EXPERIMENTAL & CLINICAL EPILEPSY AND RELATED COMORBIDITIES

EDITED BY: Mohd Farooq Shaikh, Teresa Ravizza, Jafri Malin Abdullah, Ayanabha Chakraborti and Terence John O'Brien

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frontiers Research Topics

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EXPERIMENTAL & CLINICAL EPILEPSY AND RELATED COMORBIDITIES

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Editorial: Experimental & Clinical Epilepsy and Related Comorbidities

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Keywords: epilepsy, comorbidities, experimental-animal models, clinical epilepsy, antiepileptics drugs

Editorial on the Research Topic

Experimental & Clinical Epilepsy and Related Comorbidities

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Epilepsy is a serious neurological condition affecting about 65 million people around the world, and 80% of this population is based in developing countries. Affected individuals have an increased risk for developing various comorbid conditions like cognitive, behavioral, physical, mental, and psychosocial disabilities that can adversely impact the quality of life. Many antiepileptic drugs are found to have a role in aggravating physical and/or psychiatric symptoms. This Research Topic aimed to highlight basic, clinical, and translational research involved in studying epilepsy and associated comorbidities. Translational research is an important aspect of epilepsy research and expected to play a key role in improving the quality of life of the patients. It was also expected to help in understanding the complexity of the condition and its comorbidities. Advances in the discovery of novel potential antiepileptics and their mechanism of action are also being included in this special topic.

This Research Topic has gathered 10 articles, including one brief research report, one mini-review, one hypothesis and theory, two reviews, and five original research contributions from prominent scientists in the field. The collection of papers on this Research Topic provides an up-to-date insight into current knowledge and overview of different approaches in experimental and clinical epilepsy studies as well as related comorbidities. The content of each of these articles is summarized below.

Anti-epileptic drugs (AEDs) are successful in controlling epilepsy but are reported to worsen cognitive status in a significant proportion of patients. The research conducted by Kundap et al. investigated the effectiveness of embelin (EMB), a benzoquinone derived from the plant *Embelia ribes* against pentylenetetrazole (PTZ) induced acute seizures and its associated cognitive dysfunction. This study suggests that EMB suppresses seizure-like behavior through the $GABA_A$ receptor pathway and positively influences cognitive functions in zebrafish.

Decreased bone health in epilepsy patients can be commonly attributed to AED intake; therefore, Brady et al. assessed whether bone abnormalities occur in epilepsy in the absence of AEDs by investigating mechanical characteristics and trabecular bone morphology in rats with chronic temporal lobe epilepsy. Their study revealed a lack of overt bone abnormalities in rats with chronic temporal lobe epilepsy in the absence of AED treatment and no differences in mechanical properties of

femurs. However, this warrants further studies towards understanding the source of bone abnormalities in epilepsy patients.

Temporal lobe epilepsy is a common and often drug-resistant type of epilepsy in the adult and aging populations. However, kindling studies and kindling-induced behavioral changes in animal models remain scarce. Liu et al. attempt to shed more information in this area using a mouse model of extended hippocampal kindling. The study revealed the mice were impaired in their spatial learning and memory which suggests that the extended hippocampal kindling in middle-aged mice as a model aids in the exploration of epileptogenic mechanisms and comorbidities that could be relevant to temporal lobe epilepsy.

A rare but severe myoclonic epilepsy in infants known as Dravet syndrome (DS) is caused by heterozygous mutations of the *Scn1a* gene. Recently, a glucagon-like peptide-1 (GLP-1) analog, liraglutide has emerged as a potential treatment modality for patients with nervous system diseases. The study by Liu et al. elucidates the neuroprotective role of liraglutide in mouse and cell models of *Scn1a* KO-induced epilepsy. It was found that liraglutide reduces seizure susceptibility and cognitive dysfunction in the DS mouse model with anti-apoptotic and neuroprotective effects in *Scn1a* KO mice and cells.

In a brief research report, Nobili et al. elucidate the outcome of early-chronic carbamazepine (CBZ) administration on both seizure activity and brain damage in methylazoxymethanol-pilocarpine (MP) rat model, whereby occurrence of status epilepticus and subsequent spontaneous seizures induce progressive brain damage. Their data suggest there are no differences between treatment groups, indicating that early-chronic CBZ in food administration to MP rats does not affect convulsive motor seizures.

The identification of an experimental model of absence epilepsy with predictive validity is important for the investigations of its mechanisms and evaluation and justification of experimental treatment alternatives. The review article by van Luijtelaar and van Oijen provides an overview on establishing drug effects through the design of drug evaluation studies, type of rat, traditional and novel electroencephalogram (EEG) variables, monitoring and quantification of rat behavior, limitations in data interpretation, and developments in EEG technology in genetic rat models; WAG/Rij strain and GAERS, for genetic absence epilepsy model.

The review on general early life insults linking to development of epilepsy by Semple et al. describes comprehensive preclinical evidence which demonstrates that early-life immune challenges such as those sustained during early postnatal life, prenatal immune activation, and perinatal injuries influence neuronal hyperexcitability and predispose an individual to epilepsy in later life. The review highlights neuroinflammatory mechanisms, and other indirect variables such as genetics and environment following early-life insults underlie long-term epilepsy risk. Hence, it could provide insight into the development of immunological anti-epileptogenic therapy alternatives.

Autism spectrum disorder (ASD) is a neurodevelopmental disorder with characteristics such as social communication impairments and restricted and repetitive behaviors and interests. Identifying the genetic background could be a vital feature for the diagnosis and treatment of ASD. The research by Lee et al. reports on utilizing next-generation sequencing (NGS) as a tool to analyze multiple genes simultaneously for autism genetics in the laboratory and clinical settings. The research showed VOUS genes are frequently related to ASD or other neurodevelopmental disorders such as epilepsy.

In a mini-review Ogaki et al. discuss the relationships between epilepsy and brain vascular abnormalities such as vascular malformation, blood–brain barrier dysfunction, and excessive angiogenesis. The review highlights the potential role of vascular endothelial growth factor (VEGF) and VEGF signaling as a therapeutic strategy by modulating the structure and function of the neurovascular unit in the epileptic brain.

The gut–brain-axis influence poses a hypothesis that links both epilepsy and associated comorbidities. The review by Shaikh et al. serves to highlight the possible influence of the gut–brain-axis in the manifestation of depressive symptoms in epilepsy. There is indirect evidence that revealed some specific bacterial strains that might cause depression in epilepsy which suggests a correction of this dysbiosis through probiotics supplementation might be beneficial in treating both epilepsy and related depression.

The diversity of these papers has contributed greatly to the library of basic research and clinical studies in the field of clinical epilepsy and associated comorbidities. We are grateful and hopeful that these papers would benefit and inspire other researchers to work and advance this exciting research field. Further studies that contribute towards a better understanding of the pathogenesis of such conditions can potentially lead to novel development of effective and safer treatment options for clinical epilepsy and associated comorbidities.

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MS took the initiative for the editorial write-up. AC, TR, TO'B, and JA also contributed to revising and proofreading. All authors contributed to the article and approved the submitted version.

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ORIGINAL RESEARCH
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Impaired Spatial Learning and Memory in Middle-Aged Mice With Kindling-Induced Spontaneous Recurrent Seizures

Haiyu Liu[1,2†], Kurt R. Stover[2†], Nila Sivanenthiran[2†], Jonathan Chow[2], Chloe Cheng[2], Yapeng Liu[2], Stellar Lim[2], Chiping Wu[2], Donald F. Weaver[2,3,4], James H. Eubanks[2,5,6], Hongmei Song[1,2] and Liang Zhang[2,4*]*

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Temporal lobe epilepsy is the most common and often drug-resistant type of epilepsy in the adult and aging populations and has great diversity in etiology, electro-clinical manifestations, and comorbidities. Kindling through repeated brief stimulation of limbic structures is a commonly used model of temporal lobe epilepsy. Particularly, extended kindling can induce spontaneous recurrent seizures in several animal species. However, kindling studies in middle-aged, aging, or aged animals remain scarce, and currently, little is known about kindling-induced behavioral changes in middle-aged/aging animals. We therefore attempted to provide more information in this area using a mouse model of extended hippocampal kindling. We conducted experiments in middle-aged mice (C57BL/6, male, 12–14 months of age) to model new-onset epilepsy in adult/aging populations. Mice experienced twice daily hippocampal stimulations or handling manipulations for 60–70 days and then underwent continuous electroencephalogram (EEG)-video monitoring to detect spontaneous recurrent seizures. Extended kindled mice consistently exhibited spontaneous recurrent seizures with mean incidences of 6–7 events per day, and these seizures featured EEG discharges and corresponding convulsions. The handling control mice showed neither seizure nor aberrant EEG activity. The two groups of mice underwent the Morris water maze test of spatial learning and memory 1–2 weeks after termination of the kindling stimulation or handling manipulation. During visible platform trials, the kindled mice took a longer distance and required more time than the control mice to find the platform. During hidden platform trials, the kindled mice showed no improvement over 5-day trials in finding the platform whereas the control mice improved significantly. During probe tests in which the hidden platform was removed, the kindled mice spent less time than the controls searching in the correct platform location. There were no significant differences between the kindled and control mice with respect to swim speed or total locomotor activity in an open-field test. Together, these observations indicate that the extended kindled mice with spontaneous recurrent seizures are impaired in spatial learning and memory as assessed by the Morris water maze test. We postulate that

the extended hippocampal kindling in middle-aged mice may help explore epileptogenic mechanisms and comorbidities potentially relevant to new-onset temporal lobe epilepsy in adult and aging patients. Limitations and confounds of our present experiments are discussed to improve future examinations of epileptic comorbidities in extended kindled mice.

Keywords: epilepsy, hippocampus, kindling, mice, memory

INTRODUCTION

Epilepsy is a disease characterized by an enduring predisposition to generate epileptic seizures and by the neurobiological, cognitive, psychological, and social consequences of this condition (2014 definition, International League Against Epilepsy). Temporal lobe epilepsy is the most common and often drug-resistant type of epilepsy in the adult and aging populations and has great diversity in etiology, electro-clinical properties, and comorbidities (Hauser, 1992; Engel, 1996; Brodie et al., 2009; Ferlazzo et al., 2016). It has been increasingly recognized that the treatment of epilepsy is not restricted to the achievement of seizure freedom but must also include the management of comorbid medical, neurological, psychiatric, and cognitive comorbidities (Kanner, 2016). Kindling through repeated brief stimulation of limbic structures has long been used to model temporal lobe epilepsy and comorbidities (see reviews by Brooks-Kayal et al., 2013; Gorter et al., 2016; Löscher, 2016; Mazarati, 2017; Sutula and Kotloski, 2017). However, kindling studies in middle-aged, aging, or aged animals remain scarce (de Toledo-Morrell et al., 1984; Fanelli and McNamara, 1986; Stover et al., 2017). Currently, little is known about kindling-induced behavioral changes in middle-aged/aging animals.

Classic or extended kindling protocols have been used in previous studies. While classic kindling lasting a few weeks does not induce spontaneous recurrent seizures (SRS), extended kindling is able to induce SRS in monkeys (Wada and Osawa, 1976), dogs (Wauquier et al., 1979), cats (Wada et al., 1974: Gotman 1984; Hiyoshi et al., 1993), rats (Pinel and Rovner, 1978; Pinel, 1983; Milgram et al., 1995; Michalakis et al., 1998; Sayin et al., 2003; Brandt et al., 2004), and mice (Song et al., 2018). SRS induction by extended kindling is generally not associated with gross brain injury, but rather a loss of subgroups of GABAergic interneurons in the hippocampal hilar region (Sayin et al., 2003; but see Brandt et al., 2004). The lack of observed gross brain injury in extended kindled animals is different from poststatus epilepticus models in which SRS emergence is accompanied with pronounced brain damage (Dudek and Staley, 2017; Gorter and van Vliet, 2017; Henshall, 2017; Kelly and Coulter, 2017). As such, the extended kindling model may help explore epileptogenesis and comorbidities in the absence of major brain pathology as is seen in many patients with temporal lobe epilepsy (Ferlazzo et al., 2016).

To date, two published studies are available concerning behavioral changes in extended kindled rats with SRS. One study demonstrated an increase of body weight in amygdala-kindled rats (Löscher et al., 2003) and the other examined performances of amygdala/performant-path-kindled rats in the Morris water maze (MWM) and open-field tests (Cammisuli et al., 1997). Kindled rats in the latter study were impaired during the initial training on hidden platform acquisition but not in retention of platform location in the MWM test. In addition, these animals showed a transient increase in open-field activity. Together, these findings indicate that the extended kindling of the amygdala/performant path in adult rats disrupted the acquisition phase of a spatial memory task and induced a transient elevation in open-field locomotor/exploratory behaviors. The issue remains open as to whether extensive kindling at other limbic structures and in middle-aged/aging animals may lead to more pronounced behavioral deficits.

We explored these issues in the present experiments using a mouse model of extended hippocampal kindling (Song et al., 2018). Specifically, we conducted experiments in middle-aged mice (12–14 months old) to model new-onset epilepsy seen clinically in adult/aging populations. Mice received chronic kindling stimulation or handling manipulations (controls) and then underwent continuous EEG-video monitoring before and following MWM and open-field tests. Our data indicate that middle-aged mice with kindling-induced SRS have pronounced deficits in spatial learning and memory.

METHODS

Animals

Male C57 black mice (C57BL/6N) were obtained from Charles River Laboratory (Saint-Constant, Quebec, Canada) and housed in a local vivarium. The vivarium was maintained at room temperature between 22°C and 23°C with a 12-h light on/off cycle (lights on at 6:00 am). Mice were housed in group (up to four mice per cage) with food and water *ad libitum*. All experimentations described below were reviewed and approved by the Animal Care Committee of the University Health Network in accordance with the Guidelines of the Canadian Council on Animal Care.

C57 black mice have a maximal lifespan up to 36 months but aging/aged mice often encounter health-related complications including skin lesions, ear infections, and tumors (Flurkey et al., 2007). We therefore chose to start kindling in middle-aged mice (ages 11–12 months) in an attempt to model new-onset epilepsy and comorbidities as seen clinically in adult/aging populations (Brodie et al., 2009; Ferlazzo et al., 2016) while minimizing the health-related complications that are common in aging/aged mice.

Electrode Construction and Implantation

Electrode construction and implantation were performed as previously described (Jeffrey et al., 2014; Bin et al., 2017). All electrodes were made of polyamide-insulated stainless steel wires (110-μm outer diameter; Plastics One, Roanoke, Virginia, USA). Twisted bipolar electrodes were used for stimulation and/or recording. Surgeries were performed under isoflurane anesthesia. A stereotaxic frame and micromanipulators were used for electrode placement. Implanted electrodes were secured onto the skull using the glue-based method. Each mouse was implanted with two pairs of bipolar electrodes; one positioned to the hippocampal CA3 (bregma −2.5 mm, lateral 3.0 mm, and depth 3.0 mm; Franklin and Paxinos, 1997) for kindling stimulation and local recordings and other targeted to the ipsilateral/contralateral parietal cortex (bregma −0.5 mm, lateral 2.0 mm, and depth 0.5 mm). A reference electrode was positioned to a frontal area (bregma +1.5 mm, lateral 1.0 mm, and depth 0.5 mm). The locations of implanted electrodes were verified by later histological assessments when suitable.

Hippocampal Kindling

A train of stimuli at 60 Hz for 2 s was used for hippocampal kindling stimulation (Reddy and Rogawski, 2010; Jeffrey et al., 2014; Bin et al., 2017; Stover et al., 2017; Song et al., 2018). Constant current pulses with monophasic square waveforms, a pulse duration of 0.5 ms, and current intensities of 10–150 μA were generated by a Grass stimulator and delivered through a photoelectric isolation unit (model S88, Grass Medical Instruments, Warwick, Rhode Island, USA). An ascending series was used to determine the threshold of evoked afterdischarges in individual mice. In the assessing series, a stimulation train with incremental current intensities (10 μA per step) was applied every 30 min. The lowest current that elicited an afterdischarge event of ≥5 s was considered the afterdischarge threshold. Stimulations on subsequent days used a stimulation current intensity at 25% above the threshold value (Reddy and Rogawski, 2010). Our goal was to keep constant stimulation intensity throughout the extended kindling period. However, the initial stimulation intensity often became inconsistent in evoking afterdischarges after ≥45 days of kindling experiments, which might be a result of contamination of the implanted electrodes. Due to this this complication, stronger stimulation intensities (40–80 μA above the initial intensities) were used in subsequent experiments.

Kindling stimuli were applied twice daily between 10 AM and 5 PM and ≥5 h apart (Pinel and Rovner, 1978; Pinel, 1983; Milgram et al., 1995; Michalakis et al., 1998; Sayin et al., 2003; Brandt et al., 2004). Each stimulation episode lasted for several minutes during which the mouse was placed in a glass container for EEG-video monitoring (Stover et al., 2017; Song et al., 2018). Control mice were handled twice daily for 60 consecutive days.

Continuous EEG-Video Monitoring

Concurrent EEG recordings and video monitoring were done as previously described (Bin et al., 2017). Briefly, an implanted mouse was placed in a modified cage with food and water *ad libitum*. A slip-ring commutator was mounted atop the cage and connected to the mouse *via* flexible cables. A webcam was placed near the cage to capture mouse's motor seizures. Dim lighting was used for webcam monitoring during the lights-off period. EEG and video data were collected roughly 24 h daily and for 2–3 consecutive days per session. A cursor auto-click program (Mini Mouse Macro program; http://www.turnssoft.com/mini-mouse-macro.html) was used to save data every 2 h.

Baseline EEG-video monitoring of 24 h was performed ≥1 week after electrode implantation. Similar monitoring was made after the 80th, 100th, 120th, and/or 140th stimulation to assess SRS commencement. No further kindling stimulation was applied if ≥2 SRS events/day were detected, and additional monitoring for 24–48 h was performed to assess initial SRS daily incidences. Continuous EEG-video monitoring for 24–72 h was performed after behavioral tests to determine whether SRS persisted in individual mice. The control mice were monitored for 24 h after 60 days of handling manipulations.

Differential recordings through twisted bipolar electrodes were used to sample local EEG signals (Jeffrey et al., 2014; Bin et al., 2017; Stover et al., 2017; Song et al., 2018). Mono-polar EEG recordings were used only if the differential recordings were unsuccessful. Signals were collected using two-channel or one-channel microelectrode AC amplifiers with extended head-stages (model 1800 or 3000, AM Systems; Sequim, Washington, USA). Evoked afterdischarges of the stimulated hippocampal CA3 were captured using the model 3000 amplifier *via* TTL-gated switches between recording and stimulating modes. These amplifiers were set with an input frequency band of 0.1–1,000 Hz and amplification gain of 1,000. Amplifier output signals were digitized at 5,000 Hz (Digidata 1440A or 1550, Molecular Devices; Sunnyvale, California, USA). Data acquisition, storage, and analyses were done using pCLAMP software (Version 10; Molecular Devices).

Measurements of Discharges, Interictal Spikes and Motor Seizures

Evoked afterdischarges and spontaneous ictal discharges were recognized by repetitive spike waveforms with amplitudes approximately two times of background signals and durations of ≥5 s (Jeffrey et al., 2014; Bin et al., 2017; Stover et al., 2017; Song et al., 2018). Interictal spikes were recognized by large amplitudes (≥8 times of standard deviation of background signals) and simple/complex waveforms and durations of 30–250 ms. Spikes were measured from 30-min EEG segments for individual mice. These segments were collected at ≥2 h after an ictal discharge to minimize post-ictal influences on interictal activities. The event detection function (threshold search method) of pCLAMP software was used to automatically detect spikes, and detected events were then visually inspected, and false events were rejected (El-Hayek et al., 2013; Song et al., 2018). EEG data analyses were made independently by three researchers (HYL, HMS, and LZ). Consensus on disputed events was reached after discussion.

Evoked and spontaneous motor seizures were scored using the Racine scale modified for mice (Racine, 1972; Reddy and Rogawski, 2010). Briefly, stage 0—no response or behavioral arrest, stage 1—chewing or facial movement, stage 2—chewing

and head nodding, stage 3—unilateral or bilateral forelimb clonus, stage 4—bilateral forelimb clonus and rearing, and stage 5—rearing and falling with limb clonus. Evoked and spontaneous motor seizures were assessed independently by several researchers (JC, NS, CC, YPL, and SL). The concordance rates for recognizing stage 4–5 seizures were ≥90% among these researchers.

Morris Water Maze (MWM) Test

A dark blue pool with 120 cm in diameter, and 50 cm in depth was used. The pool was placed in a quiet room and filled with white-colored water. The water was equilibrated to room temperature (between 22°C and 23°C) for at least 48 h before the MWM test. Colored papers with a variety of different shapes were posted around the pool as visual cues. A platform of 10 cm in diameter was used. For hidden platform trials, the platform was positioned 1.5 cm below water surface. A Panlab tracking system (Harvard Apparatus, Quebec, Canada) was used to monitor animal's behaviors in the pool. The MWM test was performed in the period of 11AM–3PM to minimize circadian effects. The extended kindled and control mice were tested in same days, and testing sequences for individual mice were altered in each test day.

The MWM test was performed 1–2 weeks after ending the kindling stimulation or handling manipulation. A protocol with 3 days of visible platform trials, 5 days of hidden platform trials, and two probe tests on hidden days 3 and 5 were employed (Vorhees and Williams, 2006; Stover and Brown, 2012; **Figure 2A**). In the visible and hidden platform trials, individual mice underwent four trials per day, and the maximal time for each trial was 90 s. If mice did not find the platform within 90 s, they were guided to the platform by the experimenter's hand and allowed to stay on the platform for 15 s. For the probe tests in which the platform was removed from the pool, individual mice underwent a trial of 60 s. If mice exhibited convulsions shortly before or during a trial, they were allowed to recover for 20–30 min before next trial. Any trial interfered with convulsions were excluded from analysis. Before the third day of the hidden phase and the day following the last hidden trials, the mice were subjected to a probe trial where the platform was removed, and the mice were allowed to swim in the pool for a single 60-second trial. Distances and latencies to find the platform, swim speed during the visible and hidden platform trials, and the time in searching the correct quadrant during the probe trials were analyzed. Group data for the extended kindled and control mice were compared.

Open-Field Test

The open-field test of 1-h duration was conducted 4–7 days after the MWN test and in the period of 10AM–1PM to minimize circadian effects. Each mouse was examined only once to prevent acclimation to the open-field apparatus. Care was taken to clean the plexiglass arena with 75% alcohol and water before each test to avoid odor interference from preceding test.

We used a plexiglass open-field arena (20×30 cm; Jugloff et al., 2008; Wither et al., 2013). The arena is surrounded by a housing frame that accommodates an array of 24 infrared beams forming a grid across two levels. The lower grid measures X–Y movement while the upper grid measures rearing movement. As the mouse moves, a beam is broken and registered as an activity count, depending on the timing and number of concurrent beams broken; different types of activity are recorded. Several behavioral parameters are assessed using this system. Total static or mobile count means total beam breaks in which changes in mouse's position are below or above the mobile threshold. Total rearing or central rearing count denotes the upper beam breaks detected in entire arena or in arena center. Total activity count represents total beam breaks by static, mobile, and rearing behaviors. With respect to static, mobile, or rearing time, the measure is incremented every time as the mouse is engaged in a type of activity in any given second. Active time means the total activity time including static, mobile, and rearing.

Statistical Analyses

Statistical tests were conducted using Prism 6 (GraphPad Software, San Diego, California, USA) or SigmaPlot software (Systat Software Inc., San Jose, California, USA). Student's t-test or Mann–Whitney rank-sum test was used for two-group comparisons. A one-way ANOVA or one-way ANOVA on ranks was used for multiple group comparisons, followed by a multiple comparison Holm–Sidak or Tukey test. A mixed repeated ANOVA was used for within group comparisons. Pearson or Spearman rank-order test was used for correlation analysis. Data are presented as means and standard error of the mean (SEM) throughout the text and figures. Statistical significance was set at $p < 0.05$.

RESULTS

General Behaviors and SRS Assessments

We collected data from 12 kindled mice and 12 handling control mice. The hippocampal kindling or handling manipulation over 9–10 weeks did not cause evident disruption in ambient cage behaviors or significant changes in body weights. Measured body weights were 29.1 ± 1.1 and 28.0 ± 0.8 g ($p = 0.408$) before kindling and after ending the kindling stimulation and 30.9 ± 1.4 and 29.4 ± 1.7 g ($p = 0.487$) before handling and after ending the handling manipulation. There is a discrepancy between our present observations and the previous study demonstrating excessive body weight gain in rats following extended amygdala kindling (Löscher et al., 2003). This may be due largely to a difference in kindling sites as the amygdala is known to play an important role in regulating food intake although the hippocampus may complement amygdala functions (Coppin, 2016).

We used a kindling protocol with twice daily stimulations in the present experiments. Mice were fully kindled following 19 ± 1.7 hippocampal stimulations (ranging 9 to 27 stimuli, $n = 12$) as indicated by three consecutively evoked stage-5 motor seizures (Jeffrey et al., 2014; Stover et al., 2017; Song et al., 2018). These mice exhibited SRS following 107.8 ± 5.1 hippocampal stimulations (ranging from 85 to 140 stimuli). Evoked hippocampal afterdischarges cumulative to SRS were

3,779.2 ± 399.0 s (ranging from 2,712 to 6,882 s), and cumulative Racine scales for corresponding motor seizures were 551.2 ± 56.8 (ranging from 390 to 948). Detected *via* continuous EEG-video monitoring in the first 2–3 days after ending the kindling stimulation, SRS incidences were 7.1 ± 0.7 events/day (ranging from 3.5 to 11 daily events), corresponding hippocampal ictal discharges were 45.6 ± 1.4 s in duration, and motor seizure scores were 3.54 ± 0.06 on the modified Racine scale. There was no significant correlation between the stimulation numbers needed to reach the fully kindled or SRS state and the initial SRS incidence ($p > 0.05$). The cumulative measures of evoked after discharges or motor seizures were also uncorrelated with the initial SRS incidences ($p > 0.05$). **Figures 1A**, **B** illustrates representative EEG discharges and video-captured images collected from a kindled mouse in the first day after termination of kindling stimulation.

In addition to the ictal discharges, the kindled mice exhibited repetitive interictal spikes that manifested during immobility or sleep but were also detectable in movement or exploratory behaviors (Song et al., 2018). We measured hippocampal interictal spikes as they were more frequent and robust than cortical spikes. Spike analyses were made in 30-min data segments that were collected during immobility/sleep and in the first 2–3 days after ending the kindling stimulation. The mean inter-spike intervals were in a range of 2.1 ± 0.053 to 12.9 ± 1.7 s (12 mice).

The kindled mice exhibit 6.6 ± 0.6 SRS events/day while being monitored 1–2 weeks after the behavioral tests. There was no significant difference between the initial and later SRS incidence measures ($p = 0.672$). Hippocampal interictal spikes were observed in all the kindled mice following the behavioral tests, but spike amplitudes and background signals were decreased. The latter might have largely resulted from contamination in implanted electrodes which complicated spike detection. Hippocampal interictal spikes were reliably measured from four kindled mice before and following the behavioral tests, showing no significant difference in inter-spike intervals (5.8 ± 2.4 and 4.3 ± 0.8 s, $n = 4$, $p = 0.568$). Together, these observations suggest that SRS and interictal spikes might persist in extended kindled mice.

In contrast to the extended kindled mice, the control mice showed neither seizure nor aberrant EEG activity while being examined *via* 24-h EEG-video monitoring after ending the handling manipulation.

Morris Water Maze (MWM) Test

The MWM test was conducted 1–2 weeks after the initial EEG-video monitoring. We used a protocol with 3 days of visible platform trials, 5 days of hidden platform trials, and 2 probe tests on days 3 and 5 of the hidden platform trials (**Figure 2A**).

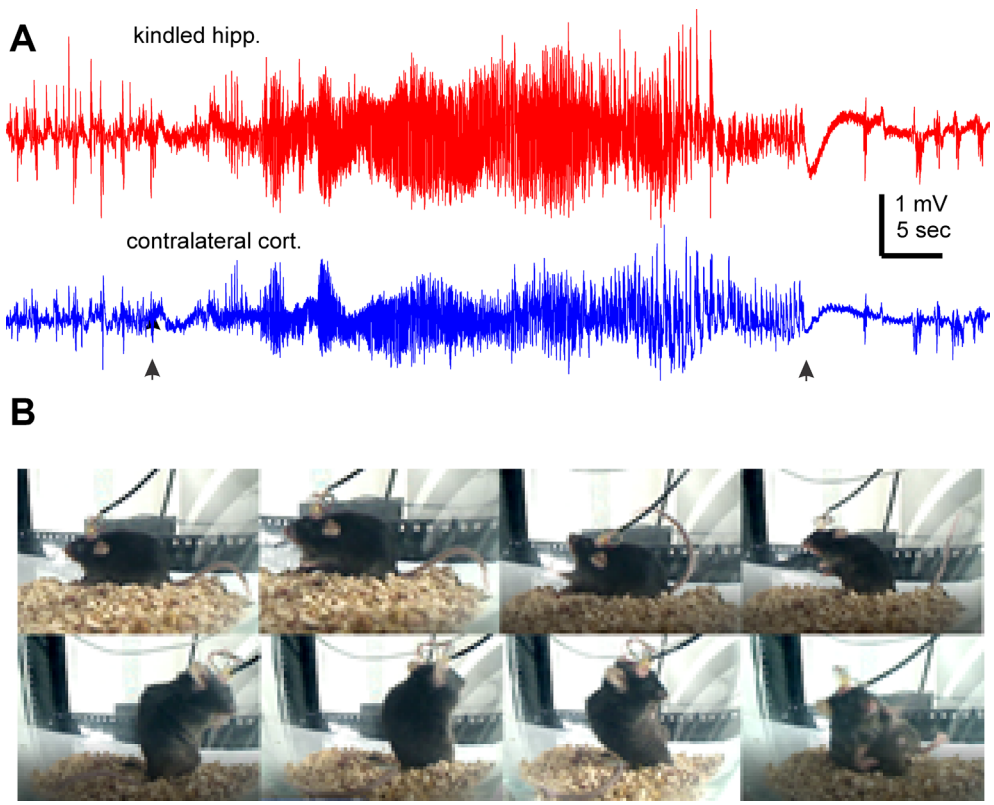

FIGURE 1 | Representative EEG discharges and corresponding motor seizure. EEG traces and images were collected from a mouse the first day after termination of kindling stimulation. **(A)** Ictal discharges were recorded from of the kindled hippocampus and contralateral parietal cortex. Filled arrows denote the onset and tarnation of discharges. **(B)** Sequential images (from top-left to bottom right) show a stage-5 seizure.

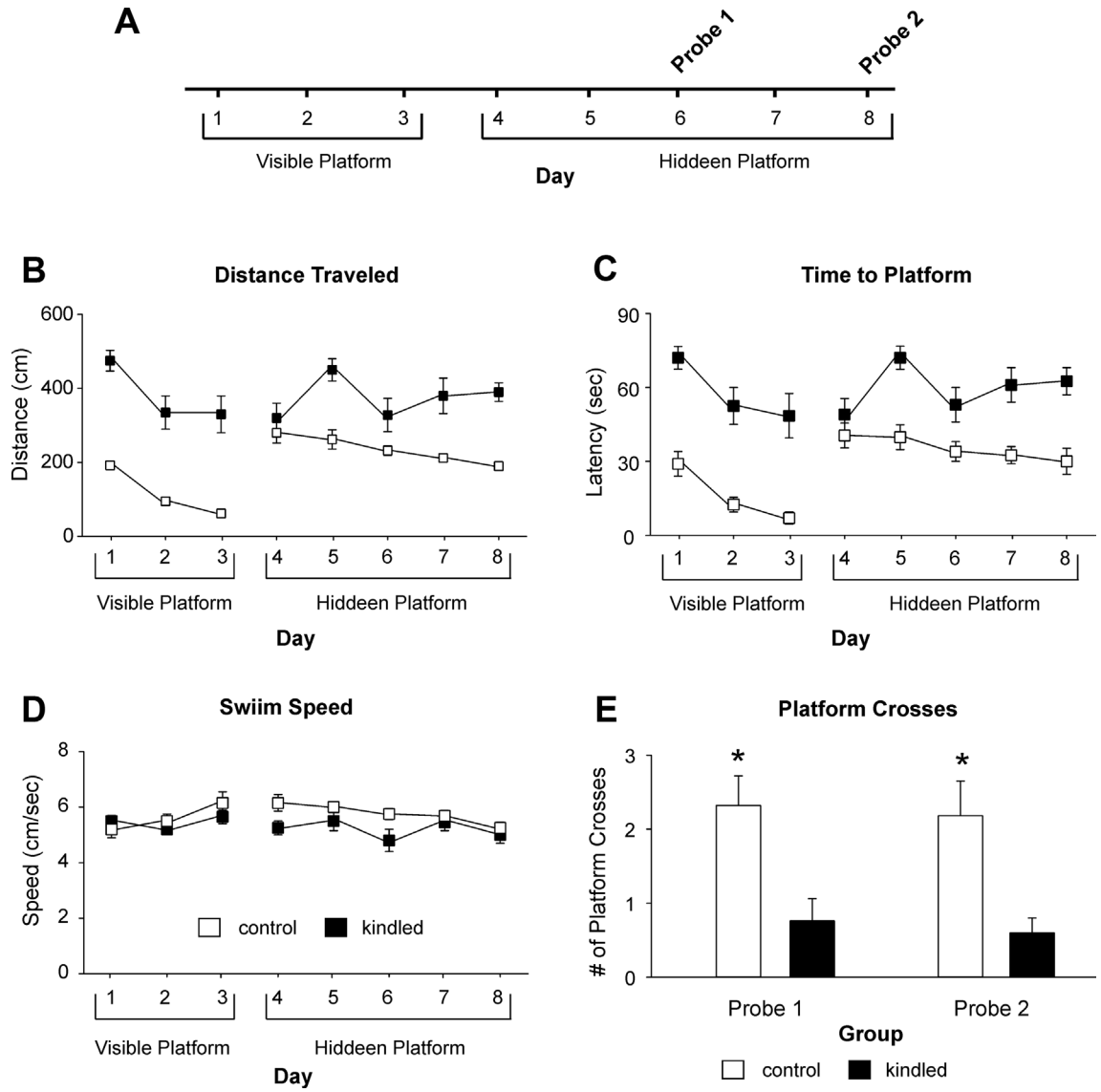

FIGURE 2 | Distances and latency Measures in the MWM test. **(A)** A schematic layout of the MWM protocol. The visible and hidden platform trials consisted of four trials per day and maximal 90 s per trial. The probe tests were one trial per day and 60 s per trial with the platform removed. Data in **(B**, **E)** (means ± SEM) were collected from 12 extended kindled mice and 12 handling control mice. **(B**, **C)** Distances and latencies needed to reach the platform. There were significant group differences in the distance and latency measures during the visible and hidden platform trials. **(D)** Swim speeds measured during the visible and hidden platform trials. There were no significant group differences in these measures. **(E)** Crosses of the platform location during the first and second probe test. The kindled mice crossed the platform location significantly less frequently (denoted by *) than the control mice.

During the visible platform trials, both the kindled and control mice were significantly improved in performance over the 3-day trials, as there were day/trial-dependent reductions in swim distance [$F(2,44) = 16.089$, $p < 0.001$] and time [$F(2,44) = 15.055$, $p < 0.001$] in finding the platform (**Figures 2B, C**). However, the kindled mice swam significantly longer distances [$F(1,22) = 28.161$, $p < 0.001$] and took significantly longer times [$F(1,22) = 30.671$, $p < 0.001$] relative to the controls to find the platform.

During hidden platform trials, the kindled mice also swam significantly longer distances and spent more time than the control mice to reach the platform [$F(1,22) = 17.280$ and 17.086; $p < 0.001$]. Over the 5-day trials, the kindled mice showed no performance improvement as there were no day/trial-dependent reductions in swim distance and time [$F(4,88) = 1.888$ and 8.208, $p = 0.120$ and 0.074], whereas the control mice improved moderately but significantly over the 5-day trials [$F(4,88) = 2.770$, $p = 0.032$; **Figures 2B, C**].

In both the visible and hidden platform trials, there was no significant difference between the kindled and control mice with respect to swim speed [$F(1,22) < 1$ and 2.034, $p = 0.973$ and 0.168] nor speed changes over the 3-day or 5-day trials [$F(4,88) = 1.994$ and < 1, $p = 0.102$ and 0.543; **Figure 2D**].

During the probe tests 1 and 2 where the platform was removed from the south-west quadrant, the extended kindled mice crossed the south-west quadrant significantly less frequently than the control mice [$F(1,22) = 10.704$ and 9.210, $p = 0.003$ and 0.006] (**Figures 2D, E**). For swim distance per quadrant in the first probe test, there was a significant effect of kindling group [$F(1,22) = 7.505$, $p = 0.012$], quadrant [$F(3,66) = 3.151$, $p = 0.031$], and a kindling group by quadrant interaction [$F(3,66) = 3.418$, $p = 0.022$], as the control mice travelled a greater distance in the correct quadrant than then control mice (**Figure 3A**). There was no effect of kindling group [$F(1,22) < 1$] or quadrant [$F(3,66) = 1.710$, $p = 0.174$] in time spent in each quadrant during the first probe trial, but there was a kindling group by quadrant interaction [$F(3,66) = 3.815$, $p = 0.014$] as the kindled mice spent less time in the correct quadrant than the control mice while there was no difference in time spent in other quadrants (**Figure 3B**). This indicated that the kindled mice did not remember the platform location during the first probe test.

For both swim distance [$F(3,63) = 9.011$, $p < 0.001$] and time [$F(3,63) = 6.479$, $p < 0.001$] per quadrant in the second probe test, there was only an effect of quadrant, as all mice favored the correct quadrant, and there were no significant differences between kindling groups or interactions (**Figures 3C, D**). This indicated that all mice remembered the platform location equally well.

Open-Field Test

The open-field test was conducted 4–7 days after the MWM test. We used a protocol with 1-h duration (Jugloff et al., 2008; Wither et al., 2013) and obtained multiple measures from the open-field test (**Table 1**). Data were collected from 9 of the 12 kindled mice and from 10 of the 12 control mice because 3 kindled mice encountered convulsive seizures during the open-field test, and data acquisition were disrupted for 2 control mice. Overall, there were no significant differences between the kindled and control mice with respect to total activity counts, total activity time, and total distances traveled, but significantly fewer static counts and shorter static time for the kindled mice than the control mice. Total rearing counts were not significantly different between the two groups, but the kindled mice had fewer central rearing counts than the control mice.

The kindled and control mice showed similar acclimation during the 1-h open-field test. When open-field activities were analyzed every 5 min, time-dependent decreases in activity were evident for both the kindled and control mice. Significantly

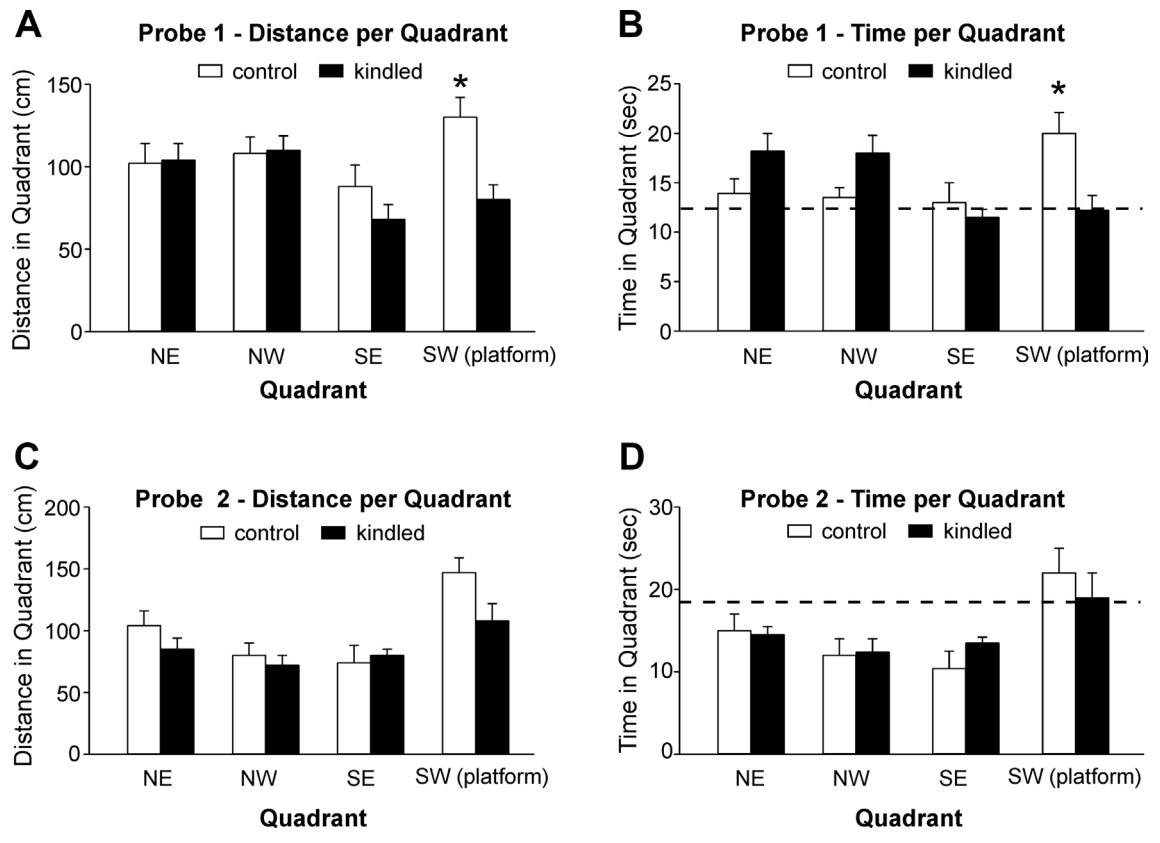

FIGURE 3 | Measures for probe tests. Data (means ± SEM) were collected from the extended kindled and handling control mice (n = 12 each group). **(A, B)** Distances traveled and time spent in each of the quadrants during the first probe trial. **(C, D)** The same measures collected during the second probe trial. Dotted lines represent chance performance. Note that the kindled mice traveled shorter distances and spent less time in the correct quadrant as compared to the controls. Significant differences between the control and extended kindled mice were denoted by *.

TABLE 1 | Measures of the open field test.

	Control	Extended kindled	P values
Total activity counts	154.8 ± 15.9	124.5 ± 22.3	0.383
Total static counts	101.1 ± 8.9	63.1 ± 9.2	0.008*
Total mobile counts	53.7 ± 8.5	61.4 ± 16.2	0.677
Total rearing counts	26.3 ± 5.4	20.8 ± 4.5	0.444
Total central rearing counts	2.3 ± 0.7	0.3 ± 0.3	0.009*
Active time (s)	111.6 ± 11.1	92.3 ± 14.6	0.307
Static time (s)	82.8 ± 7.2	57.1 ± 6.7	0.018*
Mobile time (s)	28.8 ± 4.7	35.3 ± 9.2	0.535
Rearing time (s)	39.3 ± 7.3	27.5 ± 5.6	0.218
Inactive time (s)	188.4 ± 11.1	207.7 ± 14.6	0.307
Distance traveled (m)	10.3 ± 1.2	11.3 ± 2.5	0.791

*Data (means ± SEM) were collected from 10 control mice and 9 extended kindled mice with SRS. Total counts and times were summed from a 1-h monitoring period. Significant differences between the control and extended kindled mice were denoted by *.*

higher or lower activities in the kindled mice were noted only in 5–10-min or 50–55-min periods (**Figure 4**).

DISCUSSION

Old age is associated with high incidence of seizures and epilepsy. Temporal lobe epilepsy is the most common type of epilepsy seen in the adult and aging populations. While stroke, dementia, and brain tumors are recognized risk factors, the etiology is unknown for many aging/aged individuals with new-onset epilepsy (Hauser, 1992; Brodie et al., 2009; Ferlazzo et al., 2016). It is therefore important for pre-clinical studies to examine epileptogenesis and comorbidities in middle-aged/aging animals (Kelly, 2010), but such studies remain limited (de Toledo-Morrell et al., 1984; Fanelli and McNamara, 1986; Hattiangady et al., 2011; Stover et al., 2017). Currently, there is no published information concerning hippocampus-dependent memory functions in epileptic middle-aged/aging animals with epileptic seizures. Here, we provide original information in this area using a mouse model of extended hippocampal kindling.

There were four main observations in our present experiments. 1) Both the kindled and control mice demonstrated decreases in time and distance to find the platform in the visible trials, suggesting that they could learn the task. 2) The kindled mice performed significantly worse than the controls in both the visible and hidden platform trials of the MWM test compared to control mice. 3) The kindled mice crossed the pervious location of the hidden platform fewer times than the control mice during both probe tests and had a memory deficit as measured by time and distance spent in the correct quadrant during the first probe test but not the second probe test. These observations suggest that, with increased training, the deficit in kindled mice may be lessened. 4) The kindled and control mice demonstrated similar locomotor activity in the open-field test as measured by total activity count and total distance traveled, but evident anxiety-like behaviors were noticeable in the kindled mice as they had fewer central rearing count and more static count and

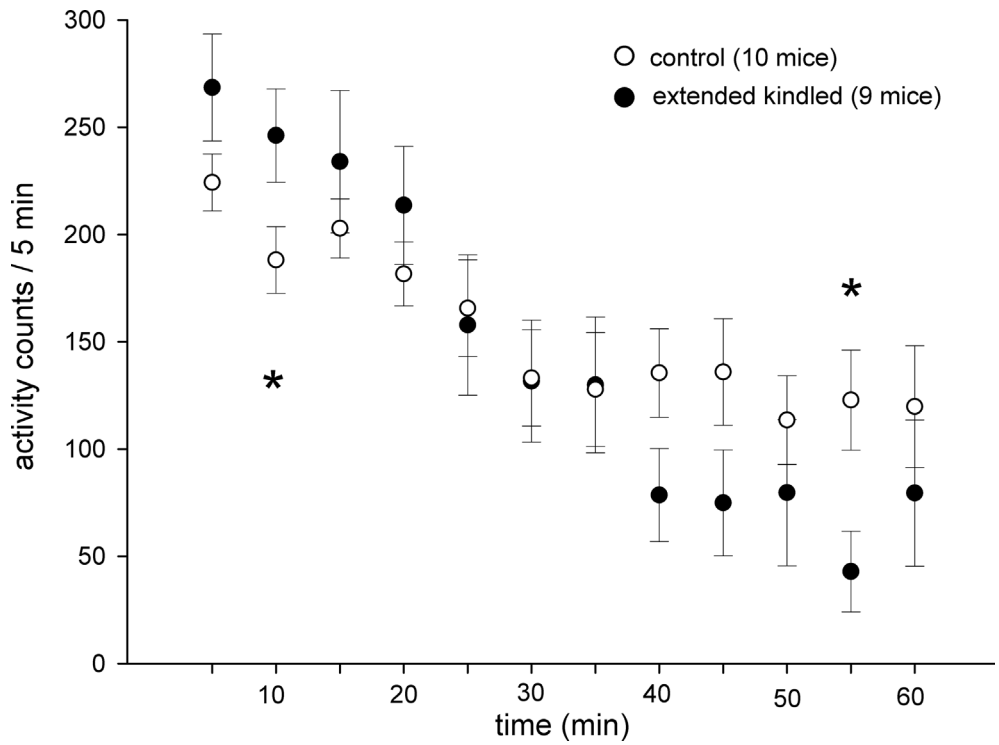

FIGURE 4 | Time-dependent changes in open field activities. Data (means ± SEM) were collected from 10 control mice and 9 extended kindled mice with SRS. Individual mice were monitored for 1 h in the open field arena. The activities detected from each 5 min period were summed and presented sequentially. Significant differences between the control and extended kindled mice were denoted by *.

time (Seibenhener and Wooten, 2015). Overall, these observations suggest that the extended kindled mice have a modifiable deficit in hippocampal-dependent learning and memory in the MWM test.

The spatial learning and memory deficit we observed from mice following extended hippocampal kindling appear to be more pronounced than that recognized in adult rats following extended amygdala/performant-path kindling (Cammisuli et al., 1997). This may be due to multiple experimental factors including differences in animal species and ages (Löscher et al., 2017), kindling sites, SRS, and interictal spike incidences. Specifically, we conducted hippocampal kindling in middle-aged C57 black mice (11–13-month-old). C57 black mice ages 8–16 months have been shown to perform poorly relative to younger mice of 3–6 months old in the MWM test (de Fiebre et al., 2006; Shoji et al., 2016; but see Benice et al., 2006; Boyer et al., 2019). A potential memory decline in the middle-aged mice used in our experiments may partly count for the moderate improvement of the control mice as well as the non-improvement of the kindled mice during the hidden platform trials. In addition, SRS with mean incidences of 6–7 events per day were observed from the extended kindled mice before and following the MWM tests, whereas SRS incidences were variable in the amygdala/performant-path-kindled rats (Cammisuli et al., 1997; Michael et al., 1998). As pronounced deficits in spatial learning and memory have been observed in other epilepsy models with SRS (Scantlebury et al., 2005; Brandt et al., 2007; Gröticke et al., 2008; Müller et al., 2009; Seeger et al., 2011; Niquet et al., 2016; Pascente et al., 2016; Vrinda et al., 2017), it is likely that frequent SRS in the extended kindled mice may lead to more severe impairment in spatial learning and memory. Moreover, frequent interictal spikes were consistently observed from extended kindled mice before and following the behavioral tests. These interictal spikes might also have strong detrimental impacts on hippocampal cognitive process (Holmes, 2013; Gelinas et al., 2016).

Several previous studies have examined the effects of hippocampal kindling on spatial learning and memory in adult rats (Gilbert et al., 1996; Leung et al., 1996; Sutherland et al., 1997; Gilbert et al., 2000; Hannesson et al., 2001a, Hannesson et al., 2001b; Hannesson et al., 2004; Leung and Shen, 2006). Fully kindled rats performed more poorly than controls in visible and/or hidden platform trails but not in probe tests (Gilbert et al., 1996; Gilbert et al., 2000) or showed deficits in hidden platform trials but not in visual platform trials and probe tests of the MWM test (Hannesson et al., 2001b). Hippocampal kindling did not affect performance (hidden platform trails) in the early phase of a delayed-match-to-place maze test but disrupted performance during the delay phase of the test (Hannesson et al., 2001a). Partial kindling did not affect initial acquisition in the MWM test or a radial-arm maze test but induced deficits in rats that were trained prior to kindling and retested after variable delays (Leung et al., 1996; Sutherland et al., 1997; Leung and Shen, 2006). There are noticeable differences between these studies and our present observations with respect to performance in the MWM test. In particular, extended kindled mice performed poorly relative to controls in visible and hidden platform trials and in the first probe test, whereas fully kindled rats showed variable impairments in these measures. In addition, extended kindled mice did not show trial-dependent improvement in the hidden platform trails, whereas such improvement was evident in fully kindled rats (Gilbert et al., 1996; Gilbert et al., 2000; Hannesson et al., 2001b). In light of the notion that a few localized hippocampal seizures disrupted spatial cognition (Hannesson et al., 2004) and cause distributive alterations of the entorhinal–hippocampal circuit responses (Leung and Shen, 2006), cumulative seizure activities induced during extended kindling, in addition to persistent SRS and interictal spikes, may worsen spatial learning and memory outcomes in our model.

Other studies have conducted long-term kindling (66–99 stimulations) of the amygdala, hippocampus, or caudate nucleus in adult rats (Kalynchuk et al., 1998; Magyar et al., 2005; Fournier et al., 2013). While the long-term kindling did not induce SRS, kindled rats presented deficits or abnormalities in open-field activity, fear memory, and/or sexual behaviors. It remains to be tested whether similar behavioral deficits/abnormalities occur in extended kindled mice with SRS.

Our present experiments have some limitations. Regarding the MWM test, we did not analyze swim trajectories due to errors in data acquisition, which prevents assessment of whether the kindled mice employed different strategies relative to the control in searching the platform. In addition, middle-aged C57 black mice, particularly those experienced extended hippocampal kindling, may have visual impairments (Vorhees and Williams, 2006), which might complicate their performances in the visible platform trials. The uses of other spatial tasks less dependent upon visual cues than the MWM test may help address this complication in our model. Furthermore, we used a small open-field arena relative to those employed in other studies (see review by Kraeuter et al., 2019). The impacts of this confound on our present observations need to be verified. The lack of assessment of a causal relation between SRS and spatial learning and memory deficit is a major weakness of our present study, which is particularly important for exploring potential management strategies for alleviating epilepsy comorbidities. Despite these limitations and weaknesses, it is our hopes that our present works may help future studies that examine epilepsy comorbidity in middle-aged/aging animals using the mouse model of extended hippocampal kindling.

DATA AVAILABILITY

All datasets generated for this study are included in the manuscript/supplementary files.

ETHICS STATEMENT

The animal study was reviewed and approved by Animal care committee of University Health Network.

AUTHOR CONTRIBUTIONS

HL, KS, HMS, and LZ participated in experimental design, data discussion, and interpretation. DW and JE participated in data discussion. NS, JC, CC, YL, SL, CPW, KS, and HMS conducted experiments and/or data analysis. KS and LZ participated in manuscript assembling and writing.

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Embelin Protects Against Acute Pentylenetetrazole-Induced Seizures and Positively Modulates Cognitive Function in Adult Zebrafish

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Purpose of the research: Epilepsy is a continuous process of neurodegeneration categorized by an enduring tendency to generate uncontrolled electrical firing known as seizures causing involuntary movement all over the body. Cognitive impairment and behavioral disturbances are among the more alarming co-morbidities of epilepsy. Anti-epileptic drugs (AEDs) were found to be successful in controlling epilepsy but are reported to worsen cognitive status in patients. Embelin (EMB) is a benzoquinone derived from the plant *Embelia ribes* and is reported to have central nervous system (CNS) activity. This study aims to evaluate the effectiveness of EMB against pentylenetetrazole (PTZ) induced acute seizures and its associated cognitive dysfunction. This was done *via* docking studies as well as evaluating neurotransmitter and gene expression in the zebrafish brain.

The principal results: Behavioral observations showed that EMB reduced epileptic seizures and the T-maze study revealed that EMB improved the cognitive function of the fish. The docking study of EMB showed a higher affinity toward gamma-aminobutyric acid ($GABA_A$) receptor as compared to the standard diazepam, raising the possibility of EMB working *via* the alpha subunit of the GABA receptor. EMB was found to modulate several genes, neurotransmitters, and also neuronal growth, all of which play an important role in improving cognitive status after epileptic seizures. Healthy zebrafish treated with EMB alone were found to have no behavioral and biochemical interference or side effects. The immunohistochemistry data suggested that EMB also promotes neuronal protection and neuronal migration in zebrafish brains.

Major Conclusions: It was perceived that EMB suppresses seizure-like behavior *via* $GABA_A$ receptor pathway and has a positive impact on cognitive functions. The observed effect was supported by docking study, T-maze behavior, neurotransmitter and gene expression levels, and immunohistology study. The apparatus such as the T-maze and seizure scoring behavior tank were found to be a straightforward technique to score seizure and test learning ability after acute epileptic seizures. These research findings suggest that EMB could be a promising molecule for epilepsy induced learning and memory dysfunction.

Keywords: embelin, epilepsy, cognitive disorder, T-maze behavior, zebrafish

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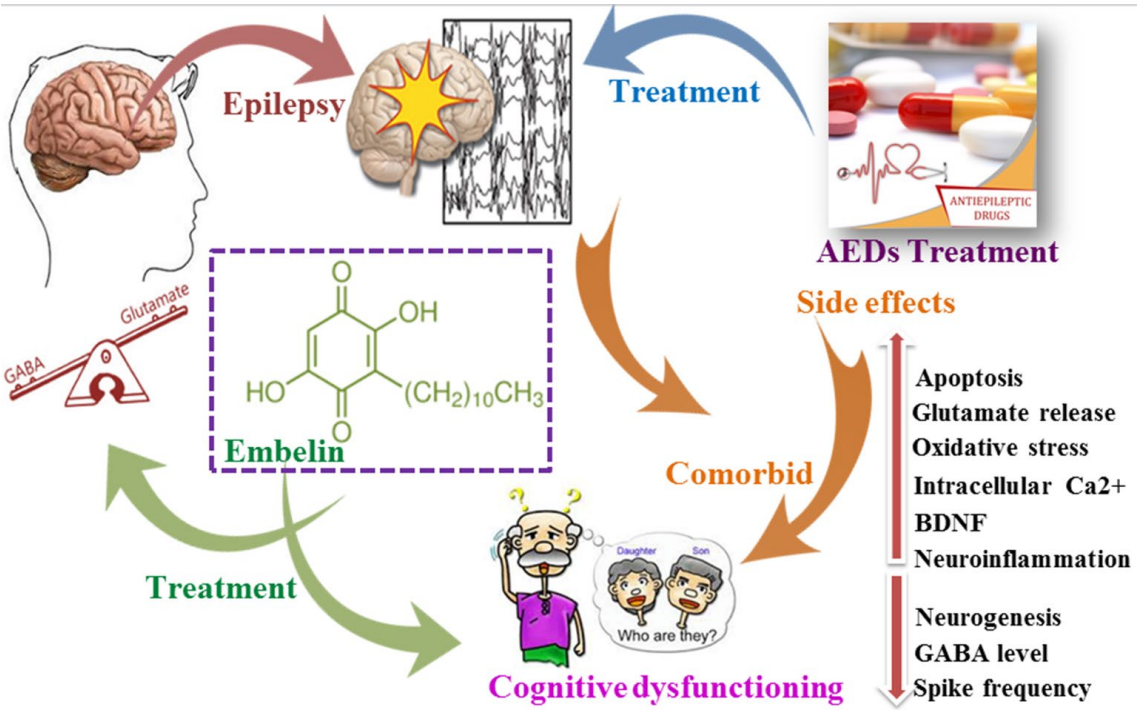


GRAPHICAL ABSTRACT | Impact of epilepsy and antiepileptic drugs on cognitive dysfunction.

BULLET POINT SUMMARY:

1) What is already known:

- Epilepsy related comorbidities such as cognitive dysfunction are common in many people with epilepsy.
- Most of the anti-epileptic drugs help in preventing seizures but have a negative impact on cognitive functions.
- A crude extract of the plant *Embelia ribes* is used to treat epilepsy in alternative medicine.

2) What this study adds:

- Embelin isolated from *Embelia ribes* was found to be effective against seizures and prevented memory decline in zebrafish.
- Embelin modulates neurotransmitters and gene expression as well as exhibits a neuroprotective effect.
- A docking study of EMB shows that it has a high affinity toward the gamma-aminobutyric acid ($GABA_A$) receptor and possibly works *via* the alpha subunit of the GABA receptor.

INTRODUCTION

Epilepsy is a neurological condition with complications associated with diverse neurobiological and behavioral alterations characterized by recurrent, spontaneous epileptic seizures (Galanopoulou et al., 2012). It is the fourth most common neurological disorder (Newton and Garcia, 2012), affecting over 70 million people of all ages around the world (Copmans et al., 2017). The significant feature of epilepsy are the seizures, but it also affects cognition, leading to a poor quality of life (Van Rijckevorsel, 2006; Kim and Ko, 2016). The prevalence of memory problems in patients with epilepsy is 40–45%, and they experience difficulties in problem-solving and learning as well as have psychomotor retardation (Mula, 2015). A notion exists that the rate/effects of seizures and the dose of AEDs play a key role in the cognitive decline of epileptic patients (Lagae, 2006; Park and Kwon, 2008). Adverse effects due to cognitive impairment are a major problem associated with AEDs as they alter the role of different genes that are associated with epileptogenesis and memory function (Gupta et al., 2017) (Gayatri and Livingston, 2006).

Few studies have investigated the role of genes such as neuropeptide Y (NPY) gene, CREB1, and brain-derived neurotrophic factor (BDNF) that interact with each other to control epilepsy and enhance long term memory (Gøtzsche and Woldbye, 2016; Luo et al., 2017). BDNF is a small protein secreted in the brain that binds to its p75 and TrKB receptors and has a remarkable role in memory, survival, and differentiation of neurons in the brain (Roopra et al., 2012). It has been known that BDNF is linked with the inception of epileptogenesis, and the epileptic condition is avoided by the upregulation of BDNF indicator in the brain (Binder, 2004). The cAMP response element (CRE) is associated with several genes responsible for epileptogenesis in the form of a promoter, and it has been

identified to phosphorylate cAMP response to generate epilepsy (Zhu et al., 2012). Theories about the cAMP-response element binding protein (CREB) and memory are still evolving. Study in mice shows that decreased CREB levels have 50% decrease in seizures episode and require more electrical kindle stimulations (Zhu et al., 2012). Earlier study has reported that chronic spontaneous seizures are reduced by NPY in a temporal lobe epilepsy model of rats (Noe et al., 2008). The anticonvulsant effect of NPY gene overexpression in rat brains shows the important role in controlling epilepsy of NPY (Noe et al., 2010). NPY has been recently proposed for gene therapy, depending upon patient data, to control epilepsy using various viral vector carriers. These trials were successful in various transgenic rodent models (Sørensen and Kokaia, 2013).

In recent years, the primary focus has turned toward non-mammalian epilepsy models for behavior testing, due to numerous factors. These factors including greater cost-effectiveness, high genetic correlation with humans, and rapid breeding (Arief et al., 2018). The cost and time required to carry out gene expression and brain cell genesis studies in zebrafish are less as compared to rodents (Mussulini et al., 2013). The main limitation in executing research in epilepsy and cognitive abnormalities is the lack of precise and reproducible animal models for the testing of new drug molecules (Yam Nath Paudel et al., 2018). To overcome this limitation, our laboratory has developed a zebrafish model of epilepsy induced cognitive dysfunction and confirmed the hypothesis that both epilepsy and AEDs affect cognitive functions in zebrafish (Kundap et al., 2017b). Although a study is conducted using zebrafish, the findings can still be translated to mammals, particularly humans, as over 70% of zebrafish genes are substantially similar to their mammalian orthologues (Macrae and Peterson, 2015). Another reason is that 70% of zebrafish genes also have one or more human orthologues, and 47% of human genes have a one to one relationship with a corresponding zebrafish gene (Howe et al., 2013). There are also studies that have established that zebrafish brain regions show homologous functions to their mammalian counterparts, despite the substantial neuroanatomical differences (Fontana et al., 2018). Recent studies have shown that many different genetically modified zebrafish models (Samarut et al., 2018) are used as initial *in vivo* screening tools for compounds derived from natural sources (Samarut et al., 2019).

Numerous natural products, including derivatives of quinone which are thought to have better efficacy and safety profiles, are known for their central nervous system (CNS) related activity (Durg et al., 2017). Embelin (EMB) exhibits favorable chemical and physical properties, and its capacity for penetrating the blood–brain barrier (BBB) (Bhuvanendran et al., 2018) makes it a suitable candidate for the treatment of CNS related complications (Pathan et al., 2009). EMB is a water-insoluble compound with a LogP value of 4.71 (octanol–water) (Xu et al., 2005), meaning that the compound is highly lipophilic and can reach the brain to exert its effect. Phytochemical and pharmacological investigations discovered that the presence of EMB is a vital element in treating CNS disorders (Kundap et al., 2017a).

Therefore, the primary goal of this study was to evaluate the effectiveness of EMB against pentylenetetrazole (PTZ)-induced seizures and associated cognitive dysfunction using adult zebrafish as an animal model. In this study, we tried to use a simple T-maze apparatus to measure the memory function of the fish after a single administration of PTZ at an acute dose. We hypothesized that EMB may work by modulating several genes that cause epilepsy, the levels of GABA, and also possibly *via* the $GABA_A$ receptor as it has a higher affinity for the receptor as compared to the standard drug diazepam. In spite of the proven role of EMB in epilepsy related genes such as NPY, BDNF, and cAMP-responsive element-binding protein 1 (CREB_1) genes (Kundap et al., 2017a), we attempted to elucidate the overall activity of EMB to support our hypothesis by using molecular docking, immunohistochemistry, and pharmacological, biochemical, and behavioral experimentations. Exploring the cellular and molecular mechanism of compounds from natural sources against epilepsy induced cognitive dysfunction will pave the way for further research and can be a potential alternative to mainstream AEDs.

TECHNIQUES AND MATERIALS

Experimental Equipment and Chemical List

All reagents used were analytical grade unless specified otherwise. Water was purified and filtered with a specific LC-MS filter using a Milli-Q system from Millipore (Bedford, MA, USA). Glutamate (Glu), gamma-amino butyric acid (GABA), acetylcholine (Ach), diazepam (DZP), PTZ, paraformaldehyde (PFA), phosphate buffered saline (PBS), benzocaine (BZ), and bromodeoxyuridine (BrdU) were purchased from Sigma–Aldrich (USA). Ethanol 95% (EtOH) was purchased from KOLIN Chemicals Co. Ltd., Korea, methanol (MeOH), chloroform (CHCl3), isopropanol (IPA), and formic acid (FA) were purchased from Friedemann Schmidt Chemicals, Parkwood 6147, Western Australia. The pure form of plant extract of EMB was purchased from YUCCA Enterprises, Mumbai, India (purity—97.90%, moisture—1.90%). Fish tanks (10 L capacity) were purchased from Petco Pet Keeper, Malaysia. The Sony Handycam (AVCHD 5x) recorder, Sony Camcorder stand, Smart 3.0.05 Tracking Software (Panlab, Harvard Apparatus), Hamilton Syringe 700–702 series 25 µl, BD Disposable Needle (30G), FSC 22 Frozen Section Media (Leica Biosystems, Nussloch, Germany), Leica CM1860 Cryostat (Leica Biosystems, Nussloch, Germany), and Agilent Infinity 1290 UHPLC, coupled with Agilent 6410 Triple Quad LC/MS and the Applied Biosystems StepOnePlus™ Real-Time PCR Systems, were also used in this work.

Zebrafish (*Danio rerio*) Care and Maintenance

Adult zebrafish (*Danio rerio*) of heterozygous wild-type-AB stock (standard short-fin phenotype) were obtained from the Institute of Molecular and Cell Biology (IMCB), 61 Bioplis Drive Proteos, Singapore 138673. All fish were kept in the Monash University Malaysia fish facility at 28°C, with a 10/14 h dark/light cycle (white incident light off at 10 pm, white incident

light on at 8 am) under aquarium conditions. Care was taken to maintain the system water pH between pH 6.8 and 7.1 by using an electronic pH pen (Classic PH Pen Tester, Yi Hu Fish Farm Trading, Singapore 698950), and the intensity of light was maintained uniformly all over the housing area at 250 Lux. Fish were nourished with TetraMin® Tropical Flakes twice a day and livestock of Artemia from Bio Marine (Aquafauna, Inc. USA) once a day to ensure a constant source of the food supply with *ad libitum* feeding. Zebrafish tanks with dimensions of 36 cm x 26 cm x 22 cm were used to house the fish, and the tanks were equipped with a water circulation system for constant aeration (Kundap et al., 2017b). Group housing was used, whereby 10–12 fishes/tank, males, and females were housed separately. The Monash Animal Research Platform (MARP), Australia, approved all the zebrafish experimental procedures (MARP-2015-084).

Drug Treatment and Groups

The treatment group of animals were initially injected with the vehicle/test drug and then injected with PTZ to check their seizure behavior. Later, their cognitive status was examined using a T-maze. EMB and standard DZP were dissolved in 10% dimethyl sulfoxide (DMSO). The animals were divided into the following groups, with each group having n = 10 unless mentioned otherwise as shown in **Figure 1**.

Set-1 (Only Emb Treatment)

Group I: vehicle control (VHC—10% DMSO) + distilled water; group II: DZP 1.25 mg/kg + distilled water: group III: EMB-0.156 mg/kg + distilled water; group IV: EMB-0.312 mg/kg + distilled water; group V: EMB-0.625 mg/kg + distilled water

Set-2 (Epilepsy Behavior Test)

Group I: vehicle control (VHC—10% DMSO) + distilled water; group II: VHC + PTZ 170 mg/kg; group III: DZP 1.25 mg/kg + PTZ (170 mg/kg); group IV: EMB-0.078 mg/kg + PTZ (170 mg/kg); group V: EMB-0.156 mg/kg + PTZ (170 mg/kg); group VI: EMB-0.312 mg/kg + PTZ (170 mg/kg); group VII: EMB-0.625 mg/kg + PTZ (170 mg/kg); group VIII: EMB-1.25 mg/kg) + PTZ (170 mg/kg); group IX: EMB-2.5 mg/kg + PTZ (170 mg/kg); group X: EMB-5 mg/kg + PTZ (170 mg/kg); group XI: EMB-10 mg/kg + PTZ (170 mg/kg)

Set-3 (Brdu Immunochemistry)

Group I: vehicle control (10% DMSO); group II: VHC + PTZ (negative control group); group III: EMB 0.156 mg/kg + PTZ (170 mg/kg); group IV: EMB 0.625 mg/kg + PTZ (170 mg/kg)

All the drugs were administered *via* intraperitoneal injection as per the procedure described previously by Kundap et al. (2017b).

Procedure for a Zebrafish Anesthesia and Intraperitoneal Injection

PTZ and EMB were intraperitoneally infused into the zebrafish as specified by the protocol given by Kundap et al. (2017b) and is given beneath. When different intraperitoneal infusions were required, the infusions were given at alternating lateral ends, instead of the midline between the pelvic fins.

Each zebrafish was caught individually using a fish holding net and after that moved into an anesthesia preparation (30 mg/L BZ). The fish were kept in the anesthesia water for 30 s until they stop moving. The zebrafish were taken out once anesthetized and weighed afterward to calculate the dose and subsequently the infusion volume. A delicate sponge roughly 20 mm in height was soaked with water and set inside a 60 mm Petri dish. A cut between 10 and 15 mm was made in the sponge to control and hold the fish for the intraperitoneal infusion. The intraperitoneal infusion was given while utilizing a dissection microscope by embedding the needle into the midline between the pelvic fins. An appropriate volume was then injected into the zebrafish, after considering the body weight of the zebrafish.

All intraperitoneal infusions were administered into the stomach pit at an area midline to the pelvic fins, utilizing a 10 µl Hamilton syringe (700 series, Hamilton 80400) (Stewart et al., 2011). The experiment was performed in a separate behavior room, with the room temperature kept between 26 and 30°C and humidity between 50 and 60%. All zebrafish were acclimatized in the said behavior room for 2 h prior to experiment to minimizing any novel tank response. Other precautions taken include using a small injection volume of 10 µl per gram of fish and using a 35-gauge needle. The zebrafish were restrained in a water saturated sponge under BZ anesthesia to reduce the distress inflicted on the zebrafish (Júnior et al., 2012). This intraperitoneal injection technique was found to be effective in zebrafish (Kundap et al., 2017b) and did not cause any mortality throughout the experiment. After the intraperitoneal injection, the zebrafish was immediately transferred to an observation tank.

Zebrafish Behavior Study for Epilepsy and Cognition

Dose Determination Study for Embelin

A wide range of EMB doses were tested, which were from 0.078 to 10 mg/kg. The zebrafish were divided into 11 groups containing 12 fish in each group. DZP was used as a positive control, and the fish received a 1.25 mg/kg dose. The vehicle-treated group received 10% DMSO, followed by a distilled water injection. To induce epileptic seizures, all the animals were first treated individually with the vehicle/DZP/EMB and then injected with 170 mg/kg of PTZ. The resulting seizure behavior was observed for seizure scoring for a period of 10 min post-PTZ injection. The seizure score was used as a parameter to determine the EC_{50} value of the EMB (Vincent, 2010). A graph was plotted as the log of drug concentration against the percentage (%) effect of the drug, giving a familiar sigmoidal shaped graph, to select the effective dose of EMB (Mensor et al., 2001).

Epilepsy Behavior and Seizure Score Recording

Adult zebrafish were tested in an observation tank, with the seizure intensity being measured using a special scoring system as per a previously developed protocol (Kundap et al., 2017b). Seizure score, seizure onset time (min), total distance traveled (cm), as well as time spent (s) in upper and the lower halves of the tank were noted. After seizure score analysis, the fish were tested for their cognitive ability using a T-maze test

(Banote et al., 2013). Animals administered with a specific PTZ concentration demonstrate different seizure scores, seizure frequencies, seizure profiles, and latencies to reach the seizure scores. Seizures normally last for 10 min after administration of 170 mg/kg of PTZ and progressively decreases with time (Desmond et al., 2012).

T-Maze Test

The T-maze is an apparatus which consists of a "T" shaped box containing one straight long arm and two short arms on the left and right-hand side (Wenk, 2001). The right short arm has a bigger opening known as the "deeper chamber," which acts as a favorable environment for the fish and thus the fish tend to spend a maximal amount of time there. The detailed specifications regarding the maze were as per a previous study. Transfer latencies (TL) were recorded at 0, 3, and 24 h post-PTZ administration. Inflexion ratios (IR3 h) = (L0–L1)/(L1) and (IR24 h) = (L0–L2)/(L2) were calculated, where L0 is the initial latency(s) at 0 h, and L1 and L2 are the latency(s) at the 3 h and 24 h trial, respectively. The behavior recordings during seizure activity and the T-maze test were analyzed to track the locomotor patterns. Tracking of the locomotor pattern was done by using the computer software SMART v3.0—Panlab Harvard Apparatus® (Kundap et al., 2017b).

Biochemical Estimation

Brain Harvesting

The zebrafish brains were harvested at the end of behavior study in order to determine the molecular changes in the brain. The animal groups were divided into two halves, and each brain was then transferred into TRIzol® for gene expression studies and another half into MeOH for LC-MS/MS studies. The whole process of brain harvesting was done under ice-cold conditions, and the brains were immediately transferred into dry ice. All the brains were stored at −80°C until further use.

Neurotransmitter Analysis

Glutamate, GABA, and Ach are the significant neurotransmitters in investigating epilepsy and cognition. These neurotransmitters were analyzed using the Liquid Chromatography–Tandem Mass Spectrometry (LC-MS/MS) technique. All the standard neurotransmitters were prepared in MeOH (0.1% FA) as a stock solution of 1 mg/ml and were kept at 4°C until use. Standards for calibration were prepared from the original stock solution. Serial dilutions from 100–2,000 ppb were used for calibration. The brain was homogenized in 200 µl of ice-cold MeOH (0.1% FA). The homogenate was vortex-mixed for 1 min and then centrifuged at 18, 000 × g for 10 min at 4°C. Finally, for LC-MS/MS analysis, the supernatant was pipetted and placed into vials.

LC-MS/MS was run on an Agilent 1290 Infinity UHPLC, coupled with an Agilent 6410 Triple, Quad LC/MS, ZORBAX Eclipse Plus C18 RRHD 2.1 x 150 mm, 1.8-micron (P/N 959759-902) auto-sampler system (Agilent Technologies, Santa Clara, CA, USA). The samples were separated on a SMol-RRHD-Eclipse-C18-8 (15) UHPLC-160129-00011-Pos-DMRM used at 30°C. The mobile phase consisting of 0.1% FA in water (solvent A) and acetonitrile with 0.1% FA (solvent B) was used with a gradient elution: 0–3 min, 50% B; 3–6 min, 95% B; and 06–07 min, 95% B, at a flow rate of 0.1 ml/min. ESI-MS/MS conditions were set as follows: ESI ion source, positive ion polarity, gas temperature 325°C, drying gas flow 9.0 L/min, nebulizer pressure 45 psi, and Vcap 4,000 V. MS acquisition of GABA, Glu, and ACh was performed in electrospray positive ionization multiple reaction monitoring (MRM) mode.

Gene Expression

Gene expression studies were carried out for the NPY, BDNF, and cAMP-responsive element-binding protein 1 (CREB_1) genes. All the brain samples were placed in ice-cold 200 µl TRIzol® reagent (Invitrogen, Carlsbad, CA, USA) and immediately stored at −80°C until further usage. The study was divided into three steps, namely, isolation of mRNA, synthesis of cDNA strand, and then real-time PCR to estimate the level of the gene expressed.

Isolation of RNA and First Strand cDNA Synthesis

The mRNA was isolated by following the protocol provided by the kit's manufacturer. In brief, brain tissue was properly homogenized in TRIzol® reagent, mixed with CHCl3, and centrifuged at 13,500 rpm (revolutions per minute) for 15 min at 4°C. The upper aqueous supernatant was transferred into new tubes, and IPA was added, mixed, and incubated for 10 min at room temperature and later centrifuged for 10 min at 13,500 rpm at 4°C. The supernatant was discarded, and the pellets were subjected to rinsing with 75% ethanol. The pellets were air-dried for 5 to 8 min. Finally, nuclease-free water was added to each tube to dissolve the mRNA pellet. The concentration and purity of the isolated mRNA were measured using a NanoDrop Spectrophotometer (Implen NanoPhotometer 190–1,100 nm, Galileo, Madrid, Spain). The mRNA samples were converted into cDNA using the QuantiTect Reverse-Transcription Kit (Qiagen) according to the manufacturer's protocol.

StepOne® Real-Time PCR

The gene expression of NPY, BDNF, and CREB_1 were measured by real-time quantitative RT-PCR (StepOne Applied Biosystems) using QuantiTect SYBR Green Dye (Qiagen, Valencia, CA). All the primer sets were provided by Qiagen (npy: Dr_npy_1_SG QuantiTect Primer Assay (QT02205763) , bdnf: Dr_bdnf_1_SG QuantiTect Primer Assay (QT02125326), and CREB_1: Dr_CREB_1 bpa_1_SG QuantiTect Primer Assay (QT02197503). The PCR mixture contained 1X SYBR Green PCR Master Mix (Qiagen), 0.7 µM of both forward and reverse primers, and 1 µl of sample cDNA. The samples were incubated at 95°C for 2 min prior to thermal cycling (40 cycles of 95°C for 5 s and 60°C for 15 s). Relative expression values of the above genes were obtained by normalizing the threshold cycle (Ct) values of genes of interest against Ct value of eef1a1b (housekeeping gene) (2 ^ [Ct eef1a1b—Ct gene of interest]).

Molecular Modeling Studies

All molecular docking studies were performed in BIOVIA Discovery Studio 4.5 (www.accelrys.com). Since the 3D structure

of the gamma-aminobutyric acid receptor ($GABA_A$ subunit β3) of zebrafish is not available, we performed molecular docking studies of EMB on the human GABA subunit β3 to predict and correlate *in vivo* results. The x-ray crystal structure of the human $GABA_A$ subunit β3 complexed with the agonist benzamidine was retrieved from the Protein Databank (PDB code: 4COF) (Miller and Aricescu, 2014). The water molecules were deleted, and hydrogen atoms were added. Finally, the protein was refined with a CHARMM force field at a physiological pH. To validate the docking reliability, a co-crystallized ligand (benzamidine) was first re-docked to the binding site of GABA. Subsequently, DZP and EMB were docked into the same active site, and 30 conformations of each compound were obtained through CDOCKER. The conformation with the lowest energy was selected as the most probable binding conformation for each ligand.

The CDOCKER is CHARMM-based docking algorithm that uses the CHARMM family of force fields and offers all the advantages of full ligand flexibility (including bonds, angles, and dihedrals) and reasonable computation times (Brooks et al., 1983). The CDOCKER algorithm adopts a strategy involving the generation of several initial ligand orientations in the active site of the target protein, followed by molecular dynamics based simulated annealing and final refinement by energy (Mo et al., 2012). The CDOCKER was used for the docking of all compounds. The molecular docking study was carried out to understand the binding mode of EMB within the active site of the $GABA_A$ β3 subunit using the Discovery Studio suit 4.5 software.

Bromodeoxyuridine (BrdU) Immunohistochemistry

Mode of Administration

Adult fish were anesthetized in system water tank (1 L) containing 0.016% BZ (pH 7.0; Classic PH Pen Tester, Yi Hu Fish Farm Trading, Singapore 698950). All the fish from each group were individually injected twice with BrdU (100 mg/kg) intraperitoneally (Mao et al., 2009), with a time interval of 4 h. The vehicle/EMB/PTZ was injected intraperitoneally as per the dose described. The animal in the treatment group was injected with the vehicle/EMB doses and habituated for 15 min in an observation tank. To induce epileptic seizures, fish from the EMB and PTZ group were individually exposed to a 170 mg/kg dose of PTZ. The postinjection survival period ranged between 2 h to 15 days, before the brain was extracted for fixation. Later on, after 2 h of PTZ treatment, animals from each group (n = 6) were euthanatized, and brain samples were harvested for immunochemistry studies to check the neuronal loss at day 0. The remaining n = 6 animals were housed separately in the individual tanks for next 15 days to check the survival and the proliferating ability of neurons.

Zebrafish Brain Fixation

Immunohistochemistry was performed on the whole brain of adult zebrafish. The animals were deeply anesthetized by immersing them into BZ dissolved in system water. The brain was extracted completely by opening the skull and was fixed overnight at 4°C in 4% PAF in saline phosphate buffer (pH 7.4). The specimen was then soaked into 10% sucrose solution at room temperature until brain sinks to the bottom of the tube (up to 5–6 h). Later, the 10% sucrose solution was replaced with a 20% sucrose solution, and the specimen was soaked overnight at 4°C. Later, the 20% sucrose solution was replaced with a 30% sucrose solution at 4°C and left for up to a week, until used for cryostat sectioning (Ekström et al., 2001).

Preparation of Cryostat Brain Section

To prepare cryo-sections, the cryostat machine was set at −20°C (Ekström et al., 2001). Specimens were molded into a cryomold block by using FSC 22 frozen section media inside the cryostat machine at −20°C. The cryostat was set to 20 microns section thickness, and each alternate section was placed on two equally divided glass slides. The sections could adhere to the slide at room temperature for at least 1 h, and later, these slides were stored at −20°C until the immunochemistry procedure.

Immunohistochemistry Procedure for the Adult Zebrafish Brain

Day-1 procedure: The protocol followed was described earlier by (Malberg et al., 2000). The sections were thoroughly washed three times in PBS with an interval of 5 min. The sections were then immersed in a 50% formamide solution (Vivantis, Inc. USA) for 2 h at 65°C. After 2 h of incubation, the section slides were washed once with 2X-SSC (Sodium Citrate; 0804-4L, Ameresco) for 5 min. Then, sections were incubated in 2N HCl for 30 min at 37°C and washed in a solution of 0.1 M boric acid for 10 min. Blocking solution was added to block the section with 10% normal horse serum (Gibco, Thermo Fisher Scientific, USA) dissolved in PBS containing 0.1% Triton-X-100 (Amresco, Ohio) for 1.5 h. Subsequently, the sections were incubated with mouse anti-BrdU antibody (1:500; Roche Diagnostics, IN, USA) in 10% horse serum with 0.5% bovine serum albumin (BSA) (Sigma Life Science, USA) in PBS containing 0.1% Triton-X-100 for 18 h at 4°C.

Day-2 procedure: Sections were washed three times with PBS at the interval of 5 min and incubated for 2 h with the biotinylated horse anti-mouse secondary antibody (1:250; BA-2001, Vector Laboratories, Burlingame, U.S.A). After incubation, the sections were followed by three washes with PBS and incubated with avidin-biotin complex (1:55; VECTASTAIN ABC Kit, Vector Laboratories, CA, U.S.A.) for 2 h at room temperature. After three washes with PBS, the sections were visualized with diaminobenzidine solution (DAB, D4293; Sigma), which was prepared in 0.1 M phosphate buffer (pH 7.4) for 4–5 min. The DAB reaction was stopped by adding PBS when a minimum background color appeared. The sections were then re-rinsed first in PBS and then in distilled water, ordered, mounted onto poly-lysine-coated slides, and dried overnight. Finally, they were dehydrated through ascending grades of alcohol and cleared in xylene before being covered in DPX (Sigma Life Science, USA) and a glass cover slip. The slides were observed for analysis later.

Cell Quantifications/Cell Counting

To quantify BrdU-positive cells, 10-μm-thick serial coronal plastic sections through the whole tectal region were prepared

as described above (n = 6). BrdU-positive cells were counted on a BX50 microscope (Olympus) with an UPlanFLN 20×/10×/4× (NA0.90) objective lens. Since the size of the teleost (zebrafish) brain is slightly different among samples of the same age, we divided all sections into 10 groups along the rostro-caudal axis (six sections/groups) and calculated the mean cell number of each group in each region in the brain. This mean cell number was compared with the corresponding group of samples.

Statistical Analysis

For statistical analyses, GraphPad Prism 5 software (GraphPad Software, Inc.) was used. Data are represented as mean and standard errors of the mean (SEM). The results acquired were analyzed by one-way ANOVA and subsequent Dunnett's multiple comparison tests to assess the differences in seizure, latency, inflexion ratio, neurotransmitter levels, and gene expression levels between treatments and also to assess the difference in cell count at day 0 and the number of cells migrated to different regions of the brain at 15 days post-PTZ injection. For all analyses, differences between a treatment group and the equivalent negative-control group were considered statistically significant if the p-value was below 0.05 ($p < 0.05$).

RESULTS

Seizure Analysis and Onset Latency

All the animals in the PTZ only treatment group showed full blown seizures up to score 4 during the 10 min recording. All the animals pretreated with EMB displayed an average seizure score of not more than 1.5, in a dose-dependent manner. It was also observed that animals treated with EMB-2.5 mg/kg to EMB-10 mg/kg (+PTZ) display seizure scores that are similar to the PTZ treated group. DZP was used as a standard drug for the epilepsy study, and it was found to suppress seizures in the DZP + PTZ treated group as shown in **Figure 2**.

Latency to seizure score 4 is the time required by the fish to reach seizure score 4 in a given time frame of 10 min. The time taken by all the animals to reach seizure score 4 from the negative control group (PTZ treated group) was 160–180 s after PTZ injection. In animals treated with DZP + PTZ, we found that the latency to reach seizure score 4 was more than 500 s. On the other hand, the onset latency was also delayed in animals treated with EMB in a dose-dependent manner. As seen in **Figure 2B**, the EMB-0.078 mg/kg to EMB-0.312 mg/kg (+PTZ) groups showed a significant increase in seizure onset latency as compared to the PTZ treated group. As the dose of the EMB increases, the activity of the drug was found to decrease and

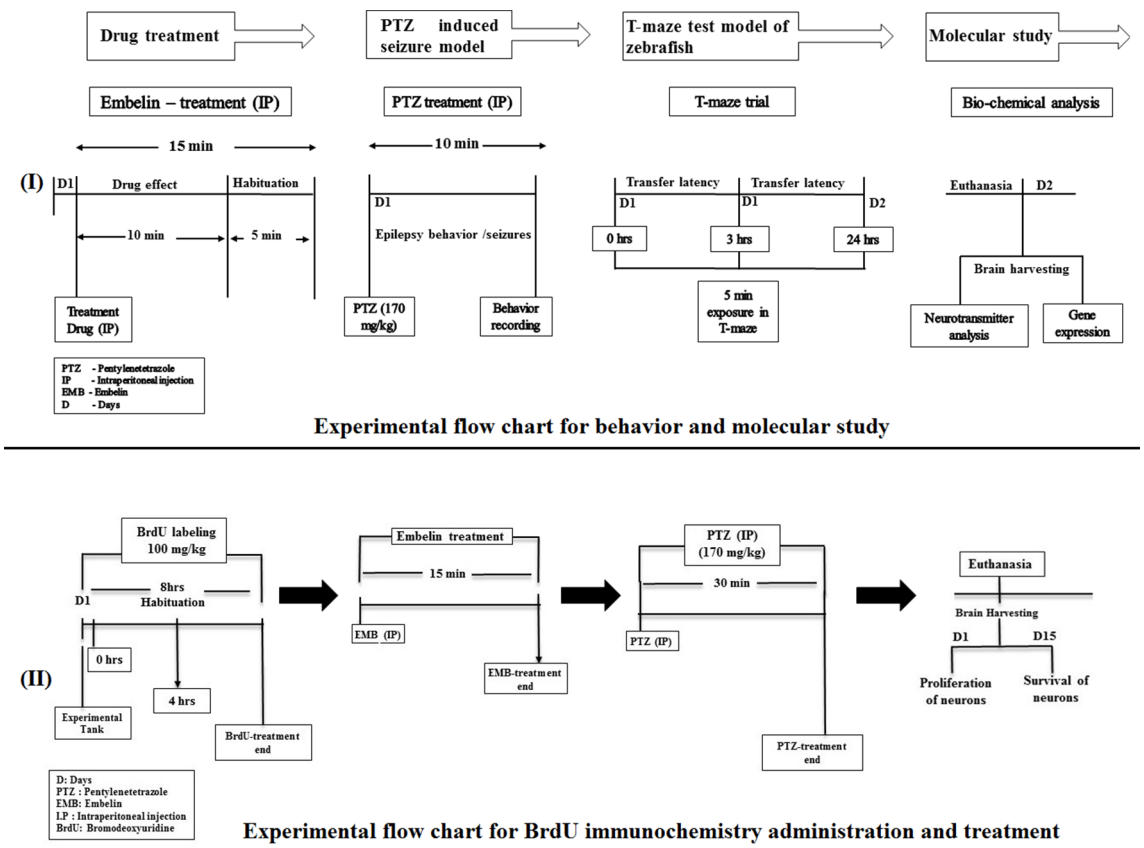


FIGURE 1 | Experimental setup and design procedure. The flowchart represents the scheme for drug treatment, PTZ administration, and behavior recording for epilepsy and T-maze used in the study.

FIGURE 2 | Seizure score and onset latency for epileptic seizures: **(A)** represents the effect of embelin on PTZ induced seizures in adult zebrafish. **(B)** represents onset of seizure latency score 4 for EMB and DZP treated fish when compared with PTZ treated group. Data are represented as mean ± SEM, n = 10, and statistically analyzed by one-way ANOVA followed by Dunnett's test *P ≤0.05, **P ≤ 0.01, and ***P ≤ 0.001.

could not delay the seizure onset latency in the EMB-0.625 mg/kg to EMB-10 mg/kg (+PTZ) group.

Locomotor Pattern

A normal swimming pattern was seen in the vehicle control group, in which the tracking pattern shows swimming all over the tank. These control fish were found to spend an equal amount of time all over the tank as shown in **Figure 3A**. An involuntary, rapid movement of the body that includes a corkscrew (spiral) swimming pattern and hyperactivity was seen in the negative control group (PTZ treated) fish. PTZ provoked seizures are a spontaneous behavior which produced the tremor and jittery locomotion tracking pattern seen mainly at the bottom of the tank. The locomotor tracking pattern for most of the EMB treated groups challenged with PTZ exhibited improved tracking patterns which were almost similar to the control group. It was found from the tracking pattern of the EMB-0.156 mg/kg to EMB-0.625 groups that fish from these groups swam all over the tank, in all directions and in both the halves of the tank, without any unwanted seizure and circular movements. However, the EMB-0.078 mg/kg group tracking pattern was similar to the PTZ treated group as the fish spent more time in the lower half of the tank, with many visible circular movements. DZP was used as a standard drug and zebrafish treated with it showed a swimming pattern from the left to the right side of the tank as seen in **Figure 3A**.

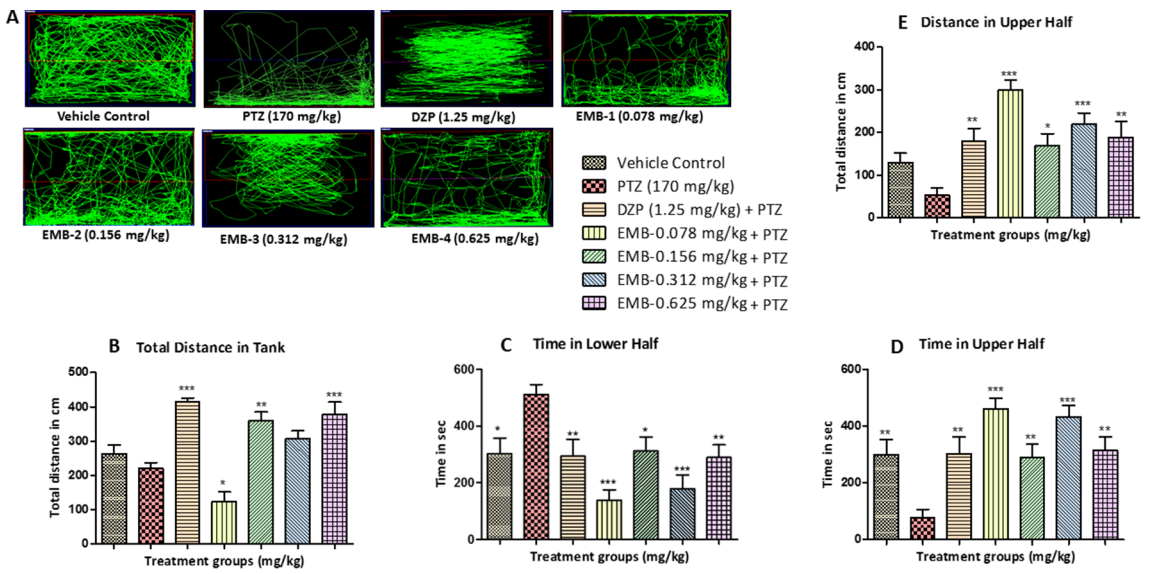


FIGURE 3 | Locomotor pattern and behavior analysis of embelin treatment against pentylenetetrazole (PTZ) induced seizures: **(A)** represents the tracking pattern of locomotor behavior for the control and PTZ treated and EMB treated groups. **(B)** represents the total distance traveled by fish in each group during locomotor behavior tracking. **(C, D)** represent the time spent in total by each fish in the lower and upper half of the behavior tank. **(E)** represents the total distance traveled in the upper half of the tank. The data are represented as mean ± SEM, n = 10, and statistically analyzed by one-way ANOVA followed by Dunnett's test *P ≤0.05, **P ≤ 0.01, and ***P ≤ 0.001.

The total distance traveled by the fish in the DZP, EMB-0.156 mg/kg, and EMB-0.625 mg/kg groups was significantly higher as compared to the PTZ group. The total distance traveled in the EMB-0.312 mg/kg and the control group was higher as compared to the PTZ treated group, but it was not statistically significant. But in the EMB-0.078 mg/kg treated group, the distance traveled was significantly less than the PTZ treated group, as shown in **Figure 3B**. It was found that the control fish spent nearly equal amount of time in both the halves of the tank; on the other hand, fish from the PTZ group were found to spend significantly more time in the lower half of the tank. The EMB treatment (EMB-0.312 mg/kg to EMB-0.625 mg/kg) reversed the seizure behavior, and all the fish were found to spend more time in the upper part of the tank as shown in **Figure 3**. The total distance traveled in the upper half of the tank was also found to be significantly more in all the EMB treated groups as compared with PTZ treated group, as shown in **Figure 3E**.

Zebrafish T-Maze Test

In the T-maze test, fish from the vehicle control group exhibited an improved memory function in the absence of seizures. The PTZ treated group exhibited a negative effect, i.e., worsened memory function, which is depicted by a decreased inflexion ratio at both 3 and 24 h. In contrast to the PTZ treated group, EMB exhibited a better improvement of memory and showed an increased inflexion ratio in a dose-dependent manner. An increase in inflexion ratio was seen in the EMB treated group, but the results were not significant after the 3 h trial. EMB-0.312 mg/kg to EMB-0.625 mg/kg showed a significant increase in the inflexion ratio as compared to the PTZ treated group at the 24 h trial but not at 3 h. The inflexion ratio was found to be low in the DZP and EMB-0.078 mg/kg treated groups and was not found to be significant when compared to the PTZ treated-group as shown in **Figures 4B, C**.

As the inflexion ratio was low, the time required for the fish to reach the deeper chamber and the time spent in the wrong arm (left turn) was found to be higher in the PTZ group as compared to the control. The control group showed little to no repeated entry into the wrong arm and thus time spent, and distance traveled to reach the deeper chamber was found to be less. Fish from the PTZ treated group frequently failed to navigate their way to the deepest chamber and had more wrong entries into the left arm (wrong arm) before entering the deepest chamber. As the inflexion ratio of fish treated with EMB (+PTZ) was high, the time taken and distance travelled to reach the deeper chamber were less and found to be significant as compared to the PTZ treated group, which ultimately decreased the total distance traveled and time spent in the wrong arm, as shown in **Figures 4C, D**.

The locomotor pattern of the healthy adult zebrafish in the vehicle control group was found to be normal, with a single right turn toward the deepest chamber. The locomotor pattern of adult zebrafish treated with DZP-1.25 mg/kg showed repeated back turns into the long arm and thus spent more time in the T-maze before reaching the deepest chamber. The fish treated with EMB-0.156 mg/kg and EMB-0.312 mg/kg showed similar locomotor activity as that of the control group and was found to travel toward deeper chamber, with fewer entries into the wrong arm. However, the fish treated with the EMB-0.625 mg/kg dose showed some weird behavior as they spent more time in the T-maze before entering the deepest chamber as shown in **Figures 5A–E**. On the other hand, the EMB-0.156 mg/kg and EMB-0.312 mg/kg treated groups showed a positive effect on the 3 h and 24 h inflexion ratios when compared with the control group. EMB-0.625 mg/kg failed to show increased an inflexion when compared to the control group. DZP-1.25 mg/kg showed a slight increase in the inflexion ratio at 3 h but was found to

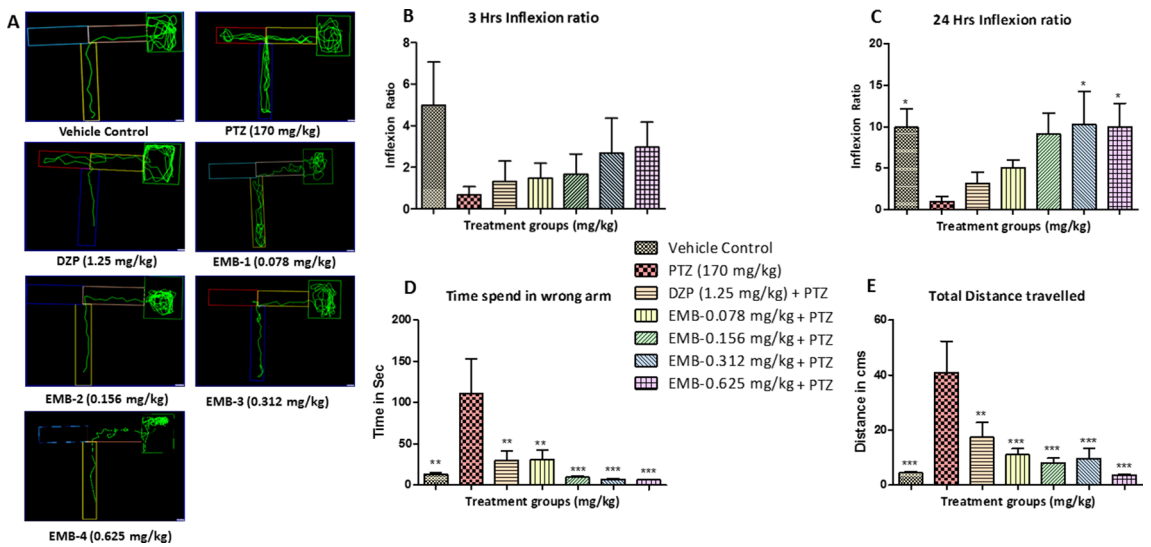


FIGURE 4 | T-maze tracking pattern and behavior analysis of embelin treatment against pentylenetetrazole (PTZ) induced seizures and cognitive dysfunction. **(A)** represents the T-maze tracking pattern of locomotor behavior for the control and PTZ treated and EMB treated groups. **(B, C)** represent the graph plot of the inflection ratio at the 3 h and 24 h T-maze trial. **(D, E)** represents the time spent in the wrong arm and the total distance traveled by each fish to reach the deepest chamber of T-maze behavior tank. Data are represented as mean ± SEM, n = 8, and statistically analyzed by one-way ANOVA followed by Dunnett's test *P ≤0.05, **P ≤ 0.01, and ***P ≤ 0.001.

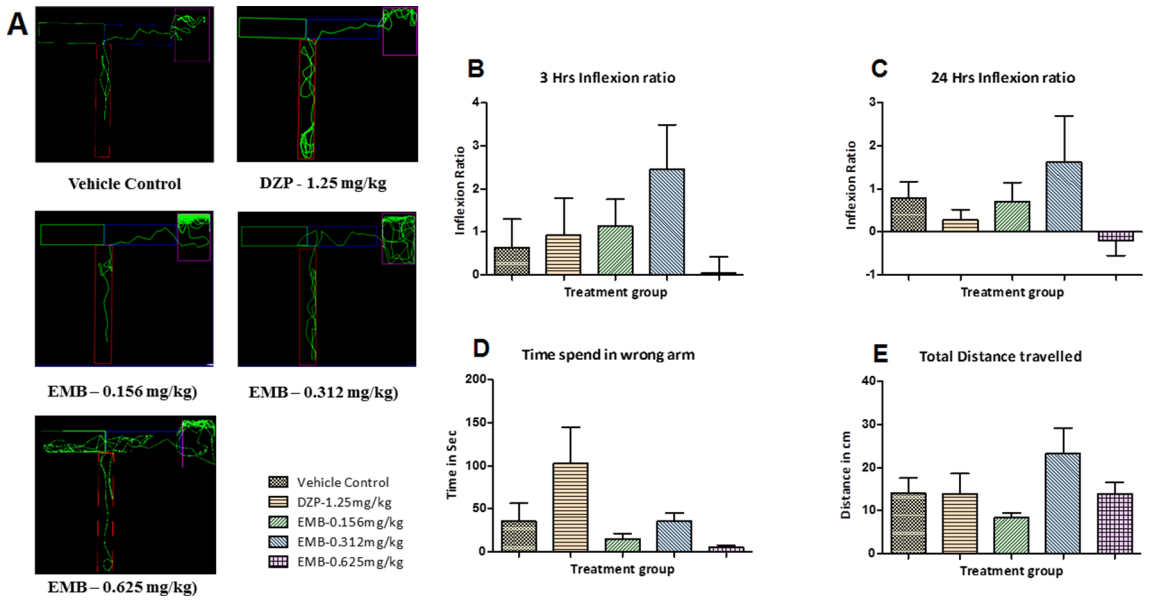


FIGURE 5 | T-maze tracking pattern and behavior analysis of embelin treatment in adult healthy zebrafish: **(A)** represents the T-maze tracking pattern of locomotor behavior for the control and DZP treated and embelin (EMB) treated groups. **(B**, **C)** represent the graph plot of the inflection ratio at 3 and 24 h. T-maze trail in adult healthy zebrafish. **(D**, **E)** represent the time spent in the wrong arm and total distance traveled by each fish to reach the deeper chamber of the T-maze in adult healthy zebrafish. Data are represented as mean ± SEM, n = 8, and statistically analyzed by one-way ANOVA followed by Dunnett's test.

decrease the 24 h inflexion ratio when compared to the control group. The time spent in the wrong arm was found to be high in the DZP treated group. The EMB-0.156 mg/kg and EMB-0.312 mg/kg groups showed an inflexion ratio similar to that of the vehicle-treated fish as shown in **Figures 5B, C**. There was no significant difference found in total distance traveled to reach the deeper chamber in all the groups when compared with the vehicle control group, as shown in **Figure 5E**.

Estimation of Neurotransmitters by LC/MS-MS

Neurotransmitter analysis by LC/MS-MS demonstrated elevated levels of GABA in the control group when compared to the PTZ treated groups. A significant increase in the level of GABA was found in the EMB-0.078 mg/kg—EMB-0.156 mg/kg treated groups when compared to the PTZ group. Low levels of GABA were found in the DZP and EMB-0.312 mg/kg and EMB-0.625 mg/kg treated groups as shown in **Figure 6A1**. The level of glutamate was found to be high in the PTZ treated group in comparison to the vehicle-treated group. Fish treated with EMB were protected against PTZ induced seizures due to the decreased level of glutamate. It was found that all the fish treated with EMB-0.078 mg/kg to EMB-0.625 mg/kg had a significantly lowered glutamate levels as shown in **Figure 6A2**. The level of glutamate was also found to be low in the control group and the DZP treated group. It was found that brain Ach levels were decreased in the PTZ group and were lower than the control group. DZP and the EMB-0.625 mg/kg treated groups showed an increased level of Ach as compared to the PTZ group. There was a slight increase in the level of Ach in EMB-0.312 mg/kg group, but this was not found to be significant, as shown in **Figure 6A3**.

The level of GABA in healthy zebrafish treated with EMB-0.312 mg/kg and EMB-0.625 mg/kg was found to be significantly lower when compared with the vehicle control group. However, the level of GABA was not significantly lower in EMB-0.156 mg/kg and DZP-1.25 mg/kg treated group when compared to the vehicle control group, as shown in **Figure 6B1**. The level of glutamate in the EMB-0.625 mg/kg only treated group was found to be significantly lower when compared with the vehicle control group. The level of glutamate was not significantly lower in the EMB-0.156 mg/kg, EMB-0.312 mg/kg, and DZP-1.25 mg/kg treated groups when compared to the vehicle control group, as shown in **Figure 6B2**. Similarly, there was no significant difference found in the level of Ach in all the EMB treated groups and the DZP treated group when compared with the vehicle control group, as shown in **Figure 6B3**.

Estimation of Gene Expression by RT-PCR

BDNF mRNA expression was upregulated in the control treated group as compared to the PTZ treated group. A significant elevation in the expression level of BDNF mRNA was observed in the EMB-0.625 mg/kg group. However, the DZP and EMB-0.156 mg/kg and EMB-0.312 mg/kg treated groups also demonstrated elevated levels of BDNF mRNA expression but was not significantly upregulated as compared to PTZ treated group. There was no increase in BDNF mRNA expression in the EMB-0.078 mg/kg treated group as compared to the PTZ treated group as shown in **Figure 7A1**. CREB_1 mRNA expression

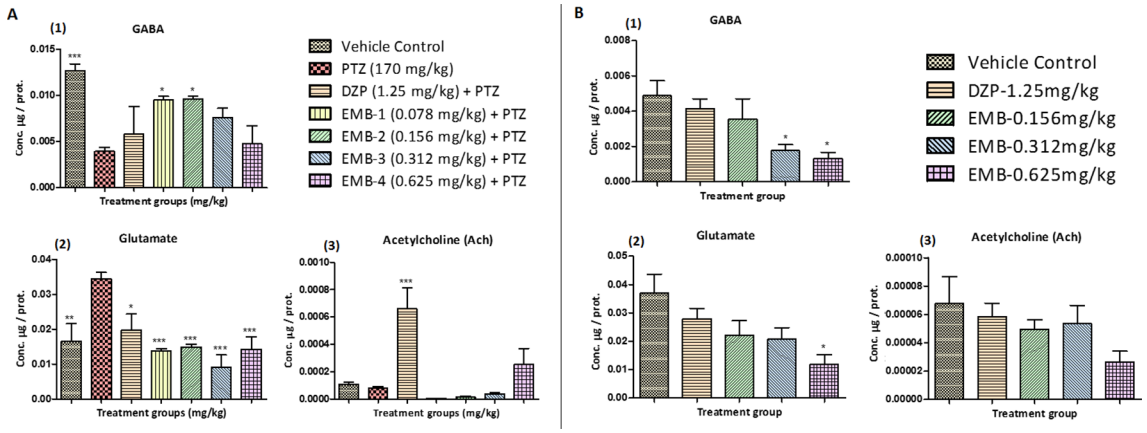


FIGURE 6 | (A) Neurotransmitters analysis in epileptic zebrafish brains after embelin treatment and 24 h. T-maze behavior: **(A1)** represents concentration of GABA in the zebrafish brain. **(A2)** represents concentration of glutamate in the zebrafish brain. **(A3)** represents concentration of acetylcholine (Ach) in the zebrafish brain. Data are represented as mean ± SEM, n = 6, and statistically analyzed by one-way ANOVA followed by Dunnett's test *P ≤0.05, **P ≤ 0.01, and ***P ≤ 0.001. **(B)** Neurotransmitters analysis in healthy zebrafish brains after embelin treatment and 24 h T-maze behavior: **(B1)** represents concentration of GABA in the zebrafish brain. **(B2)** represents concentration of glutamate in the zebrafish brain. **(B3)** represents concentration of acetylcholine (Ach) in the zebrafish brain. Data are represented as Mean ± SEM, n = 6, and statistically analyzed by one-way ANOVA followed by Dunnett's test *P ≤0.05, **P ≤ 0.01, and ***P ≤ 0.001.

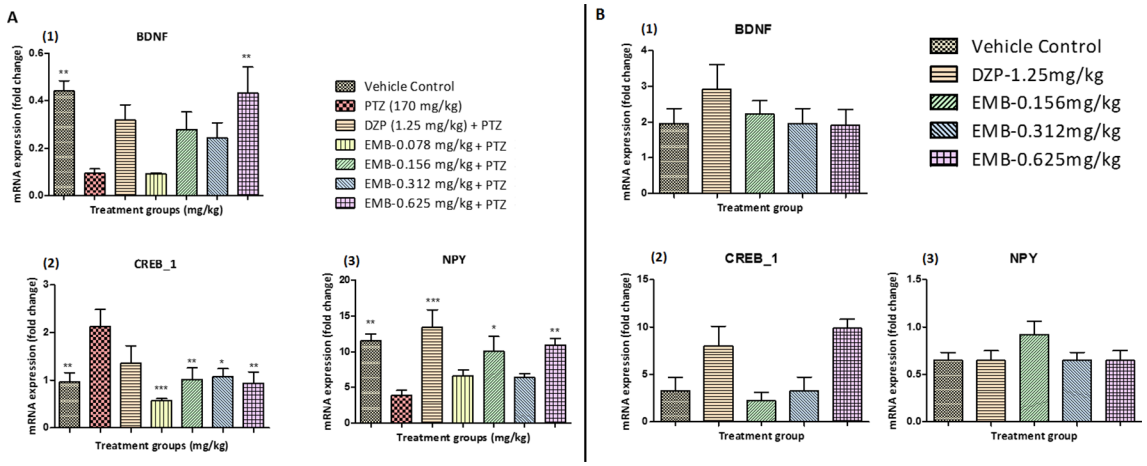


FIGURE 7 | (A) Gene expression analysis in epileptic zebrafish brains after embelin treatment and 24 h T-maze behavior: **(A1)** represents graph plot for BDNF mRNA expression in the zebrafish brain. **(A2)** represents graph plot of CREB_1 mRNA expression level in the zebrafish brain. **(A3)** represents graph plot of NPY mRNA expression in the zebrafish brain. Data are represented as mean ± SEM, n = 6, and statistically analyzed by one-way ANOVA followed by Dunnett's test *P ≤ 0.05, **P ≤ 0.01, and ***P ≤ 0.001. **(B)** Gene expression analysis in adult healthy zebrafish brains after embelin treatment and 24 h T-maze behavior: **(B1)** represents graph plot for BDNF mRNA expression in the zebrafish brain. **(B2)** represents graph plot of CREB_1 mRNA expression level in the zebrafish brain. **(B3)** represents graph plot of NPY mRNA expression in the zebrafish brain. Data are represented as mean ± SEM, n = 6, and statistically analyzed by one-way ANOVA followed by Dunnett's test *P ≤ 0.05, **P ≤ 0.01, and ***P ≤ 0.001.

was down-regulated in the control group as compared to the PTZ treated group. In all the EMB treated groups, the level of CREB_1 mRNA expression was found to be significantly down-regulated as compared to the PTZ treated group, in epileptic fish. CREB_1 mRNA expression in the DZP treated group was found to be statistically insignificant when compared to the PTZ group as shown in **Figure 7A1**. NPY mRNA expression was up-regulated in the control group when compared with PTZ treated group. However, the up-regulation of NPY mRNA was improved by DZP and EMB-0.156 mg/kg and EMB-0.625 mg/kg pre-treatment as compared with the PTZ group. There were no significant differences observed with EMB-0.078 mg/kg and EMB-0.312 mg/kg pre-treatment when compared with the PTZ treated-group, as shown in **Figure 7A3**. On the other hand, there was no significant BDNF mRNA fold change observed in the EMB treated group when compared to the vehicle control group. The level of BDNF was high in the DZP treated group but was not significant when compared with the control-treated group as shown in **Figure 7B1**. The level of CREB_1 mRNA expression was found to be high in the DZP and EMB-0.625 mg/kg treated

groups but was not significantly higher when compared to the control group. The level of CREB_1 mRNA expression in the EMB-0.156 mg/kg and EMB 0.625 mg/kg treated groups had no significant difference when compared with the vehicle control group as shown in **Figure 7B2**. Similarly, the EMB and DZP treated fish did not show any significant change in mRNA expression fold change of the NPY gene when compared to the vehicle control group, as shown in **Figure 7B3**.

Molecular Docking Predictions for Embelin

The docked conformation of benzamidine is shown in **Figure 8** (I). As described by Miller and Aricescu (2014), our result also showed that the phenyl ring of benzamidine forms π-stacking with Phe200 while the amidinium group interacts with Glu155, Ser 156, and Tyr 157 *via* hydrogen bonds. Furthermore, the electrostatic cation-π interaction was also observed with Tyr 205. The RMSD and CDOCKER interaction energy (CDIE) were found to be 0.85Å and −25.06 kcal/mol, respectively. Since DZP is used as a standard drug for the treatment of epilepsy, we have used it as a positive control. Therefore, we have docked DZP into the same active site of the $GABA_A$ receptor. DZP also fits well in the active site, showing a CDIE of −20.28 kcal/mol. As illustrated in **Figure 8** (II), it formed hydrogen bonds with Tyr97, Tyr157, and Glu155 residues, in a similar manner to benzamidine and showed hydrophobic interaction with Ala201. When EMB was docked in the active site of the receptor, a highly favorable lower CDIE of −42.09 kcal/mol was obtained. As depicted in **Figure 8** (III), it formed a hydrogen bond and an electrostatic salt bridge

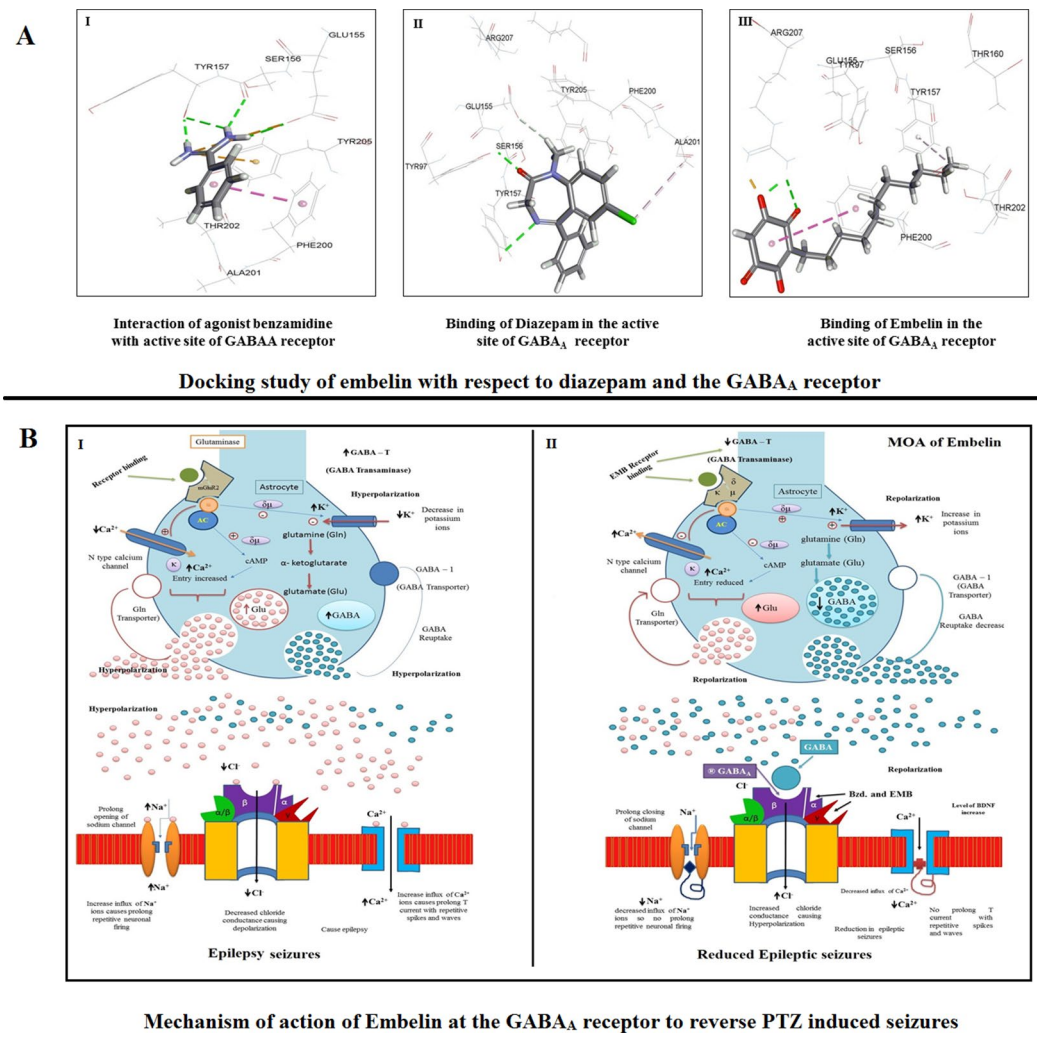


FIGURE 8 | (A) Docking study of embelin with respect to diazepam and the $GABA_A$ receptor. **(B)** Proposed mechanism of action of embelin at the $GABA_A$ receptor to reverse PTZ induced seizures. 8B1—During a seizure, a prolonged opening of the voltage-gated Na^+ channel causes an influx of sodium ions which leads to depolarization and repetitive neuronal firing. The opening of $Ca2^+$ channels increases the influx of positive calcium ions causing prolonged spikes and T current waves. As there is no affinity for the GABAA receptor, the decreased Cl^- influx causes epileptic excitation inside the neuron cell. 8B2—EMB shows higher affinity for $GABA_A$ receptor binding; it facilitates GABA mediated Cl^- channel opening with cell hyperpolarization and reduced seizure frequency, prolonged inactivation of the voltage-gated neuronal Na+ channel, prevented intracellular Na+ accumulation, reduced $Ca2^+$ influx, and inhibited glutamate.

with Arg207. Similarly, to the agonist benzamidine, the phenyl ring of EMB stacked with Phe200 and Tyr157. Thus, molecular docking studies indicated a good relationship between IC_{50} values and CDIE, thus supporting the biological results seen later.

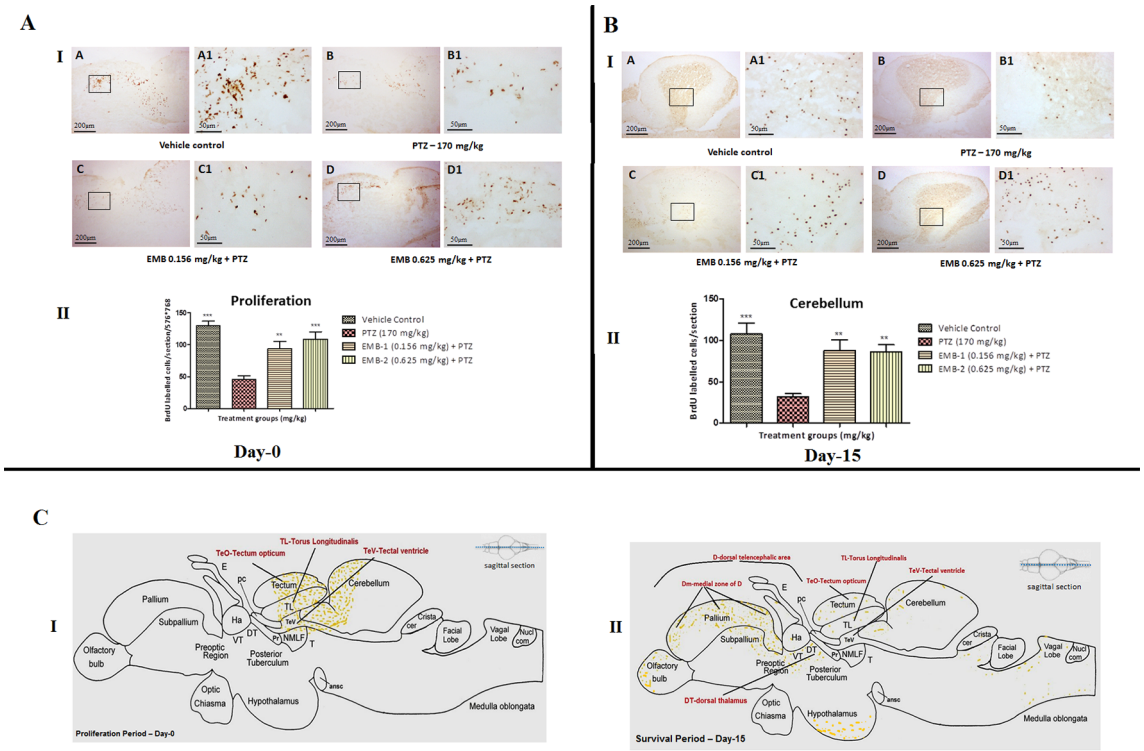
Zebrafish Brain Cell Genesis Study—BrdU Labeling

BrdU Labeled Cells and Proliferation Zone At Day 1

To identify proliferation zones in the brain of adult zebrafish, the distribution of BrdU-labeled cells, as obtained after intraperitoneal injection of labeling reagent and by employing a post-administration survival time of 1 h on day 1, was analyzed. To categorize proliferation zones in the encephalon of adult zebrafish, the scattering of BrdU-labeled cells, as obtained after intraperitoneal injection of BrdU reagent and by employing a post-administration survival time of 1 h on day 1, was analyzed. At day 0, it was found that the number of BrdU positive labeled cells was significantly higher in the control group as compared to the PTZ treated group. EMB in a dose-dependent manner significantly protect neurons from PTZ seizures, such that a significant increase in BrdU positive cells was found in the EMB treated group when compared to the PTZ treated group. It was also found that, at day 0, newborn cells tagged with the BrdU label were found to be originating from the tectum opticum (TeO), torus longitudinal (TL), tectal ventricle (TeV), and cerebellum region of the zebrafish brain as shown in **Figure 9A**.

Distribution of Positive BrdU-Labeled Cells After 15 Days of Survival

To find out a possible survival and migration pattern of the new cells after EMB and PTZ administration, the distribution of BrdU-positive cells was analyzed in five brain regions olfactory



Graphical representation of cell migration in zebrafish brain

FIGURE 9 | (A) BrdU immunohistochemistry analysis of the protective effects of embelin at the proliferation (day 0) and survival (day 15) stage against pentylenetetrazole-induced epilepsy the zebrafish brain. (A I) BrdU-positive staining revealed labeling of mitotic cells of immature neurons in the subgranular zone of the periventricular gray zone of tectum opticum with BrdU. Photomicrographs of the sagittal section of treatment groups were (A) control, (B) PTZ 170 mg/kg, (C) EMB 0.156 mg/kg + PTZ, and (D) EMB 0.625 mg/kg + PTZ. Representative photomicrographs were taken at magnifications of 40X and 200X. (II) Quantification of BrdU positive cells**.** Data are expressed as means mean ± SEM, n = 6, and statistical analysis by one-way ANOVA followed by Dunnett's test $^{**}P < 0.01$, and $^{***}P < 0.001$. (A II) BrdU immunohistochemistry analysis of the effects of embelin in improving neurogenesis and migration of cells generated during 15 days from the molecular layer to the granular layer within the dorsal zone of the periventricular hypothalamus against pentylenetetrazole-induced epilepsy the zebrafish brain. (I) BrdU-positive staining revealed labeling and migration of cells at day 15 of cell maturation and differentiation stage within the valvula cerebelli zone of cerebellum with BrdU labeling. Photomicrographs of the sagittal section of treatment groups were (A) control, (B) PTZ 170 mg/kg alone, (C) EMB 0.156 mg/kg + PTZ, and (D) EMB 0.625 mg/kg + PTZ. Representative photomicrographs were taken at magnifications of 40X and 100X. (II) Quantification of BrdU population: Data are expressed as mean ± SEM, n = 6, and statistical analysis by one-way ANOVA followed by Dunnett's test $^{**}P < 0.01$, and $^{***}P < 0.001$. **(B)** Graphical representation of cell migration in zebrafish brain. (I) represents location of positive BrdU cells at day 0. (II) represents migration and location of positive BrdU cells at day 15.

bulb, cerebellum (rhombencephalon), TeO (mesencephalon), telencephalic area (telencephalon), and hypothalamus (diencephalon). As seen, most of the cells from molecular layers migrated toward the granular layer and were found to be high in control and EMB treated brains when compared to PTZ treated fish brains. The number of positive BrdU cells found in the olfactory bulb, cerebellum, TeO, telencephalic area, and hypothalamus was significantly higher in the control and EMB treated group as compared to PTZ treated the group as shown in **Figure 10**.

DISCUSSION

A common method for inducing animal epilepsy are chemoconvulsants, which are chemical agents that can produce seizures (Choo et al., 2018). In a dose deciding study, we found that three doses of EMB (0.625 mg/kg, 0.312 mg/kg, and 0.156 mg/kg) had significant seizure protection activity. Further increasing the EMB dose reduced its seizure protection activity. This suggests that EMB has a small therapeutic window, which corroborates findings in a rodent study (Bhuvanendran et al., 2018). PTZ treated fish showed anxiety behavior as they mainly resided at the bottom half of their tank, in contrast to control fish which spent equal amounts of time in both halves. EMB treatment of up to 0.625 mg/kg reduced PTZ-induced seizures and treated fish exhibited normal swimming movements consisting of repeated short swims.

The T-maze is a standardized learning and memory paradigm (Hamilton et al., 2016) whereby zebrafish attempt to reach a deeper chamber using their spatial memory for a spacious and favorable environment (Lamb et al., 2012). The current study suggests that epileptic zebrafish treated with EMB had the ability to successfully learn, navigate, and discriminate between the wrong and right arms to reach the deeper chamber. A similar effect of EMB was also observed in healthy adult zebrafish. The 3 and 24 h inflexion ratios showed a significant increase in memory function in the EMB-0.312 mg/kg and EMB-0.625 mg/kg groups as compared to the PTZ treated group. In the zebrafish brain, the

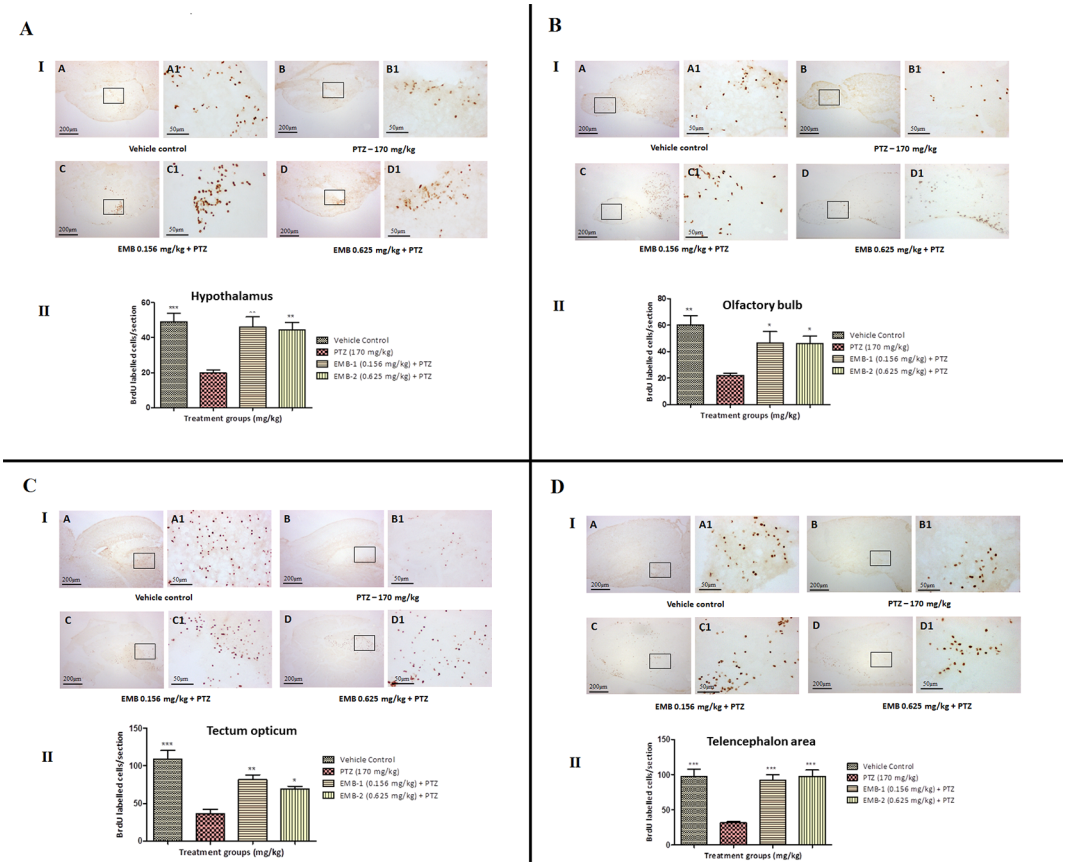


FIGURE 10 | BrdU immunohistochemistry analysis of the effects of embelin in improving neurogenesis and migration of cells generated during adulthood from the molecular layer to the granular layer within the molecular zone of the hypothalamus, the internal cellular layer of the olfactory bulb of the periventricular gray zone of TeO, and the medial zone of the dorsal telencephalic area against pentylenetetrazole-induced epilepsy the zebrafish brain. (I) BrdU-positive staining revealed labeling and number of migration of cells at day 15 of maturation and differentiation stage at the molecular zone of each section of zebrafish brain with BrdU labeling. Photomicrographs of the sagittal section of treatment groups were **(A)** control, **(B)** PTZ 170 mg/kg alone, **(C)** EMB 0.156 mg/kg + PTZ, and **(D)** EMB 0.625 mg/kg + PTZ. Representative photomicrographs were taken at magnifications of 40X and 100X. (II) Quantification of BrdU population: Data are expressed as mean ± SEM, n = 6, and statistical analysis by one-way ANOVA followed by Dunnett's test *P < 0.05, **P < 0.01, and ***P < 0.001.

olfactory bulb in the telencephalon is responsible for governing egocentric navigating myopia that helps the fish reach the deeper chamber (Lindsey et al., 2014). During an epileptic episode, the connection between the olfactory bulb and the telencephalon is likely impaired, and this may affect the learning and memory ability of the fish. But when the fish is pre-treated with EMB, it prevents the fish from becoming epileptic and also increases its learning ability, which leads to the increased 24 h inflexion ratio.

Abnormal neurotransmitter levels are related to many neural diseases including epilepsy (Levin and Cerutti, 2009). GABA is the primary neuroinhibitor in the CNS (Kaila et al., 2014) and plays a well-studied and important role in epilepsy as well as learning and memory (Luo et al., 2011). In the current study, we reported increased GABA levels in all EMB groups, proving the role of GABA in learning and memory. EMB may have some ability to suppress Ca^{2+} influx and increase chloride conductance as epileptogenesis is aided by increased Ca^{2+} influx (Zamponi et al., 2010), reduced chloride conductance, and decreased GABAergic presynaptic inhibition (Werner and Covenas, 2011). Extremely augmented excitatory glutamate release is one of the leading causes of epileptic seizures (Rowley et al., 2012). Glutamate toxicity is associated with neuronal death in neurodegenerative disorder cases (Guerriero et al., 2015), and glutamate also affects memory and learning (Izquierdo and Medina, 1997). In the present study, a lower level of glutamate was found in all groups as compared to the PTZ treated group. PTZ acts at the $GABA_A$ receptor and reduces chloride conductance (Zhang et al., 2017), leading to glutamate excitation (Banote et al., 2013). Fish getting repeatedly lost in the wrong arm of the T-maze in the PTZ treated group shows a relation between memory loss and glutamate toxicity, which was reversed by EMB treatment. Modulating memory formation is a key role of Ach, which also alters neuron excitability, prompts synaptic plasticity, and controls the firing of groups of neurons (Picciotto et al., 2012). An increase in Ach levels was also observed in the EMB treated groups but was not significant when compared to the PTZ treated group. DZP is reported to be an acetylcholinesterase (AChE) inhibitor and thus the level of Ach was high in the DZP treated group as the level and duration of Ach in the brain is increased (Lundgren et al., 1987).

BDNF has a remarkable role in synaptic plasticity, survival, and differentiation of neurons in the brain (Roopra et al., 2012) as well as increasing the persistence of short- and long-term memory storage (Bekinschtein et al., 2008). It has been discovered that BDNF is linked to the inception of epileptogenesis, and the epileptic condition can be avoided by BDNF upregulation (Binder, 2004). In the present study, we found that BDNF mRNA expression was down-regulated in the PTZ treated group when compared with the control but increased in the EMB treated groups, indicating memory improvement, neuronal survival, and differentiation of new growing neurons (Choi et al., 2018). CREs are promoters that are phosphorylated to generate epilepsy (Zhu et al., 2012). Studies in rodents show that a decrease in CREB levels has a 50% chance to decrease seizure episodes (Zhu et al., 2012) and that CREB_1 modulates synaptic plasticity for the intrinsic excitability of the neurons (Benito and Barco, 2010). CREB_1 mRNA expression was high in PTZ treated fish, confirming its role in epileptogenesis. In all the EMB treated groups, CREB_1 mRNA expression was found to be down-regulated, which indicates that EMB helps in reducing epilepsy and enhances memory. NPY has an important role in regulating physiological processes such as various brain events, memory, and learning (Kas et al., 2005). Earlier rodent studies reported that chronic spontaneous seizures are reduced by NPY in a temporal lobe epilepsy model of rats (Noe et al., 2010) and that high levels of brain NPY are crucial for memory and learning (Gøtzsche and Woldbye, 2016). The level of NPY mRNA expression in the PTZ treated group was significantly downregulated as compared to the control group. However, EMB treated fish showed increases in NPY expression, indicating that it plays a crucial role in modulating neurotransmitters, increasing neuronal growth, and preventing epilepsy in zebrafish brains (Vezzani and Sperk, 2004).

Docking studies are mainly performed to determine the affinity of a particular compound for a receptor or a similar molecule (Meng et al., 2011). We found that EMB has a high affinity for the benzodiazepine active binding site of the $GABA_A$ receptor at the β3 subunit. During epileptic seizures, the extra release of glutamate and increased breakdown of GABA causes neuronal firing and seizure episodes (Engel, 2013). By decreasing GABA release, there is much less binding of GABA to the $GABA_A$ receptor, decreasing the influx of Cl^- ions and hence causing excitability of the neuron cell. EMB's high affinity toward the GABA receptor may help in GABA release and in reducing cellular excitability by prolonged inactivation of voltage-gated neuronal Na^+ channels, preventing intracellular Na^+ accumulation and inhibiting high-frequency discharge. A reduction in Ca^{2+} influx and inhibition of glutamate may also take place after EMB treatment (Eraković et al., 2000). As EMB binds to the benzodiazepine G-protein coupled receptor at the α or γ subunit, it facilitates GABA mediated Cl^- channel opening with cellular hyperpolarization and a reduction in seizure frequency (Benarroch, 2007). This could serve as a clue toward unraveling the precise mechanism of EMB against epilepsy and related cognitive dysfunction.

Generation of neurons from neuronal stem cells is known as the process of neurogenesis (Fuchs and Gould, 2000). During neurodegeneration, the subgranular zone (SGZ), which is the part of the hippocampus, the dentate gyrus, and the subventricular zone (SVZ) are active continuously (Ming and Song, 2011). Proliferation and neurogenesis are not clearly understood in adult zebrafish. There is only one study that describes the proliferative zones and the migration of cells in the telencephalon, preoptic region, thalamus, hypothalamus, midbrain, and cerebellum (Kaslin et al., 2008). A similar result was found in the present study, where newborn cells at day 0 were found to be tagged in abundance with BrdU at TeO, TL, TeV, and the cerebellum region of the brain. The number of BrdU tagged cells was found to be high in control and EMB treated groups as compared to the PTZ treated group. Epileptic seizures induced by PTZ causes increased oxidative stress and inflammation, which leads to neuronal death in the brain (Naseer et al., 2014).

Labeling of dividing cells with the BrdU thymidine analog provides insight into EMB's effect on cell proliferation and migration after a 15 days survival period (Grandel et al., 2006). Mitotic activity was specifically pronounced in the olfactory

bulb, hypothalamus, telencephalic area (dorsal telencephalon), preoptic area of the diencephalon, optic tectum of the mesencephalon, TL, and in all three cerebellum subdivisions (Zupanc et al., 2005). In the zebrafish optic tectum, there was a lack of evidence for a long-distance migration of the new cells from their proliferation zones. Among all brain regions, the different subdivisions of the cerebellum demonstrated a maximal number of mitotic cells in control and EMB treated zebrafish. A similar result has been reported in other teleosts including guppies and the brown ghost (Rubenstein and Rakic, 2013). As the administration of PTZ damages newly born cells at the s4 phase of mitosis, the number of cells which migrated to different parts of the brain was significantly lower. Evidence of PTZ causing neurodegeneration was also previously observed (Park et al., 2006). In spite of extended neuronal growth after the epileptic episode, studies have shown that an aberrant neuronal circuit causes more severe damage to the brain structure. As the fish were pre-treated with EMB in this study, they protected the brain cell during the PTZ insult and caused less epileptic episodes and more neuronal survival. On the other hand, as the newly tagged BrdU cells in PTZ treated group were damaged after the PTZ insult, the numbers of cells at day 0 and day 15 were found to be less as compared to the control and EMB treated groups.

CONCLUSION

EMB is reported to have various activities such as being an anti-oxidant and anti-inflammatory and can cross the BBB. Findings from the current study demonstrate that EMB can also suppress seizure-like behavior and improve cognitive function in zebrafish. The docking study showed that EMB has a higher affinity toward the $GABA_A$ receptor. In addition, the current study also explored the basic mechanism of EMB, its possible site of receptor binding and its ability to promote cell migration and differentiation. The study conducted on healthy zebrafish with only EMB treatment showed similar memory behavior and no alteration in gene expression level affecting the memory status of the fish. This implicates that EMB does not interfere with the normal functioning of body processes and has no adverse effect. T-maze data, behavioral study, immunohistochemistry staining, and biochemical analysis supported the observed effect of EMB. Herein, we suggest that EMB could be a promising candidate against epilepsy induced learning and memory dysfunctions. Further investigations utilizing different seizure models are warranted and will strengthen the potential of EMB toward clinical translation. Current findings shed light on the utilization of plant-based natural compounds against epilepsy and related cognitive impairment. These will overcome the limitations of mainstream AEDs and will be an economical option as well.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The experimental protocol was approved by the Monash Animal Research Platform (MARP) Animal Ethics Committee, Monash University, Australia (MARP-2015-084).

AUTHOR CONTRIBUTIONS

UK performed most of the experimental procedure along with analysis of the results and writing of the manuscript. BC helped in performing behavior recording, data analysis, writing of the manuscript, and proofreading. NA contributed to the molecular docking study. YK contributed to designing the gene expression study and result analysis. IO contributed to LC-MS/MS study. MS conceptualised the idea, contributed to study design, result interpretation, analysis, manuscript writing, and proofreading.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Bone Health in Rats With Temporal Lobe Epilepsy in the Absence of Anti-Epileptic Drugs

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Rationale: Epilepsy patients often exhibit reduced bone mineral density and are at an increased risk of bone fracture. Whether these bone abnormalities are due to the use of anti-epileptic drugs (AED's) or the disease itself is unknown. For example, although decreased bone health in epilepsy patients is generally attributed to the use of AED's, seizures can also trigger a number of physiological processes that have the potential to affect bone. Therefore, to assess whether bone abnormalities occur in epilepsy in the absence of AED's, the current study investigated mechanical characteristics and trabecular bone morphology in rats with chronic temporal lobe epilepsy.

Methods: Ten-week old male Wistar rats underwent kainic acid-induced status epilepticus (SE; n = 7) or a sham procedure (n = 9). Rats were implanted with EEG recording electrodes at nine weeks post-SE, and video-EEG was continuously recorded for one week at 10- and 22-weeks post-SE to confirm that SE rats had spontaneous seizures. Open-field testing to assess locomotion was conducted at 23-weeks post-SE. At 24-weeks post-SE, rats were euthanized and tibia were extracted to determine trabecular morphology by micro-computed tomography (μCT), while femurs were used to investigate mechanical properties *via* 3-point bending.

Results: All post-SE rats had spontaneous seizures at 10- and 22-weeks post-SE, while none of the sham rats had seizures. μCT trabecular analysis of tibia revealed no differences in total volume, bone volume, bone volume fraction, trabecular number, or trabecular separation between post-SE or sham rats, although post-SE rats did have increased trabecular thickness. There were also no group differences in total distance travelled in the open field suggesting that activity levels did not account for the increased trabecular thickness. In addition, no differences in mechanical properties of femurs were observed between the two groups.

Conclusion: There was a lack of overt bone abnormalities in rats with chronic temporal lobe epilepsy in the absence of AED treatment. Although further studies are still needed, these findings may have important implications towards understanding the source (e.g., AED treatments) of bone abnormalities in epilepsy patients.

Keywords: bone, epilepsy, status epilepticus, animal model, micro-computed tomography

INTRODUCTION

Epilepsy is a complex group of neurological disorders that affects ~50 million people worldwide. The disease is defined as when the brain demonstrates an enduring tendency to have recurrent seizures (Fisher et al., 2014). Epilepsy is associated with several co-morbidities; however, a commonly underappreciated consequence of the condition is impairment in bone health (i.e., reduced bone quantity and/or impaired bone quality) (Petty et al., 2016). Indeed, numerous studies have reported that patients with epilepsy have an increased risk of bone fractures both during seizures and also in the absence of seizures, (Vestergaard et al., 2004; Nakken and Taubøll, 2010) which is in part due to the reduced bone health commonly observed in these patients (Petty et al., 2016).

Reduced bone health and increased risk of fracture are often attributed to the use of antiepileptic drugs (AED's) (Vestergaard, 2015; Rahimdel et al., 2016). The majority of epilepsy patients are treated with AED's and there is some evidence that certain AED's may be associated with reduced bone health (e.g., levetiracetam, (Nissen-Meyer et al., 2007; Aksoy et al., 2016; Hakami et al., 2016) oxcarbazepine, (Aksoy et al., 2016) sodium valproate, (Sato et al., 2001; Nissen-Meyer et al., 2007) and phenytoin, (Välimäki et al., 1994; Nissen-Meyer et al., 2007), while others have yet to be rigorously studied. However, whether these bone abnormalities are due to the use of anti-epileptic drugs (AED's) or the disease itself remains unknown (Petty et al., 2016). It is important to consider that seizures can trigger a number of central and systemic physiological processes that have potential to affect bone health. Therefore, it is possible that recurrent spontaneous seizures contribute to the reduced bone health observed in patients with epilepsy (Petty et al., 2016). For example, during and after seizures there is a period of sympathetic hyperactivity (Devinsky, 2004; Poh et al., 2012; Picard et al., 2017).This increased sympathetic outflow has potential to increase activation of β2 adrenergic receptors on osteoblasts (i.e., bone forming cells) (Kondo et al., 2012). The activation of these osteoblastic receptors has been found to stimulate osteoclast induced bone resorption (Kondo et al., 2012). Further, seizures induce an inflammatory cascade that is characterized by up-regulation of pro-inflammatory cytokines and chemokines, and the activation and migration of immune cells (Kumar and Loane, 2012). The disruption of the blood brain barrier (BBB) that occurs during and following seizures (Librizzi et al., 2012; Van Vliet et al., 2015) may facilitate the migration of these inflammatory mediators from the brain into the peripheral circulation where they could influence bone metabolism (Van Vliet et al., 2015). In particular, it has been well established that increased systemic inflammation is capable of activating osteoclasts, triggering bone resorption (Lee et al., 2008; Ciucci et al., 2015). Furthermore, the generation and migration of oxidative stress mediators during/post seizures may suppress bone formation and promote osteoclastic differentiation (Garrett et al., 1990; Kondo et al., 2012; Williams et al., 2015).

It is difficult to study the effect of recurrent spontaneous seizures on bone health in humans as most patients are treated with AED's and also present with a number of confounding factors (e.g., comorbidities, socioeconomic/lifestyle factors) (Petty et al., 2016). In addition, changes in bone mass in patients with epilepsy take years to manifest (El-Hajj Fuleihan et al., 2008; Nakken and Taubøll, 2010). Animal models of epilepsy allow for rigorous investigation of the effect of seizures on bone in a time- and cost-efficient manner (Brady et al., 2018). Therefore, the aim of this study was to examine the effect of recurrent spontaneous seizures on the quality and quantity of the tibia in a rat model of chronic temporal lobe epilepsy.

METHODS

Subjects

Ten-week old male Wistar rats were bred and housed in the Department of Medicine, University of Melbourne Biomedical Research Facility. Rats were housed individually under a 12 h light/dark cycle and given access to food and water *ad libitum* for the duration of the experiment. All experimental procedures were approved by The Florey Animal Ethics committee (Ethics number: 16-047 UM).

Kainic Acid-Induced Post-Status Epilepticus

Rats were randomly assigned to receive either sham (n = 9) or kainic acid (KA)-induced status epilepticus (SE; n = 7). The post-SE rat model of temporal lobe epilepsy is well characterized and mimics the epileptogenic processes observed in humans (Van Nieuwenhuyse et al., 2015). A repeated low dose KA administration protocol modified from Hellier and colleagues was used (Hellier et al., 1998; Bhandare et al., 2017). Rats in the SE group were given an i.p. injection of 7.5 mg/kg KA in 3 ml of saline, while shams were injected with saline only. Rats were subsequently monitored for behavioural seizures as assessed *via* the Racine scale. Briefly, the Racine scale categorises seizure severity into 5 classes: Class I is defined by mouth and facial movements; Class II by head nodding; Class III by forelimb clonus; Class IV by bilateral forelimb clonus and rearing; Class V by rearing and falling (Racine, 1972). If no self-sustained seizure

activity was observed (i.e., at least five class IV Racine scale seizures), another i.p. dose of 2.5 mg/kg of KA was administered up to a maximum of 15 mg/kg. Rats were excluded from the experiment if they did not show a stable self-sustained SE after the maximum KA dose. After 4 h of sustained behavioral seizures the rats were given diazepam (5 mg/kg/dose) to stop the SE.

Electrode Implantation

Rats were implanted with EEG recording electrodes at 9-weeks post-SE under isoflurane induced anaesthesia. Each rat received a subcutaneous injection of carprofen analgesic (5 mg/kg; Rimadyl; Pfizer Australia). Six burr holes were drilled through the skull. relative to bregma, one electrode was positioned at each of the following six co-ordinates: I) AP+2.0mm; II) AP-2.0mm; III) AP-4.5mm, ML+2.5mm; IV) AP-4.5mm, ML-2.5mm; V) AP-8.0mm, ML+2.0mm; and VI) AP-8.0mm, ML-2.0mm. the recording electrodes were embedded by applying dental cement around the electrodes and over the skull.

Video-EEG Recordings and Seizure Analysis

As previously described, (Shultz et al., 2013; Liu et al., 2016; Casillas-Espinosa et al., 2019a; Santana-Gomez et al., 2019) rats underwent video-EEG recordings continuously (i.e., 24 h/day) for one week at 10- and 22-weeks post-SE. Video-EEG recordings were obtained using Profusion 3 software (Compumedics, Australia) unfiltered and digitized at 512 Hz. EEG analysis was performed by an investigator blinded to the experimental groups. All EEG recordings were screened for seizures using automated software (Assyst, Australia) (Casillas-Espinosa et al., 2019a). Seizure events were visually confirmed using Profusion 3 software. A seizure was defined as an episode of rhythmic spiking activity that was three times the baseline amplitude and a frequency > 5 Hz that lasted at least 10 s (Pitkänen et al., 2005; Van Nieuwenhuyse et al., 2015; Liu et al., 2016; Casillas-Espinosa et al., 2019b). The end of a seizure was determined as the last spike. The average number of seizures per day, average seizure duration and seizure class (i.e. severity) were analysed.

Open-Field Testing

At 23 weeks post-SE, locomotion was assessed using an open-field as previously described (Shultz et al., 2013; Shultz et al., 2014). Rats were placed in the centre of a circular open-field arena (100 cm diameter) enclosed by walls 20 cm high, and allowed to freely explore for 5 min. Behaviour in the open-field was recorded by an overhead camera, and *Ethovision Tracking Software* (Noldus, Netherlands) quantified the total distance travelled as well as the number of entries and time spent in the centre area (66 cm diameter) of the arena.

µCT

Rats were euthanized at 24-weeks post-SE and the right tibia was extracted and fixed in 4% paraformaldehyde for 24 h, then washed in PBS and stored in 70% ethanol at 4°C (Brady et al., 2014; Brady et al., 2016a; Brady et al., 2016b; Brady et al., 2016c;). Before scanning, tibia were rehydrated in 0.9% saline solution for 14 h to avoid any changes in medullary density in the trabecular region. Images were acquired using a Scanco µCT 50 scanner (Scanco Medical AG, Switzerland), with a tube voltage, current and integration time of 70 kV, 144 µA and 300 ms, respectively, and isotropic voxels of 10 µm (Stauber and Müller, 2008; Schambach et al., 2010). A 0.5 mm aluminium filter was used to reduce beam hardening artefacts, two scanning iterations were used to reduce noise and tibia specimens were immersed in saline solution during scanning to prevent dehydration (Irie et al., 2018).

The region of interest (ROI) was designated as being a 2.5 mm region, beginning 1 mm from the growth plate and extending distally (Nishiyama et al., 2010; Campbell et al., 2011; Campbell and Sophocleous, 2014; Liu et al., 2015). Trabecular bone morphology within the ROI was computed using the evaluation scripts available in the Scanco IPL software (v6.1, Scanco Medical AG, Switzerland) with the following settings: threshold 220–1,000, Gaussian noise filter: Sigma 0.8, support 1. Trabecular bone parameters computed included: Total volume (TV), bone volume (BV), bone volume fraction (BV/TV), connectivity density (Conn.D), trabecular number (Tb.N), trabecular thickness (Tb.Th), and trabecular separation (Tb.Sp).

Mechanical Testing

Biomechanical properties of the diaphysis of the right femur (mediolateral bending) were compared between sham and post-SE rats at 24-weeks post-SE using a three-point bending apparatus (Brady et al., 2016a; Brady et al., 2016c; Leppanen et al., 2006). Load and deflection data were recorded continuously using transducers connected to an x–y plotter by preamplifiers. Peak force to failure and stiffness were calculated from the load deflection data.

Statistical Analyses

Statistical analyses of data were performed using IBM SPSS Statistics version 25 (Armonk, New York, USA). An independent-samples t-test was used to compare bone parameters in both experimental groups. Statistical significance was set at $p < 0.05$.

RESULTS

Video-EEG Recordings Analysis

At 10-weeks post-SE, post-SE rats averaged approximately 1 seizure per day with a mean seizure duration of 57 s and a mean seizure severity of 4.8, assessed *via* the Racine scale (see **Table 1**). At 22-weeks post-SE, rats 3 seizures per day, average duration of 58 s and a mean seizure severity of 4.8. No seizures were observed in shams rats at either recovery time.

Locomotor Activity

Locomotor activity was assessed at 23 weeks post-SE in the open-field. There were no differences in distance travelled in the open-field between sham and post-SE rats (**Figure 1**). However,

TABLE 1 | Seizure analysis in shams and post-SE rats.

		Week 10 post-SE			Week 22 post-SE		
		Seizures/day	Seizure severity	Seizure duration (s)	Seizures/day	Seizure severity	Seizure duration (s)
SHAM *n* = 9	Mean ± SEM	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Post-SE *n* = 7	Mean ± SEM	1.09 ± 0.50	4.81 ± 0.12	57.4 ± 6.47	3.02 ± 1.07	4.83 ± 0.09	58.3 ± 3.73

All post-SE rats had spontaneous seizures at 10- and 22-weeks. There was no evidence that sham rats had spontaneous seizures at either of the recovery times.

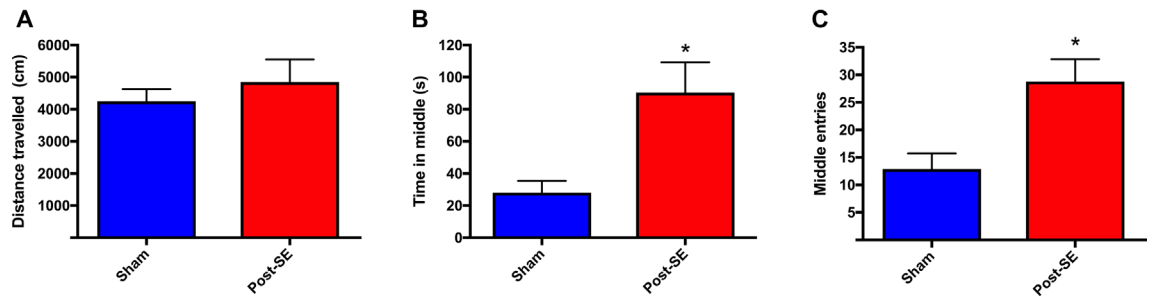

FIGURE 1 | Distance travelled in an open-field. No difference in total distance travelled during open-field session was observed between groups **(A)**. Post-SE rats spent more time in the middle **(B)** and had significantly more middle entries **(C)** when compared to sham. Bars represent means ± SEM and * represents $p < 0.05$.

post-SE rats spent more time in the middle of the arena, and had significantly more middle entries when compared to sham rats ($p < 0.05$).

µCT Analysis

µCT analysis revealed no between-group differences in total volume, bone volume, bone volume fraction, trabecular number, or trabecular separation between post-SE or sham rats. There was however, a significant increase in trabecular thickness in SE rats compared to shams (**Figure 2** g; $p < 0.05$).

Mechanical Testing

No differences in peak force to failure or stiffness was observed between femora from post-SE rats when compared to shams (see **Table 2**).

DISCUSSION

Few studies have investigated bone health in epilepsy in the absence of AED's. Therefore, here the microarchitecture of the tibia and biomechanical properties of the femur were assessed in a rat model of chronic temporal lobe epilepsy without AED treatment. SE rats averaged one seizure/day at 10 weeks post-SE and three seizures/day at 22 weeks post-SE, whereas there was no evidence of seizures in sham rats. µCT analysis revealed that there were no overt bone microstructural abnormalities present in SE rats when compared to shams, as evidenced by a lack of differences in trabecular bone parameters including: total volume, bone volume, bone volume fraction, trabecular number or trabecular separation. Although there was a subtle increase in trabecular thickness in post-SE rats, this is not considered a marker of decreased bone health (Nilsson et al., 1986; Fonseca et al., 2014). No differences were observed in mechanical properties of the femoral midshaft. It is possible that ambulatory state could confound bone outcomes, as increased mechanical loading of bone is associated with bone formation, while reduced loading can trigger bone resorption (Vicente-Rodríguez et al., 2005). However, in this study, and others, (Inostroza et al., 2012) SE rats did not display a prolonged decrease in locomotion, suggesting that decreased activity levels did not account for the subtle increase in trabecular thickness. Taken together, the present findings suggest that this experimental model of acquired epilepsy does not cause changes in bone morphological parameters that may be detrimental to bone health.

Changes in bone mass in patients with epilepsy typically occur over a 1–5 year period, (El-Hajj Fuleihan et al., 2008; Nakken and Taubøll, 2010) although, this can differ due to a number of factors (e.g., medication, age, gender, type of epilepsy, and nutritional status). (Ahmad et al., 2017) Therefore, it is possible that decreased bone health in the SE rats may take longer to manifest and may have occurred at time-points not featured in this study. However, when one takes into account the life-span of the rat (1–4 years) and that the SE rats had evidence of severe seizures for > 5 months, it appears unlikely that significant changes would have occurred at more chronic stages. Moreover, our finding that trabecular thickness was actually increased in post-SE rats may further indicate that post-SE rats were unlikely to have bone loss at a later stage. Further studies examining

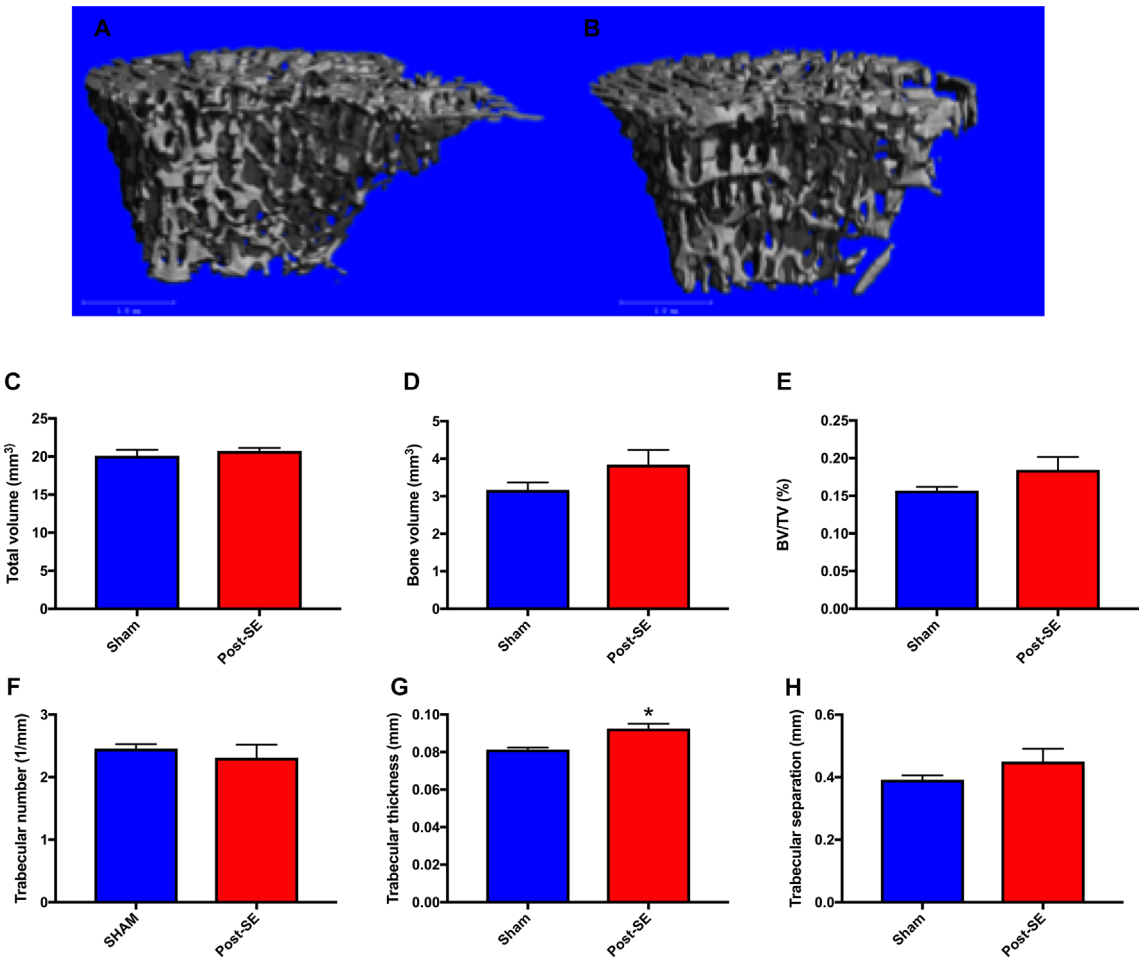

FIGURE 2 | Representative μCT reconstructions of trabecular bone regions from sham **(A)** and Post-SE rats **(B)**. There were no differences between post-SE rats when compared to shams on the measures of total volume **(C)**, bone volume **(D)**, bone volume fraction **(E)**, trabecular number **(F)** or trabecular separation **(H)**. However, trabecular thickness **(G)** was significantly increased in post-SE rats when compared to shams. Bars represent means ± SEM and * represents $p < 0.05$.

TABLE 2 | Mechanical characteristics of femora from sham and post-SE rats.

Group	Peak Force (N)	Stiffness (x 10^4 Nm^2)
Sham n = 9	407.2 ± 14.73	76.21 ± 4.50
Post-SE n = 7	422.3 ± 26.97	77.97 ± 7.48

There were no differences in mechanical properties at between sham and post-SE groups. Values are means ± SEM.

gene and protein expression, are required to determine the precise mechanism through which post-SE rats had increased trabecular thickness and its potential biological significance. However, several studies have reported that serum levels of leptin are significantly increased following amygdala electrical kindling (a model of temporal lobe epilepsy) in rats (Bhatt et al., 2004; Bhatt et al., 2005; Hum et al., 2009). Given that peripherally-acting leptin stimulates bone formation it is possible that leptin may have played a role in the increased trabecular thickness observed in the current study (Reseland et al., 2001; Gordeladze et al., 2002; Wei et al., 2008; Wang et al., 2011). The administration of kainic acid may have influenced bone metabolism. For example, treatment of osteoclasts with 10–100 μM of NBQX a kainic acid receptor antagonist decreased osteoclastic bone resorption *in vitro* (Szczesniak et al., 2005). However, the exact effect that kainic acid treatment had on bone in this study requires further investigation. It is also possible that the increased trabecular thickness may have been due to increased loading caused by locomotion in post-SE rats. However, the current study and others (Inostroza et al., 2012), found no differences in distance travelled between post-SE rats and shams. These findings suggest that that the lack of overt bone abnormalities between the two groups was not due to differences in mechanical loading of the bones, which may influence bone volume and thickness. However, it is important to consider that although we observed no differences in locomotion between the two groups, it is possible that post-SE rats were more active at time-points not featured in this study. Furthermore, it should be noted that some studies have reported that post-SE rats travel further in the open field, (Petkova et al., 2014) while other studies have reported post-SE rats travel less in the

open field (Yu et al., 2018). Future studies would benefit from monitoring physical activity of all rats in their home-cages to directly determine activity levels of sham and post-SE rats.

This study also found no differences in biomechanical properties of the femoral midshaft between sham and post-SE rats. It is likely that this is due to the slow remodelling rate of this region that is comprised predominantly by cortical bone (Fonseca et al., 2014). When compared with trabecular bone, cortical bone has a much lower surface-to-volume ratio and hence remodels at a much lower rate (Szulc and Seeman, 2009). Accordingly, changes in bone parameters typically manifest first in trabecular bone. Taken together with the absence of changes in trabecular bone microstructure, it is unlikely that changes occurred in bones not featured in this study. However, future studies should examine both trabecular and cortical bone microstructure, mechanical characteristics at various locations (e.g., tibia, femur and vertebrae), as well as blood-based markers of bone turnover to enhance the understanding of the effect of seizures on bone. Examination of bone mineral content and collagen cross-linking *via* Fourier transform infrared microspectroscopy, as was done in the initial Garip studies, (Szulc and Seeman, 2009; Rolvien et al., 2017) would also be informative.

A recent study used Wistar Albino Glaxo/Rijswijk (WAG/Rij) rats, a polygenic rat model of genetic generalised epilepsy with absence seizures, and assessed changes in bone mineral content and mineral matrix ratios following a 5-week audiogenic kindling regime (Garip et al., 2013). Fourier transform infrared microspectroscopy (FTIRM) analysis revealed that rats with epilepsy had reduced mineral content and collagen crosslinks, while B type carbonate was increased (Garip et al., 2013). The aforementioned findings have all been associated with reduced biomechanical properties and suggest that seizures in WAG/Rij rats may compromise bone health (Garip et al., 2013). However, WAG/Rij rats which, have an unknown inherited mutation, (Garip et al., 2013) were compared to healthy Wistar rats. Given that genetic mutations that result in seizures have previously been associated with bone loss and skeletal fragility, (Rolvien et al., 2017) it is unclear whether the bone abnormalities observed in WAG/Rij rats were due to the seizures or the unknown inherited mutation (Rolvien et al., 2017; Garip Ustaoglu et al., 2018).

In a follow-up study by the same group, however, WAG/Rij rats that had seizures induced by audiogenic kindling were compared to WAG/Rij rats that did not experience seizures when exposed to the same stimuli (Garip Ustaoglu et al., 2018). Following 5-weeks of kindling, rats that had seizures displayed reduced mineral and matrix properties in cortical and trabecular bone regions of the tibia, femur and spine when compared to rats that did not have seizures, suggesting that the seizures induced abnormalities in bone (Garip Ustaoglu et al., 2018). Furthermore, it was also demonstrated that treatment with the AED carbamazepine reduced mineral and matrix properties in cortical and trabecular regions of the tibia, femur and spine compared to rats that were not susceptible to audiogenic kindling (Garip Ustaoglu et al., 2018). The difference in findings between the current study and work by Garip et al. is uncertain but, may be due to differences in seizure mechanisms which cause non-convulsive absence seizures induced by audiogenic kindling and the tonic clonic recurrent spontaneous seizures that occur in the post-SE model of acquired epilepsy. Additionally, a potential limitation of FTIRM is that in the context of analysing bone it cannot be performed *in vivo* (Rolvien et al., 2017; Garip Ustaoglu et al., 2018). Therefore, although it is unlikely that there is a link between audiogenic seizure susceptibility and bone mineral content, to further demonstrate that the changes observed were due to seizures, a longitudinal study is required to confirm that WAG/Rij rats that have audiogenic seizures do not have reduced mineral content at baseline compared to those that do not. Although this study and others have provided insight into the effect of epilepsy on bone health, a limitation of this work so far is that only male rats have been studied. Evidence suggests that, post-menopause, females with epilepsy may have an increased risk of developing bone abnormalities (Ensrud et al., 2004; Lyngstad-Brechan et al., 2008). Therefore, it would be beneficial to examine bone health in aged or ovariectomized female rats (post-menopausal rodent models) with epilepsy. An additional limitation of this study is that μCT scans were performed post-mortem, hence there were no pre-seizure baseline measurements of bone. Further studies utilising serial *in vivo* μCT analysis pre-SE and at multiple time-points post-SE to assess changes in bone growth and morphology overtime may provide further insight how epilepsy may affect bone.

Several human studies have reported that AED's are associated with reduced bone health, particularly in patients with acquired temporal epilepsy. For example, levetiracetam, (Nissen-Meyer et al., 2007; Aksoy et al., 2016; Hakami et al., 2016) oxcarbazepine, (Aksoy et al., 2016) sodium valproate, (Sato et al., 2001; Nissen-Meyer et al., 2007) and phenytoin, (Välimäki et al., 1994; Nissen-Meyer et al., 2007) have been associated with bone loss in patients with epilepsy (Beniczky et al., 2012; Aksoy et al., 2016; Hakami et al., 2016). In addition to AED's, patients with epilepsy often have a lack of exposure to sunlight, limited physical activity and a high prevalence of vitamin D deficiency which all contribute to reduced bone health (Beerhorst et al., 2013). Furthermore, patients with epilepsy have an increased risk of seizure related trauma (e.g., slips and falls) which increases the likelihood of fracture (Beerhorst et al., 2013; Petty et al., 2016). The novel findings of the current study suggest that rats with chronic acquired temporal lobe epilepsy in the absence of AED's do not have overt changes in bone morphological parameters, or mechanical properties indicative of decreased bone health. Whilst the results suggest that the bone loss observed in patients with acquired temporal epilepsy may be due to the use of AED's, other comorbidities, or socioeconomic/lifestyle factors, future studies are still required to determine the cause of bone deficiencies in epilepsy patients and how they can be prevented.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/supplementary material.

ETHICS STATEMENT

The animal study was reviewed and approved by The Florey Animal Ethics committee (Ethics number: 16-047 UM).

AUTHOR CONTRIBUTIONS

All authors contributed to the writing of the manuscript. RB, PC-E, TO'B, RM, and SS conceptualized and designed the experiments. PC-E completed the seizure analysis. KW, DR, RB, and PL completed the μCT scanning and analysis.

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Vascular Abnormalities and the Role of Vascular Endothelial Growth Factor in the Epileptic Brain

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Epilepsy is a chronic neurological disorder generally defined to be caused by excessive neuronal activity. Thus, excessive neuronal activity is the main target of the currently used antiepileptic drugs (AEDs). However, as many as 30% of epileptic patients show drug resistance to currently available AEDs, which suggests that epilepsy should be attributed not only to neuronal cells but also to other brain cells, such as glial cells and vascular cells. Astrocytes, pericytes, and endothelial cells in particular comprise the blood–brain barrier (BBB), which tightly regulates the exchange of substances between the brain parenchyma and the circulating blood. It has been proposed that BBB dysfunction, especially barrier leakage, exacerbates epileptic progression, and conversely, that epileptic seizures induce barrier leakage. Furthermore, several studies have shown that BBB dysfunction is one of the main causes of drug resistance in epilepsy. To better understand the mechanisms that link BBB dysfunction and intractable epilepsy to gain insights for the future development of treatments, we review and discuss the relationships between epilepsy and brain vascular abnormalities, mainly by focusing on vascular malformation, BBB dysfunction, and excessive angiogenesis. Because these abnormalities have been reported to be caused by vascular endothelial growth factor (VEGF) in the ischemic brain, we discuss the possible role of VEGF in vascular abnormalities in the epileptic brain, in which the upregulation of VEGF levels has been reported. Both glial cells and endothelial cells express VEGF receptors (VEGFRs); thus, these cells are likely affected by increases in VEGF during seizures, which in turn could cause vascular abnormalities. In this review, we review the possible role of VEGF in epilepsy and discuss the mechanisms that link vascular abnormalities and intractable epilepsy.

Keywords: antiepileptic drug, neurovascular abnormalities, astrocyte, pericyte, endothelial cell, blood–brain barrier

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INTRODUCTION

Epilepsy is a chronic neurological disorder accompanied by spontaneous and frequent seizures, which are induced by the aberrant hyperactivity of neurons. The main effect of existing antiepileptic drugs (AEDs) is the prevention of the excessive activity of neurons, mainly by activating ion channels expressed in neurons; however, as many as 36% of epileptic patients show drug resistance

(Kwan and Brodie, 2000). Drug-resistant epilepsy is defined as the "failure of adequate trials of two tolerated and appropriately chosen and used AED schedules (whether as monotherapies or in combination) to achieve sustained seizure freedom" (Kwan et al., 2010). Temporal lobe epilepsy (TLE) is a common drug-resistant epilepsy, and surgical treatments are often used for the treatment of TLE (Jallon et al., 2001). Therefore, new drug targets are needed.

Approximately 20 years ago, the concept of the neurovascular unit (NVU) was proposed (Harder et al., 2002). According to this concept, neuronal activity is regulated not only by neurons themselves but also by other cells surrounding blood vessels, including glial cells, pericytes, and endothelial cells (**Figure 1**). Glial cells, especially astrocytes, provide lactate and oxygen to support neuronal health. Pericytes have actin–myosin systems that are associated with cell contraction, which enables the regulation of vessel diameters and changes in blood flow that could affect neuronal activity. Endothelial cells produce vasoactive factors to regulate vessel diameters, possibly resulting in changes in neuronal activity (Muoio et al., 2014). These changes in the brain microenvironment together could affect neuronal activity and brain function.

In this review, in considering possible new therapeutic targets for epilepsy, we mainly focus on the NVU, which is comprised of glial cells, pericytes, and endothelial cells. In the epileptic brain, several studies have suggested that the NVU exhibits abnormal characteristics. Although the topic on vascular structure abnormalities in epilepsy has been reviewed elsewhere (Morin-Brureau et al., 2012; Marchi and Lerner-Natoli, 2013; Josephson et al., 2015), here we specially focused on vascular malformation, blood–brain barrier (BBB) dysfunction, and excessive angiogenesis (**Figure 2**). In a brain with vascular malformation, vessel aggregations, for example, compress the brain parenchyma, resulting in abnormalities in the layer structures of neurons in the cerebral cortex. The malformation of neuronal layers could induce the hyperactivity of neurons, which results in epilepsy. In a brain with BBB dysfunction, albumin leaked from cells in the blood is taken up by astrocytes and attenuates the adjustment of extracellular ion concentrations by astrocytes, possibly resulting in neuronal hyperactivity. Additionally, BBB leakage could induce excessive angiogenesis and increase blood flow. These abnormalities are possibly induced by vascular endothelial growth factor (VEGF), whose expression is reported to be increased in the brains of patients with TLE (Rigau et al., 2007; Castañeda-Cabral et al., 2019). VEGF, which is produced and secreted from several types of cells, such as fibrocytes, myocytes, and astrocytes, under pathological conditions (de Vries et al., 1992), modulates the function of several types of cells, especially endothelial cells (Lee et al., 2007). VEGF induces the proliferation and migration of endothelial cells, which results in the promotion of angiogenesis (Hoeben et al., 2004). Therefore, we will describe vascular abnormalities in the epileptic brain, mainly by focusing on the roles of VEGF in their development.

VASCULAR MALFORMATION

It is commonly known that epileptic seizures are often associated with vascular malformations (Stefan and Hammen, 2004), and vascular malformations themselves can be a cause of epilepsy in some cases. Intracranial vascular malformations, which have been suggested to be a cause of epilepsy, are divided into two major types: arteriovenous malformations (AVMs) and cerebral cavernous malformations (CCMs) (Brown et al., 2005). These malformations often develop in the fetus between 3 and 8 weeks and are induced by the abnormal differentiation of the mesoderm. In AVMs, abnormal structures called "niduses" are found, in which arteries and veins are connected with blood flow in tumors without capillaries in the brain parenchyma (**Figure 2A**). In CCMs, aggregated venous blood vessels are observed in the brain, and these aggregations are often expanded and accompanied by slight hemorrhaging. AVMs and CCMs are common causes of intracerebral hemorrhage (ICH) in young adults; therefore, ICH itself could result in the induction of epileptic seizures. However, even AVMs and CCMs without symptomatic ICH can induce epileptic seizures. Eight percent of patients with unruptured AVMs were found to exhibit their first seizures within 5 years after the first detection of unruptured AVMs, and 58% of individuals with seizures develop epilepsy within 5 years (Josephson et al., 2011). In individuals with CCMs, there have been few studies describing the risk of seizures or epilepsy development mainly because it is difficult to detect CCM; however, one study reported that the risk of occurrence of the first seizure is 4% within 5 years after the first detection of CCMs and that the risk of development of epilepsy is 94% in CCM patients (Josephson et al., 2011). It has also been reported that patients with CCMs more likely develop epilepsy than patients with other massive lesion diseases (Lv et al., 2010).

The pathological mechanisms of epilepsy induced by AVMs and CCMs remain largely unclear. AVMs that are associated with seizures exist in various brain regions, but they are generally found in the temporal lobe (Josephson et al., 2011), and AVMs cause epileptic seizures through ischemia and impairment of the perinidal vascular reserve (Fierstra et al., 2011). One of the remarkable mechanisms that links the induction of epileptic seizures and CCMs is the deposition of hemosiderin (Leone et al., 2011). Hemosiderin, which is a pigment that includes iron derived from hemoglobin leaked from endothelial cell junctions, is rarely deposited in the healthy brain. Around deposits of hemosiderin, gliosis is often promoted, which may contribute to epileptogenesis. Thus, it is possible that AVM- and CCM-associated hemorrhage induces the deposition of hemosiderin, which includes iron, and iron could greatly contribute to the formation of focal epilepsy (Zhang et al., 2014; Josephson et al., 2015). Iron deposition causes lipid peroxidation and the alternation of protein function and could induce the disruption of the neuronal excitatory versus inhibitory balance, likely *via* disrupting the intracellular and extracellular homeostasis of neurotransmitters.

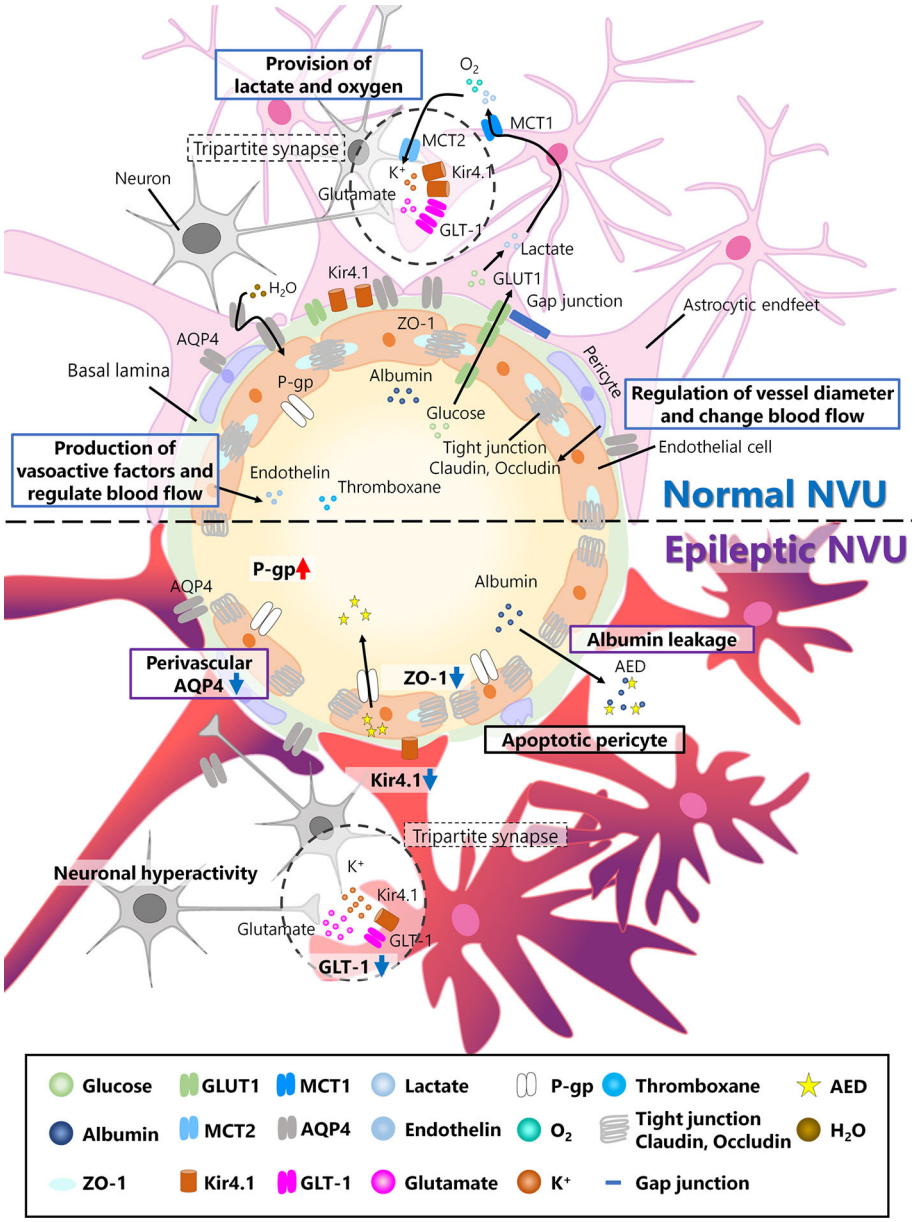

FIGURE 1 | Neurovascular unit (NVU) in the normal and epileptic brain. The NVU is comprised of neurons, astrocytes, pericytes, and endothelial cells. The basal lamina also exists between endothelial cells and astrocytes and is covered with astrocytic endfeet in the normal NVU. In the normal NVU, pericytes regulate the vessel diameter and change in blood flow. Endothelial cells are connected by tight junction proteins such as claudin and occludin, and ZO-1 is a type of occludin. Glucose is transported from the blood flow to astrocytes via glucose transporter type 1 (Glut1), metabolized into lactate, and discharged into extracellular space via monocarboxylate transporter 1 (MCT1). This lactate is transported to neurons via MCT2. Additionally, oxygen is also provided to neurons by astrocytes. Astrocytes and pre/post synapses comprise tripartite synapses. At tripartite synapses, potassium ions and glutamate released from neurons flow into astrocytes via inwardly rectifying potassium (Kir) 4.1 channels or glutamate transporter 1 (GLT-1), both of which are expressed around astrocytic endfeet. Additionally, aquaporin (AQP) 4, which is also expressed in astrocytes, regulates water transport. Endothelial cells produce vasoactive factors, such as thromboxane and endothelin, and regulate blood flow. In epileptic NVUs, ZO-1 expression is downregulated, leading to the apoptosis of pericytes, which induces blood–brain barrier (BBB) leakage. Albumin released from the blood flow into the brain parenchyma in the epileptic brain can be a cause of drug resistance. Kir 4.1 and GLT-1 are downregulated in astrocytes, which fail to regulate ion and neurotransmitter homeostasis. Perivascular AQP4 downregulation induces an imbalance in water influx. P-gp, which is expressed in endothelial cells and is associated with antiepileptic drug (AED) excretion, is upregulated in the epileptic NVU.

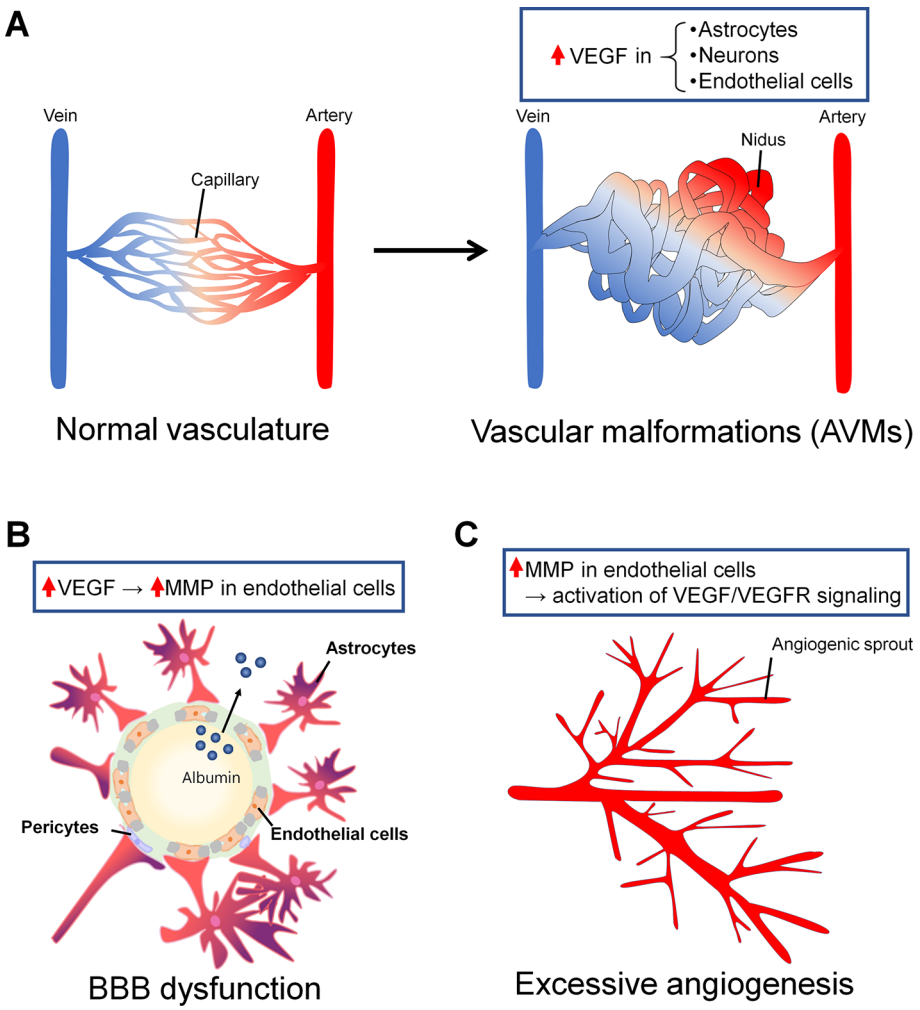

FIGURE 2 | Vascular abnormalities in epilepsy associated with vascular endothelial growth factor (VEGF). **(A)** Arteriovenous malformations (AVMs) are one of the major vascular malformations found in the epileptic brain. In normal vasculature, arteries and veins are connected by capillaries (right). In AVMs, there are some regions in which arteries and veins are not connected by capillaries and are instead directly connected by blood tumors called niduses (left). In astrocytes, neurons, and endothelial cells around and in niduses, VEGF expression is increased. **(B)** BBB dysfunction can be a cause or a consequence of epilepsy, or it can be both a cause and a consequence at the same time. BBB dysfunction induces albumin leakage and resistance to antiepileptic drugs (AEDs). VEGF induces an increase in MMP levels in endothelial cells, leading to BBB leakage. Albumin leaked from the blood flow binds AEDs and promotes drug resistance. **(C)** Angiogenesis is associated with BBB dysfunction in the epileptic brain. Neo vessels sprout from the pre-existing vessels. Matrix metalloproteinase (MMP) overexpression in endothelial cells could induce increases in VEGF/VEGFR signaling levels and angiogenesis.

It remains unclear how AVMs and CCMs develop, but it is likely that excessive angiogenesis underlies the formation of both AVMs and CCMs (Leblanc et al., 2009). In addition, it has been suggested that the formation and progression of AVMs and CCMs are induced by the local overexpression of VEGF (Li et al., 2018; Park and Park, 2016). VEGF, which is widely recognized as the main factor that induces angiogenesis, is locally overexpressed in and around AVMs in the brain (Koizumi et al., 2002). Specifically, VEGF-C and -D and the VEGF receptors (VEGFRs) Flt-1 and -4 are overexpressed in and around niduses. In contrast, the VEGF concentration in the plasma of AVM patients was lower than the control level, and it recovered to the control level after the treatment of AVMs (Kim et al., 2008). The reason why the VEGF concentration in plasma in AVM patients is lower while the concentration in and around niduses is higher than control levels remains unknown. In AVMs, VEGF overexpression is found in astrocytes, neurons, and endothelial cells; therefore, it is possible that these cells secrete VEGF to support angiogenesis and the formation of AVMs (Li et al., 2018), although direct evidence that supports this hypothesis has not been reported.

The concentration of plasma VEGF in CCM patients is higher than the control level, and it is decreased to the control level after CCM treatment (Park and Park, 2016). The expression levels of VEGF and VEGFR in CCMs are also higher than the control levels (Rothbart et al., 1996; Uranishi et al., 2001). Furthermore,

when *CCM3*, one of the genes responsible for CCM, is deleted in mice, normal VEGFR2 signaling, which is necessary for angiogenesis, is attenuated (He et al., 2010). Thus, it is possible that the mutation of *CCM3* destabilizes the VEGFR signaling pathway, inducing excessive angiogenesis and the formation of CCMs.

BBB DYSFUNCTION

The BBB in the brain capillary consists of endothelial cells, basement membrane, pericytes, and astrocytes. In the BBB, tight junctions formed by endothelial cells regulate paracellular flux, and the basement membrane, which is comprised of extracellular matrix secreted by endothelial cells and pericytes, is associated with vascular signaling; in addition, pericytes regulate blood flow and the infiltration of immune cells. Finally, astrocytes, which express the water channel aquaporin 4 (AQP4), are crucial for water homeostasis in the central nervous system (CNS; Daneman and Prat, 2015). The main function of the BBB is the regulation of the substances that are allowed to enter the brain parenchyma from the systemic blood flow.

The relationship between BBB dysfunction, especially BBB leakage, and epilepsy have long been studied (Oby and Janigro, 2006). BBB dysfunction has been found both in the brains of patients with epilepsy and in the corresponding animal models. In brains that undergo status epilepticus (SE), it was reported that albumin leakage was found surrounding all vessels in the hippocampus and cortex (van Vliet et al., 2007). In the hippocampus of TLE patients, albumin was also found in neurons and astrocytes that exist around vessels (van Vliet et al., 2007). The causal relationship between epileptic seizures and BBB leakage is consistent with findings from animal models of epilepsy. When rats were injected with pilocarpine to induce SE, BBB leakage was found in the hippocampal CA3 region and the dentate gyrus (Marchi et al., 2007). Furthermore, when rats were injected with kainic acid (KA) to induce SE, erythrocyte leakage in the piriform cortex and amygdala induced by SE was attenuated when rats were treated with an inhibitor of mammalian target of rapamycin (mTOR), rapamycin, which has been experimentally shown to exert anticonvulsant effects (van Vliet et al., 2016). Additionally, when BBB leakage was artificially induced by administrating hypertonic mannitol into rats, the number of seizures was increased in electric stimuli-induced SE rats (van Vliet et al., 2007).

To date, it is still debatable whether BBB dysfunction is a consequence or a cause of epilepsy. Neurons have been shown to exhibit epileptiform discharges induced by serum that is leaked from the blood flow (Seiffert et al., 2004). Furthermore, several mechanisms have been suggested that explain the leakage of thrombin and albumin from plasma into the brain parenchyma (Lee et al., 1997; van Vliet et al., 2007). It has also been suggested that BBB dysfunction triggers drug resistance in epilepsy. BBB leakage causes albumin to flow into the brain parenchyma from the plasma. Albumin decreases the concentrations of nonbinding AEDs (Marchi et al., 2009), likely decreasing the medical efficacy in terms of pharmacokinetics. Furthermore, drug excretion from the brain may be enhanced, as the overexpression of P-gp protein, which is one of the ABC transporters (ATP-binding cassette transporters) associated with drug excretion, was found to be induced after seizures (Zhu and Liu, 2004).

Several molecules, such as matrix metalloproteinase (MMP), have been suggested to induce BBB dysfunction in the epileptic brain. MMP is known to cleave extracellular matrix and degrade tight junction proteins (Lischper et al., 2010). In the cortex and hippocampus of patients with intractable epilepsy due to focal cortical dysplasia, MMP upregulation was reported (Konopka et al., 2013). In particular, the prominent upregulation of MMP-9, one of the MMP family members that is expressed in brain capillaries, was observed. In the brains of pilocarpine-induced SE rats, the increased release of glutamate by neurons increased MMP levels, leading to BBB dysfunction (Rempe et al., 2018). VEGF is another molecule associated with BBB dysfunction in the brain (Croll et al., 2004; Rigau et al., 2007). VEGF administration induced BBB leakage in rodents, and there was a positive correlation between VEGF-induced leakage and the increased activity of MMP-9 in the ischemic brain (Valable et al., 2005; Jiang et al., 2014). It remains unclear whether and how VEGF-induced BBB leakage and upregulated MMP-9 activity synergistically affect the epileptic brain.

Other molecules suggested to be associated with BBB dysfunction include platelet-derived growth factor (PDGF) and its beta receptor (PDGFRβ). PDGF is secreted from platelet and macrophages, whereas PDGFRβ is expressed in endothelial cells. PDGFRβ overexpression in endothelial cells induced the development of pericyte-microglia scar in the epileptic brain (Milesi et al., 2014; Klement et al., 2018; Klement et al., 2019). Because pericytes are essential components of BBB, pericyte-microglial scar would result in BBB leakage. Moreover, when seizure-like activities were induced in organotypic hippocampal slice cultures, the treatment of the cultures with imatinib, an inhibitor of PDGFRβ, decreased the reactivity between PDGFRβ-positive cells and capillaries which was triggered by seizure-like activities (Klement et al., 2019).

EXCESSIVE ANGIOGENESIS

Angiogenesis, the process of new vessel formation from pre-existing vessels, can be observed under both physiological, i.e., developmental, and pathological conditions. The density of vessels in the hippocampi from TLE patients was approximately two times higher than that in hippocampi from nonepileptic controls (Rigau et al., 2007). Moreover, the frequency of seizures was correlated with the density of the vessels in the hippocampi of TLE patients; the density of vessels in patients who had seizures less than once a month was significantly lower than that in patients who had seizures 1~20 times a month. It should also be noted that the existence of hippocampal sclerosis (HS) did not affect the density of vessels in the hippocampi of TLE patients. The increase in the density of

brain vessels has been reproduced in animal models of TLE. The vessel density in the hippocampus of lithium-pilocarpine-induced SE rats was increased compared to that of the control group, especially in the chronic phase (Rigau et al., 2007). By using rat hippocampal slices cultured with KA to induce seizure-like events (SLE), it was found that the vessel density and the number of vessel branches were increased at 24 h after KA application, and these phenomena were suppressed by inhibiting neuronal activity with tetrodotoxin (Morin-Brureau et al., 2011).

Several studies have suggested that angiogenesis in the epileptic brain is correlated with VEGF-associated BBB dysfunction (Rigau et al., 2007; Morin-Brureau et al., 2012). VEGF and VEGFR2 mRNA were upregulated, and ZO-1, which is a tight junction-related protein, was down-regulated in KA-treated slice cultures (Morin-Brureau et al., 2011). Treatment with anti-VEGF antibodies attenuated the increases in vessel density and branch numbers and the decrease in ZO-1 expression. The authors also found that VEGF/VEGFR2 signaling likely induces angiogenesis and BBB dysfunction through the proto-oncogene tyrosine protein kinase Src pathway.

Endothelial cells express VEGFR, and several reports have suggested that MMP activates the VEGF/VEGFR signaling pathway in angiogenesis under pathological conditions (Ferrara, 1995; Bergers et al., 2000; Hollborn et al., 2007). However, whether and how these systems contribute to angiogenesis in the epileptic brain remain to be studied. It is possible that MMP upregulation induces an increase in VEGF, leading to angiogenesis in the epileptic brain. Even in cultured normal tissues, in which pancreatic islets and endothelial cells were cocultured, that were treated with MMP, an increase in the VEGF concentration in the culture medium and angiogenesis in endothelial cells were observed (Bergers et al., 2000). Additionally, when cancer model mice were treated with a VEGFR inhibitor or an MMP inhibitor, angiogenesis, which is commonly observed in animal models of cancer, was inhibited (Hollborn et al., 2007). These data suggest that angiogenesis may also be prevented by inhibiting VEGFR or MMP in the epileptic brain.

DISCUSSION

Mechanisms underlying synchronized bursting of neurons have been therapeutic targets for epilepsy. However, about one-third of patients with epilepsy remain resistant to AEDs, which suggests that the role of brain cells other than neurons should be also studied in the epileptic brain. NVU, which consists of multiple cell types including neurons, astrocytes, pericytes, and endothelial cells, can be an essential therapeutic target for epilepsy. In physiological conditions, each cell type that consists NVU plays multiple roles: pericytes regulate the vessel diameter and modulate blood flow; endothelial cells produce vasoactive factors and modulate blood flow; astrocytes provide lactate and oxygen to support neuronal survival and growth (**Figure 1**). Specifically, endothelial cells, being tightly adhered each other as a part of NVU, play an essential role to regulate the exchange of substances between brain and blood flow in BBB. However, in the epileptic NVU, the regulatory systems of substance exchange are frequently disrupted because the adhesion between endothelial cells is attenuated, phenomena referred to as BBB breakdown. BBB breakdown results in the release of albumin and its binding to AEDs, which is a cause of drug resistance. Additionally, BBB breakdown results in the excessive release of potassium and glutamate. To make matters worse, the astrocytic expression of inwardly rectifying potassium (Kir) 4.1 channels and glutamate transporter1 (GLT1) is downregulated in the epileptic brain, which could result in dysregulation of ion and neurotransmitter concentration in tripartite synapses.

VEGF/VEGFR signaling is essential for angiogenesis in CNS (Mancuso et al., 2008). VEGFR stimulation in endothelial cells activates phosphatidilinosytol-3-kinase (PI3K) and following Rac/Rho signaling as well as growth factor binding protein2 (GRB2), which leads to the activation of Cdc42. Both Rac/Rho and Cdc42 have been shown to be associated with cell proliferation. The activation of VEGFR also induces the activation of MAP kinase signaling, which is also related to cell proliferation. Thus, VEGF-triggered cell proliferation is considered to be the basis of CNS angiogenesis.

In the current review, we have mainly focused on the negative effects of VEGF/VEGFR signaling in the epileptic brain, including vascular malformations, BBB dysfunction, and excessive angiogenesis. However, it should be noted that VEGF/VEGFR signaling also has neurotrophic effects in CNS including the induction of neurite outgrowth and suppression of neuronal death (Rosenstein et al., 2010). Furthermore, several studies have reported that VEGF has antiepileptic effects in pilocarpine-induced SE rats (McCloskey et al., 2005; Han et al., 2017; Lenzer-Fanara et al., 2017). VEGF treatment reduced spontaneous discharges of neurons in brain slices (McCloskey et al., 2005) and promoted hippocampal neurogenesis *in vivo*, improving recognition ability (Han et al., 2017). Additionally, VEGF treatment changed the morphology of astrocytes, especially their branches (Lenzer-Fanara et al., 2017), which may affect hippocampal function after seizures because astrocytic feet are essential components of tripartite synapse (**Figure 1**).

Enhanced VEGF/VEGFR signaling may contribute to attenuating cognitive deficits after epileptic seizures by promoting adult neurogenesis in the dentate gyrus (Rosenstein et al., 2010; Han et al., 2017). The relationship between adult neurogenesis and the NVU has been studied (Goldman and Chen, 2011), and a recent study reported that microvascular hemodynamics modulate adult neurogenesis (Shen et al., 2019). Furthermore, VEGF administration suppressed neuronal loss in the hippocampal CA1 region 24 h but not one month after pilocarpine-induced SE in rats (Nicoletti et al., 2010). Additionally, when rats were treated with VEGF and angiopoietin-1, VEGF-induced vascular permeability was suppressed without affecting neuroprotective effects of VEGF

(Nicoletti et al., 2008). Therefore, the use of andiopoetin-1 can be a therapeutic strategy to suppress vascular abnormalities in the epileptic brain.

The donor and recipient cells of VEGF remain to be studied. Neurons do not express VEGFR2, which mainly plays a role in angiogenesis under normal conditions, but some studies have reported that it does play a role under abnormal conditions, such as in ischemia (Croll et al., 2004). Thus, it is proposed that cells around the NVU, including neurons, astrocytes, and endothelial cells, could express VEGFR2 after SE. The enhanced VEGF/VEGFR2 signaling in these cells may be neuroprotective in the acute phase and may contribute to angiogenesis in the chronic phase through the activation of the Src pathway and subsequent ZO-1 downregulation. Additionally, VEGF released from activated microglia in the epileptic brain may contribute to enhanced neurogenesis (Dudvarski Stankovic et al., 2016).

Overall, VEGF and VEGF signaling are potential therapeutic targets for epilepsy through the modulation of NVU structure and function. However, the donor–recipient relationships between cells that comprise the NVU should be more specifically defined in the future. It will also be important to pay attention to the opposing effects of VEGF in modulating epileptogenesis, which likely depend on the phase of epileptogenesis.

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REVIEW
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Immune Challenges and Seizures: How Do Early Life Insults Influence Epileptogenesis?

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The development of epilepsy, a process known as epileptogenesis, often occurs later in life following a prenatal or early postnatal insult such as cerebral ischemia, stroke, brain trauma, or infection. These insults share common pathophysiological pathways involving innate immune activation including neuroinflammation, which is proposed to play a critical role in epileptogenesis. This review provides a comprehensive overview of the latest preclinical evidence demonstrating that early life immune challenges influence neuronal hyperexcitability and predispose an individual to later life epilepsy. Here, we consider the range of brain insults that may promote the onset of chronic recurrent spontaneous seizures at adulthood, spanning intrauterine insults (e.g. maternal immune activation), perinatal injuries (e.g. hypoxic–ischemic injury, perinatal stroke), and insults sustained during early postnatal life—such as fever-induced febrile seizures, traumatic brain injuries, infections, and environmental stressors. Importantly, all of these insults represent, to some extent, an immune challenge, triggering innate immune activation and implicating both central and systemic inflammation as drivers of epileptogenesis. Increasing evidence suggests that pro-inflammatory cytokines such as interleukin-1 and subsequent signaling pathways are important mediators of seizure onset and recurrence, as well as neuronal network plasticity changes in this context. Our current understanding of how early life immune challenges prime microglia and astrocytes will be explored, as well as how developmental age is a critical determinant of seizure susceptibility. Finally, we will consider the paradoxical phenomenon of preconditioning, whereby these same insults may conversely provide neuroprotection. Together, an improved appreciation of the neuroinflammatory mechanisms underlying the long-term epilepsy risk following early life insults may provide insight into opportunities to develop novel immunological anti-epileptogenic therapeutic strategies.

Keywords: epilepsy, seizure, immune response, cytokines, interleukin-1, brain injury, neuroinflammation, development

INTRODUCTION

Epilepsy may develop later in life following a prenatal or early postnatal insult such as cerebral ischemia, stroke, brain trauma, or infection. These so-called "acquired epilepsies" account for approximately one-third of all human epilepsies (Engel, 1996; Thomas and Berkovic, 2014), and present clinically after a latent period of variable length (months to years) following the precipitating insult. During this time the brain undergoes progressive changes in neuronal connectivity and intrinsic excitability to ultimately result in an increased propensity for spontaneous recurrent seizures (a process known as "epileptogenesis") to occur (Lowenstein, 1996; Herman, 2002). While diverse in nature, early life insults that have been associated with the subsequent development of epilepsy share common pathophysiological pathways involving innate immune activation, including neuroinflammation, which is proposed to play a critical role in epileptogenesis (Vezzani et al., 2011a; Becker, 2018).

The developing brain undergoes significant dynamic changes during fetal and early postnatal life, rendering it particularly vulnerable to insults and stressors which can have either transient or permanent effects on neuronal function (Herlenius and Lagercrantz, 2004). Indeed, the developing brain at baseline has an increased propensity for seizure activity compared to the adult brain (Hauser, 1995), thought to be attributed at least in part to the abundant excitatory circuits but fewer inhibitory circuits in the neonatal brain (Nardou et al., 2013). Further, the developing brain appears to be primed to respond to immune challenges in such a way that predisposes the brain towards seizure induction (Bilbo and Schwarz, 2012; Nasr et al., 2019). Immune challenges and the subsequent immune response, including neuroinflammation, are increasingly recognized as an important factor in the pathophysiology of seizure generation, seizure-related neuropathology, and epileptogenesis (Galic et al., 2008; Riazi et al., 2010; Vezzani et al., 2011a).

Several mechanisms have been proposed to explain how and why prenatal, perinatal, and postnatal insults, such as those described above, result in a vulnerability to develop acquired epilepsy later in life. For example, modulation of gene transcription and epigenetic programming, acquired channel and synaptopathies, and neuronal network connectivity all likely play an important role, as discussed elsewhere (Becker, 2018). Here, we focus on evidence surrounding the hypothesis that inflammation promotes epileptogenesis, elaborating on data regarding soluble inflammatory mediators as well as cellular contributions in this process.

Neuroinflammation, defined as an inflammatory response within the brain or spinal cord, is a common consequence of brain injuries, insults, and immune challenges (Disabato et al., 2017). Characterized by the release of inflammatory mediators including pro- and anti-inflammatory cytokines, complement proteins and danger signals, activation of innate immune cells, astrocytes and microglia, and recruitment of blood-derived leukocytes into the central nervous system (CNS), neuroinflammation is also a common feature of temporal lobe epilepsy (TLE) in both patients and animal models (Ravizza et al., 2011; Vezzani et al., 2011a; De Vries et al., 2016). As reviewed in depth elsewhere, increasing evidence suggests that inflammation represents a causal mechanism that can also initiate and perpetuate seizure activity (Vezzani et al., 2011a; Webster et al., 2017), contributing to both ictogenesis (the onset of a seizure) and epileptogenesis (Vezzani et al., 2019).

In this review, we will consider the most common early life insults linked to the development of epilepsy later in life—including prenatal immune activation, perinatal injuries, and immune challenges sustained during early postnatal life (such as infections, neurotrauma, and even seizures themselves) (**Figure 1**). While not all of the described insults are purely immune-mediated —and indeed, are known to involve other biological mechanisms (e.g. environmental stress and neurotrauma)— we have incorporated these insults here in review to highlight how a range of distinctly different insults during early life can similarly yield a propensity for later life epilepsy. Much of the mechanistic evidence to date is preclinical, utilizing rodent models at postnatal day (p) 0–5 to correspond roughly to the third trimester prenatal

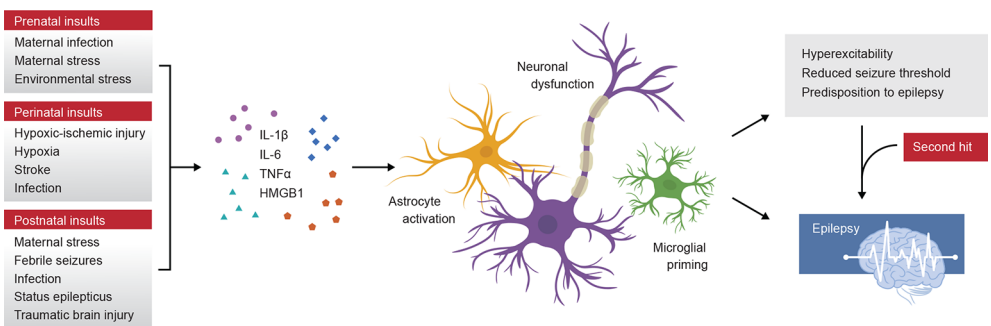

FIGURE 1 | Schematic summary of prenatal, perinatal, and postnatal insults to the developing human brain that initiate an inflammatory immune response, including the release of pro-inflammatory cytokines interleukin (IL)-1β, tumor necrosis factor alpha (TNFα), IL-6 and others. Experimental models have revealed that these cytokines promote astrocyte and microglial reactivity, and contribute to neuronal dysfunction by several mechanisms including alterations in neurotransmitter receptor subunit expression. These changes may lead to hyperexcitability or a reduced seizure threshold, resulting in an increased vulnerability to epilepsy. Epilepsy may develop over time and can be accelerated or triggered by a second-hit insult, such as a later life immune challenge.

in humans, p7–12 equivalent to the human infant at birth, and p21 to model the transition to early childhood (Semple et al., 2013). Specifically, we focus on evidence of the neurobiological mechanisms underlying the chronic consequences of such insults; in particular, the neuroinflammatory response. Together, this review provides a wide-ranging overview of how and why epilepsy may develop after insults to the developing brain *via* neuroimmune modulation. Such an understanding is necessary to inform the development and appropriate application of novel therapeutic agents targeting the relevant biological mechanisms, with the goal of disrupting and preventing the epileptogenic process from occurring.

PRENATAL INSULTS

Prenatal life is a time of unique immunological status for a developing fetus, which is intricately associated with maternal health status. A large and growing body of literature provides evidence that infections and other immune challenges sustained during pregnancy can influence fetal brain development, with *in utero* exposure to infections and/or inflammation considered to be an environmental risk factor for neurodevelopmental and psychiatric disorders including autism and schizophrenia (Solek et al., 2018; Guma et al., 2019).

Epidemiological data has suggested a relationship between maternal infections and a high incidence of childhood epilepsy in offspring (Norgaard et al., 2012). Several large population-based cohort studies have reported the greatest risk of epilepsy in the offspring of mothers who sustained infections resulting in fever during early to mid-pregnancy (Sun et al., 2008; Sun et al., 2011). Experimentally, this scenario can be modeled in rodents by evoking an infection-like immune challenge to pregnant dams, then assessing the seizure susceptibility of the resulting offspring. Lipopolysaccharide (LPS), a component of the cell wall of gram-negative bacteria and commonly used experimental immunogen to model a bacterial infection, results in persistent changes in neuronal excitability *in vitro* (Gullo et al., 2014), and exacerbates hippocampal excitability in electrical kindling models *in vivo* (Auvin et al., 2010b). When embryos are exposed to LPS *via* inoculation of the pregnant dam at gestational days 15–16, a second challenge at p21—injection of the L-glutamate analog kainic acid (KA)—revealed increased seizure susceptibility compared to those exposed to saline control (Yin et al., 2015). This finding was associated with exacerbated, long-lasting astrogliosis, and worsened spatial learning ability when assessed at adulthood (Yin et al., 2015). Astrocytes, as the most numerous glial cells in the CNS, play many essential roles in tissue homeostasis, synaptic transmission, and neuroimmune responses (Farina et al., 2007; Clarke and Barres, 2013). Accumulating compelling evidence suggests that aberrant astrocyte activation contributes to the pathophysiology of epilepsy (De Lanerolle et al., 2010; Yin et al., 2015; Patel et al., 2019). Together with epidemiological evidence that systemic inflammation increases an individuals' susceptibility to seizures by lowering their seizure threshold (Yuen et al., 2018), these studies provide the foundation for the hypothesis that inflammation is a critical modulator of brain excitability.

Polyinosinic:polycytidylic acid (poly I:C) is an experimental substrate frequently used to mimic viral infections. When administered to gestating animals in a model known as maternal immune activation (MIA), this toll-like receptor 3 (TLR3) agonist results in long-lasting physiological perturbations (Meyer, 2014). Poly I:C administration to pregnant mice between embryonic days 12 to 16 results in the offspring exhibiting increased vulnerability to hippocampal kindling, with strong evidence supporting a role for the cytokines interleukin (IL)-6 and IL-1β in these effects (Pineda et al., 2013). The dependence of these effects on signaling *via* TLR3 was demonstrated by use of TLR3 gene deficient mice, albeit at adulthood, which show a reduced propensity to develop epileptic seizures after administration of the proconvulsant pilocarpine (Gross et al., 2017).

Several cytokines are known to have both acute and long-lasting effects on neuronal excitability, with IL-1β being the best characterized to date. Systemic or intracerebral administration of LPS or poly I:C to the mother rodent (or offspring; see the section *Infections in Postnatal Life*) provokes an acute elevation of pro-inflammatory cytokines *via* gene transcription of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) in neurons and glia of the mother and offspring (Pineda et al., 2013). By triggering a systemic immune response in the mother, gestational infections mimicked by LPS or poly I:C appear to also comprise the fetal placental barrier, allowing entry of maternally derived cytokines and other molecules (e.g. glucocorticoids) into the fetal circulation, where they can influence the developing fetal brain (Meyer, 2014). The extent to which maternally derived factors cross the placenta—a process known as "vertical transfer"—is incompletely understood, but appears to be cytokine-specific, and may be *via* either direct or passive transport (Zaretsky et al., 2004; Gilmore et al., 2005; Dahlgren et al., 2006). Regardless, abundant studies have demonstrated that a wide range of cytokines are increased in the fetal brain within hours after MIA in pregnant rodents, including IL-1β, tumor necrosis factor alpha (TNFα), and IL-6 (Solek et al., 2018). Activated microglia are likely to be a major source of inflammatory mediators in this context (Perry and Holmes, 2014). These cytokines can then act both directly and indirectly to modulate neuronal excitability and neurotransmission—for example, by altering the subunit composition of glutamatergic and gamma-aminobutyric acid (GABA)-ergic receptors (Vezzani et al., 2011a; Vezzani et al., 2011b; Vezzani and Viviani, 2015).

In the poly I:C MIA model, antibody blocking experiments were used to demonstrate that both IL-1β and IL-6 are required for an increased propensity for recurrent seizures (Pineda et al., 2013). Further evidence that IL-1β has a causative or modulatory role in network excitability stems from studies demonstrating anticonvulsive effects when IL-1β levels are reduced; either by intracerebral injection of an IL-1 receptor antagonist, transgenic overexpression of the receptor antagonist, or inhibition of interleukin-converting enzyme (Ravizza et al., 2008; Vezzani et al., 2011b).

MATERNAL AND ENVIRONMENTAL STRESS THROUGHOUT DEVELOPMENT

Environmental stress during pregnancy, and early postnatal life, may promote the development of epilepsy during later life. Increasing evidence suggests that early life stress is an important modulator of limbic epilepsy, likely *via* effects on endocrine function, neuroplasticity, neurotransmission, and cellular electrophysiology (Koe et al., 2009). MIA and other forms of maternal stress, such as separation of dam from pups or early handling of pups, may also be considered as an early life stressor which aggravates epileptogenesis in both status epilepticus (SE) and kindling models (Salzberg et al., 2007). Additionally, stress resulting from transport of dams during pregnancy, as well as maternal behaviors and early postnatal malnutrition, have all been demonstrated to promote susceptibility to KA or amygdala kindling-induced seizures of the offspring during later life (Salzberg et al., 2007; Moriyama et al., 2013; Simao et al., 2016).

Maternal glucocorticoids as a consequence of a hyperactive hypothalamic–pituitary–adrenocortical (HPA) axis have been proposed to be one of the key mechanisms by which maternal stress mediates network reorganization and epileptogenesis in the developing offspring (Koe et al., 2009; Kumar et al., 2011; Jones et al., 2014; Wulsin et al., 2016). Another mechanism is *via* disruption of the GABA switch, a developmentally regulated functional change in GABA signaling from excitatory to inhibitory that occurs in the first 1–2 postnatal weeks (Ben-Ari, 2002). Neonatal stress from maternal separation has been found to delay the timing of the GABA switch in the mouse hippocampus, with consequences for behavior at adolescence (Furukawa et al., 2017). This study suggests that early life insults can disrupt or modify this essential step in GABAergic maturation and the resulting neuronal excitation/inhibition balance—which could have consequences for seizure propensity also. Indeed, the poly I:C MIA model has also been shown to delay the GABA switch in mice, resulting in hyperexcitability of neuronal networks and higher susceptibility to seizures at adulthood (Corradini et al., 2018).

Importantly, alterations in the stress response—resulting in elevated stress hormone levels—have been shown to promote chronic priming and activation of microglia, which then generate increased cytokines IL-1β, IL-6, and TNFα in response to a secondary immune challenge (Frank et al., 2014). This mechanism by which stress promotes increased neuroinflammation may thereby contribute to epileptogenesis in adulthood (Salzberg et al., 2007).

PERINATAL INSULTS

Hypoxic–ischemic injury (HI) is a significant cause of brain damage in newborn infants, and is associated with a high incidence of neurodevelopmental disabilities. Neonatal hypoxic–ischemic encephalopathy, defined as a syndrome of disturbed neurological function during the first days of life, often occurs following moderate to severe HI, and is the most common cause of neonatal seizures (Volpe, 1989; Zupanc, 2004). These acute seizures likely result from excitotoxic neuronal damage and cell death, following compromised oxygen and glucose supply to the developing brain. Epilepsy is reported in 9–33% of term infants with neonatal HI (Glass et al., 2011), with injury to the motor cortex, hippocampus, and occipital lobe being identified as risk factors for epilepsy in this context (Xu et al., 2019). Ischemic stroke is another common disorder affecting approximately one in every 4,000 live births, which is associated with both acute seizures and the subsequent development of epilepsy (Lynch and Nelson, 2001).

Animal models of perinatal hypoxia, HI, or stroke have suggested that the propensity for both acute seizures and epileptogenesis after injury is age-dependent (Jensen et al., 1998). Using the well-established Rice–Vannucci model of perinatal HI to p7 pups (Rice et al., 1981), acute seizures are associated with the extent of brain damage (Bjorkman et al., 2010), and spontaneous recurrent seizures have been reported in a subset of animals (Williams et al., 2004; Williams and Dudek, 2007). In another stroke model, involving induction of a photothrombotic lesion in the sensorimotor cortex of p7 rodents, seizure vulnerability was evaluated in response to the $GABA_A$ receptor antagonist and pro-convulsive agent pentylenetetrazol (PTZ), an agent widely used to assess brain excitability (Kliioueva et al., 2001). From electroencephalogram (EEG) analysis performed at 5 and 18 days post-injury (p12 and p25), early life stroke was found to result in an exacerbated response to PTZ, with a higher proportion of animals exhibiting clonic seizures, as well as longer response duration (Brima et al., 2013).

Hypoxia alone has also been shown to induce spontaneous tonic–clonic seizures in rodents, when induced at p10–12, but not in older (p15–60) or younger (p5) rats (Jensen et al., 1991; Jensen et al., 1992). These animals also displayed increased susceptibility to convulsant-induced seizures at adulthood, while hippocampal slices collected postmortem demonstrated chronic changes in CA1 hippocampal network excitability. Of note, these abnormalities were evident in the absence of overt histopathological damage or chronic neurobehavioral deficits (Jensen et al., 1991). In subsequent studies, hypoxic injury and HI at p7 have been shown to result in an increased vulnerability to KA challenge at 6 weeks post-injury (Rodriguez-Alvarez et al., 2015), as well as spontaneous epileptiform discharges and recurrent motor seizures by 2–12 months of age—but typically only in a subset of injured animals with cerebral cystic infarcts (Kadam et al., 2010; Peng et al., 2015). The frequency and severity of spontaneous behavioral and electrographic seizures increases over time, highlighting the progressive nature of epileptogenesis.

The contribution of inflammation to development of epilepsy in this context was recently demonstrated, by use of a novel therapeutic drug Vitexin. This anti-inflammatory botanical flavonoid was found to reduce cytokine release, neutrophil infiltration, and blood–brain barrier (BBB) permeability alongside a reduction in epilepsy susceptibility after HI in

neonatal rats (Luo et al., 2018). In the clinic, in a small cohort study of patients with neonatal HI, elevated IL-6, TNFα, and IL-1β were found to be associated with the subsequent onset of epilepsy (Numis et al., 2019), suggesting that these cytokines may hold value as predictive biomarkers of later life epilepsy risk.

POSTNATAL INSULTS

Hyperthermia-Induced Febrile Seizures

Febrile seizures (FS), typically provoked by fever, are common during infancy and early childhood, affecting approximately 3–5% of children between 6 months and 5 years of age (Berg and Shinnar, 1996; Jensen and Baram, 2000). When recurrent or prolonged (approximately one-third of FS), these complex seizure events can lead to sustained modification and dysfunction of hippocampal neurons, which is proposed to underlie a heightened risk of subsequent epileptogenesis and neurocognitive dysfunction during later life (Dube et al., 2007; Huang and Chang, 2009). Epidemiological studies have linked prolonged FS during childhood with the development of TLE (Chen et al., 1999; Saltik et al., 2003; Fukuda et al., 2015), although whether this relationship is indeed causal, and the underlying mechanisms, remains unclear. Susceptibility to the convulsant effects of hyperthermia decreases with age in both humans and rodents, such that investigation into this phenomenon may provide insight into the mechanisms that govern this developmentally specific vulnerability during early life (Jensen and Baram, 2000).

Experimentally, rodent models of hyperthermia-induced FS typically utilize p10–14 animals (Heida et al., 2004; Heida and Pittman, 2005), consistent with the time period thought to represent the neurodevelopmental transition during the first 2 weeks of life in the human (Gottlieb et al., 1977; Semple et al., 2013). This model results in increased seizure susceptibility by adulthood, evident as a reduction in seizure threshold and increased susceptibility to seizure-induced cell death after a KA second-hit (Dube et al., 2000; Van Gassen et al., 2008). Approximately 40% of these animals develop spontaneous TLE alongside neuropathology in the cortex and hippocampus. Several factors have been proposed as mechanisms of epileptogenesis in this context, including the effects of altered brain temperature, changes in the endocannabinoid system, altered $GABA_A$ subunit composition, and inflammation (Feng and Chen, 2016). In terms of the inflammatory response, the release and subsequent actions of IL-1β have also been strongly implicated (Feng and Chen, 2016). In patient populations, specific IL-1β polymorphisms have been associated with sporadic development of FS (Kira et al., 2005). Supporting this, administration of IL-1β after an induced FS in juvenile rats leads to a significant increase in seizure incidence compared to saline-treated controls (Fukuda et al., 2015), while IL-1β alone can mimic the effects of FS on adult seizure susceptibility (Feng et al., 2016). In another study, only rats in which IL-1β was elevated chronically went on to develop spontaneous limbic seizures after FS (Dube et al., 2010). In contrast, administration of the IL-1R antagonist is anticonvulsive (Heida and Pittman, 2005), while IL-1R–deficient mice are resistant to FS, independent of the genetic background strain (C57Bl or 129/Sv) (Dube et al., 2005). This mechanism holds strong promise for clinical translation, with two case reports demonstrating that treatment with the IL-1R antagonist reduced seizure burden and relapse in children with febrile infection-related epilepsy syndrome (Kenney-Jung et al., 2016; Dilena et al., 2019).

Infections in Postnatal Life

Epidemiological evidence has demonstrated that CNS and systemic infections are another major cause of acquired epilepsy (Annegers et al., 1988; Marks et al., 1992). Indeed, an episode of viral encephalitis resulting from, for example, herpes simplex or cytomegalovirus, has been reported to increase the risk of subsequent unprovoked seizures by 16-fold (Annegers et al., 1988). These seizures are also associated with concurrent neurological consequences, and the increased risk for both epilepsy and neurobehavioral complications may persist even after the infection has resolved for at least 20 years (Annegers et al., 1988; Raschilas et al., 2002; Chen et al., 2006). In the juvenile rodent, poly I:C and LPS have been extensively utilized to model infection-like immune challenges during early postnatal life, and examine the effect of such insults on brain excitability.

Postnatal Poly I:C

In addition to use in the MIA model of a prenatal immune challenge, poly I:C is regularly employed to investigate infection-like immune challenges during early postnatal life. When injected directly into the rat hippocampus at p13–14, poly I:C induces fever and a local increase in IL-1β levels (Galic et al., 2009). Poly I:C facilitates electrical kindling epileptogenesis, as evident by an increased number of observed limbic seizures (Dupuis et al., 2016). Animals administered poly I:C at p13 demonstrated a faster seizure onset and prolonged kindled state compared to when the immune challenge was induced at adulthood (p74), again highlighting the age-dependent vulnerability of the early postnatal brain to hyperexcitability (Dupuis et al., 2016). Although microglia were hypothesized to play a role in these observations, administration of the tetracycline antibiotic minocycline, which has reported microglial suppressive effects (as well as other effects), prior to kindling, failed to reverse the pro-epileptogenic effects of poly I:C (Dupuis et al., 2016). In another study, animals exposed to poly I:C at p14 were found to be more susceptible to lithium-pilocarpine and PTZ-induced seizures at adulthood, and exhibited memory deficits in a fear conditioning paradigm (Galic et al., 2009). These chronic changes were coincidental with persistently altered levels of glutamate receptor subunits messenger ribonucleic acid (mRNA) expression, which were able to be suppressed by neonatal systemic minocycline, implicating a role for microglial activation as an underlying mechanism (Galic et al., 2009). Increased seizure susceptibility observed in adult rodents following poly I:C administration to young pups, similar to in the MIA context, is understood to depend at least in part upon early life activation of IL-1β signaling (Galic et al., 2009). Together, these studies demonstrate that poly I:C exposure is

pro-epileptic in the early postnatal brain, similar to the prenatal brain, although the precise mechanisms (and whether these are age-dependent) remain incomplete.

Postnatal LPS

Perhaps the most common experimental model of postnatal infection involves administration of LPS, typically to the periphery (intraperitoneal) to p10–16 rodent pups (Galic et al., 2008; Auvin et al., 2010a). Depending upon the dose, LPS induces a transient inflammatory response within the first 12–24 h post-injection which largely resolves thereafter. Of note, a persistent increase in seizure susceptibility is evident following low dose LPS at p7–14—compared to when LPS was administered earlier (p1) or later (p20) (Galic et al., 2008). This time period coincides with the developmental peak in synaptogenesis and synaptic pruning, yielding considerable changes in neuronal circuitry which likely underlies this critical window of vulnerability. This paradigm has since been used to demonstrate that LPS exposure increases susceptibility to KA-induced seizures at p35, alongside impairments in long-term potentiation and exacerbated hippocampal neurodegeneration (Chen et al., 2013), and pilocarpine-induced seizures at 2 months of age (Setkowicz et al., 2017). In another study, KA was administered simultaneously with LPS to p14 pups, revealing long-lasting molecular changes alongside increased seizure excitability by adulthood. This was enhanced further in the ~50% subset of LPS + KA–treated animals that exhibited an overt behavioral seizure response FS compared to those that did not, as evidenced by increased *in vitro* excitability as well as modified N-methyl-D-aspartate (NMDA) and $GABA_A$ receptor subunit protein expression in the hippocampus (Reid et al., 2013).

Similarly, LPS has been shown to potentiate fever-induced FS at p14 in both rats and mice, at least acutely (Eun et al., 2015). Administered peripherally 2 h prior to hyperthermia-induced seizure onset, LPS promoted susceptibility of animals to seizures, alongside enhanced pro-inflammatory cytokine production and microglial activation. These findings suggest that peripheral inflammation works synergistically with hyperthermia to potentiate seizures and exacerbate the resultant immune response. Whether this combinatorial challenge also predicts heightened vulnerability to chronic epilepsy has not yet been examined.

Several studies have noted that the inflammatory challenge during early life is typically transient; for example, LPS-induced acute inflammation rarely lasts more than 24 h following injection. Thus significant changes in the reactivity to seizures observed in adulthood do not directly result from inflammation *per se,* but rather indirectly from the long-term effects of the acute inflammatory response on the immature brain, and its subsequent developmental trajectory (Kosonowska et al., 2015; Janeczko et al., 2018).

LPS administration triggers the abundant release of IL-1β and TNFα, which can act on receptors such as IL-1R on the hippocampal dentate gyrus to facilitate enhanced epileptiform activity (Gao et al., 2014). A central role for these cytokines has been demonstrated in the context of epileptogenesis following LPS in the p14 rat, which can be partially prevented by administration of either IL-1R antagonism or an anti-TNFα antibody (Galic et al., 2008; Auvin et al., 2010b). LPS-induced seizure susceptibility was also recently shown to involve TLR4 activation, signaling *via* extracellular signal–regulated kinases 1 and 2 (Erk1/2), in a manner dependent on myeloid differentiation primary response 88 (MyD88) (Shen et al., 2016). Constitutive activation of Erk1/2 in astrocytes alone was sufficient to enhance excitatory synaptogenesis, while deleting MyD88 or suppressing Erk1/2 in astrocytes was able to ameliorate seizure sensitivity, providing direct evidence for a developmental role of astrocytes in predisposing towards epileptogenesis (Shen et al., 2016).

As noted earlier, a particular window of enhanced vulnerability to seizures has been identified in rodent models during the second week of postnatal life, coincidental with significant synaptogenesis and synaptic pruning (Galic et al., 2008). As microglia are known regulators of synaptic remodeling (Tremblay et al., 2011), and peak in cell density at around p14–28 (Kim et al., 2015), microglia may also govern this age-dependent susceptibility (**Figure 2**). Heightened reactivity due to normal developmental changes render microglia particularly poised to mount an exaggerated inflammatory response to early life seizures or other immune challenges that are encountered at this time; and this over-reactive immunity may exacerbate acute neuronal injury thereby contributing to long-term epileptogenic effects (Tremblay et al., 2011; Kim et al., 2015).

Postnatal Status Epilepticus

SE is defined as a condition in which abnormally prolonged seizures occur, which may have long-term consequences including neuronal loss and altered neuronal networks (Trinka et al., 2015). In experimental animals, a transient episode of SE can "convert" a previously normal brain into an epileptic one, providing a model in which to explore mechanisms of epileptogenesis (Lothman and Bertram, 1993). Of note, vulnerability to KA appears to be age-specific, with younger animals (p5–15) exhibiting more severe SE with a shorter latency, and higher mortality, compared to older animals (p20–60) (Holmes and Thompson, 1988; Stafstrom et al., 1992).

Microglia, which mediate a significant proportion of the innate immune capacity of the CNS, are critical for immune surveillance in the steady state, as well as the response to injury and disease (Disabato et al., 2017). Chronic microglial activation is a common component to a wide range of neurodegenerative conditions including multiple sclerosis, Alzheimer's disease, and traumatic brain injury (TBI), likely contributing to neuronal dysfunction and cell loss to facilitate disease progression. KA-induced SE triggers a time-dependent microglial activation response including the release of pro-inflammatory cytokines TNFα and IL-1β (Wyatt-Johnson et al., 2017), which appears to precede the appearance of neuronal damage (Rizzi et al., 2003; Ravizza et al., 2005). In this context, IL-1 signaling has again been implicated, with experiments in which IL-1β was administered prior to KA reported an increase in the time spent in seizures *via* an NMDA receptor–dependent mechanism (Vezzani et al., 1999).

In some instances, in response to an immune challenge, microglia are induced to a "primed" state—not activated *per se*, but in an intermediate phenotype which renders them able to

respond more rapidly when subsequently activated, including the production of greater quantities of pro-inflammatory cytokines compared to normally activated, non-primed (or quiescent) microglia (Sparkman and Johnson, 2008). This appears to be the case after early life KA-induced seizures in p15 rats, followed by a second-hit exposure of KA at p45. Animals that were exposed to both KA doses had greater microglial activation, associated with elevated pro-inflammatory cytokine levels and increased susceptibility to seizures compared to saline-control animals that received the KA only at p45 (no prior exposure) (Somera-Molina et al., 2009). Treatment with Minozac, a small novel therapeutic compound that inhibits pro-inflammatory cytokine production, attenuated these effects. These results implicate cytokines produced by activated microglia as one mechanism by which early life seizures contribute to increased vulnerability to neurological insults in adulthood (Somera-Molina et al., 2009). Similarly, administration of minocycline following KA at p25 in mice has been shown to reduce vulnerability to a second SE event at p39, likely to be attributed to the suppression of microglial activation—providing further evidence that early life insults such as seizures act to prime microglia for a subsequent immune challenge (Abraham et al., 2012).

Early Life Neurotrauma

TBI during early childhood is another well-known cause of epilepsy. This post-traumatic epilepsy (PTE) has a reported incidence of up to 35% after severe TBI (Annegers et al., 1980; Ates et al., 2006; Arndt et al., 2013). While several well-characterized experimental models have been utilized in adult rodents to explore PTE after TBI to the mature brain (D'ambrosio et al., 2004; Bolkvadze and Pitkanen, 2012; Kelly et al., 2015; Ostergard et al., 2016), there has been a lack of age-appropriate models to consider the complex interaction between ongoing brain development and epileptogenesis that occurs after a TBI during early childhood.

An established model of experimental TBI to the p21 mouse, utilizing the controlled cortical impact model of unilateral injury to the parietal lobe, results in progressive neuropathology and chronic neurobehavioral and neurocognitive dysfunction consistent with what is commonly observed in toddler-aged children after TBI (Tong et al., 2002; Pullela et al., 2006). This model has recently been demonstrated to also reproduce many of the features characteristic of PTE in humans, including histopathological evidence of circuitry reorganization, interneuron loss, and hippocampal gliosis (Semple et al., 2017). Brain-injured mice exhibit both an increased vulnerability to PTZ-evoked seizures, evident as early as 2 weeks post-TBI, suggesting that epileptogenesis is underway at this time creating an environmental primed for the development of PTE. A proportion of TBI mice were reported to develop at least one spontaneous seizure within a 7-day video-EEG recording period by 4–6 months post-injury—from 15% after a moderate injury severity up to over 90% incidence after a severe injury involving considerable hippocampal pathology (Semple et al., 2017; Webster et al., 2019).

Although the mechanisms underlying PTE remain unclear, several lines of evidence point towards a prominent role of cytokine signaling, particularly *via* IL-1 (Webster et al., 2017). Experimentally, administration of the IL-1R antagonist attenuates both sub-acute and chronic susceptibility to PTZ-induced seizures after pediatric TBI in the mouse (Semple et al., 2017). Genetic data from patient populations has also implicated specific IL-1β polymorphisms with the risk of PTE after a TBI (Diamond et al., 2015).

This latter point raises and somewhat addresses an important question—why do some individuals, a minority, respond to an early life insult with epilepsy, while others who sustain a similar insult do not? Our understanding of how environmental factors (such as an early life insult) interact with genetics to promote epileptogenesis remains in its infancy. However, there is increasing evidence that genetic predisposition to epilepsy will increase an individuals' likelihood of developing late-onset seizures after an acquired insult. For example, a higher risk of post-stroke epilepsy was recently reported in individuals with a family history of epilepsy compared to those without a family history, even when adjusted for stroke injury severity (Eriksson et al., 2019). Similarly, in a population-based cohort study of more than 1.6 million Danish adults and children, a family history of epilepsy was associated with an approximately 10-fold higher risk of developing late-onset seizures following a severe brain injury (Christensen et al., 2009). Limited studies to date have specifically probed for gene associations with acquired epilepsy risk, as recently reviewed (Leung et al., 2019). Consistent with the abovementioned evidence on IL-1β polymorphisms and PTE risk, a meta-analysis found that specific alleles of both IL-1β and IL-1α have also been associated with risk of epilepsy after FS (Saghazadeh et al., 2014). Further investigation into other genes involved in the inflammatory response, and in a range of patient populations, are needed to determine the extent to which genetic variance contributes to an individual's risk of epilepsy after an early life insult.

INFLAMMATORY PRECONDITIONING—PROTECTION *AGAINST* EPILEPSY?

Contrary to the above-described literature, there is also preclinical evidence that an early life insult inducing a modest inflammatory response can alternatively attenuate the response to a second-hit insult. This phenomenon, termed "preconditioning," occurs when the brain develops resistance to injury after exposure to a low dose, typically subthreshold stimuli, such as brief ischemia, hypoxia, or low dose endotoxin. Preconditioning in the context of brain insults has been well documented in adult animals, but less so in the immature brain. Even fewer studies have considered the effect on neuronal excitability and seizure vulnerability.

Administration of a low dose of LPS (typically in the 0.05–1.0 mg/kg range) (Hickey et al., 2011) is one of the best-characterized approaches to yield neuroprotection *via* preconditioning. Acting *via* TLR4, LPS is thought to reprogram the intracellular response to a subsequent insult, resulting in broad neuroprotection *via* activation of anti-inflammatory factors, alongside the downregulation of NF-κB

(Hickey et al., 2011; Wang et al., 2015; Amini et al., 2018). One study compared young rats exposed to systemic low dose LPS at p6 and p30, followed by pilocarpine injection at 2 months of age, and reported that LPS at p30 only resulted in a reduction in acute seizures alongside ameliorated seizure-induced changes in microglial morphology (Kosonowska et al., 2015). Anti-ictogenic effects have also been reported following TLR3 activation in adult mice, whereby intraventricular poly I:C administered 6 h prior to a KA challenge was found to prevent the anticipated increase in hippocampal excitability (Kostoula et al., 2019). This effect was mimicked by administration of the cytokine interferon gamma, suggesting that activation of downstream signaling *via* interferon regulatory factor 3 is involved (Kostoula et al., 2019). Of note, poly I:C administered at 15 min, 1 h, or 24 h prior to KA failed to elicit anti-ictogenic effects, suggesting that the timing is crucial and likely involves the activation of transcriptional rather than posttranslational mechanisms to influence neuronal excitability.

A role for astrocytes in modulating the relative levels of pro- versus anti-inflammatory mediators has been reported as a biological mechanism associated with this phenomenon, as well as microglial priming (Kosonowska et al., 2015). In addition to modulation of the inflammatory response, several other cellular and molecular mechanisms have been proposed to underlie preconditioning neuroprotection, for example, changes in calcium binding, transcriptional regulation, apoptosis, growth, and development processes (Friedman et al., 2013; Friedman and Hu, 2014). The apparent paradox regarding why an early life insult may induce either seizure susceptibility or resistance (alongside neuroprotection) is poorly understood. However, it may be that TLR3 and TLR4 have dual roles whereby activation of alternative pathways in different cell types yields differential consequences. It is clear that the preconditioning phenomenon is both age and dose dependent (Hickey et al., 2011). Further, the time interval between the first and second insult is likely to be an important determinant. For example, although seizure susceptibility was not examined, one study found that low dose LPS administered at 48 h before HI in p7 rats was neuroprotective, whereas administration earlier at 72 h before HI instead increased the extent of brain damage (Hickey et al., 2011).

CONCLUSIONS

In this review, we have summarized and critically discussed the most common known causes of acquired epilepsy following injury or insult during early life, from maternal infection exposure through to TBI during young childhood. We have excluded from our discussion some other causes of acquired epilepsy, such as brain tumors (Weisman et al., 2018) or malformation of cortical development (e.g. focal cortical dysplasia) (Crino, 2015), based on the observation that these factors often persist throughout a patients' life span—rather than being a transient early life insult that resolves with time, in the face of persistent seizure susceptibility, as we have focused on in this review.

All of the described early life insults induce activation of the innate immune response, eliciting reactivity of glial cells, release of pro-inflammatory mediators, and neuronal or network changes in favor of a more excitable CNS microenvironment, which appears to facilitate the process of epileptogenesis and subsequent emergence of spontaneous recurrent seizures (or increased vulnerability to evoked seizures) at a later time (**Figure 1**). To date, the evidence supports that early life challenges act as risk factors for epilepsy, but do not necessarily cause epilepsy *per se*; indeed, genetic predisposition, environmental factors, and interactions between all of these variables are likely to determine an individual's risk status (Koe et al., 2009). Experimental models have been invaluable to

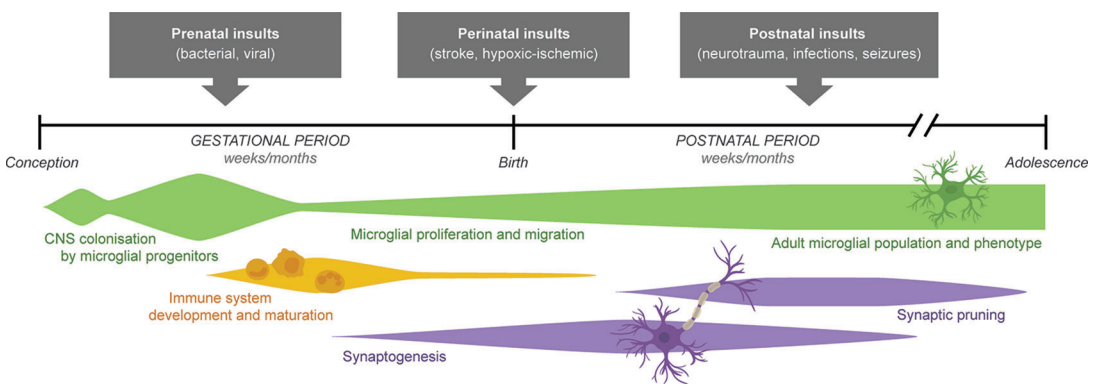

FIGURE 2 | Schematic timeline illustrating key neurodevelopmental processes ongoing through gestational and postnatal periods in the mammalian brain. A wide range of prenatal, perinatal, and postnatal insults influence the developing brain both acutely but also chronically, driving an increased propensity for neuronal hyperexcitability and seizure susceptibility during later life. Age-dependent vulnerability to these chronic consequences is thought to be determined, at least in part, by the state of microglial development (changes in number, phenotype, and activity), as well as maturation of neuronal circuits (a product of synaptogenesis and synaptic pruning over time). Adapted from Semple et al. (2013) and Lenz and Nelson (2018).

TABLE 1 | Key inflammatory mediators implicated in epileptogenesis after early life insults: experimental evidence.

Mediator	Insult	Model	Species/Age	Effect and Potential Mechanisms	Reference(s)
IL-1β	Bacterial infection (MIA; postnatal infections)	Systemic or intracerebral LPS administration	Rat, p14 Rat, g15–16	• After p14 LPS, increased IL-1β production in response to KA at adulthood • After LPS + sub-convulsive KA, i.c.v. IL-1β increased the proportion of animals with seizures, while IL-1R antagonist was anticonvulsive • IL-1R modifications associated with hyperexcitability, upregulation of NF-κB, and altered GABAergic subunit expression	(Heida and Pittman, 2005; Vezzani et al., 2008; Chen et al., 2013)
	Preterm HI injury	In utero HI + LPS administration	Rat, g18	• Increased placental IL-1β acutely and sub-acutely associated with fetal neuroinflammation and neuronal injury	(Maxwell et al., 2015)
	Viral infection (MIA; postnatal inoculation)	Systemic or intracerebral poly I:C administration	Mouse, g12–16 Rat, p13–14	• Chronic epilepsy phenotype in offspring prevented by antibodies to IL-1β and IL-6 (when combined) • Increased IL-1β associated with kindling epileptogenesis	(Galic et al., 2009; Pineda et al., 2013; Dupuis et al., 2016)
	Infantile FS	Hyperthermia induction ± intranasal IL-1β	Rat, p10–12 Mice, p14–15	• Addition of intranasal IL-1β increased seizures following KA at p70–73, associated with hippocampal cell loss in CA3 region • Hippocampal IL-1β levels only in rats that developed late-onset seizures • Exogenous IL-1β exacerbates FS, while IL-1R–deficient mice show resistance to FS • mRNA levels of IL-1R correlates with epilepsy-predictive MRI signal changes	(Dube et al., 2005; Dube et al., 2010; Fukuda et al., 2014; Fukuda et al., 2015; Patterson et al., 2015)
	Status epilepticus	KA kindling; lithium-pilocarpine	Rat, p9–15	• Upregulated IL-1β and IL-1R acutely and chronically (to 8 weeks), associated with glial activation	(Holmes and Thompson, 1988; Rizzi et al., 2003; Omran et al., 2012)
	Trauma	Controlled cortical impact	Mouse, p21	• Upregulation of IL-1β and IL-1R acutely; prevention of chronic seizure susceptibility by treatment with IL-1R antagonist	(Semple et al., 2017)
IL-6	Bacterial infection	Systemic or intracerebral LPS administration	Mouse, p10–14 Rat, p6	• Increased IL-6 acutely post-LPS associated with chronically activated microglia	(Kosonowska et al., 2015)
	Infantile FS	Hyperthermia induction ± IL-6 administration	Rat, p23–28	• IL-6 dose-dependently reduced hyperthermia-induced seizures —anticonvulsive effect	(Fukuda et al., 2007)
	Viral infection (MIA)	Systemic or intracerebral poly I:C administration	Mouse, g12–16	• Chronic epilepsy phenotype in offspring prevented by antibodies to IL-1β and IL-6 (when combined)	(Pineda et al., 2013)
	Prenatal immune challenge (IL-6)	Systemic IL-6 administration	Mouse, g12–16	• In combination with IL-1β, increased propensity to hippocampal kindling, associated with social deficits	(Washington et al., 2015)
	Status epilepticus	KA kindling	Rat, p9–21	• Upregulated IL-6 acutely after KA, associated with glial activation	(Rizzi et al., 2003)
TNFα	Bacterial infection	Systemic or intracerebral LPS administration	Rat, p6–7, p14	• Increased TNFα acutely post-LPS, and in response to KA at adulthood • Response to lithium-pilocarpine, KA, and pentylenetetrazol at adulthood was mimicked by i.c.v. recombinant TNFα and blocked by an anti-TNFα antibody	(Galic et al., 2008; Chen et al., 2013; Kosonowska et al., 2015)
	Bacterial infection (meningitis)	*S. pneumoniae* inoculation	Rat, p11	• TNFα-converting enzyme attenuates incidence of seizures and exerts neuroprotection	(Meli et al., 2004)
	Preterm HI	In utero HI + LPS administration	Rat, g18	• Increased placental IL-1β acutely and sub-acutely associated with fetal neuroinflammation and neuronal injury	(Maxwell et al., 2015)
	Status epilepticus	KA kindling; lithium-pilocarpine	Rat, p9–21 Rat, p25	• Upregulated TNFα acutely after KA associated with glial activation • Upregulated TNFα and chronically in pilocarpine model of TLE, associated with astrocyte activation	(Rizzi et al., 2003; Ashhab et al., 2013)

BBB, blood–brain barrier; DAMP, damage-associated molecular pattern; g, gestational day; GABA, gamma-aminobutyric acid; FS, febrile seizure; HCN, hyperpolarization-activated cyclic nucleotide-gated channel; HI, hypoxic–ischemic injury; HMGB1, high-mobility group box protein-1; HPA, hypothalamic–pituitary axis; i.c.v., intracerebroventricular; IL, interleukin; IL-1R, interleukin-1 receptor; KA, kainic acid; LPS, lipopolysaccharide; mRNA, messenger ribonucleic acid; MIA, maternal immune activation; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; NMDA, N-methyl-D-aspartate; NMDAR, N-methyl-D-aspartate receptor; p, postnatal day; poly I:C, polyinosinic:polycytidylic acid; PTZ, pentylenetetrazol; S. pneumoniae, Streptococcus pneumoniae; TBI, traumatic brain injury; TLE, temporal lobe epilepsy; TLR, toll-like receptor; TNFα, tumor necrosis factor alpha.

determine particular developmental windows of increased vulnerability to insult, whereby the brain is rendered immunologically primed and more reactive to a second-hit insult should one occur (**Figure 2**). Transient cytokine release (including IL-1β, TNFβ, and IL-6; see **Table 1**), microglial priming, and astrocyte reactivity are all mechanisms by which early life immune challenges can yield long-lasting effects on seizure threshold. Several other cytokines, chemokines, and

damage-associated molecular patterns including IL-6 and high mobility group box protein-1 have also been implicated in seizure ictogenesis and epileptogenesis, as reviewed extensively elsewhere (Vezzani et al., 2008; Vezzani and Viviani, 2015; Webster et al., 2017; Vezzani et al., 2019). However, few studies to date have studied these mediators in the context of how early life immune challenges promote later onset epilepsy.

Future studies to identify and characterize the key factors mediating the chronic consequences of such insults may allow for the development of predictive tests to more readily identify those individuals at greatest risk. Further, novel immune-based therapies may provide therapeutic benefit by aborting the epileptogenesis process prior to the onset of spontaneous recurrent seizures, or even mitigating its severity after the onset of epilepsy (i.e. disease modifying).

AUTHOR CONTRIBUTIONS

BS conceptualized and drafted the manuscript. LD and TO'B provided critical revision and intellectual input, and LD generated the figure. BS and LD incorporated reviewer feedback to revise the manuscript for resubmission. All authors approve the manuscript and are accountable for its content.

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frontiers in Pharmacology

BRIEF RESEARCH REPORT
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Early Chronic Carbamazepine-in-Food Administration to MAM/Pilocarpine Rats Does Not Affect Convulsive Motor Seizures

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Antiepileptic drug-resistance is a major health problem in patients with cortical dysplasia (CD). Whether drug-resistant epilepsy is associated with progressive brain damage is still debated. We previously generated a rat model of acquired CD, the methylazoxymethanol-pilocarpine (MP) rat, in which the occurrence of status epilepticus and subsequent spontaneous seizures induce progressive brain damage (Nobili et al., 2015). The present study tested the outcome of early-chronic carbamazepine (CBZ) administration on both seizure activity and brain damage in MP rats. We took advantage of the non-invasive CBZ-in-food administration protocol, established by Ali (2012), which proved effective in suppressing generalized convulsive seizures in kainic acid rat model of epilepsy. MP rats were treated immediately after the onset of the first spontaneous seizure with 300 mg/kg/day CBZ formulated in pellets for a two-months-trial. CBZ-treated rats were continuously video-monitored to detect seizure activity and were compared with untreated epileptic MP rats. Despite CBZ serum levels in treated rats were within the suggested therapeutic range for humans, CBZ affected spontaneous convulsive seizures in 2 out of 10 treated rats (responders), whereas the remaining animals (non-responders) did not show any difference when compared to untreated MP rats. Histological analysis revealed cortical thinning paralleled by robust staining of Fluoro-Jade$^+$ (FJ$^+$) degenerating neurons and diffuse tissue necrosis in CBZ-non-responder *vs* CBZ-responder rats. Data reported here suggest that MP rat model represents suitable experimental setting where to investigate mechanisms of CD-related drug-resistant epilepsy and to verify if modulation of seizures, with appropriate treatment, may reduce seizure-induced brain damage.

Keywords: malformation of cortical development, cortical dysplasia, double-hit model, seizures, drug-resistance, drug-in-food protocol, brain damage

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INTRODUCTION

Malformations of cortical development are the results of different pathologic events occurring during the process of cortical ontogenesis (Barkovich et al., 2012) and represent the neuropathologic substrate of a large proportion of drug-resistant epileptic patients. In particular, focal cortical dysplasia (FCD) is the most common brain malformation in patients undergoing epilepsy surgery for the relief of intractable seizures (Fauser et al., 2006; Lerner et al., 2009; Blümcke et al., 2011; Battaglia et al., 2013). Drug-resistance represents a still unsolved major clinical issue since the only determined intervention for pharmaco-resistant epileptic patients is the surgical resection of the epileptic focus, whenever possible. For this reason, the identification of drug-resistance mechanisms and the development of new therapeutic strategies overcoming resistance problem are crucial issues that need to be investigated in order to improve epileptic patients' outcome.

Prenatal administration of methylazoxymethanol (MAM) in rats at E15 is capable of inducing microcephaly, alteration of apical and basal dendritic morphology, loss of lamination and para-/intra-hippocampal heterotopia in all newborns, therefore MAM rats have been long used to model human brain malformations (Chevassus-au-Louis et al., 1998; Colacitti et al., 1999; Calcagnotto and Baraban, 2005; Battaglia et al., 2017). However, MAM rats suffer from the virtual lack of seizure occurrence and, despite studies exploring and demonstrating hyperexcitability, they rarely develop spontaneous seizures (Harrington et al., 2007; Battaglia et al., 2017). To study the mechanisms of epileptogenicity in the malformed brain, we previously developed a rat model of acquired cortical dysplasia based on prenatal MAM exposition and post-natal pilocarpine treatment (MAM-pilocarpine or MP rat), leading to status epilepticus (SE) and subsequent spontaneous recurrent seizures [SRS; (Colciaghi et al., 2011)]. MP rat model recapitulates both pathological conditions of human CD, i.e., abnormal cortical structure and SRS. We showed that SRS in this experimental model are associated with progressive cellular and molecular brain abnormalities (Colciaghi et al., 2011; Colciaghi et al., 2014). More recently, we showed that cell death after SE, in MP rat model, takes place during the entire epilepsy course, and we reported a temporal and regional specific pattern of brain damage, that is more evident in neocortex and thalamus in the early epilepsy stages (3 days after SRS onset), and becomes predominant in hippocampal CA layers in the later chronic stages (3–6 months of SRS), supporting the hypothesis that the widespread neurodegenerative process, may be related to seizure recurrence (Nobili et al., 2015). The question whether epilepsy is a static or progressive disease has been long-debated and still unsolved (Cole, 2000; Sutula et al., 2003; Finardi et al., 2013; Rossini et al., 2017). In fact, although recurring seizures can potentially contribute to neuronal reorganization and eventually cell death, data from both experimental and human studies are somewhat controversial. At the same time, evidence of progressive neurodegeneration in resistant epilepsy would support early surgical intervention (Haneef et al., 2010). To address this issue, appropriate anti-seizure treatment should be designed to independently evaluate long-term effects of chronic seizures on brain structure/function from those related to SE occurrence (Holmes et al., 1998). The comparison in a given experimental model between SE/no-SRS and SE/SRS animals should demonstrate the effect of seizures in the chronic epileptic phase on the brain.

Therefore, here we tested the outcome of early-chronic carbamazepine (CBZ) administration on both seizure activity and brain damage in MAM-pilocarpine rats, taking advantage of the non-invasive CBZ-in-food administration protocol [300 mg/kg body weight (bwt)/daily] which proved effective in completely suppressing generalized convulsive seizures in non-malformed kainic acid rat model of epilepsy (Ali et al., 2012).

MATERIALS AND METHODS

Ethical Statement

According to the ARRIVE guidelines, procedures were carried out to minimize discomfort and pain to treated rats, in compliance with National (D.L. 116 Suppl 40/1992 and D.L. 26/2014) and International guidelines and laws (2010/63/EU Legislation for the protection of animals used for scientific purposes). The experimental protocols were approved by the Ethics Committee of the Fondazione IRCCS Istituto Neurologico C. Besta and by the Italian Ministry of Health (protocol numbers: BR1/2012 and 961/2016-PR). Rats were housed in groups of two or three per cage under controlled conditions (22 ± 2°C and 12:12 light–dark cycle, lights on at 7 a.m.) until pilocarpine-SE induction. From this moment onwards, they were individually housed in cages and they were kept separated for all the duration of the trial. The animals were given free access to food and water.

MAM and Pilocarpine Administration

Pregnant Sprague-Dawley rats (n = 4; Charles River, Calco, Italy) received two intraperitoneal (ip) doses of MAM (15 mg/kg maternal body weight, in sterile saline, MRI Global, Kansas City, Missouri, USA) 12 h apart at embryonic day E15, as previously reported (Colacitti et al., 1999). Twenty-eight young adult litters (280–350 g, 2–3 months old) of MAM-treated dams were used for inducing SE with pilocarpine (270 mg/kg ip), as previously described (Colciaghi et al., 2011). Thirty minutes before pilocarpine, rats received N-methylscopolamine (1 mg/kg, ip) to minimize peripheral cholinergic activation (Clifford et al., 1987).

SE and Seizure Assessment

Pilocarpine treated rats were monitored by two researchers that rated pilocarpine-induced symptoms. Briefly, behavioral seizures were graded as non-convulsive seizures (NCS) and convulsive motor seizures [CMS; tonic-clonic, tonic, and clonic; (Williams et al., 2009)] according to a modified Racine's scale (Sharma et al., 2018; Colciaghi et al., 2019). SE onset was defined by the time of pilocarpine injection to the occurrence of the first CMS (stage 3–5) followed by continuous seizure activity: once rats reached stage 3–5, the behavioral activity fluctuated between

NCS (stage 1–2) to CMS (stage 3–5). Rats that experienced either one isolated stage 3–5 seizure or stage 1–2 NCS only and that did not develop continuous seizure activity were excluded from the study (n = 6 rats, 22% of pilocarpine-treated rats). Diazepam (10 mg/kg ip) was administered 90 min after SE onset to alleviate seizures and decrease mortality rate. Death occurring during SE (n = 6 rats, 27% of MP rats) could not be anticipated on the basis of external monitoring. MAM-pilocarpine treated rats were continuously video-recorded using an infrared camera system (24h/day for 2 months) to detect the onset of spontaneous seizures and to quantify SRS occurrence. SRS were graded as follows: stage 0 through 5, as outlined by Racine (1972); stage 6, cluster of multiple stage 5 seizures; stage 7, violent jumping and running; stage 8, stage 7 plus tonic hindlimb extension and tail rigidity (Pinel and Rovner, 1978a; Pinel and Rovner, 1978b). Two researchers blinded to rats' treatment observed the videos in a fast-forward mode. In case of any seizure-like activity, the videos were stopped, reversed and watched at normal speed. Since we could just rely on video-monitoring, we chose to restrict seizure quantification to generalized convulsive motor seizures (stages 4-7). Stage 8 seizures were never observed during video-monitoring of chronic epileptic rats. Total amount of generalized convulsive motor seizures were quantified for 50 days of video-recording/each rat. However, during video-monitoring analysis, any event suggestive of focal seizure (Racine stage 1-3, such as orofacial automatisms, head nodding, anterior limb clonus with lordotic posture) together with wild running/jumping behavioral not eventually evolving in CMS, were noted for each rat.

Carbamazepine-in-Food Treatment

Randomly chosen epileptic MP rats surviving SE were treated immediately after the onset of the first spontaneous seizure with a daily dose of 300 mg/kg (bwt) CBZ formulated in food pellets for two-months (n = 10, hereafter referred to as MP-CBZ rats), according with protocol previously established by Ali and colleagues (Ali et al., 2012). Since the amount of food-intake for the maintenance of body weight of an adult rat is about 60 g/kg bwt/day, CBZ was formulated in food pellets as 5 mg CBZ per 1 g pellet (Mucedola Srl, Milano, Italy). CBZ-containing food (60 g pellet/kg bwt) was supplied in a single feeding each day at 10 a.m. for a total of 60 days (60 administrations). CBZ-treated rats were weighed every 3–5 days to determine any possible effect of CBZ-containing pellet on body weights. Further, the residual pellet possibly left was weighed every day in order to calculate the exact amount of pellet and CBZ dose taken by the individual rat/each day. In order to estimate the bioavailability of the drug, the CBZ serum level was estimated in each individual rat the last day of treatment using the Carbamazepine assay on the ARCHITECT c Systems™ (iCARB-Enzyme immunoassay, Abbott Laboratories), according to product insert and routinely used to monitor CBZ serum level in patients.

Remaining MP rats (n = 6) were used as proper epileptic untreated rat group and received normal pellet (hereafter referred as MP-untreated rats). Additional n = 4 age-matched naïve MAM rats, neither experiencing SE nor SRS, were used as untreated non-epileptic controls (MAM-CTR). At the end of the two-months-trial, all rats were sacrificed for histological/morphometric analysis (see below).

Morphologic Analysis

Morphologic analysis was performed as previously described (Colciaghi et al., 2019). Briefly, animals were killed with overdose of chloral hydrate and then perfused with 4% paraformaldehyde in 0.1 M phosphate buffered saline at pH 7.2. Brains were removed from the skull, post-fixed overnight, and cut with vibratome (Leica Biosystem, Wetzlar, Germany) into 40 to 50 μm thick coronal sections that were collected in serial order. As morphologic/neuroprotective read-outs of chronic CBZ-therapy, we evaluated the cortical thickness in thionine stained sections (see data analysis section, below) and the Fluoro-Jade (FJ; Histo-Chem Inc., Jefferson, AR, USA) labeling in cortex and hippocampus (Nobili et al., 2015). One series of coronal sections (1 out of 7 sections) was counterstained with 0.1% thionine and at least 6 sections per rat (from -0.3 to -5.8 mm from bregma) were processed for FJ analysis as previously reported (Nobili et al., 2015).

Data Analysis and Statistical Evaluation

Latency to SE onset and SE behavioral evolution were analyzed in MP-untreated *vs* MP-CBZ rat group and statistically compared by means respectively of Mann-Whitney non parametric U-test and Student's t-test. For CMS (stage 4–7) quantification, since MP rats exhibited spontaneous seizure activity with a non-normal distribution (regardless of control or CBZ treatment), a Mann-Whitney U-test was used to compare the total number of seizures between the two experimental groups.

Cortical thickness was measured in 3 serial thionine-stained coronal sections from i) the rostral cortex (anterior commissure, at ~ −0.3/−0.8 mm from bregma), ii) the somatosensory frontoparietal cortex (−2.8/−3.8 mm from bregma), iii) the temporal or posterior cortex (−4.8/−5.8 mm from bregma) (Paxinos and Watson, 1982). Selected sections were digitized by means of Aperio CS2 slide scanner (Leica Biosystems Nussloch GmbH) at 20x magnification and cortical thickness was measured in each section at 0° (1 mm lateral to the midline), 45° and 90° from the midline, as previously described (Colciaghi et al., 2011; Colciaghi et al., 2014) and indicated in **Figure 2A**. The three measures per section were averaged to a single value and the obtained measures from the 3 serial sections from each area were averaged again to a single value to obtain the mean cortical thickness of rostral, sensorimotor and posterior cortex for each rat. N = 4 MAM-CTR, n = 8 MP-CBZ and n = 6 MP-untreated rats were analyzed. Differences among groups were statistically analyzed for each neocortical area by means of one-way analysis of variance (ANOVA) followed by Tukey HSD as post-hoc comparison test.

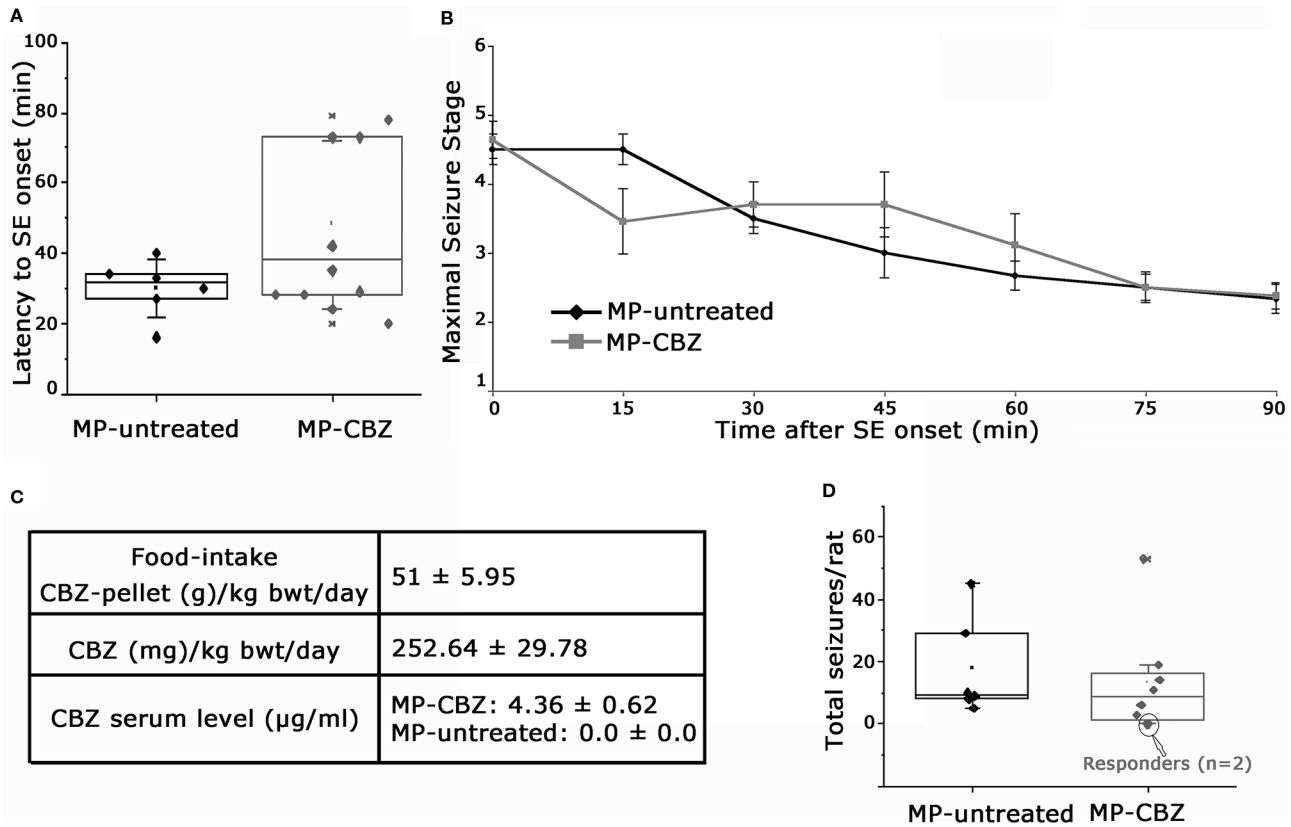
All measurements were performed independently by two operators blind the to the animal treatment; data were expressed as mean ± SD (or SEM when indicated) and differences were considered significant with $p < 0.05$. The sample size of rats necessary to detect a difference of 15% with a power of 80% and alpha 0.05 (Machin et al., 1997) between MP

untreated and MP-CBZ groups was estimated using variance values obtained in previous similar cortical thickness determinations (Colciaghi et al., 2014; Nobili et al., 2015) and data of video-monitoring seizure analysis in chronic epileptic pilocarpine/kainic rats treated with CBZ reported by other groups (Chakir et al., 2006; Polli et al., 2014).

RESULTS

Seizure Assessment and Selection of CBZ-Responders and Non-Responders

MP rats were randomly assigned to untreated- or CBZ-experimental group: a retrospective analysis of SE evolution of single rats was conducted to verify possible difference of SE severity between the two groups (**Figures 1A, B**). SE onset time ranged between 16–70 min after pilocarpine injection. We did not observed differences in regards to latency to SE onset between MP-untreated and MP-CBZ rat groups (respectively 30 ± 8.25 min *vs* 46 ± 23.77 min, p = 0.242 **Figure 1A**). All rats were scored every 15 min after the onset of the first CMS (stage 3–5, *Time 0* in **Figure 1B**): no differences in seizure development and intensity according to the behavioral scoring of CMS were observed between the CBZ-treated and untreated-rats (**Figure 1B**). The estimated SRS onset was not significantly different between the two groups: 6.43 ± 1.50 and 11.15 ± 4.30 (day after SE ± SD), respectively for MP-CBZ and MP-untreated rats. The day after SRS onset, rats assigned to MP-CBZ experimental group (n = 10) entered the two-months CBZ trial. Two out of 10 MP-CBZ treated rats died due to stage 7 seizures, respectively



Food-intake CBZ-pellet (g)/kg bwt/day	51 ± 5.95
CBZ (mg)/kg bwt/day	252.64 ± 29.78
CBZ serum level (μg/ml)	MP-CBZ: 4.36 ± 0.62 MP-untreated: 0.0 ± 0.0

FIGURE 1 | Behavioral assessment of status epilepticus and SRS and data of CBZ-in-food trial. **(A)** Box plot representation of latency (min) to onset of pilocarpine-induced SE in MP-untreated and MP-CBZ treated rats. No significant difference was observed between MP-CBZ and MP-untreated rats as assessed by means of Mann-Whitney U-test (respectively 30 ± 8.25 min *vs* 46 ± 23.77 min, p = 0.242). **(B)** Behavioral assessment of convulsive-motor seizure (stage 3-5) throughout status epilepticus, rated every 15 min over a 90 min observation period. No differences were observed between MP-untreated and MP-CBZ rat group at each time point considered as assessed by student t-test (SE onset, 0 min: p = 0.71; 15 min: p = 0.125; 30 min: p = 0.45; 45 min: p = 0.276; 60 min: p = 0.435; 75 min: p = 1; 90 min: p = 0.884; n = 6 rats in MP-untreated group; n = 10 rats in MP-CBZ group). Data are expressed as mean ± SD. **(C)** CBZ administration started after the first spontaneous seizure and the drug was administered in food every 24 h (at 10 a.m.) for 2 months. The food intake corresponded to a normal caloric diet. Average CBZ dose per day was 252.64 ± 29.78 mg/kg/day (mean ± SD) and serum CBZ levels were within the range suggested for humans (4–12 μg/ml). **(D)** Box plot showing the total number of stage 4-7 CMS/per rat during the trial. No difference was observed between the two groups as assessed by means of Mann-Whitney U-test (mean SRS number ± SEM: 17.15 ± 6.48 and 13.23 ± 6.16, respectively for MP-untreated and MP-CBZ rat group, p = 0.560). Encircled dots in panel D represent 2 out of 8 MP-CBZ rats (MP-CBZ/responders) in which CBZ treatment totally affected convulsive stage 4-7 seizures. In A and D, the box ranges indicate the 25th and 75th percentile and whiskers represent SD values.

3 and 5 days after SRS onset. Data obtained in the CBZ trial (n = 8 rats) are reported in **Figure 1C** and can be summarized as follows: all rats ate the CBZ-containing food throughout the 24 h day irregularly but continuously, with feeding episodes concentrated in the first 2 h after providing the CBZ pellet (10–12 a.m.) and in the first 2–3 h of the dark period (7–10 p.m.). The total amount of food consumed per day corresponded to a normal caloric diet (food-intake, **Figure 1C**), and no difference was detected in the progressive increase of body weight among the treated rats during CBZ treatment (data not shown). Mean CBZ dose taken/day per rat was 252.64 ± 29.78 mg/kg/day (mean ± SD) while serum CBZ level in treated rats was 4.36 ± 0.60 μg/ml, within the suggested therapeutic range for humans [4–12 μg/ml; (Patsalos et al, 2018)].

The quantification of stage 4–7 convulsive motor seizures (Pinel and Rovner, 1978a; Pinel and Rovner, 1978b) during 2 months of video recording was not significantly different between MP-untreated and MP-CBZ rats (**Figure 1D**). SRSs occurred within clusters followed by seizure-free period for both groups; no differences were found between treated *vs* untreated rats [mean SRS cluster interval (days) ± SD: MP-untreated: 1.84 ± 1.13; MP-CBZ: 2.35 ± 1.80; n.s. p = 0.56; mean inter-cluster interval (days) ± SD: MP-untreated: 15.30 ± 5.8; MP-CBZ: 10.11 ± 5.96; n.s. p = 0.429]. However, CBZ treatment affected stage 4–7 CMS in 2 out of 8 MP-CBZ rats (encircled dots in panel D), hereafter referred to as MP-CBZ/responders. SRS onset time of the two MP-CBZ/responders was respectively 5 and 6 days post-SE.

It's noteworthy that all rats, regardless of the treatment, daily exhibited events suggestive of focal seizures, such as orofacial automatisms, head nodding and anterior limb clonus with lordotic posture, together with periods of spontaneous and intense wild running or jumping-like behavior without generalized convulsion mainly concentrated in the dark period. In the absence of electroencephalography (EEG) monitoring these events were not included in seizures' quantification.

Morphological Assessment of SRS and CBZ Treatment Effect on the Epileptic Brain

We previously showed that the occurrence of SE plus SRS affects the cytoarchitecture of the chronic epileptic MAM/pilocarpine rat brain, significantly reducing both cortical thickness and hippocampal volume after 3 months of SRS (Colciaghi et al., 2011; Colciaghi et al., 2014). We therefore evaluated the effect of recurrent seizures on brain morphology by comparing the cortical thickness of non-epileptic naïve MAM (MAM-CTR), MP-untreated and MP-CBZ rats (**Figure 2**). Representative serial coronal thionine stained sections from each experimental group are reported in **Figures 2A-L**. The mean cortical thickness was significantly reduced in both MP-untreated and MP-CBZ rats when compared to non-epileptic MAM-CTR rats, mainly affecting the posterior temporal and entorhinal cortex (**Figure 2M**). In contrast, no significant difference was obtained between MP-CBZ and MP-untreated rats.

To evaluate the localization and extent of degenerating neurons, FJ labeling was performed in MP-CBZ/responder and non-responder rats (**Figure 3**). We did not detect any FJ^+ neuron in CBZ-responders (**Figures 3B, D, F**), while CBZ/non-responder rats (**Figures 3A, C, E**) exhibited a FJ^+ pattern very similar to that previously demonstrated in chronic epileptic MP rats (Nobili et al., 2015). In particular, evident labeling was obtained in deep cortical layer (arrowheads, **Figure 3A**) paralleled by intense staining of hippocampal CA pyramidal neurons (arrows **Figures 3A, E**) and by the presence of diffuse necrosis in entorhinal cortex (yellow arrowheads, **Figure 3C**).

DISCUSSION

The present study extends the characterization of MAM/pilocarpine rat model of epileptogenic cortical dysplasia, previously established in our lab (Colciaghi et al., 2011; Colciaghi et al., 2014; Nobili et al., 2015), revealing no effect of early chronic administration of CBZ 300 mg/kg/day (2-months trial) on generalized convulsive motor seizures and on brain damage in most treated rats (8 out of 10) when compared to untreated epileptic MP rats.

Limitations of the Study

The findings of this study have to be seen in light of some limitations. The primary concerns the use of video-monitoring for spontaneous seizure assessment. In fact, since we could not perform video-EEG monitoring to indisputably identify focal seizures (Racine 1–3 seizure stage) that could be confused with non-epileptic movements related to normal rat activity, the anti-seizure efficacy of CBZ was restricted to the quantification of generalized CMS (stage 4–7). Similarly, activities suggestive of a seizure, such as wild-running behavior without falling, lordosis, tail erection were included in our quantification only when evolving into convulsive tonic-clonic seizures. The exclusion of <3 Racine seizures (and the consequent under-sampling of seizures) is likely the main reason for the relative low number of reported seizures in our limited setting (without video-EEG). The analysis of CMS by video-monitoring was the most reliable readout available to us. Thus, we believe that under-sampling in our experimental conditions was a necessity. However, taking into account that CBZ-in-food-protocol (300 mg/kg/day) was totally effective in suppressing any event suggestive of a seizure in epileptic kainic acid rat model (Ali et al., 2012), that CMS frequency obtained in untreated-MP rat model is similar to what reported by other groups in long-term (50-60 days) video-monitoring of chronic epileptic pilocarpine and kainic rats (Capella and Lemos, 2002; Chakir et al., 2006; Polli et al., 2014), and that early-chronic treatment with CBZ (120 mg/kg/day) in naïve pilocarpine rats was effective in suppressing Racine stage 4-5 CMS (Chakir et al., 2006), we believe that, although preliminary, the present data could offer an experimental paradigm to investigate mechanisms of resistance to specific AED.

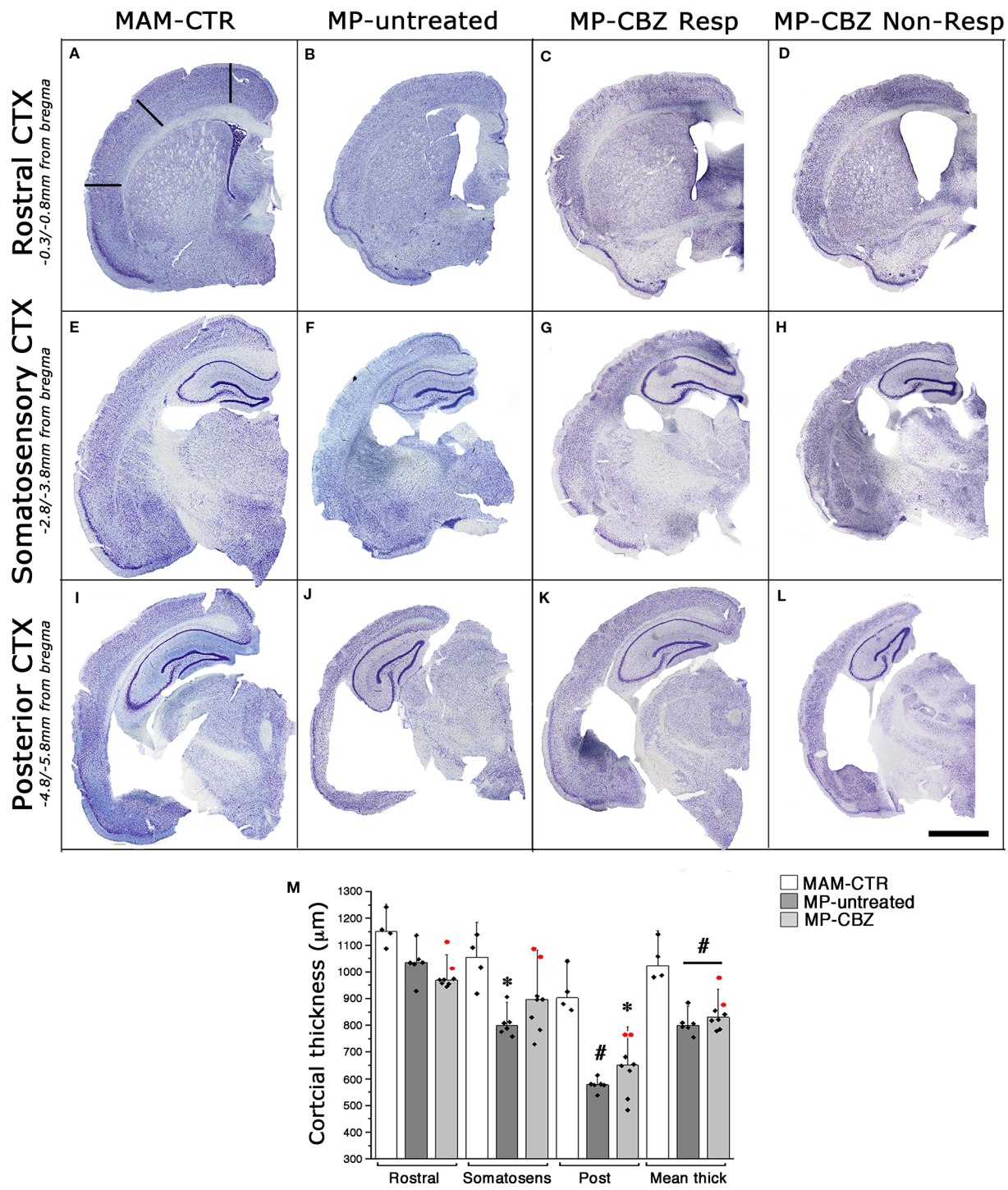


FIGURE 2 | Effects of seizures and CBZ treatment on neocortical atrophy. **(A–L)** Low-power thionine-stained coronal sections from rostral **(A–D)**, somatosensory **(E–H)** and posterior **(I–L)** cortical areas in representative non-epileptic naïve MAM (MAM-CTR; **A, E, I)**, epileptic MP-untreated **(B, F, J)**, MP-CBZ/responder **(C, G, K)** and MP-CBZ/non responder rats **(D, H, L)**. Scale-bar: 2mm **(M)** Bar-chart with dot-plots of single measurements of cortical thickness analyzed in different cortical region in MAM-CTR (n = 4), MP-untreated (n = 6) and MP-CBZ rat (n = 8) groups. Cortical thickness was significantly affected in the posterior cortex (Post) in both MP-untreated ($^{\#}p < 0.01$) and MP-CBZ (* $p < 0.05$) *vs* MAM-CTR rats. Mean cortical thickness (Mean thick) was significantly decreased in both MP-untreated and MP-CBZ rats when compared to MAM-CTR rats ($^{\#}p < 0.01$). A trend to decrease, although not significant, was observed in the rostral and somatosensory cortical areas in MP-CBZ *vs* MAM-CTR. Differences never emerged by comparing MP-CBZ with MP-untreated rats. Red-dots indicate the single cortical measurements of the 2 rats in which CBZ treatment totally affected convulsive stage 4–7 seizures. Data were expressed as mean ± SD.

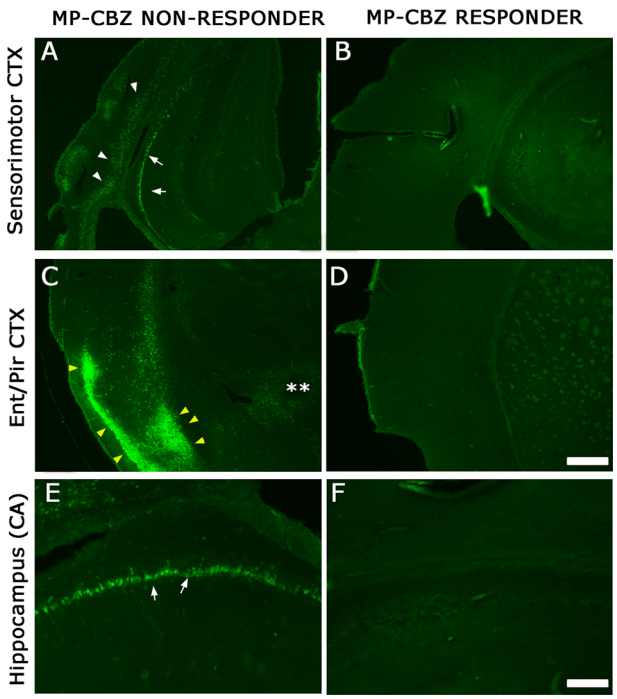


FIGURE 3 | FJ labeling in neocortical and hippocampal areas in MP-CBZ/non-responders and MP-CBZ/responders. FJ staining of representative MP-CBZ/non-responder **(A**, **C**, **E)** and MP-CBZ/responder rat **(B**, **D**, **F)** in the frontoparietal sensorimotor **(A, B)**, and entorhinal **(C, D)** cortical areas, and in the hippocampus **(E, F)**. Note the numerous FJ^+ neurons in all neocortical areas in MP-CBZ/non-responder when compared to MP-CBZ/responder rats. FJ^+ degenerating neurons were found in deep cortical layers of sensorimotor cortex (white arrowheads, **(A)** of MP-CBZ/non-responder rats, paralleled by diffused necrotic areas at the entorhinal/piriform cortical area (yellow arrowheads in **(C)**. At the hippocampal level many FJ positive neurons were detected in CA1/2 regions of MP-CBZ/non-responder rats (arrows in **A**, **E**). By contrary, FJ^+ degenerating neurons were never found in MP-CBZ/responder rats in any region considered (2 out of 8; **B**, **D**, **F**). Scale-bars: 300 μm in A-D; 100 μm in E-F.

MAM/Pilocarpine Rat, Model of Pharmaco-Resistant CD

The matter of the drug-resistant epilepsy associated with cortical dysplasia has been broadly addressed in the last years in experimental models, including MAM rats, by assessing the efficacy of conventional AEDs (Smyth et al., 2002; Jellett et al., 2015; Battaglia et al., 2017). In general, most of the studies showed a basic medically refractoriness of MAM rats to these conventional AEDs but virtually nothing is known about chronic AED treatment on seizure expression and seizure-related brain damage in epileptic malformed rats. *In-vitro* experiments showed that epileptiform activity induced by potassium channel blocker 4-Aminopyridine (4-AP) in hippocampal slices from MAM rats was relatively refractory to phenobarbital, CBZ, valproate (VPA), ethosuximide (ESM) and lamotrigine. Accordingly, *in vivo* experiments demonstrated that VPA was not effective in prolonging latency to SE in MAM animals after acute administration of kainic acid, in contrast to what observed in control animals (Smyth et al., 2002) or when seizures were induced by flurothyl inhalation (Jellett et al., 2015). In 2004, Serbanescu and colleagues, by combining prenatal MAM exposure with postnatal treatment with cholesterol biosynthesis inhibitor AY-9944, generated the two-hit MAM-AY rat model of chronic atypical absence seizures (Serbanescu et al., 2004). ECoG analysis of slow spike-and-wave discharge (SWD) duration did not show any difference between naïve-AY and MAM-AY rats but revealed medical refractoriness of MAM-AY rats to conventional anti-absence drugs (namely ESM and VPA). However, the analysis was restricted to a single drug administration and SWD quantification was limited to 1h recording period.

As CD patients are characterized by pharmaco-resistant epilepsy, the most valid experimental model, where screening long-term efficacy of AEDs, should rely on chronic and spontaneous seizure activity associated with brain malformation. In our opinion, this is a central issue, never addressed so far. In fact, as properly suggested by Potschka (2012), whether disease-associated alterations occur as consequences of repeated seizures, that—in turn—could contribute to therapeutic failure, should be verified in chronic model with SRS and not merely in acute seizure model, e.g., as latency to seizure onset.

To the best of our knowledge, this is the first study testing the outcome of early-chronic AED administration on both seizure activity and brain damage in CD model. As previously reported by Dudek's group, the once-per-day non invasive CBZ-in-food administration protocol used in the present study offered the evident advantage of minimizing the handling of epileptic rats (Grabenstatter et al., 2007; Ali et al., 2012), thus avoiding the stress and pain unavoidably associated with ip injections, which in turn could induce seizures (Jöels, 2009) thus resulting in a very practicable approach for preclinical drug screening. As pilot, the present study does not claim to get deep into the possible mechanisms of AED-resistance associated with cortical dysplasia, rather it was originally established to verify whether and to what extent SRS could be controlled in MAM/pilocarpine rats, and to dissect the eventual effect of SE from that of SRS on brain damage. Additional trials, on larger cohort of animals treated with different AEDs, will be necessary to address the last issue. In this regard, preliminary unpublished data obtained from few MP rats (n = 4) from our lab show that spontaneous seizure activity of MP-CBZ non-responder rats was not affected either when rats entered in a subsequent trial with clobazam (CLB)-in food (40 mg/kg/day, for 4 weeks). Taken together these data indicate that MAM/pilocarpine rat can be considered a model of medically-intractable seizure or, at least, very poor response to CBZ.

The dysfunction of blood brain barrier (BBB) and the transporter hypothesis have been proposed as possible contributing factors of such a drug resistance in MAM rats. In fact, an intrinsic BBB leakage is a typical feature of the heterotopic regions of MAM rat brain while no changes were observed in tissue with normal cytoarchitecture (Marchi et al., 2006). Further, the occurrence of pilocarpine-induced SE in

MAM rats worsens the BBB damage and induces the up-regulation of the multidrug resistance P-glycoprotein 1 (P-gp) in the perivascular astrocytes (Marchi et al., 2006), similarly to what described in human brain specimens of FCD patients (Sisodiya et al., 1999; Sisodiya et al., 2001; Sisodiya et al., 2002). Additional experiments will be necessary to verify whether dynamic changes in BBB function may determine the pharmacological responses of the epileptic malformed brain to AEDs and whether the BBB is preserved in AED-responder rats.

Brain Damage in Epilepsy Models: Related to SE Only?

The question whether repeated seizures might be associated with progressive alterations of the brain has been long debated and as yet unresolved. In the current opinion, that a prolonged seizure damages the brain is widely accepted (Sloviter et al., 2006; Sloviter et al., 2007; Noè et al., 2019; Zhang et al., 2012), whereas the hypothesis that seizure recurrence is associated with cumulative brain damage is much more debated, even if supported by recent MRI studies and meta-analysis of MRI morphometry studies in pharmaco-resistant temporal lobe epilepsy patients [TLE; (Bernhardt et al., 2009; Caciagli et al., 2017; Galovic et al., 2019)]. The analysis of convulsive epilepsy models in which seizures are prevented by appropriate treatments would provide a more reliable evaluation of the effect of SE on the brain cellular and molecular features.

MAM/pilocarpine rats represent a convulsive model of chronic epilepsy, since the development of SE is the necessary pre-requisite to determine the later onset of SRS. In this model, therefore, as in most post-SE epilepsy models currently in use, it is difficult to dissect the pathologic effect of SE on the cellular and molecular features of the malformed brain from that of subsequent SRS.

The comparison of brain damage, through already developed morphologic and functional read-outs (Colciaghi et al., 2011; Nobili et al., 2015) revealed that CBZ-responder rats, that experienced frank SE but not stage 4–7 CMS, were similar to non-epileptic MAM-CTR rats. Indeed, FJ^+ degenerating neurons were never detected in MP-CBZ/responder rats in any region considered. Conversely, the degree of brain damage in CBZ-non responders, that fully developed spontaneous convulsive seizures after SE, was similar to MP-untreated rats but significantly different from MAM-CTR rats. The small number of MP-CBZ responder rats (n = 2) did not allow a statistical comparison of seizure-induced brain damage *vs* other groups. We are aware that the lack of evident brain damage observed in the 2 CBZ-rats could be interpreted as a fortuitous event. However, we believe it's worth pointing out since it may also suggest that even infrequent spontaneous CMS, and not merely the occurrence of SE, could eventually contribute to brain damage.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/supplementary material.

ETHICS STATEMENT

Procedures were carried out to minimize discomfort and pain to treated rats in compliance with National (D.L. 116 Suppl 40/1992 and D.L. 26/2014) and International guidelines and laws (2010/63/EU Legislation for the protection of animals used for scientific purposes). The experimental protocols were approved by the Ethics Committee of the Fondazione IRCCS Istituto Neurologico Carlo Besta and by the Italian Ministry of Health (protocol numbers: BR1/2012 and 961/2016-PR).

AUTHOR CONTRIBUTIONS

GB and FC designed and supervised the study research. FC and MC wrote the paper. PN, CC and FC performed animal treatment. FC, PN, and AC analyzed video-recordings. PN and AC performed/analyzed morphological experiment. FC performed stat analysis. UG analyzed drug plasma level.

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The Glucagon-Like Peptide-1 Analogue Liraglutide Reduces Seizures Susceptibility, Cognition Dysfunction and Neuronal Apoptosis in a Mouse Model of Dravet Syndrome

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Dravet syndrome (DS) is a refractory epilepsy typically caused by heterozygous mutations of the *Scn1a* gene, which encodes the voltage-gated sodium channel Nav1.1. Glucagon-like peptide-1 (GLP-1) analogues, effective therapeutic agents for the treatment of diabetes, have recently become attractive treatment modalities for patients with nervous system disease; however, the impact of GLP-1 analogues on DS remains unknown. This study aimed to determine the neuroprotective role of liraglutide in mouse and cell models of *Scn1a* KO-induced epilepsy. Epileptic susceptibility, behavioral changes, and behavioral seizures were assessed using electroencephalography (EEG), IntelliCage (TSE Systems, Bad Homburg, Germany), and the open field task. Morphological changes in brain tissues were observed using hematoxylin and eosin (HE) and Nissl staining. Expression of apoptosis-related proteins and the mammalian target of rapamycin (mTOR) signaling pathway were determined using immunofluorescence and western blotting in *Scn1a* KO-induced epileptic mice *in vitro*. *Scn1a* KO model cell proliferation was evaluated using the Cell Counting Kit-8 assay, and the effect of liraglutide on cellular apoptosis levels was examined using Annexin V-FITC/PI flow cytometry. Apoptotic signal proteins and mTOR were assessed using reverse transcription - quantitative polymerase chain reaction (RT-qPCR) and western blotting. Our results showed that liraglutide significantly increased mRNA ((0.31 ± 0.04) $*10^{-3}$ vs. (1.07 ± 0.08) $* 10^{-3}$, $P = 0.0004$) and protein (0.10 ± 0.02 *vs.* 0.27 ± 0.02, $P = 0.0006$) expression of *Scn1a* in *Scn1a* KO-induced epileptic mice. In addition, liraglutide significantly alleviated electroencephalographic seizures, the severity of responses to epileptic seizures (96.53 ± 0.45 % vs. 85.98 ± 1.24 %, $P = 0.0003$), cognitive dysfunction, and epileptic-related necrotic neurons (9.76 ± 0.91 % vs. 19.65 ± 2.64 %, $P = 0.0005$) in *Scn1a* KO-induced epileptic mice.

Moreover, liraglutide protected against *Scn1a* KO-induced apoptosis, which was manifested in the phosphorylation of mTOR (KO+NS: 1.99 ± 0.31 vs. KO+Lira: 0.97 ± 0.18, $P = 0.0004$), as well as the downregulation of cleaved caspase-3 (KO+NS: 0.49 ± 0.04 vs. KO+Lira: 0.30 ± 0.01, $P = 0.0003$) and restoration of the imbalance between BAX (KO+NS: 0.90 ± 0.02 vs. KO +Lira: 0.75 ± 0.04, $P = 0.0005$) and BCL-2 (KO+NS: 0.46 ± 0.02 vs. KO+Lira: 0.61 ± 0.02, $P = 0.0006$). Collectively, these results show that liraglutide reduces seizure susceptibility and cognitive dysfunction in the mouse model of Dravet syndrome, and exerts anti-apoptotic and neuroprotective effects in *Scn1a* KO mice and cells.

Keywords: apoptosis, Dravet syndrome, epilepsy, GLP-1, mTOR, neuroprotection, SCN1A

INTRODUCTION

Dravet syndrome (DS), also known as severe myoclonic epilepsy in infants, is a rare childhood epileptic encephalopathy characterized by early onset seizures, multiple types of seizures, anxiety-like behavior, severe cognitive deficits, and resistance to antiepileptic drug treatment (Brunklaus et al., 2012; Dutton et al., 2017; Knupp and Wirrell, 2018). Approximately 70–80% of DS patients have been found to have mutations in the *SCN1A* gene encoding a neuronal voltage-gated sodium channel Nav1.1 subunit and more than 90% of these *SCN1A* mutations are *de novo* (Escayg and Goldin, 2010; Brackenbury and Isom, 2011; Verbeek et al., 2011).

Glucagon-like peptide-1 (GLP-1) is generally considered as a peripheral incretin hormone that is secreted from intestinal L-cells after food ingestion. GLP-1 binds to and activates its receptor (GLP-1R) in the pancreatic islets, stimulates insulin secretion, and inhibits glucagon release in a glucose-dependent manner and improves glycemic control (Katsurada and Yada, 2016). Therefore, GLP-1 analogues such as liraglutide are currently used as a second-line therapy to treat type 2 diabetes. GLP-1 and liraglutide can pass through the blood brain barrier (BBB) (Kastin et al., 2002; McGovern et al., 2012). In addition, GLP-1 can also be synthesized by preproglucagon neurons in the brainstem including the caudal nucleus tractus solitarii (NTS) (Baggio and Drucker, 2007; Christian, 2012). The GLP-1 receptor (GLP-1R) is widely expressed in neurons of several brain subregions such as hypothalamus, hippocampus, and cortex (Cork et al., 2015; Yoshino et al., 2015), suggesting the GLP-1 signaling could modulate a variety of neuronal functions (Korol et al., 2015; Prashant et al., 2018). Indeed, it has been reported that GLP-1 analogues exert neuroprotective effects in mouse models of acute and chronic epilepsy (Koshal et al., 2018; Wang et al., 2018; Hussein et al., 2019). Previous studies have shown that different apoptosis signaling pathways including BCL-2 associated X protein (BAX)/B cell lymphoma 2 (BCL-2) proteins are involved in seizure-induced neuronal death (Guo et al., 2017; Rubio et al., 2019), and liraglutide treatment could reduce the expression of an apoptotic marker caspase-3 in the brain of a Pentylenetetrazole (PTZ)-induced kindled rat model (Hussein et al., 2019).

In addition, the mammalian target of rapamycin (mTOR) signaling pathway has attracted interest due to its critical role in regulating neuronal function, proliferation, apoptosis, and other cellular processes associated with epileptogenesis (Maiese et al., 2013; Bockaert and Marin, 2015; Citraro et al., 2016). The expression of mTOR signaling-related proteins is abnormally increased in animal models of epilepsy (Citraro et al., 2016). GLP-1 analogues can regulate mTOR expression *via* the AMP-activated protein kinase (AMPK) pathway (He et al., 2016; Zhang et al., 2017). Although several studies have shown the anticonvulsant potential of GLP-1 analogues (Koshal and Kumar, 2016; Wang et al., 2018; de Souza et al., 2019), the effect of liraglutide on the epileptogenesis and cognitive dysfunction in the DS mouse model has not been examined. In the present study, we investigated the possible anti-epileptic effect of liraglutide in *Scn1a* KO-induced epileptic mice and its potential effect on both Bcl-2 regulate apoptotic pathway and the mTOR pathway that are involved in epileptogenesis.

MATERIALS AND METHODS

Animals

Mice with heterozygous loss-of-function mutations in *Scn1a* ($Scn1a^{+/-}$) recapitulate many features of DS and provide a useful disease model. Homozygous mice ($Scn1a^{-/-}$) display tremors, ataxia, seizures, and loss of righting reflex after postnatal day 9 and die by postnatal day 16. On the solely 129S6/SvEvTac strain, heterozygous mice have no overt phenotype. If mice of this 129S6/SvEvTac strain are crossed with C57BL/6, F1 heterozygotes exhibit spontaneous seizures and premature lethality (Miller et al., 2014; Kang et al., 2019). *Scn1a* KO mice created with 129S6/SvEvTac background were provided by Dr. Long-Jun Wu (Department of Cell Biology and Neuroscience, School of Arts and Sciences, Rutgers University, USA). The F1 heterozygotes (4 weeks, male) and their Wild Type (WT) littermates were used in all experiments. All of the animals were maintained in a 12-h light/dark cycle with a constant room temperature and housed in groups (five to six mice per cage) with

Abbreviations: SCN1A, NaV1.1 sodium channel; CNS, central nervous system; DS, Dravet syndrome; GLP-1, glucagon-like peptide-1; GLP-1R, glucagon-like peptide-1 receptor; HE, hematoxylin and eosin; IF, immunofluorescence; KO, knockout; Lira, liraglutide; mTOR, mammalian target of rapamycin; PCR, polymerase chain reaction; RT-qPCR, Reverse transcription-quantitative polymerase chain reaction; WB, western blotting; WT, wild type.

food and water *ad libitum*. The mice were handled according to the guidelines approved by the Institutional Animal Care and Use Committee of Ningxia Medical University (IACUC Animal Use Certificate No.: SCXK [Ning] 2015-0001). All efforts were made to minimize the number of animals used and their suffering.

Chemicals and Reagents

Liraglutide (HYP0014, purity 99.96%) was purchased from MedChemExpress (MCE, USA). Rabbit anti-SCN1A (Abcam, ab24820), p-mTOR (Abcam, ab84400), mTOR (Abcam, ab2732), Cleaved-Caspase3 (Abcam, ab2302), BAX (Abcam, ab53154), BCL-2 (Abcam, ab196495), and β-actin (Abcam, ab8227) antibodies were used as the primary antibodies based on the validation results by the manufacturers. Secondary antibodies including AlexaFluor 488-conjugated goat anti-rabbit IgG (Abcam, ab150077) were purchased from Abcam. Goat anti-rabbit IgG (LI-COR, USA) were purchased from LI-COR Bioscience (Lincoln, NE, USA).

Genotyping the *Scn1a* KO Mice

DNA was isolated from mice tail biopsies using the TIANamp Genomic DNA kit according to the manufacturer's instructions (Tiangen Biotech, Beijing, China). The DNA concentration was determined using a spectrophotometer (Nanodrop 2000; Thermo Fisher Scientific, Waltham, MA, USA). The presence of the *Slcn1a* exon 1 deletion was identified by the standard polymerase chain reaction (PCR) technique using the enzyme Hot Start High-Fidelity DNA Polymerase (New England Biolabs, Ipswich, MA, USA) and specific primers. The sequences of the primers used for mouse genotyping were as follows: common primer 5′-AGTCTGTACCAGGCAGAACTTG-3′, wild type reverse primer 5′-CCCTGAGATGTGGGTGAATAG-3′, mutant reverse prime 5′-AGACTGCCTTGGGAAAAGCG-3′. The PCR reaction was performed in a mixture containing 2.5 μl common primer and 1.25 μl wild type reverse primer and 1.25 μl mutant reverse primer, 3 μl nuclease-free water, 12 μl DNA polymerase, and 5 μl DNA. After the initial denaturation for 30 s at 98°C, 35 PCR cycles were performed (98°C for 10 s, 56°C for 30 s, and 72°C for 2 min), followed by a final extension at 66.5°C for 2 min. The PCR products were observed after the electrophoresis on a 2% agarose gel stained with GelRed (Biotium, Fremont, CA, USA) and loaded with a 700bp DNA ladder (Biomed, Beijing, China). The genotype results were confirmed by sequencing of the PCR products (Sangon Biotech Co., Ltd., Shanghai, China).

Animal Experimental Protocol

A total of 120 WT mice and F1 heterozygous *Scn1a* KO mice were randomly divided into five groups: (I) Liraglutide treated WT group (WT + Lira, N = 24), which received liraglutide treatment (150 μg/kg, i.p) once daily for 14 days (During et al., 2003; Yoshino et al., 2015). (II) Normal saline treated WT group (WT + NS, N = 24), which received the same volume of 0.9% normal saline once daily for 14 days. (III) Normal saline treated F1 heterozygous *Scn1a* KO mice group (KO + NS, N = 24). (IV) Liraglutide treated F1 heterozygous *Scn1a* KO mice group (KO + Lira, N = 24). (V) Valproic acid treated F1 heterozygous *Scn1a* KO mice group (KO + VPA, N = 24), which received the same volume of valproic acid once daily for 14 days. The time point at which the mice were sacrificed to take out brain tissues was consistent with the liraglutide administration time (**Supplementary Figure 1**).

Blood Glucose Measurements

We collected blood samples from the tail vein and measured the fasting blood glucose. Blood glucose levels were measured by the Sannuo blood glucose meter (Sinocare Inc. China) before treatment and before sacrifice at different time points (12 h, 1 d, 3 d, 7 d, 14 d after the liraglutide administration). The measurements were repeated three times to reduce errors and the average value was calculated as the blood glucose level.

Electroencephalography (EEG) Measurement

Mice were anesthetized with 1% pentobarbital (40 mg/kg, i.p) and maintained at normal body temperature on a feedback-controlled heating blanket (TR-200, Safebio, Shanghai, China). The mice were then transferred to a stereotactic frame and a midline scalp incision was made to expose the skull. EEG electrodes were skull-mounted on the left amygdala (coordinates from the bregma: AP = −1.2 mm and L = −9 mm) and the frontal cortex (coordinate from the bregma: AP = 2 mm and L = −2 mm). Behavioral seizures and EEG were observed and recorded by a biomedical signal acquisition and processing system (BL-420 N, Techman Software, Chengdu, China). A novel definition of High-Voltage Sharp Waves (HVSWs) was used: sharp waves with a high amplitude at least three times the EEG baseline, a duration of at least 5 s, and a frequency of at least 2 Hz (Twele et al., 2016).

Evaluation of Behavioral Seizures

The severity of seizures was assessed and classified according to the Racine stage (Racine, 1972): stage 0: normal behavior; stage 1: mouth and facial movement; stage 2: head nodding; stage 3: forelimb clonus; stage 4: rearing with forelimb clonus; and stage 5: rearing and falling with forelimb clonus. In addition, stage 5 seizures had a definite termination before onset, and status epilepticus was defined as the duration of stage 5 seizures unless intentionally stopped. First abnormal behavior was referred to facial automatism and excessive salivation. The severity of seizures can be evaluated using the sum score of each mouse and the duration of each experiment according to the following formula: Seizure severity = Σ (total scores of one specified mouse)/time of experiment.

IntelliCage

IntelliCage system program settings and experimental protocol: (1) Adaptation and Free exploratory stage: All mice could move freely in the cage, the valves in the four corners were open, and the mice were free to choose the corner to drink water. The number of visiting and nosepoke in each corner of each mouse was recorded to assess the animal's cognitive ability in the new environment, which was set to 5 days. (2) Nosepoke learning stage: At this stage, the program was set to the four corners where the valves were closed and the mouse must learn to open the

valve to drink water. After the nosepoke learning, the valve will be closed after the end of the visiting. This stage was set to 5 days. (3) Position learning stage: The corner with the least number in the nosepoke learning stage was defined as the "correct" corner, and the remaining three corners were defined as the "wrong" corners. All mice can only open and drink water when they nosepoke the corner that was defined as "correct". The error visit rate of each mouse was recorded to evaluate the position learning ability, which was set to 7 days. (4) Reversal position learning stage: The corner corresponding to the diagonal of the "correct" corner in the position learning stage was defined as the "correct" corner, and the remaining corners were defined as the "wrong" corners. The error visit rate of each mouse was also recorded to evaluate the reversal position learning ability, which was set to 7 days.

The Open Field Task

Mice were placed in a closed planar area (length: 50 cm; width: 50 cm; depth: 40 cm) for 10 min and mice activity was recorded using Smart 3.0 video tracking software (Panlab, Spain). A 50 cm (length) × 50 cm (width) planar area was digitally divided into 20 quadrants of the same size (6 central quadrants and 14 peripheral quadrants). The 6 central quadrants are collectively referred to as the central region, and the 16 peripheral quadrants are collectively referred to as the peripheral regions. The system automatically recorded the travel distance (cm) and the time (s) that mice spent in the central area and the surrounding area, and analyzed the data.

Immunofluorescence Staining

Mice were anesthetized in 1% pentobarbital (40 mg/kg, i.p) and perfused with saline through the heart, then followed by 4% paraformaldehyde. After the decapitation, the mouse brain was quickly removed, and post fixed in 4% paraformaldehyde for 15 h at room temperature (RT). The brains were soaked in the 30% sucrose solution for cryopreservation for 24 h. Brain tissues were cut using a cryostat (Leica, Wetzlar, Germany) and 30-μm-thick sections were collected. Non-specific binding was blocked for 1 h at RT using 3% normal goat serum and 0.1% Triton-X-100 in phosphate-buffered saline (PBS). Rabbit anti-SCN1A (1:500), p-mTOR (1:500), mTOR (1:500), Cleaved-Caspase3 (1:500), BAX (1:500), and BCL-2 (1:500) antibodies were used as the primary antibodies based on the validation results from the manufacturers. After washing in PBS 3 times, the sections were incubated with the secondary antibody AlexaFluor 488-conjugated goat anti-rabbit IgG (1:500) for 1 h at RT. The sections were then washed, counterstained with 4,6-diamidino-2-phenylindole (DAPI) (ZLI-9557, ZSGB-BIO, Beijing, China), and covered with a coverslip. The images were captured with a Leica DM6 fluorescence microscope (Leica, Germany). Image-pro plus 6.0 software (Media Cybernetics, Bethesda, MD, USA) was used for quantification of the average optical density (AOD) of images.

Hematoxylin and Eosin Staining

Hematoxylin & Eosin (HE) staining was conducted according to a conventional protocol. Briefly, 30-μm-thick sections were stained with hematoxylin solution for 5 min and then immersed five times in 1% acidic ethanol (1% HCl in 70% ethanol) followed by rinsing in distilled water. The sections were further stained with eosin solution for 3 min, then dehydrated with a gradient alcohol and cleared in xylene. The mounted slides were examined and photographed using a Leica DM6 fluorescence microscope (Leica, Germany).

Nissl Staining

The rehydrated mouse cortex sections were stained in a cresyl violet solution at 56°C for 1 h and then washed with deionized water. The sections were further maintained in the Nissl differentiation solution for at least 2 min until a colorless background was observed under the microscope, dehydrated (each in 50, 60, 70, 80, 95, and 100% ethanol for 3 min), washed in xylene, and fixed with neutral balsam. The sections were photographed using a Leica DM6 fluorescence microscope (Leica, Germany) and cells in the region of interest from the Nissl stained image were counted with Image-pro plus 6.0 software (Media Cybernetics, Bethesda, MD, USA).

Scn1a-Knockout Cell Line

The mouse hippocampal neuronal cell line (HT22) with the knockout of *Scn1a* was constructed and stored in the liquid nitrogen at the Ningxia Key Laboratory of Cerebrocranial Diseases (Shi et al., 2019). The revived cells were passaged with a 0.25% trypsin-EDTA solution (Solarbio, China). The cells were maintained in dulbecco's modified eagle medium (DMEM) (Bioind, USA) containing 10% fetal bovine serum (FBS) (Bioind, USA) and 1% penicillin-streptomycin (Solarbio, China) in an incubator with 5% CO_2 at 37°C.

The cells were divided into four experimental groups: (I) HT22 cell, medium-treated group (HT22 Control), (II) HT22 cell, liraglutide-treated group (HT22 + Lira), (III) *Scn1a* KO HT22 cell, medium-treated group (KO Control), (IV) *Scn1a* KO HT22 cell, liraglutide-treated group (KO + Lira). Subsequently, the cells were seeded into a 96-well plate at a density of 1×10^5 cells/well, and then liraglutide at a concentration of 8, 10, or 12 nM was added to the cells. The cells were further cultured in the incubator, and then collected at 24, 48 and 72 h. An inverted phase contrast microscope (Leica, Germany) and cell counter (Bio-RAD, USA) were used to observe and measure the cell numbers under different conditions.

Cell Proliferation Assay

The Cell Counting Kit-8 assay (Dojindo, Japan) was used to evaluate the cell proliferation. Briefly, at 24, 48, and 72 h, the *Scn1a* knockout HT22 cells were treated with different concentrations of liraglutide and cultured in the 96-well plate at a density of 1×10^4 cells/well. The blank wells with the culture medium were used for the background detection, and three duplicate wells were set for each group. After each time point of harvest and washing once with PBS, Cell Counting Kit-8 solution (10%) was added to individual wells, and the plate was incubated at 36°C for 1 h. Absorbance at 450 nm was measured using a microplate reader (Bio-τeck, USA). The measurement was repeated and the optical density (OD) values were averaged.

The cell proliferation rate was calculated using the formula: Cell proliferation rate (%) = ((treated cell OD)) − (blank OD))/((control group OD) − (blank OD)) × 100.

Flow Cytometry

Flow cytometry was used to assess the effect of liraglutide on the apoptosis of *Scn1a* knockout HT22 cells. Annexin V-FITC/Propidium Iodide (PI) double staining cell apoptosis detection kit was purchased from BestBio (BestBio, Shanghai, China). The cells were seeded in the 96-well plate at a density of 1×10^5 cells/well. Each group consisted of three duplicate wells and cultured for 48 h. The cells were detached by EDTA- trypsin and centrifuged at 2,000 rpm for 5 min, and washed twice with PBS and 500 μl of binding buffer. AnnexinV-FITC and PI were added to the cells and further incubated at RT for 15 min in the dark. The apoptosis rate of each group was analyzed by Cytoflex flow cytometer (BECKMAN COULTER) when the number of cells was up to $1 \times 10^{4/}$tube.

Reverse Transcription-Quantitative Polymerase Chain Reaction (RT-qPCR)

The total RNA was extracted from cells or brain tissues using the RNA extraction kit manufactured by Omega according to the manufacturer's protocol. First-strand cDNA was generated using reverse transcription kit (RR036A) manufactured by TAKARA. First-strand cDNA samples were used as the PCR templates with gene-specific forward and reverse primers (**Supplementary Table 1**, primers synthesized by Shanghai Shenggong Bioengineering Co., Ltd). The cDNA was amplified using qPCR kit (RR820A, TAKARA). The PCR parameters were set as follows: denaturation at 95°C for 2 min, followed by 40 cycles of denaturation at 95°C for 10 s, annealing at 58°C for 30 s, and extension at 72°C for 30 s. After the PCR amplification, data analysis was performed using Bio-Rad IQ5 software and *Gapdh* was used as a reference gene. The relative quantification ($2^{-\triangle Ct}$ or $2^{-\triangle\triangle Ct}$) method was used to present the data. All experiments were performed in triplicates.

Western Blotting Assay

Total proteins from the brains and *Scn1a* KO HT22 cells were prepared and extracted using the BCA Protein Extraction Kit (KGP2100, KeyGEN, Nanjing, China). Protein concentration was measured using the BCA Protein Assay Kit (KGP902, KeyGEN, Nanjing, China). Equal amounts of protein (50 μg per lane) were resolved on 8 or 10% sodium dodecyl sulfate (SDS) - polyacrylamide gel (SDS-PAGE), and then transferred onto 0.22 μm polyvinylidene fluoride (PVDF) membrane (Millipore, USA). When the protein transfer was completed, membranes were blocked with 5% non-fat milk for 1 h, followed by incubation overnight at 4°C with rabbit anti-SCN1A (1:500), p-mTOR (1:500), mTOR (1:500), Cleaved-Caspase3 (1:500), BAX (1:500), BCL-2 (1:500), or β-actin (1:1,000) primary antibody based on the validation results from the manufacturers. After incubation with primary antibody, membranes were washed with TBST three times for 5 min each time. β-actin served as internal references. Membranes were further incubated with the corresponding secondary antibody goat anti-rabbit IgG (1:1,000; LI-COR, USA) for 1.5 h. Quantification of bands was performed from optical density values using the Odyssey CLX instrument system (LI-COR, USA). All experiments were performed in triplicates.

Statistical Analysis

Statistical analysis was performed using PRISM 6 (GraphPad Software, California, USA). The data were presented as mean ± standard deviation (SD). Two-group comparisons were evaluated with two-tailed Student's *t* test. To compare three or more groups, a one-way or two-way ANOVA followed by a Dunnett, Newman-Keuls, or Bonferroni *post hoc* test was used. $P < 0.05$ was considered statistically significant. At least three independent experiments were performed for each condition.

RESULTS

Genotype Verification of *Scn1a* KO Mice and the Increase of Scn1a Expression in the Brain of *Scn1a* KO Mice After Liraglutide Treatment

Heterozygous mice ($Scn1a^{+/-}$, 129S6/SvEvTac strain) were crossed with WT mice (C57BL/6 strain), and F1 generation mice were genotyped. Based on the exon 1 sequence of mouse *Scn1a* gene, a common primer, WT reverse primer, and mutant reverse primer were designed to amplify DNA fragments using PCR. Exon 1 of the mouse *Scn1a* gene was replaced by a neomycin resistance cassette; as such, the expected PCR products were 357 bp for WT mice, and 200 bp and 357 bp for heterozygous mice (**Figure 1A**). According to the genotyping results, the successful generation of *Scn1a* KO animals (F1 generation) was achieved. The F1 heterozygotes (4 weeks, male) and their Wild Type (WT) littermates were used in all experiments. RT-qPCR, western blotting (WB), and immunofluorescence staining were used to further assess the effect of replacing exon 1 of the mouse *Scn1a* gene with a neomycin resistance cassette on Scn1a expression in the mouse brain. As shown in **Figure 1B**, WT and *Scn1a* KO littermates exhibited clearly different *Scn1a* mRNA expression in the brain. Statistical analysis revealed a significant decrease in the level of *Scn1a* mRNA expression in the *Scn1a* KO group compared with the WT group (WT: $(8.55 \pm 0.35) *10^{-3}$ vs. $(0.81 \pm 0.27) * 10^{-3}$, $P = 0.0001$, **Figure 1B**). In addition, SCN1A protein expression levels in age-matched littermates were detected using WB with whole-cell protein extracts from the brain. As expected, *Scn1a* KO mice demonstrated no evidence of SCN1A protein production, as the band corresponding to SCN1A was almost totally absent (WT: 1.02 ± 0.02, KO: 0.10 ± 0.01, $P < 0.001$, **Figures 1C, D**).

The specific distribution of SCN1A in the mouse brain was also examined by immunofluorescence staining. Strong SCN1A-like immunoreactivity was observed in neurons, mostly in the cortex and hippocampus (**Figure 1E**). Interestingly, compared with *Scn1a* KO mice that were given normal saline, the

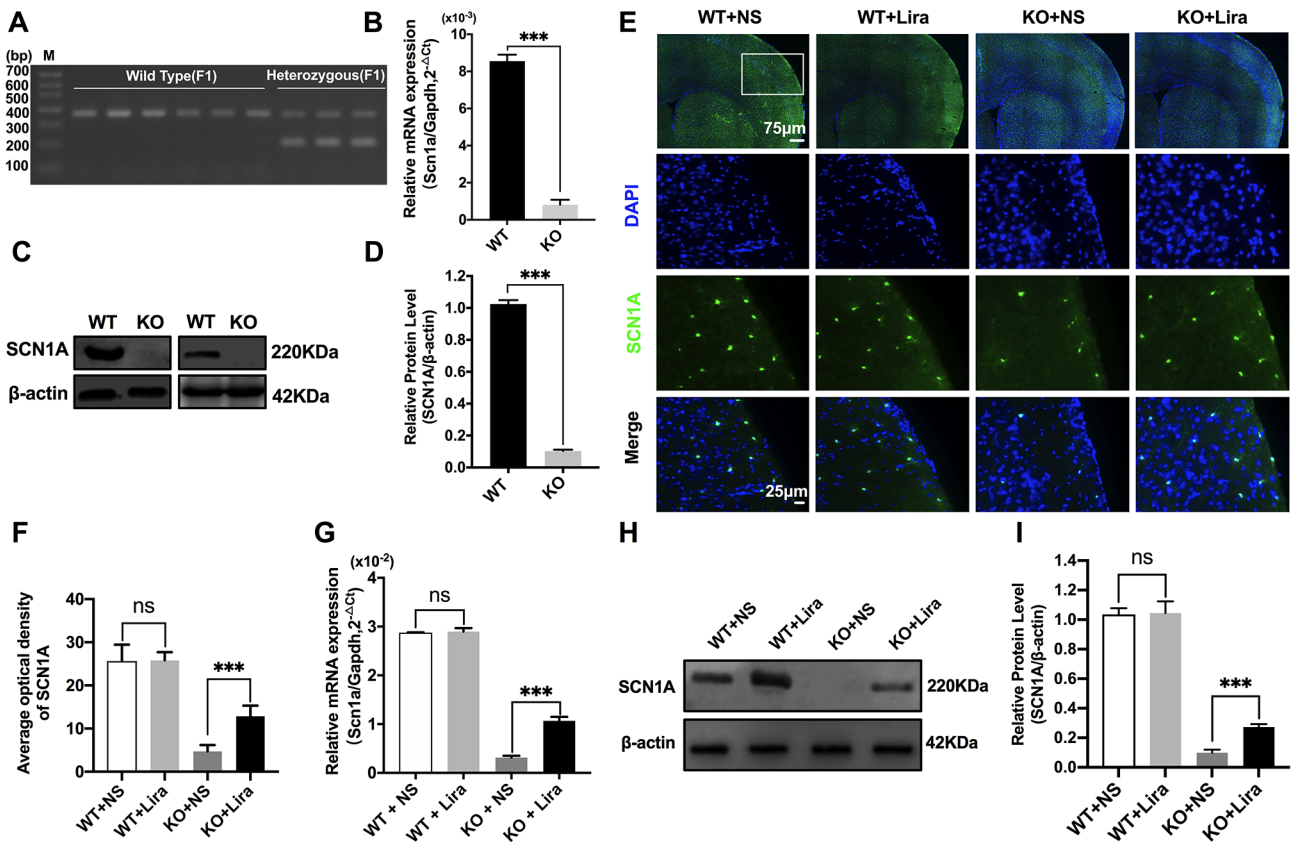

FIGURE 1 | Phenotype verification of *Scn1a* KO mice and the increase of SCN1A expression after liraglutide treatment in the brain of *Scn1a* KO mice. **(A)** PCR was used to identify wild-type and *Scn1a* KO heterozygous (F1) genotypes. (M: DNA marker). **(B)** RT-qPCR analysis of the *Scn1a* mRNA expression from brains of wild-type (WT) and *Scn1a* KO heterozygote (KO) mice (N = 24 mice per group; *Student's t-test*, $P < 0.001$). **(C)** Representative western blotting showing SCN1A protein expression in brains of WT and *Scn1a* KO mice. **(D)** Summary graph of western blotting analysis demonstrating a significant reduction in the SCN1A protein in brains of *Scn1a* KO as compared to WT mice (N = 24 mice per group; *Student's t-test*, $P < 0.001$). **(E)** Representative images of immunofluorescent staining with SCN1A (green), and DAPI (blue) in the ipsilateral side of the cortex of the WT and *Scn1a* KO mice. Scale bar: 75 μm for the full-scale images and 25 μm for the magnified images. **(F)** Summary graph of the average optical density of SCN1A fluorescent staining measured in images **(E)** demonstrating a profound increase of SCN1A in the cortex of *Scn1a* KO mice after liraglutide administration (N = 24 mice per group; *Student's t-test*, $P < 0.001$). **(G)** RT-qPCR analysis of the SCN1A mRNA expression after liraglutide administration in brains of WT and *Scn1a* KO mice (N = 24 mice per group; *One-way ANOVA, Student's t-test*). **(H)** Representative western blotting showing SCN1A protein expression in brains of the WT and *Scn1a* KO mice. **(I)** Summary graph of western blotting analysis demonstrating a dramatic increase in the SCN1A protein from brains of *Scn1a* KO after liraglutide administration (N = 24 mice per group; *One-way ANOVA, Student's t-test*). The relative expression of SCN1A was normalized to reference controls *Gapdh* and β-actin in RT-qPCR and Western blotting, respectively. Data were presented as mean ± SD; ***, $P < 0.001$; ns, not significant. All experiments were performed in triplicate. WT, Wild-type; KO, *Scn1a* Knockout heterozygotes (F1); NS, Normal saline; Lira, Liraglutide.

expression of SCN1A was increased in the same brain region in *Scn1a* KO mice after Lira administration (KO+NS: 4.73 ± 1.46, KO+Lira: 12.85 ± 2.48, $P < 0.001$, **Figures 1E, F**). RT-qPCR and WB were used to further verify this result. Both Scn1a mRNA and protein levels in the brain of *Scn1a* KO mice were significantly increased after Lira administration (mRNA, KO +NS: (0.31 ± 0.04) $*10^{-2}$, KO+Lira : (1.07 ± 0.08) $* 10^{-2}$, **Figure 1G**; protein, KO+NS: 0.10 ± 0.02, KO+Lira: 0.27 ± 0.02, $P < 0.001$, **Figures 1H, I**). In contrast, lira administration did not change the mRNA and protein level in the brain of WT mice (**Figures 1E–I**). Lira had no effects on blood glucose levels in either WT or *Scn1a* KO mice that were tested before euthanization at different time points (**Supplementary Figure 2**).

Liraglutide Reduces Seizure Susceptibility and Cognitive Dysfunction in *Scn1a* KO-Induced Epileptic Mice

Behavioral Seizures and EEG Recording

The functional significance of Lira in the susceptibility to *Scn1a* KO induced-epileptic seizures was investigated. Seizure behavior was evaluated and compared between WT and *Scn1a* KO mice after Lira administration. Seizure stage scores were used for latency and duration of seizures to assess epilepsy susceptibility. As shown in **Figure 2A**, *Scn1a* KO mice exhibited seizure behavior for more than 2 h and exhibited severe tonic-clonic seizures before status epileptic seizures to gradually reach stage 5 seizures. However, the duration of seizure behavior in *Scn1a* KO mice after Lira injection was prolonged

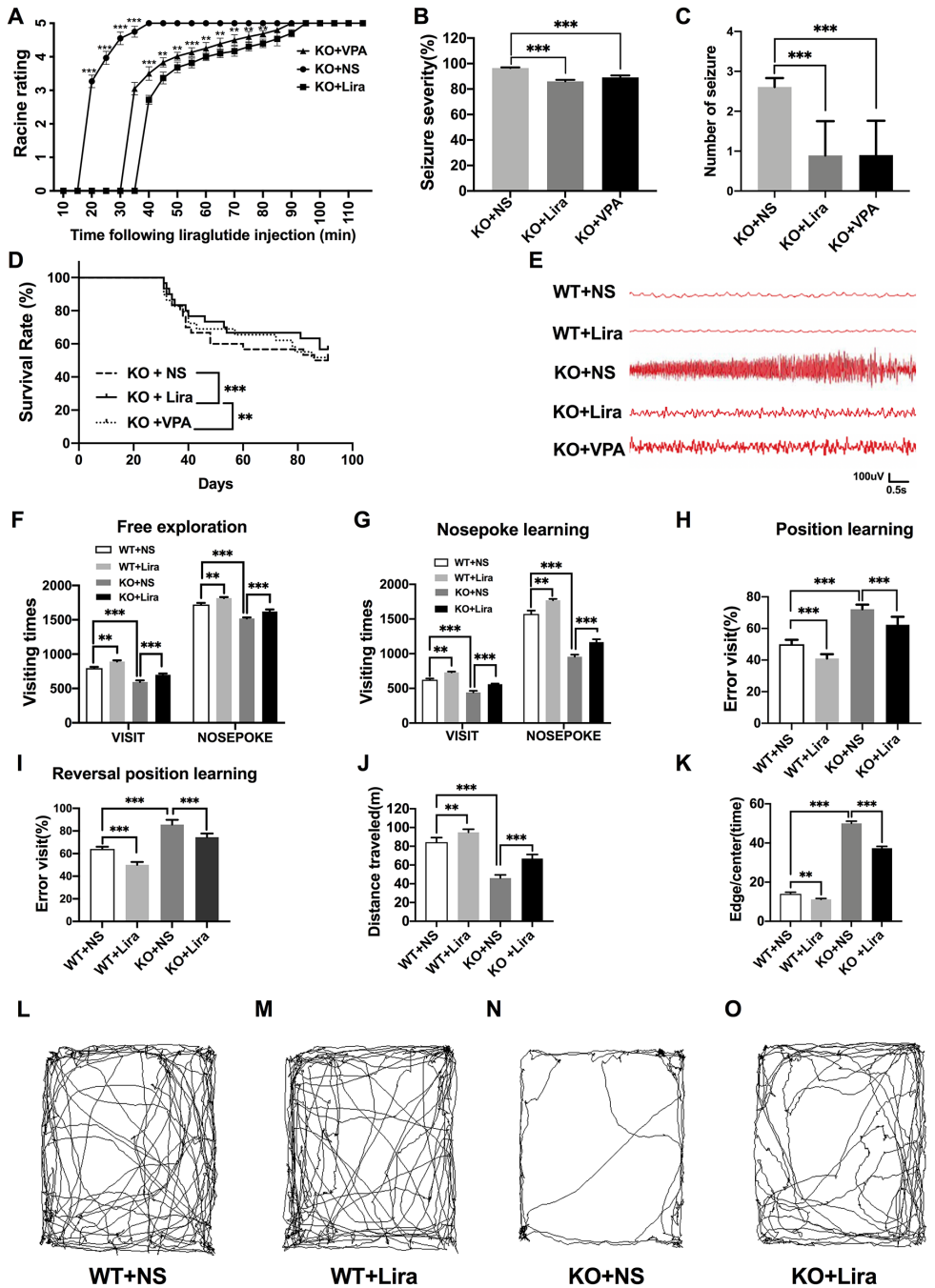

FIGURE 2 | Effects of liraglutide on behavioral performance in *Scn1a* KO-induced epileptic mice. **(A)** Behavioral seizure progression on the racine scale (N = 24 mice per group; *Two-way ANOVA*). **(B)** The total seizure severity evaluation (N = 24 mice per group; *Student's t-test*, $P < 0.001$). **(C)** The number of seizures daily in each group over the duration of the experiment (N = 24 mice per group; *Student's t-test*, $P < 0.001$). **(D)** Survival curves showing a statistically significant difference in survival in each group over the duration of the experiment (N = 24 mice per group; *One-way ANOVA, Student's t-test*). **(E)** A representative EEG recording of seizure activity in the WT and *Scn1a* KO mice. **(F)** Free exploratory (N = 24 mice per group; *Two-way ANOVA, Student's t-test*). **(G)** Number of visits and nosepoke in nosepoke learning (N = 24 mice per group; *Two-way ANOVA, Student's t-test*). **(H)** Number of correct visits in position learning (N = 24 mice per group; *One-way ANOVA, Student's t-test*). **(I)** Number of correct visits in reversal position learning (N = 24 mice per group; *One-way ANOVA, Student's t-test*). **(J)** Significant impairment was found in total distanced traveled (N = 24 mice per group; *One-way ANOVA, Student's t-test*). **(K)** Significant impairment was found in total Edge/Centre time (N = 24 mice per group; *One-way ANOVA, Student's t-test*). **(L–O)** Difference in thigmotaxis behavior between NS and Lira in WT or *Scn1a* KO mice was found, respectively, which was shown in a computer-generated trace of the animal's movements over 10 min. Data were presented as mean ± SD; **, *** represent $P < 0.01$, and $P < 0.001$, respectively. All experiments were performed in triplicate. WT, Wild-type; KO, *Scn1a* Knockout heterozygotes (F1); NS, Normal saline; Lira, Liraglutide.

($P < 0.001$; **Figure 2A**) and the duration of seizures was reduced ($P < 0.001$; **Figure 2A**), followed by seizures that appeared to be relatively mild. Compared with the Lira group, the duration of seizure behavior was reduced and the Racine rating was higher in VPA group ($P < 0.001$; **Figure 2A**). Both lira and VPA administration markedly reduced seizure severity in *Scn1a* KO mice compared to NS control mice (KO+NS: 96.53 ± 0.45, KO+Lira: 85.98 ± 1.24, KO +VPA: 89.12 ± 1.61, $P < 0.001$, **Figure 2B**). As shown in **Figure 2C**, the number of seizures was significantly reduced in both Lira and VPA group (KO+NS: 2.63 ± 0.84, KO+Lira: 1.01 ± 1.15; KO+VPA: 1.02 ± 1.19, $P < 0.001$, **Figure 2C**). Survival curves showed that Lira markedly reduced the mortality compared with *Scn1a* KO NS control group and VPA group (**Figure 2D**).

In addition, cortical electrodes were implanted to measure the EEG seizure activity in mice. As shown in **Figure 2E**, *Scn1a* KO control mice (KO+NS) exhibited a higher frequency and magnitude of electroencephalographic seizure activity compared with WT mice. The administration of Lira or VPA markedly reduced the frequency and amplitude of EEG seizure activity (**Figure 2E**).

Cognitive Performance

We evaluated the cognitive performance of WT and *Scn1a* KO induced epileptic mice before and after the Lira administration with the IntelliCage system (adaptation and free exploration test, nose poke learning stage test, position learning stage test, and reversal position learning stage test) and smart 3.0 mouse behavioral system (the open field task).

Compared with WT mice, *Scn1a* KO mice exhibited significantly shorter visiting time (WT+NS: 796.33 ± 17.67, KO +NS: 593.83 ± 22.99, $P < 0.001$, **Figure 2F**) and the number of nose pokes was significantly reduced in the adaptation and free exploration test (WT+NS: 1722.83 ± 23.12, KO+NS: 1519.33 ± 15.18, $P < 0.001$, **Figure 2F**). The administration of Lira significantly increased visiting times (WT+NS: 796.33 ± 17.67, WT+Lira: 892.83 ± 16.51, $P < 0.01$, **Figure 2F**; KO+NS: 593.83 ± 22.99, KO+Lira: 699.33 ± 18.96, $P < 0.001$, **Figure 2F**) and the number of nose pokes in both WT and *Scn1a* KO mice (WT+NS: 1722.83 ± 23.12, WT+Lira, 1815.83 ± 14.85, $P < 0.01$, **Figure 2F**; KO+NS: 1519.33 ± 15.18, KO+Lira: 1621.01 ± 30.38, $P < 0.001$, **Figure 2F**).

In the nose poke learning stage test, *Scn1a* KO mice had significantly shorter visiting times (WT+NS: 625.01 ± 16.09, KO +NS: 441.16 ± 22.67, $P < 0.001$, **Figure 2G**) and the number of nose pokes was significantly reduced (WT+NS: 1572.51 ± 48.78, KO+NS: 956.67± 29.92, $P < 0.001$, **Figure 2G**), as compared with WT mice. Compared with *Scn1a* KO mice, the Lira group had significantly longer visiting times (WT+NS: 625.01 ± 16.09, WT+Lira, 730.33 ± 11.07, $P < 0.01$, **Figure 2G**; KO+NS: 441.16 ± 22.67, KO+Lira, 560.01 ± 9.19, $P < 0.001$, **Figure 2G**), and the number of nose pokes was significantly increased (WT+NS: 1572.51 ± 48.78, WT+Lira: 1770.67 ± 19.02, $P < 0.01$, **Figure 2G**; KO+NS: 956.67± 29.92, KO+Lira: 1166.83 ± 41.57, $P < 0.001$, **Figure 2G**).

In both position and reversal position learning stage tests (**Figures 2H, I**), *Scn1a* KO mice had significantly higher error visiting rate compared with WT mice (position test, WT+NS: 50.01 ± 2.82, KO+NS: 72.17 ± 2.93, $P < 0.001$; reversal position test, WT+NS: 64.02 ± 2.01, KO+NS: 85.67 ± 4.18, $P < 0.001$). Lira administration significantly decreased error visiting rates in WT and *Scn1a* KO mice in both tests (position test, WT+NS: 50.01 ± 2.82, WT+Lira: 41.01 ± 2.76, $P < 0.001$; reversal position test, KO+NS: 85.67 ± 4.18, KO+Lira: 74.5 ± 3.27, $P < 0.001$; **Figure 2I**).

We further investigated the cognition-related behavior of WT and *Scn1a* KO mice administered with NS or Lira using the open field task experiment. Compared with WT mice, the distance traveled by *Scn1a* KO mice decreased significantly (WT+NS: 84.33 ± 4.93, KO+NS: 46.01 ± 3.46, $P < 0.001$, **Figures 2 J, L, N**), and the residence time in the central region was significantly prolonged (WT+NS: 14.01 ± 2.09, KO+NS: 50.01 ± 2.83, $P < 0.001$, **Figures 2K, L, N**). Lira treatment significantly increased traveled the distance (WT+NS: 84.33 ± 4.93, WT+Lira: 94.83 ± 3.31, $P < 0.01$; KO+NS: 46.01 ± 3.46, KO+Lira: 66.83 ± 4.41, $P < 0.001$; **Figures 2J–O**), and prolonged the residence time in the edge region (WT+NS: 14.01 ± 2.09, WT+Lira: 11.17 ± 1.17, $P < 0.01$; KO+NS: 50.01 ± 2.83, KO+Lira: 37.33 ± 2.25, $P < 0.001$; **Figures 2K–O**) in both WT and *Scn1a* KO mice.

Collectively, these results suggest that *Scn1a* KO deficiency significantly aggravated the susceptibility and severity of seizures and exacerbated cognitive dysfunction.

Liraglutide Alleviates Neuronal Damage Following *Scn1a* KO-Induced Status Epilepticus

To investigate *Scn1a* KO-induced neuron damage and whether this damage was alleviated by administration of Lira, the lesion conditions of the cortex after 14 days following *Scn1a* KO-induced epilepsy were analyzed using HE and Nissl staining. Compared with the WT group (WT+NS, **Figure 3A**), the HE staining showed that cortical necrosis in the *Scn1a* KO group (KO+NS, **Figure 3A**) was severe, and the cells were disorderly arranged with unclear edges. Nucleus pyknosis, cytoplasmic staining, and cell body shrinkage were observed in cortical cells of the *Scn1a* KO mice (KO+NS, **Figure 3A**). In contrast, cortical cells in the *Scn1a* KO mice treated with Lira were neatly arranged with and displayed relatively normal nuclear morphology (KO+Lira, **Figure 3A**). The number of necrotic neurons was also markedly reduced (KO+Lira, **Figure 3A**). The Nissl staining results were consistent with those of the HE staining. Compared with the WT mice, the apparent neuron loss featured as loose, widened, and diffuse cell layers was observed in similar cortex brain regions of the *Scn1a* KO mice (KO+NS, **Figure 3B**). After the Lira treatment, the neuron loss was significantly reduced in similar cortex brain regions of the *Scn1a* KO mice (KO+NS: 9.76 ± 0.91, KO+Lira: 19.65 ± 2.64, $P < 0.001$, **Figures 3B, C**). These results suggest that *Scn1a* KO deficient mice exhibit varying degrees of aggravated neuronal lesions in the cortex. However, Lira intervention exerts a neuroprotective effect to alleviate this neuronal damage.

Liraglutide Inhibits Phosphorylation mTOR Hyperactivation in the Brain of *Scn1a* KO-Induced Epileptic Mice

Activation of the mTOR pathway has been reported to be related to epileptogenicity. Furthermore, mTOR hyperactivation was observed in genetic and acquired epilepsy syndromes (Citraro et al., 2016). The mTOR signaling pathway was examined in cortical neurons using

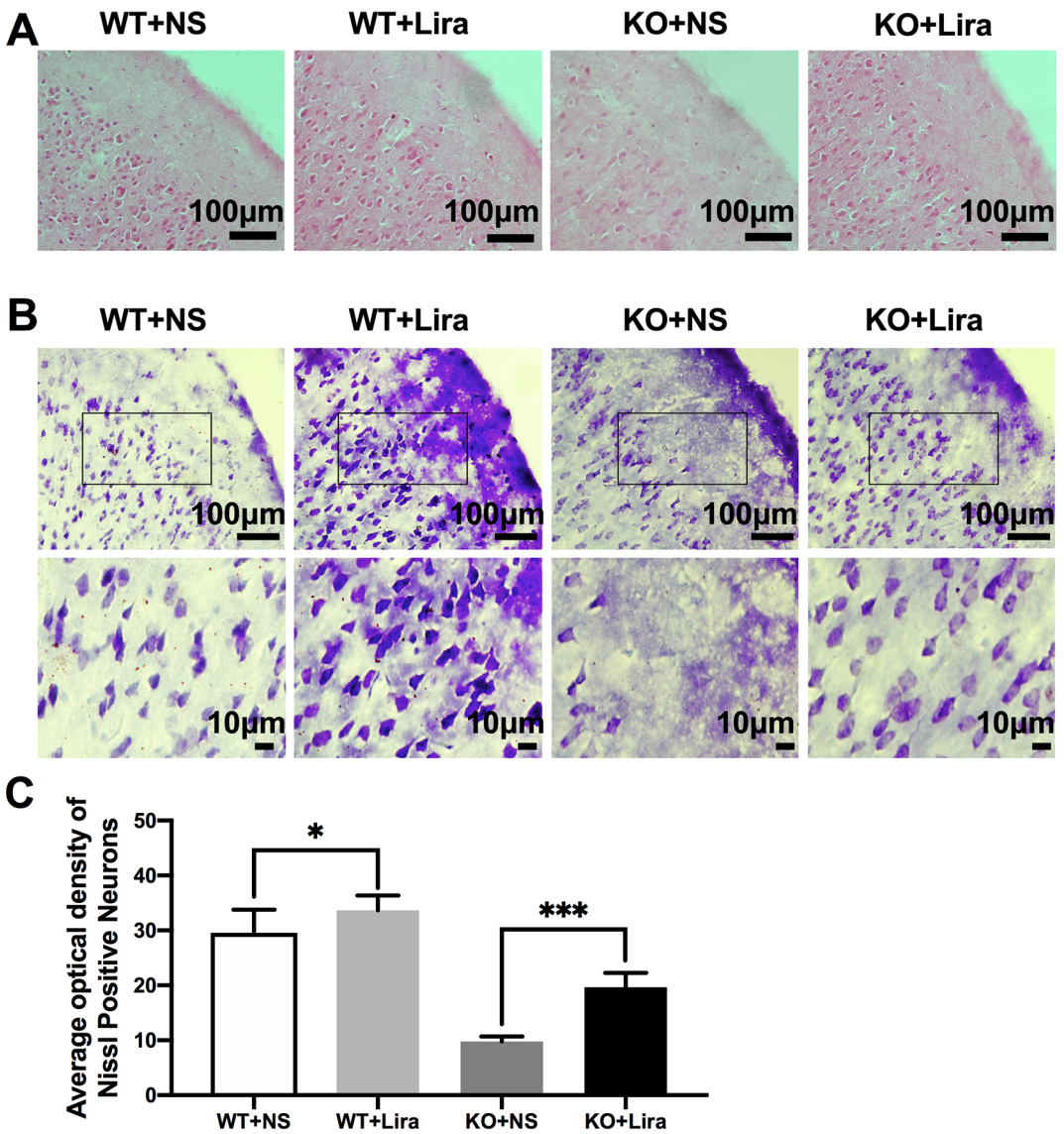

FIGURE 3 | HE staining and Nissl staining showed neuron damage in the cortex. **(A)** Representative images of HE staining of the cortex. Scale bar: 100 μm. **(B)** Representative images of Nissl staining of the cortex. Scale bar: 100 and 10 μm. **(C)** Histograms of the Nissl-positive neurons obtained *via* Nissl staining in the cortex (N = 24 mice per group; *One-way ANOVA, Student's t-test*). Data were presented as mean ± SD; *, *** represent $P < 0.05$, and $P < 0.001$, respectively. All experiments were performed in triplicate. WT, Wild-type; KO, *Scn1a* Knockout heterozygotes (F1); NS, Normal saline; Lira, Liraglutide.

immunofluorescence staining. The results revealed that mTOR fluorescence intensity was significantly enhanced in *Scn1a* KO mice compared with WT mice (WT+NS: 15.08 ± 2.24, KO+NS: 32.18 ± 5.07, $P < 0.001$, **Figures 4A, B**). However, treatment with Lira significantly reduced fluorescence intensity in both WT (WT+NS: 15.08 ± 2.24, WT+Lira: 7.41 ± 1.77, $P < 0.05$) and *Scn1a* KO + Lira groups (KO+NS: 32.18 ± 5.07, KO+Lira: 20.94 ± 1.19, $P < 0.001$, **Figures 4A, B**). RT-qPCR was used to evaluate mTOR mRNA expression in WT and *Scn1a* KO mice and the results were consistent with those from immunofluorescence staining (**Figure 4C**). We further measured the protein expression ratio of p-mTOR/mTOR in WT and *Scn1a* KO mice by western blotting. The p-mTOR/mTOR ratio was significantly higher in the brain of *Scn1a* KO mice compared to that of WT mice (WT+NS: 0.11 ± 0.01, KO+NS: 1.99 ± 0.31, $P < 0.001$, **Figures 4D, E**). Importantly, Lira treatment significantly decreased the expression ratio of the p-mTOR/mTOR in *Scn1a* KO mice (KO+NS: 1.99 ± 0.31, KO+Lira: 0.97 ± 0.18, $P < 0.001$, **Figures 4D, E**), indicating that Lira can inhibit the mTOR signaling pathway in *Scn1a* KO-induced epileptic mice.

Liraglutide Reduces Apoptosis in the Brain of *Scn1a* KO-Induced Epileptic Mice

Apoptosis is a well-known and important feature of epilepsy-related diseases. Apoptosis was examined using immunofluorescence

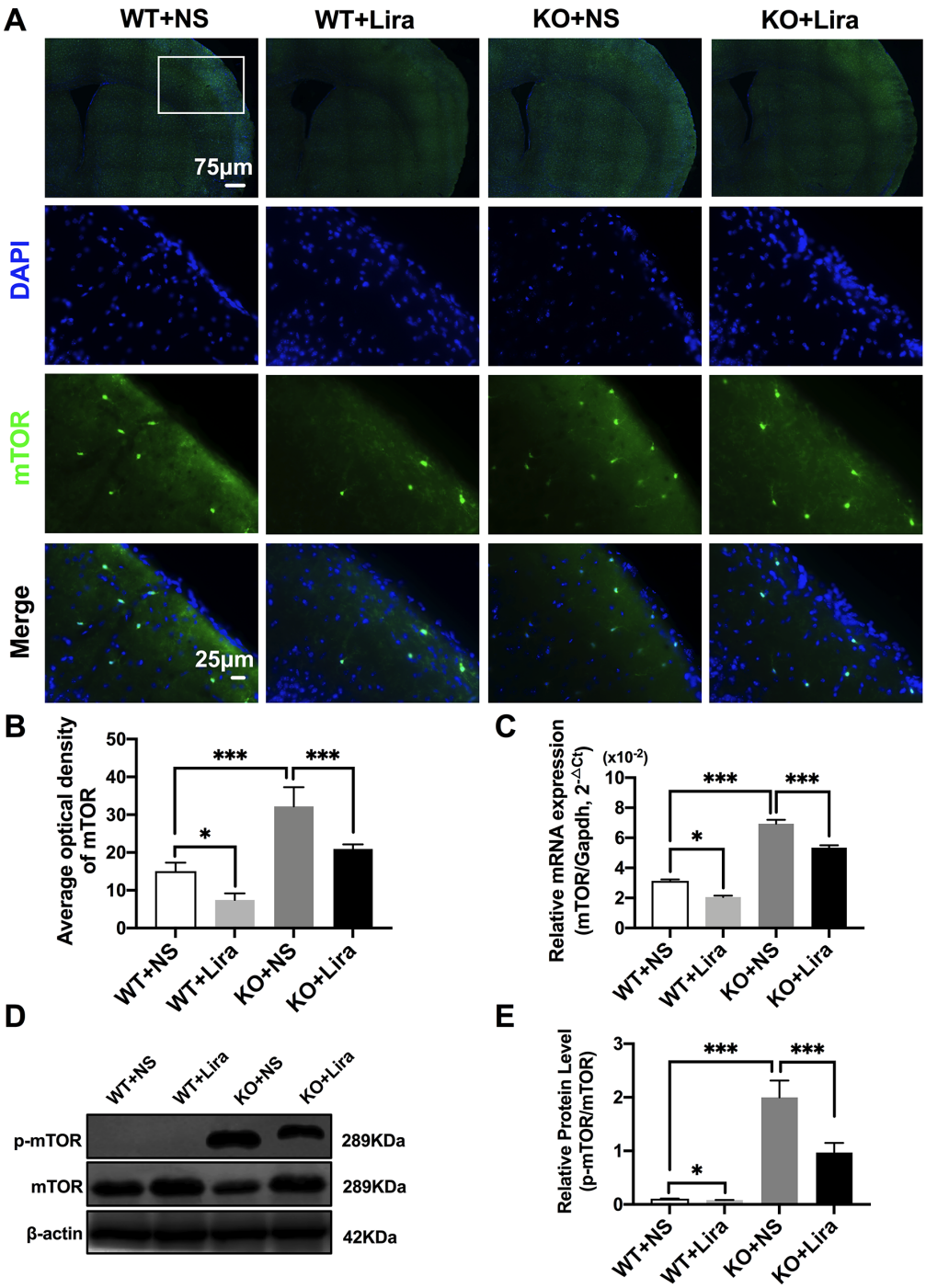

FIGURE 4 | Liraglutide inhibited phosphorylation mTOR hyperactivation in *Scn1a* KO-induced epileptic mice. **(A)** Representative images of immunofluorescent staining with mTOR (green), and DAPI (blue) in the ipsilateral side of the cortex of the WT and *Scn1a* KO mice. Scale bar: 75 μm for the full-scale images and 25 μm for the magnified images. **(B)** Summary graph of the average optical density of mTOR fluorescent staining measured in images (A) demonstrating a decrease of mTOR in the cortex of *Scn1a* KO mice after liraglutide administration (N = 24 mice per group; *Student's t-test, P* < 0.001). **(C)** RT-qPCR analysis of the mTOR mRNA expression from brains of wild-type (WT) and *Scn1a* KO heterozygote (KO) mice (N = 24 mice per group; *One-way ANOVA, Student's t-test*). **(D)** Representative western blotting showing mTOR protein expression in brains of WT and *Scn1a* KO mice. **(E)** Summary graph of western blotting analysis demonstrating a significant reduction in the mTOR protein in brains of *Scn1a* KO as compared to WT mice after liraglutide administration (N = 24 mice per group; *Student's t-test*, *P* < 0.001). The relative expression of SCN1A was normalized to reference controls *Gapdh* and β-actin in RT-qPCR and Western blotting, respectively. Data were presented as mean ± SD; *, *** represent *P* < 0.05, and *P* < 0.001, respectively. All experiments were performed in triplicate. WT, Wild-type; KO, *Scn1a* Knockout heterozygotes (F1); NS, Normal saline; Lira, Liraglutide.

staining with regard to BCL-2, BAX, and cleaved caspase-3. The results revealed that BCL-2 fluorescence intensity was significantly enhanced in the *Scn1a* KO + Lira group compared with the *Scn1a* KO group (KO+NS: 8.66 ± 0.48, KO+Lira: 20.4 ± 2.78, $P < 0.001$, **Figures 5A, B**). Moreover, BAX and cleaved caspase-3 fluorescence intensity was significantly reduced in the *Scn1a* KO + Lira group compared with the *Scn1a* KO group (BAX, KO+NS: 33.72 ± 2.01, KO+Lira: 23.7 ± 1.22, $P < 0.001$; Cleaved caspase-3, KO+NS: 36.83 ± 1.84, KO+Lira: 21.94 ± 1.8, $P < 0.001$; **Figures 5C–F**). The mRNA and protein expression level of these apoptosis-related proteins was further evaluated using RT-qPCR and WB in the brain of both WT and *Scn1a* KO mice.

RT-qPCR revealed that the BAX/BCL-2 ratio and cleaved caspase-3 mRNA levels were significantly downregulated in the *Scn1a* KO + Lira group compared with the *Scn1a* KO group (BAX/BCL-2, KO+NS: 2.45 ± 0.17, KO+Lira: 1.59 ± 0.11, $P < 0.001$; Caspase-3, KO+NS: 1.74 ± 0.12, KO+Lira: 1.24 ± 0.11, $P < 0.001$; **Figure 5G**). Moreover, Lira treatment significantly decreased the ratio of BAX/BCL-2 and the expression of the apoptosis-related protein, cleaved caspase-3 in *Scn1a* KO mice (BCL-2, KO+NS: 0.46 ± 0.02, KO+Lira: 0.61 ± 0.02, $P < 0.001$; BAX, KO+NS: 0.90 ± 0.02, KO +Lira: 0.75 ± 0.04, $P < 0.001$; Cleaved caspase-3, KO+NS: 0.49 ± 0.04, KO+Lira: 0.30 ± 0.01, $P < 0.001$; **Figures 5H, I**). These data suggest that Lira administration can inhibit apoptosis in the brain of *Scn1a* KO mice.

Liraglutide Promotes Proliferation and Reduces Apoptosis by Inhibiting mTOR Hyperactivation in *Scn1a* KO Cells

We examined the effect of Lira on the apoptosis and proliferation in a *Scn1a* knockout cell line model. Given the previous work in the authors' laboratory (Shi et al., 2019), the *Scn1a* knockout in HT22 cell line was established using Crispr/cas 9 technology, and RT-qPCR and WB were used to verify the *Scn1a* KO cell line. As shown in **Figure 6A**, the level of SCN1A mRNA expression in the *Scn1a* KO cells was significantly lower compared with the HT22 control cells (HT22: 2.05 ± 0.16, *Scn1a* KO: 0.12 ± 0.02, $P < 0.001$, **Figure 6A**). In addition, the markedly decreased SCN1A protein expression level in *Scn1a* KO cells was measured by WB (HT22: 0.98 ± 0.03, *Scn1a* KO: 0.02 ± 0.01, $P < 0.001$, **Figures 6B, C**). The viability of *Scn1a* KO cells under different concentrations (8, 10, and 12 nM) of Lira was determined using the CCK-8 assay at 24, 48 and 72 h, respectively. The total number of *Scn1a* KO cells were measured using a cell counter (**Figures 6D, E**). Based on the results, Lira did not exhibit toxicity at lower doses ($P > 0.05$; **Figure 6F**) and hardly impacted cell viability at higher doses when its concentration was lower than or equal to 10 nM (**Figures 6D–F**); thus, 48 h was the appropriate time for drug treatment. Therefore, 10 nM Lira was used in subsequent experiments.

Furthermore, Annexin V-FITC/PI flow cytometry was used to evaluate apoptosis in the *Scn1a* KO cell model, and revealed that the apoptotic rate of both HT22 control and *Scn1a* KO cells was significantly decreased after Lira treatment (HT22 control: 1.75 ± 0.03, HT22+Lira: 1.40 ± 0.07, *Scn1a* KO: 2.68 ± 0.19, *Scn1a* KO+lira: 1.59 ± 0.10, $P < 0.001$; **Figures 7A–E**). The expression level of apoptosis-related proteins was also evaluated using RT-qPCR and WB in both HT22 control and *Scn1a* KO cells. RT-qPCR revealed that the BAX/BCl-2 ratio and cleaved caspase-3 mRNA levels were significantly downregulated in the *Scn1a* KO cells treated with Lira (mRNA: BAX/BCL-2, *Scn1a* KO: 2.29 ± 0.05, *Scn1a* KO+Lira: 1.56 ± 0.05; Caspase-3, *Scn1a* KO: 1.75 ± 0.13, *Scn1a* KO+Lira: 1.22 ± 0.1; $P < 0.001$; **Figure 7F**). Moreover, Lira treatment significantly decrease the ratio of BAX/BCL-2 and the expression of the apoptosis-related protein, cleaved caspase-3 in *Scn1a* KO cells (protein: BCL-2, *Scn1a* KO: 0.07 ± 0.02, *Scn1a* KO+Lira: 0.36 ± 0.03; BAX, *Scn1a* KO: 0.65 ± 0.02, *Scn1a* KO+Lira: 0.51 ± 0.04; Cleaved Caspase-3, *Scn1a* KO: 0.28 ± 0.03, *Scn1a* KO+Lira: 0.21 ± 0.02; $P < 0.001$; **Figures 7G, H**).

We further evaluated whether mTOR activation was affected by Lira in both HT22 control and *Scn1a* KO cells. RT-qPCR was used to evaluate mTOR mRNA expression in HT22 and *Scn1a* KO cells. Compared with the *Scn1a* KO group, mTOR mRNA was significantly downregulated in the *Scn1a* KO + Lira group (*Scn1a* KO: 2.14 ± 0.18, *Scn1a* KO+Lira: 1.52 ± 0.16, $P < 0.001$, **Figure 7F**). Compared with the *Scn1a* KO group, the p-mTOR/mTOR protein ratio was significantly downregulated in the *Scn1a* KO + Lira group (*Scn1a* KO: 1.13 ± 0.11, *Scn1a* KO +Lira: 0.45 ± 0.07, $P < 0.001$, **Figures 7G, H**). These data suggest that Lira modulates *Scn1a* KO-induced apoptosis in TH22 cells *via* the mTOR signaling pathway *in vitro*.

DISCUSSION

The results of the present study demonstrate that Lira reduces seizure susceptibility, minimizes cognitive dysfunction, and inhibits apoptosis in neurons in *Scn1a* KO-induced epileptic mice and, moreover, decreases the levels of mTOR hyperactivation. Behavioral seizures in *Scn1a* KO-induced epilepsy models show that SCN1A deficiency increases susceptibility to seizures; however, treatment with Lira can reduce susceptibility and severity. In addition, *Scn1a* KO deficiency exacerbates the typical pathological manifestations of epilepsy in *Scn1a* KO mice. However, Lira intervention can ameliorate this pathological change. Neuroprotective effects were also observed in *Scn1a* KO HT22 cell model after Lira treatment. Hence, these findings suggest that the inhibition of apoptosis *via* inactivation of mTOR phosphorylation by Lira may partially contribute to its effects on epileptogenesis, seizure relief, and neuroprotection.

DS is a severe epileptic encephalopathy, most often resulting from *de novo SCN1A* mutations (Lorincz and Nusser, 2010), and typically begins in infancy with seizures provoked by fever, including status epilepticus cognitive impairment (Sugiura et al., 2012; Beck et al., 2019), and poor response to available antiepileptic drugs. Voltage-gated sodium channels are protein complexes consisting of one alpha subunit and one or more beta subunits to mediate action potentials in excitable tissues

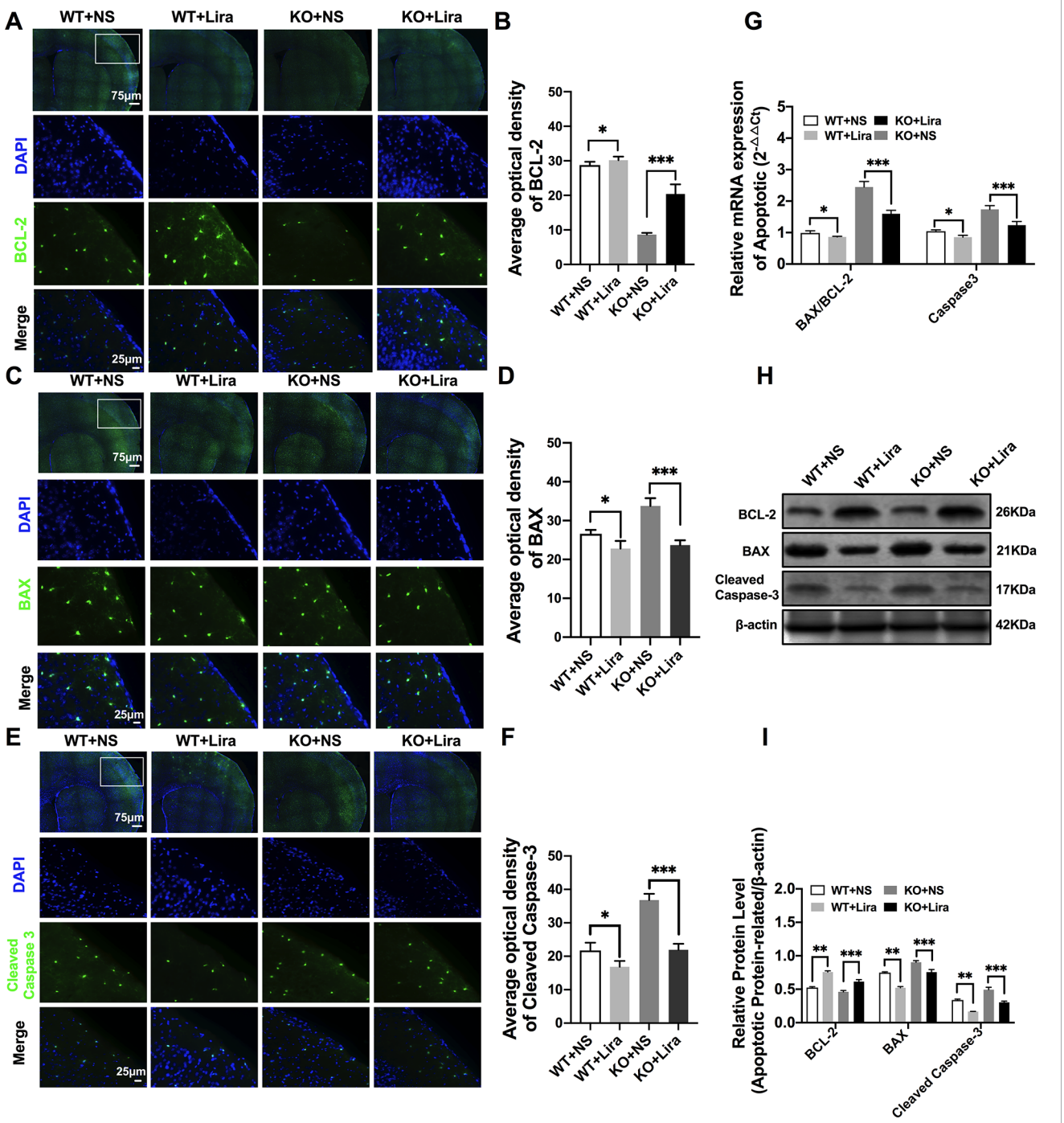

FIGURE 5 | Liraglutide blocked apoptosis in *Scn1a* KO-induced epileptic mice. **(A, C, E)** Representative images of immunofluorescent staining with BCL-2/BAX/Cleaved Caspase-3 (green), and DAPI (blue) in the ipsilateral side of the cortex of the WT and *Scn1a* KO mice. Scale bar: 75 μm for the full-scale images and 25 μm for the magnified images. **(B, D, F)** Summary graph of the average optical density of BCL-2, BAX, and Cleaved Caspase-3 fluorescent staining measured in images (A, C, E) in the cortex of mice (N = 24 mice per group; *One-way ANOVA, Student's t-test*). **(G)** RT-qPCR analysis of the BAX/BCL-2 and Caspase-3 expression from brains of wild-type (WT) and *Scn1a* KO heterozygote (KO) mice (N = 24 mice per group; *One-way ANOVA, Student's t-test*). **(H)** Representative western blotting showing BAX/BCL-2 and Cleaved Caspase-3 protein expression in brains of WT and *Scn1a* KO mice. **(I)** Summary graph of western blotting analysis demonstrating a significant reduction in the apoptosis-related protein in brains of *Scn1a* KO as compared to WT mice after liraglutide administration (N = 24 mice per group; *Student's t-test*, $P < 0.001$). The relative expression of apoptosis-related protein was normalized to reference controls *Gapdh* and β-actin in RT-qPCR and Western blotting, respectively. Data were presented as mean ± SD; *, **, *** represent $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively. All experiments were performed in triplicate. WT, Wild-type; KO, *Scn1a* Knockout heterozygotes (F1); NS, Normal saline; Lira, Liraglutide.

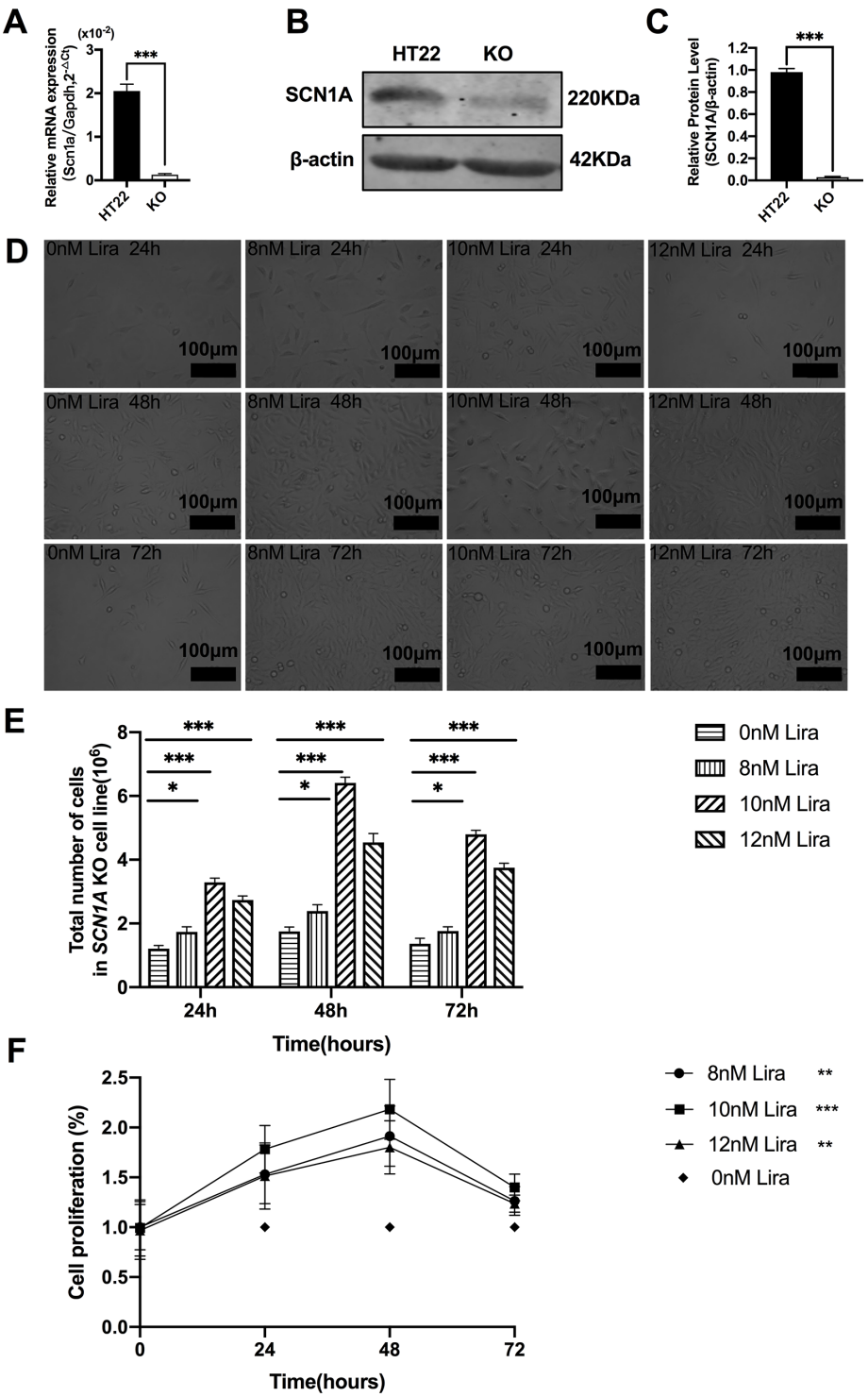

FIGURE 6 | Liraglutide promoted the proliferation of *Scn1a* knockout HT22 cell in a time-and-dose relationship. **(A)** RT-qPCR analysis of the *Scn1a* mRNA expression from HT22 and *Scn1a* KO cell (*Student's t-test*, $P < 0.001$). **(B)** Representative western blotting showing SCN1A protein expression in the HT22 and *Scn1a* KO cell. **(C)** Summary graph of western blotting analysis demonstrating a significant reduction in the SCN1A protein in *Scn1a* KO as compared to HT22 cell (*Student's t-test*, $P < 0.001$). **(D)** Morphological changes of *Scn1a* KO cell in different time treated with different concentrations of liraglutide. **(E)** Total number of cells in *Scn1a* KO cell line (*Two-way ANOVA*). **(F)** Cell proliferation by CCK-8 (*Two-way ANOVA*). The relative expression of SCN1A was normalized to reference controls *Gapdh* and β-actin in RT-qPCR and Western blotting, respectively. Data were presented as mean ± SD; *, **, *** represent $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively. All experiments were performed in triplicate. WT, Wild-type; KO, *Scn1a* Knockout in HT22 cell; Lira, Liraglutide.

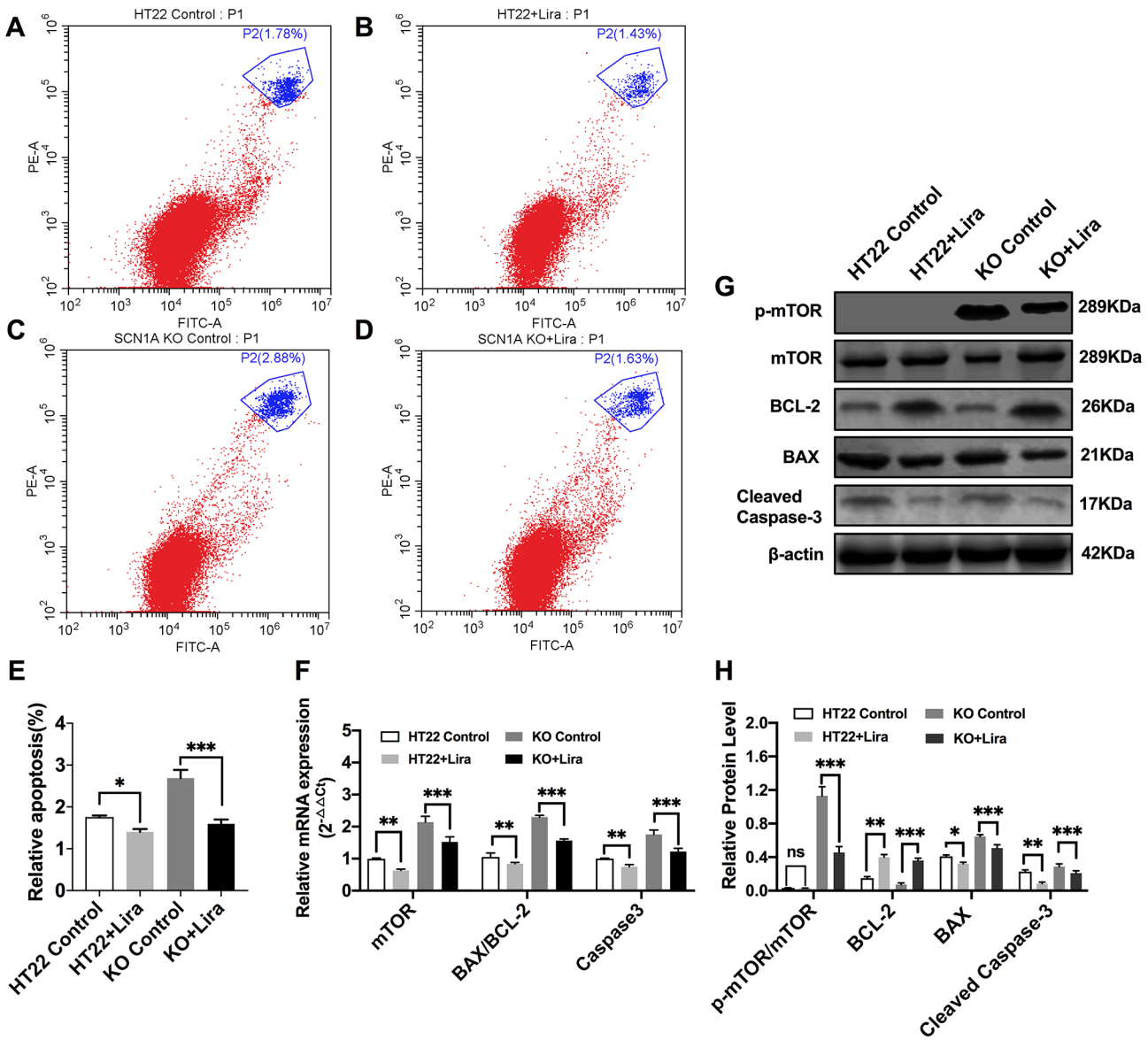

FIGURE 7 | Liraglutide promoted proliferation and reduced apoptosis by inhibiting phosphorylation mTOR hyperactivation in *Scn1a* KO cell. **(A–D)** Annexin V-FITC/PI flow cytometry evaluating apoptosis in *Scn1a* KO cell model. **(E)** The percentage of induction of apoptosis in cell treated with the 10 nM Lira in comparison with the control group and 48 h of incubation (*One-way ANOVA*). **(F)** RT-qPCR analysis of the BAX/BCL-2, Caspase-3, and mTOR expression from HT22 and *Scn1a* KO cell (*One-way ANOVA, Student's t-test*). **(G)** Representative western blotting showing BAX/BCL-2, Cleaved Caspase-3, and p-mTOR/mTOR protein expression in the HT22 and *Scn1a* KO cell. **(H)** Summary graph of western blotting analysis demonstrating a significant reduction in the apoptosis-related and mTOR protein in *Scn1a* KO as compared to HT22 cell after liraglutide administration (*Student's t-test*, $P < 0.001$). The relative expression was normalized to reference controls *Gapdh* and β-actin in RT-qPCR and Western blotting, respectively. Data were presented as mean ± SD; *, **, *** represent $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively. All experiments were performed in triplicate. WT, Wild-type; KO, *Scn1a* Knockout in HT22 cell; Lira, Liraglutide.

(Whitaker et al., 2001; Tsukamoto et al., 2017). The mammalian genome has nine homologs of sodium channel (SCN1A-SCN5A and SCN8A-SCN11A) encoding nine sodium channel alpha subunits (Nav1.1–Nav1.9), respectively. The alpha subunit is the major component of the sodium channel and is the functional unit. Nav1.1 channels are expressed in the cortex, hippocampus, cerebellum, and olfactory bulb (Escayg and Goldin, 2010; Lorincz and Nusser, 2010; Brackenbury and Isom, 2011). Scn1a$^{+/-}$ heterozygous KO mice mimic features of DS and provide a potential screening platform to investigate novel therapeutics (Kalume et al., 2013; Miller et al., 2014; Mistry et al., 2014; Kang et al., 2019). We used the F1 heterozygous *Scn1a* KO mouse model,

and the clinical presentation and identification were consistent with previous literature reports (Cheah et al., 2012; Miller et al., 2014; Mistry et al., 2014). *Scn1a* KO mice were injected with Lira for 14 days and its effects were evaluated. EEG, IntelliCage, and the open field task experiment has shown that Lira could reduce seizure susceptibility and cognitive dysfunction in *Scn1a* KO-induced epileptic mice. Recurrent seizures are often concomitant with neuronal loss in human patients and in animal models (Curia et al., 2014). Thus, we used HE and Nissl staining to observe neuronal death and degeneration and evaluate the effects of Lira in *Scn1a* KO-induced epileptic mice. HE and Nissl staining revealed that Lira alleviated neuronal damage following *Scn1a* KO-induced status epilepticus. Furthermore, IF results demonstrated that Lira increased the expression levels of *Scn1a* in *Scn1a* KO-induced epileptic mice, which was further supported by RT-qPCR and WB results. These findings suggest that Lira treatment delays the epileptic developmental process, reduces seizure susceptibility, alleviates neuronal damage, and minimizes behavior and cognitive deficits in *Scn1a* KO-induced epileptic mice.

Apoptosis is a type of the programmed cell death, and two major pathways can induce apoptosis, namely, the mitochondrial apoptotic pathway (intrinsic pathway) and the death receptor apoptotic pathway (extrinsic pathway) (O'Neill et al., 2016; Green, 2017; Wang et al., 2017). Increasing membrane permeability can mediate the release of pro-apoptotic proteins from mitochondria to activate the intrinsic apoptotic pathway controlled by the Bcl-2 superfamily. Combined transmembrane death receptors with ligands, such as Fas ligand (FasL) and tumor necrosis factor (TNF), can activate the extrinsic pathway. Both pathways converge at the level of effector caspase, and then activate caspase-3 and caspase-7 to induce apoptosis (Rongvaux et al., 2014; Kayagaki et al., 2015). Seizures can cause apoptosis, which in turn can promote the progression of epilepsy (Engel et al., 2010; Fu et al., 2010). Considering the effects of apoptotic mechanisms, the results are not surprising, and apoptosis induction may be the most common target for epilepsy treatment. In our experiments, we used the expression levels of cleaved caspase-3, BCL-2 associated X protein (BAX), and B cell lymphoma 2 (BCL-2) to reflect the extent of apoptosis. We demonstrated that the administration of Lira downregulated the expression of cleaved caspase-3 and the ratio of BAX to BCL-2 in *Scn1a* KO models. Therefore, Lira can reduce neural apoptosis in *Scn1a* KO-induced epileptic mice potentially *via* inhibition of BAX/BCL-2 signaling.

mTOR is a serine/threonine kinase, divided into two multimeric active forms: rapamycin-sensitive mTOR complex (mTORC1) and mTOR complex 2 (mTORC2) (Lipton and Sahin, 2014; Citraro et al., 2016). The core molecules of mTORC1 and mTORC2 are, respectively, the regulatory-associated protein of mTOR (Raptor) and (Rictor). mTORC1 is sensitive to nutrients and regulates protein synthesis and cell growth through the downstream molecules 4E-BP1 and S6K. mTORC2 is regulated *via* PI3K and growth factor signaling and is responsive to growth factor signaling by phosphorylating the C-terminal hydrophobic motif of some AGC kinases such as Akt and SGK (Linke et al., 2017; Hua et al., 2019; Yang et al., 2019). mTOR is a key regulatory node in growth-factor signaling across evolution. Its importance in a wide array of physiological processes, including insulin signaling, cell growth, immunity, and brain development, has been confirmed in animal models and in humans (Goncalves et al., 2018). Moreover, mTOR hyperactivation has been observed in genetic and acquired epilepsy syndromes (Liu et al., 2014). In the present study, we found that Lira inhibited the hyperactivation of mTOR in *Scn1a* KO mice. Therefore, Lira can exert neuroprotective effects in *Scn1a* KO-induced epileptic mice by inhibiting the mTOR signaling pathway.

GLP-1 is an incretin hormone used to treat diabetes mellitus. GLP-1R are not only distributed in intestinal L cells, but also in the central nervous system (CNS) (Richards et al., 2014; Katsurada and Yada, 2016). We hypothesize that this widespread distribution of GLP-1R may be the anatomical basis for GLP-1 analogues to exert neuroprotective effects. It has been reported in some research that GLP-1 analogues demonstrate neuroprotective effects in acute and chronic epileptic mouse models. GLP-1 analogs may exert anti-epileptic effects through apoptotic pathways in epileptic mouse models (Koshal and Kumar, 2016; Hussein et al., 2019; Wen et al., 2019). Moreover, the mTOR signaling pathway has been shown to be involved in regulating neuronal function, proliferation, apoptosis, and other cellular processes associated with epileptogenesis. Studies have shown that the activity of the mTOR signaling pathway is abnormally increased in epilepsy models, and that GLP-1 analogs can regulate mTOR expression *via* the AMPK pathway (Hurtado-Carneiro et al., 2012; Kong et al., 2018). In our experiments, we found that apoptosis and mTOR expression were increased in *Scn1a* KO mice and cell models. However, this effect can be reversed after intervention with GLP-1 analogue.

In summary, the current study has demonstrated that Lira can significantly inhibit hyperactivation of the mTOR signaling pathway and reduce neural apoptosis in *Scn1a* KO mice and cell models. Although experimental animal and cell models have been used for elucidating these correlatives, further experimental research and rigorous clinical investigations should be conducted in human-based, clinical studies.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/ **Supplementary Material**.

ETHICS STATEMENT

The mice were handled according to the guidelines prescribed by the Institutional Animal Care and Use Committee of Ningxia Medical University (IACUC Animal Use Certificate No.: SCXK (Ning) 2015 – 0001).

AUTHOR CONTRIBUTIONS

Study design: TS, FW, ZJ. Experiment implementation: SL, SR, WH. Paper writing: SL, YZ, ZJ. Data analysis: SL, ZJ, YZ, SR, KS, LX, XL, DW, and ND. All authors read and approved the final 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/fphar.2020.00136/full#supplementary-material

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The Gut-Brain-Axis on the Manifestation of Depressive Symptoms in Epilepsy: An Evidence-Driven Hypothesis

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Epilepsy is a severe neurological disorder involving 70 million people around the globe. Epilepsy-related neuropsychiatric comorbidities such as depression, which is the most common, is an additional factor that negatively impacts the living quality of epilepsy patients. There are many theories and complexities associated with both epilepsy and associated comorbidities, one of which is the gut-brain-axis influence. The gut microbiome is hypothesized to be linked with many neurological disorders; however, little conclusive evidence is available in this area. Thus, highlighting the role will create interest in researchers to conduct detailed research in comprehending the influence of gut-brain-axis in the manifestation of depressive symptoms in epilepsy. The hypothesis which is explored in this review is that the gut-brain-axis do play an important role in the genesis of epilepsy and associated depression. The correction of this dysbiosis might be beneficial in treating both epilepsy and related depression. This hypothesis is illustrated through extensive literature discussion, proposed experimental models, and its applicability in the field. There is indirect evidence which revealed some specific bacterial strains that might cause depression in epilepsy.

Keywords: epilepsy, depression, gut-brain-axis, gut microbiome, vagus nerve

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INTRODUCTION

Epilepsy is a disease of the central nervous system (CNS) which incurs episodes of epileptic seizures, accompanied by psychiatric consequences. In 2005, the Task Force of the International League Against Epilepsy (ILAE) expressed both theoretical definitions of "seizures" and "epilepsy" and the operational definitions which were used for clinical diagnosis. For a theoretical definition, epilepsy is characterized by epileptic seizures, with cognitive and neurobiological consequences. An epileptic seizure is an event of abnormal brain neuronal activity. In contrast, the operational or clinical definition requires the following situations: 1) At least two reflex seizures occurring more than 24 h

apart 2) One unprovoked seizure and a probability of further seizures (≥60%) after two unprovoked seizures, occurring over the next ten years and 3) have been diagnosed with an epilepsy syndrome (Fisher et al., 2014).

The prevalence of this complex illness affects nearly 70 million people worldwide in a variety of forms and severity. In advanced countries, the incidence is around 40–70 per 100,000/year, whereas in less advanced countries, it is higher, approximately 100–190 per 100,000/year (Sander, 2003). Epilepsy (idiopathic or primary) incidence is prominent in early childhood and after adolescence in developed countries (Stephen and Brodie, 2000) while, incidence is higher in developing countries of the childhood group (Klein et al., 2003; Olafsson et al., 2005). Epilepsy, both incidence and prevalence, is observed to be elevated in males than females in both developed and developing countries. This could arguably be due to the presence of female sex hormones, estrogen, and progesterone, which may affect the threshold of seizure to some extent (Lavados et al., 1992). Few studies are available about the prevalence of epilepsy based on race and ethnicity. Prevalence of epilepsy is high in African-Americans (8.2/1,000) and non-South Asians (7.8/1,000) as compared to Caucasians (5.4/1,000) and South Asians (3.6/1,000) (Wright et al., 2000; Banerjee et al., 2009). However, it is not conclusive as it can be subjected to the lack of control of the socioeconomic status between the groups, posing a limitation to the study.

The etiology of epilepsy includes a variety of CNS disorders from parasitic infections. A common parasitic infection which affects the intracranial region is neurocysticercosis, from pork tapeworm larva, *Taenia solium* infection. It is a frequent parasitosis of the CNS with seizures as a typical manifestation (Medina et al., 1990). It was revealed that the proportion of neurocysticercosis in people with active epilepsy displayed consistency, estimated to be 29% from several studies performed in endemic areas of Latin America, Sub-Saharan Africa, and Southeast Asia (Ndimubanzi et al., 2010; Nash, 2014). This suggests the significance of neurocysticercosis as an etiology of epilepsy. Other microbial infections include bacterial such as tuberculous meningitis, whereby epilepsy manifests in the form of intracranial tuberculomas (Bahemuka and Murungi, 1989).

Another etiology of epilepsy for adults is head or brain injuries leading to cranial trauma whereas, in children, it is mainly febrile convulsions which are acute neurological disturbances. Genetic risk factors are also involved. Individuals with a first-degree epileptic relative have a threefold risk of developing one too (Senanayake and Roman, 1993). Seizure syndromes may arise from ion-channel disorders, progressive myoclonus epilepsies, neurogenetic disorders from developmental abnormality, energy metabolism defects or metabolic disturbances, as well as neuronal migration disorders (Steinlein, 2008). Lastly, environmental causes of epilepsy can be contributed by potential neurotoxic agents such as benzene hexachloride pesticide, utilized as a food grain preservative in the Lakhimpur Kheri district of India (Khare et al., 1977). There are several other seizurogenic chemicals such as the organophosphorus (OP) nerve agent and dichlorodiphenyltrichloroethane (DDT) which disrupts the modulation of sodium ion channels by delaying the action potential falling phase, inducing the intermittent release of nerve impulse. This causes tremors and seizures (Jett, 2012).

The gut microbiome is hypothesized to be linked with many neurological disorders; however, little conclusive evidence is available in this area. Thus, highlighting the role will create interest in researchers to conduct detailed research in comprehending the influence of gut-brain-axis in the manifestation of depressive symptoms in epilepsy. The hypothesis which is explored in this review is that the gut-brain-axis do play an important role in the genesis of epilepsy and associated depression. The correction of this dysbiosis might be beneficial in treating both epilepsy and related depression.

Depression in Epilepsy

Among the many psychiatric disorders documented, depression has prevailed to be the most common comorbidity in epileptic patients (Grover, 2017). Depression is associated with loss of mood/interest, anhedonia, appetite or weight alterations, disrupted sleep and psychomotor activity, repetitive thoughts of death, and reduced energy and fatigue that persist. Despite epilepsy being a risk for depression, a bi-directional relationship is suggested whereby patients with clinical depression history is observed to have a higher likelihood of epilepsy, about four to six times (Kanner, 2003). Depressive disorders in epilepsy patients can generally be classified into three types—major depressive disorder (MDD), dysthymic disorder, and depressive disorder, which includes minor depression (Runeson and Rich, 1994). MDD and dysthymic disorder differ in terms of severity, persistence, and chronicity, but share common symptoms as mentioned above. MDD is diagnosed when there are recurring major depressive episodes for at least 2 weeks, whereas dysthymic disorder is a chronic disorder with persisting symptoms for ≥ 2 years. Minor depression is suggested if patients have less than five symptoms (Moore and Brown, 2012).

The active prevalence of depression in epileptic patients is not precise due to variations in study settings, demographic differences, and debatable methodologies employed to detect depression or epilepsy. However, the prevalence was found to be 23.1% for active depression in patients with epilepsy (Fiest et al., 2013). The lifetime prevalence of depression in epileptic patients is approximately 6% and 30% in population-based studies, and as high as 55% observed in patients of tertiary centers (Kanner, 2003; Gnanavel, 2017). Hence, the risk of depressive disorders is elevated by twofold in epilepsy compared to the standard population. The prevalence of depressive symptoms was assessed in epileptic Nigerians using the Beck Depression Inventory (BDI) for quantitative assessment and Hamilton Rating Scale for Depression (HRSD) was carried out. It was revealed the frequency of depressive symptoms was 42% and 45% respectively. There was a crucial difference between patients and controls on both scales and higher depression scores in women compared to men (Ogunrin and Obiabo, 2010).

The link between depression and epilepsy is significantly more profound when compared to other chronic medical conditions. A study examined the depression prevalence in epileptic patients as compared to patients with asthmatic conditions or healthy control individuals delineated that

depressive symptoms are common in epileptic people (36.5%) as opposed to asthmatic people (27.8%) and healthy controls (Ettinger et al., 2004). The presence of depressive symptoms could be related to recurrent seizures in epileptic patients. Additionally, depression occurs in only 4% of patients free from seizures, approximately 10% in those with rare occasions of seizure attacks (less than once a month), whereas these percentages are elevated to 21% in patients having more seizure attacks and uncontrollable epilepsy. Consistent findings further strengthen the relationship whereby epileptic patients and persistent seizures have a predisposition to experience depression compared to patients in remission (Jacoby et al., 1996; O'Donoghue et al., 1999).

The bi-directional relationship, although not causal, between depression and epileptic patients could suggest common pathogenesis in both states, whereby one disorder may potentially expedite the development of another. The neurobiology perspective of depressive epileptic patients involves common pathogenetic mechanisms which could be abnormal activity or levels of neurotransmitters such as serotonin, noradrenaline, dopamine, GABA, and glutamate. The study involving genetic epilepsy-prone rats found that the serotonergic and noradrenergic pre- and postsynaptic transmission deficit rats exhibited severe seizures. When compared to the rats, MDD patients displayed similar abnormalities in the endocrine system (Jobe et al., 1995). Other studies investigated the effect of pharmacologic reduction in serotonin on seizures in humans. It was found that monoamines depletion such as serotonin has increased rate and gravity of seizures in epileptic patients (Kanner, 2005). Other potential contributing factors include the abnormalities in certain neuroanatomic regions, based on its structure and function, in depression and epileptic seizure, commonly associating with comorbid depression.

Based on neuroimaging, studies, in primary MDD, there exist alterations in the various neuroanatomic structures morphologically and volumetrically, especially frontal atrophy which includes medial prefrontal cortex, frontal cortex, and dorsolateral prefrontal cortex atrophy (Zhao et al., 2014; Bludau et al., 2016). Meta-analyses have also recorded a decrease in volume of the cingulate cortex found in MDD patients compared to healthy individuals (Rodriguez-Cano et al., 2014) and depressive symptoms cause hippocampal atrophy (Buddeke et al., 2017). It was documented that patients with temporal lobe epilepsy have frontal lobe dysfunction. This dysfunction leads to a reduction in inferofrontal metabolism and interferes serotonergic neuron transmission. Hence, it is speculated that frontal lobe dysfunction can predispose the patient to depression (Menzel et al., 1998). Studies have also revealed the prevalence rate of depression ranges from 19% to 65% among epileptic patients of mesial temporal or frontal lobe origin (Kanner and Balabanov, 2002). Overall, depression in epilepsy may arise from neurotransmitter dysfunction and neuroanatomic changes.

Current Treatments for Depression in Epilepsy

With the high prevalence and the burden of the disease, which resulted in the mortality of depression manifested in suicide, many patients often go undiagnosed and without proper treatment. In a clinical assessment of 174 patients with chronic epilepsy, MDD was a common diagnosis when they were interviewed using the Mini-International Neuropsychiatric Interview (MINI) and mood disorders modules. It was also found less than half were given antidepressant medications as a treatment (Jones et al., 2005).

Frequently prescribed anti-depressants to epileptic patients are from classes such as the selective serotonin reuptake inhibitors (SSRIs) and serotonin-noradrenaline reuptake inhibitors (SNRIs). They act as primary drugs or treatment for depression due to greater tolerability and decreased adverse effects as compared to classic tricyclic antidepressants (TCAs) which have been known to worsen seizure activity and decreasing seizure threshold in a dose-dependent manner (Dailey and Naritoku, 1996; Elger et al., 2017). The mechanism of action for both inhibitors blocks different transporters at the presynaptic neuronal membrane and elevate serotonin and/or noradrenaline levels in the synapse (Cardamone et al., 2013). Citalopram treatment for four consecutive months at 20 mg per day for depression in epilepsy patients reduced the total number of seizures from 1.32 to 0.82 seizures/month, and 67% of patients in the study experienced considerable improvement or remission (18%) of depressive symptoms (Specchio et al., 2004).

The pro-convulsant effect of venlafaxine (SNRI) was absent, and as the dose increased from 20 to 40 mg/kg/d, it did not significantly lower seizure threshold (Ahern et al., 2006). However, venlafaxine overdose can induce seizures, but at therapeutic dosage, it is safe to be used (Pisani et al., 2002). Available literature suggests that long-term SSRI/SNRI anti-depressant treatment does not exacerbate seizure frequencies in patients and even some experience complete freedom from seizure activity during the anti-depressant treatment. There were only a few exceptional cases reported whereby individuals did experience worse seizure frequencies which could be reversed by removal of anti-depressant or increasing anti-epileptic medications (Specchio et al., 2004; Okazaki et al., 2011). It was also recorded the anti-depressants effects on seizure activity from approximately 75,000 epileptic patients which revealed a significant reduction in seizure incidence of depressed patients on anti-depressants as compared to placebo-treated, suggesting a potent anti-seizure effect when given at therapeutic doses (Alper et al., 2007).

There exist another class of medication known as the noradrenergic and specific serotonergic anti-depressants (NaSSAs) which are psychiatric drugs used primarily as anti-depressants. In the NaSSAs class, mirtazapine was studied for its risks of epileptic events in adults (20–64 years) while on this anti-depressant. A follow-up of 5 years was conducted and it was found that mirtazapine, sertraline, and escitalopram did not

heighten the risk of seizure activity out of the 11 drugs examined (Hill et al., 2015). Similar evidence was observed in another large study which utilized the drug safety data information. It was concluded that SSRIs and the mirtazapine anti-depressants might pose as a better candidate to treat depression in patients with enhanced seizure risk as compared to tricyclic anti-depressants (Koster et al., 2013).

Nevertheless, there exist some limitations with employing anti-depressants into the medication regime of an epileptic patient. There could be a pro-convulsant potential of the anti-depressants as mentioned earlier, which often leads to lowering the seizure threshold and thus exacerbating seizure activity. The seizure risk associated with anti-depressant administration also varies between the type of drugs whereby maprotiline and amoxapine has a greater risk compared to doxepin, at smaller chances. However, the SSRI class is believed to have a reduced risk of inducing seizures (Pisani et al., 1999; Habibi et al., 2016). Another limitation includes the potential pharmacokinetic interactions between the anti-epileptic and anti-depressant medication *via* the cytochrome P-450 pathway involved in drug metabolisms. Anti-epileptic medications, including phenytoin and barbiturates, can induce P-450 isoenzymes (CYP1A2 and CYP3A4) which depresses the level of the antidepressant medications. In contrast, SSRIs such as sertraline and fluoxetine inhibit isoenzyme metabolism and elevate anti-epileptic blood levels (Trivedi and Kurian, 2007).

The common mode of actions anti-depressants share is their modulation of biological chemicals in the body, such as serotonin and norepinephrine. The beneficial effect of administering anti-depressants can be reaped by initiating the treatment in patients with epilepsy with careful surveillance of plasma drug levels of both types of medications, anti-epileptic, and depressant as well as for any pharmacokinetic interactions. The selection of antidepressants with the slightest interaction may be appropriate to develop a pharmacological tool to treat depressive symptoms in epileptic patients (**Table 1**).

TABLE 1 | Type of anti-depressant drugs and their effect on seizures.

Drug	Class	Mode of action	Effect on seizures
Imipramine Desipramine Amoxapine Doxepin	TCAs	Inhibit the uptake of norepinephrine and serotonin in adrenergic and serotonergic neurons	Lower the seizure threshold
Sertraline Fluoxetine Citalopram	SSRIs	Blocks the SERT which induces selective inhibition of serotonin uptake at the presynaptic neuronal membrane	Helps in seizures control
Desvenlafaxine Duloxetine Venlafaxine	SNRIs	Blocks the NET	Decrease in seizure frequency
Mirtazapine	NaSSAs	Antagonizing the adrenergic α2-autoreceptors and α2-heteroreceptors, blocks 5-HT_2 and 5-HT_3 receptors	Safe in epilepsy

TCAs, tricyclic antidepressants; SSRIs, selective serotonin reuptake inhibitors; SERT, serotonin reuptake transporter; SNRIs, serotonin-noradrenaline reuptake inhibitors; NET, noradrenaline transporter; NaSSAs, noradrenergic and specific serotonergic anti-depressants.

Depression in Epilepsy and the Gut-Brain-Axis

Depression and epilepsy may no longer be considered diseases of the CNS only. They appear more complex than that. Depression is also a co-morbidity of several other disorders such as obesity, irritable bowel syndrome (IBS), chronic fatigue syndrome, and type-2 diabetes mellitus (Slyepchenko et al., 2017). Emerging shreds of evidence have suggested a potential role of the gut microbiota in the pathophysiology of depression (Jiang et al., 2015; Zheng et al., 2016). However, there is limited literature which reviews the link between depression to gut microbiota by different groups of researchers (Slyepchenko et al., 2017; Patist et al., 2018; Schachter et al., 2018) (**Figure 1**).

The type of food an individual consumes will have its influences on the gut and its immune system. A high-fat diet (HFD) changes and interacts with the microbiota composition, activates intestinal mast cells, and elevates the secretion of pro-inflammatory cytokines from the macrophages (C, 2013). The intestinal microbiota may play a part impacting cognition, mood, anxiety, and depression, as well as CNS physiology. The microbial imbalance or maladaptation, also known as dysbiosis, have been suggested to correlate with various neurodegenerative disorders (Paoli et al., 2014; Jiang et al., 2017; Parashar and Udayabanu, 2017), autism (Evangeliou et al., 2003), depression (Murphy et al., 2004), multiple sclerosis (Storoni and Plant, 2015), seizure susceptibility in epilepsy (Dahlin and Prast-Nielsen, 2019), and cancer (Scheck et al., 2012; Allen et al., 2014).

Gut Microbiota-Depression

The promising effects of probiotics in depression were reported in few studies. Rats induced to have depression had decreased brain noradrenaline levels, and increased peripheral IL-6 secretion and amygdala corticotrophin-releasing factor mRNA levels. However, probiotic *Bifidobacterium infantis* treatment reversed the behavioral deficits, reinstate basal noradrenaline concentration, and normalized immune response (Desbonnet et al., 2010). A different probiotic strain *Lactobacillus rhamnosus* modulated the GABAergic system of normal, healthy mice (Bravo et al., 2011). The *L. rhamnosus* reduced $GABA_{B1b}$ mRNA expression in the hippocampus, amygdala, and *locus coeruleus* of the mice suggesting an antidepressant-like effect of $GABA_B$ receptor antagonists (Slattery et al., 2005). *L. rhamnosus* also altered the levels of hippocampal expression of $GABA_{A\alpha1}$ and $GABA_{A\alpha2}$ mRNAs, which may have contributed to the antidepressant-like behavior of the animals. The effects of *L. rhamnosus* were abolished in vagotomized mice, reinforcing the contribution of the vagus nerve in relating the gut and the brain (Bravo et al., 2011). Germ-free (GF) mice exposed to forced swimming test have decreased depression-like behavior compared with specific pathogen-free mice. However, GF mice with microbiota originating from MDD patients, have elevated depression-like behavior compared to GF mice with microbiota derived from normal, healthy individuals. The microbiota of MDD patients was characterized by considerable changes in *Firmicutes*, *Actinobacteria*, and *Bacteroidetes* abundance (Zheng

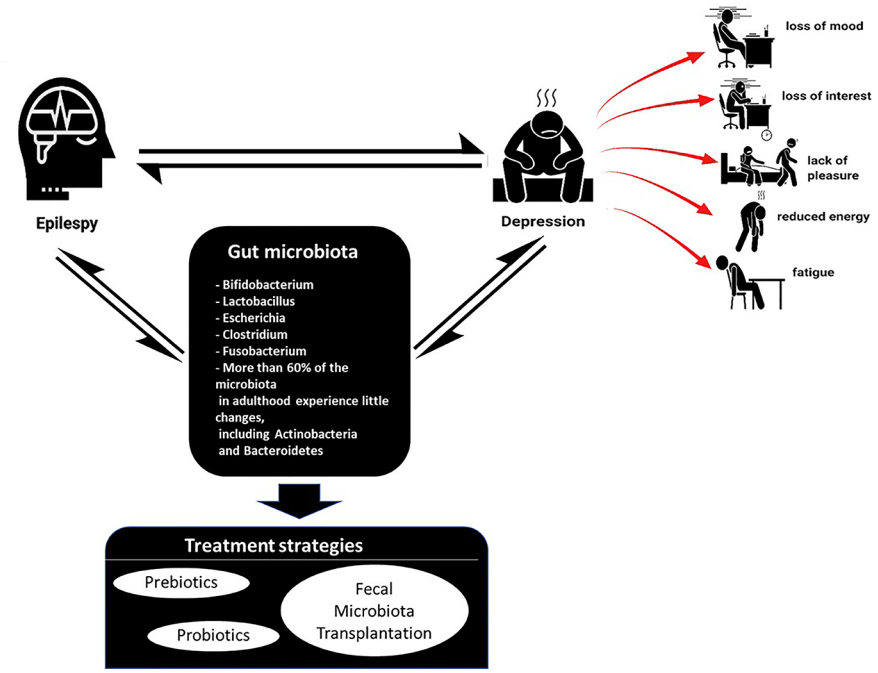

FIGURE 1 | Commonly found gut microbiome in epilepsy and depression & possible treatment strategies.

et al., 2016). Analysis of fecal microbiota of MDD patients by other groups of researchers also showed changes in a relative abundance of *Bacteroidetes* although the shift in microbial compositions regarding healthy controls differed across studies (Naseribafrouei et al., 2014; Jiang et al., 2015).

Several labs have shown that anxiety-, depression-like behavior, and cognitive impairment occurring from gastrointestinal disease, a high-fat diet or antibiotic usage linked with abnormalities in the gut microbiota. However, supplementation by specific *Lactobacillus fermentum* NS9 strain restored and regulated the microbiome of the gut (Hu et al., 2015; Wang et al., 2015). The likelihood of depressive symptoms rooted in abnormal gut microbiota is further explored, whereby it was found that depressed rats possessed different microbiota as compared to control rats. Citalopram was given and it alleviated the defects but did not restore the microbiota, whereas *Lactobacillus helveticus* NS8 treatment improved both situations (Liang et al., 2015), suggesting the pivotal role of commensal microbiota in mental disorders.

Gut Microbiota-Epilepsy

There is evidence which suggests a relationship of gut microbiota in epilepsy. A case-report involving a 22-year-old Crohn's disease patient received fecal microbiota transplantation (FMT) treatment and displayed seizure-free events despite discontinuation of antiepileptic drug with sodium valproate during the 20 months follow-up (He et al., 2017). It was also revealed that probiotic treatment decrease seizure frequencies (at least 50%) in 28.9% of the patients with drug-resistant epilepsy (Gomez-Eguilaz et al., 2018). Moreover, a study illustrated the differences or the alterations of gut microbiome composition in patients with drug-resistant epilepsy (n = 42) and patients with drug-sensitive epilepsy (n = 49) whereby in drug-resistant patients, there were more abundant rare bacteria from the phylum *Firmicutes* as compared to the latter. Through this study, it was also found that *Bifidobacteria* and *Lactobacilli* incur less than four seizure events per year (Peng et al., 2018).

Additionally, a diet that has shown positive effects on neurological disorders and neurodegenerative diseases is known as the ketogenic diet (KD). Of note, KD is an established therapy alternative for therapy-resistant epilepsy treatment or medically refractory epilepsy in children only. KD is a high in fat with adequate protein intake, and low-carbohydrate diet, at the ratio of fat to protein and carbohydrates, 3:1. Hence, approximately 70–90% of the energy intake will be obtained from fat. Treatment with KD resulted in a more than 50% reduction in the seizure in about half of the treated children (Freeman et al., 1998; Neal et al., 2009). The mode of action behind the KD is the diet induces ketosis. Subsequently, the ketones are utilized as a substitute substrate for cellular ATP production (Dahlin and Prast-Nielsen, 2019). The metabolic shift incurs biochemical, hormonal, and metabolic changes which may cause a decrease in neuronal excitation and seizures events. However, ketosis may not be solely responsible for seizure response and there could exist several other hypotheses of the modes of action in KD which could play a more prominent role.

Through this diet, it may affect gut health. It was recently found that the relative abundance of the beneficial gut bacteria, bifidobacteria, *E. rectale,* and *Dialister* decreases while *E. coli*

increases in a therapy-resistant epileptic patient after KD diet for 3 months (Lindefeldt et al., 2019). Prebiotics can restore the number of bifidobacteria, while reducing *E. coli* and enterococci in human trials (Turroni et al., 2016) and probiotics reduced the number of seizures by more than 50% of epileptic patients receiving probiotics as a complementary treatment (Gomez-Eguilaz et al., 2018). Therefore, there is a need to understand the effect of KD-induced changes in gut microbiota on the therapeutic effects of KD, the influence of KD on the overall gut health, and if concurrent intake of prebiotics or probiotics is required during a KD treatment.

Gut Microbiota-Depression in Epilepsy

Gut microbiota alteration has distinctively shown to be parallel with the pathophysiology of depression and epilepsy. From present evidence, the link between microbiota profile in MDD and epilepsy remains speculative. However, there is indirect evidence drawn from other studies that may provide insight into common, specific bacteria strain indicative of depression in epilepsy. As mentioned earlier, MDD is a co-morbidity of IBS. Importantly, IBS elevates epilepsy risk (Chen et al., 2015). Similar to MDD, IBS patients have increased gut permeability (Zhou et al., 2009; Gecse et al., 2012), resulting in an influx of LPS (Dlugosz et al., 2015), and elevated pro-inflammatory cytokines (Liebregts et al., 2007; An et al., 2016). The alterations of the fecal microbiome under the influence of KD in children with epilepsy were investigated. After 3 months on KD intervention, it was revealed that the relative abundance of *Bifidobacterium* was considerably lowered in patients through whole metagenomic sequencing, suggesting the role of the microbiota in seizure susceptibility and the potential anti-seizure efficacy in KD treatment (Lindefeldt et al., 2019).

IBS patients who also have MDD are less responsive to psychotherapy and antidepressants (Whorwell et al., 1987; Drossman et al., 2000), hence maintaining the makeup of gut microbiota through prebiotics and probiotics appears to be an ideal treatment for depression in epilepsy. In support of this notion, research has indicated that depression, anxiety, and panic attacks (Schnorr and Bachner, 2016; Liang et al., 2018a) may be from abnormal gut microbiota. The robust link between depressive symptoms to the gut microbiota can be seen in research conducted whereby the GF or microbiota-depleted animals presented defective brain development and abnormal mental growth (Luczynski et al., 2016; Chen et al., 2017), indicating that both neuroplasticity and myelin plasticity is regulated by the gut microbiota. In light of this, regulation or rectifying the microbiota abnormalities could alleviate the disorder by improving the microbiota-gut-brain axis and growth of the brain and behavior. Hence, the remedial or beneficial effects from microbiota regulation or therapies targeting the microbiota have continuously gained popularity through attempted treatments by microbiota intervention using probiotics, prebiotics, and fecal microbial transplantation.

Overall, the possible link between the gut microbiota and depression in epilepsy has enhanced the research interest in the gut microbiota as a therapeutic agent. The gut microbiota plays an important role in regulating the host's cognitive functions, mood, and emotion through the microbiota-gut-brain axis, consisting of three pathways 1) nerve, 2) neuroendocrine, and 3) immune pathways (Liang et al., 2018b). The gut microbiota is able to regulate neurotransmitter synthesis *via* modification in neurotransmitter-based metabolism pathways and possibly impacting the expression of neurotransmitter-related genes. Different bacteria strains synthesize different neurotransmitters such as Bacillus and few lactic acid bacteria (LAB) strains, synthesize acetylcholine and catecholamines (Wall et al., 2014) whereas Candida, Streptococcus, Escherichia, and Enterococcus can synthesize 5-HT (Holzer and Farzi, 2014), which makes up 90% of the body's 5-HT (Margolis et al., 2014). Various coryneform and LAB strains make glutamate (Glu), and synthesize GABA (Mazzoli and Pessione, 2016). The neuroactive property of gut microbiota regulation could be further exploited as a potential therapy or treatment in depression in epilepsy as the root of both depression and epilepsy is speculated to originate from dysfunction in neurochemistry.

Alternative Treatment Therapies for Depression in Epilepsy

Alternative treatments also include various psychotherapies, pharmacotherapies, and their combinations. The efficacy of psychotherapies in depression was reported to be possibly better than anti-depressants in decreasing relapse (Thompson et al., 2010; Walker et al., 2010). It was also shown that stimulation of vagal nerve was efficient for treatment-resistant epilepsy and depressive patients with sustained effects over time by elevating brain-derived neurotrophic factor (BDNF) expression in the rat brain by phosphorylation of TrkB (Furmaga et al., 2012). However, the combination of drug- and therapy-based treatment such as vagus nerve stimulation needs to be carefully considered. It has been demonstrated by a mouse model study whereby it employed a combination of SSRIs pharmacotherapy, fluoxetine, and fear-extinction training. This combination eliminated conditioned fear as compared to individual treatment which was ineffective and did not elicit any beneficial response (Karpova et al., 2011).

The supplementation of probiotics as adjuvant therapy for alleviating symptoms of depression in epilepsy may be proposed to manage the disorder better. As depression in epilepsy encompasses is a heterogeneous disorder, manipulation of the gut microbiota may offer a similarly effective treatment as compared to drug-based medication. Despite the immediate occurrence of the physiological effects in most antidepressants, the therapeutic effect is only apparent after continued usage for weeks and in some patients (15–30%), the associated side effects may discontinue its usage (Gartlehner et al., 2005).

Lately, a new term has been coined, encephalobiotics, consisting of probiotics, prebiotics, postbiotics, microbes, microbial parts, or agents that could manipulate the microbiota for improvement of cognition (Prescott and Logan, 2016). In contrast, psychobiotics are live bacteria (probiotics) that confer remedial mental benefits upon ingestion in patients suffering from psychiatric illnesses by interacting with the

commensal gut bacteria (Sarkar et al., 2016). By applying these therapies to alleviate the symptoms of depression in epilepsy, it could indeed eliminate certain barriers for effective treatment. However, the mechanism of action of these therapies as a treatment for depression in epilepsy needs to be further elucidated. One may propose the mode of action involves the regulation of inflammatory markers and neurotransmission (Wallace and Milev, 2017). It would be worthwhile to investigate the connection between the central and enteric nervous systems as they may work in tandem to produce the beneficial effects of biotics supplementation. More future studies including more in-depth animal and human studies alike, are needed before these therapies can be regarded as a front-line treatment for alleviating the symptoms of depression in epilepsy.

Despite the new strategies, the process of selecting the type of therapy and its suitability to the patient is complex as it takes into account many playing factors such as the specific syndromes experienced by the patient, records of the illness and its corresponding treatment, physical comorbidities, risk of inducing drug interactions, and lastly, patient compliance toward the treatment. Moreover, it is imperative to provide considerable evidence regarding any additive and synergy of combined therapies and medication for depression in epilepsy treatment. Epileptic patients who experience emotional and mental deterioration must be recognized promptly and diagnosed accordingly. This is possible through dependable screening instruments and executing the appropriate medical support or referral to a professional such as a psychiatrist. Overall, the present paper has analyzed the hypothesis which proposed the influence of gut microbiota in depression in epilepsy. Detailed studies are needed for conclusive evidence to understand further the role and the manipulation of the gut microbiota in the gut-brain axis to increase its applicability and relevance in treating patients with depression in epilepsy using alternative treatment strategies.

AUTHOR CONTRIBUTIONS

MS and CL conceptualized the idea. MS, CL, WC, and FS had contributed in the literature review and manuscript writing. MS and WC have revised and edited the manuscript.

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REVIEW
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Establishing Drug Effects on Electrocorticographic Activity in a Genetic Absence Epilepsy Model: Advances and Pitfalls

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The genetic rat models such as rats of the WAG/Rij strain and GAERS were developed as models for generalized genetic epilepsy and in particular for childhood absence epilepsy. These animal models were described in the eighties of the previous century and both models have, among others, face, construct and predictive validity. Both models were and are currently used as models to predict the action of antiepileptic medication and other experimental treatments, to elucidate neurobiological mechanisms of spike-wave discharges and epileptogenesis. Although the electroencephalagram (EEG)/electrocorticogram (ECoG) is imperative for establishing absence seizures and to quantify the for absence epilepsy typical spike-wave discharges, monitoring the animals behavior is equally necessary. Here an overview is given regarding the design of drug evaluation studies, which animals to use, classical and new EEG variables, the monitoring and quantification of the behavior of the rats, some pitfalls regarding the interpretation of the data, and some developments in EEG technology.

Keywords: WAG/Rij's rats, antiepiletic drugs, EEG-behavioral relationship, genetic absence models, spike-wave discharges (SWDs)

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INTRODUCTION

Predictive validity is the key word in preclinical animal models. This implies that results of putative new treatment in humans, often new drugs, can be predicted correctly based on results obtained in the models. This was illustrated in the GAERS and WAG/Rij models by showing that the classical anti-absence drugs such as ETX and VPA, as well as benzodiazepines, correctly predicted their action in patients with absences. The outcomes of the studies in the models can be considered as correct predictions, since these antiepileptic drugs suppress dose and time dependently, absence seizures, better, the more easily quantifiable EEG hallmarks of absences, spike-wave discharges (SWDs) in the models. A good EEG signal is imperative for the evaluation of drug studies on SWDs. For an overview of some of the most commonly used antiepileptic drugs that were evaluated in these two models see e.g. Depaulis and van Luijtelaar (2006). Since then very few new antiepileptic drugs were introduced in the market, and none new antiepileptic drugs were specific aimed for the treatment of absence epilepsy. The models were also successfully used to predict an aggravation of SWDs in patients, good examples are the GABA-mimetics tiagabine and vigabatrin, and various

sodium channel blockers, such as lamotrigine and the classical anticonvulsants phenytoin and carbamazepine. These results as obtained in the genetic models can be considered as correct rejections based on predictions from the models. There is some discussion regarding whether the results as obtained with lamotrigine were correctly predicted in the models, or whether it is a false rejection, implying that it is effective in patients, but not in the genetic models. Lamotrigine has some efficacy in patients although less than VPA and ETX (Glauser et al., 2010), in WAG/Rij rats a decrease in the incidence of SWDs was found as well, but only at a sedative dose of the compound (van Rijn et al., 1994).

Many other drugs and ligands were tested in the models for studying mechanism and processes affecting absence seizures, such as cytokines (van Luijtelaar et al., 2012a; Kovács et al., 2015; Russo et al., 2014), antidepressant and antipsychotic drugs (Russo et al., 2016), different ion channel openers and blockers, NO synthase inhibitors or NO donors (Przewlocka et al., 1996), nucleosides (Kovács et al., 2014), CB1 receptor agonists (van Rijn et al., 2010; Citraro et al., 2013), positive and negative allosteric modulators of mGluR (Celli et al., 2019; Ngomba and van Luijtelaar, 2018), and neuropeptides (for review see e.g. van Luijtelaar and Zobeiri, 2014; Russo et al., 2016). Currently the models are used to predict the effects of new T-type channel blockers (Tringham et al., 2012; Remen et al., 2016), for the study of antiepileptogenesis (Blumenfeld et al., 2008; Sarkisova et al., 2010; van Luijtelaar et al., 2013; Russo et al., 2016; Leo et al., 2019), for the odyssey toward ultimate causes of absence epilepsy in this model, and for experimental treatment techniques for absence epilepsy such as various types of non-invasive and invasive electrical or magnetic stimulation (Godlevsky et al, 2006; van Luijtelaar and Zobeiri, 2014; van Luijtelaar et al., 2017).

Absence Seizures

The presence of these absences can only reliably be established with an EEG; absences manifest themselves in the EEG as bilateral synchronized, asymmetric around the zero-line, and generalized SWDs. There are clinical concomitants of SWDs in rodents, but analog to what can be seen in children, these absences remain often unnoticed. Accelerated breathing, rhythmic movements of the vibrissae, incidental eye-blinks, very mild facial myoclonus, and head tilting in an otherwise immobile animal can be noticed concomitant to the SWDs in the EEG in the WAG/Rij model (van Luijtelaar and Coenen, 1986), see **Figure 1**. The trains of SWDs lasting from 1 to up to 30 s, in WAG/Rij the mean duration is about 5 s, sometimes a bit longer (7–8 s, in some labs shorter, ca 3 s) and WAG/Rij's at 6 months have about 16–20 SWDs per hour, adding several hundred SWDs per 24 h. Shorter than 1 s SWDs are not included by us, since their clinical relevance is not certain, or their morphology remains embryonic. The awareness of elapsing time is disturbed during the SWDs in WAG/Rij rats (van Luijtelaar et al., 1991), suggesting a decrease in the level of consciousness. Here we describe in some detail methods for the evaluation of drugs on

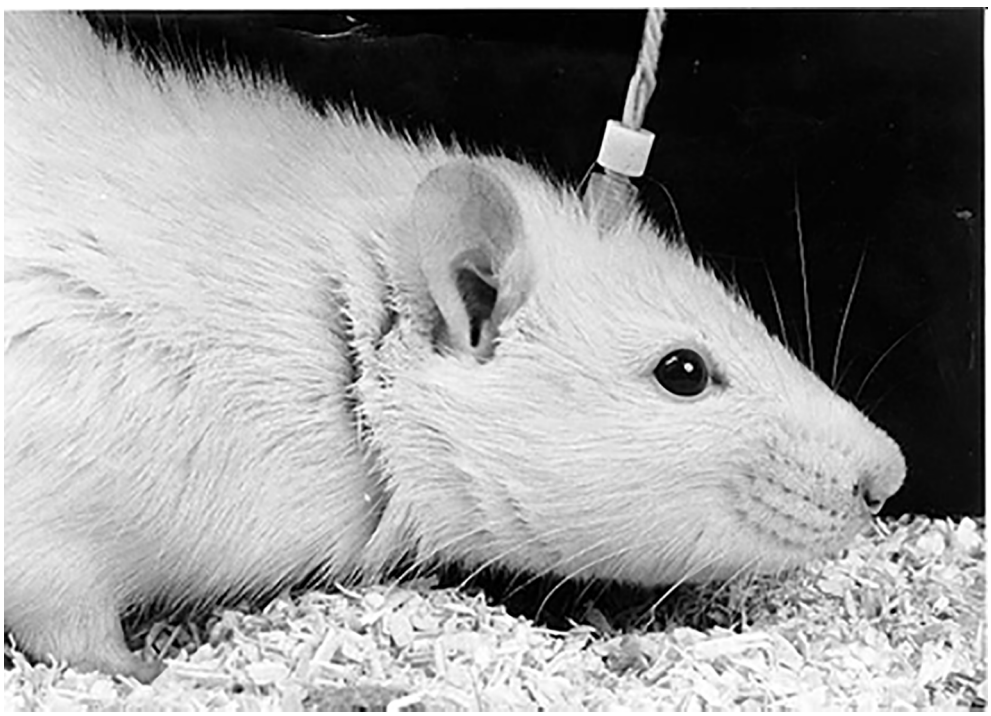

FIGURE 1 | The free moving WAG/Rij rat equipped with a chronically implanted tri-polar Plastic One electrode set connected to a preamplifier and amplifier allowing to quantify number of spike-wave discharges (SWDs) before and after the administration of compounds (Photo H. van de Sluis).

the occurrence of the for absence epilepsy typical SWDs with the aid of the EEG, and some pitfalls regarding the interpretation. The most straightforward parameters will be mentioned as well some auxiliary ones, as well as some other approaches regarding data analyses. Finally older and more recent EEG recording techniques in free moving animals will be briefly discussed.

THE DESIGN OF THE STUDY: WHICH RATS TO USE

It is crucial that the rats used for the evaluation of a compound have a sufficient amount of SWDs, allowing to establish an increase, and a decrease in seizure activity. In case rats have a low number per time unit, than it might be difficult to further decrease their number. This was the case after rats were chronically exposed to ETX in order to induce antiepileptogenesis. In that case the drug VU0360372 showed only a rather small decrease compared to the action of the same drug in the untreated sham control group (D'Amore et al., 2016). Considering that 2 months old WAG/Rij have few SWDs, and not all animals show clear and unambiguous SWDs, and the age dependent increase in incidence of SWDs, it is recommended to use WAG/Rij's at an age of 6 months; than it is our experience that they have a sufficient amount of SWD's to find drug and dose related effects and both an increase and decrease in number and mean duration of SWDs can be found (van Luijtelaar and Coenen, 1986; Schridde and van Luijtelaar, 2004; Bouwman et al., 2007; Remen et al., 2016). In case strong anti-absence effects are anticipated, slightly younger, so 5 months old animals could be used as well. It is not known whether younger animals could be used to establish a pro-absence action. It is our experience though that well known proabsence drugs do not show their pro-absence action in presymptomatic (ca. 2 months old) WAG/Rij rats. GAERS have more SWDs, and they occur more early during ontogeny compared to WAG/Rij rats (Jarre et al., 2017). This has the practical consequence that the base-line (=pre-drug) EEG recording period might be shorter (often 20 min periods were used), and that younger GAERS were successfully used for the evaluation of putative antiepileptic medication. A note of warning is that the incidence of SWDs may vary between labs as a consequence of long term housing in another environment, and genetic drift. Mainly early environmental factors were described affecting incidence and duration in WAG/Rij rats, e.g. maternal (Peeters et al., 1992b; Sitnikova et al., 2016; Sarkisova and Gabova, 2018) and housing effects (Schridde and van Luijtelaar, 2004). Moreover, large differences in incidence were described in the GAERS model between labs in different continents as well (Powell et al., 2014).

Male Versus Female Rats

The incidence in children with absence epilepsy is larger in girls than in boys, this suggests for a role of the sex chromosomes. Female WAG/Rij rats have as much SWDs per hour as male rats (Coenen and van Luijtelaar, 1987), the same results were obtained in the GAERS model (van Luijtelaar et al., 2014). All the genes involved in absence epilepsy are not discovered as yet and absence epilepsy has a polygenetic origin, none of the known genes are localized at the sex chromosomes. This fact does not preclude the use of female rats. The sex ratio in humans point toward the a sex linked genetic contribution in humans, which is, as mentioned, not present in both Wistar derived models. Female rats and mice are five times less often studied within the field of pharmacology (Beery and Zucker, 2011). Most often indeed male rats of the WAG/Rij and GAERS strain were used to establish effects of drugs; a small disadvantage of the usage of female rats is that the physiological fluctuations in the concentration in the ovarian hormone progesterone in the reproductive 4–5 days cycle modulate the number of spontaneously occurring SWDs in WAG/Rij rats (van Luijtelaar et al., 2001). For about 6 h at proestrus day the number of SWDs is increased compared to the same hours of the other days of the cycle. Specifically at these hours the plasma level of progesterone is enhanced. To control for the ovarian cycle is rather laborious. The higher number of SWDs in some hours of the proestrus days may contribute to a higher between subject variability in acute studies and a higher within subject variability in chronic studies. However, whether this intrinsically more variable number of SWDs in female rats is a sufficient reason for not using females for the evaluation of putative new anti-absence drugs is doubtful since the desired effects of a putative new drug should be robust and a small increase in within and between subjects variability should hardly matter the conclusions whether the new compound is effective or not. Therefore, although female GAERS and WAG/Rij rats were rarely used for drug studies, there are no apriori reasons to use only male subjects. The usage of female subjects for pharmacological studies will also contribute to reducing the number of surplus animals.

Should independent groups of rats be used? Although it is rather common to use independent groups of animals for different doses of a systemically administered compound in an acute pharmacological study, this is not always absolutely necessary. Once rats are implanted with EEG recording electrodes, they can be used repeatedly, among others for different doses of a compound. If this is indeed done, one should control for order effects in order to avoid that order of the drug administration is mixed with dose of the drug. A cross-over block design or counterbalancing for order and dose might prevent this. Next, a sufficient large enough washout period should be used before the animals can be used for a second dose, depending on the half-life of the compound. It is not uncommon to use a 48 h period for this. By starting every new dose with a base-line (=pre-drug) recording session, the putative cross-over, neuroplastic, and wash out effects can be noticed and be controlled for.

It is our experience that a group size of eight animals per dose is generally sufficient to establish reliably the effects of a (dose of a) compound. A solvent control in studying the effects of a compound is very necessary for two reasons: handling animals for the injection, as well as restraining them to connect them with the EEG recording leads, or putting them in a clean EEG recording cage may induce some stress for the animals (especially when mice are used) and stress affects the level of corticosteroids, also in epileptic rats (Tolmacheva et al., 2012). Both stress and steroid hormones are known to have a biphasic effect on SWDs and even the anticipation

to an upcoming stressor affects the number of SWDs (Tolmacheva et al., 2012). This might be relevant in case of repeated drug administration studies in which animals get daily injections and the anticipation of this may influence the incidence of SWDs. Once more, a predrug administration EEG recording session will give insight whether there are cross-over effects of a previous treatment or other effects might have happened. A second reason for having a solvent control group is that some solvents, e.g. Tween 80 and a mixture of saline/ethanol/propylene glycol, even when administered systemically, increase the incidence of SWDs (Peeters et al., 1992a). But also i.c. administration of solvents might have an effect on the incidence of SWDs (Tolmacheva and van Luijtelaar, 2007).

In case different doses are used and the relevant doses needs to be explored, it is common to have three different doses, differing a factor 3 from each other. Most often, the effects of a compound vary in time, and dependent on the T max and half-life of a compound the time epochs in which the SWDs are quantified: some drugs have a quick T max and short half-life, and this demands for example 15 min time intervals (as was the case after tiagabine, Coenen et al., 1995) and a post drug recording of 90 min, while other drugs are more slow to be eliminated and the number and duration per hour describes adequately the dynamics of the drug response, this has been the case in for example vigabatrin, some of its effects on incidence lasted 6 h (Bouwman et al., 2007). Therefor it can be expected that other effects of this compound such as a change in spectral power density or mean duration will last more than 48 h. Another example is the experimental anti-absence drug RO0711401, a mGlu1R PAM: it showed a more than 6 h lasting effect on SWD incidence (Ngomba et al., 2011).

Adaptation the animals to the recording leads and cage (for example 24 h) and to the recording room and handling them for a few minutes per day prior to the actual day of the start of the EEG recording session contributes to obtain a reliable and representative EEG measurement. It is our experience that a 2 h base-line recording period prior to the administration of the compound yields a good indication about a subjects hourly number of SWDs and this serves as a solid base to determine changes in the SWD occurrence. Since environmental noise or sudden changes in noise level or ambient light may interrupt ongoing spontaneous behavioral activity, sleep and also SWD, the environment of the recording conditions is critical, without and shielded from environmental noise, and without the presence of equipment and the experimenter in the EEG recording room.

The recording cages should be placed in a light-dark controlled room, similar regarding light on and off as in the vivarium; if not, the animals should be given time to adapt to the shifted LD cycle, it is thought that the speed of adaptation of sleep and SWDs is about 1 h per day (Smyk et al., 2019b).

TIME OF DAY EFFECTS

The states of vigilance, such as wakefulness, slow wave sleep, or REM sleep, highly influence the occurrence of SWDs. The SWDs preferentially appear during passive wakefulness or during instable vigilance periods such as changes from active wakefulness to passive wakefulness to light slow wave sleep (Lannes et al., 1988; Coenen et al., 1991; Drinkenburg et al., 1991; Smyk et al., 2019a). SWDs rarely occur during deep slow wave sleep, active wakefulness, or REM sleep. Especially deep slow wave sleep, occurring at the beginning of the light period in nocturnal animals, is notorious for its incompatibility with SWD occurrence and during these hours of the 24 L-D cycle rats have indeed few SWDs (van Luijtelaar and Coenen, 1988). The beginning of the light period with its low incidence is an unfavorable period to establish effects of a compound on SWDs. Therefore it is recommended to test the putative anti-absence effects during the dark period, and especially in the first few hours of the dark period the daily maximum in the number of SWDs is reached and the putative anti- or proabsence action can be found more easily than during the light period. Off note, it is imperative to test all animals and all doses at the same time of day in order to control for circadian effects on the occurrence of SWDs, next to the effects of sleep, as well that the compound's efficacy might depend on the time of day. Circadian rhythmicity on the occurrence of SWDs, albeit difficult to disentangle from the effects of sleep, are well known to be present in absence patients as well as in WAG/Rij rats (van Luijtelaar and Coenen, 1988; Smyk et al., 2011).

The Technique of EEG Recording

The principles of the EEG recording techniques as used in freely moving animals, reviewed by Coenen et al., (2004), have not changed much, while quite some technical advancements were made. The techniques, as described by Coenen et al. are still state of the art, giving the aims of the recordings, that is establishing whether an acute or chronic administered drug has putative anti- or proabsence effects and on whether the EEG is modified. The latter requires a clean EEG without movement artefacts. However, some technical innovations may open the way for more robust, longer, and more channels recordings.

The nature of the weak electrical signal (microvolts) from the brain demands that it has be amplified before it can be monitored, stored and processed in an adequate way. Commonly, a differential amplification is applied, implying that the differential voltage between the active electrode and ground and between the reference electrode and ground, is used yielding a signal representing the voltage difference between the active and reference electrode (differential recording). The advantage is that the common signals that appear simultaneously and in-phase in both inputs from the active and reference electrodes, often artefacts, are suppressed. This suppression of the common signals is expressed in the "common mode rejection ratio" of the amplifier. Operational amplifiers (OpAmps) are commonly used as EEG amplifiers: they are high-gain electronic voltage amplifiers with an input from an active electrode and one from a reference electrode, both measured against ground, and a single-ended output, which is displayed as the EEG signal between the active and reference electrode. In this configuration, an OpAmp produces an output potential (relative to circuit ground) that is typically hundreds or thousands of times larger than the potential difference between its input

terminals. Nowadays OpAmps are available with rail to rail capabilities: this means that they can handle analog signals up to the supply voltages on both the positive and negative rails. A "rail" is a boundary that a signal has to work within, giving it a wide range in amplitudes. Rail to rail implies that OpAmps are able to operate at low supply voltages, relevant in long lasting wireless EEG studies.

Before the EEG signal can be obtained from the OpAmp, a so-called pre-amplifier or front-end amplifier, placed as closely as possible to the signal source, is often used to transfer the small signals from a high resistant circuitry, which is sensitive to all kinds of interfering signals and noise, to a low resistant circuitry, insensitive for disturbing signals. We put the pre-amplifier in the cable plug to the headstage. Also the moving EEG cables, connecting the animals with a swivel, due to grooming, or during an epileptic insult, become relatively insensitive for the induction of interfering signals. Finally, the amplifier should be completely isolated from earth, which guarantees an optimal safety for experimental subjects.

The EEG signal has in principle an infinite bandwidth. Commonly, however, the interest is in a restricted bandwidth in which the relevant phenomena occur. For the EEG this is generally in the range between low frequencies (>0.1–1.0 Hz) and high frequencies (<40–100 Hz). Hence, band-pass filtering is necessary with a high-pass (blocking low frequencies) and a low-pass (blocking high frequencies) filter. Frequencies below 0.1 Hz are difficult to measure in a reliable way, due to gradual changes in properties of recording electrodes or areas around the electrode tips. These slowly changing voltages, called DC-offset or drift, are the reason that by preference an AC ("alternating current") amplifier is used, rejecting these slow voltages. For a DC ("direct current") recording (from 0 Hz on) extra measures to reduce offset and drift have to be taken (e.g. by the use of non-polarization electrodes). DC measurements become more and more available and there are indications that seizures, including the generalized SWDs, characterizing absence seizures, are accompanied or preceded by DC shifts.

Depending of the specific experimental goals, adjustment of the high-pass filter from 0.1 to 1.0 Hz is acceptable in most cases, although the rather slow oscillations previously often not recognized or ignored, might have a function in coordinating other electrographic sleep waves such as epochs of delta activity, sleep spindles, and gamma oscillations in recurring groups (Crunelli and Hughes, 2010). The low-pass filter was traditionally set as low as possible, beginning with 30 Hz, over the years more often at 70 Hz or recently higher (200 Hz) to measure also gamma-oscillations (40–80 Hz). Restriction of the bandwidth is done in order to reduce noise, which is not only dependent of circuitry-resistance, but also of bandwidth. The noise of the 50 or 60 Hz electrical net ("hum") is another complication. Shielding of all cables reduces the problem and the use of a cage of Faraday, an electrically shielded and often sound attenuating chamber in which no electromagnetic radiation can enter, protects the biological signals further from disturbances in the environment. Another solution is a "notch"-filter, specifically designed to block the 50 or 60 Hz hum of the electric network. Nowadays often non-filtered signals are collected; the presence and availability of excelled digital filters allows to filter signals off line, and in some cases on-line. The advantage of recording of non-filtered signals is that apriori no information is removed (=filtered), a disadvantage could be that it is difficult to check the quality of the EEG signals and to monitor a smooth EEG recording session with unfiltered EEG signals. In case high end computers are used, on-line filtering can be performed on the recorded raw EEG and be displayed directly on the monitor. Digital filtering is preferred above analog filtering given that analog filters cause more phase delays, the delays encountered by the digital filtering can be minimal and depend on the quality and speed of the soft-ware.

The unfiltered EEG is sensitive for DC shifts. The amplitude of DC shifts might be up to milliV, and much larger that the amplitude of the EEG (microV). It is our experience, that the direction of DC shift may vary in direction and amplitude across time and space and so it could be different for different electrodes; the DC at the reference electrode could be positive, and simultaneously negative at the active electrodes. Then EEG clipping can easily occur and its accompanying loss of the EEG signals.

The signals from the animal are *via* a connecting cable connected to a swivel, allowing free movement of the animal. Various types of swivels are available, non-motorized and motorized, all with a minimal rotational torque. In case of a large number of channels (> 8), a motorized commutator might be considered. These swivels prevent the connecting cables from tangling and it permits continuous full rotations in any horizontal direction. By adding a spring in the suspension of the EEG cables and an extra loop there are no restrictions toward movements in the vertical direction (rearing). An elegant way to compensate for the weight of the cable in case of mice or young rats are used, is to have a counter weight or counter balance arm allowing the animal to move up and down with its head, lie in different sleeping positions, or stand on its hind legs while still keeping enough tension on the cable to lift or lower the cable, minimizing the cable putting strain onto the animals head, head connector plug or precious headstage.

Finally, the EEG can be recorded and the analog signals have to be digitized. In order to ensure an adequate analysis of the EEG the sampling frequency should be at least twice the highest frequency of interest in the recording (Nyquist's theorem). The signal is best sampled with a frequency of a power of 2 (2n) (e.g. 256, 512, 1,024 Hz) for a standardized frequency distribution in the spectral analysis. A common sample-frequency at a cut-off of 70 Hz is 256 samples per second. The AD converter should have a resolution of 12 bit. Digitization and data storage are done with a computer using for example a Windaq (Dataq Instruments) acquisition systems, which allows the simultaneous recording and monitoring of multiple channels or animals. Usually there are differences in activity between different brain structures, and

the epileptic activity might be generalized to appear only local. Therefore, multiple electrodes may be used to give an adequate reflection of the underlying activity of these different structures.

Due to better electronic component developments, miniaturization, and design of dedicated special purpose components, multichannel-analog amplification, analog-digital converter, encoder, and transmitter might be combined in one single ultra low power component. This allows the design of wireless biosignal transmitters that have adequate size for implantation in small rodents (including mice). The data can be transmitted *via* radiotelemetry and picked up by antenna's placed around on under the cage, or *via* WiFi or Bluetooth technology *via* a small microcontroller. This microcontroller can already enhance the recorded data by controlling the active ground or reference up to differentiating (compare) local EEG channels up to generating feedback stimuli toward the test animal.

Wireless technology provides the possibility of recording EEG while the animal is engaged in a behavioral test or social interaction test without being hampered by conventional connecting cables, during tonic-clonic seizures without the risk of interfering cables or outside the lab environment (Vyssotski et al., 2006). Wireless recordings prevent mechanical problems as present in hardwired system that makes contact with the free moving animal, and are electrically safe. For a recent example of a multichannel wireless device see Ball et al. (2014).

Instead of the traditional passive electrodes, active electrodes that use active feedback to each electrode can be used. The signal that is sent to active electrodes is the signal from the reference electrode. It can be either a fixed reference electrode, or the mean of a number of active electrodes, called a common mode reference. It is possible to adjust recording settings like sensitivity and or sample rate by sending these adjustments back to the headstage of the animal. Reducing amplification and sample rate can slow down computations in the headstage and lower the amount of data that has to be transmitted. These reductions will result in an enormous reduction of power consumed by the headstage electronics, thus increasing the operational recording time in case wireless technology is used by a longer battery life running time.

Currently the limitations are still the power supply (batteries) of the headstage electronics in wireless systems. Systems are currently under development that can work with a wireless charging of the batteries. However it is unknown whether long time and close exposure to energy-field necessary to charge the batteries could be hazardous for the health and over all wellbeing of the animal. It might also influence the normal natural behavior of the animal (depending on charging performance in relation to magnetic field strength and used charging frequency next to the magnetic field and power of the WiFi- and Bluetooth-frequencies used at close ranges).

Ideally an EEG system would consist of a DC capable measuring multi channel system with high impedance connected to a fast multi channel and high-bit resolution analog to digital converter (with a minimum of 12-bits per channel) recorded by a powerful computer. Data are recorded and stored on hard disk and data are filtered/rectified/analyzed/presented (on screen)/fed back to an active ground/or trigger feedback for subjects evoking a response, such as in case with closed loop deep brain electrical stimulation.

EEG Electrode Implants

Since the SWDs are generalized and bilaterally symmetrical, registration in a single hemisphere is sufficient, moreover, the exact locations of the cortical EEG recording electrodes do not matter for a large extent after it is clearly established that the oscillations are genuine SWDs, and indeed generalized and bilateral symmetrical, and have the wave form of spike-and waves, as was the case in WAG/Rij rats (Meeren et al., 2002; Sitnikova and van Luijtelaar, 2007). Sleep spindles or some high voltage spindles may appear locally. Many details regarding the surgery can be found elsewhere, regarding the location of the EEG electrodes it can be mentioned that we have excellent experiences with a simple and economical surface electrode implantation and recording system with very few cable movement artefacts. First, we implant three epidural electrodes, most common one is implanted above the frontal cortex, (2 mm anterior to bregma and 2 mm lateral). This location above the frontal cortex is excellent for recording delta waves, which are more clear expressed at the frontal cortex than at the parietal cortex, as well as for recording the most clear SWDs as the amplitude of the spikes of the SWDs in WAG/Rij rats is most pronounced expressed at the frontal cortex (Midzianovskaia et al., 2001; Meeren et al., 2002). A second electrode is preferential at the parietal cortex, ca 4 mm lateral and 6 mm posterior to bregma. It serves as a second active electrode or as a reference and it records theta activity originating from the hippocampus beneath the cortex. This might be necessary in case sleep and REM sleep need to be analyzed. For a clean EEG, a third, earth, electrode is helpful, it enables a good electrical potential measurement of all other (active and reference) electrodes. The usage of an earth electrode enhances also the common mode suppression and in combination with an active and reference it gives a way more stable signal. It is connected to the ground of the amplifier. We have good experiences pacing this electrode above the cerebellum. Plastic One Inc., Roanoke, USA, tripolar electrode sets MS 333/2 were often used by us. Alternatively, different types of pre-fabricated or in-house made electrodes can be used, for example for recording more than two to six cortical areas (see Meeren et al. (2002) from an epidural grid, from a set of (24) screws in the cranium (Ding et al., 2019), from a set of pre-prepared electrodes glued in a pedestal for cortical and subcortical recording (Lüttjohann and van Luijtelaar, 2015), or with Neurnexus probes or macroarrays (Jonak et al., 2018), or with self-designed alternatives allowing 24 EEG channel recordings in free moving mice (Wasilczuk et al., 2016; Ding et al., 2019). These can be custom made regarding locations and depth and prefabricated. Electrodes can be constructed from fine stainless steel wire, connected to a pin with a small insert. A large number of electrodes that are individually wired and connected with an external recording

system quickly demands space on the animals head and this may be challenging. A solution can be found by the usage of multiplexed headstages: they overcome this limitation by combining the signals from many electrodes to a smaller number of connections directly on the animal's head (Wang et al., 2017).

Once the EEG electrode set is implanted, and the animals are given a recovery period of 1 to 2 weeks, the animals are handled for a few days and habituated to the recording conditions by connecting them *via* a swivel. Next, the EEG can be recorded in freely moving animals for several hours or days. In order to see whether the EEG signals are of a sufficient quality and have a biological origin (instead of filtered noise), it might be necessary to see whether the amplitude of the EEG signal increases when the rats are passive and asleep, or when behaviorally active, theta activity might be present, dependent on whether the electrode is placed in or above the large hippocampal area in rats.

For methodologies that require a complete immobility of the animals [e.g., *in vivo* extra and intracellular electrophysiology, magnetic resonance imaging (fMRI)], most anesthetic drugs cannot be used as they suppress SWD. Instead, neurolept-analgesia (Hypnorm, a mix of fentanyl and fluanisone, see Inoue et al. (1994) for details and different doses), and local injections of lidocaine have been used to allow the contention of the animals into a stereotaxic frame (Pinault et al., 1998). Using this preparation, spontaneous SWD and extracellular unit activity can be recorded with the same pattern and occurrence as in the awake freely moving rats (Pinault et al., 2001; Deransart et al., 2003), although an increase in number of SWD and a decrease in the interspike frequency has been found in WAG/Rij rats after anesthetic doses of Hypnorm (Inoue et al., 1994). Moreover, a putative new anti-absence drug should be investigated in freely moving animals while the behavior being quantified.

The Dependent Variables

The EEG's are analyzed regarding the presence of SWDs. An example of a SWD, as measured in cortex and thalamus, is depicted in **Figure 2**. The parameters that are analyzed and reported are the number of SWDs (incidence), mean duration of SWDs, SWD-index, total time of SWD activity, and all per time unit, are most commonly used to quantify SWDs. SWD index and total time of SWD activity are less favorable since both measures are a composite of the number/incidence and mean duration. Moreover, the number of SWDs is mainly determined by the excitability of the cortex, the mean duration is determined by different endogenous mechanisms involved in aborting ongoing SWDs in which the reticular thalamic nucleus plays a

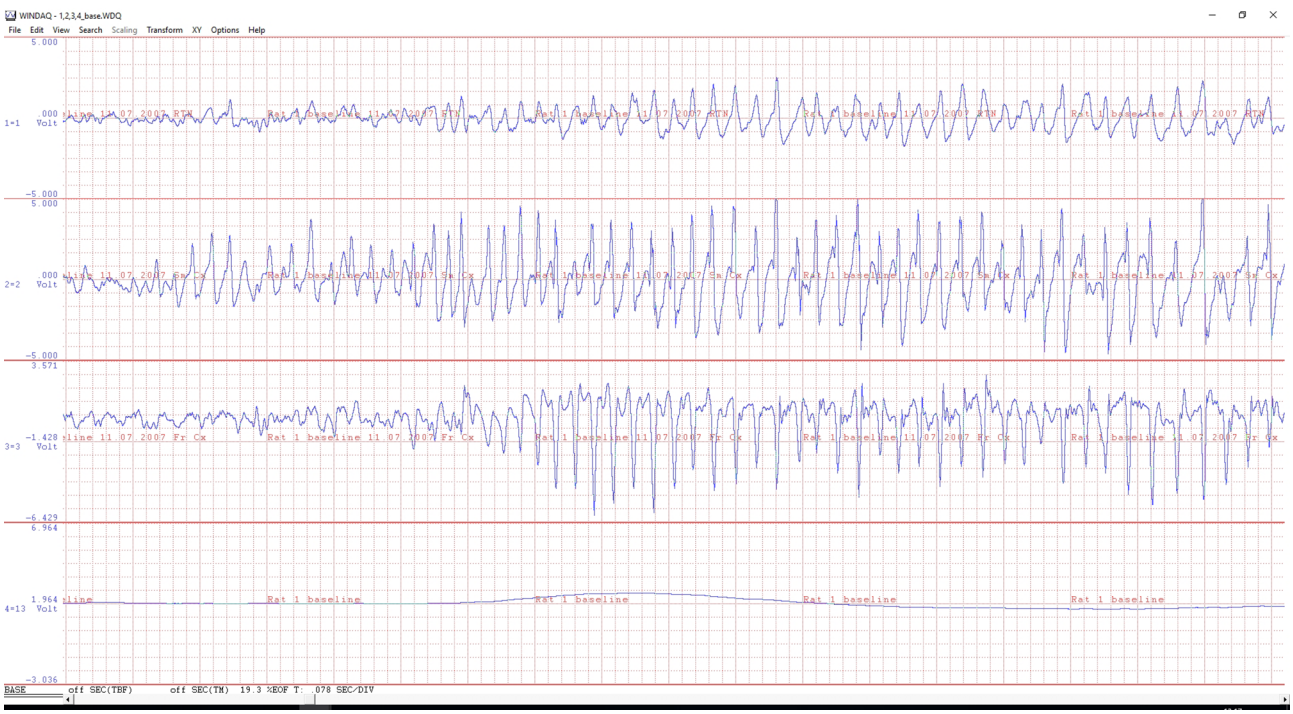

FIGURE 2 | An example of a ECoG of a WAG/Rij rat with electrodes in the reticular thalamic nucleus (upper channel), sensory motor (middle) and frontal (bottom ECoG) cortex. The beginning of a 8 Hz spike-wave discharge (SWD) is illustrated. Note the differences in morphology between the three EEG traces during the generalized SWD. The fourth signal is from an infrared movement detector (PIR); the line is flat when the animal is motionless, while showing large and steep changes during active movements (not illustrated). A minor body movement is seen at the beginning of the SWDs. The time scale of the X-axis is 0.078 sec/division. The amplitude, voltage, is on an arbitrary scale for monitoring purposes only.

major role (Lüttjohann and van Luijtelaar, 2015). By reporting the composite parameter, one loses the occasion to report on the different processes controlling SWDs. Some drugs might have specific effects on the number of SWDs; other drugs might influence their mean duration. The hazard rate of the durations of the inter-SWD intervals has been used by e.g. Bouwman et al. (2007): it indicates the instantaneous probability that the SWD will stop as a function of time and reveals information about the underlying SWD stopping mechanism (Maris et al., 2006). It was changed after the anticonvulsant and proabsence drug vigabatrin, leading to an enhancement of rather short SWDs as well as an increase in the number of rather long SWDs.

The scoring of the SWDs is most often done by visual inspection, which is still the golden standard. For criteria and see van Luijtelaar and Coenen, 1986; and the next paragraph for a discussion. Automated scoring systems for SWD quantification in WAG/Rij rats were developed by us (Westerhuis et al., 1996 and adapted to SpikeWave Complex Finder software by PLC van den Broek, Radboud University, Nijmegen). This and other automated system should be considered as auxiliary tools for the analyses of large amounts of data. Considering the rather stereotypical appearance of SWDs it is not difficult to develop a sensitive, selective, and reliable SWD quantification system (for review see van Luijtelaar et al., 2016; Casillas-Espinosa et al., 2019).

In all cases it is recommended to observe the rat's behavior after the administration of an unknown drug since the animal's behavior may changes qualitatively (signs of psychogenic behavior), and stereotyped behavior and clonic seizures might show the same quantitative activation pattern as grooming. This difference cannot always be appreciated by fully automated movement detection or registration systems. The possibility exists that a putative antiabsence drug or an experimental drug may reduce SWDs because the compound changes the animal's behavior. An illustrative example is cocaine; nobody would claim that it is a good anti-epileptic or anti-absence drug although it reduced dose-dependently SWDs in WAG/Rij rats. Cocaine eliminated explorative and automatic, and passive behavior, whereas various stereotypical activities such as uncoordinated head and body movements and head swaying emerged. In total bodily activity was increased by cocaine, and similar observations can be seen after the administration of amphetamine. This was detected by systematic behavioral observations and quantification of the observed behavior (van Luijtelaar et al., 1996). In these cases the reduction of SWDs is secondary to the increase in physical/bodily activity of the animal induced by the drug. Another example would be the central stimulant apomorphine. It does reduce the number of SWDs (Midzianovskaia et al., 2001), but this decrease is secondary to the effects of apomorphine on behavior.

In case an increase in passive behavior is found, than it might be worthwhile to analyze whether the drug is affecting sleep quality and quantity. If it does, than the benefits of good seizure control should outweigh the negative effects on sleep quality and or quantity. Therefore, it is imperative to get an impression whether the drug has sedative effects or activating effects on behavior. Although this information can be to some extend be obtained from the EEG recordings, REM sleep can be determined in rats only if together with the EEG and electroomyogram (EMG) or an index of the animals behavior (active, such as walking, explorative behavior, grooming, eating, drinking, or being passive) or passive (lying, sitting with eyes open or closed) is available. A simple, reliable, and cheap alternative to determine changes in behavior (either a decrease or increase) of a compound is to have an infrared movement detector (PIR, Passive Infrared Recorder) above each recording cage. In case a new compound has sedative or hypnotic effects, the PIR will register less bodily activity. In that case it is recommended to analyze the EEG together with the PIR in terms of sleep parameters. Sleep can be either qualitatively of quantitatively affected. Quantitative aspects are e.g. described by percentages total sleep, non-REM sleep, or REM sleep and by the number and duration of intermittent wakefulness periods, or sleep efficacy, for a study of the effects of the anti-absence drug ETX on sleep see van Luijtelaar et al. (2012b). A major qualitative aspect of non-REM sleep is the amount of delta activity, by visual inspection of the sleep EEG it is difficult to establish the amount of specifically deep slow wave sleep. Spectral analyses of non-REM sleep is then indicated with a focus on the amplitude of delta activity (in rats 1–5 Hz). Amplitude or power can be expressed as percentage of total power over 0.5–100 Hz to normalize the data across animals. Delta power can be increased or decreased compared to control treatment, benzodiazepines, or its partial agonists are known to reduce delta power (Coenen et al., 1992), the pro-absence drug tiagabine facilitates delta power (Lancel et al., 1998). The nuchal EMG is most often used for visual and automatic sleep wake classification in rats, a good replacement for the nuchal EMG is the signal obtained from a PIR. As mentioned, it monitors the amount of bodily activity of an animal in a recording cage; the amplitude of the analog signal from the PIR is high when the animals are physically active, and the amplitude is low when the animal is passive. The analyses of the amplitude of this signal (the area under the curve) from a PIR will yield the first indication whether the behavior of the animals is changed as a consequence of the administration of the drug.

Are All 8 Hz Oscillations SWDs?

A relevant question that was recently raised again is whether all oscillations in the 8 Hz domain, mimicking to some extent the SWDs in GAERS and WAG/Rij rats and are known for a long term to be present in different outbred strains of rats (Willoughby and Mackenzie, 1992; Marescaux et al., 1984; van Luijtelaar et al., 1994) and now noticed in wild rats, should be indeed considered as genuine epileptic SWDs (Taylor et al., 2017; Taylor et al., 2019). First, WAG/Rij rats have clinical concomitants during the SWDs, they are mild, and might be easily overlooked. A combined EEG video study showed that during the cortical expressed SWDs WAG/Rij rats show head tilting, twitching of the vibrissae, accelerated

breathing, and occasional eye blinks, and facial myoclonus in otherwise immobile animals (van Luijtelaar and Coenen, 1986). Close inspecting of the EEG oscillations as presented in Taylor et al. (2019) shows that only a minority of the illustrated SWDs in the wild rats could classify as SWDs according to our criteria and characteristic features. Relevant and typical is that SWDs appear as bilateral symmetrical generalized, minimal duration 1 s, asymmetric morphology with a sharp large amplitude negative peak with a duration between 25 and 35 ms, this peak is most pronounced expressed at the frontal cortex and a clear negative wave (40–60 ms), less well expressed at the frontal cortex but more at the thalamic VPM, with a "sudden" appearance from a normal appearing background and without a waxing and waning pattern). The possibility that the 8 Hz oscillations as seen in the wild rats represent a local sensory motor rhythm, often described in cats (it was also called mu-rhythm) but in some rodents strains as well, characteristic for attentive wakefulness, remains to be explored. Although we have never seen a clear sensory motor rhythm in WAG/Rij rats, it is likely to be present in other outbred lines such as Long Evans rats. In GAERS, the SWDs seem to be driven by a 5–9 Hz oscillation, as was originally proposed by Pinault (2003). Moreover, WAG/Rij do not have SWDs while being attentive or aroused (Osterhagen et al., 2010).

We did describe (Drinkenburg et al., 1993) also 8 Hz SWD-like phenomena in the cortical EEG of WAG/Rij rats during drowsiness and light slow wave sleep, not fully meeting our rather strict criteria of SWDs and named them spiky phenomena; their appearance is in between sleep spindles and SWDs, less sharp spikes, more symmetric than SWDs, poorly expressed wave, and a waxing and waning pattern, a shorter duration than SWDs, rarely exceeding 2–3 s, mimicking also some of the high voltage spindles, as were introduced by Buzsáki and were recorded during alert immobility (Buzsáki et al., 1988; Jandó et al., 1995). The fact that quite some WAG/Rij rats have spiky phenomena next to genuine SWDs, demonstrates that there is a continuum regarding the morphology of the 8 Hz oscillations. Moreover, sometimes a genuine appearing SWDs transfers into a spiky-phenomenon. The spiky phenomena are less well studied, and it is not known whether they are indeed generalized, are bilateral symmetrical have a similar cortical origin as SWDs, and whether they are accompanied by clinical concomitants. The fact that the "peaks" of the spiky phenomena are less sharp than in genuine SWDs casts serious doubts about them being epileptic (an epileptic spike is per definition sharp). The sharpness of the peak of the SWDs is mostly clearly revealed by the presence of the second and higher harmonics of the dominant frequency spectrum of SWDs, their amplitude, or power is lower in spiky phenomena and sleep spindles compared to SWDs. In fact the energy in the 30–80 Hz band (Ovchinnikov et al., 2010) and the slope of the frontal peaks of the SWDs (SWD detection and quantification program developed by P. van den Broek, and used by us for more than 15 years and by the group of Terence O'Brien, Melbourne) were empirically found as the most sensitive and reliable parameters for the automatic detection of SWDs and to separate SWDs from other 8 Hz oscillations, among others the intermediate sleep spindles characterizing the transition between nonREM and REM sleep (Gottesmann and Gandolfo, 1986). But there are many other methods to detect SWDs in rodents, for review see van Luijtelaar et al. (2016). Another way to distinguish epileptic SWDs from non-epileptic 8 Hz oscillations is the presence of a second small negative spike in a SWD, just before the large amplitude negative spike dominating SWDs. It appeared in SWDs at the occipital cortex and in VPM, this small spike survived an SWD averaging process, suggesting that it is genuine (Sitnikova and van Luijtelaar, 2007). This small spike characterized SWDs in 44% of the patients, as was described in a classical study by Weir (1965). Finally, a single Morlet based model could reliable classify SWDs from other 8 Hz oscillations (Sitnikova et al., 2009), demonstrating a different morphology than the other 8-Hz oscillations as can be found in the cortical EEG of WAG/Rij rats. Besides the morphological differences between SWDs and other 8 Hz oscillations, there is the predictive validity based on the evaluation of at least 12 different anti-epileptic drugs of the models, there is little doubt about that the SWDs seen in the WAG/Rij and GAERS models are truly epileptic.

Some New Principles for EEG Data Analyses

The EEG was used classically for basic research and clinical applications on the field of epilepsy and sleep; and tended to become less relevant for basic neuroscience research through the availability of functional MRI and other brain imaging techniques, an analyses of PubMed showed that over the years the number of rodent EEG papers is increasing again. The classical way to analyze the EEG in case of clinical applications in the field of epilepsy is still visual inspection ("the golden standard"), mainly regarding the description of aberrant EEG activity regarding morphology and spatial temporal distribution, and frequency and duration of occurring of interictal spikes, or other epileptoformic electroencephalographic characteristics, including the SWDs. The availability of computers and software for single channel EEG analyses allows to describe the spectral content of the EEG and the effects of drugs, including the effects of antiepileptic drugs on the spectral content of the EEG drugs. Of later development are the spectral decomposition techniques allowing to describe its changes from moment to moment, e.g. by wavelet analyses. These spectral analytical techniques have revealed the dynamics within SWDs (Bosnyakova et al., 2007), but also differences between preictal, ictal, and interictal EEG activity. The presence of multichannel EEG, at least more than one single channel, allows a new category of analyses, network analyses. It allows evaluating neuronal synchrony in EEG data, either obtained with invasive as the case with epidural or depth electrodes commonly used in rodent EEG based sleep and epilepsy research, or non-invasive recording techniques in human EEG or MEG studies. Different methods can be used, for a tutorial review see Bastos and Schoffelen (2016), such as coherence, a robust measure which is commonly used as an index of the non-directed functional coupling between different brain structures at various frequencies (Thatcher, 2012), linear and non-linear correlation function (Lopes da Silva et al.,

1989), phase synchronization index, mutual information function, transfer entropy, and partial directed coherence, and Granger causality. It allows to describe, in the time domain, but sometimes as well in the frequency domain, the relation between the activity in two or more EEG channels, as well, depending on the method, the directed functional relationships, in the sense that it can be established which channel influences the other channel(s) and at which strength, and its dynamics in case a sliding window is used. We have successfully applied some of network analyses techniques to describe dynamics in coupling and based on a non-linear regression technique a cortical onset zone was described in the WAG/Rij rats, which was the bases for the cortical focus theory (Meeren et al., 2002; Meeren et al., 2005; Westmijse et al., 2009; van Luijtelaar et al., 2011). Next, different thalamic nuclei have a different function in the maintenance and abortion process of SWDs, as was established with different network analyses techniques (Lüttjohann and van Luijtelaar, 2015; Sysoeva et al., 2016). Drug related studies with network analyses methods of antiepileptic drugs are scares, in other domains they were already successfully applied (Ahnaou et al., 2014) and there are good reasons to assume that new mechanisms and new drug targets can be found with these new approaches acknowledging that epilepsy and certainly genetic generalized epilepsy, are network types of brain dysfunctions (Stefan and Lopes da Silva, 2013; Lüttjohann and van Luijtelaar, 2015).

Limitations and Strength.

A limitation of the WAG/Rij model is that this model, the same holds for all other genetic absence models, does not show the spontaneous remission (different studies show a range from 21%–74%), or change to generalized convulsive seizures occurring in ca 40% of children with childhood absence epilepsy (Tenney and Glauser, 2013). Another limitation is that it has a single genotype, and the heterogeneousness regarding genetic causes, commonly found in children, is missing in this inbred strain. The strength is that the genetic models are well characterized, have face, predictive, and construct validity, that the rats (WAG/Rij) are widely available (also commercially at Charles River), that different groups report the same epileptic phenotype. Epigenetic effects are described, and comorbidities, present in epilepsy patients such as dysthymia, are modeled as well (Sarkisiva et al., 2010). Finally, the WAG/Rij and GAERS models have no other obvious neurological deficits and allow the study and the development of seizures in a genuine epileptic brain.

AUTHOR CONTRIBUTIONS

GvL and GvO compiled and wrote the manuscript.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Next-Generation Sequencing in Korean Children With Autism Spectrum Disorder and Comorbid Epilepsy

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Autism spectrum disorder (ASD) is a neurodevelopmental disorder characterized by impairments in social communication and restricted and repetitive behaviors and interests. Identifying the genetic background may be one of the key features for the future diagnosis and treatment of ASD. With the tremendous development in genetic diagnosis techniques, next-generation sequencing (NGS) can be used to analyze multiple genes simultaneously with a single test in laboratory and clinical settings and is well suited for investigating autism genetics. According to previous studies, there are two types of genetic variants in ASD, rare variants and common variants, and both are important in explaining pathogenesis. In this study, NGS data from 137 participants with ASD were reviewed retrospectively with consideration for comorbid epilepsy. Diagnostic yield was 17.51% (24/137), and pathogenic/likely pathogenic variants were seen more frequently in female participants. Fourteen participants were diagnosed with comorbid epilepsy, six of them had pathogenic/likely pathogenic variants (43%). Genes with variants of unknown significance (VOUS) which have one or more evidence of pathogenicity following the American College of Medical Genetics (ACMG) criteria were also reviewed in both ASD and ASD with comorbid epilepsy groups. We found that most frequently found VOUS genes have previously been reported as genes related to ASD or other developmental disorders. These results suggest that when interpreting the NGS results in the clinical setting, careful observation of VOUS with some pathological evidence might contribute to the discovery of genetic pathogenesis of neurodevelopmental disorders such as ASD and epilepsy.

Keywords: autism spectrum disorder, epilepsy, next-generation sequencing, clinical exome sequencing, autism genetics

INTRODUCTION

Autism spectrum disorder (ASD) is a neurodevelopmental disorder with core symptoms of persistent deficits in social communication and restricted, repetitive patterns of behavior, interests, or activities (APA, 2013). According to recent reports, ASD no longer seems to be a rare disease; the overall prevalence is 16.8 per 1,000 children aged 8 years, with overall male-to-female ratio of 4:1 in the United States (Baio et al., 2018). Autism spectrum disorder is not simply a disorder but a significant social problem because the annual costs for ASD patients are tremendous (Lavelle et al., 2014). Costs may include medical and nonmedical costs and indirect costs such as parental productivity loss (Buescher et al., 2014). Accurate diagnosis and treatment guidance might substantially impact treatment of the disorder and reduce annual costs.

Autism spectrum disorder is associated with various coexisting factors including those related to genetics, the prenatal environmental, and the postnatal environmental (Lord et al., 2018). While knowledge about the neurobiological basis of ASD is still insufficient, genetic factors are regarded as crucial components according to previous studies (Rosenberg et al., 2009; Hallmayer et al., 2011). Studies of monozygotic twin concordance and sibling recurrence rates clearly reveal that genetic factors play important roles in the development of ASD (Geschwind, 2011; Sandin et al., 2014; Tick et al., 2016). In this context, identifying the genetic background of each ASD patient could be the 'cornerstone' of proper diagnosis and individualized treatment. In general, genetic diagnosis has the benefits of informing prognoses and preventing further superfluous invasive testing, leading to tailored treatment and family counseling (Han et al., 2018). In particular, genetic testing may be a key component in the development of precision medicine, in hope of predicting treatment outcomes on an individual basis. Tools for genetic analyses are rapidly developing and the collection of genetic information is accelerating tremendously (Geschwind and State, 2015; Loth et al., 2016).

However, there are inevitable obstacles to defining a genetic basis of ASD. First, the majority of ASD cases cannot be explained by a single gene mutation. Previous reports have indicated that only 10% of ASD cases originate from a rare variant in a single gene (Persico and Napolioni, 2013). Moreover, ASD can occur as a result of a combination of common variants (Gaugler et al., 2014). The fact that common variants are not causal for disease makes it harder for researchers to define their clinical importance (Geschwind, 2011; Persico and Napolioni, 2013). Second, several characteristics of autism genetics, such as an extremely heterogeneous genetic contribution, many different loci underlying disease, variable phenotypic expression, and lack of specificity make it difficult to understand the neuropathology of the condition (Persico and Napolioni, 2013). Solving heterogeneity may be the most important future task for ASD researchers.

It is clear that traditional candidate gene studies are not suited to investigate common gene variants. As next-generation sequencing (NGS) technologies developed, whole exome sequencing (WES) was used to identify diverse genetic variants, including common variants in ASD (Sanders et al., 2012; Persico and Napolioni, 2013). The use of NGS, with its ability to simultaneously analyze multiple genes (in case of WES) in a single test, is currently being established in clinics and laboratories (Yohe and Thyagarajan, 2017).

From a genetic perspective, an ASD patient should be monitored for comorbid epilepsy because ASD and epilepsy are known to share genetic backgrounds, which may be related to neuropathophysiology during brain development (Tuchman and Rapin, 2002; Tuchman and Cuccaro, 2011). Autism spectrum disorder patients with rare gene variants related to genetic syndromes such as Rett's syndrome, are strongly suspected to have comorbid epilepsy (Canitano, 2007). Previous studies show that epilepsy in ASD is highly related to intellectual disability (ID) (Amiet et al., 2008) and associated with severity of ASD (Ko et al., 2016). To reveal the genetic background of neurodevelopment, examining the discriminative characteristics of genetic components of ASD with epilepsy and ID and comparing them with ASD with no comorbidities is essential. To investigate genetic variants associated with ASD and ASD with comorbid epilepsy, we planned a retrospective review of the medical records and NGS data of ASD patients.

MATERIALS AND METHODS

Participants

We reviewed medical records of ASD patients who underwent NGS for genetic evaluation, who visited a specialist in an outpatient clinic for autism at the Severance Children's Hospital, from January 1, 2016. In clinical settings, we recommend NGS to parents when patients show severe autistic symptoms, morphological problems, or other medical or neurological comorbidities. Data from 141 patients were collected, and four patients among them were excluded due to lack of clinical assessment and follow-ups which are vital for diagnosing ASD. One hundred and thirty-seven enrolled participants were clinically diagnosed as ASD by a specialized psychiatrist on the basis of diagnostic criteria suggested in DSM-5, and several clinical assessments (see *Clinical Assessments*) support the diagnosis. As data were reviewed retrospectively and it was impossible to fulfill assessments not performed in the clinical setting, there were missing scores for Intelligence-Quotient (IQ), the Social Responsiveness Scale (SRS), and the Social Communication Questionnaire (SCQ), in several participants. We examined not only NGS and clinical assessment data, but also checked for comorbid epilepsy and other comorbidities, history of seizure, and electroencephalogram (EEG) reports. For the cases with comorbid epilepsy, we followed the diagnostic decision of neurologists in the Severance Children's Hospital. This study was approved by the applicable institutional Review Boards for research with human subjects at Severance Hospital, Yonsei University College of Medicine, where this study was performed.

Clinical Assessments

All participants had a previous clinical diagnosis of ASD by a specialized child psychiatrist. The diagnosis of ASD was established using Autism Diagnostic Interview-Revised (ADI-R) and Autism Diagnostic Observation Schedule (ADOS), the gold standard for ASD diagnosis. Clinical autistic characteristics of the participants were supplemented by the CARS, SRS and SCQ.

The Childhood Autism Rating Scale (CARS) is a 15-item behavioral rating scale, developed to distinguish ASD and other developmental disorders and to assess the severity of ASD. Each item is scored from one to four points, and midpoint scores are also possible. Higher scores indicate more severe ASD symptoms. The cut-off score, which distinguishes ASD from other developmental disorders, is 30 points (Amiet et al., 2008). The reliability and validity of the Korean version of CARS (K-CARS) have been verified (Shin and Kim, 1998).

The SRS is a 65-item questionnaire that asks parents and/or teachers about the characteristics of the social interactions shown by children over the past 6 months (Constantino et al., 2000). Each question is scored from zero to three points, depending on the frequency of the action described in each item. Higher scores mean a lower social function. We previously confirmed the clinical validity of the SRS in Korean children and provided the Korean T-score norm (Cheon et al., 2016). In the present study, we used total T-score of the participants.

The SCQ is a 40-item screening instrument that is based on ADI-R, a tool for more in-depth assessment of ASD symptoms, and selects key items that deviate from normal development (Chandler et al., 2007). The Korean version of the Social Communication Questionnaire (K-SCQ) was verified as a reliable and valid instrument for screening autistic symptoms in the Korean population (Kim et al., 2015). Each question is answered with yes or no. Higher scores indicate more severe symptoms associated with ASD.

The ADI-R is a semi-structured parent interview tool for parents of children aged 2 years and older (Lord et al., 1994). This is generally conducted in conjunction with ADOS, which directly monitors and assesses the child, and is used to complement the interpretation of results (Lord et al., 2000).

To assess the cognitive levels of participants, we used the Korean-Wechsler Intelligence Scale for Children-IV (K-WISC-IV) and the Korean Wechsler Preschool and Primary Scales of Intelligence-IV (K-WPPSI-IV). We also used Korean-Bayley-III for children who were unable to perform the Wechsler's intelligence scales because of their age or development status.

Next-Generation Sequencing

The xGen Inherited Diseases Panel (Integrated DNA Technologies, Coralville, IA, USA) including 4,503 candidate genes was used for exome sequencing. Genes associated with various neurodevelopmental disorders such as ASD, epilepsy, seizure disorder, and X-linked ID are included in this panel.

Genomic DNA extracted from individuals' samples was used for library preparation and target capture using a custom panel targeting candidate genes. Massively parallel sequencing was performed using the NextSeq 550Dx System (Illumina, San Diego, CA, USA). Quality control and sequence analysis was carried out using our custom analysis pipeline. Copy number analysis was carried out using our custom analysis pipeline (Kim et al., 2019). The GRCh37 (hg19) build was used as the reference sequence for mapping and variant calling while using Burrows-Wheeler alignment (BWA) tool (version 0.7.12). HaplotypeCaller and MuTect2 in the GATK package (3.8-0) and VarScan2 (2.4.0) were used to identify single nucleotide variations (SNV) and insertion and deletions (indels). Databases used for analyses and variant annotation include Online Mendelian Inheritance in Man (OMIM), the Human Gene Mutation Database (HGMD), Clinvar, dbSNP, 1000 Genomes, the Exome Aggregation Consortium (ExAC), the Exome Sequencing Project (ESP), and the Korean Reference Genome Database (KRGDB). Classification of variants followed the standards and guidelines established by the American College of Medical Genetics (ACMG) (Richards et al., 2015), with a scoring algorithm implemented in the DxSeq Analyzer (Dxome, Seoul, Korea). All pathogenic and likely pathogenic variants were further confirmed by Sanger sequencing.

Genetic variants that are not met for pathogenic/likely pathogenic nor benign/likely benign are classified as variants of unknown/uncertain significance (VOUS) according to the ACMG guideline. Benign and likely benign variants were excluded in our NGS clinical reports. If VOUS had one or more evidence of pathogenicity but unmet criteria for pathogenic/likely pathogenic, they were regarded as VOUS with a relatively high probability of pathogenicity. The VOUS with high probability of pathogenicity were selected by physicians in laboratory medicine referencing the criteria on evidence of pathogenicity in the ACMG guideline. Among VOUS with high probability of pathogenicity, we selected five or less variants for analysis.

Statistics

To compare demographic characteristics and results of clinical assessments between patients with and those without pathogenic/likely pathogenic variants, we used the Chi-squared test and the independent t-test. Statistical significance was defined at $p < 0.05$. Analyses were performed using the Statistical Package for the Social Sciences software (version 25.0; SPSS Inc., Chicago, IL, USA).

RESULTS

Among 137 patients, only three patients showed no pathogenic/likely pathogenic variants nor VOUS according to our NGS clinical reports. Seven cases were identified with pathogenic variants, and 17 participants had likely pathogenic variants. The diagnostic yield acquired from the total NGS data was about 17.51% (24/137). Differences in demographic information and clinical assessment results are presented in **Table 1**. The proportion of females to males was significantly higher in the pathogenic/likely pathogenic variants group (62.5%, $p = 0.006$). The pathogenic/likely pathogenic variants

TABLE 1 | Demographic data of participants.

	Patients with pathogenic/likely pathogenic variants (n = 24)	Patients without pathogenic/likely pathogenic variants (n = 113)	*p*-value	Total (n = 137)
Male: Female (female/male ratio, %)	9:15 (62.5%)	76:37 (32.7%)	**0.006**	85:52
Age (months)	65.21	60.58	0.463	61.39
IQ	50.88	53.51	0.395	53.02
SRS (total T-score)	86.79	86.29	0.933	86.39
SCQ	17.67	16.82	0.639	16.99
CARS	33.184	31.669	0.402	31.935
Comorbid ID	20 (83.3%)	84 (74.3%)	0.349	104 (75.9%)
Comorbid epilepsy	6 (25%)	8 (7.1%)	**0.008**	14 (10.2%)

Pathogenic/likely pathogenic variants appeared more frequently in the female group (62.5%) than in the male group (p-value = 0.006). There were no remarkable differences in age and clinical assessments (IQ, SRS, SCQ, CARS) between the two groups. Comorbid intellectual disability was prominent in both groups, while comorbid epilepsy was more frequently diagnosed in pathogenic/likely pathogenic variants group (p = 0.008). IQ, Intelligence quotient; SCQ, Social Communication Questionnaire; CARS, The Childhood Autism Rating Scales; ID, Intellectual disability. ***BOLD: p < 0.05****.*

group was associated with higher incidence of comorbid epilepsy (25%, $p = 0.008$). There were no between-group differences in age and clinical assessment scores (IQ, SRS T-score, SCQ, CARS). Characteristics of epilepsy and reports of electroencephalogram (EEG) in ASD with comorbid epilepsy were listed in **Supplementary Material 1**.

By comparing males with females (**Table 2**), we found that females appear to have higher scores for the SRS total T-score ($p = 0.024$) as well as frequently detected pathogenic/likely pathogenic variants ($p = 0.006$). The CARS score was also slightly higher in females ($p = 0.045$), while age and other scores (IQ, SCQ) showed no significant statistical differences.

Genes that harbored pathogenic variants included *SHANK3, PTEN, NSD1, PAFAH1B1*, and *RAI1*. Mutation types include exon deletion and nonsense mutations. We also identified copy number variants (CNV), chromosome 8p23.2 duplication, and chromosome 15q11.2–q13.2 duplication. These variants were expected to lead to loss of genetic function (Richards et al., 2015) which may play a role in pathogenesis of disease. Genetic information from OMIM were also described in **Table 3**. Among pathogenic variants, only *PAFAH1B1* was not previously reported to be related to neurodevelopmental disorder including ASD, ID, and epilepsy.

Likely pathogenic variants showed various types of mutations such as copy number variants, exon deletion, nonsense mutation and missense mutation (**Table 4**). Both patients with variants in *TSC2* were diagnosed with tuberous sclerosis clinically. Likewise, both patients with variants in *MECP2* were diagnosed with Rett's syndrome in clinical setting. While most of genes are known to be related to ASD, ID or epilepsy, *ABCC2, CCDC50* and *SLC26A4* were not reported to be related to neurodevelopmental disorder according to OMIM. Likely pathogenic group showed significantly lower SRS T-score compared to pathogenic group (**Supplementary Material 2**).

Importantly, pathogenic or likely pathogenic gene variants were found in approximately 43% (6/14) of participants with comorbid epilepsy. 8p23.2 duplication was the only pathogenic variant, and variations in Xp22.2p22.33 and the genes *NLGN4X, TSC2, MECP2, SYNGAP1*, were classified as likely pathogenic. Suspected genetic variants of each patient with comorbid epilepsy were shown in **Table 5**. There was no significant differences in IQ, SRS T-score, SCQ and CARS between patients with pathogenic/likely pathogenic variants and with VOUS (**Supplementary Material 3**).

All patients had 0 to 37 VOUS genetic variants, 11.45 variants on average in our NGS clinical reports. There were several genes commonly observed with various variations. *TSC2, ADGRV1, RAI1, CDH7, RELN,* and *NSD1* were the most commonly reported genes with variants of unknown origin regardless of mutation types. Genes with VOUS were repeatedly identified about 1.8 times on average in our data, with standard deviation of 1.79. **Figure 1** shows the most frequently identified genes presenting VOUS in our patients without considering the variant type. More specifically, we also examined variants of unknown significance, including types of mutation and locations of the variants. As shown in **Figure 2**, an identical missense mutation in the *FOXP1* gene was found three times among 137 patients, and other missense mutations were seen twice. These results suggest that large portion of genes with VOUS were restricted to missense mutation and have already been reported as genes related to ASD according to OMIM and SFARI database.

TABLE 2 | Male–Female comparison.

	Male	Female	*p*-value
Pathogenic/likely pathogenic variants	9 (10.6%)	15 (28.8%)	**0.006**
Age	61.93	60.52	0.792
IQ	53.48	52.24	0.616
SRS (total T-score)	82.86	93.91	**0.024**
SCQ	16.58	17.75	0.434
CARS	31.16	33.25	**0.045**
Comorbid ID	65 (76.5%)	39 (75.0%)	0.845
Comorbid epilepsy	6 (7.1%)	8 (15.4%)	0.118

There were more females who have pathogenic/likely pathogenic variants (p = 0.006). Females showed higher SRS T-score (p = 0.024) and CARS score (p = 0.045) on average. There were no significant differences in age, IQ, SCQ, and comorbidity of ID, epilepsy. IQ, Intelligence quotient; SCQ, Social Communication Questionnaire; CARS, The Childhood Autism Rating Scales; ID, Intellectual disability. ***BOLD: p < 0.05****.*

DISCUSSION

Among 137 patients, only three patients showed no pathogenic, likely pathogenic variants and VOUS. This might be because patients who had severe symptoms or signs suggesting a genetic etiology in the clinical setting underwent NGS. Severe symptoms are usually related to genetic burden in ASD (Pizzo et al., 2019).

According to previous studies, diagnostic yields vary case by case (Yang et al., 2014; Tammimies et al., 2015; Rossi et al., 2017). Our yield of 17.51% may be within the predicted range, but the remarkable differences between males and females are concerning. The diagnostic yield of the female group was

TABLE 3 | Genetic characteristics: genes with pathogenic variants.

No.	Gene	Accession	Nucleotide	Amino acid	Diseases (OMIM)	Zygosity	Inheritance[a] (OMIM)	ACMG
5	*SHANK3*		Deletion (exon 9–22)		**Phelan–McDermid syndrome** {Schizophrenia 15}	Hetero		
6	**8p23.2 duplication (2.25 Mb)							
16	**PAFAH1B1* (*LIS1*)	NM_000430.3	Exon 4 deletion		Lissencephaly 1; Subcortical laminar heterotopia	Hetero	AD	
17	*RAI1*	NM_030665.3	Exon 6 deletion		**Smith–Magenis syndrome**	Hetero	AD	
46	*PTEN*	NM_000314.4	c.249C > A	p.Cys83Ter	**Cowden syndrome 1** **Macrocephaly/autism syndrome** Bannayan–Riley–Ruvalcaba syndrome; Endometrial carcinoma, somatic; {Glioma susceptibility 2}; Lhermitte–Duclos syndrome; Malignant melanoma, somatic; {Meningioma}; PTEN hamartoma tumor syndrome; {Prostate cancer, somatic}; Squamous cell carcinoma, head and neck, somatic; VATER association with macrocephaly and ventriculomegaly	Hetero	AD,AR	PVS1, PM2, PM6
68	15q11.2q13.2 duplication (9.5Mbp)							
136	*NSD1*	NM_022455.4	c.6349C > T	p.Arg2117Ter	**Sotos syndrome 1** Beckwith–Wiedemann syndrome; Leukemia, acute myeloid	Hetero	AD	PVS1, PM2, PP5

*Pathogenic variants that were found in seven participants. OMIM, Online Mendelian Inheritance in Man; ExAC, population frequency from The Exome Aggregation Consortium; KRGDB, population frequency from the Korean Reference Genome Database; AD, Autosomal dominant; AR,Autosomal recessive; XD, X-linked dominant; XR, X-linked recessive; ACMG, The American College of Medical Genetics and Genomics guideline (Richards et al., 2015); PVS, Very strong evidence of pathogenicity; PM, Moderate evidence of pathogenicity; PP, Supporting evidence of pathogenicity. [a]Inheritance of the gene described in OMIM. *not previously reported to be associated with neurodevelopmental disorders (ASD, ID, epilepsy). **genetic variants in ASD with comorbid epilepsy.* ***BOLD: Clinical syndromes and diseases related to neurodevelopmental disorders (ASD, ID, epilepsy)***.

28.8% which was significantly higher than that of the males. Higher SRS T-scores and CARS scores that indicate severity of autism symptoms were also significantly high in females. Though females are less prevalent in ASD (Baio et al., 2018), genetic burden and symptom severity can be higher than males. Females with ASD are known to have more genetic load than males (Lai et al., 2015), and severe clinical conditions also tend to be related with genetic variants (Lovato et al., 2019). Such reports support our results which highlight the importance of genetic evaluation in females with ASD.

Rare Genetic Variants in Autism Spectrum Disorder

Most pathogenic variants were found in genes such as *SHANK3, PTEN, NSD1*, and the 8p23.2 duplications that have already been reported to be associated with ASD. Most pathogenic variants are related to specific neurodevelopmental syndromes. Variants in *SHANK3* can accompany Phelan–McDermid syndrome (Berg et al., 2018); *PTEN,* Cowden syndrome (Goffin et al., 2001); *NSD1*, Sotos syndrome (Kurotaki et al., 2002); and *RAI1*, Smith–Magenis syndrome (Slager et al., 2003; Laje et al., 2010). These syndromes are often reported to be related with ASD (Goffin et al., 2001; De Rubeis et al., 2014; Connolly et al., 2017). In the case of the 15q11.2–q13.2 duplication, a previously reported duplication in 15q11–13 was associated with ASD, and if variants are inherited from the father, Prader–Willi syndrome should also be considered (Bolton et al., 2004; Veltman et al., 2005). Autism spectrum disorder with these syndromes related genes should be monitored with caution, regarding comorbidities. However, unlike other genes, variants of *PAFAH1B1* have not been previously reported as rare variants that affect ASD development. An animal study demonstrated that mutation in the murine ortholog of this gene contributes to diminished social interaction in mice (Sudarov et al., 2013). As the gene plays a role in synaptogenesis and nervous system development (Wall et al., 2009; Sudarov et al., 2013), the possibility of ASD pathogenicity should not be neglected. The 8p23.2 duplication is described in *Rare Genetic Variants in Autism Spectrum Disorder With Comorbid Epilepsy*.

Most genes containing likely pathogenic variants were reported to be associated with neurodevelopmental disorders. The copy number variants, Xp22.2p22.33 deletion, 15q24 deletion (Adam et al., 2018), and 14q31.3–32.12 deletion (Crkvenac Gornik et al., 2019) were also reported to be related to developmental delay. Otherwise, some genes which had not been considered as ASD related genes were discovered. *ABCC2* is known to trigger Dubin–Johnson syndrome, which causes an increase in conjugated bilirubin levels (Keitel et al., 2003). The condition is characterized by black pigment in the liver. Mutations in *SLC26A4* have been related to Pendred syndrome, leading to sensorineuronal hearing loss (Landa et al., 2013). The participant with history of comorbid hearing loss might be due to genetic mutation in high probability, but whether this genetic mutation is also responsible for ASD development or not is unclear due to lack of evidence. *CCDC50* with a duplication in exon 11 was reported to be associated with progressive hearing loss in the Spanish group (Modamio-Høybjør et al., 2007), but to our knowledge, no reports were found to be related to *CCDC50* with neurodevelopmental disorders. Further studies are needed to understand the relationship between these genes and ASD.

TABLE 4 | Genetic characteristics: genes with likely pathogenic variants.

No.	Gene	Accession	Nucleotide	Amino acid	Diseases (OMIM)	Zygosity	Global frequency (ExAC)	Korean frequency (KRGDB)	Inheritance[a] (OMIM)	ACMG
39	**ABCC2*	NM_000392.3	c.2443C > T	p.Arg815Ter	Dubin–Johnson syndrome	Hetero	0.00002826		AR	PVS1, PM2
	**ABCC2*	NM_000392.3	c.2302C > T	p.Arg768Trp	Dubin–Johnson syndrome	Hetero	0.00007539	0.000803859	AR	PP3
57	*MECP2*	NM_004992.3	c.403A > G	p.Lys135Glu	**{Autism susceptibility, X-linked 3}** **Mental retardation** **Rett syndrome** Encephalopathy, neonatal severe	Hetero			XR, XD	PM2, PP3, PP5
60	**Xp22.2p22.33 deletion					Hetero				
	***NLGN4X*	NM_020742.3	Whole gene deletion		**Asperger syndrome susceptibility** **Autism susceptibility** **Mental retardation**	Hetero				
63	Xp22.31p22.33 deletion					Hetero				
	NLGN4X	NM_020742.3	Whole gene deletion		**Asperger syndrome susceptibility** **Autism susceptibility** **Mental retardation**	Hetero				
66	*DLGAP2*	NM_004745.4	Whole gene duplication		**Autism spectrum disorder**	Hetero				
69	*AUTS2*	NM_015570.2	c.2962dleG	p.Glu988LysfsTer37	**AUTS2 syndrome Mental retardation**	Hetero			AD	PVS1, PM2
75	*SCN2A*		Exon 15-16 deletion		**Epileptic encephalopathy, early infantile**	Hetero			AD	
76	*KAT6A*	NM_006766.3	c.3456G > A	p.Trp1152Ter	**Mental retardation**	Hetero				PVS1, PM2
	**CCDC50*	NM_178335.2	c.82_83dupAC	p.Leu29ProsfsTer40	?Deafness,	Hetero			AD	PVS1, PM2
87	*HUWE1*	NM_031407.5	c.693+1G > A		**Mental retardation, syndromic, Turner type**	Hetero				PVS1, PM2
94	***TSC2*	NM_000548.3	c.4744_4746del	p.Ile1582dle	**Tuberous sclerosis-2** Lymphangioleiomyomatosis, somatic	Hetero			AD	PM2, PM4, PM6
95	***TSC2*	NM_000548.3	c.2838_122G > A		**Tuberous sclerosis-2** Lymphangioleiomyomatosis, somatic	Hetero			AD	PM2, PM6, PP5, PP4
96	*CACNG2*	NM_006078.3	c.437-2A > G		Mental retardation	Hetero			AD	PVS1, PM2
98	15q24 deletion (2.2Mb)									
121	***MECP2*	NM_004992.3	c.455C > G	p.Pro152Arg	**{Autism susceptibility, X-linked 3}** **Mental retardation** **Rett syndrome** Encephalopathy, neonatal severe	Hetero			XR, XD	PM2, PP5, PP3, PP5
133	14q31.3-32.12 deletion					Hetero				
138	**SLC26A4*	NM_000441.1	c.2168A > G	p.His723Arg	Deafness with enlarged vestibular aqueduct; Pendred syndrome	Hetero	0.0001	0.00401929	AR	PP3,PP2,PP5
	**SLC26A4*	NM_000441.1	c.919-2A > G		Deafness with enlarged vestibular aqueduct; Pendred syndrome	Hetero	0.0003	0.000803859	AR	PVS1, PP5
142	***SYNGAP1*	NM_006772.2	c.980T > C	p.Leu327Pro	**Mental retardation**	Hetero	–	–	AD	PM2, PP5

Seventeen participants showed likely pathogenic variants. OMIM: Online Mendelian Inheritance in Man; ExAC, population frequency from The Exome Aggregation Consortium; KRGDB, population frequency from the Korean Reference Genome Database; AD, Autosomal dominant; AR. Autosomal recessive; XD, X-linked dominant; XR, X-linked recessive; ACMG, The American College of Medical Genetics and Genomics guideline (Richards et al., 2015); PVS, Very strong evidence of pathogenicity; PM, Moderate evidence of pathogenicity; PP, Supporting evidence of pathogenicity. [a]Inheritance of the gene described in OMIM. ***BOLD: Clinical syndromes and diseases related to neurodevelopmental disorders (ASD, ID, epilepsy).***

TABLE 5 | Genetic characteristics: genes with most suspected variants to be related to ASD with epilepsy.

No.	ACMG classification	Gene	Accession	Nucleotide	Amino acid	Diseases (OMIM)	Zygosity	Global frequency (ExAC)	Korean frequency (KRGDB)	Inheritance (OMIM)	ACMG
6	Pathogenic	8p23.2 duplication (2.2Mb)									
20	VOUS	*SCN3A*	NM_006922.3	c.5873C > G	p.Thr1958Arg	**Epilepsy, familial focal**	Hetero			AD	PM2
24	VOUS	*MECP2*	NM_004992.3	c.602C > T	p.Ala201Val	**Epileptic encephalopathy, early infantile {Autism susceptibility, X-linked 3}** **Mental retardation** **Rett syndrome** Encephalopathy, neonatal severe	Hemi	0.0015	0.00643087		PP3, PP5
34	VOUS	*GRIN2A*	NM_000833.4	c.3059C > G	p.Ser1020Cys	**Epilepsy, focal, with speech disorder and with or without mental retardation**	Hetero				PM2
38	VOUS	*SCN1A*	NM_006920.4	c.2556+9_2556+10insG		**Dravet syndrome** **Epilepsy, generalized, with febrile seizures plus, type 2** **Febrile seizures, familial, 3A** Migraine, familial hemiplegic	Hetero			AD	PM2
60	Likely pathogenic	Xp22.2p22.33 deletion					Hetero				
	Likely pathogenic	*NLGN4X*	NM_020742.3	Whole gene deletion		**Asperger syndrome susceptibility** **Autism susceptibility** **Mental retardation**	Hetero				
84	VOUS	*ROBO1*	NM_002941.3	c.3229C > T	p.Gln1077Ter		Hetero				PVS1, PM2
94	Likely pathogenic	*TSC2*	NM_000548.3	c.4744_4746del	p.Ile1582del	**Tuberous sclerosis-2** Lymphangioleiomyomatosis, somatic	Hetero			AD	PM2, PM4, PM6
95	Likely pathogenic	*TSC2*	NM_000548.3	C.2838-122G > A		**Tuberous sclerosis-2** Lymphangioleiomyomatosis, somatic	Hetero			AD	PM2, PM6, PP5, PP4
121	Likely pathogenic	*MECP2*	NM_004992.3	c.455C > G	p.Pro152Arg	**{Autism susceptibility, X-linked 3}** **Mental retardation** **Rett syndrome** Encephalopathy, neonatal severe	Hetero			XR, XD	PM2, PM5, PP3, PP5
122	VOUS	*ZEB2*	NM_014795.3	c.2494G > A	p.Ala832Thr	Mowat–Wilson syndrome	Hetero				PM2
142	Likely pathogenic	*SYNGAP1*	NM_006772.2	c.980T > C	p.Leu327Pro	**Mental retardation**	Hetero				PM2, PP5
143	VOUS	*LRP2*	NM_004525.2	c.5314G > A	p.Val1772Ile	Donnai–Barrow syndrome	Hetero				PM2, PP3
144	VOUS	*TUBGCP6*	NM_020461.3	c.4009G > A	p.Gly1337Arg	Microcephaly and chorioretionpathy	Hetero	0.00001048			PM2

Most suspected genetic variant of each ASD patients with comorbid epilepsy. OMIM, Online Mendelian Inheritance in Man; ExAC, population frequency from The Exome Aggregation Consortium; KRGDB, population frequency from the Korean Reference Genome Database; AD, Autosomal dominant; AR, Autosomal recessive; XD, X-linked dominant; XR, X-linked recessive; ACMG, The American College of Medical Genetics and Genomics guideline (Richards et al., 2015); PVS, Very strong evidence of pathogenicity; PM, Moderate evidence of pathogenicity; PP, Supporting evidence of pathogenicity. [a]Inheritance of the gene described in OMIM. ***BOLD: Clinical syndromes and diseases related to neurodevelopmental disorders (ASD, ID, epilepsy)****.*

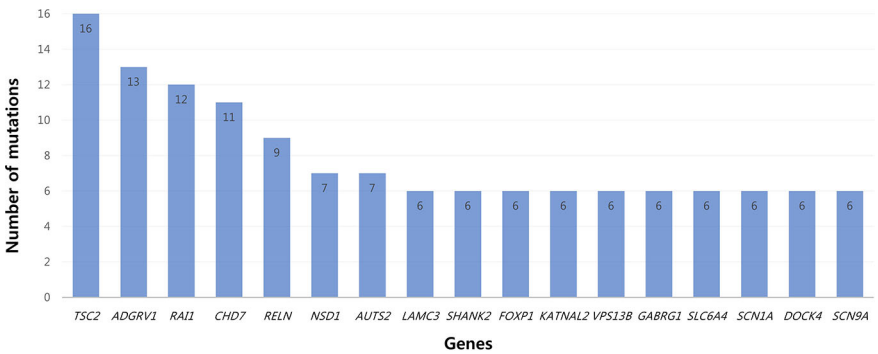

FIGURE 1 | Most common genes with variants of unknown significance. Among genes with VOUS, *TSC2* (16 times) was most frequently observed. After *TSC2*, common genes appeared in the order of *ADGRV1* (13), *RAI1* (12), *CHD* (11), *RELN* (9), *NSD1/AUTS2* (7) and *LAMC3/SHANK2/FOXP1/KATNAL2/VPS13B/GABRG1/SLC6A4/SCN1A/DOCK4/SCN9A (6)*.

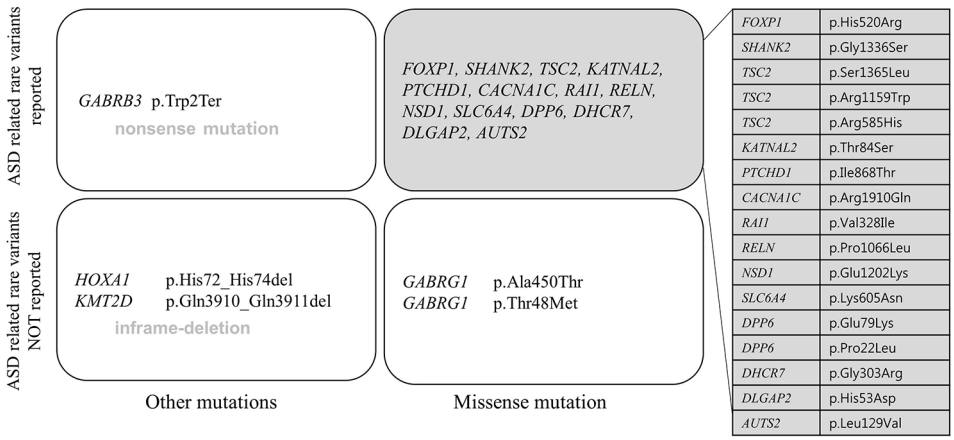

FIGURE 2 | Repeatedly reported variations of unknown significance. A missense mutation (p.His520Arg) of *FOXP1* gene was most commonly found. Only *FOXP1* gene variation appeared three times in 137 patients, the rest of missense mutations were found twice each. In perspective of mutation type, one nonsense mutation (p.Trp2Ter in *GABRB3* gene), two in-frame deletions (p.Gln3910_Gln3911del in *KMT2D* gene, p.His72_His74del in *HOXA1* gene), 21 missense mutations were related to each gene. Most genes have already been reported to be related to ASD according to OMIM and SFARI database.

Rare Genetic Variants in Autism Spectrum Disorder With Comorbid Epilepsy

In our study, two cases with *TSC2* variation and one case with *MECP2* variation were diagnosed as tuberous sclerosis and Rett's syndrome among ASD patients with comorbid epilepsy, upon evidence of general appearance, clinical manifestation, and brain magnetic resonance imaging. Heterozygous *SYNGAP1* gene mutations have been associated with ASD, ID, several forms of idiopathic generalized epilepsy, and delay in psychomotor development (Pinto et al., 2010; Klitten et al., 2011). Though some variants (8p23.2 duplication, Xp22.2p22.33 deletion, and *NLGN4X*) classified as likely pathogenic, the association with epilepsy has not been reported so far.

We confirmed a 2.25 Mb duplication in the short arm of chromosome 8 (8p23.2), including the *CSMD1* gene, by additional microarray examination. Though various size duplications of 8p23.2 are known to be associated with ASD and developmental delay, such as speech delay and learning difficulties (Glancy et al., 2009; Fisch et al., 2011), there was no evidence of association with epilepsy. The complement pathway is tightly controlled in the brain and disruption of microglia-specific complement receptor 3(CR3)/C3 signaling results in sustained deficits in synaptic connectivity. It is believed that deregulation of complement activity could induce aberrant synaptic elimination, which may influence susceptibility to both neurodegenerative and psychiatric disorders (Schafer et al., 2013). Although the mechanism of pathogenesis of epilepsy is not established, recent studies have reported that synaptic connectivity is associated with the development of epilepsy (Chu et al., 2010; Karoly et al., 2018). To date, there is

insufficient evidence to explain the direct relationship between duplication of *CSMD1* and epilepsy. However, we suggest that the overexpression of *CSMD1* due to 8p23.2 duplication leads to abnormal synaptic connectivity and it may contribute to the occurrence of epilepsy.

It is known that deletion of chromosome Xp is associated with ID and ASD (Shinawi et al., 2009; Willemsen et al., 2012). More than 100 genes are known to be located on Xp22.2-p22.33. Males with deletions encompassing Xp22 exhibit a phenotype consistent with the loss of one or more of the genes located in this region (Melichar et al., 2007). In females, there are only a few reports that show *de novo* chromosomal deletions of Xp22 are associated with ASD and developmental delay (Thomas et al., 1999; Chocholska et al., 2006). In addition to the loss of genes located in Xp22, unfavorable X-inactivation of the intact chromosome would be another mechanism for the expressed phenotype in females (Shinawi et al., 2009). Additional tests to confirm the exact location and extent of the Xp deletion were recommended, but not performed in our case. *NLGN4X* is a gene located in the short arm of the X chromosome and is also known to be related to ASD and ID (Jamain et al., 2003; Macarov et al., 2007), but not with epilepsy. In an animal study, *Nlgn4* knock-out (KO) mice showed decreased network response and increased protein expression of synaptic proteins, such as N-methyl-D-aspartate receptor (Nmdar) subunit 1 (Nr1), and metabotropic glutamate receptor 5 (mGluR5), which are involved in synaptic plasticity and excitatory circuit rewiring (Delattre et al., 2013). Imbalance between excitatory and inhibitory synapses is one of the main hypotheses explaining the pathogenesis of epilepsy (Matsumoto and Ajmonemarsan, 1964). Furthermore, excitation/inhibition imbalances resulting from neurodevelopmental deficits have been suggested as pathogenic mechanisms for both ASD and epilepsy (Bozzi et al., 2018).

Interestingly, though estimated as VOUS, some previously reported epilepsy genes (*SCN1A, SCN3A, MECP2,* and *GRIN2A*) were also detected. Genes may have different mutation types or location, which leads to different effect sizes on ASD or epilepsy development, but they still have an important impact on disease occurrence.

Variants of Unknown Origin

In the development of ASD, multiple loci tend to show relatively weak genotype–phenotype correlations and act additively (Persico and Napolioni, 2013). This means that common variants with low effect sizes should not be ignored considering the heterogeneity of ASD. Unfortunately until now, studies such as genome-wide association studies (GWAS), which focus on the contribution of common variants to disease, have not yield consistent results (Geschwind, 2011). According to the ACMG guideline, the VOUS variant can also have one or more evidence of pathogenicity even it was classified as VOUS. So the NGS results should be interpreted carefully, as it is possible to suggest new common variants relevant to ASD pathogenesis.

Except two genes (*ADGRV1* and *GABRG1*), 15 genes frequently classified as VOUS were also reported multiple times with rare variants in ASD (Geschwind, 2011; Persico and Napolioni, 2013; Sener et al., 2016). As shown in **Figure 2**, except one nonsense mutation and two in-frame deletions, most VOUS were missense mutations. That is, the usual types of VOUS mutation are less likely to disrupt function of gene. For this reason, even if a mutation occurs in the same gene, the effect on the development of ASD might be different depending on the mutation type.

Likewise, *ROBO1* which was identified in ASD with comorbid epilepsy was implicated in developmental dyslexia and dysfunction of language acquisition system (Hannula-Jouppi et al., 2005; Bates et al., 2011). In addition to the roles in guiding axons and the Slit/Robo signaling pathway, *ROBO1* is also involved in cellular processes such as cell migration and immune cell activation during neuroinflammatory responses (Mirakaj and Rosenberger, 2017). Recently, it has been suggested that inflammation and autoimmunity play important roles in childhood seizures and epilepsies (Korff and Dale, 2017). In our case, the patient with a nonsense mutation in *ROBO1* was diagnosed with ASD and had clinical manifestations of focal seizure. This case suggests that although a genetic variation does not satisfy the criteria of pathogenic/likely pathogenic variants, it might affect an individual's phenotype.

Through these results, it is possible to surmise that genes with known pathogenic variants may often appear with VOUS also. As variants affect genetic functions such as synaptic and neuronal plasticity (Ben-David and Shifman, 2012), the influence on ASD would be exerted when loss of function occurred, even though the effect may vary by location and type of mutation. It is necessary not to overlook genes with VOUS if the gene has been previously reported with pathogenic variants in neurodevelopmental disorders, including ASD.

Heterogeneity has been a great challenge for developing tailored treatment of ASD as there are a large number of genes related to ASD, and loss of function differs according to each type of mutations. Through the advancement of genetic analysis technology, NGS results are being used in clinical fields, but it is still difficult to interpret and identify the clinical significance. To provide proper management to ASD individually, discrimination of the pathogenic variant among multiple variants should be achieved. Our results show that it is necessary to notice genes with VOUS although their function is not clearly defined yet. Especially in ASD presenting heterogeneous clinical manifestation and frequent comorbid disorders, results of genetic analysis should be performed with caution. The VOUS in ASD related genes involved with unclear mutation, or non-ASD related genes with clinically relevant phenotype may be of primary importance in investigating genetic data (Lovato et al., 2019). Efforts to identify the function of genes with VOUS will lead to discovering genetic pathogenesis of neurodevelopment disorder in the future.

There are several limitations to this study. First, as this study reviewed medical records retrospectively, clinical assessment could not be performed without bias. This may have influenced the statistical results of the demographic data. Second, in cases of age under 4 years, we could estimate intellectual disability only by Bayley Scales of Infant Development. Third, as our participant group mostly showed severe phenotypes (SRS T-score 86.39 on average), further studies are needed to compare differences in genetic components

according to severity of ASD phenotype. Furthermore, as we analyzed the clinical NGS reports retrospectively, we could not show data from typically developing control group. To define the pathogenicity of variants of genes, comparing the result with that of normal population might be helpful. Finally, as medical records were reviewed cross-sectionally, we could not evaluate the development of comorbidities including epilepsy.

Despite the limitations mentioned above, there are several strengths in our study. First, to our knowledge, this is the first NGS study in ASD patients with or without comorbid epilepsy in Korea. As all patients are Korean, our results are not confounded by population genetic heterogeneity. Second, because NGS was carried out only in ASD patients who had severe phenotypes, comorbid disorders, or suspicious general appearance in a clinical setting, genetic variants thought to impact ASD development were able to be easily obtained. Third, we found some genes that have not previously been reported but are possibly pathogenic in ASD. Finally, by considering comorbid epilepsy, we confirmed genetic overlaps in ASD and epilepsy, even though genetic variations are currently known to be related just with either ASD or epilepsy.

In conclusion, we suggest that rare variants (pathogenic/likely pathogenic) and common variants (VOUS) are both necessary in investigating individuals' genetic characteristics in ASD and epilepsy. Pathogenic/likely pathogenic variants might be useful in confirming genetic syndrome, predicting comorbidity, and treatment planning. The VOUS might also influence the phenotype characteristics of ASD and epilepsy, even though the evidence and possibility are not strong enough. Careful efforts in interpreting the VOUS might contribute to understand the genetic cause of ASD and epilepsy.

DATA AVAILABILITY STATEMENT

The datasets generated for this study will not be made publicly available because it includes the patient's genetic data for clinical purpose.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Institutional Review Boards at Severance Hospital, Yonsei University College of Medicine. Written informed consent from the participants' legal guardian/next of kin was not required to participate in this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: JL, SH, K-AC. Performed and analyzed NGS: S-TL, S-GP, SS, JC. Analyzed data: JL, SH, S-GP, SS. Wrote the manuscript: JL, SH, S-TL, S-GP, SS, JC, K-AC.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2020.00585/full#supplementary-material

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