plays a crucial role in maintaining the balance of excitation and inhibition in the central nervous system. Abnormalities in the GABAergic systems lead to declined inhibitory function in the brain, resulting in the dominance of excitation.

Federico et al. examined the role of GABA and glutamate receptor subtypes in the spread of epileptic activity. They confirmed that $GABA_A$ but not $GABA_B$ receptors were essential in seizure propagation (28). Perfusion of $GABA_A$ receptor antagonist bicuculline could lead to spontaneous seizure activity in the isolated guinea pig brain (29). The role of GABA receptors was further verified in a clinical study. Bortolato et al. reported a patient with neural damage in the right frontal lobe and a mirror focus in the contralateral hemisphere. After the resection of the primary focus, the density of $GABA_A$/benzodiazepine receptor binding in the left frontal lobe significantly increased (56). A recent study has further reported the role of inhibition defects in the formation of a secondary epileptic focus. The application of bicuculline enhanced contiguous seizure propagation and focal bicuculline microinjection into the regions distant to the 4-AP injection site leading to a secondary, non-synchronous epileptic discharge (67).

Additionally, excitatory GABA action induced by high chloride concentration contributed to seizure generation (68–70). After experiencing recurrent seizures, GABA transmitters directly depolarized neurons due to a persistent increase of extracellular chloride ions termed as a shift of GABA function from inhibitory to excitatory. This early elevated concentration of chloride ions is due to two chloride co-transporters: NKCC1, which imports the chloride ions, and KCC2, which extrudes them. In epileptic conditions, the changed expression of KCC2 and NKCC1 would occasionally influence the ionic homeostasis of chloride ions and contributes to secondary epileptogenesis (71, 72). For example, the GABA-acting ASD phenobarbital is a first-line drug to treat neonatal seizures. However, it would be less efficient after recurrent seizures because phenobarbital exacerbated the high intracellular chloride mediated by a combined action of NKCC1 and the downregulation of KCC2 in an established mirror focus (38). Moreover, although the NKCC1 antagonist bumetanide could not prevent the seizure propagation to the contralateral hippocampus and the formation of the mirror focus, it could block the spontaneous epileptiform activities and partly reduce the excitatory action of GABA in the isolated mirror focus (39). In conclusion, these results suggest the excitatory action mediated by chloride co-transporters can cause a longlasting shift in the depolarizing direction of the actions of GABA and ultimately induce secondary epileptogenesis.

The formation of the secondary focus is different from that of the primary one and is mainly dependent on synaptic transmission. According to the currently available evidence, the excitatory transmission may mediate the early stage of the secondary focus (dependent and intermediate phase proposed by Morrell). In contrast, the abnormality of inhibitory transmission may further mediate the consolidation stage of the secondary focus (the independent phase). Therefore, the role of these two kinds of synaptic transmission cannot be completely dissected (**Figure 1**), and further integrating studies are needed to dissect the mechanism of secondary epileptogenesis contributed by both excitatory and inhibitory synaptic transmission.

Other Molecules Participate in Secondary Epileptogenesis

In the central nervous system, besides the excitatory/inhibitory neurotransmission, which is also the main target of available ASDs, neurons directly connect the cytoplasms of adjacent cells through channels docking of two hemichannels called gap junction (73). It is a common direct pathway for intercellular communication between glial cells and neurons. Connexin 36 (Cx36) mediated gap junction communication had been certified to participate in epileptogenesis and emerge as a potential target for epilepsy (74). Gajda et al. investigated the role of Cx36 mediated gap junction communication in the maintenance and propagation of epileptic discharges in both the primary focus and the mirror focus. They reported that the Cx36 channels also promoted secondary epileptogenesis (35). The above function

partially mediated the formation of the mirror focus. They also demonstrated that the level of Cx36 mRNA was significantly increased after experiencing 25–30 spontaneous seizures (33).

On the other hand, cumulative studies demonstrated that epileptic pathogenesis might be associated with other factors which indirectly regulate neural excitability. Such as neurodegeneration, neurogenesis, and neuroinflammation, of which neuroinflammation gradually attracts researchers' attention (75–77). The inflammatory mediators play a significant role in the development of chronic spontaneous seizures (41). Early experiencing febrile seizures can induce the formation of secondary adult epileptogenesis in some cases, which might be mediated by neuroinflammation (78, 79). As Choi et al. reported, the level of HMGB1 showed a significant increase in the serum of patients with febrile seizures at first (80). It significantly contributed to the pathogenesis of hyperthermia-induced seizures and the following epileptogenesis. The application of HMGB1 further aggravated the acquired epilepsy after experiencing febrile seizures, which suggested that it played a vital role in acquired secondary epileptogenesis (81). One of the possible mechanisms may be that HMGB1 induces the expression of P-glycoprotein (P-gp) (82), which is directly related to drug resistance and epileptogenesis (83, 84).

In addition to neuroinflammatory cytokines, other molecules have also been reported to be involved in secondary epileptogenesis. Sulfated octapeptide of cholecystokinin (CCK-8) was a kind of neuropeptide that could increase the firing frequency of the action potentials in the neurons of the hippocampal CA1 (85). Moreover, the decreased calbindin staining might lead to decreased excitability and firing rate of pyramidal cells (86). The unilateral injection of KA into the dorsal hippocampus induced acute status epilepticus, followed by a latent phase with ipsilateral neuronal degeneration, which could generate epileptic seizures. Arabadzisz et al. investigated the expression and distribution of some specific neuromodulators in the hippocampus contralateral to the injection site in this process. The labelings of CCK-8 and calbindin were selectively decreased in the latent phase (34). The authors suggest that such changes in CCK-8 and calbindin expression may relate to the formation of epileptic seizures in the contralateral hippocampus.

Decades of studies have already revealed changes of some crucial molecules in secondary epileptogenesis. Nevertheless, the brain works as an interconnected network. The microscopic molecular mechanisms may not reflect the whole dynamic processes in secondary epileptogenesis. Macroscopical perspectives should be taken into account in different experimental designs.

CELLULAR MECHANISMS

Synaptic Plasticity in Secondary Epileptogenesis

In addition to molecular mechanisms, changes in synaptic function were also related to secondary epileptogenesis. The concept "seizures beget seizures" has been widely known for years. Moreover, cumulative studies have proved that repetitive seizures lead to more severe chronic epilepsy (87). In that process, synaptic plasticity certainly plays a key role. The long-term potentiation and depotentiation (LTP and LTD) of synaptic transmission are forms of long-lasting synaptic plasticity in the mammalian brain. During the process of LTP, synaptic strength gradually increases with the repetitive excitatory stimulation, while the situation is inverse in LTD. Both LTP and LTD mediate diverse forms of experience-dependent plasticity, including learning and memory, emotional feelings, and epilepsy (88). The phenomenon of LTP was first found in dentate granule excitatory neurons, which was essential in the stabilization and elimination of synapses during the development and adjustment of neural circuits (89). It is reasonable that the genesis of the secondary epileptic focus is associated with LTP due to that the process of these two phenomena is very similar-both of the two processes require repeated stimulation and reinforcement (90). An electrophysiological study performed by Beldhuis et al. analyzed bilateral hemispheres epileptiform activities on the amygdala kindling rats. The analysis of the linear and non-linear association functions showed that the connection between the two amygdalas was strengthened after daily kindling, and the excitability of the contralateral amygdala increased along with the kindling process (25). Further immunohistochemical studies illustrated that the activation of the neocortical areas contralateral to the primary focus was the result of synaptic connections, repeatedly strengthened synapses could lead to the spread of seizures (31, 55).

Given that the changes in synaptic plasticity are closely related to drug addiction (91). Kirkby et al. provided direct evidence about the role of enhanced synaptic plasticity caused by drug addiction in the secondary kindling process. They used amphetamine as a pretreatment agent to induce addiction in rats, thereby enhancing synaptic plasticity in the brain. Then the relationship between amphetamine pretreatment and the rate of kindling acquisition was studied. They demonstrated that the amphetamine pretreatment would lead to a much faster procedure of the kindling process in the secondary but not the primary epileptic focus (24).

Synaptic plasticity changes eventually lead to the remodeling of neural circuits and can be considered the connection from macroscopic brain network to microscopic synaptic function and transmitters, which provides another perspective for studying secondary epileptogenesis.

Other Cellular Changes

Other alternative mechanisms for secondary epileptogenesis which were occasionally reported include selective loss of interneurons, formation of excitatory synapses, etc. (87). Also, both mossy fibers sprouting and astrogliosis are reported as biomarkers of aberrant excitatory synaptogenesis, and they were observed in the unilateral KA model and electrical stimulation model (92). Meanwhile, postepileptic lesions showed changes in neuronal density, reactive astrogliosis, and sclerosis of critical structures that might cause secondary epileptogenesis. A histopathological study showed that most patients with TLE would have the characteristics of severe neuronal loss in the amygdala (93). However, the relatively small sample size of these

studies limits the utility of these findings, and further systematic studies are required.

Circuitry Views for Secondary Epileptogenesis

With significant developments in neuroscientific experimental techniques such as optogenetics, trans-synaptic viral tracing, and large-scale single-unit recordings, epilepsy is gradually considered a circuitry disease caused by the formation of abnormal brain networks (94, 95). The study of the time sequence of seizure initiation had demonstrated that the seizures were not just synchronized events in a single region but involved many crucial circuits originating from seizure focus to its downstream regions. The formation of abnormal excitatory circuits usually leads to the generation of seizures. For example, our previous studies have demonstrated that the microcircuit in the subiculum gates the genesis of the generalized seizures and further pharmacoresistance in TLE (68, 96, 97). It can be deduced that the heterogeneity of seizure spread sequence and excitation amplitudes may further lead to the formation of the secondary focus.

After reviewing decades of researches on secondary epileptogenesis, a conclusion can be drawn that the secondary focus was most likely to occur at the homotopic area of the contralateral hemisphere. Because the two hemispheres of the brain, especially in the temporal lobe regions, have the densest neural projection. Our previous study demonstrated that the unilateral kindled amygdala could promote the process of kindling acquisition in the contralateral amygdala (42). Also, it has long been deemed that the secondary epileptic focus can occur not only in the contralateral homotopic areas but also in the other regions of the ipsilateral hemisphere. The independent epileptic discharge could be detected in both the amygdala and the globus pallidus in an epileptic model whose seizures originate from the hippocampus (98). In unilateral amygdaloid overkindled cats, seizures could originate from both the amygdala and the ipsilateral frontal cortex (26). These studies suggest that the secondary epileptic foci do not form in isolation. Generalization of epileptic excitability is usually accompanied by the evolution of epileptic circuits.

Neuroimaging is a valuable tool to visualize the microstructural changes of epileptic circuits. Moreover, isotopic indicators in positron emission tomography (PET) have become one of the most commonly used methods to estimate glucose utilization of a particular nucleus. Handforth et al. compared the behavioral severity with autoradiography anatomic patterns in amygdala-induced status epilepticus. The spread of seizure activities from the amygdala to other limbic and non-limbic structures existed long before the appearance of motor seizures. This network first recruited the direct amygdala projection areas, then the contralateral structures (99). As Bankstahl et al. reported, by applying a novel PET protocol targeting the overactivity of P-gp, seizure-induced regional changes in P-gp activity can be identified (100). The overexpression of P-gp is closely related to epileptogenesis, by which method, the potential secondary epileptic focus can be detected. Diffusion tensor imaging (DTI) changes in patients with TLE were evaluated, and evidence for the microstructural changes of the hippocampus was also provided. Less-robust abnormalities of DTI suggested the secondary involvement of the thalamus in epilepsy. This structure was recruited into the hippocampal epileptic network (54). Additionally, Pustina et al. compared the role of three interhemispheric white matter pathways in generating contralateral epileptiform spikes during interictal activity. Diffusion tensor imaging was used to measure the integrity of those pathways in the temporal lobes: the tapetum, the anterior commissure, and the body of the fornix. These data suggested that the tapetum pathway could cause the emergence of contralateral spikes, and it was not due to the containment of callosal fibers (101).

Growing evidence suggests that understanding the mechanisms in neurological functions and diseases, especially in epilepsy, cannot be focused solely on the microscopic molecular level. Macroscopic circuitry views provide a more accurate and dynamic mechanism perspective. Although a few studies have already suggested that the formation of the secondary focus is related to the circuits, systematic studies are still needed to elucidate the circuitry mechanism of secondary epileptogenesis (**Figure 2**).

AVAILABLE AND POTENTIAL TREATMENTS FOR SECONDARY EPILEPTOGENESIS

Epilepsy Surgery

In the early 1960s, the development of neurosurgical intervention was just getting started. At that period, ASDs and surgical resection were the main available treatments for epilepsy. For patients with bilateral epileptic foci, neither medication nor surgery can achieve a satisfactory curative effect. However, given that corpus callosum was reported to play a critical role in bilateralization and symmetrization of seizures (30). Some clinicians would choose corpus callosotomy for patients with intractable epilepsy. The curative effect of corpus callosotomy on secondary epileptogenesis was initially demonstrated in animal models. The effect of corpus callosotomy was firstly demonstrated in a motor cortical kindling model. Kudo et al. divided twelve cats into two groups, five with the corpus callosotomy. The corpus callosotomy could significantly delay the seizure progression from focal to generalized convulsive seizures and decline the transfer effect of epileptic seizures (30). Callosotomy was mainly chosen for those patients with complex focal seizures or Lennox-Gastaut syndrome (102). Ono et al. reported that 63.2% of the patients with bilateral discharges showed desirable outcomes after callosotomy for intractable epilepsy. While after correlating postoperative outcomes with EEG data, it turned out that the patients with lateralized seizure discharges often had superior effects compared with those who had bilateral discharges (103).

However, besides the therapeutic effects, side effects should not be ignored. The split brain syndrome as a side effect of callosotomy had been reported due to brain

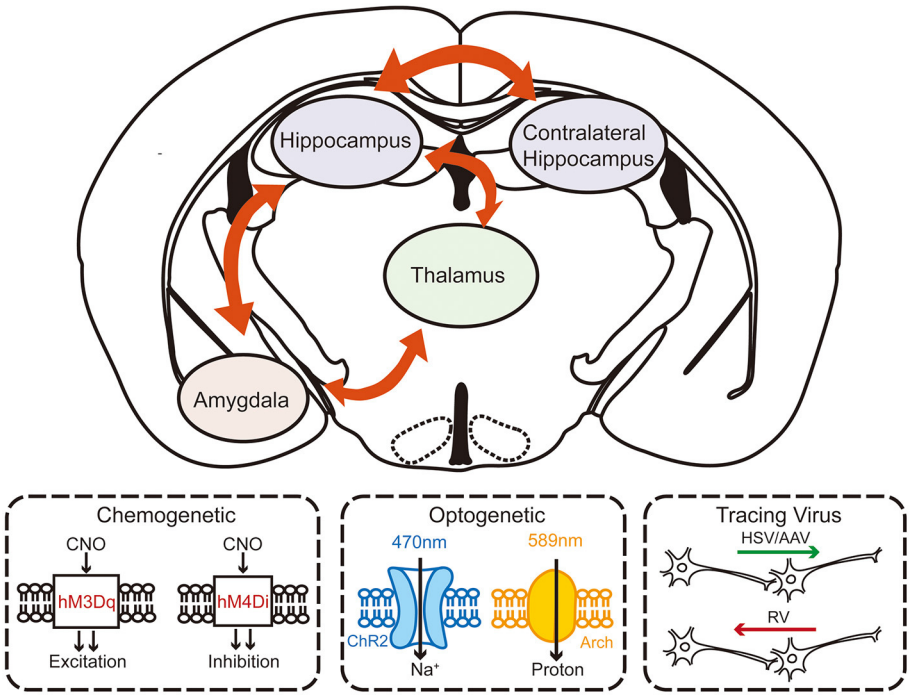

FIGURE 2 | The schematic diagram of investigating the circuity mechanism of secondary epileptogenesis in temporal lobe epilepsy by using advanced experimental approaches. Combined multifaceted techniques including chemogenetics, optogenetics, and viral tracing can help to reveal the possible circuitry basis of secondary epileptogenesis.

asymmetry. Dyssynchrony of consciousness in the two hemispheres can be observed and further affects the self-care abilities of patients. Sometimes, callosotomy can even ameliorate both generalized seizure and status epilepticus (102). Thus, the callosotomy must be performed with special caution and regarded as the last choice when no alternative options exist.

Pharmacotherapy

Available ASDs

Most of the first and second generation ASDs like phenytoin, valproic acid, and benzodiazepines are used to control seizures in patients with or without secondary focus. Those ASDs act on diverse molecular targets, including voltage-gated sodium/calcium channels and $GABA_A$ receptors. Taken that almost no available ASDs can interfere the epileptogenesis (3), the ineffectiveness of these ASDs on secondary epileptogenesis is imaginable.

However, some third-generation drugs act differently. Levetiracetam (LEV) is a pyrrolidone derivative which can be used as both anticonvulsant and antiepileptogenic medication. It does not target postsynaptic receptors or membrane ion channels but acts by combining with the component factor of the synaptic vesicle (SV2A) and further blocks the transmission of excitatory neurotransmission (104). The antiepileptogenic effect of LEV was firstly demonstrated on animal models and can persist after drug withdrawal (105). Yang et al. demonstrated that early administration of LEV could prevent posttraumatic epileptogenesis both *in vivo* and *in vitro*. It also significantly raised the stimulus intensity required to trigger epileptiform bursts (106). Furthermore, combined use of LEV and topiramate could also significantly retard the epileptogenesis in rats after pilocarpine-induced status epilepticus (107). Although direct evidence was still lacking, it can be deduced that LEV might also interfere with the genesis of secondary epileptogenesis.

To sum up, the complete abolishment of secondary epileptogenesis by current ASDs is still not evident. Available ASDs, which mainly target ion channels and GABA receptors, turn out to be invalid for epileptogenesis. Future drug designs should focus on molecules and mechanisms closely related to secondary epileptogenesis.

Future Potential Medications

Novel mechanisms findings of secondary epileptogenesis would, in turn, provide potential therapeutic targets for it. For example, both preclinical and clinical evidence has highlighted the importance of neural inflammation on epileptogenesis in recent years. There is positive feedback between the pro-inflammatory factors and the epileptic activities. The biosynthesis of inflammatory cytokines and prostaglandins will be activated after epileptic stimuli and, in turn, enhance the epileptic excitability (108). Thus, the inflammatory inhibitors may have potential antiepileptic effects. We previously reported that targeting the caspase-1-interleukin-1β inflammatory pathway

could reduce neuronal excitability and suppress secondary epileptic susceptibility caused by febrile seizures (79, 109). Besides, as mentioned above, the inhibitor of HMGB1 may also have the potential to treat secondary epileptogenesis (81). Additionally, the phenomenon of LTP is based on the function of AMPA receptors and NMDA receptors. (88). Consequently, the AMPA and NMDA receptor antagonist application delays the enhancement of synaptic connection and may prevent the formation of the secondary focus.

Aside from approaches targeted on the molecules closely related to secondary epileptogenesis, directly retarding the primary epileptogenesis may also be helpful (110). New directions of ASDs development should address both the primary and secondary epileptogenesis rather than merely seizure control. With the development of pharmacogenomics and the discovery of accurate biomarkers, precise individualized therapy for secondary epileptogenesis will be possible and could certainly help those patients.

Other Treatment Options

Neurostimulations, including deep brain stimulation, vagus nerve stimulation, and transcranial magnetic stimulation, are effective for neurological diseases (1). Among these, deep brain stimulation has gradually evolved as an effective alternative treatment in epilepsy, with the advantages of reversibility and controllability. For secondary epileptogenesis, our previous research firstly reported that low frequency stimulation (LFS, 1 Hz) at the primary focus could significantly retard the secondary kindling acquisition of the mirror focus (**Figure 3**). Then we further specified the time window of LFS for secondary epileptogenesis treatment. The LFS would have a better effect before developing into a generalized seizure (42). Similarly, Couturier et al. determined the relative efficacy of different protocols of brain stimulation for secondary epileptogenesis. By comparing the antiepileptic effects of LFS on the corpus callosum and high frequency stimulation (HFS) at both primary focus and anterior nucleus, a conclusion can be drawn that the LFS at the corpus callosum can significantly reduce the seizure frequency of both primary and secondary focus (111). These results provided direct evidence to confirm the promising therapeutic effect of LFS for secondary epileptogenesis.

Recently, the brain-responsive neurostimulator (RNS System, NeuroPace Inc.) has been approved by the FDA as an adjunct treatment for refractory epilepsy, including patients who had more than one epileptic foci (112). In a case report, by implanting an RNS System into a patient whose left temporal seizure focus overlapped with language areas which led to the residual of epileptic structures after surgery, Geller et al. reported that this adjunct treatment achieved a desirable curative effect in that patient (113). Transcranial focal stimulation (TFS), a noninvasive neuromodulation strategy, has been shown to reduce seizure activities and avoid P-gp overexpression in different experimental models. According to these, it is indicated that TFS may also represent a new neuromodulatory strategy to revert secondary epileptogenesis (114).

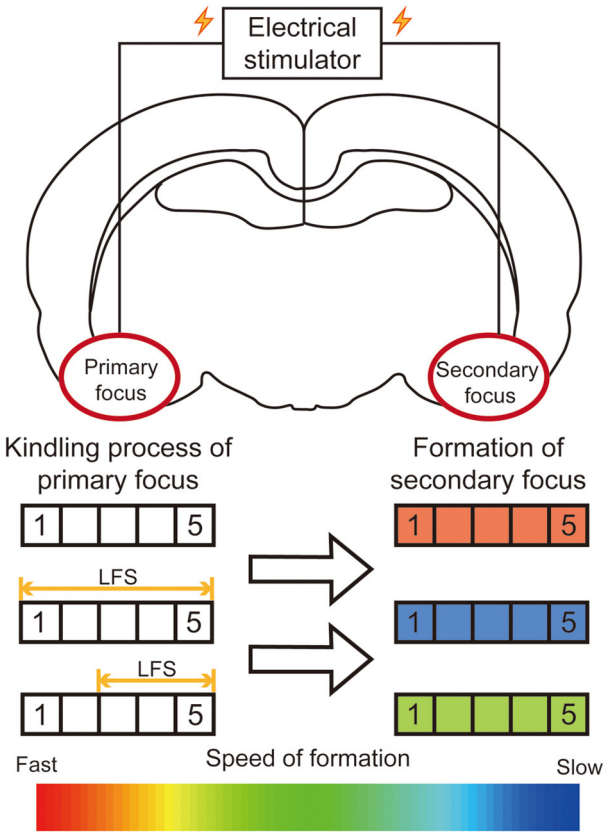

FIGURE 3 | Summary diagram of efficacy of LFS on the primary focus for secondary epileptogenesis. In the kindling model of secondary epileptogenesis, bilateral amygdalae were kindled successively. The application of LFS at the primary focus significantly retards the epileptogenesis of the secondary focus. The rectangle on the left represents the stage to which the primary focus was kindled. The color of the rectangle on the right represents the relative speed of the formation of the secondary focus.

Despite the promising results, neuromodulation is limited by its invasive nature (associated with device implantation) and battery-related problems. Future studies should focus on the crucial brain regions involved in secondary epileptogenesis and develop more biocompatible and continuable devices.

CONCLUSION AND FUTURE PROSPECTIVES

To sum up, secondary epileptogenesis is a longlasting issue that remains unsolved in epilepsy. Decades of clinical and experimental evidence have confirmed its existence and gradually revealed the possible mechanisms ranging from the molecular to the circuitry level. Both excessive activation of excitatory receptors and reduced inhibition of GABA receptors eventually lead to the formation of

the secondary epileptic focus. Other molecules such as HMGB1, caspase-1, and CCK-8 may also contribute to this process. Currently, surgery seems to be the optimal option for secondary epileptogenesis. However, both neuroinflammation inhibitors and DBS show great potential in retarding secondary epileptogenesis. More importantly, with the development of optogenetics and chemogenetics, treatments targeting crucial circuits show great potential in interfering with secondary epileptogenesis. The combination of new neurobiological techniques can bring new insights to illustrate the mechanism of this longlasting problem and novel therapeutic approaches as well.

AUTHOR CONTRIBUTIONS

YS wrote the manuscript. YG, YR, CX, and ZC edited the manuscript. All authors contributed to the article and approved the submitted version.

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VEGF Modulates Neurogenesis and Microvascular Remodeling in Epileptogenesis After Status Epilepticus in Immature Rats

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Neurogenesis and angiogenesis are widely recognized to occur during epileptogenesis and important in brain development. Because vascular endothelial growth factor (VEGF) is a critical neurovascular target in neurological diseases, its effect on neurogenesis, microvascular remodeling and epileptogenesis in the immature brain after lithium-pilocarpine-induced status epilepticus (SE) was investigated. The dynamic changes in and the correlation between hippocampal neurogenesis and microvascular remodeling after SE and the influence of VEGF or SU5416 injection into the lateral ventricles at different stages after SE on neurogenesis and microvascular remodeling through regulation of VEGF expression were assessed by immunofluorescence and immunohistochemistry. Western blot analysis revealed that the VEGFR2 signaling pathway promotes phosphorylated ERK and phosphorylated AKT expression. The effects of VEGF expression regulation at different stages after SE on pathological changes in hippocampal structure and spontaneous recurrent seizures (SRS) were evaluated by Nissl staining and electroencephalography (EEG). The results showed that hippocampal neurogenesis after SE is related to microvascular regeneration. VEGF promotion in the acute period and inhibition in the latent period after SE alleviates loss of hippocampal neuron, abnormal vascular regeneration and inhibits neural stem cells (NSCs) ectopic migration, which may effectively alleviate SRS severity. Interfering with VEGF via the AKT and ERK pathways in different phases after SE may be a promising strategy for treating and preventing epilepsy in children.

Keywords: VEGF, neurogenesis, microvascular remodeling, epileptogenesis, SE

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INTRODUCTION

Convulsive disorders are the most common emergency in pediatric neurology. The incidence of convulsions is higher in children than in adults (1), and children are more prone to severe convulsions and status epilepticus (SE), which is associated with more serious long-term nervous system sequelae such as epilepsy, cognitive impairment, and emotional disorders and thus greatly affects the quality of life of children (2–4). However, at present, the specific

mechanism of epileptogenesis after SE is not fully understood, and there is no effective method for repairing convulsion-induced brain damage or effectively preventing epilepsy.

Epileptogenesis is a pathological process characterized by spontaneous recurrent seizures (SRS) that gradually emerge after SE. The progression from acute convulsive brain injury to epileptogenesis can be divided into the acute phase, latent phase and chronic phase according to the different physiological and pathological changes that occur in the brain and the occurrence of SRS. In animal model, 0–3 days after SE were identified acute period, 3–7 days after SE were identified the latency period and approximately after 7–10 days was chronic early stage. SRS appears from chronic early stage. Of course, the onset and duration of such changes are approximated (2). Studies have found that a series of molecular pathological changes occur in the brain during the latent phase (5) and that SE-induced brain damage in the immature brain is different from that in the mature brain. It may be possible to prevent epileptogenesis by identifying important targets related to the mechanism of epilepsy in the immature brain.

Previous research has widely recognized that SE increases neurogenesis both in animal models of epilepsy and human temporal lobe epilepsy (TLE) (6, 7). It is known that the immature brain has a greater regenerative capacity than the mature brain and SE-induced endogenous neurogenesis may compensate for hippocampal neuronal loss. However, our previous study revealed that hippocampal neurons in the immature brain undergo excessive apoptosis after SE and that endogenous neural stem cells (NSCs) in the subgranular zone (SGZ) of the hippocampal dentate gyrus (DG) exhibit a proliferative response and abnormal migration as in the mature brain (8). The abnormal migration of newborn granule cells leads to brain development disorders and increased susceptibility to convulsions through forming functional excitatory synapses in the molecular layer of the DG (MoDG) and excitatory recurrent circuits (9). SE-induced neurogenesis is a complicated process and may be regulated by numerous other factors. The microenvironment of NSCs is closely related to the proliferation, migration, differentiation, and survival of NSCs. Microvessels are some of the most important components of the NSCs microenvironment. In addition to providing nutritional support to NSCs, blood vessels affect the migration of NSCs in normal brains. Some studies have found that microvascular injury is characterized by blood-brain barrier (BBB) destruction and excessive angiogenesis after SE (10–14). Thus, we hypothesized that pathological angiogenesis after SE might affect the migration and integration of NSCs and thus cause neurons to migrate ectopically and glial cells to establish abnormal synaptic connections, form a pathological neural network, promote abnormal excitation of neurons, and participate in epileptogenesis (15). An increasing number of studies have confirmed that continuous proliferation of hippocampal NSCs and microvascular remodeling in pathological changes are involved in the pathogenesis of epilepsy, but the specific mechanism is still unclear (16–18).

Vascular endothelial growth factor (VEGF), as an important common factor between the nervous system and the vascular system, is a promising neurovascular molecular target (19); however, research on its role in epilepsy is limited. VEGF is a dimeric glycoprotein that was first isolated and purified from cultured bovine pituitary astrocytes in 1989 by Ferrara et al. (20). Its molecular weight is 34–45 kDa. VEGF is secreted by neural tube cells in the developing brain, and it mainly binds to its receptor to perform its biological functions. The members of the VEGF family that have been discovered thus far include VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E, and placental growth factor. VEGF commonly refers to VEGF-A. The known VEGF receptors (VEGFRs) are VEGFR1 (Flt1), VEGFR2 (Flk-1), VEGFR3 (Flt-4), NP-1 and NP2 (21–23). The localization and functions of different VEGFRs are distinct. VEGF-A, which plays an important role in angiogenesis, endothelial cell proliferation and migration and the proliferation of NSCs, mainly exerts its biological functions by binding to VEGFR2 in the nervous system (24). Because VEGF has dual effects on blood vessels and nerves in the nervous system, some researchers believe that VEGF acts as a double-edged sword in epilepsy. It promotes the proliferation of blood vessels and contributes to the development of epilepsy. On the other hand, it inhibits hippocampal cell apoptosis and promotes the proliferation of NSCs, the survival of neurons, and the repair of damaged nerves (25–27). Previous research revealed that the dynamic expression of VEGF and VEGFR2 fluctuates during the development of epilepsy after SE, which is consistent with the trend in SRS (8, 28). VEGF can promote the regeneration of hippocampal NSCs in the acute phase after SE (8), suggesting that it may be involved in epileptogenesis. Does VEGF, in addition to promoting the proliferation of NSCs after SE, affect the regeneration of microvessels in the brain? Does VEGF have distinct effects in different stages of epilepsy development after SE? Does the temporal regulation of VEGF expression and receptor signaling pathways after SE have different effects and influence the development of epilepsy?

Since the proliferation of hippocampal NSCs after SE is not synchronized with vascular remodeling, we speculated that VEGF expression is regulated differently during different time windows after SE. Therefore, based on our previous studies, we performed the present study to investigate the critical role of VEGF and VEGFR-2 inhibitor SU5416 expression regulation and the time-dependent effect of the VEGF on neurogenesis and microvascular remodeling in the immature brain after SE to explore the role of VEGF in the development of epilepsy and to provide new ideas for the prevention and treatment of epilepsy.

MATERIALS AND METHODS

Animals

Healthy male specific pathogen-free (SPF) Sprague-Dawley (SD) rats aged 18 and 20 days were provided by the Experimental Animal Center of Chongqing Medical University. All animal experiments were performed in accordance with protocols approved by the Animal Care Committee of Chongqing Medical University, Chongqing, China. The animals were housed in a controlled environment at a humidity of 60% and a temperature of 21 ± 1°C on a 12 h light/dark cycle (lights on from 7:00 AM−7:00 PM) and provided food and water ad libitum.

Lithium Chloride-Pilocarpine-Induced Rat Model of SE

SE was induced in 20-d-old male SD rats. Seizures were scored as stages 1–5 based on Racine's five-point seizure scale (29). SE was induced *via* intraperitoneal (i.p.) injection of pilocarpine (30 mg/kg, Sigma) 18–20 h after lithium chloride injection (127 mg/kg, i.p., Solarbio, Beijing, China). Atropine (Shuanghe, Beijing, China) was administered via i.p. injection (1 mg/kg) 30 min after the onset of seizure. Sixty min after SE, all rats received a single dose of diazepam (10 mg/kg, i.p.). Rats that did not display SE and those that died during SE were excluded. Control rats were treated with the same volumes of saline.

The study was divided into two parts. In the first part, thirty male postnatal day 20 rats were randomly divided into the SE ($n = 15$) and control groups ($n = 15$). Pilocarpine was administered in accordance with the protocol described above. The rats were sacrificed by decapitation under deep anesthesia with 10% chloral hydrate (3 ml/kg, i.p.) at various time points (7, 14 or 28 d after SE, $n = 5$ in each group at each time point). In the second part, ninety-five postnatal day 18 rats were randomly divided into the control group ($n = 13$), the SE group ($n = 82$), the acute-phase SE VEGF intervention (SV0) group (injection of VEGF into the lateral ventricle from 2 h to 2 d after SE), the acute-phase SE SU5416 intervention (SU0) group (injection of SU5416, a VEGFR2 inhibitor, into the lateral ventricle from 2 h to 2 d after SE), the latent-phase SE VEGF intervention (SV5) group (injection of VEGF into the lateral ventricle from 5 d to 7 d after SE), and the latent-phase SE SU5416 intervention (SU5) group (injection of SU5416 into the lateral ventricle from 5 d to 7 d after SE). A catheter was implanted into the lateral ventricle on the 18th day after birth, and SE was induced 21 d after birth. The model rats were administered VEGF or SU5416 via the catheter inserted into the lateral ventricle from 2 h or 5 d after SE induction. The rats were sacrificed 2, 7, or 28 d after SE ($n \geq 14$ in each group). The experimental procedure is presented in **Figure 1**.

Pump Implantation and Intracerebroventricular Drug Injection

The rats were anesthetized via ether inhalation and placed in a stereotactic frame. The anterior fontanelle was used as the origin. A Hamilton syringe was placed in the right lateral cerebral ventricle at predetermined coordinates: 1.2 mm posterior to and 1.2 mm lateral from lambda and a depth of 3.8 mm. A drill was used to create a small hole in the skull, a catheter was inserted (Roanoke Corporation, USA) into the lateral ventricle and fixed with glass ionomer cement, and a tube was inserted into the catheter to seal it after the cement solidified. After the operation was completed, the rats were placed in cages and kept warm. After the operation, an i.p. injection of 300,000 units/kg penicillin was administered to prevent infection.

Rats with SE were randomly divided into the SV0, SU0, SV5, and SU5 groups and received injection of VEGF 165 40 ng [8 ng/μl, dissolved in phosphate-buffered saline (PBS),Peprotech, Rocky Hill, USA] or SU5416 5 mM [1 mM/μl, dissolved in dimethylsulfoxide (DMSO), Selleck, USA] per rat for 3 continuous days (2 h, 1 d, and 2 d or 5 d, 6 d, and 7 d after SE) at a rate of 0.5 μl/min. After injection, the microsyringe was kept in place for 5 min until the liquid was fully diffused. Then, the injection needle was removed, and the cap was closed.

Immunofluorescence and Immunohistochemistry

Anesthetized rats were perfused through the left ventricle with 4% paraformaldehyde. Hippocampal coronal slices were cut into consecutive sections at a thickness of 40 μm and immediately placed in cryoprotectant solution [150 g of $C_{12}H_{22}O_{11}$ and 150 ml of $(CH_2OH)_2$ dissolved in 500 ml of PBS] at 4°C until immunofluorescence was performed. The following primary antibodies were used: anti-VEGF (rabbit polyclonal antibody, Abcam, 1:200), anti-VEGFR2 (rabbit polyclonal antibody, Abcam, 1:200), anti-Dcx (rabbit polyclonal antibody, Abcam, 1:200), anti-CD31 (mouse monoclonal antibody, Abcam, 1:200), and anti-polysialic acid-neural cell adhesion molecule (PSA-NCAM) (mouse monoclonal antibody, Millipore, 1:80). Nis-element BR3.2 software was used to capture images of the hippocampal DG area and blindly analyzed the average fluorescence intensity from 2 sections per animal ($n = 5$ for each group) selected randomly.

Brain tissues were embedded in paraffin after dehydration, and the paraffin sections were cut coronally at a thickness of 4 μm. The sections were soaked in xylene for at least 1 h for dewaxing and then blocked in 5% BSA. A CD31 primary antibody (mouse monoclonal antibody, Abcam, 1:200) was used, DAB was used for color development (Zhongshan, China), and the sections were counterstained with hematoxylin. The slices were imaged under a microscope with an oil immersion lens. Vessels with a diameter of <10 μm were considered microvessels. The microvessel density (MVD) in the hilar area of the DG was calculated with NIS software according to the following formula: MVD = number of microvessels (N)/area of interest (mm^2). The averaged MVD from 4 sections per animal ($n = 5$ for each group) selected randomly was assessed in each group.

Western Blot Analysis

The hippocampus of each rat was rapidly dissected and stored in liquid nitrogen immediately after dissection. Protein concentrations were determined using a Bio-Rad protein assay kit (Bio-Rad Laboratories, USA). An equal amount (30 μg) of each sample was loaded on 6% and 10% SDS polyacrylamide gels. The proteins were separated by electrophoresis and then transferred to polyvinylidene difluoride membranes (0.22 μm, Millipore Corp, Billerica, MA, USA). The membranes were blocked with PBS containing 10% milk for 1 h and then incubated with the following specific primary antibodies overnight at 4°C: anti-VEGFR2 (rabbit polyclonal antibody, Abcam, 1:1000), anti-AKT (rabbit monoclonal antibody, Abcam, 1:2000), anti-phospho-akt (rabbit monoclonal antibody, Cell Signaling Technology, 1:2000), anti-ERK (rabbit monoclonal antibody, Cell Signaling Technology, 1:2000), and anti-phospho-erk (rabbit monoclonal antibody, Cell Signaling Technology, 1:2000). In parallel, the Western blots were probed with an anti-β-actin antibody (mouse monoclonal antibody, 1:500, Boster) for

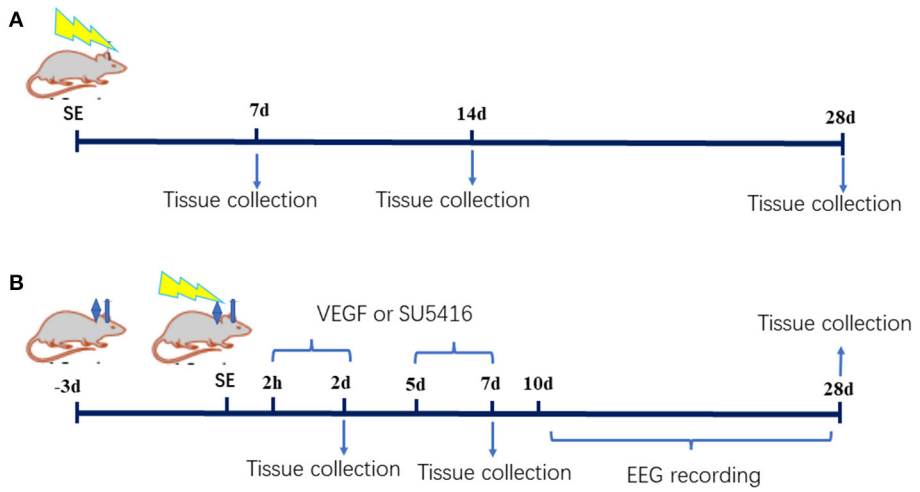

FIGURE 1 | Animal experiment procedure. **(A)** The first part **(B)** the second part.

data normalization. The proteins were visualized using Clarity Western Electrochemiluminescence (ECL) Substrate (Bio-Rad, USA), and the gray values were analyzed using a Syngene imaging system.

Nissl Staining

Samples were processed in the same way as described for immunohistochemistry, and paraffin sections were cut coronally at a thickness of 5 μm and subjected to Nissl staining. The sections were stained with cresyl violet for the subjective evaluation of hippocampal damage.

Electroencephalography

A 1.00-mm-diameter hole was made in the parietal skull of each rat ($n = 3$ for every group) on the left and right sides of the sagittal suture, and a hole was drilled above the telencephalon for grounding. Then, simple homemade electrodes with 0.1-mm nickel-chromium wires were inserted so that the wires touched the dura mater for scalp EEG (**Supplementary Figure 1**). After 3 days of recovery, SE was induced in rats to establish the SE model, and EEG was performed during modeling and then for 2 h per day until 28 days after SE with a radiophysiological signal detection system (Telemetry ReSCarch Limited, New Zealand). The rats in the other intervention groups were subjected to EEG for 2 h per day for 18 days starting after drug administration (10 days after SE). EEG was mainly used to record changes in SRS. An ADL instrument amplifier was used with the following parameters: low pass filter at 100 Hz, digitization rate of 2 kHz and amplification rate of 1,000 times. According to a previous study (24), seizures were defined as discharges with a frequency >5 Hz, an amplitude >2 times than baseline and a duration of 10 s. pClampfit software was used to record the average number of daily spontaneous seizures, identify the 10-min period with the most severe seizures, and analyze the frequency of seizures and the duration of each seizure in a 10-min period.

Statistical Analysis

All data are presented as the means ± SD and were analyzed with SPSS 17.0 statistical software. The data were analyzed using independent sample Student's *t*-tests or one-way analysis of variance (ANOVA) followed by the LSD *post hoc* test or Dunnett's T3 test. $p < 0.05$ was considered statistically significant.

RESULTS

SE Induction and EEG Recording

According to Racine's five category classification of seizures (29), SE was successfully induced in a total of 111 rats after pilocarpine administration, and the SE was interrupted after 60 min by injection with diazepam. Fourteen rats that failed to develop SE or died during SE were excluded. EEG recording was performed to detect epileptic discharge from SE to epileptogenesis (**Figure 7A**). The electroencephalograms of SD rats in the control group mainly consisted of α and β waves, with scattered θ waves and no SRS. Lithium chloride-pilocarpine induced SE in immature rats, and the electroencephalograms of these rats mainly showed persistent high-amplitude sharp waves and spike waves during convulsive states. However, in the latent phase after SE, electroencephalograms were almost normal, showing a few sharp waves and spikes. In the chronic phase, electroencephalograms mainly showed scattered sharp waves and spine waves lasting from a few seconds to tens of seconds, which represented spontaneous repeated epileptic discharges. The rats exhibited stage 2–4 epileptic seizures on the Racine scale, as unilateral or bilateral forelimb clonus was common during the seizures, and generalized convulsions were rare.

Relationship Between NSCs and Neovascularization

PSA-NCAM is a single-chain transmembrane glycoprotein that mediates cell-cell and cell-matrix adhesion and is a marker of

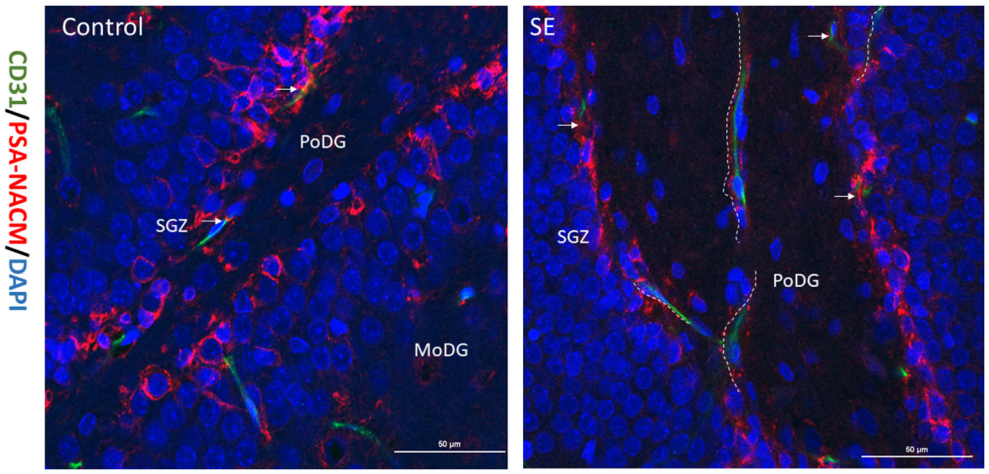

FIGURE 2 | Relationship between migrated NSCs and neovascularization. The expression of CD31 and PSA-NACM in the hippocampal DG at 7 days post SE in immature rats. PSA-NACM was stained prominently in the granule cells of SGZ (arrows) and is tributions of CD31 at the polymorphic layer of the DG (PoDG) or molecular layer of the DG (MoDG) in the hippocampus. PSA-NACM colocalized with CD31 expressed in hundreds of microns in the direction of PoDG after SE. It indicated that NSCs migrated hundreds of microns from the SGZ in the direction of new blood vessels. CD31, green; PSA-NACM, red; scale bar, 50 μm.

NSCs migration. Double labeling immunofluorescence for PSA-NACM (red fluorescence) and CD31 (green fluorescence) was used to assess the spatial relationship between hippocampal NSCs and blood vessels in the latent period after SE. As shown in the merged image in **Figure 2**, CD31 colocalized with PSA-NACM are concentrated in the SGZ and arranged in chains along CD31 after SE. It indicates that NSCs along with newly blood vessel gradually migrating into the granule cell layer (GCL) even arrived at molecular layer of the DG (MoDG) or the polymorphic layer of the DG (PoDG) from SGZ after SE.

Effect of Differential Regulation of VEGF During Different Time Periods on Neurogenesis During Epileptogenesis After SE

Doublecortin (Dcx) is a specific marker of neuronal precursor cells that can act on tubulin to polymerize microtubules, stabilize the cytoskeleton, and directly impact the initiation of neuronal migration. Immunofluorescence was conducted to assess the dynamic changes in Dcx expression from the latent period to the chronic period of epileptogenesis after SE (**Figure 3A**). Dcx (green fluorescence) was expressed on the cell membrane in the shape of a flocculent ribbon or ring and was mainly expressed in the hippocampal SGZ in a chain arrangement. In the control group, almost all of the Dcx-positive cells in the hippocampus were distributed in the hippocampal SGZ. The fluorescence intensity of Dcx in the hippocampal DG area showed a decreasing trend from 28 to 49 days after birth, which was consistent with the process of brain development. Dcx expression also gradually decreased from 7 to 28 days after SE in the SE group, but the Dcx fluorescence intensity was higher in the SE group than in the control group at each time point, and the Dcx fluorescence intensity was significantly higher in the SE group than in the control group at 7 days after SE. The difference was statistically significant (control: 1,132.03 ± 106.10; SE: 1,328.69 ± 94.81, $P < 0.01$), as shown in **Figure 3B**. In addition, Dcx-positive cells were not confined to the SGZ in the SE group. Dcx-positive cells were distributed in the GCL of the DG at 7 days after SE and showed a tendency to migrate across the GCL to the PoDG. This finding suggests that NSCs that proliferate in the acute phase after SE tend to migrate during the latent period and that the latent period is critical for neurogenesis and the migration of NSCs (**Figure 3A**).

We further interfered with the expression of VEGF in the acute and latent phases after SE and evaluated the migration of NSCs (**Figure 3C**). ANOVA revealed a significant effect of VEGF at different time periods on DCX [$F_{(5,54)} = 50.746$, $P < 0.001$]. In the SV0 group, Dcx was still mainly located in the SGZ and GCL, but the fluorescence intensity of Dcx was significantly higher in the SV0 group than in the SE group, and the difference was statistically significant ($P < 0.001$). In the SV5 group, Dcx-positive cells were not confined to the SGZ and GCL, and many Dcx-positive cells were scattered in the PoDG and MoDG. The Dcx fluorescence intensity was significantly lower in the SV5 group than in the SV0 group ($P < 0.01$), but the difference in Dcx fluorescence intensity between the SV5 group and the SE group was not significant. However, in the SU0 and SU5 groups, Dcx-positive cells were mainly distributed in the SGZ, and the fluorescence intensity was much lower in the SU0 and SU5 groups than in the SE group (**Figures 3C,D**). These data suggest that promotion of VEGF expression in the acute phase after SE can significantly promote the proliferation of NSCs, that the promotion of VEGF expression during the latent phase after SE may lead to abnormal migration of NSCs. Becides, the inhibition of VEGF expression in the acute phase or the latent phase after SE may inhibit neurogenesis.

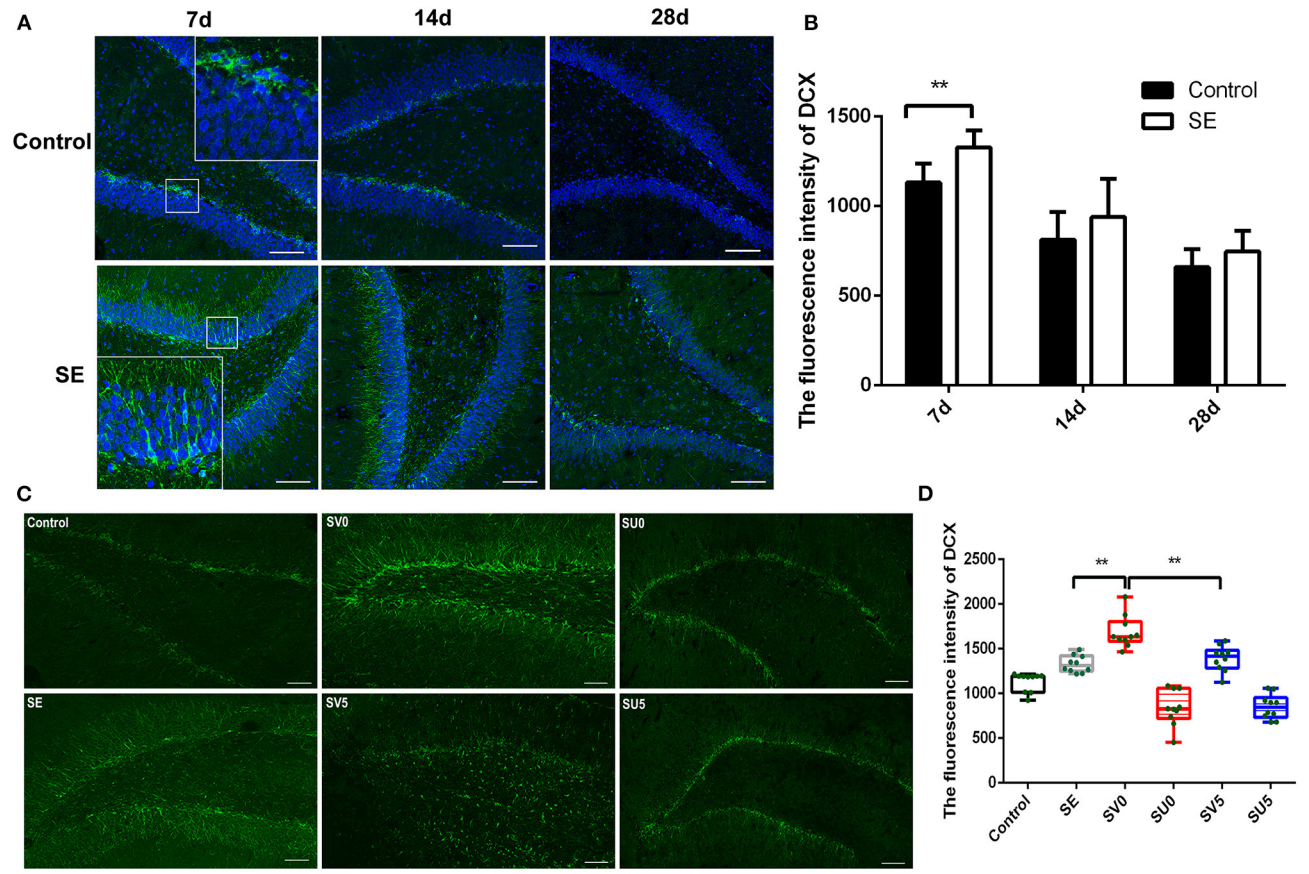

FIGURE 3 | The effect of VEGF on neurogenesis during epileptogenesis after SE **(A)** The immunofluorescence of DCX in the DG of the hippocampus after SE (Dcx: green; DAPI: blue; 200×, scale bar: 100 μm). **(B)** Dcx fluorescence intensity in the DG of the hippocampus after SE. The mean fluorescence intensity of DCX prominently increased at 7 days post SE compared to the control, $^{**}P < 0.01$. **(C)** Effect of VEGF-based interventions on neurogenesis in different phases after SE (SV, VEGF injection; SU, application of a VEGFR2 inhibitor. Dcx: green, scale bar: 100 μm). **(D)** Dcx fluorescence intensity in the VEGF intervention groups in different phases after SE. Values were mean ± SD, $^{**}P < 0.01$, $n = 5$ for each group.

Effect of Differential Regulation of VEGF During Different Time Periods on Microvascular Regeneration During Epileptogenesis After SE

Platelet endothelial cell adhesion molecule-1 (PECAM-1/CD31), which is present in the tight junctions of vascular endothelial cells, is a marker of neovascularization. Immunohistochemistry was used to detect the dynamic changes in CD31 expression from the latent phase to the chronic phase after SE. The results, which are shown in **Figure 4A**, showed changes in microvascular regeneration.

During the development of the immature brain (28 d-49 d after birth), the expression of CD31 in the hippocampus was not significantly correlated with age in the control group, while the expression of CD31 in the hippocampal DG gradually increased from 7 to 28 days after SE in the SE group. The microvessel density (MVD) in the hilar area of the DG was calculated with NIS software according to the following formula: MVD = number of microvessels (N)/area of interest (mm2). The MVD in the SE group was significantly higher than that in the control group at each time point, and the difference was statistically significant (Control-7d: 334.62 ± 154.46, SE-7d: 521.62 ± 156.75, $P < 0.01$; Control-14d: 339.54 ± 121.48, SE-14d: 684.01 ± 147.97, $P < 0.01$; Control-28d: 354.30 ± 86.86, SE-28d: 1033.39 ± 190.25, $P < 0.01$), as shown in **Figure 4B**. This result suggests that vascular proliferation occurs during the latent phase after SE, gradually increases during epileptogenesis, and peaks in the chronic phase after SE.

We further interfered with the expression of VEGF in the acute and latent phases after SE and investigated the changes in the density of new microvessels in the hippocampus in the chronic phase after SE (**Figures 4C,D**). ANOVA revealed a significant effect of VEGF at different time periods on CD31 and MVD [$F_{(3,76)} = 79.087$, $P < 0.001$]. However, the expression of CD31 and MVD in the hippocampus in the chronic phase after SE was not significantly different between the groups treated in acute-phase SE (the SV0 and SU0 groups) and the SE group. The expression of CD31 in the chronic phase after SE and the MVD were significantly higher in the SV5 group, in which

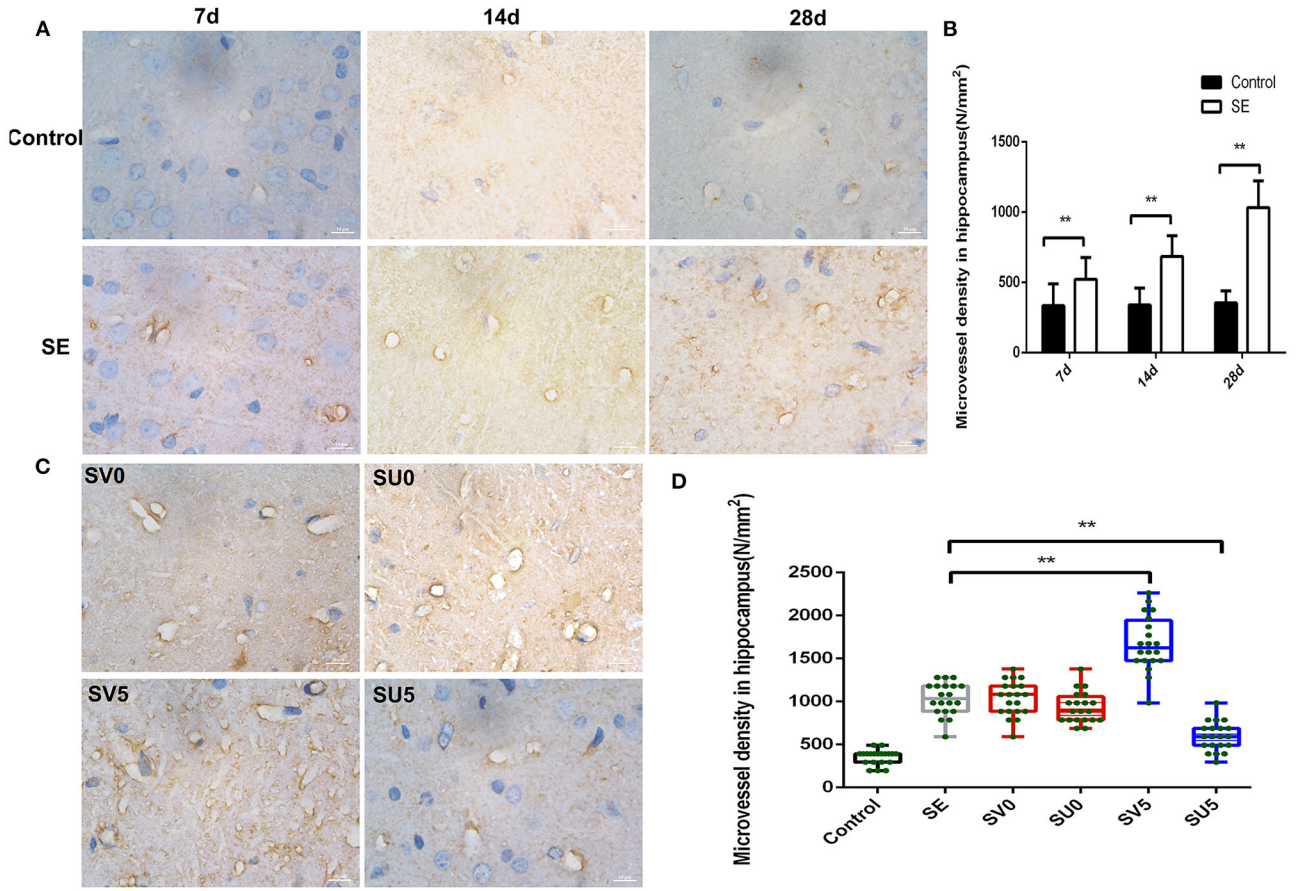

FIGURE 4 | Effect of VEGF on microvascular regeneration during epileptogenesis after SE. **(A)** Expression of CD31 in the hippocampus after SE (1,000 ×, scale bar: 10 μm). **(B)** MVD in the hippocampus after SE (N/mm2). The MVD in the SE group was significantly higher at each time point than that in the control group, $^{**}P < 0.01$. **(C)** Effect of VEGF intervention in different phases after SE on microvascular regeneration (1,000 ×, scale bar: 10 μm). **(D)** MVD in the hippocampus after VEGF intervention in different phases after SE. The MVD of SV5 was much higher than that of the SE group; however, the MVD of SU5 was much lower than that of the SE group. $^{**}P < 0.01$, $n = 5$ for each group. SV, VEGF injection; SU, application of a VEGFR2 inhibitor.

intervention occurred in the latent phase after SE, than in the SE group. The difference was statistically significant ($P < 0.01$). This finding suggests that the best time window for regulating microvessel regeneration after SE is the latent phase. Inhibition of VEGF expression in the latent phase after SE can inhibit the regeneration of microvessels after SE.

Molecular Mechanism of VEGF Regulation in Different Phases After SE

Western blotting was used to determine the activation status of VEGFR2, phospho-protein kinase B (P-akt), and phospho-extracellular signal-regulated kinase (P-erk) to study the influence of VEGF regulation in different stages after SE on its downstream signaling pathways. The expression levels of kinase B (AKT) and extracellular signal-regulated kinase (ERK), which are downstream of the VEGFR2 protein, were significantly higher in the SE group than in the control group. The protein expression of VEGFR2 in the hippocampus was upregulated in the SV0 group and SV5 group compared with the SE group [$F_{(3,20)} = 29.801$, $P < 0.001$], and the expression levels of the downstream proteins P-akt [$F_{(3,20)} = 6.270$, $P = 0.004$] and P-erk were increased to varying degrees [$F_{(3,20)} = 6.581$, $P = 0.003$]. VEGFR2 and P-erk protein levels in the SU0 group were significantly lower than those in the SE group, and the difference was statistically significant ($P < 0.01$). P-erk and P-akt protein levels in the SU5 group were significantly lower than those in the SE group, and the difference was statistically significant ($P < 0.01$, $P < 0.05$), as shown in **Figure 5**. The results indicate that the AKT and ERK signaling pathways are activated in the acute phase after SE. The AKT and ERK signaling pathways downstream of VEGFR2 are activated to varying degrees and may participate in epileptogenesis.

Nissl Staining for the Assessment of Hippocampal Structure in the Chronic Phase After SE

As shown in **Figure 6**, in the control group, the size and morphology of neurons in the CA1 and CA3 regions of the

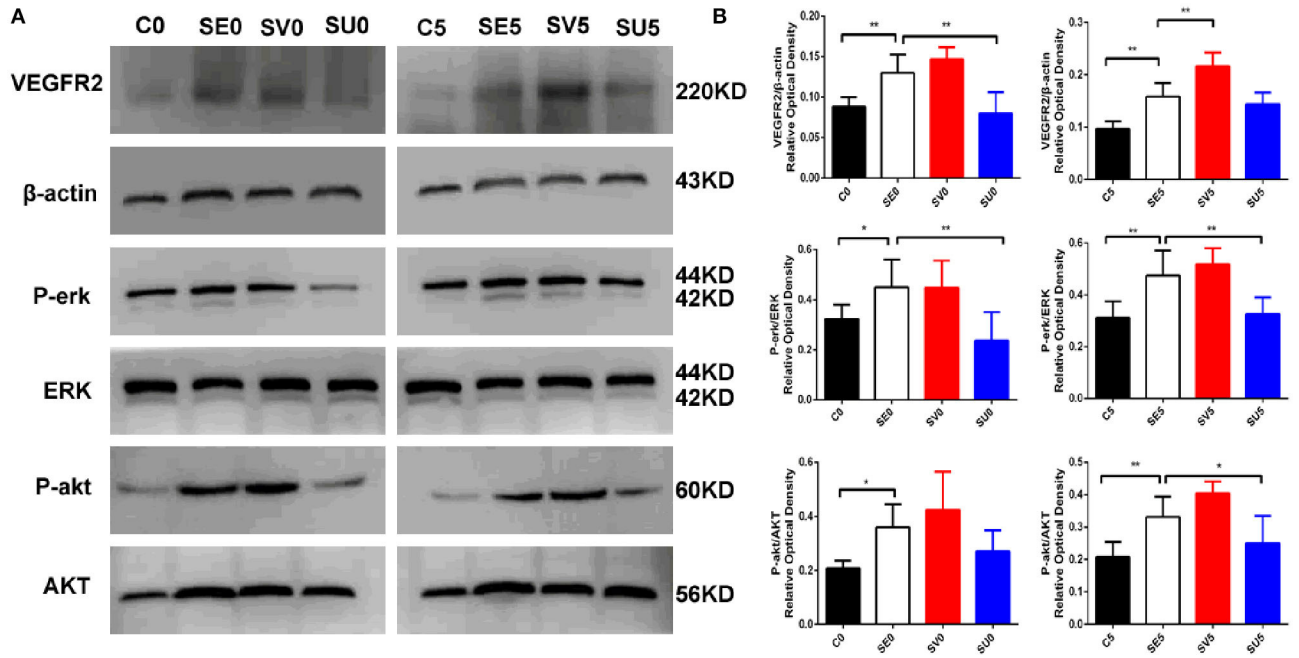

FIGURE 5 | Western blotting results showing the effects of VEGF regulation in different phases after SE on downstream signaling pathways. **(A)** Representative results with the corresponding antibodies. VEGFR2, P-akt, P-erk, AKT and ERK expression levels in the hippocampi of SE rats following regulation of VEGF in different phases after SE. **(B)** Quantitative analyses of the protein expression level in each group. The relative optical densities of VEGFR2, P-erk and P-akt were upregulated post-SE $^{*}P < 0.05$, $^{**}P < 0.01$, $n = 6$ for each group. C, control; SV, VEGF injection; SU, application of a VEGFR2 inhibitor.

hippocampus were neurons; the neurons were neatly and closely arranged and structurally intact, the Nissl bodies were clearly visible, and there was no obvious neuronal loss. The hippocampal structure was disrupted in the chronic phase after SE, the cell arrangement was loose, the CA1 and CA3 regions were missing, Nissl bodies were missing, and scattered nuclear lysis was observed (shown as arrowhead). In the SV0 and SV5 groups, the pyramidal cell of CA1 and CA3 areas were almost tightly organized and ordered. The Nissl bodies were visible in the SV0 group. In addition, vascular infiltration was observed in the CA1 region in the SV5 group (shown as arrow). However, in the SU0 and SU5 groups, the CA1 and CA3 regions showed obvious Nissl bodies and neuronal loss that were same as observed SE group.

EEG Manifestations in Different Phases of Epileptogenesis in Immature Rats

EEG discharges were recorded daily in the different intervention groups during epileptogenesis from 10 to 28 days after SE, and spontaneous epileptic discharges of varying degrees, which manifested as scattered sharp waves and spike waves lasting tens of seconds, were detected in all experimental rats in the chronic phase after SE (**Figure 7B**). This result indicates that inhibiting or promoting VEGF expression in different phases after SE does not significantly reduce or increase the incidence of epileptic discharges in the chronic phase after SE.

The duration, frequency and average number of SRS per day in each intervention group were further analyzed. The treatment factor had a significant effect on the seizure duration [$F_{(4,190)} = 35.918$, $P < 0.0,01$], seizure frequency [$F_{(4,160)} = 28.851$, $P < 0.001$], and number of discharges [$F_{(4,65)} = 10.737$, $P < 0.001$]. There were no significant differences between the SV0 and SU0 groups and the SE group; however, the duration, frequency, and average number of seizures per day were significantly higher in the SV5 group than in the SE group, and the difference was significant ($P < 0.01$). The main behavioral manifestations of SV5 group rats during SRS were facial twitching, rhythmic nodding and chewing, general rigidity, raised unilateral forelimb clonus, and bilateral forelimb clonus with hindlimb standing in the most severe cases. Interestingly, the average duration of each seizure ($P < 0.05$), seizure frequency ($P < 0.01$), and average number of seizures per day ($P < 0.05$) were significantly lower in the SU5 group than in the SE group, and the difference was statistically significant. Based on observation by the naked eye, the behavioral manifestations, which mainly included spontaneous eye gaze and facial muscle twitching and rarely included unilateral or bilateral forelimb clonus, were significantly less severe in the SU5 group than in the SE group. These results suggest that regulation of VEGF expression in the latent phase after SE has a greater impact on the degree of epilepsy. Inhibiting the expression of VEGF in the latent phase after SE can reduce the severity of EEG epileptic discharges.

DISCUSSION

SE is more likely to impair the development of neural networks and thus lead to intractable epilepsy in infants and young

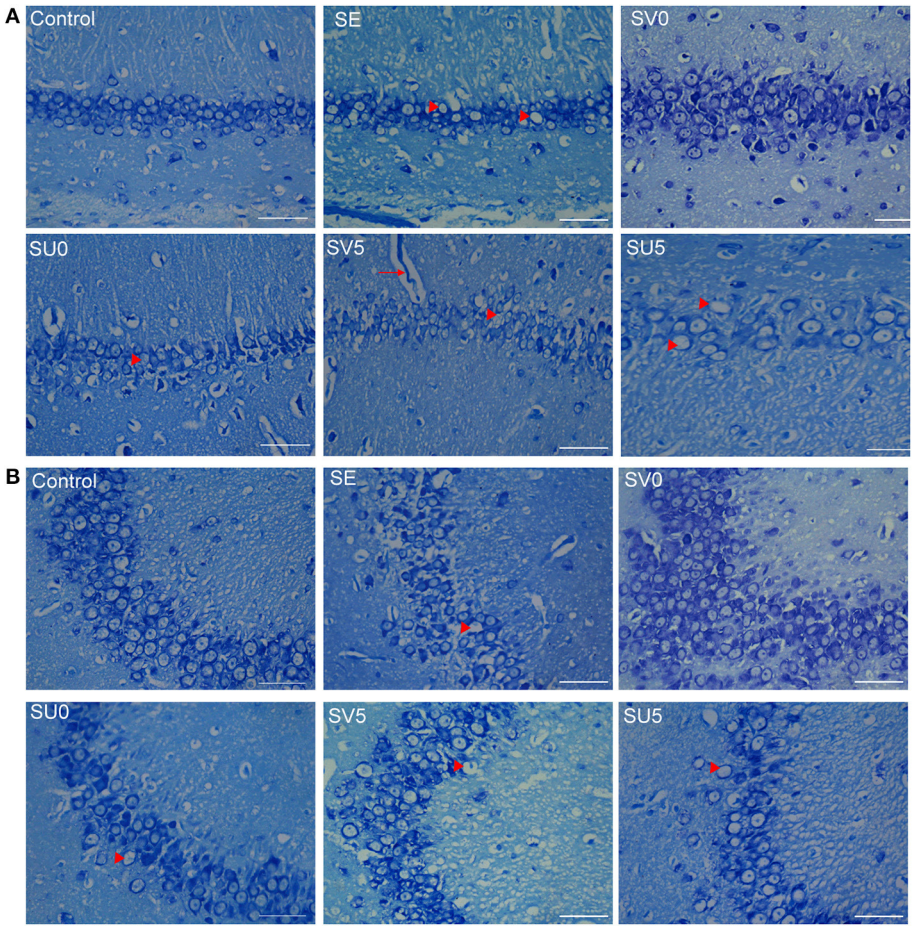

FIGURE 6 | Morphological structure of the hippocampus in different groups in the chronic phase after SE. **(A)** Morphological structure of the hippocampal CA1 region and **(B)** CA3 region in the different groups in the chronic phase after SE. Vascular infiltration was observed in the CA1 region in the SV5 group, as shown by arrows (scale bar: 50 μm).

children than in older individuals. In general, pathological changes in the brain occur in acute, latent phase and chronic phases after convulsive brain injury (3). Neurogenesis and remodeling of microvessels are important role in the process of epileptogenesis (17).

Studies have found that hippocampal neurogenesis can be induced within at least 1–2 weeks after an acute seizure. In turn, the SRS could also stimulate the proliferation of NSCs in the DG (30–33). New neurons in the subventricular zone (SVZ) of the adult hippocampus migrate only a short distance to the GCL to replace apoptotic neurons and reintegrate with neurons in the GCL under normal conditions. However, in the mature brain after SE, the NSCs show abnormal migration, with neurons directly entering the DG or even passing through the GCL to enter the MoDG. The axons of mature neurons in the MoDG follow the pathway of moss fibers of granular cells in the hippocampal DG into the CA3 region after SE, forming abnormal synaptic connections with CA3 neurons (34). The synapses formed by moss fibers exhibit high excitability and low inhibition and are prone to abnormal synchronous discharges; thus, they increase excitability, lead to SRS, form abnormal excitatory neural circuits associated with epilepsy and are the pathological basis of refractory temporal lobe epilepsy (35, 36).

Our data suggest that the latent phase of SE is a critical period for the migration of NSCs in the immature brain. Unfortunately, early migration-related regulatory signals are often lacking or incorrect in this period and can thus easily cause ectopic migration of neurons instead of contributing to their correct integration in the GCL as in the mature brain. Besides, the ectopic NSCs destined for apoptosis even before integrating into the DG circuit during the acute period post-SE. Besides, it has a widely recognized that vascular regeneration is involved in epilepsy pathogenesis (14, 37). In our study, we also found that seizures could affect microvessel proliferation that the density of hippocampal microvessels gradually increased after the incubation period and continued to increase until the chronic phase. According to our data, neovascularization acted as a stent for NSC migration, and pathological angiogenesis affected the direction of migration and abnormal integration of NSCs and contributed to the development of epilepsy, which is consistent

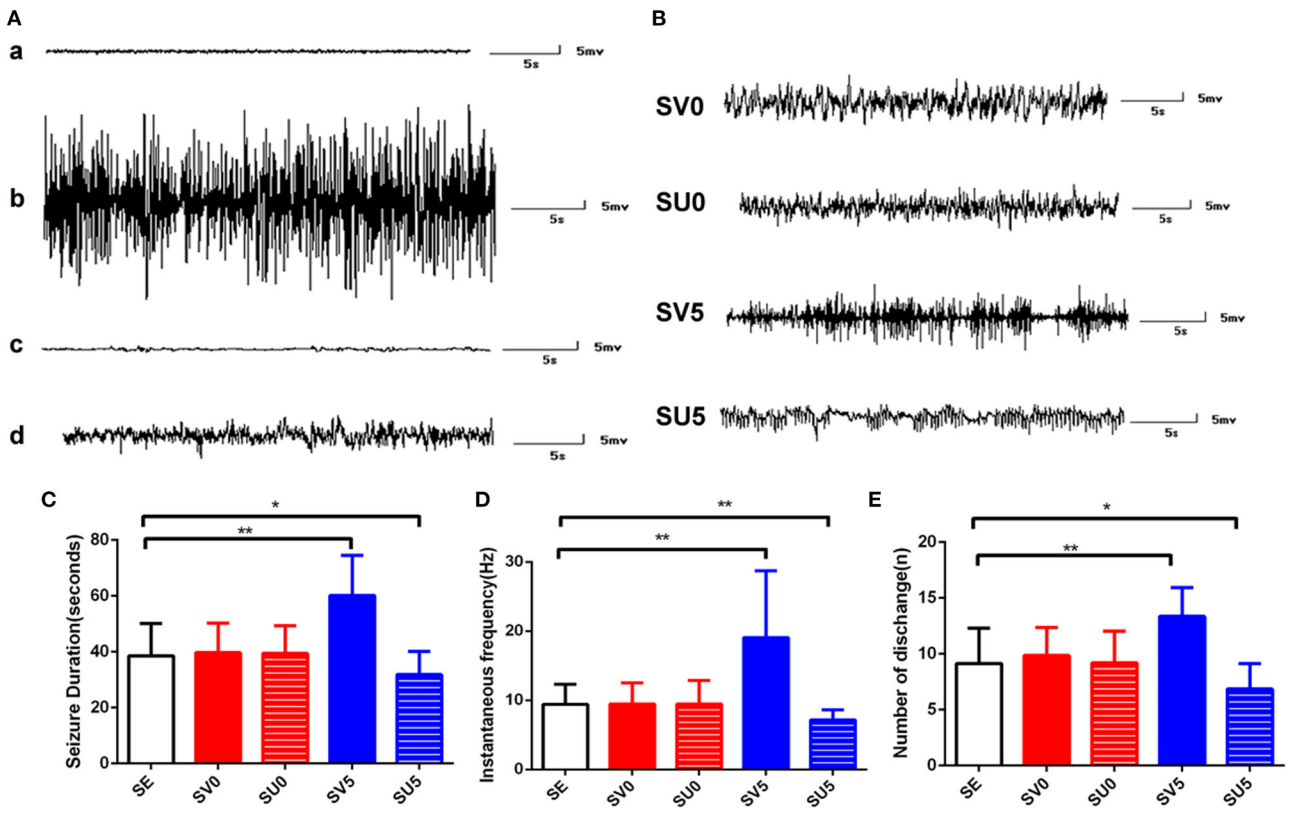

FIGURE 7 | EEG recordings of rats from different groups during different periods. **(A)** EEG recordings from rats in the SE group during epileptogenesis. (a) EEG recording from a normal immature rat. (b) EEG recording from a rat in the acute phase after SE. (c) EEG recording from a rat in the latent period of epileptogenesis after SE. (d) EEG recording of SRS in the SE group. **(B)** EEG recordings of SRS in the different intervention groups. **(C)** SRS duration, **(D)** seizure frequency and **(E)** the number of discharges during the SRS phase after SC. $^{*}P < 0.05$, $^{**}P < 0.01$.

with previous research (13, 17, 38). If the survival of hippocampal NSCs is protected during the acute period after SE and the regeneration of microvessels is inhibited during the latent period after SE, the formation of epileptic networks could be prevented.

The interaction of VEGF has been indicated to be involved in neurogenesis and angiogenesis in normal adult brain. It has been confirmed in cerebral ischemia models that VEGF has different effects in different periods after brain injury. Blocking VEGF expression in the early stage of ischemia can reduce cerebral edema. Promoting VEGF expression in the subacute stage can improve cerebral vascular perfusion. VEGF can improve cerebral vascular perfusion 48 h after cerebral ischemia and can promote angiogenesis and coordinate neurogenesis at the injury site but does not further increase or disrupt vascular penetration (39). A study (40) revealed that injection of exogenous VEGF into the lateral ventricle in the subacute stage after brain injury, i.e., 7 days after successful modeling of stroke in neonates, promotes the proliferation of endothelial cells, increases the total number of blood vessels and protects the brain. Our previous studies showed that the peak expression of VEGF in the hippocampus was in the acute phase after SE, which promotes the proliferation of NSCs and reverses cognitive deficits (8). Considering the dual role and time-dependent effects of VEGF in pathological changes after brain injury, safe and practical application of VEGF after SE is the key to treatment. The results of our study could identify the appropriate time window for regulating vascular regeneration by targeting VEGF after SE.

In this study, by regulating the expression of VEGF in different periods after SE, we found that upregulation of VEGF expression in the acute phase after SE significantly promotes the proliferation of NSCs and can significantly prevent the loss of neurons in the CA1 and CA3 regions but has no obvious effect on microvascular regeneration. However, promoting the expression of VEGF during the latent period after SE further aggravates the ectopic migration of NSCs and significantly increases MVD. However, if VEGFR2 is blocked in the acute phase, it has little effect on the proliferation of NSCs and no obvious effect on the microvasculature. Blocking VEGFR2 in the latent phase can inhibit the remodeling of blood vessels and the ectopic migration of NSCs.

SRS emergence is a key indicator of the occurrence and development of epilepsy, and our data showed that downregulation of VEGF expression in the latent phase after SE could effectively reduce the severity of epileptic discharges during SRS by shortening the duration of each seizure and reducing the frequency and number of seizures, suggesting that the key

regulatory period of epileptogenesis is the latent phase. This finding is consistent with previous reports (28, 41) that selective inhibition of neurogenesis after SE would be valuable to reduce the severity of SRS. The present study also indicated that timig is very important to regulating the frequency and severity of SRS. We speculated that in the latent phase after SE, VEGF expression is inhibited, the level of hippocampal newly ectopic cell could be reduced, and these changes may interfere with the formation of abnormal excitatory synaptic connections and epileptic networks.

VEGFR2 is a tyrosine kinase receptor that can activate multiple downstream signaling pathways, causing neurogenesis, angiogenesis, neural circuit reconstruction and abnormal neural network formation. The PI3K/Akt signaling pathway and the MAPK/ERK signaling pathway are important signaling pathways in cells that play an important role in cell proliferation, differentiation, and apoptosis (42, 43). Both of these pathways may play a role in the development of epilepsy. A previous study (44) showed that the key pathophysiological mechanism underlying the ability of VEGF to protect against focal cerebral ischemia and increase the permeability of the BBB is the PI3K/Akt signaling pathway. Researchers (45) found that electrical convulsive stimulation can induce VEGF expression to regulate P-akt in the treatment of acute cerebral infarction. In addition, a previous study revealed that ERK is activated immediately after severe convulsions and that P-erk expression is upregulated and maintained at a high level from 28 d to 3 months after SE (46). In fact, it has been found that the crosstalk exists between AKT and ERK pathways. In the hippocampus, AKT could be activated first and then initiate ERK pathway to induct cell proliferation (47–49). In this study, the AKT and ERK signaling pathways were activated to varying degrees when VEGF/VEGFR2 signal transduction was interfered with in the acute phase or the latent phase after SE. This finding indicates that the molecular mechanism by which VEGF regulates hippocampal neurogenesis and vascular regeneration after SE may be these two signaling pathways, which are downstream of VEGFR2.

In summary, the present study demonstrated that VEGF, as a common target in nerves and blood vessels, might be closely related to neurogenesis and vascular regeneration in the immature brain during the development of epilepsy after SE. VEGF and VEGFR2 receptor may be valuable therapeutic targets for the prevention of epilepsy. An important aspect may be the proper therapeutic timing. Selective promotion the proliferation of hippocampal NSCs and inhibition of neurogenesis after SE, alleviation of the loss of neurons in the hippocampal and ectopic migration of newborn nerve cells caused by pathological regeneration, which contributes to effectively alleviate SRS severity. The upstream and regulatory factors of VEGF and the mechanism by which VEGF-mediated neovascularization affects the migration of NSCs were not investigated in this study and need to be further explored.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by the Animal Care Committee of Chongqing Medical University, Chongqing, China.

AUTHOR CONTRIBUTIONS

WH and LJ conceived and designed the experiments. WH and HC performed the experiments and analyzed the data. XS, TL, and LC contributed reagents, materials, and analytical tools. WH wrote the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fneur.2021.808568/full#supplementary-material

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SYSTEMATIC REVIEW
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Targeting Inflammatory Mediators in Epilepsy: A Systematic Review of Its Molecular Basis and Clinical Applications

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Introduction: Recent studies prompted the identification of neuroinflammation as a potential target for the treatment of epilepsy, particularly drug-resistant epilepsy, and refractory status epilepticus. This work provides a systematic review of the clinical experience with anti-cytokine agents and agents targeting lymphocytes and aims to evaluate their efficacy and safety for the treatment of refractory epilepsy. Moreover, the review analyzes the main therapeutic perspectives in this field.

Methods: A systematic review of the literature was conducted on MEDLINE database. Search terminology was constructed using the name of the specific drug (anakinra, canakinumab, tocilizumab, adalimumab, rituximab, and natalizumab) and the terms "status epilepticus," "epilepsy," and "seizure." The review included clinical trials, prospective studies, case series, and reports published in English between January 2016 and August 2021. The number of patients and their age, study design, specific drugs used, dosage, route, and timing of administration, and patients outcomes were extracted. The data were synthesized through quantitative and qualitative analysis.

Results: Our search identified 12 articles on anakinra and canakinumab, for a total of 37 patients with epilepsy (86% febrile infection-related epilepsy syndrome), with reduced seizure frequency or seizure arrest in more than 50% of the patients. The search identified nine articles on the use of tocilizumab (16 patients, 75% refractory status epilepticus), with a high response rate. Only one reference on the use of adalimumab in 11 patients with Rasmussen encephalitis showed complete response in 45% of the cases. Eight articles on rituximab employment sowed a reduced seizure burden in 16/26 patients. Finally, one trial concerning natalizumab evidenced a response in 10/32 participants.

Conclusion: The experience with anti-cytokine agents and drugs targeting lymphocytes in epilepsy derives mostly from case reports or series. The use of anti-IL-1, anti-IL-6, and anti-CD20 agents in patients with drug-resistant epilepsy and refractory status epilepticus

has shown promising results and a good safety profile. The experience with TNF inhibitors is limited to Rasmussen encephalitis. The use of anti-α4-integrin agents did not show significant effects in refractory focal seizures. Concerning research perspectives, there is increasing interest in the potential use of anti-chemokine and anti-HMGB-1 agents.

Keywords: adalimumab, anakinra, canakinumab, cytokines, neuroinflammation, epilepsy, tocilizumab, rituximab

INTRODUCTION

Epilepsy affects more than 50 million people worldwide and represents an unsolved public health problem (1). In 2017 ILEA published an operational classification of seizures ad epilepsies which gave a remarkable contribution in improving epilepsy diagnosis and treatment (2, 3) (**Figure 1**). Despite the significant advances made in its treatment, about 7–20% of the children and 30–40% of the adults develop drug-resistant epilepsy (DRE) (4), which can be reasonably defined as an incompletely controlled disease despite the trial with two appropriate antiseizure medications (ASM; whether as monotherapy or in combination) at the correct posology) (5, 6). One of the most severe clinical complications observed in epileptic individuals is status epilepticus (SE), defined by ILEA (7) (**Table 1**). Refractory status epilepticus (RSE) is defined by a SE in which the administration of a benzodiazepine bolus and another ASM does not resolve the clinical picture (13). Among the research areas to improve the therapeutic strategy against drug-resistant epilepsy and RSE, there is increasing interest in the role of agents targeting neuroinflammation. Neuroinflammation is a non-specific biologic response of the brain and spinal cord innate immune system (14), which shows a bidirectional relationship with seizures. Indeed, seizures themselves can be responsible for neuronal and glial damage followed by an inflammatory response, while experimental studies investigating the effects of the administration of pro-inflammatory molecules in the central nervous system (CNS) evidenced that inflammation significantly reduces the seizure threshold (15). Although different authors have focused on the identification of the molecular mechanisms responsible for the inflammatory-dependent induction of seizures and the research for potential therapeutic agents targeting neuroinflammation for the treatment of epilepsy (16, 17), there is no uniform agreement on their use, and data from large cohorts of patients and randomized studies are missing.

In this work, we review the known molecular mechanisms linking neuroinflammation and epilepsy, including the role of the main inflammatory mediators involved in the process of epileptogenesis. For epileptic syndromes cited and terminology related to epilepsy we used the new ILEA operational practical clinical definition of epilepsy (**Table 1**) (8). Moreover, we provide a systematic review of the clinical experience with anti-cytokine agents (anti-IL-1, anti-IL-6, and anti-TNF agents) and with agents targeting the effectors of adaptive immunity in the treatment of epilepsy, with a focus on DRE and RSE. Finally, in the last section of this work, we discuss the main research perspectives in this field, including anti-chemokine agents. This paper aims to help reduce the knowledge gap in the field of neuroinflammation in epileptic individuals and represents the first systematic review performed on the role of anti-cytokine and anti-lymphocyte agents.

SEIZURES AND NEUROINFLAMMATION—AN OVERVIEW

Neuroinflammation develops as a consequence of different stimuli, such as CNS trauma, infection, ischemic and hemorrhagic diseases, and seizures, although it can be also the consequence of a systemic inflammatory response spreading to CNS (14). Additionally, neuroinflammation is partly responsible for the neuronal damage and clinical manifestations of CNS disorders primarily featured by uncontrolled adaptive immune function, including autoimmune encephalitis (Anti-NMDR, Anti-AMPAR, Anti-GABA/AR and Anti-GABA/BR, Anti-VGKC, Anti-GAD, Anti-GlyR, Anti-DPPX, Anti-mGluR5, Anti-IgLON5) (18, 19) and Rasmussen syndrome (10) (**Table 1**), and others. Although inflammatory mediators are central in maintaining brain homeostasis, being implicated in the initiation of tissue repair after CNS injury, the process of neurogenesis, neuronal plasticity, and in the behavioral responses related to stress, neuroinflammation can be responsible for neuronal damage, altered cellular function, and impaired secretion and response to neurotransmitters, thus participating to the process of epileptogenesis (14). The CNS inflammatory response depends on the activity of the resident innate immune cells (particularly, microglial cells and astrocytes) and the release of inflammatory mediators, including cytokines, chemokines, prostaglandin, nitric oxide (NO), and reactive oxygen species (ROS) (17). Among the cellular effectors of neuroinflammation, microglial cells play a central role. They function as sensors for different triggers, having macrophage-like activity, and are implicated in the recognition of pathogens and cellular debris, and the secretion of cytokines and chemokines (20). The function of immune surveillance of microglial cells depends on multiple cellular receptors, with toll-like receptors (TLR) being directly involved in the recognition of pathogen-associated molecular patterns (PAMPS) (21). Other cellular subtypes are involved in CNS inflammation and include endothelial cells and perivascular macrophages, which can act increasing vascular permeability and, through the expression of adhesion molecules, contribute to leukocyte chemotaxis (14, 22). The main cytokines participating in the CNS inflammatory response and epileptogenesis are interleukin 1β (IL-1β), IL-6, and tumor necrosis factor-α (TNF-α) (**Figure 2**). An elevation of the serum

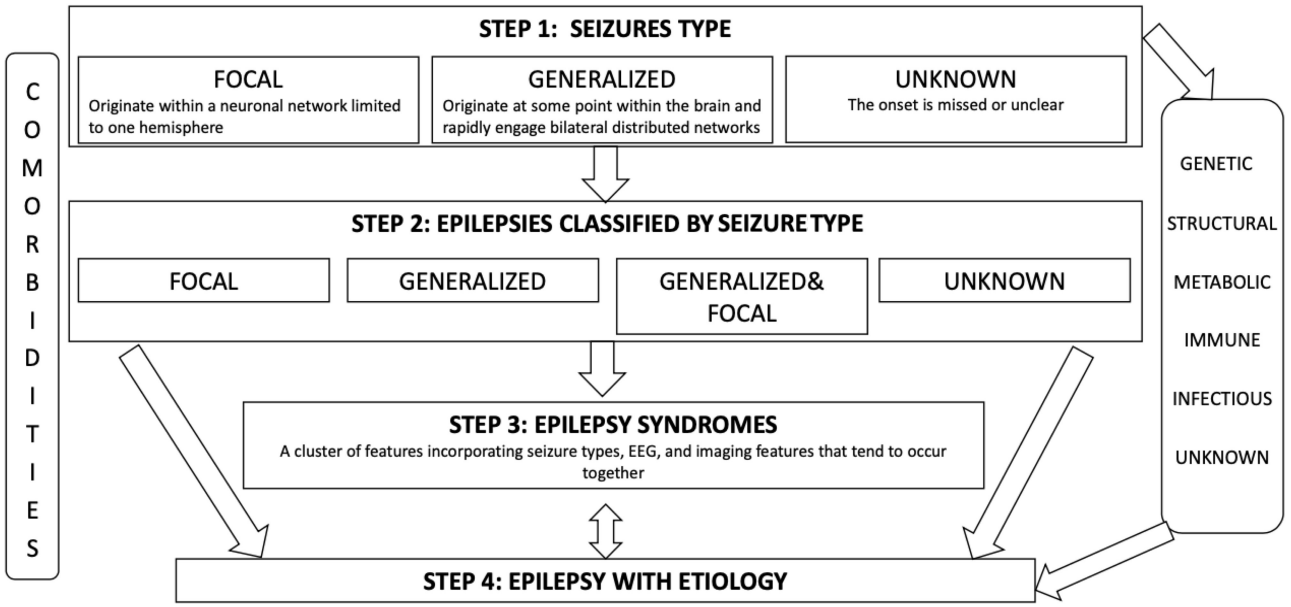

FIGURE 1 | Framework for epilepsy classification designed to allow diagnosis at multiple levels depending on the informationand resources available. At all levels of diagnosis, we should consider more broadly the etiology of the patient's epilepsy. A range of six etiological groups has been recognized: genetic, structural, metabolic, immune, infectious, and unknown.

and cerebrospinal (CSF) levels of these cytokines in individuals with epilepsy has been demonstrated in different studies (23), as well as the higher expression of genes encoding for these mediators in the brain tissue from patients who underwent brain resection for drug-resistant focal epilepsy (17). Interestingly, the levels of inflammatory mediators are higher in patients with drug-resistant epilepsy (24), suggesting a contribution of neuroinflammation in the development of drug resistance. Additionally, the demonstration that in animal models the intrathecal administration of IL-1β causes a reduction of the seizure threshold pointed out the direct role of cytokines in seizure and epileptogenesis (15).

Among soluble inflammatory mediators, the danger signal molecule high-mobility group box-1 (HMGB-1) also plays a central role in neuroinflammation, being involved in the TLR4-IL-1β axis, and in the enhancement of the CNS inflammatory response (25). Chemokines and their receptors participate in CNS inflammation favoring leukocyte migration to the site of inflammation and endothelial adhesion but are also directly implicated in epileptogenesis through the interaction with neurotransmitters and neuropeptides (26), as further discussed. Finally, the role of the blood-brain barrier (BBB) in neuroinflammation and epileptogenesis is of extreme clinical and research interest (25). Inflammatory mediators, produced both in CNS and systemically, can enhance its permeability and BBB leakage allows the spread of systemic inflammatory mediators within the CNS, thus amplifying the inflammatory process (17).

IL-1 and Epilepsy: Molecular Basis

IL-1β is mostly produced by cells of the innate immune system, including monocytes, macrophages, and dendritic cells, following a wide range of triggers, such as infections and cellular damage (i.e., oxidative stress) (27). In CNS, IL-1β is produced by glial cells and other cellular sources, including endothelial cells (28). The main molecular mechanism leading to the synthesis of IL-1β is the assembly and function of the macromolecular complex of the inflammasome, in a process that requires the recognition of the inflammatory trigger (i.e., *via* toll-like receptors) leading to the activation of caspase-1, finally allowing the release of active IL-1β (29). Once released, IL-1β acts as an initiator of the inflammatory response, promoting the synthesis of other pro-inflammatory cytokines (such as IL-6), induces fever, and acts as a direct effector of inflammatory organ damage (30). High serum and CSF levels of IL-1β have been demonstrated in individuals suffering from epilepsy, including those affected by developmental epileptic encephalopathies, with even higher concentrations in patients with drug-resistant epilepsy (17). Although the mechanisms linking IL-1β and epileptogenesis are far to be fully understood, pieces of evidence suggest that neuronal excitation and excitotoxicity secondary to the enhanced effect of glutamate play a significant role (31). Indeed, IL-1β has been demonstrated to influence the calcium influx across the N-methyl-D-aspartate (NMDA) receptor, reduce glutamate uptake by astrocytes and increase glutamate release by glial cells (32). On the other hand, studies on the influence of IL-1β on GABA-ergic transmission show conflicting results (32).

Currently, the agents targeting IL-1β comprise the IL-1 receptor antagonist anakinra, the anti-IL-1 monoclonal antibodies canakinumab (human antibody) and gevokizumab (humanized antibody), and the IL-1 inhibitor rilonacept, which consists of a fusion protein composed of the Fc portion of human IgG and the extracellular domain of IL-1 receptor (33,

TABLE 1 | Definitions.

Operational (practical) clinical definition of epilepsy (8)
Epilepsy is a disease of the brain defined by any of the following conditions: • 1. At least two unprovoked (or reflex) seizures occurring >24 h apart • 2. One unprovoked (or reflex) seizure and a probability of further seizures similar to the general recurrence risk (at least 60%) after two unprovoked seizures, occurring over the next 10 years • 3. Diagnosis of an epilepsy syndrome Epilepsy is considered to be resolved for individuals who had an age-dependent epilepsy syndrome but are now past the applicable age or those who have remained seizure-free for the last 10 years, with no seizure medicines for the last 5 years.
Status epilepticus (7)
"A condition resulting either from the failure of the mechanisms responsible for seizure termination or from the initiation of mechanisms, which lead to abnormally, prolonged seizures (after time point t_1). It is a condition, which can have long-term consequences (after time point t_2), including neuronal death, neuronal injury, and alteration of neuronal networks, depending on the type and duration of seizures."
Super refractory status epilepticus (7)
SE continues for more than 24 h after the first administration of general anesthesia.
Autoimmune encephalitis (9)
Autoimmune encephalitis encompasses a wide variety of protean pathologic processes associated with the presence of antibodies against neuronal intracellular proteins, synaptic receptors, ion channels and/or neuronal surface proteins.
Rasmussen encephalitis (10)
Unilateral hemispheric encephalitis whose main clinical features include refractory focal epilepsy or epilepsia partialis continua, hemiparesis, and progressive cognitive decline.
New-onset refractory status epilepticus (NORSE) (11)
NORSE is a clinical presentation, not a specific diagnosis, in a patient without active epilepsy or other preexisting relevant neurological disorder, with new onset of refractory status epilepticus without a clear acute or active structural, toxic or metabolic cause.
Febrile infection-related epilepsy syndrome (FIRES) (11)
FIRES is a subcategory of NORSE, applicable for all ages, that requires a prior febrile infection starting between 2 weeks and 24 h prior to onset of refractory status epilepticus, with or without fever at onset of status epilepticus.
Electrical status epilepticus in sleep (ESES) (12)
Electrical status epilepticus in sleep (ESES), a childhood-onset epileptic encephalopathy, is characterized by epilepsy, cognitive regression, and marked activation of epileptiform activity during non-rapid eye movement (NREM) sleep to produce an electroencephalography (EEG) pattern of near-continuous spike-wave discharges.

34). Since their introduction, anti-IL-1 agents have become a cornerstone in the treatment of autoinflammatory disorders, and have also been used in other rheumatologic disorders, such as rheumatoid arthritis (RA), gout, adult-onset Still diseases and systemic juvenile idiopathic arthritis (sJIA), and cytokine storm syndromes (35), with anakinra and canakinumab being the most widely used drugs. The use of gevokizumab is still experimental, since the drug has not been approved by the Food and Drug Administration.

IL-6 and Epilepsy: Molecular Basis

IL-6 has a pivotal role in enhancing and maintaining the inflammatory response and activating adaptive immunity. Systemically, the release of IL-6 is followed by the production of acute-phase proteins (C-reactive protein, serum amyloid protein, fibrinogen), the release of platelets, angiogenesis, and an increase in vascular permeability (36). Additionally, IL-6 participates in driving the differentiation of T CD4+ cells in T helper 17 (Th17) cells, promotes the differentiation and expansion of T CD8+ cytotoxic cells, and inhibits the proliferation of regulatory T cells (Tregs) (36). In the CNS, this cytokine is mostly produced by glial cells, with a mechanism triggered by multiple factors, including the binding of IL-1β and TNF-α to specific surface receptors on glial cells (37). Additionally, IL-6 can be secreted by astrocytes and neurons and can be released by perivascular and brain endothelial cells in response to inflammatory and infectious stimuli (37). Increased serum and CFS levels of IL-6 have been demonstrated in patients with refractory epilepsy, and an association between IL-6 levels and the degree of neuronal apoptosis was evidenced in patients undergoing surgery for refractory temporal lobe epilepsy (38). Notably, the serum cytokine levels have been shown to decrease after surgery (38). Apart from the induction of apoptosis, a direct role of IL-6 in epileptogenesis has been suggested (31). It is known that IL-6 interferes with GABA-A receptor functioning, while recent data suggest that the cytokine could reduce glutamate signaling and consequent neuronal toxicity (32).

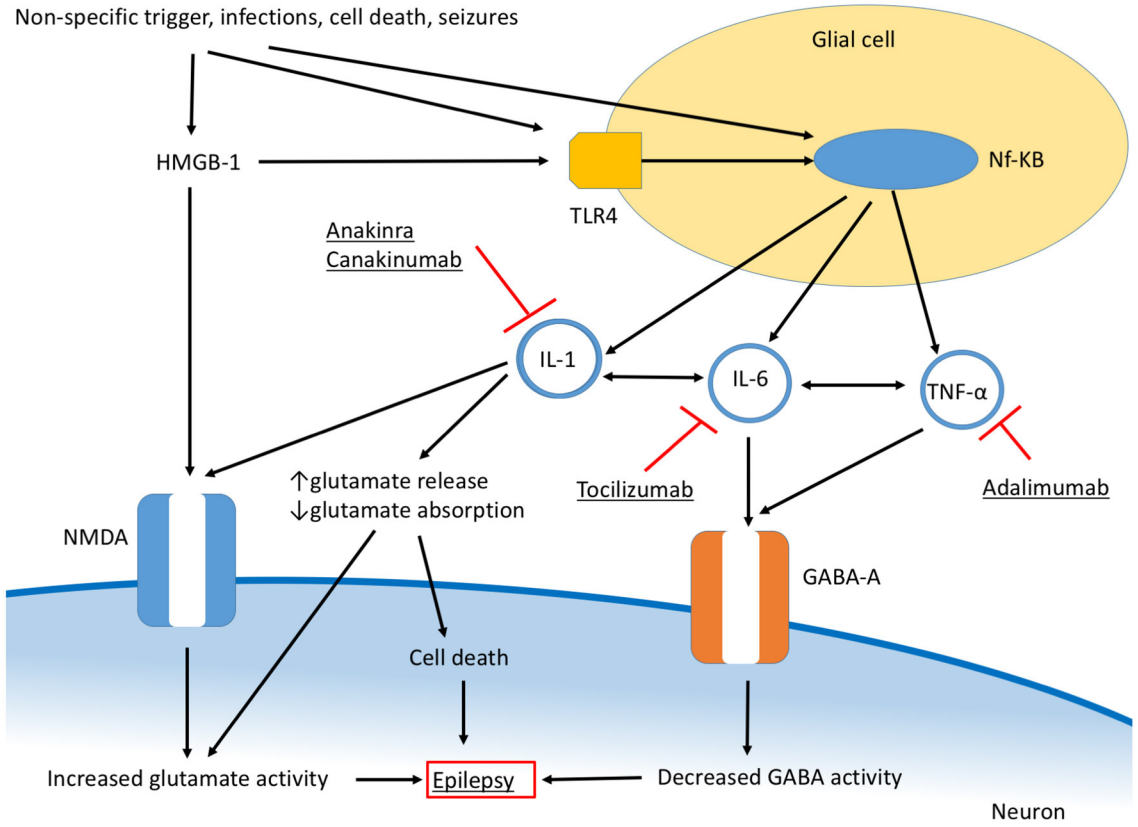

FIGURE 2 | Overview of inflammatory pathways involved in epilepsy and main therapeutic targets.

The most widely used anti-IL-6 therapeutic agent is tocilizumab, a humanized monoclonal anti-IL-6 receptor antibody that is part of the therapeutic armamentarium against autoinflammatory disorders, cytokine storm syndromes, and autoimmune diseases (36, 39). Concerning neurological diseases, tocilizumab is used in autoimmune encephalitis and different systemic inflammatory disorders with CNS involvement, including linear scleroderma "*en coup de sabre*" (LSCS), and neuro-Behcet's disease (40, 41). The human monoclonal anti-IL-6 antibody sarilumab is a therapeutic option in patients with RA and different authors suggested a role in the treatment of severe COVID-19 (42, 43).

TNF-α and Epilepsy: Molecular Basis

Tumor necrosis factor-alpha (TNF-α) is a pleiotropic effector cytokine of the TNF superfamily that plays a role in the regulation of cell homeostasis and of the immune-inflammatory pathways (28). TNF-α is produced by a wide range of cells (including microglia, astrocytes, and neurons) (44, 45) and interacts with two transmembrane glycoprotein receptors, TNF receptor 1 (TNFR1) and TNF receptor 2 (TNFR2), that differ in their cellular expression profiles, ligand affinities and signaling pathways (45). The main downstream effectors are Nuclear Factor Kappa-B, C-Jun N-terminal Kinase, p38, and the pathway of sphingomyelinase/ceramide. The exact role of TNF-α in epileptogenesis is not yet understood (46). The cytokine may operate by direct interaction with neurons or by influencing the expression of neurotransmitter receptors on glial cells (47, 48). TNF-α can also alter the permeability of the blood-brain barrier (28, 49). Reportedly, the expression of TNF-α in astrocytes may promote an inflammatory and degenerative outcome, while the cytokine expression in neuronal and microglial cells would be associated with tissue repair and remyelination (50–52). This dual role of TNF-α reflects the subtype of receptor that is preferentially involved in the signaling: TNFR1 is thought to induce an epileptogenic and proinflammatory phenotype through a series of post-translational mechanisms that regulate the expression and the turnover of AMPA, GABAA, and NMDA-NR1 receptors, while TNFR2 shows an anticonvulsant and neurotrophic orientation. Indeed, a study conducted on hippocampal tissues from patients with intractable temporal lobe epilepsy documented the predominance of TNFR1 pathways. Specifically, the involvement of TNFR1 caused the activation of apoptosis pathways capable to perpetrate the seizure-induced brain injury (53).

All molecules with TNF-inhibitory action that are currently approved for therapeutic use are monoclonal antibodies (mAbs) obtained by mutation and gene-splicing techniques (54, 55). These drugs can act both with an antagonistic effect, blocking the cellular functions mediated by TNF-receptors, or with an

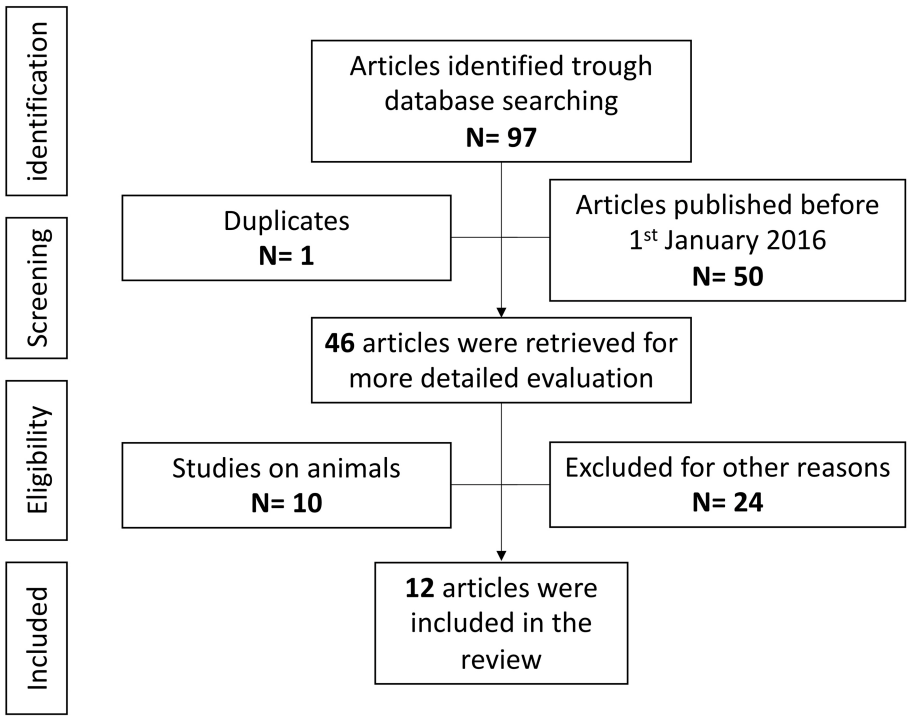

FIGURE 3 | Anti-IL-1 agents and epilepsy, literature review.

agonistic function through reverse signaling mediated by the transmembrane portion of TNF-α (56). In the spectrum of CNS disorders, the use of anti TNF-α agents has been specifically studied for multiple sclerosis (MS) (57, 58). However, two clinical trials evidenced discouraging results, since patients showed clinical and radiological signs of progression of the disease during treatment (57, 58). Moreover, a possible correlation between the exposure to anti-TNF-α agents and the occurrence of inflammatory demyelinating and non-demyelinating events emerges from different cases and case-series of patients treated for non-CNS related disorders (59–69). We speculate that the adverse neurological effects observed in patients treated with TNF-inhibitors for other diseases influence and limit the choice of these therapies for the treatment of many neurological conditions. Despite a causal correlation between demyelination and the use of anti TNF-α agents is uncertain, some mechanisms have been proposed to explain the pathogenesis of the adverse events observed, including the fact that the use of TNF-inhibitors may reduce the expression of TNFR2 receptors within the brain tissues, impairing the course of reparative processes (70–73). In the particular case of epilepsy, animal models highlighted how the effects of the TNF-α can be different depending on which receptor subtype governs the signaling pathway. Hence, future therapeutic agents may be targeted to the mechanisms that govern the predominance between TNFR1 and TNFR2, to downregulate the former without reducing the expression of the latter (74).

Adaptive Immunity and Epilepsy: Molecular Basis

Adaptive immunity plays an important role in the immune surveillance within the CNS through a series of mechanisms, considering the status of immune privilege of the CNS (75). Nonetheless, cells of adaptive immunity have also been implied in the pathogenesis of different immune-mediated diseases of the CNS (76). Moreover, experimental studies conducted in models of epilepsy suggested that adaptive immunity is actively involved in this process (77). Following seizures, the alteration of the BBB leads to the release of chemokines and drives the infiltration of lymphocytes and other cells of the innate immunity (i.e., monocytes) (78). Within the CNS, B and T cells exert their effector functions with different mechanisms. The understanding of the pathogenesis of certain forms of immune-mediated epilepsy revealed that B and T cells can respond to stimuli originating directly by neuronal antigens or by molecular mimicry between the antigens of infectious agents (mainly viruses) and components of the CNS tissues (79, 80). To date, a wide range of antibodies directed toward extracellular domains have been described and reunited under the term "Neuronal Surface Antibodies." The presence of such antibodies underlies several CNS syndromes characterized by seizures (81). In other conditions, identified as paraneoplastic syndromes, the production of antibodies is rather an epiphenomenon and the pathogenesis recognizes a role of cytotoxic T cells (82). Xu et al. investigated the involvement of cell-mediated immunity in patients with intractable forms of epilepsy and demonstrated

TABLE 2 | Systematic review of anti-IL-1 agents in epilepsy.

References	Disease	*N*, (age)	Study design	Intervention	Timing	Efficacy	Safety
Sa et al. (105)	FIRES	2 Pt 1: 1.9 years Pt 2. 2.5 years	Case Report	Pt 1 Anakinra 5 mg/Kg/day s.c for 14 days	Start: Day 43	From Day 51 seizures decreased in frequency and on Day 60 these stopped. After 3 weeks new onset of seizures (2–5/month)	No adverse effects
				Pt 2 Anakinra 10 mg/Kg/day s.c for 90 days	Start: day 22 Discontinued after 3 months of treatment	No improvement	No adverse effects
Yang et al. (106)	FIRES	1 (6 years old)	Case Report	Anakinka 100 mg s.c. twice daily for 1 year	Start: Day 28	Resolution of seizures after 4 days. Stop ketogenic diet after 9 months. Follow-up: 1 seizure/month	No adverse effects
Kern-Smith et al. (107)	NORSE	1 (5 years old)	Case Report	Anakinra for 13 days (posology not specified)	Start: Day 12 Stop: Day 24	Stop midazolam infusion, without return of electrographic status epilepticus, after 2 days	No adverse effects
Dilena et al. (108)	FIRES	1 (10 years old)	Case Report	Anankinra 2.5 mg/kg/day (100 mg) s.c and, 3 days after, 2.5 mg/kg twice daily	Start: after 18 months from diagnosis Stop: 7 months after	Full seizure control after 3 days	No adverse effects
Jyonouchi and Geng (109)	ESES	1 (6 years old)	Case Report	Anakinra 100 mg/day s.c.	Start: 25 months after diagnosis	Despite the improvement of behavioral symptoms, ESES pattern persisted.	No adverse effects
Lai et al. (110)	FIRES	25, (5–11 years old)	Retrospective	Anakinra 3–5 mg/kg/day (initial dose) Anakinra 4–9 mg/kg/day (final dose)	Start: 20 days after the onset of FIRES Stop: 86 days after	Earlier anakinra initiation after seizure onset was associated with shorter duration of mechanical ventilation, and ICU and hospital LOS. Amongst children with available seizure frequency data, 11/15 (73.3%) exhibited> 50% seizure reduction at 1 week of anakinra treatment. 3/25 (12%) died	3/25 (12%) developed DRESS 2/25 (8%) developed cytopenia 10/25 (40%) developed infections (1 discontinued anakinra for infections)
Westbrook et al. (111)	FIRES	1 (21 years old)	Case Report	Anakinra 100 mg 3 times daily s.c (Initial dose) Anakinra 100 mg twice daily s.c (after 10 days) Anakinra 100 mg once daily s.c. (after 25 days)	Start: 32 days after the diagnosis of FIRES Stop: 1 year after at which time discontinuation will be discussed	Full seizure control after 24 h	No adverse effects

(Continued)

TABLE 2 | Continued

References	Disease	*N*, (age)	Study design	Intervention	Timing	Efficacy	Safety
Kenney- Jung et al. (112)	FIRES	1 (32 months old)	Case Report	Anakinra 5 mg/kg/twice daily s.c. Anakinra 5 mg/kg/twice daily s.c.	2 cycles: Day 6–23 Day 54-ongoing	Improved seizure control in both cycles (from 5.8 to 1.3 seizure/day in the first cycle; from 8 to 0.17 seizure/day in the second). Twelve months after initial presentation, the patient experiences rare focal seizures.	Development of DRESS (day 22, followed by discontinuation)
DeSena et al. (113)	DRE	1 (14 years old)	Case Report	Anakinra 100 mg daily Anakinra 100 mg twice daily Canakinumab 300 mg every 4 weeks	Start: 2 years Stop: After 4 weeks After: 2 months	Rapid ∼80% reduction in seizure frequency (from 4 to 15/day to 4/week). No clinically evident seizures. Improvements in her fatigue, general malaise, quality of life, and academic performance. Long periods of being seizure-free, currently averaging one seizure per several months.	No adverse effects
Stredny et al. (114)	FIRES	1 (6 years old)	Case Report	Anakinra 20 mg/kg daily	From day 6 to 20 of hospitalization	No clinical response	No adverse effects
Mochol et al. (115)	RE	1 (43 years old)	Case Report	Anakinra 100 mg daily sc	26 years after disease presentation. 1st cycle: 2 months 2nd cycle: 7 months	Complete seizure control after 1 week of treatment. Relapse after 2 weeks of withdrawal 2nd cycle: full seizure control after 10 days 13 months seizure-free	Pneumonia
Choi et al. (116)	SRSE in AE	1 (38 years old)	Case Report	Anakinra 100 mg daily s.c	From week 12 Duration: 12 days	Resolution of status epilepticus Recovery in communication and walking)	No adverse effects

AE, autoimmune encephalitis; DRE, drug-resistant epilepsy; DRESS, Drug rash with eosinophilia and systemic symptoms; ESES, Encephalopathy with electrical status epilepticus in sleep; FIRES, Febrile infection-related epilepsy syndrome; NORSE, New-onset refractory status epilepticus; RE, Rasmussen encephalitis; SRSE, super-refractory status epilepticus.

localization of blood-derived, antigen-specific CD8+ and γδ T cells in the epileptogenic zone, showing how the amount of infiltrated cells correlated with seizure severity (83). Interestingly, the majority of T cells detected resulted to be memory T cells, capable to sustain an inflammatory response even in the absence of costimulatory signals. Additionally, the amount of brain infiltrating T reg cells resulted inversely correlated with seizure severity (83). The role of CD8+ cells is peculiar since under inflammatory conditions neurons can express MHC class I and II molecules perpetrating cell-mediated brain damage (84).

Studies conducted in the past years raised the awareness of how targeting the mechanisms of action of B and T cells could be of therapeutic interest. The use of monoclonal antibodies as immune modulators has substantially changed the approach to many inflammatory diseases, including some autoimmune disorders of the CNS (85). The activity of these agents consists mainly in inhibiting the production of antibodies and blocking the intrusion of effector lymphocytes in the CNS. Rituximab is a chimeric anti-CD20 mAb engineered to reduce the pool of B cells that undergo maturation and that produce antibodies, hence decreasing the entity of humoral immune response (86). Originally approved for the treatment of B cell lymphoma, to date rituximab is employed in the treatment of many immune-mediated diseases of the CNS, including multiple sclerosis and other forms of autoimmune demyelinating disorders (87, 88). This molecule represents also an off-label second line of treatment for several forms of autoimmune encephalitis (89). Particularly, the use of rituximab for the treatment of anti-NMDR has been the object of studies that evidenced a good response in terms of outcome and a lower frequency of relapses (19, 90). With a more heterogeneous degree of clinical response, the use of rituximab is also reported in cases of encephalitis associated with anti-GAD, anti-myelin oligodendrocyte glycoprotein, and anti-leucine-rich glioma inactivated 1 (LGI1) antibodies (91–93). Additionally, occasional reports in patients with RE and one patient with FIRES sustain a possible therapeutical application for rituximab in these diseases (94, 95). Natalizumab is a humanized monoclonal antibody targeting α4-integrin (CD49d) on the surface of lymphocytes (96). The interaction between this integrin and the VCAM receptor is involved in the process of diapedesis and hence crucial to lymphocyte extravasation to the CNS. Natalizumab has been prominently employed in patients with multiple sclerosis, with good therapeutic results despite the onset of progressive multifocal leukoencephalopathy (96, 97). The use of natalizumab has been studied in animal models of RE (98) and one patient with RE treated with natalizumab is reported in literature (99).

SYSTEMATIC REVIEW OF THE LITERATURE

Objective of the Systematic Review

This systematic review aims to assess whether anti-cytokine (anti-IL-1, anti-IL-6, anti-TNF), anti-CD20, and anti-α4-integrin agents could be effective and safe for the treatment of DRE and RSE.

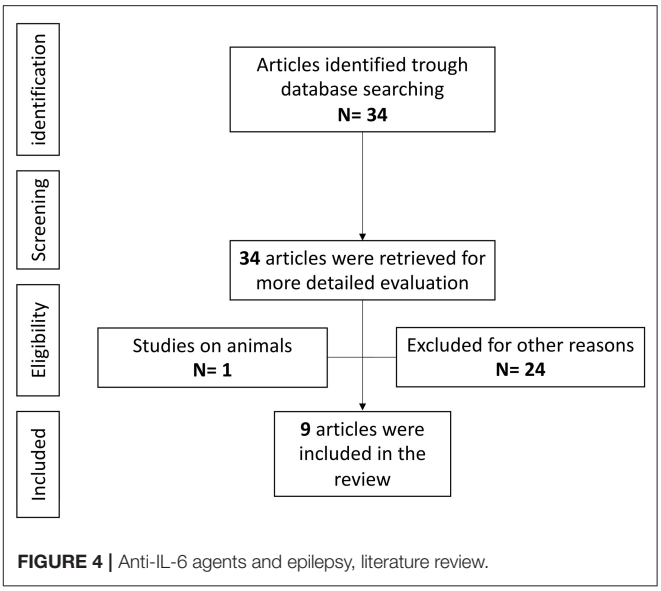

FIGURE 4 | Anti-IL-6 agents and epilepsy, literature review.

Methods

Protocol

This systematic review was performed following the preferred reporting items for systematic reviews and meta-analysis protocol (PRISMA-P) reporting guidelines (100). The review protocol has not been registered.

Inclusion Criteria

Population

The study included patients with no age restriction and diagnosed with DRE or RSE. Patients with DRE or RSE caused by specific syndromes (autoimmune encephalitis, LSCS, RE) were included.

Intervention

We included patients receiving treatment with anti-IL-1 (anakinra, canakinumab), anti-IL-6 (tocilizumab), anti-TNF (infliximab, adalimumab, etanercept), anti-CD20 (rituximab), anti- anti-α4-integrin (natalizumab) agents.

Comparators

Patients receiving ASM, corticosteroids, and other conventional immunosuppressive agents or placebo were included. Studies not including a comparator arm have also been included.

Outcomes

The primary outcomes for this review were the efficacy in decreasing or arresting seizures in RSE, controlling the epileptic phenotype in DRE, and the safety of the therapeutic agents in epileptic individuals. The secondary outcome was (when available) the analysis of the motor, behavioral, and cognitive function and recovery after treatment.

Study Types

We included all the case reports, case series, retrospective, prospective studies, and clinical trials in which the following

TABLE 3 | Systematic review of anti-IL-6 agents in epilepsy.

References	Disease	PTS *N* Age	Study design	Intervention	Timing	Efficacy	Safety
Magro et al. (117)	CNS disease and DRE associated with LSCS.	1 (22 years old)	Case Report	Tocilizumab 162 mg S.C. once a week	Start: 6 months after	Noticeable improvement in cognitive and affective symptoms with decrease in seizure frequency. Resolution of many of the enhancing lesions on brain MRI	No adverse effects
Stredny et al. (114)	FIRES	1 (6 years old)	Case Report	Tocilizumab 12 mg/kg S.C. every 2 weeks	Start: Day 20 Stop: Day 76	Reduction of seizure	No adverse effects
Donnelly et al. (118)	NORSE	1 (26 years old)	Case Report	Tocilizumab 300 mg IV. for two times	First dose: 9 weeks after the beginning of treatment Second dose: 12 weeks after the beginning of treatment	Stop seizures after 48 h	No adverse effects
Osminina et al. (119)	CNS disease and DRE associated with LSCS.	1 (2 years 10 months)	Case Report	Tocilizumab 10 mg/kg IV. once in 4 weeks	Start: 16 months after the beginning of symptoms Stop: 26 months after first infusion of Tz.	Reduction of periventricular focus; stop seizures.	No adverse effects
Jaafar et al. (120)	SRSE	1 (8 years old)	Case Report	Tocilizumab, 8 mg/kg/day S.C. divided in two doses 1 week apart	Start: 10 days after admission to hospital	Stops seizure 24 h after	No adverse effects
Cantarín-Extremera et al. (121)	NORSE	2 Pt 1: 1.9 years old Pt 2: 2.7 years old	Case Report	Pt 1 Tocilizumab 4 mg/kg once a week	Start: Day 21 Stop: Day 28	Seizures decrease in frequency, in VEEG critical patterns had disappeared.	No adverse effects
				Pt 2 Tocilizumab 4 mg/kg for 2 times	Start: Day 30 and 40	48–72 h after the first dose, the seizures began to decrease progressively in frequency and intensity, there was global neurological improvement, recovering normality in terms of language, level of consciousness, and motor capacity, but persisting hyperactivity.	No adverse effects
Jun et al. (124)	NORSE	7 [median 25 years old (22–64)]	Prospective	Tocilizumab 4 mg/kg for 2 cycles in 1-week intervals, a monthly dose (8 mg/kg) was added if needed	Start: Median day 25 (6–73)	Resolution of status epilepticus in 6/7 patients 3/6 of the survived patients showed improvement on the mRS	2/7 (2.9%) leukopenia 1/7 (1.4%) diarrhea 1/7 (1.4%) pneumonia 1/7 (1.4%) sepsis

(Continued)

TABLE 3 | Continued

References	Disease	PTS *N* Age	Study design	Intervention	Timing	Efficacy	Safety
Benucci et al. (122)	Limbic Encephalitis with Anti-CASPR2 Antibodies	1 (64 years old)	Case Report	Tocilizumab 8 mg/kg IV. once a month for 6 months, then 162 mg every week s.c	Start: 2–3 months after admission.	Full seizure control Resolution of behavioral changes and seizures Improvement in mRS	No adverse effects
Vallecoccia et al. (123)	SRSE	1 (34 years old)	Case Report	2 doses of tocilizumab 4 mg/kg at a 1-week interval	Start: day 24	Partial recovery after 7–10 days from the first administration. Resolution of the clinical picture after 1 month ("ketamine and ketogenic diet added)	Sepsis by drug-resistant pathogen

CNS, Central nervous system; DRE: drug-resistant epilepsy; FIRES, Febrile infection-related epilepsy syndrome; LSCS, Linear scleroderma "en coup de sabre;" mRS, modified Rankin scale; NORSE, New-onset refractory status epilepticus; RSE, refractory status epilepticus; SRSE, Super-refractory status epilepticus.

criteria were met: (1) original publication; (2) human studies focusing on RSE and/or DRE; (3) at least one enrolled patient with no age restriction; (4) only papers published in English; (5) only papers published between 1 January 2016 and August 2021.

Exclusion Criteria

We excluded (1) reviews, book chapters, author's replies ad commentaries; (2) animal experiments; (3) all papers published before 2016; (4) all the studies not meeting the inclusion criteria.

Search Strategy and Data Sources

A systematic review of the literature concerning the use of these drugs in patients with epilepsy was conducted in May 2021 and updated in September 2021 on the MEDLINE database (through PubMed). The search terminology was constructed using the name of specific anti-IL-1, anti-IL-6, anti-TNF, anti-CD20, and anti-α4-integrin drugs and the terms "status epilepticus," "epilepsy," and "seizure" with the use of Boolean operators [e.g., "(anakinra AND status epilepticus) OR (anakinra AND seizures)"], ["(tocilizumab AND status epilepticus) OR (tocilizumab AND seizures)"], [(anti-tumor necrosis factor-alpha) OR (anti-TNF)) OR (tumor necrosis factor inhibitor)) OR (Infliximab)) OR (Etanercept)) OR (Adalimumab)) AND ((Epilepsy) OR (seizures))], [((rituximab) OR (anti-CD20)) AND ((Epilepsy) OR (seizures))], [(natalizumab) AND ((Epilepsy) OR (seizures))]. For all searches, the respective Medical Subject Headings (MeSH) and Emtree terms were used, if available.

Study Records

Three review authors (GC, GD, AM) independently screened the titles and abstracts using the previously described inclusion and exclusion criteria. In this first step, the studies considered for potential inclusion were divided into five categories: anti-IL-1, anti-IL-6, anti-TNF agents, anti-CD20, and anti-α4-integrin. In a second step, the same three review authors (GC, DP, AM) evaluated full-text articles and assessed the eligibility for this systematic review. Any disagreement regarding eligibility was resolved through discussion with a third-party member (AO). Data were extracted using a standardized form on Microsoft Excel (Microsoft Corporation, Seattle, USA). The form was designed by GC, and data were extracted by GC, DP, and AM. Any disagreement regarding data extraction was resolved through discussion with a third-party member (AO). The following data were extracted for each study: first author, publication year, number of enrolled patients and their age, study design, specific drugs used, dosage and route of administration (if available), the timing of administration, patients outcomes (decrease in seizure frequency/arrest of seizures or status epilepticus. motor, behavioral and cognitive outcome, adverse events) and the dosage of serum and CSF cytokines. The data obtained from extraction were synthesized through quantitative and qualitative analysis.

Risk of Bias Assessment and Quality of the Evidence

The risk of bias was evaluated using the risk of bias in non-randomized studies of interventions (ROBINS-I) tool (101) for non-randomized studies and the evidence-based medicine (EBM) indications (102) for case series and reports. Each

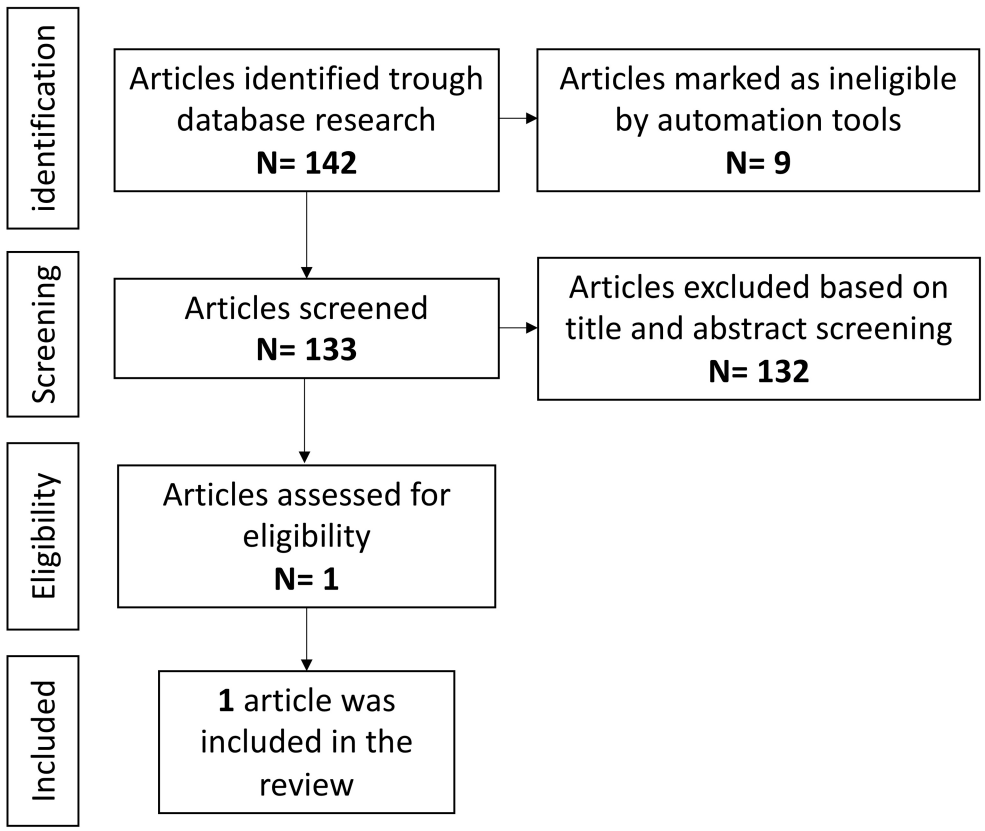

FIGURE 5 | Anti-TNF agents and epilepsy, literature review.

study was assessed by one review author (GC). To estimate the level of evidence reached by this work, the grading of recommendations, assessment, development, and evaluation (GRADE) (103) approach was used. The risk of bias, precision, consistency, directness, and publication bias was analyzed.

Systematic Literature Review: Anti-IL-1 Agents and Epilepsy

Study Selection

Overall, 97 references were identified using the search strategy. After identifying one duplicate and removing 50 articles published before 1 January 2016, the full text of 46 articles was retrieved and evaluated in detail. After a full-text assessment 34 articles were excluded: 10 studies were on animals and 24 were not relevant to the topic of the present review. One paper on multifocal neutrophilic meningoencephalitis (MNM) was excluded as did not directly analyze the effect on the epileptic phenotype (104). Twelve articles were finally included in this review (**Figure 3**), for a total of 37 cases of epilepsy with different etiologies (105–116). All publications were in English, six were from the USA, three were from Europe, one from Asia, and two were international studies, involving different countries. Eleven publications were case reports, while the last was a retrospective observational study.

Results

Among the 37 patients included in this systematic review, the median age was 7 years. Thirty-two patients suffered from FIRES; the other patients suffered from new-onset refractory status epilepticus (NORSE) ($n = 1$), super-refractory status epilepticus (SRSE) ($n = 1$), RE ($n = 1$), encephalopathy with electrical status epilepticus in sleep (ESES) ($n = 1$), and DRE ($n = 1$) (**Tables 1**, **2**).

Anakinra was administered with various posologies, ranging from 3 to 20 mg/Kg/day, with a maximum dose of 100 mg SC per administration; canakinumab 300 mg SC was used in one study; no studies were reporting the use of rilonacept. Anti-IL-1 drugs were administrated at least as third-line therapy, after ASM ($n = 37$), corticosteroids ($n = 32$), immunosuppressive agents (sirolimus, $n = 1$), ketogenic diet ($n = 23$), intravenous immunoglobulins ($n = 28$), rituximab ($n = 7$), or plasma exchange ($n = 14$). Two (=2) patients underwent deep brain stimulation and one underwent surgery for DRE. Regarding the timing of administration, anti-IL-1 was started early in 32 cases, with a median delay of 20 days from the onset of seizures. In the remaining five cases, anti-IL-1 treatment was administrated after more than 4 months. Except for five cases in which there weren't improvements (three of them died after drug discontinuation) (105, 110, 114), the use of anti-IL-1 was effective in reducing the seizure burden. Indeed, its use significantly reduced seizures

TABLE 4 | Systematic review of anti-TNF agents in epilepsy.

References	Disease	PTS *N* Age	Study design	Intervention	Timing	Efficacy	Safety
Lagarde et al. (127)	Rasmussen encephalitis.	11 [median 6.5 years (1.5–37)]	Multicenter, open-label, prospective study	Adalimumab 24 mg/m^2 s.c. (maximum 40 mg), every 14 days	Start: Median delay 31 months (range 1–192) after diagnosis	Complete response (>50% seizure frequency decrease) in 5 patients; 3 of these 5 patients had stabilization of functional deficits. One patient showed significant but transitory (6 months) improvement.	One patient discontinued adalimumab due to mild increase of creatine kinase blood levels (normalized after 3 weeks); one patient showed superficial skin infection (not requiring discontinuation of treatment).

(>50%) in 11/15 of the patients described in the cohort by Lai et al. with available clinical data (110), and in 9/12 of the patients described in case reports (**Table 2**). Concerning safety issues, the most frequently reported adverse effect was the development of infections, which have been reported in 11 patients, while four patients experienced drug rash with eosinophilia and systemic symptoms (DRESS) (110, 112). Regarding the cognitive, motor, and behavioral recovery, although in most of the patients presented in case reports (9/12) an improvement was reported, the outcome has been mostly described qualitatively. The study by Lai et al. evidenced that, among the 22 surviving patients, 50% had motor deficit, 77% attention deficit, and more than 50% of the patients had speech, memory, or executive function deficit. In this study, 45% of the surviving patients had mild or moderate disability measured using the pediatric cerebral performance category (PCPC) scale, and 22,7% had a severe disability or vegetative state. Additionally, all the surviving patients in this study have DRE at last follow-up (110). The cytokine serum and CSF levels are reported in 13/37 (35%) and 14/37(37,8%) of the patients, respectively. In the analysis of patients treated with anti-IL drugs, we evidenced some potential biases deriving from missing data, as seizure frequency was not reported for all the included patients (particularly, data are not available for 10/25 patients in the cohort by Lai et al.) (110). Additionally, the lack of use of specific assessment scales did not allow a deeper evaluation of the motor, behavioral and cognitive outcome.

Systematic Literature Review: Anti-IL-6 Agents and Epilepsy

Study Selection

Overall, 32 references were identified using the search strategy. The full text of these 34 articles was retrieved and evaluated in detail, and 25 articles were excluded based on the exclusion criteria. Nine articles were finally included in this review (**Figure 4**), for a total of 16 patients with epilepsy with different etiologies (114, 117–124). Two studies, performed on patients with autoimmune encephalitis and anti-N-methyl-D-aspartate receptor (NMDAR) encephalitis, were excluded as epilepsy was not considered as a specific outcome of the study, although the studies evidenced a global disease activity improvement after treatment with tocilizumab (125, 126). All publications were in English, three from the USA, four from Europe, and two from Asia. Eight publications were case reports, another ($n = 1$), was a prospective study.

Results

Among the 16 patients included in this systematic review, the median age was 23 years. Patients suffered from NORSE ($n = 10$), SRSE ($n = 2$), DRE associated with LSCS ($n = 2$), limbic encephalitis with anti-CASPR2 antibodies ($n = 1$), and FIRES ($n = 1$) (**Table 3**). Tocilizumab was administered in all 16 cases either SC or IV. No other anti-IL-6 was used. Tocilizumab was administered at least as a third-line therapy, after ASM ($n = 16$), corticosteroids ($n = 14$), immunosuppressive agents ($n = 2$), ketogenic diet ($n = 3$), intravenous immunoglobulins ($n = 14$), plasma exchange ($n = 5$), or others (anakinra $n = 1$, electroconvulsive therapy $n = 1$). Tocilizumab was started

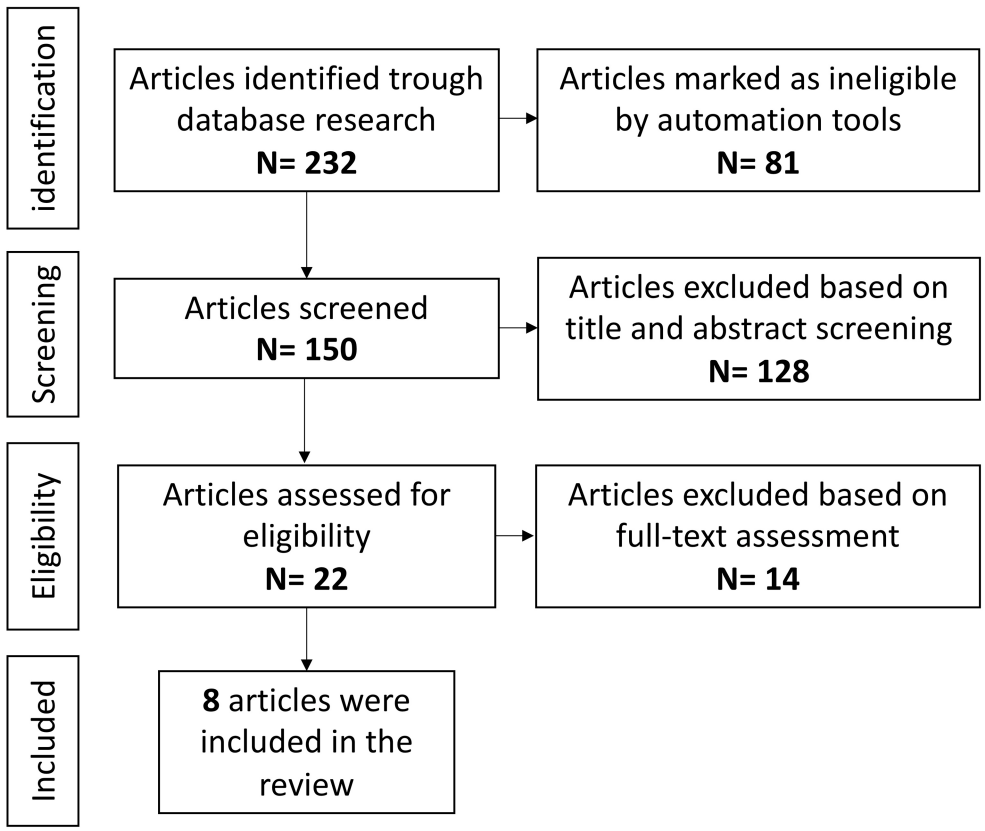

FIGURE 6 | Anti-CD20 agents and epilepsy, literature review.

within the first 6 months of illness in 15 cases (median: 25 days). In only one patient tocilizumab was administered after more than 12 months from the disease onset (**Table 3**). From the available data, it emerges that the use of anti-IL-6 was effective for partial or complete seizure control in most patients [6/7 of the patients described in the cohort study by Jun et al. (124), 9/9 of the patients described in case reports showed reduction or arrest of the seizures]. Concerning safety, although tocilizumab has been well-tolerated by the majority of the patients, our analysis evidenced three cases of infection (one pneumonia and two cases of sepsis), and two patients developed leukopenia (123, 124). The motor, behavioral, and cognitive outcome has been reported mostly with qualitative assessment, showing a reported improvement in seven out of the nine single case reports, with the persistence of behavioral dysregulation (114), mild cognitive impairment, and mild ataxia (118) in two patients. In the study by Jun et al. and the case presented by Benucci et al. the modified Rankin scale (mRS) was used, demonstrating an improvement at follow-up in 4/7 survived patients (122, 124). The dosage of serum and CSF cytokines was available each in 10/16 (62,5%) of the patients. Also in the study of patients treated with tocilizumab, the risk of biases (particularly, missing data) is present, with data being often presented only with qualitative assessment. Additionally, the high rate of therapeutic response in case reports suggests the possibility of a publication bias.

Systematic Literature Review: Anti-TNFα Agents and Epilepsy

Study Selection

Overall a total of 142 references were identified during the initial electronic search, nine of which were marked as not eligible by automation tools because they were not published in English language.

A total of 133 potentially eligible studies were selected. Among these, 132 were excluded (**Figure 5**). One study resulted eligible and was hence included in this review: a multicenter, open-label, prospective study (127).

Results

A total of 11 patients with RE were included in the study (**Table 4**). The median age at first seizure was 6.5 years (range 1.5–37 years). All patients received adalimumab SC (24 mg/m^2 with a maximum of 40 mg) every 14 days. Before adalimumab, patients received treatment with corticosteroids ($n = 11$), in addition to immunoglobulins ($n = 8$) and azathioprine ($n = 1$). The Anti TNF-α agent was started with a median delay of 31 months after the first seizure (range 1 month-16 years). The primary outcome was the decrease of seizures frequency, considering "responders" patients experiencing a decrease in seizure frequency by at least 50%. The secondary outcome measures were neurologic and cognitive outcomes and side

TABLE 5 | Systematic review of anti-CD20 agents in epilepsy.

References	Disease	*N*, (age)	Study design	Intervention	Timing	Efficacy	Safety
Cheli et al. (134)	Anti-LGI1 encephalitis with DRE	1 (54 years old)	Case Report	Rituximab 1,000 mg/day IV, 2 doses 15 days apart then one single dose after 6 months.	Start: 7 weeks after the onset of seizures. Stop: 9 weeks after the onset of seizures.	No further seizures occurred after the treatment. The neuropsychological evaluation resulted within normal range. After 6 months, the patient experienced a cognitive relapse that resolved after the administration of a single dose of Rituximab.	No adverse effects
Kurukumbi et al. (133)	Patient 1: anti-NMDR encephalitis with RSE Patient 2: anti-LGI1 encephalitis with DRE Patient 3: N-type anti-VGCC encephalitis with RSE	3 Pt 1: 32 years Pt 2: 72 years Pt 3: 19 years	Case Series	Pt 1: Rituximab 375 mg/m^2/day IV weekly for 4 weeks, then rituximab 1,000 mg IV every 6 months Pt 2: Rituximab 1,000 mg IV every 6 months Pt 3: Rituximab 1,000 mg IV every 6 months	Pt 1, start: 27 days after the onset of seizures. Pt 2, start: 12 months after diagnosis Pt 3, start: 5 days after the onset of seizures.	Pt 1: resolution of seizures and behavioral disorders, with a return to baseline cognition and personality Pt 2: electrographic and clinical seizure freedom with return of premorbid cognitive function Pt 3: abrogation of seizures and return to baseline functioning	No adverse effects
Sansevere et al. (132)	RE	1 (11 years old)	Case Report	Rituximab 375 mg/m^2 weekly for 4 weeks	Start: 5 days after diagnosis Stop: 33 days after diagnosis	No clinical response. Functional hemispherectomy with right hemisphere deafferentation was performed 3 months after the final dose of rituximab	No adverse effects
Schneider et al. (131)	Anti-NMDAR encephalitis with RSE	1 (22 years old)	Case Report	Rituximab 500 mg IV, followed by a second dose after 6 months and a third after 16 months	Start: not specified Stop: 16 months after the first administration	Complete remission of epileptic seizures and psychotic symptoms	No adverse effects
Jun et al. (124)	NORSE*	6 [median 36 years (22–61)]	Prospective	Rituximab 375 mg/m^2/day IV weekly	Not specified	Persistence of SE despite the treatment. Patients eventually received Tocilizumab	No adverse effects
El Tawil et al. (130)	RE with DRE	1 (61 years old)	Case Report	Rituximab (posology not specified)	Start: 10 years after diagnosis Stop: not specified	Clear and sustained improvement in seizure frequency and severity and patient's disabilities	No adverse effects

(Continued)

TABLE 5 | Continued

References	Disease	*N*, (age)	Study design	Intervention	Timing	Efficacy	Safety
Timarova et al. (129)	RE with RSE	1 (32 years old)	Case Report	Rituximab 375 mg/m^2/day IV weekly (two cycles 22 months apart)	Start: not specified Stop: 22 months after the first administration	Reduction of epileptic seizures with residual 2–3 partial seizures per week. Worsening 18 months after (partial seizures rose to 6 per day). After the second cycle, persistence of one partial seizure per day.	No adverse effects
Byun et al. (128)	AE**	12 [median 32 years (18–68)]	Prospective study	Rituximab 375 mg/m^2/day IV weekly for 4 weeks. The treatment was then repeated every month.	Start: 3–9 weeks after first immunotherapy cycle (with steroid or IVIg).	Remission of seizures in 8/12 patients at 6 months; seizures reduction > 50% in 1/12 patient; no clinical change in 3/12 patients.	Infusion-related reactions (headache, dizziness, chest discomfort) ($n = 2$); rash with pruritus 24 h after the infusion ($n = 1$). This patient discontinued the treatment.

AE, autoimmune encephalitis; DRE, drug-resistant epilepsy; IV, intravenous; IVIg, intravenous immunoglobulins; LGI1, leucine rich glioma inactivated 1; NMDAR, N-methyl-D-aspartate receptor; NORSE, New-onset refractory status epilepticus; RE, Rasmussen encephalitis; SRSE, super-refractory status epilepticus; VGCC, voltage-gated calcium channel.

**One patient suffered from anti-NMDAR encephalitis, in the other cases the disease was cryptogenic.*

***AE in these patients was due to anti-NMDAR (n = 8), anti-LGI1 (n = 3), and anti-Ma2/Ta (n = 1).*

effects of the treatment. Although none of the patients became seizure-free, five patients were considered as responders and another one experienced a transitory improvement in frequency of seizures. In three of the five patients, a stabilization of cognitive decline was observed and two patients had improvement of their motor deficiencies. According to the authors, the response to the treatment seemed to occur more likely in slowly progressive forms of RE and patients with concomitant autoimmune diseases (uveitis and juvenile arthritis). None of the responders underwent hemispherectomy, considering the absence of a severe motor and cognitive deficit. On the other hand, three patients experienced a severe progression of the disease, with the requirement of hemispherectomy. Concerning safety, in 1 patient adalimumab was discontinued due to an elevation of creatine kinase levels and another patient showed a superficial skin infection that did not require interruption of the treatment and was successfully controlled with a local antiseptic (127).

Systematic Literature Review: Anti-CD20 Agents and Epilepsy

Study Selection

Overall a total of 232 references were identified during the initial electronic search, 82 of which were marked as not eligible by automation tools because published before 1 January 2016 and/or not in English language. A total of 150 potentially eligible studies were selected. Among these, 128 were excluded for irrelevancy after a screening of the titles and abstracts (**Figure 6**). After a full-text assessment of the remaining 22 studies, eight of them resulted eligible and were therefore included in this review (124, 128–134). Six publications were case reports and two were prospective studies.

Results

A total of 26 patients with different epileptic disorders were included in this review (**Table 5**). The median age of the patients was 32 years (range 11–72). The patients suffered from RE ($n = 3$) and from AE due to anti-NMDAR ($n = 11$), anti-LGI1 ($n = 5$), anti-voltage-gated calcium channels (VGCC) antibodies ($n = 1$), or onconeural antibodies (anti-Ma2/Ta) ($n = 1$). Four patients presented a cryptogenic RSE. The posology of rituximab was variable within the different cases and in one case it was not specified (**Table 5**). For all patients, rituximab was started as second-line immunotherapy after the administration of IVIg and steroids. The timing of administration was variable and, given the heterogeneity of the diseases, it is difficult to determine homogeneous timepoints of reference for all the patients. Overall, the treatment with rituximab was effective in abolishing or significantly reducing the burden of seizures in 16/26 patients (61,5%), while in 10/26 patients, the therapy was ineffective. One patient with RE underwent functional hemispherectomy with remission of seizures (132). As discussed in Section Results, the six patients from the cohort of Jun et al. received Tocilizumab with the resolution of status epilepticus (124). Of the three patients in the study of Byun et al. that did not show a clinical change in the course of their disease after the administration of rituximab, one deceased for reasons not related to the therapy itself (128). The two others continued to

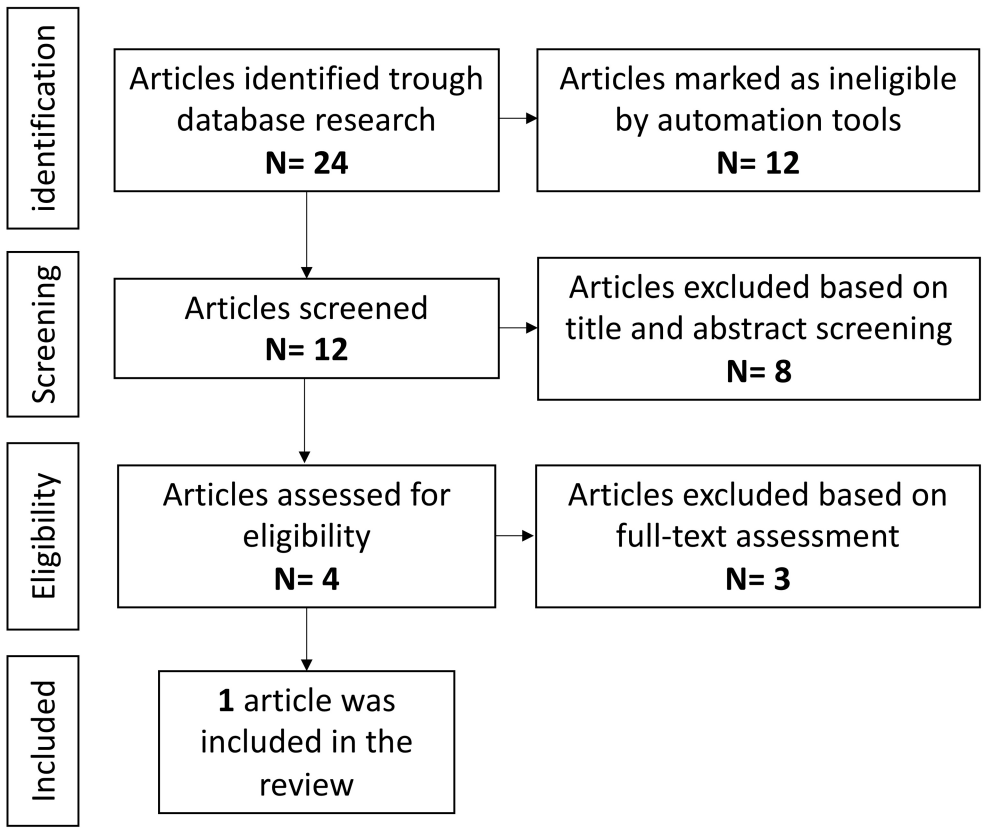

FIGURE 7 | Anti-α4-integrin agents and epilepsy, literature review.

show seizures and there is no mention of additional treatments. Data concerning the behavioral, cognitive, and motor outcomes were available for seven patients (130–134). An improvement was reported in six patients and in five of them the behavioral and cognitive functions were restored to premorbid condition. One patient with RE, albeit improved, showed residual motor and speech dysfunctions (130). In another case, the administration of rituximab resulted ineffective. Finally, only one patient had to discontinue the therapy due to a cutaneous rash associated with pruritus that appeared 24 h after the administration (128). Two other patients experienced mild infusion-related reactions and were able to continue the therapy (126). No adverse effects were reported in the other patients.

Systematic Literature Review: Anti-α4-Integrin Agents and Epilepsy

Study Selection

Overall a total of 24 references were identified during the initial electronic search, 12 of which were marked as not eligible by automation tools because published before 1 January 2016 and/or not in English language. A total of 12 potentially eligible studies were selected. Among these, eight were excluded for irrelevancy after a screening of the titles and abstracts (**Figure 7**). After a full-text assessment of the remaining four studies, three of them resulted ineligible, since the evaluation of seizures frequency and/or severity was not in the outcomes of the studies, or because studies were not concerning patients with RSE or DRE. Therefore, one single study was included in this review, a randomized, placebo-controlled, double-blinded study (phase 2 study OPUS, NCT03283371) (135).

Results

A total of 32 patients with drug-resistant focal epilepsy were included in this review (**Table 6**). The mean age was 42.8 years (±14.56). All patients received natalizumab 300 mg IV every 4 weeks for 24 weeks. The timing of administration was not specified. The primary endpoint of efficacy of the study included was to evaluate the change from baseline in seizure frequency (number of seizures per 28 days) from weeks 8 to 24 of the placebo-controlled period. Overall, the natalizumab-treated group showed a greater reduction in seizure frequency compared to placebo (−14.4%), although the predefined threshold for therapeutic success of 31% relative reduction was not achieved (135). Concerning the secondary endpoints, despite a reduction of ≥50% in seizure frequency from baseline during weeks 8–24 of treatment in 10/32 participants (31.3% compared to 17.6% of the placebo group), none of the participants that received natalizumab remained free from seizures. Reportedly, one patient experienced an inadequate treatment response and withdrew

TABLE 6 | Systematic review of anti-α4-integrin agents in epilepsy.

References	Disease	*N*, (age)	Study design	Intervention	Timing	Efficacy	Safety
French et al. (135)	DRE	32 [mean 42.8 years (±14.56)]	Randomized, placebo-controlled, double-blinded study	Natalizumab 300 mg IV every 4 weeks for 24 weeks	Start: not specified Stop: 24 weeks after the first dose.	Compared to placebo, the natalizumab-treated group showed a greater reduction in seizure frequency from baseline. 10/32 participants showed a reduction of ≥50% from baseline in seizure frequency during weeks 8–24. None of the participants remained free from seizures. One participant experienced an inadequate treatment response (e.g., did not modify ASMs after week 12 of the placebo-controlled period or did not discontinue the treatment after the active run-in period due to lack of efficacy)	24/32 participants experienced adverse effects ranging from mild (15/24), moderate (8/15) and severe (1/32). One participant experienced a serious event (urticaria) and discontinued the treatment.

ASM, anti-seizure medication; DRE, drug-resistant epilepsy; IV, intravenous.

from the treatment (135). Finally, the exploratory endpoints included change from baseline in frequencies of focal to bilateral tonic-clonic seizures and focal seizures. Participants of the natalizumab-treated group showed respectively a decrease of 21.34% and an increase of 6.61% in frequency over placebo. The endpoints of the study did not pertain to the motor, behavioral or cognitive outcome of the participants. Concerning safety, although adverse events were reported in 24/32 participants, ranging from mild (15/24) to moderate (8/15) and severe (1/32), only 2/32 showed events of special interest and only one of these participants discontinued the treatment due to urticaria (135).

Strengths and Limitations

This is the first systematic review exploring the use of anti-cytokine agents and anti-lymphocyte agents in individuals with DRE or RSE. This study has different limitations. Firstly, data on the use of anti-cytokine agents are mostly derived from isolated case reports and case series, with no randomized clinical trials, and the age range of the included patients is considerable. This significantly raises the risk of publication bias, thus favoring the publication of positive reports. Moreover, the administration of concomitant treatments (ASMs, corticosteroids, other immunosuppressive agents) can represent a considerable confounding factor. Therefore, the level of evidence is currently low. However, it is worth highlighting that the administration of anti-IL-1 agents (mainly anakinra) and tocilizumab in patients with severe DRE, such as RSE or FIRES, has shown a reduced seizure burden in most of the described patients, and a good safety profile is usually well-tolerated. Particularly, literature data report that more than 50% of the patients with FIRES respond to anakinra, while tocilizumab has been mostly used in patients with NORSE and SRSE showing partial or complete seizure control in almost all the described cases (**Figure 8**). Therefore, despite the mentioned limitations of this work, literature data suggest considering anti-IL-1 or anti-IL-6 therapeutic approaches in these conditions. Data on the use of adalimumab in patients with RE encourage further studies to assess whether this drug or other TNF-inhibitors could be effective in the treatment of those forms of RE refractory to other immunotherapies, but without the criteria for a surgical approach. Selected patients, like those with slowly progressive forms, may benefit from treatment with Anti TNF-α agents, while less is known regarding their use in other forms of epilepsy since the literature is scarce and studies on animal models are limited. Apart from the effect on seizures, anti-cytokine agents could significantly affect also motor, behavioral, and cognitive recovery, although this outcome has been heterogeneously reported in the different studies, with only four studies analyzing specific scales (110, 122, 124, 127). Notably, the serum and CSF cytokine profile was determined in <50% of the included patients. Although this investigation is not part of routine clinical practice (difficulty of interpretation, limited availability) the increasing knowledge of the involvement of cytokines in DRE and RSE could lead to a more diffuse use of this dosage, to provide a therapeutic strategy targeted on the main mediator involved in the individual patient.

The administration of Rituximab resulted to be effective in the majority of patients with DRE and RSE, with a more conspicuous

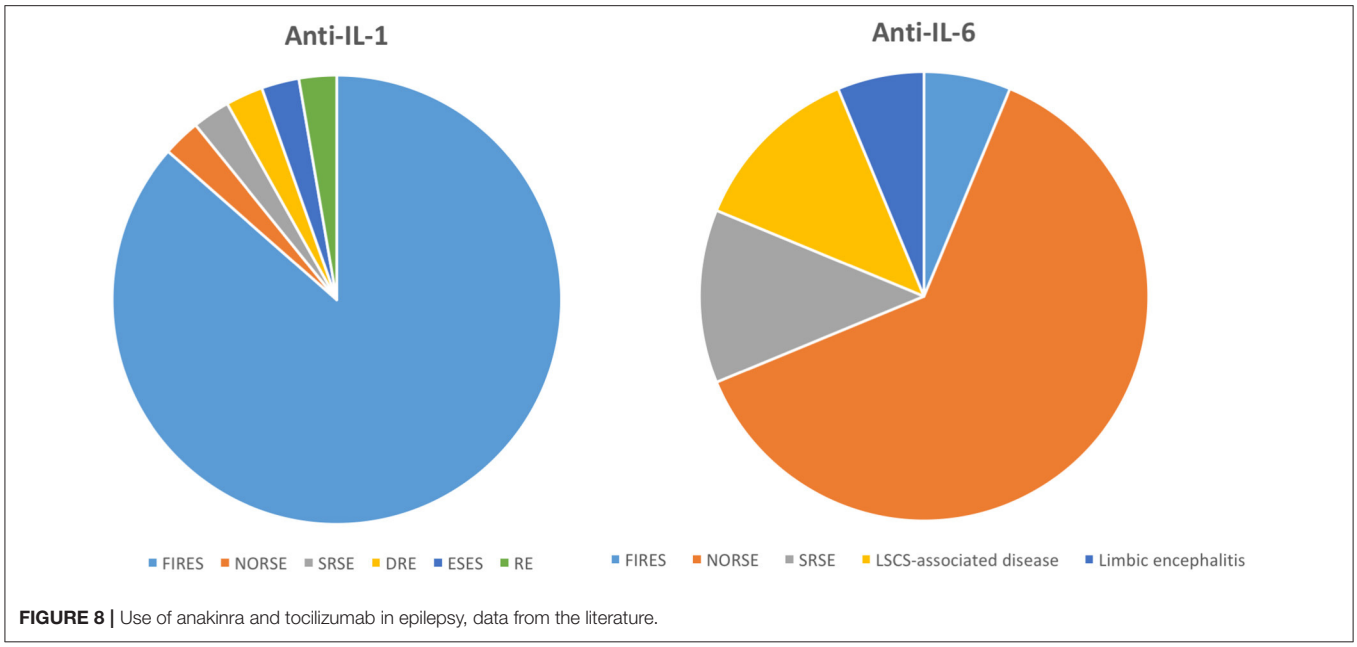

FIGURE 8 | Use of anakinra and tocilizumab in epilepsy, data from the literature.

response in individuals affected by AE-related seizures. Once more, a possible limitation and source of bias comes from the fact that studies concerning the use of Rituximab consit mainly in case reports or case series. This may also be the cause of the considerable heterogeneity in the timing and posology of the treatment that was evidenced. Future studies in this field should prioritize the definition of an appropriate treatment regimen.

Although targeting the leukocyte extravasation to brain parenchyma appeared to be a creditable therapeutic opportunity for patients with intractable epilepsy, the results of a randomized, double-blinded, placebo-controlled study showed that the administration of natalizumab did not significantly change seizure frequency in adults with drug-resistant focal epilepsy (135). Future trials, with a larger sample size, may overcome the limits of this latter study in detecting statistically significant differences.

OTHER INFLAMMATORY TARGETS AND EPILEPSY: FUTURE THERAPEUTIC PERSPECTIVES

Preclinical research is focusing on the identification of novel therapeutic targets for the treatment of neuroinflammation. In this regard, the role of agents targeting the human high-mobility group box-1 (HMGB-1) and chemokines are of particular interest. HMGB-1, a regulator of gene transcription and DNA remodeling/repair (136), mediates inflammatory responses *via* interactions with the receptor for advanced glycation end products (RAGE) and toll-like receptor (TLR) 4. It promotes the release of pro-inflammatory cytokines (e.g., TNF-α and IL-6) and acts as a key initiator of inflammation particularly within the brain (136–138). Anti-HMGB1 mAbs have proven to be effective in different mouse models of epilepsy (138–140), reducing also the chronic inflammatory pathways (upregulation of inflammation-related genes, microglial activation, and neuronal cell death). Chemokines are involved at different levels in CNS homeostasis, and have been implicated in the pathogenesis of different CNS diseases, including epilepsy (26, 141). In animal models of epilepsy, the administration of a CCL2 transcription inhibitor (Bindarit) or a selective antagonist of the CCR2 receptor (RS102895) suppressed the LPS-induced seizure enhancement (142, 143). Additionally, CX3CL1/CX3CR1 and the CC-chemokine receptor CCR5, widely expressed in the CNS microglia, have been implicated in the pathogenesis of epilepsy and represent other potential therapeutic targets (144–147). Overall, these findings, albeit resulting form preclinical experiments, might build the basis for new therapeutic strategies in the upcoming years.

CONCLUDING REMARKS

The involvement of the immune and inflammatory response in the pathogenesis of DRE and RSE are being progressively elucidated, leading to the increasing use of anti-cytokine agents in the treatment of these conditions. The experience with anti-IL-1, anti-IL-6, and anti-TNF drugs in the treatment of epilepsy is still limited and derives mostly from the observation of isolated case reports or small case series. In this work, we performed a systematic review that, despite the low evidence reached, showed promising results regarding the use of anti-cytokine agents in specific patients with DRE and RSE in terms of both efficacy and safety.

Beyond these anti-cytokine agents, there is increasing interest in the use of drugs targeting cells of adaptive immunity. The experience in the use of rituximab in RSE and DRE is fragmentary and there is a lack of a uniform regimen of

treatment but this drug resulted to be effective in the major part of patients. Concerning the employment of natalizumab, the experience is limited to a single study that did not evidence a significant advantage in patients with DRE receiving the treatment.

Hopefully, preclinical research advances will allow the identification of new therapeutic strategies targeting neuroinflammation in epilepsy, and the collection of a larger number of clinical data will help in identifying those patients who will benefit from anti-cytokine treatments.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

GC, GD, and AM: study design, data collection, statistical analysis, data interpretation, manuscript preparation, and literature search. TF, AO, DP, and AB: study design, data interpretation, and manuscript revision. PS, GM, SS, and RC: study design, supervision, and manuscript revision. AR: data collection, statistical analysis, data interpretation, and manuscript preparation. All authors contributed to the article and approved the submitted version.

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Anti-Epileptic Effect of Crocin on Experimental Temporal Lobe Epilepsy in Mice

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Temporal lobe epilepsy (TLE) is a common kind of refractory epilepsy. More than 30% TLE patients were multi-drug resistant. Some patients may even develop into status epilepticus (SE) because of failing to control seizures. Thus, one of the avid goals for anti-epileptic drug development is to discover novel potential compounds to treat TLE or even SE. Crocin, an effective component of *Crocus sativus L.*, has been applied in several epileptogenic models to test its anti-epileptic effect. However, it is still controversial and its effect on TLE remains unclear. Therefore, we investigated the effects of crocin on epileptogenesis, generalized seizures (GS) in hippocampal rapid electrical kindling model as well as SE and spotaneous recurrent seizure (SRS) in pilocarpine-induced TLE model in ICR mice in this study. The results showed that seizure stages and cumulative afterdischarge duration were significantly depressed by crocin (20 and 50 mg/kg) during hippocampal rapid kindling acquisition. And crocin (100 mg/kg) significantly reduced the incidence of GS and average seizure stages in fully kindled animals. In pilocarpine-induced TLE model, the latency of SE was significantly prolonged and the mortality of SE was significantly decreased by crocin (100 mg/kg), which can also significantly suppress the number of SRS. The underlying mechanism of crocin may be involved in the protection of neurons, the decrease of tumor necrosis factor-α in the hippocampus and the increase of brain derived neurotrophic factor in the cortex. In conclusion, crocin may be a potential and promising anti-epileptic compound for treatment of TLE.

Keywords: temporal lobe epilepsy, crocin, kindling, pilocarpine, spontaneous recurrent seizure

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INTRODUCTION

Although several new anti-epileptic drugs (AEDs) have been developed, still 30% patients (even 75% in mesial TLE) remain resistant to many kinds of AEDs and gradually progress to refractory epilepsy (Wang et al., 2018; Wang and Chen, 2019; Xu et al., 2021a). Some patients may even develop into status epilepticus (SE) because of failing to control seizures. For these patients, epileptic foci resection surgery is an available treatment; however, it is not suitable for many cases due to unacceptable neurological or cognitive impairments (Wang et al., 2021). Therefore, it is essential for seeking novel effective AEDs.

Natural products from traditional Chinese medicine have emerged to be a promising choice for the treatment of epilepsy. Several natural products have been reported to be effective on experimental

epilepsy and associated neurobehavioral comorbidities (Sucher and Carles, 2015; Bo-Qiang et al., 2018). Saffron, the dried stigmas of *Crocus sativus L. (*Iridaceae*)*, is being widely used in traditional medicine for a wide variety of neurological conditions (Singh, 2015). Crocin is considered as one of the main effective components of saffron with a low toxicity (Bostan et al., 2017). Extensive preclinical and clinical studies about its traditional use have been reported, such as anti-inflammation (Teng et al., 2021), anti-tumor (Mousavi et al., 2009), sedation and hypnosis (Masaki et al., 2012), anti-depression (Amin et al., 2015), anti-anxiety (Pitsikas et al., 2008) and anti-Parkinson's disease (Rao et al., 2016). Several papers also reported its anti-epileptic activity in experimental epilepsy. Tamaddonfard et al. (2012) found crocin significantly inhibits epileptiform activities induced by penicillin. Recently, Mazumder et al. (2017) also reported that crocin can attenuate pentylenetetrazole (PTZ) kindling development. However, Hosseinzadeh and Talebzadeh (2005) found crocin is ineffective on PTZ induced seizures. Based on this controversy, it is necessary to further evaluate the anti-epileptic effect of crocin in more epileptic models (Alavizadeh and Hosseinzadeh, 2014).

In previous report, we have found that low-dose crocin can retard the progression in hippocampus rapid kindling acquisition in C57BL/6J mice, while high-dose crocin relieved the generalized seizures (GS) in fully-kindled mice (Wang et al., 2017a). The results suggested that the crocin may have a potential anti-epileptic effect in experimental TLE. However, two questions are still unclear. The first one is that the most patients suffer the spontaneous recurrent seizures (SRS), while the effect of crocin on SRS is still unknown. A pilocarpine model of mice, which is considered to mimic SE and SRS of patients, is always utilized to study the epileptogenesis and screen new AEDs (Zhong et al., 2012; Xu et al., 2016). So we first aim to investigate the anti-epileptic effect of crocin on the SE and SRS in pilocarpine-induced model in the present study. The second one is that although several hypotheses on the underlying anti-epileptic target of the crocin have been provided by some reports, it remains not very clear (Ahmed et al., 2020). Based on our preliminary findings, we speculate that brain derived neurotrophic factor (BDNF), or some inflammatory factors, such as interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α), may be potential targets for the anti-epileptic effect of crocin. Hence, we also aim to explore the underlying anti-epileptic target of crocin in this study.

MATERIALS AND METHODS

Animals

Male ICR mice were purchased from Shanghai SLAC Laboratory Animal Co., Ltd., weighing 25–35 g, raised at the Laboratory Animal Center of Hangzhou Medicine College. Mice were housed in individual cages with a 12-h light/dark cycle (lights on from 08: 00 to 20:00). Water and food were provided ad libitum. Experiments were carried out between 10:00 and 17:00 in each day. All experiments were approved by the Hangzhou Medicine College Animal Experimentation Committee and were in complete compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Surgery

Under 1% pentobarbital sodium anesthesia (40 mg/kg, i.p.), mice were mounted in a stereotaxic apparatus (512600, Stoelting, United States). Electrodes were implanted into the right hippocampus (AP: −2.9 mm, L: −3.0 mm, V: −3.0 mm) (Zhao et al., 2020). The electrodes were made of twisted stainless steel Teflon-coated wires (diameter 0.2 mm, A.M. Systems, United States) insulated except at the tip (0.5 mm); the tip separation was about 0.5 mm. The electrodes were connected to a miniature receptacle, which was attached to the skull with dental cement. After surgery, mice were allowed 7 days of recovery.

Hippocampal Rapid Electrical Kindling Procedure

As described by the previous papers (Jin et al., 2013; Wang et al., 2017b), electrical stimulations were delivered by a constant-current stimulator (SEN-7203, SS-202J, Nihon Kohden, Japan) and electroencephalograms (EEG) were recorded with a Neuroscan system (Compumedics, Melbourne, Australia). The afterdischarge threshold (ADT) was determined by an application of a 2 s train of 1 ms monophasic square-waves at 20 Hz, beginning at 40 μA and increasing by 20% which were given at 1 min intervals until an electrographic afterdischarge (AD) lasting at least 5 s was elicited. Only the animals with an ADT less than 400 μA can be used for following experiments. Six hippocampal kindling stimulations (1 ms pulses, 20 Hz frequency, 2 s duration) at 30 min intervals with an intensity of 400 μA daily were delivered for 8 days.

Behavioral seizures were scored according to Racine's scale (Racine, 1972), as modified for the mouse: stage 0, no response or behavior arrest; stage 1, chewing or facial twitches; stage 2, chewing and head nodding; stage 3, unilateral forelimb clonus; stage 4, bilateral forelimb clonus and rearing; stage 5, rearing and falling. Seizure stages 1–3 indicate focal seizures, while stages 4–5 are considered as GS (Engel et al., 1978). When mice exhibited three consecutive stage 5 seizures, they were regarded as fully kindled. In addition, the AD duration (ADD) was recorded as well.

Assessment of the Effects of Crocin on Kindling Acquisition

Mice were randomly divided into four groups matched for their ADTs. The initial ADTs of most mice ranged from 100 to 200 μA, thus there was no significant variability in the initial ADTs of mice in each group. In group 1–3, 10, 20 or 50 mg/kg crocin (Sigma-Aldrich, St. Louis, MO, United States) was respectively delivered i.p. 30 min prior the first electrical stimulation (methods and parameters were described above) of each day. In group 4, equivalent 0.9% saline (NS) was instead delivered as a vehicle control. Schematic timeline of the experiment is shown as **Figure 1A**. Seizure stages were recorded by two experimenters

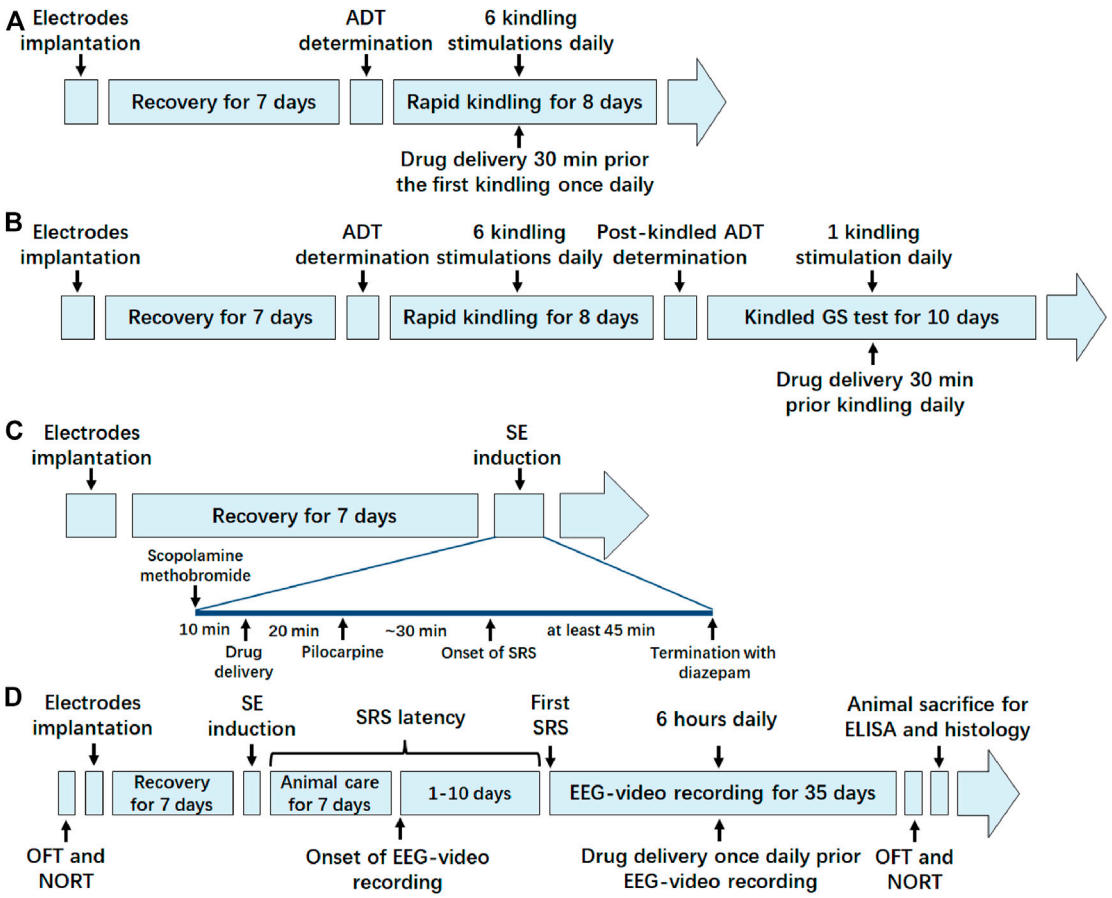

FIGURE 1 | Schematic timeline diagram of experiments in each animal model. **(A)** Timeline of the experiment in hippocampal rapid kindling model; **(B)** timeline of the experiment in fully kindled model; **(C)** timeline of the experiment in pilocarpine-induced SE model; **(D)** timeline of the experiment in pilocarpine-induced SRS model. ADT, after discharge threshold; GS, generalized seizure; SE, status epilepticus; SRS, spontaneous recurrent seizure; OFT, open field test; NORT, novel object recognization test; EEG, electroencephalograms; ELISA, enzyme linked immunosorbent assay.

who were blinded to the grouping and drug delivery after each stimulation. ADD was subsequently calculated according to EEG recorded as described above. The number of stimulations required to reach and remain at each seizure stage was also calculated to analyze the stepwise progression of kindling seizures (Jin et al., 2013; Chen et al., 2020).

Assessment of the Effects of Crocin on Fully Kindled GS

The post-kindled ADT of fully kindled mice (without any drug during kindling) was determined by the same procedure used for kindling ADT. On the next day, mice were divided into four groups. In group 1–3, 20, 50 or 100 mg/kg crocin (i.p.) was delivered 30 min prior the once-daily electrical stimulation (parameters were described above) for 10 days. In group 4, equivalent NS was instead delivered as a vehicle control. Schematic timeline of the experiment is shown as **Figure 1B**. The seizure stage and ADD were measured by the same procedures used for kindling seizures. GS duration (GSD) was also calculated from the onset to the termination of GS. In addition, the incidence of GS and average seizure stage during 10 days were calculated (Wang et al., 2017a).

Assessment of the Effects of Crocin on Pilocarpine-Induced SE

Schematic timeline of the experiment is shown as **Figure 1C**. Mice with electrodes implantation at the hippocampus were randomly divided into four groups. Scopolamine methobromide (1 mg/kg, i.p., Sigma-Aldrich, St. Louis, MO, United States) was administrated 30 min before SE induction and then crocin (50, 100 or 200 mg/kg, i.p) or equivalent NS was given 20 min prior to pilocarpine. Pilocarpine (350 mg/kg, i.p., Sigma-Aldrich, St. Louis, MO, United States) was administrated to induce SE. When mice exhibited continuous tonic-clonic seizures and epileptic spikes in EEG, usually following with stage 4–5 seizures, they were defined as the onset of SE. The surviving mice with SE lasting at least 45 min were followed by diazepam (5 mg/kg, i.p. KingYork, TianJin, China) to terminate the SE (Curia et al., 2008; Xu et al., 2020). The mice, which did not experience stage 4 or 5 seizures following the

pilocarpine injection or did not maintain the seizure intensity (less than 3 times stage 4 or 5 seizures in 45 min) were excluded. The latency of SE and the mortality of mice in each group were recorded by two experimenters who were blinded to the grouping and drug delivery.

Assessment of the Effects of Crocin on Pilocarpine-Induced SRS

Schematic timeline of the experiment is shown as **Figure 1D**. SE was induced by pilocarpine as described above. After termination with diazepam, surviving mice were rehydrated with NS (4 ml/kg, i.p.) and given special care for the next 7 days by feeding powdered feed and water (i.g. if necessary). The untreated group was given an equal volume of NS instead of pilocarpine, and the rest of the operation was performed as above. From day 8 after induction of SE, SRS of the surviving mice were recorded by EEG-video for 6 h daily (11:00–17:00). SRS was defined as repetitive epileptic spikes with a frequency >5 Hz and a duration >20 s on EEGs recorded by a Powerlab system (AD Instruments, Bella Vista, Australia), accompanied by a stage 4 or 5 behavioural seizure, which was assessed by synchronous video recordings (Curia et al., 2008; Xu et al., 2020). The mice with SRS divided into three groups: NS, sodium valproate (VPA, 200 mg/kg, i.p.) and crocin (100 mg/kg, i.p.) group. Drugs were administrated once daily for 35 days from the observation of the first SRS and an equal volume of NS intraperitoneally was injected into the mice of NS group. The mice in the untreated group were also subjected to the above-mentioned video recording and NS injection. The number of SRS, the duration of each seizure and the seizure stage were measured. The seizure stage was also referenced to the Racine score (Racine, 1972). Some mice, which experienced SE but never observed SRS during the observation period, were not significantly different in seizure severity during SE compared with SRS mice, and were divided into the non-SRS group (nSRS) as a control group in subsequent experiments.

Open Field Test

The OFT method refers to our previous study (Ye et al., 2017). Briefly, the mice were habituated the laboratory environment 1 day before OFT. Next day, mice was gently placed into the center of the OFT chamber (40 cm × 40 cm × 40 cm, Xinruan, Shanghai, China). Each mouse was allowed to habituate the chamber for 5 min, and then allowed to move freely for 5 min with video recording. The total travelling distance, the travelling distance in the central zone and the duration in the central zone were measured. OFT, which was performed on the same mice as the previous study of SRS, was respectively examined before the pilocarpine-induced SE and after completing all drugs administration.

Novel Object Recognization Test

The NORT method refers to our previous study (Zhong et al., 2020). Briefly, the mice were habituated the laboratory environment 1 day before NORT. Next day, mice was gently placed into the center of the NORT chamber (40 cm × 40 cm × 40 cm, Xinruan, Shanghai, China). Each mouse was allowed to habituate the chamber for 5 min, and then two identical objects F1 and F2 were placed in the two opposite corners of the field. Then, free movement of each mouse was recorded for 5 min (Test 1, T1), followed by returning the mouse to the cage. After 90 min, F2 was replaced with a new object N (different color from F2), and the mouse was placed into the chamber again for 5 min (Test 2, T2). The behavior of each mouse in T1 and T2 were recorded by video and the software of the NORT chamber was used to analyze the duration of exploring the three objects, and the distance from the tip of the mouse's nose to the object ≤2 mm was identified as the criterion for exploratory behavior. Discrimination ratio (DR) and discrimination index (DI) was calculated with the following formula. DR = [N or F2/(N or F2 + F1)] × 100%. DI = [(N or F2 - F1)/(N or F2 + F1)]. N, F1, and F2 in the above equations are the duration of exploring each object, respectively. NORT, which was performed on the same mice as the previous study of SRS, was respectively examined before the pilocarpine-induced SE and after completing all drugs administration as well.

Enzyme Linked Immunosorbent Assay

After the last behavioral test, half of the mice in each group were anesthetized with 1% pentobarbital sodium anesthesia (40 mg/kg, i.p.). Then the animals were sacrificed and the brain tissues were quickly removed. The left and right cortex and hippocampus were separated on the ice, weighed and homogenized in NS at a ratio of 1:9 (w/v), centrifuged at 3000 rpm for 20 min. Then, the supernatants were extracted. The commercially available ELISA kits (Elabscience Biotechnology Co., Ltd., Wuhan, China) for BDNF, TNF-α and IL-1β were used to detect the contents of the three molecules according to the instructions.

Histology and Neuronal Damage Examination

After the last behavioral test, the other half of the mice in each group were anesthetized with 1% pentobarbital sodium anesthesia (40 mg/kg, i.p.). The animals were sacrificed and the brain tissues were removed following with perfusion through the left ventricle with NS for 20 min and 4% paraformaldehyde for 30 min. The brain tissues were merged in 4% paraformaldehyde for 24 h, and dehydrated with 30% sucrose solution for 2–3 d. The sections were sliced with a thickness of 12 μm on a frozen microtome. After dried, the slices were stained with 1% cresyl violet, and graded with 0.5% hydrochloric acid alcohol, 70%, 90% and 100% alcohol. After decolorization, sealing solution (neutral gum: xylene = 1:1) was used to seal the slices and the hippocampal area was photographed with microscope to compare the neuronal damage in the same area of mice in each group.

At the end of the experiments, electrode placements were histologically verified as well. Brain sections were cut (10 μm) and stained with toluidine blue O. Only the mice with electrodes correctly lying within the hippocampus were included in the statistical analysis. In our experiments, 184 out of 217 mice had electrodes correctly located in the target.

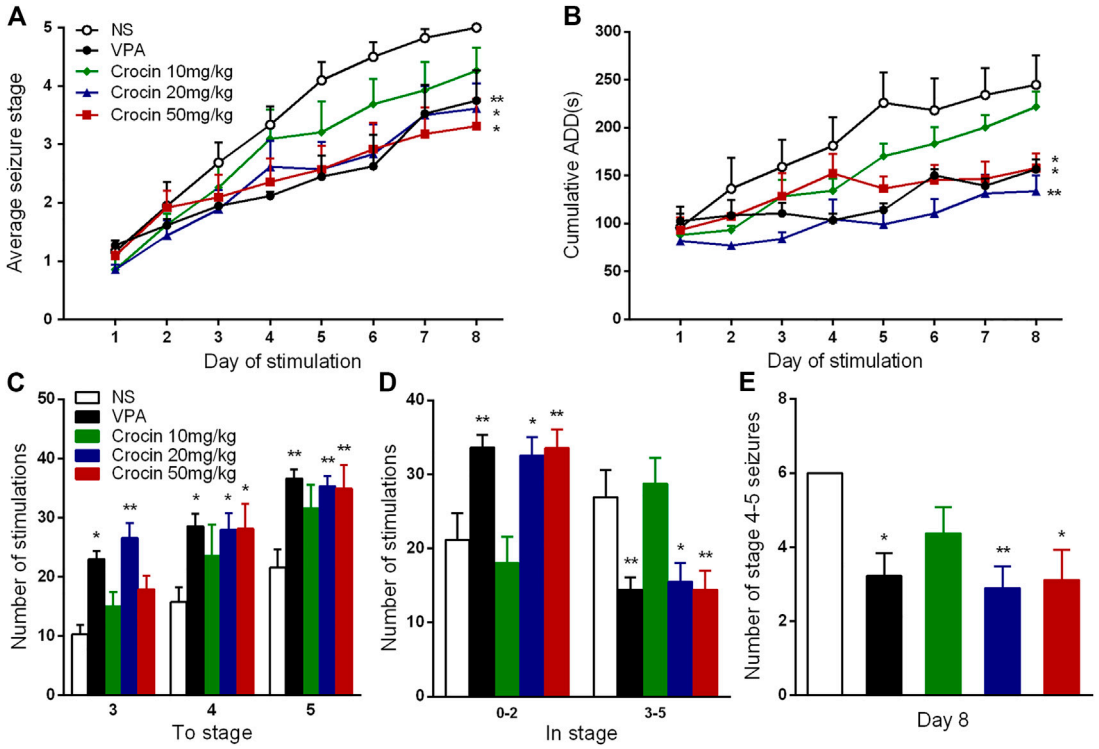

FIGURE 2 | Effects of crocin on hippocampal rapid kindling acquisition (n = 8 for each group, x±S.E.M.). **(A)** Average seizure stage; **(B)** cumulative ADD of six stimulations each day; **(C)** the number of stimulations to reach seizure stage 3, 4, 5; **(D)** the number of stimulations to stay in seizure stage 0–2 and 3–5 during kindling acquisition; **(E)** Number of stage 4–5 seizures in the 8th day. ADD, afterdischarge duration; NS, normal saline; VPA, sodium valproate. Values are from 3 independent experiments. *p < 0.05, **p < 0.01, compared with NS group. Two-way ANOVA was performed for **(A,B)**. Comparisons of the number of stimulations for each seizure stage **(C,D)** were made with the nonparametric Mann–Whitney U test. One-way ANOVA for repeated measures was used followed by Tukey's t-test for **(E)**.

Statistical Analysis

The statistical analysis was performed using SPSS 17.0 software. Two-way analysis of variance (ANOVA) was performed for repeated measures in the analysis of group differences in kindling and fully kindled model. Comparisons of the number of stimulations for each seizure stage were made with the nonparametric Mann–Whitney U test. The $\chi 2$ test was used to compare the incidence of GS and the mortality of pilocarpine-induced SE. Two-way ANOVA was also performed in the analysis of OFT and NORT data. One-way ANOVA was used with Tukey's t-test in the analysis of other data. Data are presented as mean ± standard error of mean (S.E.M.). $p < 0.05$ indicates a statistical significance.

RESULTS

Crocin Retards the Kindling Acquisition in ICR Mice

Compared with the NS group, 20 and 50 mg/kg crocin significantly retarded the progression of the daily average seizure stages ($p < 0.05$, both, **Figure 2A**) and shortened the cumulative ADD per day ($p < 0.01$ and $p < 0.05$, **Figure 2B**). In **Figure 2B**, 20 mg/kg crocin seemed to be more efffective on reducing the cumulative ADD than 50 mg/kg crocin, although there was not a significant difference. These effects were comparable to that of the positive drug VPA ($p < 0.01$ and $p < 0.05$, **Figures 2A,B**). Although the effect of 10 mg/kg crocin was yet not significant, it seemed to be a tendency to retard kindling acquisition in the late period of kindling.

To further analyze the possible therapuetic window of crocin, we also calculated the number of stimulations required to each stage and stayed in each stage. Both 20 and 50 mg/kg crocin increased the number of stimulations required to reach the stage 4 ($p < 0.05$, both, **Figure 2C**) and 5 ($p < 0.01$, both, **Figure 2C**); meanwhile, the number of stimulations in stage 0–2 was increased ($p < 0.05$ and $p < 0.01$, **Figure 2D**) and the number of stimulations in stage 3–5 was relatively decreased ($p < 0.05$ and $p < 0.01$, **Figure 2D**) in 20 and 50 mg/kg crocin group, compared with the NS group. 20 mg/kg crocin also increased the number of stimulations required to reach stage 3 ($p < 0.01$, **Figure 2C**). In the last 6 kindling stimulations during the last day, the number of stage 4–5 seizures in 20 and 50 mg/kg crocin group was significantly decreased ($p < 0.01$ and $p < 0.05$, **Figure 2E**), compared with the NS group. These data suggested that crocin may retard the progression of epilepsy in ICR mice.

Crocin Alleviates the Fully Kindled GS in ICR Mice

Compared with the NS group, 100 and 200 mg/kg crocin can significantly reduce the incidence of GS ($p < 0.001$, both,

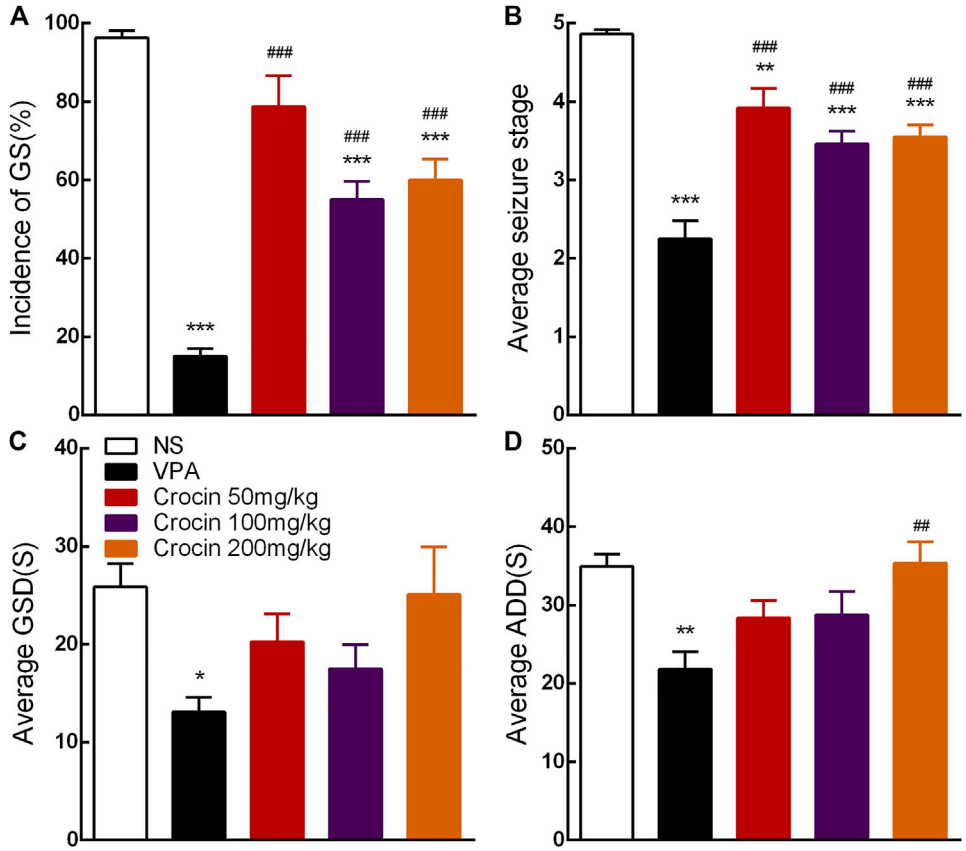

FIGURE 3 | Effects of crocin on the incidence of GS and seizure severity in fully kindled mice ($n = 8$ for each group, x±S.E.M.). **(A)** Mean incidence of GS, **(B)** average seizure stage, **(C)** average GSD and **(D)** average ADD during 10 days. GS, generalized seizure, GSD, generalized seizure duration, ADD, afterdischarge duration; NS, normal saline; VPA, sodium valproate. Values are from 3 independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, compared with NS group; $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$, compared with VPA group. One-way ANOVA was used for **(B–D)**, followed by Tukey's t-test. The $\chi 2$ test was used for **(A)**.

Figure 3A) and suppress the average seizure stage ($p < 0.001$, both, **Figure 3B**), but it did not affect average GSD ($p > 0.05$, **Figure 3C**) or average ADD ($p > 0.05$, **Figure 3D**). 50 mg/kg crocin only suppressed the average seizure stage ($p < 0.01$, **Figure 3B**). However, compared with the VPA group, the inhibitory effect of crocin was relatively weaker ($p < 0.001$, **Figures 3A,B** and $p < 0.01$, **Figure 3D**). In addition, neither unexpected behavior nor abnormal EEG was induced by crocin (even the highest dose reached 200 mg/kg) in these mice during the 10 days. These data suggested that crocin at high dose may have an inhibitory effect on the fully kindled GS in ICR mice.

Crocin Inhibits the Pilocarpine-Induced SE and SRS in ICR Mice

To confirm the effect of crocin on SE and SRS, we used the pilocarpine-induced SE and SRS model. In SE model, 100 mg/kg crocin significantly prolonged the latency of SE induced by pilocarpine ($p < 0.05$, **Figure 4A**) and reduced the mortality of SE ($p < 0.001$, **Figure 4B**), compared with the NS group. These effects were comparable to that of the positive drug VPA. 200 mg/kg crocin reduced the mortality of SE ($p < 0.01$, **Figure 4B**), however, 50 mg/kg crocin did not appear any significant effect. In SRS model, compared with the NS group, 100 mg/kg crocin significantly reduced the number of SRS ($p < 0.001$, **Figure 4C**), which was similar with positive drug VPA ($p < 0.001$, **Figure 4C**). VPA and 200 mg/kg crocin can reduce mean duration of SRS ($p < 0.05$, **Figure 4E**). However, neither crocin nor VPA could reduce the mean seizure stage ($p > 0.05$, **Figure 4D**). These data suggested that crocin at high dose may alleviate SE and SRS.

Crocin May Not Improve the Cognitive Behaviors After SRS in ICR Mice

To investigate the effect of crocin on the improvement of cognitive function after SRS in mice, NORT was examined. OFT was also performed to assess the movement ability of mice in each group. In the OFT, representative tracks of each group were shown in **Figure 5A**. The total travelling distance of SRS mice in NS group significantly increased ($p < 0.05$, **Figure 5B**), compared with that in nSRS group. VPA can significantly decrease the total travelling distance ($p < 0.01$, **Figure 5B**), compared with that in NS group and crocin

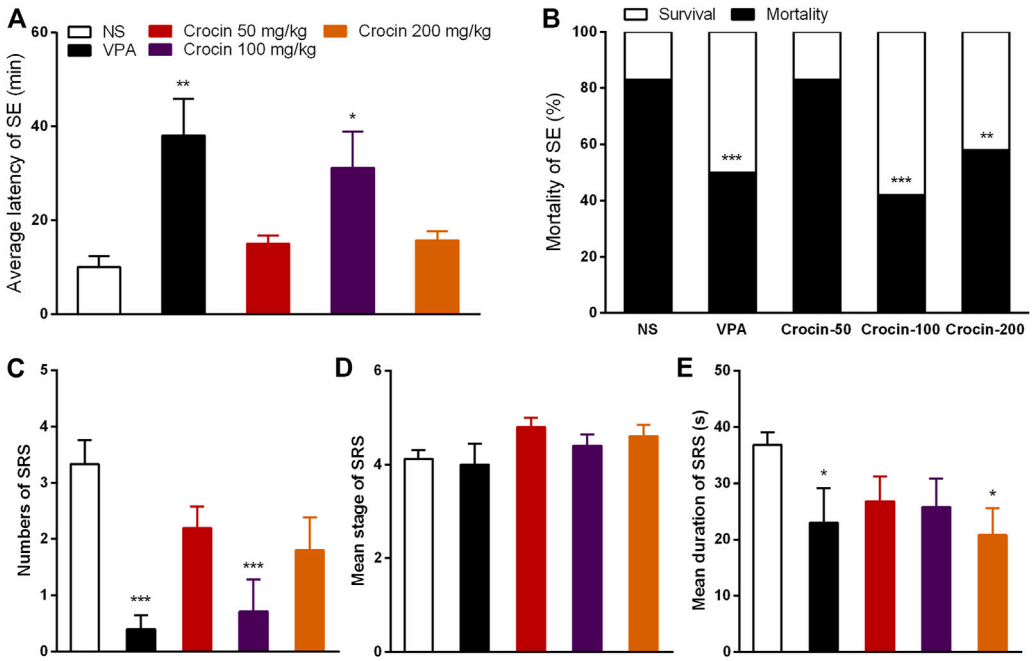

FIGURE 4 | Effects of crocin on pilocarpine-induced SE and SRS. **(A)** average latency of SE (n = 12 for each group, x±S.E.M.); **(B)** mortality rate of SE (n = 12 for each group); **(C)** number of SRS (n = 7 for each group, x±S.E.M.); **(D)** mean seizure stage of SRS (n = 7 for each group, x±S.E.M.); **(E)** mean duration of SRS (n = 7 for each group, x±S.E.M.). SE, status epilepticus; SRS, spontaneous recurrent seizures; NS, normal saline; VPA, sodium valproate. Values are from four independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001, compared with NS group. One-way ANOVA for repeated measures was used followed by Tukey's t-test for **(A,C,D)** and the $\chi 2$ test was used for **(B)**.

(100 mg/kg) had a tendency towards decrease but still higher than that in the VPA group (p < 0.05, **Figure 5B**). The movement distance of mice in the central zone after SRS in NS group was significantly increased than that in nSRS group (p < 0.05, **Figure 5C**). It was significantly reversed in the VPA group (p < 0.001, **Figure 5C**), and 100 mg/kg crocin had a tendency of decline (p > 0.05, **Figure 5C**), compared to the NS group but significantly higher than that in the VPA group (p < 0.01, **Figure 5C**). Similarily, the duration that mice stayed in the central zone after SRS in NS group was significantly increased than that in nSRS group (p < 0.05, **Figure 5D**) and it was only reversed in the VPA group (p < 0.05, **Figure 5D**). These data suggested that the movement of mice may increase after SRS due to their high excitability and VPA but not crocin (100 mg/kg) can significantly reverse the increase.

In the NORT, representative tracks of each group were shown in **Figure 5E**. As shown, both crocin and VPA had a tendency to improve the DI of mice that suffered SRS, but neither one showed a significant difference (p > 0.05, **Figure 5G**) and they did not improve the DR as well (p > 0.05, **Figure 5F**). These data suggested that crocin at 100 mg/kg may not improve the cognitive behaviors after SRS.

Crocin Decreases the Loss of Neurons in the Hippocampus After SRS in ICR Mice

To verify whether crocin has a mitigating effect on neuronal damage after SRS, the neurons in the hippocampus of mice in each group were stained and observed in this experiment. Mice in the NS group showed the loss of neuron in both CA3 and CA1 regions of the right and left hippocampus (**Figures 6C,H,M,R**), compared to the naïve (**Figures 6A,F,K,P**) and nSRS group (**Figures 6B,G,L,Q**); while VPA (**Figures 6D,I,N,S**) and 100 mg/kg crocin (**Figures 6E,J,O,T**) treatment ameliorated the neuronal damage. The quantification analysis also showed that the number of survival neurons in the NS group significantly decreased after SRS, compared with that in the naïve group (p < 0.01 in left and right CA3, p < 0.05 in left CA1, p < 0.001 in right CA1, **Figures 6U–X**) as well as that in nSRS group (p < 0.05 in right CA3, **Figure 6V**; p < 0.01 in right CA1, **Figure 6X**). In VPA and crocin (100 mg/kg) group, more neurons survived in left CA3 (p < 0.05 for both VPA and crocin, **Figure 6U**), right CA3 (p < 0.05 for both VPA and crocin, **Figure 6V**), left CA1 (p < 0.05 for crocin, **Figure 6W**) and right CA1 (p < 0.001 for VPA, p < 0.01 for crocin, **Figure 6X**) region of the hippocampus after SRS, compared with that in the NS group. These data suggested that crocin at 100 mg/kg may protect the neurons in CA3 and CA1 region of the hippocampus after SRS.

Effects of Crocin on the Concentration of BDNF, IL-1β and TNF-α in Brain Tissues of ICR Mice

To investigate whether the underlying target of crocin is concerned with BDNF, IL-1β or TNF-α, ELISA method was used to measure the concentration of them in the

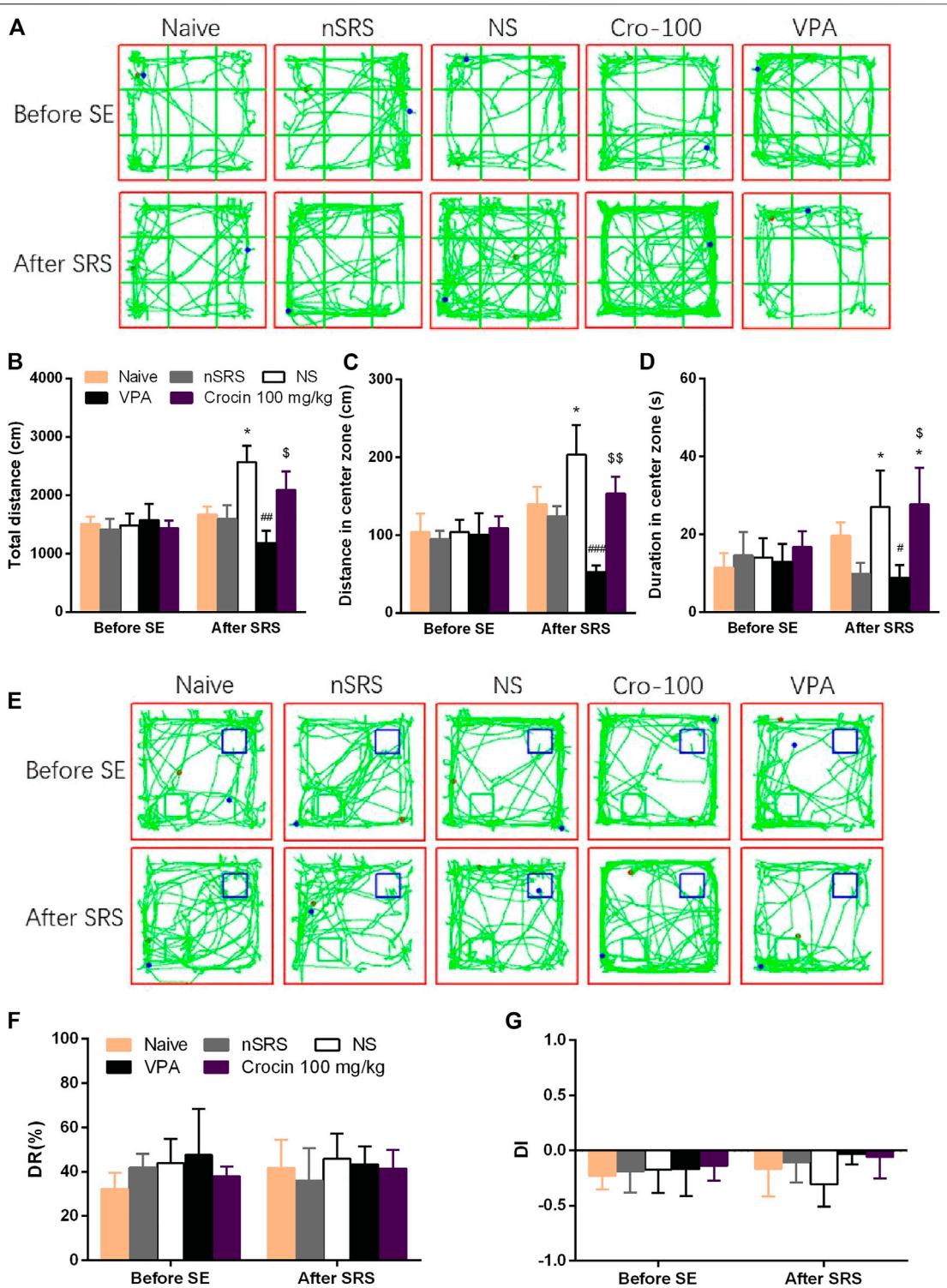

FIGURE 5 | Effects of crocin on the behaviors of mice in OFT and NORT ($n = 7$ for each group, x±S.E.M.). **(A)** representative tracks of mice in OFT; **(B)** total travelling distance of mice in OFT; **(C)** movement distance of mice in center zone in OFT; **(D)** duration of mice staying in center zone in OFT; **(E)** representative tracks of mice in NORT (blue boxes mark the location of old object and green boxes mark the location of novel object); **(F)** DR of mice in each group; **(G)** DI of mice in each group. OFT, the open field test; NORT, the novel object recognization test; DR, discrimination ratio; DI, discrimination index; SE, status epilepticus; SRS, spontaneous recurrent seizures; nSRS, non-SRS; NS, normal saline; VPA, sodium valproate. Values are from 3 independent experiments. $^{*}p < 0.05$, compared with nSRS group; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$, compared with NS group (with SRS); $^{\$}p < 0.05$, $^{\$\$}p < 0.01$, compared with VPA group. Two-way ANOVA was performed for **(B,C,D,F,G)**.

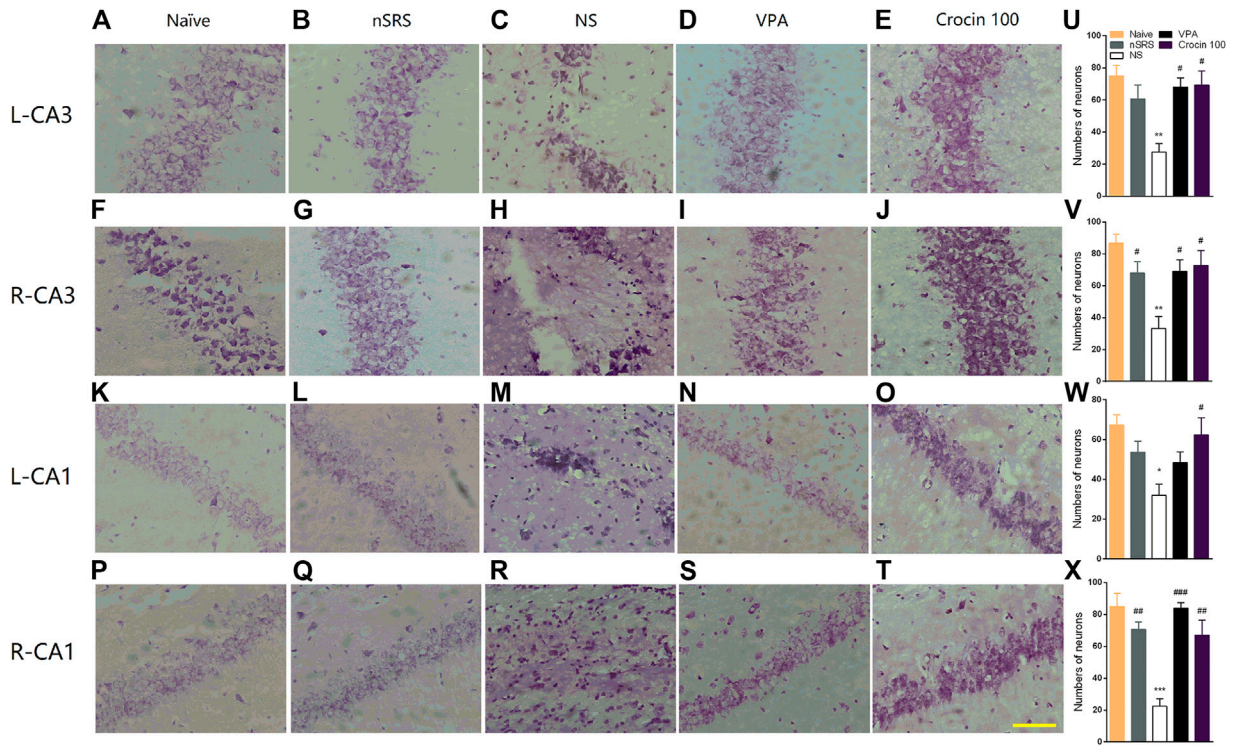

FIGURE 6 | Effects of crocin on the neural damage in the hippocampus region in each group of mice. **(A–E)** H-E staining of neurons in the left hippocampal CA3 region; **(F–J)** H-E staining of neurons in the right hippocampal CA3 region; **(K–O)** H-E staining of neurons in the left hippocampal CA1 region; **(P–T)** H-E staining of neurons in the right hippocampal CA1 region. Bar = 200 μm. **(U–X)** The quantification of survival neurons in left CA3, right CA3, left CA1 and right CA1 (n = 3 for each group, x̄±S.E.M.). SRS, spontaneous recurrent seizures; nSRS, non-SRS; NS, normal saline; VPA, sodium valproate. Values are from 3 independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, compared with naive group; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$, compared with NS group (with SRS). One-way ANOVA was used for **(U–X)**, followed by Tukey's t-test.

hippocampus and cortex after SRS. Compared with the NS group, 100 mg/kg crocin increased the BDNF concentration in the cortex of SRS mice ($p < 0.05$, **Figure 7D**), but failed to increase the BDNF concentration in the hippocampus ($p > 0.05$, **Figure 7A**). In addition, It had little effect on the concentration of IL-1β ($p > 0.05$, **Figures 7B,E**) in the hippocampus and cortex; however, there was a decrease in the concentration of TNF-α in hippocampus ($p < 0.05$, **Figure 7C**). These data suggested that crocin at 100 mg/kg may reduce the concentration of TNF-α in hippocampus and increase the concentration of BDNF in the cortex.

DISCUSSION

In the present study, we mainly found that crocin can alleviate the kindling acquisition and expression in hippocampal rapid electrical kindling model and could suppress the pilocarpine-induced SE and SRS in mice. The protective effect of crocin on neuronal damage may be involved in the inhibition of experimental epilepsy; while the alteration of the concentration of TNF-α in hippocampus and the concentration of BDNF in the cortex may also be one of underlying mechanisms for the anti-epileptic activity of crocin. These results suggested that crocin is effective on the treatment of experimental TLE in mice and it may be a potential anti-epileptic drug.

In our previous study, we have found that crocin has an inhibitory effect on the hippocampal rapid electrical kindling seizures in C57BL/6J mice (Wang et al., 2017a). However, due to the high mortality of the C57BL/6J mice in pilocarpine-induced SRS model, we have to use ICR mice in this study instead. Therefore, we first assessed the effect of crocin on hippocampal rapid electrical kindling seizures in ICR mice to clarify whether or not its effects were strain-specificial. The results illustrated that crocin can alleviate the kindling acquisition and expression in hippocampal rapid electrical kindling model of ICR mice (**Figures 2**, **3**), in accordance with the previous results in C57BL/6J mice (Wang et al., 2017a), which means the effects of crocin are not strain-specificial and it can significantly inhibit the electrical kindling-induced experimental TLE.

In the past researches, the anticonvulsant effects of crocin are controversial. Hosseinzadeh and Talebzadeh (2005) found that crocin did not exhibit anti-epileptic activity against PTZ-induced acute seizure, however, Mazumder et al. (2017) found that crocin can retard the epileptogensis in a PTZ-kindling model of mice. In addition, Tamaddonfard et al. (2012) found that crocin had a significant inhibitory effect on penicillin-induced epileptiform activities. We speculated that there may be two causes for these

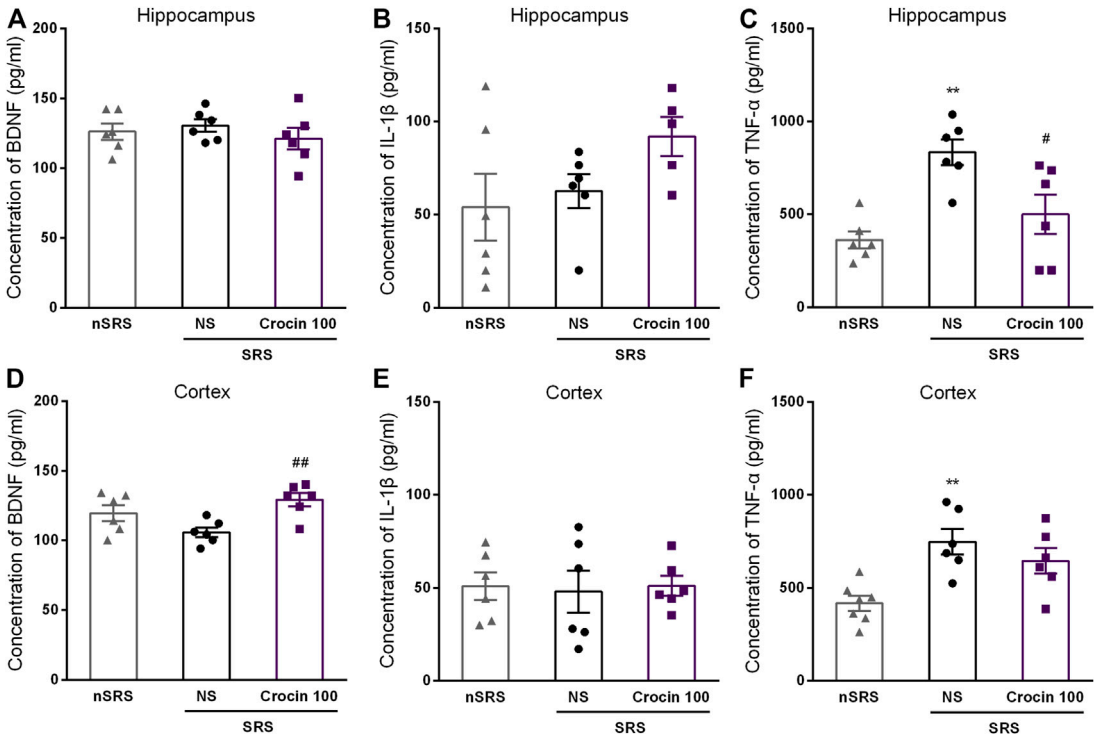

FIGURE 7 | Effects of crocin on intracerebral concentration of BDNF, IL-1β and TNF-α in each group of mice ($n = 6$ for each group, x±S.E.M.). **(A)** the concentration of BDNF in the hippocampus of mice; **(B)** the concentration of IL-1β in the hippocampus of mice; **(C)** the concentration of TNF-α in the hippocampus of mice; **(D)** the concentration of BDNF in the cortex of mice; **(E)** the concentration of IL-1β in the cortex of mice; **(F)** the concentration of TNF-α in the cortex of mice. SRS, spontaneous recurrent seizures; nSRS, non-SRS; NS, normal saline; BDNF, brain derived neurotrophic factor; IL-1β, interleukin-1β; TNF-α, tumor necrosis factor-α. Values are from 3 independent experiments. $^{**}p < 0.01$, compared with nSRS group; $\#p < 0.05$, compared with NS group (with SRS). One-way ANOVA for repeated measures was used followed by Tukey's t-test for **(A–F)**.

contradictory results. First, different models of epilepsy were chosen so that the effect of crocin is inconsistent in previous studies, which may suggest the effects of crocin on epilepsy have model selectivity. As the effect of crocin in spontaneous seizure models, which are considered to simulate the characteristics of spontaneous epilepsy in clinic, has not been investigated in previous studies, we mainly use a pilocarpine-induced SRS model to assess the effect of crocin in our present study. The results showed that 100 mg/kg crocin can significantly reduce the number of SRS (**Figure 4**) and the loss of neuron in the hippocampus (**Figure 6**), and 200 mg/kg crocin can reduce mean duration of SRS (**Figure 4**). These data suggested that crocin may be effective against the spontaneous seizures. Second, most previous studies used minor doses or short-term administration (Tamaddonfard et al., 2012; Alavizadeh and Hosseinzadeh, 2014), which may imply that the effect of crocin may be limited by insufficient number of administration or dose of crocin. Therefore, the long-term administration of crocin (daily for 35 days) and higher doses were also chosen in our present study. As we expected, the long-term administration of crocin can inhibit the pilocarpine-induced SRS, however, higher dose (100 mg/kg) rather than the highest dose of crocin showed more effective on pilocarpine-induced SE and SRS. One possible explanation is that crocin has been reported to have multiple acting targets including anti-inflammation, BDNF, and apoptosis etc. (Hosseinzadeh and Talebzadeh, 2005; He et al., 2016; Mazumder et al., 2017; Rana and Musto, 2018; Teng et al., 2021). Due to the multiple targets, different treatment doses may activate different targets and pathways, which may cause the anti-epileptic effect of crocin lacks significant dose-dependence. In addition, the activation of multiple targets may also explain why crocin is effective against multiple types of epileptic model, such as electrical kindling (**Figures 2**, **3**), PTZ kindling (Mazumder et al., 2017), pilocarpine-induced SE and SRS (**Figure 4**). At present, our data indicated that 100 mg/kg crocin may be the optimal dose and the long-term administration of crocin may be important for inhibiting the SRS. Meanwhile, the animals with crocin did not show any significant behavioral abnormalities during the 35 days, which may be a preliminary evidence of the safety of long-term administration of crocin. In general, our findings provide new and strong evidences to support the view that crocin is effective on epilepsy and it may be a promising anti-epileptic compound, which is worthy of further translational medicine research.

Due to the observed loss of neurons in the hippocampus of SRS mice (**Figure 6**), we also examined the behaviors of mice in each group by OFT and NORT to observe the influence of the neural damage and possible improvement effect of crocin. In

OFT, the activity of SRS mice significantly increased, which may result from the increase in excitability after SRS. VPA can reduce the activity and crocin just has a tendency to reduce the distance of movement in the central zone (**Figure 5**). In NORT, there were no significant differences in DR and DI among the mice in each group. These data indicated that the crocin may have a weak role of improvement the cognitive behaviors after SRS. We guess that the severity of neuronal damage caused by a few times of SRS was not sufficient to lead to significant cognitive impairment in the mice in this experiment, which is a possible explanation for this result.

The anti-epileptic mechanism of crocin is still unclear. It was thought to be related to enhancement of benzodiazepine receptor system and γ-aminobutyric acid in several previous researches (Mazumder et al., 2017; Rana and Musto, 2018), however, it still exists controversial. For example, the effect of crocin was comparable with diazepam in one report (Rana and Musto, 2018); while diazepam but not equivalent dose crocin was effective on seizures in another report (Hosseinzadeh and Noraei, 2009). So it is probably that there exist other underlying anti-epileptic mechanisms of crocin. Brain-derived neurotrophic factor (BDNF) is a small (14 kD) secreted protein that binds to the ectodomain of its cognate receptor, tyrosin kinase receptor B (TrkB) and low affinity neurotrophic factor receptor. BDNF-TrkB receptor pathway, which plays a major role in triggering a series of downstream cascade reactions, is considered to be closely related with epilepsy (Unsain et al., 2009; Heinrich et al., 2011). As we previously found, crocin can significantly promote the secretion of BDNF in the hippocampus of rats with cerebral ischemia (He et al., 2016). To confirm whether or not crocin can increase the level of BDNF, we measured the concentration of BDNF in hippocampal and cortical regions of mice. Unexpected, it was found that crocin significantly increased the concentration of BDNF in the cortex, but not the hipocampus, of the SRS mice, compared with the NS group (**Figure 7**). According to previous reports, BDNF plays an important role in the development of epilepsy and is closely related to neuronal survival and apoptosis, brain function and behavior, and synaptic plasticity (Chao, 2003). In recent years, BDNF has also been found to play a role in formation of epilepsy (Walczak et al., 2013). Some studies suggest that BDNF may facilitate or inhibit the process of epilepsy formation (Hong et al., 2013; Chiu et al., 2019), but its exact role and potential mechanisms remain unclear. Pallavi Sharma et al. suggested that the effect of changes in BDNF content on epilepsy formation depends on the brain region where the change occurs and the timing of BDNF cascade pathway activation (Sharma et al., 2019). The results of our experiments support this view. Hence, we hypothesize that increase of BDNF content in the cortex may have an epileptic suppressive effect, and the exact mechanism needs to be further investigated.

In addition, a large number of research reports have already pointed out a correlation between epilepsy and intracranial inflammatory responses (Choi et al., 2009; Riazi et al., 2010; Davis and Dalmau, 2013), where acute or chronic inflammation triggers changes in the content of certain cytokines at specific sites, ultimately leading to neurological dysfunction such as abnormal neuronal excitability, neuronal degeneration and epilepsy (Samland et al., 2003). There are also corresponding immunosuppressive therapies targeting certain inflammatory factors in clinical practice (Vezzani and Baram, 2007). It has been suggested (Dupuis and Auvin, 2015; Dey et al., 2016) that altered levels of inflammatory mediators in the brain will lead to hyperexcitability or damage of neurons and eventually lead to seizures. Then, repeated seizures in turn increase the excitability of neurons to aggravate their damage and death, both of which form a vicious circle and may be a cause of epileptogensis. Similar phenomena have also been found in various SRS animal models. For example, elevated levels of pro-inflammatory cytokines, such as IL-1β, TNF-α, etc., have also been found in the experimental SRS (Shen et al., 2019; Suleymanova, 2021). Based on the above findings, this experiment examined the levels of two pro-inflammatory cytokines, IL-1β and TNF-α, in the hippocampus and cortex. The results showed that the levels of TNF-α in the hippocampus of mice in the crocin group had a decrease, while there was no difference in IL-1β, compared with the NS group (**Figure 7**). We speculate that crocin may inhibit the inflammatory response through reducing the content of the pro-inflammatory cytokine TNF-α in the hippocampal region, thus achieving the effect of suppressing epilepsy. This is consistent with the findings of Galic et al. and others (Vezzani and Granata, 2005; Galic et al., 2008; D'Ambrosio et al., 2013), who found that anti-TNF-α monoclonal antibodies blocked the proepileptic effects of lipopolysaccharide; moreover, Choi et al. (2009) demonstrated considerable elevation of pro-inflammatory cytokines such as IL-1β in brain tissue of epileptic patients in addition to TNF-α, but these phenomena did not occur in our experiments, which may be related to the regions of the examined brain tissues. In a recent study, elevated levels of IL-1β has also been considered to probably have brain region specificity in a TLE animal model (Xu et al., 2021b). Hence, we will further explore the changes of inflammatory factors in more brain regions in our subsequent experiments. In addition, the relationship between crocin, BDNF and pro-inflammatory cytokines, and whether other signaling molecules are involved in the process also remains to be further investigated.

In conclusion, crocin can significantly retard the progression of epilepsy, alleviate GS and suppress SE and SRS in experimental TLE. Its mechanism may be involved in the protection of neurons, the decrease of TNF-α content in the hippocampus and the increase of BDNF content in the cortex. Thus, crocin may be a potential anti-epileptic compound for treatment of TLE.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusion of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by Hangzhou Medicine College Animal Experimentation Committee.

AUTHOR CONTRIBUTIONS

KZ, CQ, RL, XW, and ZH performed experiments and analyzed data. KZ, JM, and YY designed the research and analyzed data. KZ and JY wrote and revised the manuscript.

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A Comparison of Epileptogenic Effect of Status Epilepticus Treated With Diazepam, Midazolam, and Pentobarbital in the Mouse Pilocarpine Model of Epilepsy

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Status epilepticus (SE) is a medical emergency associated with acute severe systemic damage and high mortality. Moreover, symptomatic SE is one of the highest risk factors for epileptogenesis. While the antiepileptic drugs (AEDs) are chosen in favor of acute control of SE, the potential short-term and long-term effects of such AEDs have been ignored in clinics. In this study, we hypothesized that AEDs that are used to control acute SE might affect the feasibility for the chronic development of epileptogenesis after SE. Therefore, we sought to compare the epileptogenic effects of SE that are terminated by three AEDs, i.e., diazepam, midazolam, and pentobarbital, which are widely used as first-line anti-SE AEDs. For this purpose, we used a mouse model of SE induced by intraperitoneal (i.p.) injection of lithium chloride (LiCl)-pilocarpine. The pilocarpine-induced SE was terminated with diazepam, midazolam, or pentobarbital. Then we compared short-term and long-term effects of SE with different AED treatments by examining SE-associated mortality and behavioral spontaneous recurrent seizures (SRSs) and by using magnetic resonance imaging (MRI) and immunohistochemistry to evaluate pathological and cellular alterations of mice in the different treatment groups. We found that i.p. injections of diazepam (5 mg/kg), midazolam (10 mg/kg), and pentobarbital (37.5 mg/kg) were able to terminate acute pilocarpine-SE effectively, while pentobarbital treatment showed less neuroprotective action against lethality in the short phase following SE. Long-term evaluation following SE revealed that SE treated with midazolam had resulted in relatively less behavioral SRS, less hippocampal atrophy, and milder neuronal loss and gliosis. Our data revealed an obvious advantage of midazolam vs. diazepam or pentobarbital in protecting the brain from epileptogenesis. Therefore, if midazolam

provides as strong action to quench SE as other AEDs in clinics, midazolam should be the first choice of anti-SE AEDs as it provides additional benefits against epileptogenesis.

Keywords: epilepsy, epileptogenesis, status epilepticus (SE), midazolam, diazepam, pentobarbital

INTRODUCTION

Epileptogenesis refers to the pathological and pathophysiological processes, which are initiated by initial brain insults and chronically results in the development of spontaneous recurrent seizures (SRSs). The time window between the initial brain insults and the first SRS is usually called the latent period. An initiating insult can be one of the wide ranges of brain damage that include neurological infections, traumatic brain injury (TBI), ischemia, intracerebral hemorrhage, intracranial tumor, unprovoked seizures, etc. During the latent period and throughout the whole process of epileptogenesis following the initial brain insults, comprehensive changes, e.g., neuronal loss, loss of balance of neuronal excitation and inhibition, gliosis, brain tissue sclerosis, brain structural and network reorganization, chronically occur in the brain regions that are vulnerable to epileptogenesis (1, 2). It is believed that the accumulation of those pathological and pathophysiological changes after initial brain insults gives rise to the formation of the epileptic brain and the occurrence of unprovoked SRS (1, 2).

When a seizure lasts for more than 5 min or two or more seizures occur too close to allow full recovery of epileptic activities between seizures, there is a medical emergency that is called status epilepticus (SE). In practical conditions, any SE should be treated as quickly as possible because poorly controlled SE is associated with severe systemic damage and high mortality. The treatments against SE are firstly aimed to terminate seizures with antiepileptic drugs (AEDs) and then to prevent acute seizure recurrence. Diazepam, midazolam, lorazepam, phenobarbital, phenytoin, and valproate are some of the widely used AEDs for the treatment of SE (3, 4). The treatment option is usually decided according to the stages and time points of SE. For example, diazepam, lorazepam, pentobarbital, and midazolam are recommended as the first-line choices against early convulsive SE, while phenytoin and valproate are considered to be second-line choices against established convulsive SE (3, 4). Among the numerous initial brain insults, symptomatic SE is one of the highest risk factors of epileptogenesis. Approximately 1.5–17% of patients develop SRS within 30 years after TBI depending on injury severity (5), 2.4–22% of patients develop SRS within 20 years after infectious brain damage depending on the types of infection (6), while 41% of patients may become epileptic in 10 years after an acute symptomatic seizure with SE (7).

While the AEDs are chosen in favor of acute control of SE, the potential short-term and long-term effects of such AEDs on SE-triggered epileptogenesis have been ignored in clinics. Since SE is highly epileptogenic, we hypothesized that AEDs that are used to control acute SE might affect the feasibility of the chronic development of epileptogenesis after SE. Therefore, to test this hypothesis, we designed experiments to compare the epileptogenic effects of SE that terminated different AEDs. In these experiments, an intraperitoneal (i.p.) injection of pilocarpine to a mouse produces a model of SE, which was terminated with three different AEDs, i.e., diazepam, midazolam, or phenobarbital. Then we evaluated the epileptogenic effects of SE with different AED treatments by examining SE-associated mortality, behavioral SRS, and pathological and cellular alterations of mice in the three different treatment groups.

RESULTS

Pentobarbital Treatment Provides Less Neuroprotective Action Against Lethality in the Short Phase Following SE Induction

Pilocarpine-induced SE is a well-established translatable animal model mimicking human SE in behavioral symptoms, pharmacological responses, and the development of SRS of temporal lobe epilepsy after a latent period (8, 9). Therefore, we used the pilocarpine model of SE and SRS to observe the short-term and long-term changes during epileptogenesis in mice in our experiments (**Figure 1A**). SE was induced with lithium chloride (LiCl)-primed pilocarpine (60 mg/kg i.p.). At the end of the induction of SE for 2 h, SE was terminated with an i.p. injection of one of the investigated AEDs, i.e., diazepam (5 mg/kg), midazolam (10 mg/kg), and pentobarbital (37.5 mg/kg). Following the termination of SE, the mice were kept in home cages and received intensive care for 1 week. Then the mice were subjected to a set of experiments that includes behavioral monitoring for SRS, structural investigation using MRI, cellular morphology using immunohistochemistry to discriminate the effects of diazepam, midazolam, and pentobarbital on SE-induced epileptogenesis (**Figure 1A**).

In total, 91 mice were treated with LiCl-pilocarpine (**Figure 1A**). We observed that 70 (76.9%) mice showed SE without death, 15 mice (16.5%) did not show SE, and 6 (6.6%) mice had died in 2 h following pilocarpine treatment (**Figure 1B**). This is consistent with our previous studies (10, 11). The survival mice with SE were randomly allocated into 3 groups that were i.p. injected with diazepam ($n = 19$), midazolam ($n = 23$), or pentobarbital ($n = 28$) to terminate SE. The mice that did not show SE received no treatment. After SE induction, the survival of mice was daily calculated for 5 weeks. We found that none of the mice without SE ($n = 15$) died in the 5 weeks. While, in the treatment groups, the death of mice had occurred almost entirely in the first week after SE induction, reflecting the SE-associated but not SRS-associated short-term effects of treatments. Among the three treatment groups, the mice in the pentobarbital group had the highest mortality (**Figure 1C**;

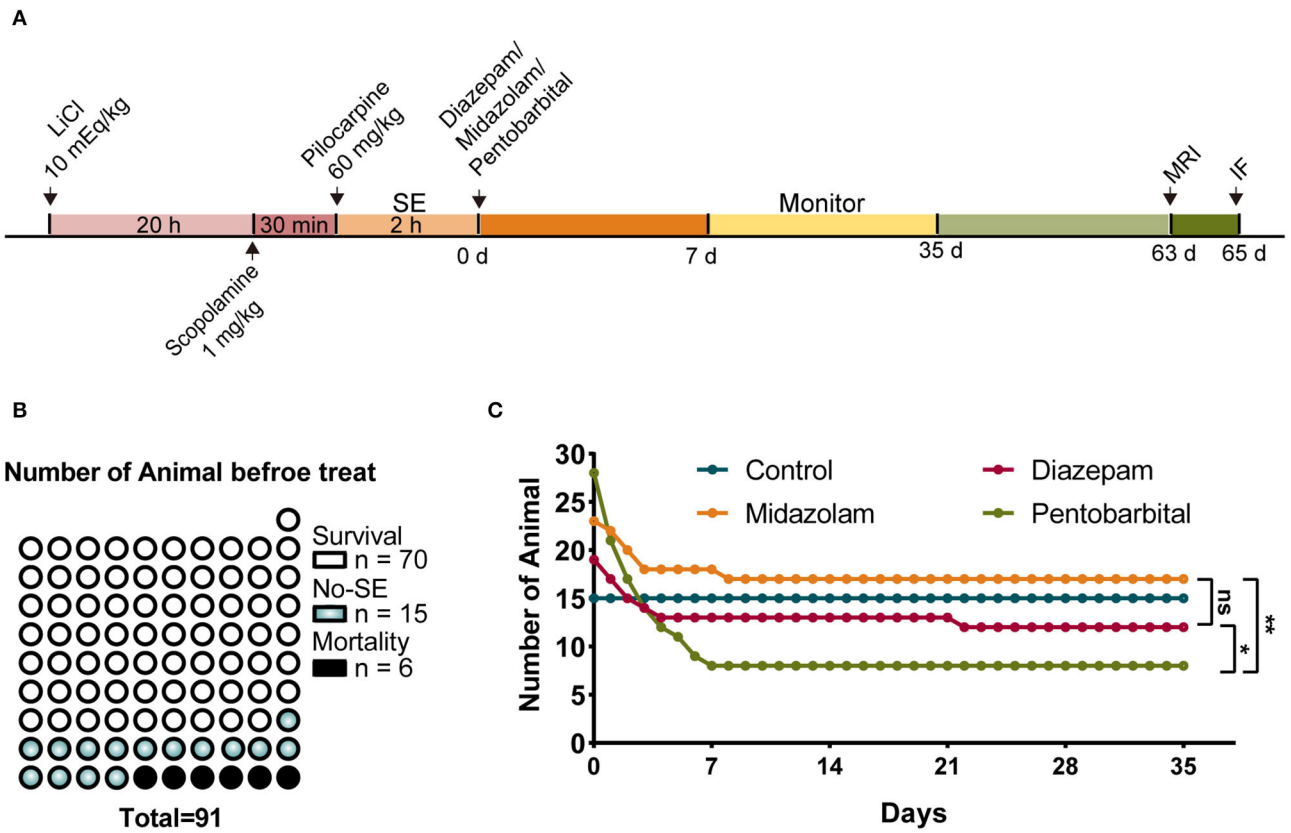

FIGURE 1 | Comparison of mortality after status epilepticus (SE) treated with diazepam, midazolam, and pentobarbital. **(A)** Experimental paradigm of SE induction by LiCl-pilocarpine and a set of experiments performed after SE induction. (**B)** A number of mice of survival from SE, no SE responses (non-SE), and mortality after pilocarpine administration and before anti-SE treatments. **(C)** Number of mice allocated to the treatments with diazepam, midazolam, and pentobarbital to terminate SE and remaining within 5 weeks after SE. Control mice were no SE and received no treatment. The number of mice at the end of the fifth week/immediately after treatments: control group, n = 15/15; diazepam group, $n = 12/19$; midazolam group, $n = 17/23$; pentobarbital group, $n = 8/28$. Pentobarbital vs. diazepam, $p = 0.0343$; pentobarbital vs. midazolam, $p = 0.0019$; diazepam vs. midazolam, $p = 0.5159$; Fisher's exact test. $^{*}p < 0.05$, $^{**}p < 0.01$, ns indicates no significant difference.

pentobarbital vs. diazepam, $p = 0.0343$; pentobarbital vs. midazolam, $p = 0.0019$; diazepam vs. midazolam, $p = 0.5159$; Fisher's exact test). Therefore, SE treated with pentobarbital provides less neuroprotective action in the short phase following SE induction.

Midazolam Treatment Provides Relative Long-Term Benefit Against the Development of SRS

From the seventh day after SE induction, the mice were subjected to video monitoring for 4 weeks (w 1–4) to allow the detection of chronic SRS (**Figure 1A**). To reduce the contamination of normal vs. epileptic behaviors, only severe seizures scored ≥ 3 according to the Racine scale (12) were included in the statistical calculation. Weekly statistics showed that midazolam treatment led to relatively milder SRS, as in the third week (**Figures 2E,F**), but not in the first 2 weeks and the last week of seizure monitoring (**Figures 2A–H** and **Supplementary Table S1**), both the number of seizures per mice per day (**Figure 2E**; diazepam 0.9762 $\pm$ 0.4876, midazolam 0.1681 $\pm$ 0.1334, $p = 0.0007$) and the percentage of mice with at least one seizure per day (**Figure 2F**; diazepam 19.05 $\pm$ 6.3%, midazolam 7.56 $\pm$ 4.445%, $p = 0.0126$) were significantly lower in the midazolam group comparing with the diazepam group. Meanwhile, the relatively milder severity of SRS in the midazolam group was illustrated by the total number of seizures in 4 weeks of seizure monitoring showing a lower occurrence of seizures per mice with high-frequency SRS (**Figure 2I**; diazepam 31.13 $\pm$ 19.97, midazolam 11.67 $\pm$ 7.089, $p = 0.0189$), but not low-frequency SRS (**Figure 2J**), comparing with the diazepam group. However, the percentage of mice with at least one SRS in weeks 1–4 of seizure monitoring was similar between the midazolam and diazepam groups. While pentobarbital treatment also led to the lower number of seizures per mice per day in week 3 (**Figure 2E**; diazepam 0.9762 $\pm$ 0.4876, pentobarbital 0.3929 $\pm$ 0.2835, $p = 0.0115$), overall, it had similar severity of SRS when compared with diazepam treatment (**Figures 2A–K** and **Supplementary Table S1**). These data suggest that midazolam treatment provides relative long-term benefits against the development of SRS.

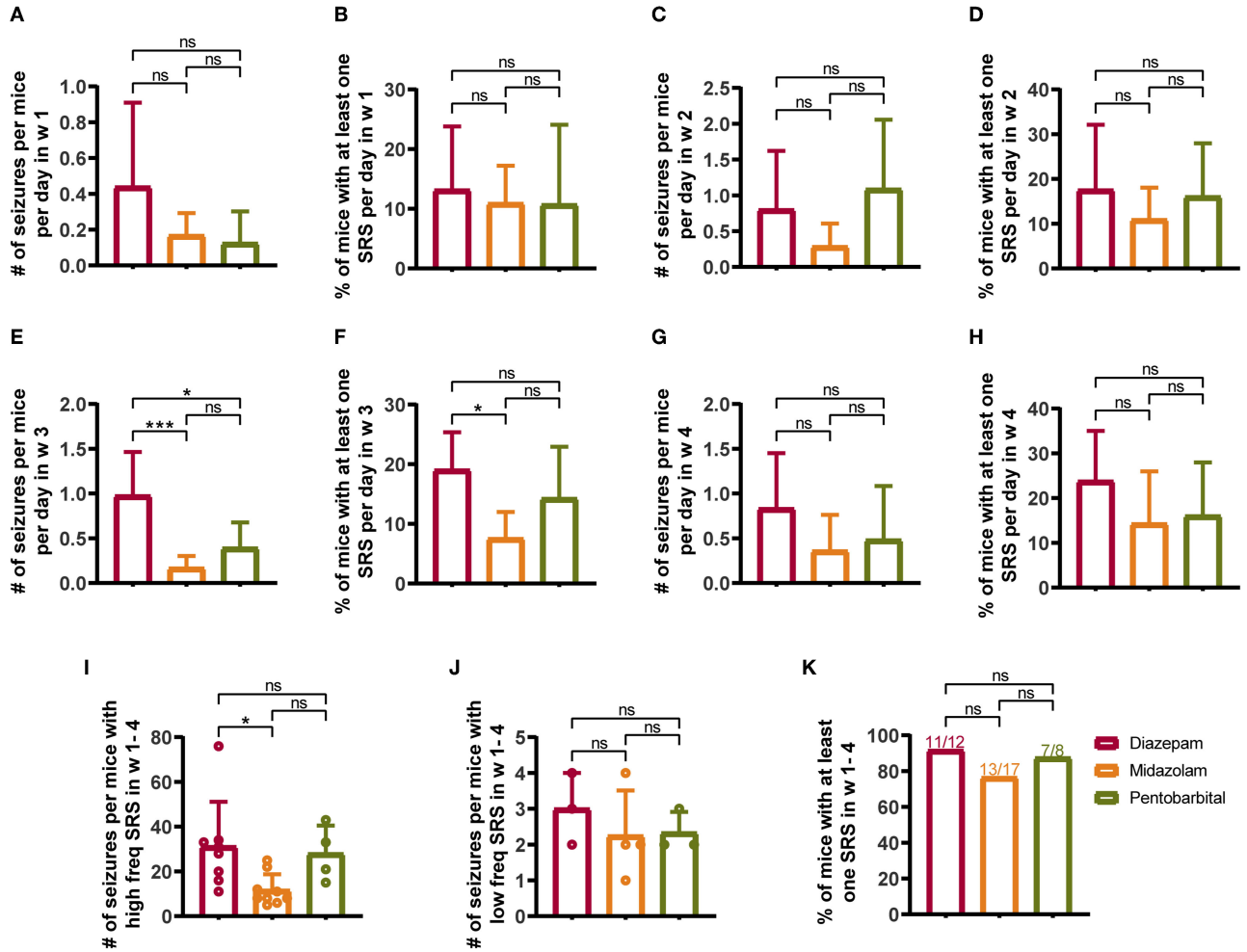

FIGURE 2 | Comparison of spontaneous recurrent seizures (SRSs) monitored for 4 weeks (W 1–4) from 7th to 35th days after SE treated with diazepam, midazolam, and pentobarbital. **(A)** Number of seizures per mouse per day in week 1 of different treatment groups. **(B)** Percentage of mice with at least one SRS per day in week 1 of different treatment groups. **(C)** Number of seizures per mouse per day in week 2. **(D)** Percentage of mice with at least one SRS per day in week 2. **(E)** A number of seizures per mouse per day in week 3. **(F)** Percentage of mice with at least one SRS per day in week 3. **(G)** Number of seizures per mouse per day in week 4. **(H)** Percentage of mice with at least one SRS per day in week 4. **(I)** A number of seizures per mouse with high-frequency SRS which was defined that the total number of seizures of a mouse was more than 4 in a week. **(J)** Number of seizures per mouse with low-frequency SRS, which was defined that the total number of seizures of a mouse was 4 or less per week. **(K)** Percentage of mice with at least one SRS in weeks 1–4. **(A–J)** One-way ANOVA with *post-hoc* Tukey's multiple comparisons test. **(K)** Fisher's exact test, $^{*}p < 0.05$, $^{***}p < 0.001$, ns indicates no significant difference. Data were presented as mean ± SD. See also **Supplementary Table 1**.

Midazolam Treatment Causes Less Hippocampal Sclerotic Atrophy

Magnetic resonance imaging (MRI) is able to reliably detect regional structural changes of the epileptic brain in relation to epileptogenesis of animal models (13, 14). Hippocampal atrophy is the most featured structural alternation of the pilocarpine-SE model of spontaneous chronic temporal epilepsy (13, 15, 16). To compare the long-term effects of diazepam, midazolam, and pentobarbital treatments antagonizing SE on brain structural changes, we performed MRI 9 weeks after SE induction. T2-weighted images showed that SE induction chronically causes hippocampal atrophy indicated by the shrinked hippocampal volume in all the treatment groups of mice when compared with the control group of mice that failed to be induced SE by pilocarpine (**Figure 3A**). However, voxel-based morphometry (VBM) analysis of T2-weighted images showed that midazolam treatment causes relatively less hippocampal sclerotic atrophy when compared to diazepam and pentobarbital treatments (**Figures 3B,C** and **Table 1**), suggesting that midazolam treatment against SE leads to less structural damages.

Midazolam Treatment Results in Milder Neuronal Loss and Gliosis

Neuronal death and reactive gliosis occurring soon after evoking SE and continuously during epileptogenesis contribute to the

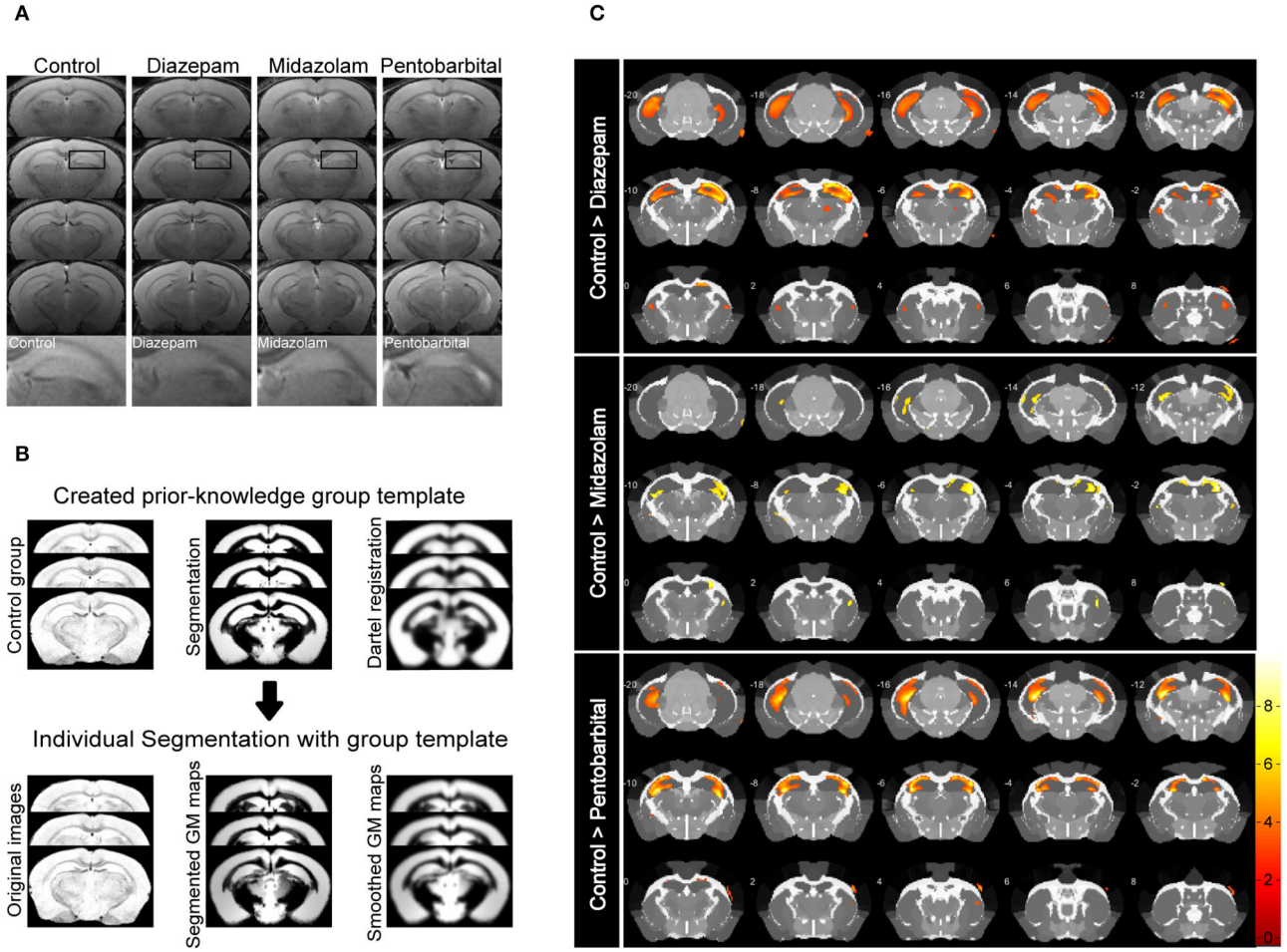

FIGURE 3 | Voxel-based morphometry (VBM) analysis of MRI T2-weighted images showing differential hippocampal atrophy 9 weeks after SE received different treatments. **(A)** Hippocampal T2 maps along the rostro-caudal axis (up-down) showing sclerotic atrophy of hippocampus of control (non-SE and no treatment), diazepam, midazolam, and pentobarbital groups of mice after SE. The images in the lowest row are the amplifications of areas in the rectangle of the second row of images. **(B)** The template creation for spatial normalization of the segmented gray matter (GM) images and VBM procedure. **(C)** Each subfigure of the treatment group showed volumetric changes of GM when compared with corresponding subfigure of control group. Hippocampal GM densities in the midazolam group were significantly higher than other groups. The color scale bar on the right represents t-value of the statistical analysis. Control $n = 6$; diazepam $n = 7$; midazolam $n = 7$; and pentobarbital $n = 4$.

formation of brain tissue atrophy and progressive development of epilepsy (2, 17). Therefore, in the final experiments, we performed immunohistochemistry staining neuronal marker NeuN, astrocytic marker Glial Fibrillary Acidic Protein (GFAP), and microglia marker Iba1 to compare the chronic cellular alterations in the hippocampus of mice treated with diazepam, midazolam, and pentobarbital against SE. Consistent with the VBM analysis of MRI images, the hippocampal shapes shown by immunohistochemistry staining exhibit different severities of hippocampal sclerotic atrophy in different treatment groups. Overall, in the midazolam-treated mice, there is relatively less tissue sclerosis and neuronal loss so that cornu ammonis (CA) pyramidal cell layer can be observed in most of the samples. In contrast, in the diazepam- or pentobarbital-treated mice, the CA pyramidal cell layer is lost in most of the cases (**Figures 4A**, **5A**).

$NeuN^+$ quantification revealed that midazolam treatment resulted in a less neuronal loss as the number of $NeuN^+$ counting was significantly higher when compared with pentobarbital treatment in the dantate gyrus (DG) (midazolam 461 ± 201.7, pentobarbital 184.5 ± 74.86, $p = 0.0224$) and non-DG regions (midazolam 899.2 ± 909.9, pentobarbital 68.38 ± 43.46, $p = 0.0111$; **Figures 4A–C**). Quantification of $GFAP^+$ area revealed that midazolam treatment resulted in lower astrogliosis in the CA1, CA3, and DG when compared with both diazepam (CA1: diazepam 27.13 ± 3.077, midazolam 16.12 ± 4.38, $p = 0.0007$; CA3: diazepam 33.6 ± 5.63, midazolam 20.22 ± 4.895, $p = 0.0011$; DG: diazepam 25.01 ± 1.75, midazolam 20.06 ± 2.873,

TABLE 1 | Summary of Voxel-based morphometry (VBM) analysis.

Regions		Control > Diazepam		Control > Midazolam		Control > Pentobarbital	
Full names of brain regions	**Abbreviations of regions**	k_E	t	k_E	t	k_E	t
Cornu ammonis, area 1	CA1	-	8.47	-	4.89	-	5.81
Cornu ammonis, area 2	CA2	-	-	3,630	5.4	-	-
Cornu ammonis, area 3	CA3	1,3927	10.05	1,272	4.34	12,546	10.28
Dentate gyrus, molecular layer	DG-mo	11,739	8.46	-	-	-	7.35
Dentate gyrus, polymorph layer	DG-po	-	5.09	-	-	-	-
Caudoputamen	CP	1,250	4.84	139	4.83	110	3.9
Alveus of hippocampus	ACAv2/3	132	4.37	-	-	-	-
Anterior cingulate area, ventral part, layer 2/3	ACAv5	186	4.57	-	-	-	-
Anterior cingulate area, ventral part, layer 5	alv	-	-	148	4.25	-	-
Anterior olfactory nucleus	AON	82	4.27	67	4.58	-	-
Ansiform lobule Crus 1	ANcr1	73	3.88	-	-	-	-
Arbor vitae	arb	-	-	46	4.04	-	-
Bed nuclei of the stria terminalis	BST	150	4.65	-	-	-	-
Cingulum bundle	cing	-	-	509	4.22	-	-
Genu of corpus callosum	ccg	32	3.47	35	3.97	-	-
Corpus callosum, body	ccb	-	-	157	3.94	-	6.8
Entorhinal area, medial part, dorsal zone, layer 1	ENTm1	57	3.8	119	4.76	-	-
Entorhinal area, lateral part, layer 2	ENTl2/3	-	-	57	3.55	-	-
Flocculus	FL	61	3.99	-	-	-	-
Internal capsule	int	608	4.65	-	-	-	-
Lateral septal nucleus, rostral (rostroventral) part	LSr	59	3.54	-	-	-	-
Lateral hypothalamic area	LHA	-	-	36	5.54	-	-
Primary motor area, Layer 6a	Mop6a	-	-	-	-	63	3.98
Orbital area, medial part, layer 1	ORBm1	581	7.61	-	-	240	5.65
Optic tract	opt	31	3.38	33	3.94	-	-
Posterior complex of the thalamus	PO	224	4.03	-	-	-	-
Sensory root of the trigeminal nerve	sV	83	4.36	-	-	-	-
Primary somatosensory area, upper limb, layer 6a	SSp-bfd6a	-	-	-	-	486	5.8
Primary somatosensory area, barrel field, layer 6a	SSs6a	-	-	-	-	-	3.6
Supplemental somatosensory area, layer 6a	SSp-ul6a	239	4.17	283	4.26	-	-
Postpiriform transition area	TR	-	-	-	-	39	5.19

K_E: larger than 30 contiguous voxels.
t: maximum T value.
Control n = 6; diazepam n = 7; midazolam n = 7; pentobarbital n = 4.
$p < 0.005$ uncorrected for multiple comparisons.

$p = 0.0186$.) and pentobarbital (CA1: pentobarbital 32.12 ± 4.085, $p < 0.0001$; CA3: pentobarbital 30.81 ± 3.366, $p = 0.0136$; DG: pentobarbital 31 ± 3.549, $p < 0.0001$) treatments (**Figures 4A,D–F**). Consistently, Quantification of $Iba1^+$ area in hippocampus also revealed less gliosis in the midazolam treatment group when compared with both diazepam (diazepam 2.883 ± 0.4546, midazolam 1.439 ± 0.5788, $p = 0.006$) and pentobarbital (pentobarbital 2.755 ± 0.9972, $p = 0.0222$) treatment groups (**Figures 5A,C**). In line with this, Sholl analysis to evaluate the microglia architecture showed that midazolam treatment led to a less pro-inflammatory M1 phenotype as the number of intersections, ending radius, and ramification index of microglia processes were higher in the midazolam treatment group when compared with both diazepam and pentobarbital treatment groups (**Figures 5B,D–G**). These data indicate that mice with SE treated with midazolam result in milder neuronal loss and gliosis chronically following SE.

DISCUSSION

Although diazepam, midazolam, and pentobarbital are able to terminate SE effectively, their short-term and long-term consequences have not been compared. In the present study we demonstrate that, in the aspect of short-term consequence, the mice with SE treated with pentobarbital have a higher mortality than midazolam and phenobarbital. While in the aspect of long-term consequence, we demonstrate that mice with SE treated with midazolam showed less hippocampal sclerosis and gliosis and result in less development of SRS.

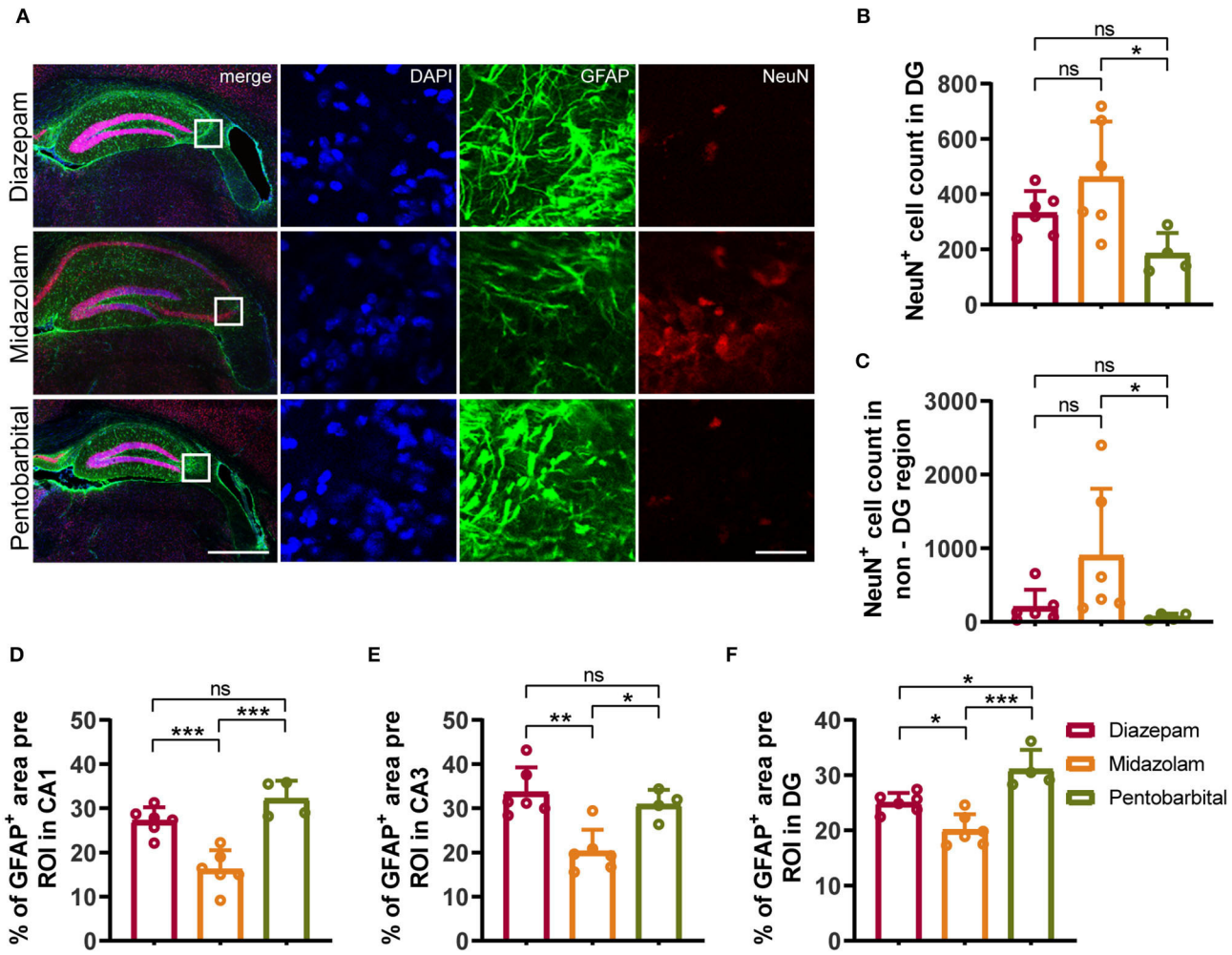

FIGURE 4 | Immunohistochemical staining showed differential hippocampal neuronal loss and astrogliosis 9 weeks after SE received different treatments. **(A)** Representative staining for neurons ($NeuN^+$) and astrocytes ($GFAP^+$) of diazepam, midazolam and pentobarbital groups. Left scale bar: 500 μm; right scale bar: 100 μm. **(B)** Statistics of $NeuN^+$ cell count in hippocampal DG area (diazepam, $n = 6$; midazolam, $n = 6$; pentobarbital, $n = 4$). **(C)** Statistics of $NeuN^+$ cell count in hippocampal non-DG area (diazepam, $n = 6$; midazolam, $n = 6$; pentobarbital, $n = 4$). **(D)** Statistics of percentage of $GFAP^+$ area per region of interest (ROI) in hippocampal CA1 area (diazepam, $n = 6$; midazolam, $n = 6$; pentobarbital, $n = 4$). **(E)** Statistics of percentage of $GFAP^+$ area per region of interest (ROI) in hippocampal CA3 area (diazepam, $n = 6$; midazolam, $n = 6$; pentobarbital, $n = 4$). **(F)** Statistics of percentage of $GFAP^+$ area per region of interest (ROI) in hippocampal DG area (diazepam, $n = 6$; midazolam, $n = 6$; pentobarbital, $n = 4$). One-way ANOVA with *post-hoc* Tukey's multiple comparisons test, $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, ns indicates no significant difference. Data were presented as mean ± SD.

Diazepam and midazolam belong to benzodiazepines, which are a class of drugs widely used as sedatives, anticonvulsants, hypnotics, muscle relaxants, and anesthetics. The actions of benzodiazepines were fulfilled by targeting $GABA_A$ receptors. When binding to the benzodiazepine side of $GABA_A$ receptors, benzodiazepines do not directly cause functional opening of the receptors, but they allosterically modulate the receptors and promote their affinity for agonist GABA (18, 19). By this means, benzodiazepines enhance neuronal inhibition and reduce neuronal excitability (20–22). However, the exact mechanisms of modulation of $GABA_A$ receptors by benzodiazepines are complicated, as there are many types of $GABA_A$ receptors with different binding affinity for GABA and benzodiazepine. This is in that each $GABA_A$ receptor comprises 5 subunits and there are up to 19 different subunits of the $GABA_A$ receptor (23, 24). Therefore, even for the same type of $GABA_A$ receptor, benzodiazepines (e.g., diazepam) may produce biphasic actions *via* separable subunit-dependent mechanisms (25). By this means, diazepam and midazolam may produce different effects on neuronal degeneration, gliosis, and neuroinflammation *via* different $GABA_A$ receptors expressed by different cell types, thus leading to different effects on epileptogenesis.

Pentobarbital belongs to barbiturates, which are primarily prescribed as anticonvulsants and sometimes used as sedatives, hypnotics, and anesthetics. Pentobarbital can not only allosterically modulate $GABA_A$ receptors (26) but also

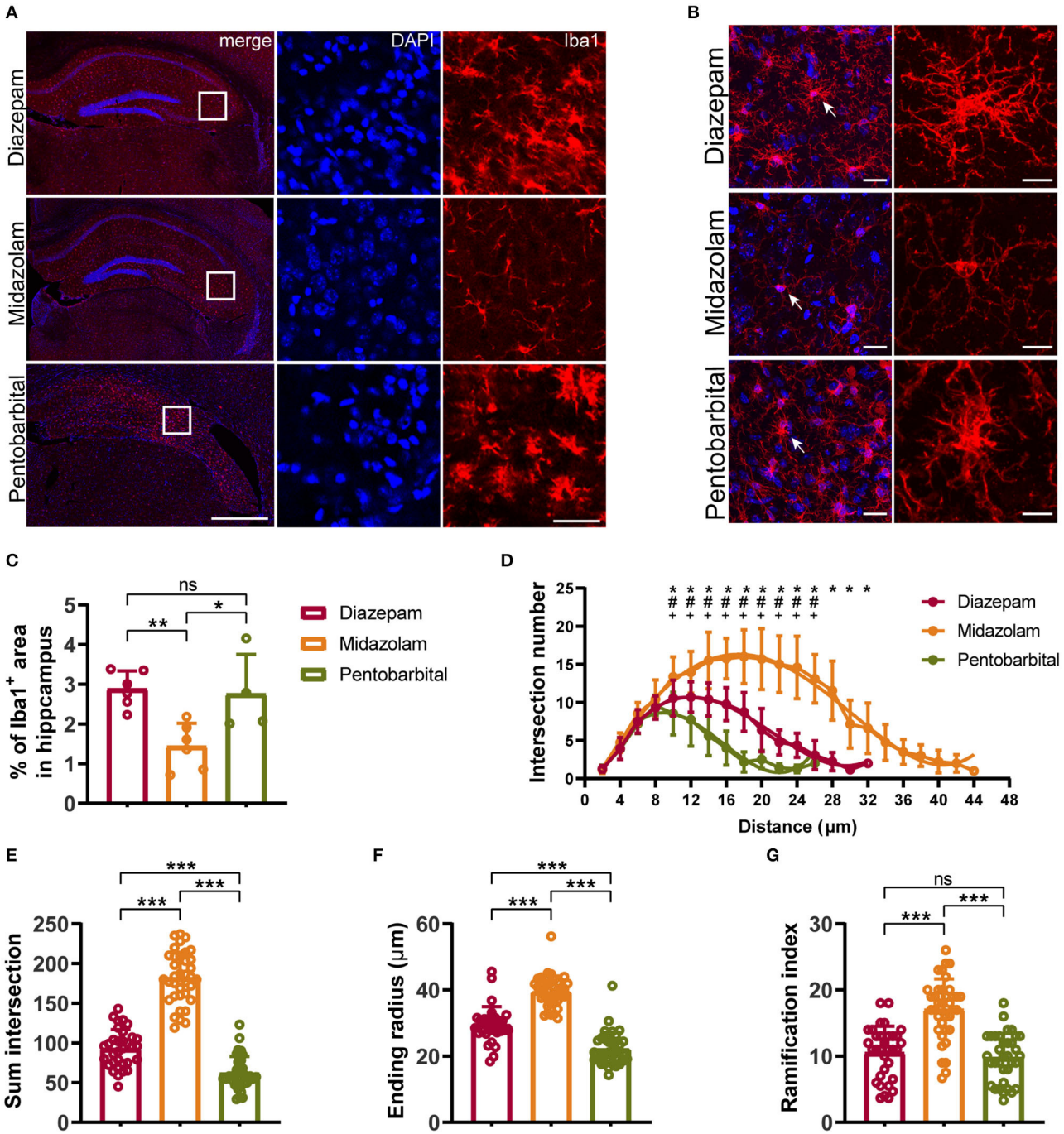

FIGURE 5 | Immunohistochemical staining showing differential hippocampal microgliosis 9 weeks after SE received different treatments. **(A, B)** Representative staining for microglia (Iba1$^+$) of diazepam, midazolam, and pentobarbital groups. Scale bar in (A): left, 500 μm; right, 100 μm. Scale bar in **(B)**: left, 20 μm; right, 10 μm. **(C)** Statistics of percentage of Iba1$^+$ area in hippocampus. **(D)** Summary number of intersections as a function of the radial distance from soma by 2 μm increments showed that Iba1$^+$ cells of midazolam group ($n = 22$) had greater numbers of ramifications than diazepam ($n = 16$) and pentobarbital ($n = 21$) groups at 10–38 μm distances from the soma. Diazepam vs. midazolam, $^*p < 0.05$; diazepam vs. pentobarbital, $\#p < 0.05$; midazolam vs. pentobarbital, $+p < 0.05$; two-way ANOVA with Tukey's multiple comparisons test. **(E)** Summary number of intersections per Iba1$^+$ cell (diazepam, $n = 33$; midazolam, $n = 37$; pentobarbital, $n = 35$). **(F)** Length of maximum branch (ending radius) of Iba1$^+$ cells (diazepam, $n = 33$; midazolam, $n = 37$; pentobarbital, $n = 35$). **(G)** Ramification index of Iba1$^+$ cells (diazepam, $n = 33$; midazolam, $n = 37$; pentobarbital, $n = 35$). One-way ANOVA with *post-hoc* Tukey's multiple comparisons test, $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, ns indicate no significant difference. Data were presented as mean ± SD.

directly activate $GABA_A$ receptors and induce GABAergic chloride currents (27). Additionally, pentobarbital is able to exert inhibition by suppressing some glutamate receptors and inhibiting glutamate release (26, 28, 29). Despite versatile inhibitory actions, barbiturates have the drawback of acute toxicity and lethality (26, 30). In line with this drawback, our data reveal that the mice with SE treated with pentobarbital have a higher mortality in the first week after SE induction when compared with midazolam and diazepam treatments. Such an acute neurotoxicity is likely to turn into chronic detriment *via* neuronal death, neuronal regeneration, gliosis, neuroinflammation, and so on. In terms of chronic epileptogenic effects of SE, although treatment with pentobarbital is followed by more neuronal loss, gliosis, and hippocampal sclerosis when compared with midazolam treatment, the statistics of SRS of mice show no significant difference between the pentobarbital and midazolam treatment groups. Presumably, this is due to insufficient animal numbers caused by SE-associated higher mortality of mice pentobarbital group.

In summary, by comparisons of epileptogenic effects of SE treated with diazepam, midazolam, and pentobarbital in the mouse LiCl-pilocarpine model of epilepsy, our data reveal an obvious advantage of midazolam vs. diazepam or pentobarbital in protecting the brain from epileptogenesis. LiCl-pilocarpine-induced murine SE represents the most well-established, clinically translatable SE model mimicking acute behavioral, electrographic, pharmacological, and pathological features and chronic epileptogenesis observed in human SE (31). Therefore, if midazolam provides as strong action to quench SE as diazepam, pentobarbital, and other AEDs in clinics, midazolam should be the first choice of anti-SE AEDs as it provides additional benefits against epileptogenesis. However, the molecular mechanisms underlying this intriguing long-term advantage and disadvantage of a single treatment with AEDs are yet to be studied in the future.

CONCLUSIONS

Our data provide evidence that pentobarbital treatment for SE showed less neuroprotective action against lethality in the short phase following SE induction and that relatively to diazepam and pentobarbital, midazolam treatment for SE resulted in long-term benefits on epileptogenesis in that such SE causes less behavioral SRS, less hippocampal atrophy, and milder neuronal loss and gliosis. Therefore, in addition to the quality of acute control of SE, the short-term and long-term consequences of AEDs chosen to control SE should be taken into account in clinics. However, our study shall be followed by clinical studies regarding this concluding point and the studies relevant to the underlying molecular mechanisms.

METHODS

Animals

Animals used in this study were purchased from Guangdong medical laboratory animal center, China. Animals were male ICR mice at 8–9 weeks of age and kept in a specific pathogen-free room with controlled temperature (22–24°C), humidity (50–75%), light conditions (12/12-h light-dark cycle, 08:00–20:00), and water and food *ad libitum*. Animals were treated according to the guidelines for Ethical Review of Animal Experiments at Southern Medical University in China, and the study was approved by the Southern Medical University Animal Ethics Committee.

LiCl-Pilocarpine Model of Epilepsy and Evaluation of Seizures

Mice were intraperitoneally (i.p.) injected with LiCl (10 mEq/kg, Sigma #213233) 20 h prior to i.p. injection of pilocarpine (60 mg/kg, MedChemExpress #HY-B0726). To reduce peripheral effects of pilocarpine, mice were given an i.p. injection of scopolamine methyl nitrate (1 mg/kg, Sigma #S2250) 30 min before pilocarpine injection. Behavioral seizure severity in response to pilocarpine was assessed by Racine's standard classification into 0–6 stages (12). SE is defined as stages 4–6 epileptic seizures lasting for at least 30 min. Mice with SE received randomly i.p. injections of diazepam (5 mg/kg, Sanchine #H23021885), midazolam (10 mg/kg, Jiangsu Enhua #H10980025), or pentobarbital (37.5mg/kg, GENIA Biotech #P3760) at 2 h after pilocarpine treatment. From the seventh day after seizure induction, mice were videotaped 12 h (8 a.m. to 8 p.m.) every day for 28 days to capture SRSs. To avoid contamination of normal activities with mild seizures (stages 1–2) of mice, only seizures scored higher than stage 3 are included in the statistics of SRS.

Immunohistochemistry

Mice were killed under anesthesia with pentobarbital sodium (75 mg/kg, i.p.). Brain slices were sectioned at 40 μm and were rinsed in phosphate-buffered saline (PBS). After washing the residual embedding medium (OCT), slices were blocked by 5% bovine serum albumin (BSA) with 1% Triton-X 100 for 1.5 h and then incubated with anti-Iba1 (1:800, Wako #019–19741), anti-NeuN (1:50, CST #24307), or anti-GFAP (GFAP; 1:800, Millipore #MAB360) in 5% BSA overnight at 4°C. After washing, slides were incubated with Alexa Fluor 488 (1:500, ZSGB-Bio #ZF-0511) or 594 (1:500, ZSGB-Bio #ZF-0513) goat anti-rabbit or goat anti-mouse secondary antibody for 1.5 h and then incubated with 4′,6-diamidino-2-phenylindole (DAPI; 1 μg/ml, Sigma #D9542) for 15 min at room temperature, washed 3 times with PBS for 15 min, and mounted onto slides. The slices were dried and mounted by coverslips with mounting medium. The samples were imaged using Nikon A1R confocal microscope. Cell counting and fluorescent area calculation were performed by ImageJ (version7). For the morphological analysis of microglia, confocal image stacks were collected using a 60× oil-objective lens with a 0.5 μm interval. Branch analysis was performed by Sholl analysis with ImageJ. Microglial ramification index ramification was defined as the ratio between maximal intersections and the number of primary branches calculated when primary branches are valid and not zero.

MRI Acquisition

A 7.0 T small animal MRI system (PharmaScan70/16 US, Bruker BioSpin MRI GmbH, Germany) was used to collect T2 data from

the mice brain with ^{1}H MRI CryoprobeTM 2 Element Array Kit for mice. The mice were anesthetized with 3% isoflurane before scanning and 1–1.5% isoflurane in oxygen during scanning. For T2 imaging, Bruker's Multi-Slice-Multi-Echo sequence was used. Scanning parameters were as follows: repetition time (TR) = 3577.557 ms, echo time (TE) = 35ms, total scan 23 slices, slice thickness = 0.5 mm, no slice gap, matrix = 256 × 256, field of view = 16 × 16 mm^2, voxel size = 0.0625 × 0.0625 × 0.5 mm^3, the scanning time was 3 min 48 s 963 ms. All the original Bruker images were converted to DICOM format with Paravision 6.0 software.

VBM

For statistical parametric mapping (SPM)-voxel-based morphometry (VBM), structural data were analyzed using SPM 12 (https://www.fil.ion.ucl.ac.uk/spm/software/spm12/) running on MATLAB R2020a (MathWorks, Inc., Natick, MA, USA). T2-weighted images were manually re-oriented, placed the origin at the anterior commissure. Subsequently, the images were segmented to identify gray and white matter based on standard tissue probability maps (Turone Mouse Brain Template, https://www.nitrc.org/) using an old Segment for SPM. Exponentiated Lie Algebra (DARTEL) was used to estimate the deformations that best align the images together by iteratively registering the imported images with their average created a study-specific template for spatial normalization of the segmented gray matter (GM) images. The remaining subjects were registered non-linearly to this template using DARTEL existing template module and then normalized to Montreal Neurological Institute (MNI) space. Images were smoothed spatially with a full width at half maximum kernel of 6 mm. The results were visualized using xjView (https://www.alivelearn.net/xjview/). The TMBTA (Brain-Atlas) was used to define anatomic regions and the voxel of each region was reported. A group comparison of hippocampal GM was performed by using a *t*-test control minus the treatment group. The resulting t-statistic maps were thresholded at values of $p < 0.005$ (uncorrected). Only clusters larger than 30 and positive contiguous voxels were considered in the analysis.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

The animal study was reviewed and approved by Southern Medical University Animal Ethics Committee.

AUTHOR CONTRIBUTIONS

YG and RC contributed to the experimental design. SL, SQ, BQ, YG, and RC established the methodology. XT performed the experiments and analyzed the data. ZZ, JZ, and SL helped to perform the experiments and maintain the animals. XT and RC wrote the manuscript. All authors contributed to the finalization and approved the content of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fneur.2022.821917/full#supplementary-material

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