EXPERIMENTAL MODELS OF EPILEPSY AND RELATED COMORBIDITIES

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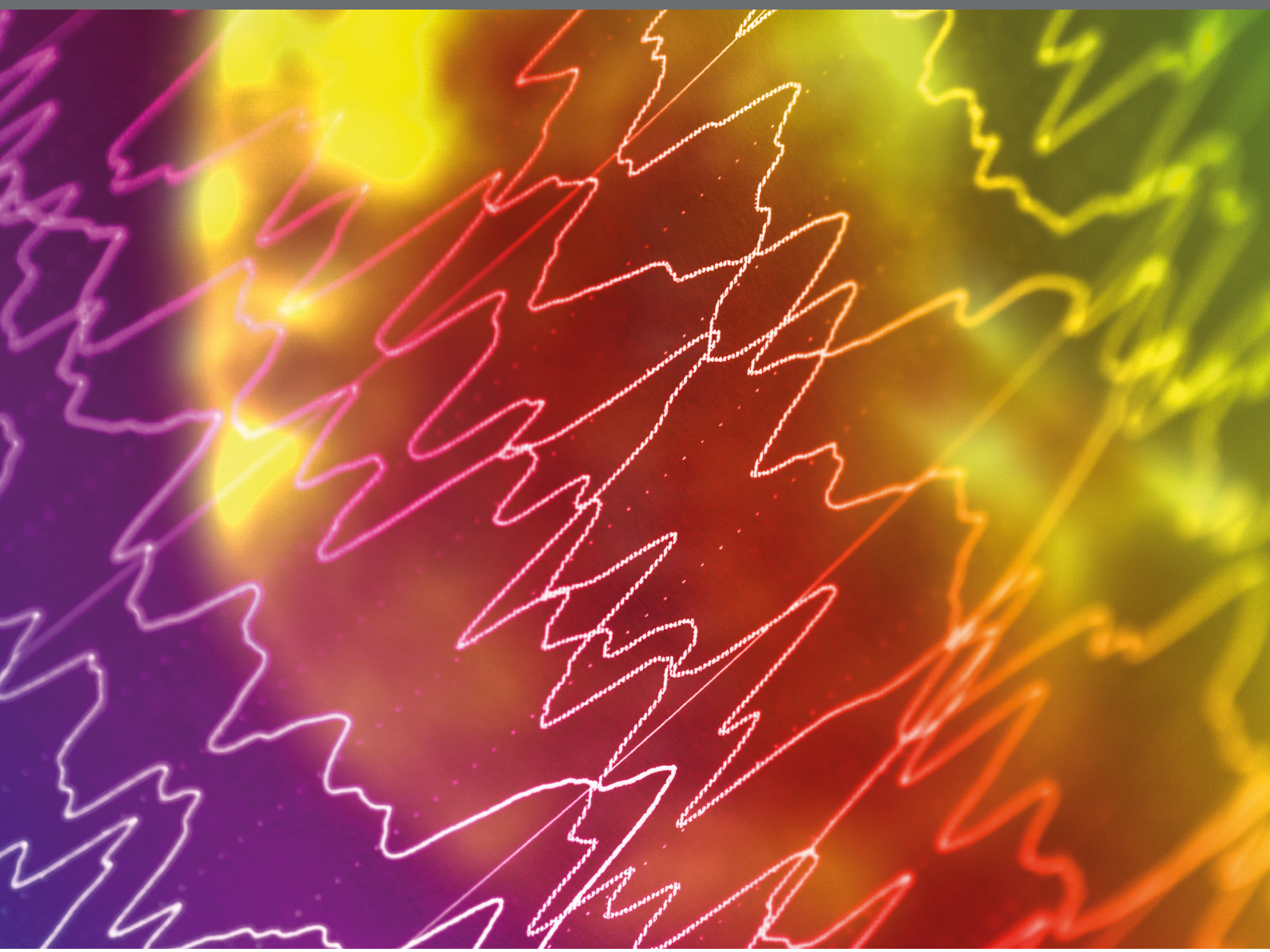

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EXPERIMENTAL MODELS OF EPILEPSY AND RELATED COMORBIDITIES

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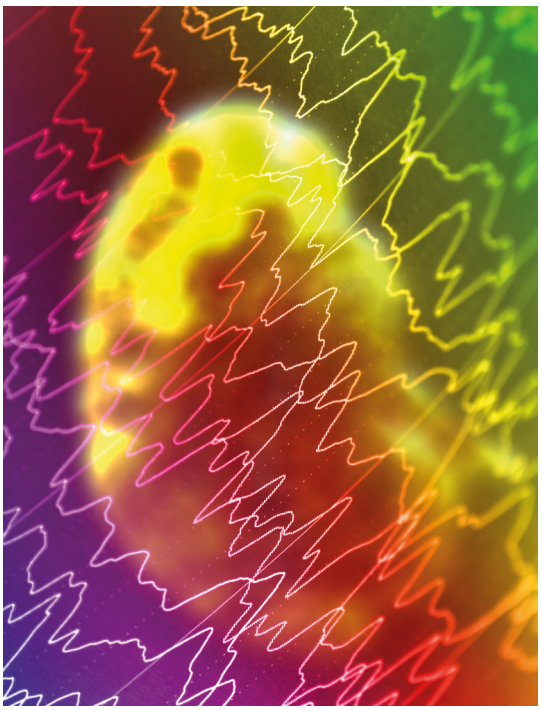

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Epileptic patients live with epilepsy-associated complications such as cognitive dysfunction, psychological discomfort, and sexual function decline, and are more likely to experience emotional and mental health issues problems, including depression and anxiety. Many antiepileptic drugs are found to have a role in aggravating psychiatric symptoms. Animal models, which inform translational questions about epilepsy comorbidities, are used to study the relationship between epilepsy and related comorbidities. The aim of this Research Topic was to highlight basic, clinical and interdisciplinary research involved in studying the disease and its comorbid effects.

Various experimental models are used to understand the mechansims of disease and to discover newer antiepileptic drugs. These experimental models combines the input from behavioral, biochemical and molecular level including genetic.

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EDITORIAL
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Editorial: Experimental Models of Epilepsy and Related Comorbidities

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Keywords: epilepsy, comorbidities, animal models, anticonvulsants, inflammation in epilepsy

Editorial on the Research Topic

Experimental Models of Epilepsy and Related Comorbidities

Epilepsy is a chronic neurological disease and patients with epilepsy have an increased risk for cognitive, behavioral, and psychosocial disorders that can adversely impact the quality of life. Various factors like common underlying predisposition, mechanisms underlying epileptogenesis, direct effects of seizures, and adverse effects of anti-seizures drugs or other non-pharmacological therapies may play a causal role in such epilepsy associated comorbidities. New and validated screening approaches as well as novel therapeutic approaches are warranted, that may help with the early detection and treatment of comorbid conditions. Experimental models of epilepsies can play a critical role for improving our understanding of not only of the fundamental mechanism of epileptogenesis but also to generate new insights regarding the relationship between epilepsy and related comorbidities.

This Research Topic compiles 12 articles, including 3 reviews, 1 mini-review, and 8 original research contributions from leading scientists in the field. The collection of papers on this topic offers an up-to-date overview of current knowledge and approaches for studying epilepsy and related comorbidities. The articles highlight the pivotal role of numerous experimental and clinical studies that have elucidated novel mechanistic insights about the pathophysiology of these conditions. Translational perspective of research in this domain as well as important methodological issues and some emerging hypotheses in the field are also highlighted. Recent progress in the pharmacotherapy and management of these conditions have also been critically analyzed and discussed. The content of each of these articles is summarized below.

The systematic review on non-mammalian models of epilepsy research by Arief et al., describes the role of non-mammalian models (i.e., Zebrafish, Drosophila, *C. elegans* etc.) for better understanding the mechanisms of epileptogenesis as well as for screening compounds with potential anti-seizure properties. This review highlights that a more comprehensive evaluation of these currently under-utilized non-mammalian models may help preclinical epilepsy research progress.

The article by Muke et al., reports on the anti-convulsive potential of nasally administered coumarin fraction of medicinal plant *Eclipta alba* using PTZ induced kindling model. Their mechanistic studies showed that the efficacy of coumarin nasal formulation (CNF) was due to its ability to reduce oxidative damage and neuroinflammation. These exciting findings suggest the promising role of CNF as a therapeutic modality for epilepsy and related comorbidities.

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Sleep disorders and epilepsy share a complex reciprocal relationship. The review article by Kumar et al., focuses on the current status of our understanding of the pathogenesis and therapeutic avenues related to three of the most common type of epilepsy that occur during sleep, namely hypermotor epilepsy (SHE), benign partial epilepsy with centrotemporal spikes (BECTS), and Panayiotopoulous syndrome (PS). The article also discusses the recent advances in the pharmacotherapy of these conditions.

Zeng et al., in their original research article elucidate the effects of Ginsenoside compound K (GCK), the key metabolite of Ginsenoside Rb1, Rb2, and Rc, on acute seizure and the potential mechanism underlying its effects. Their study showed that that GCK exerts its effect by enhancing the expression of proteins related to GABAergic inhibition.

Currently available anti-seizure drugs (AEDs) raise several concerns related to lack of efficacy in a significant proportion of epilepsy patients and adverse events as well as their limited supply in Countries with poor resources and high costs. Over the years, traditional herbal medicines have occupied a prominent role in the treatment and management of epilepsy. However, robust experimental evidence related to the effectiveness and side effects of such herbal medications are limited. The article by Moto et al. showed that aqueous extract of *C. quadrangularis,* a plant belonging to family *Vitaceae* and traditionally well-known for its anticonvulsive properties, exerts antiepileptic as well as anxiolytic effects in the pilocarpine model of epilepsy. The authors also report that the neuroprotective effect of this herbal extract is mediated by its antioxidant properties as well as through modulation of GABAergic transmission.

The genetically epilepsy-prone rat (GEPR) represents an useful animal model for studying mechanisms of epilepsy especially for genetically determined seizure predisposition. The GEPR shows heightened sensitivity to convulsant drugs as well as to kindling, hyperbaric, and hyperthermic seizures. Aguilar et al., tested male and female genetically epilepsy-prone rat (GEPR-3), a GEPR strain that exhibits inherited susceptibility to tonic-clonic seizures, in a battery of behavioral tests and showed that GEPR-3s showed increased anxiogenesis in multiple behavioral tests of anxiety. These interesting new data underscores the importance of genetic influence that may underlie both the seizure disorder and epilepsy associated comorbidities.

Dravet syndrome (DS) is a severe form of epilepsy formerly known as severe myoclonic epilepsy of infancy (SMEI) that appears in infancy or early childhood as frequent febrile seizures. As the condition progesses, it also leads to other types of seizures like monoclonus and status epilepticus. Griffin et al., presents the state-of-the-art on DS preclinical animal models with a focus on seizure phenotypes and behavioral comorbidities. The authors have critically analyzed the use of these models for drug screening with an emphasis on assay protocols and pharmacological profiles. In particular, the article provides a detailed overview of the electrophysiological and behavioral drug screening assays in zebrafish and summarizes the data on 3,000 drugs screened to date. The authors advocate that this preclinical strategy offers a modality for effective drug screening especially for genetic epilepsy.

In their original research article, Ye et al., outline new findings about the neuroprotective effect of L-3-n-butylphthalide (NBP), a compound obtained from the seed extracts of *Apium graveolens*. Using a mouse model of chronic epilepsy, they demonstrate the effectiveness of this compound in alleviating both seizure severity and the associated behavioral and cognitive alterations. Their studies further show that NBP mediates such protective effects by increasing the transcription of neuroprotective factors like, brain-derived neurotrophic factor (BDNF), and Klotho as well as by restoring the expression of neural synaptic proteins such as postsynaptic density protein 95 and glutamic acid decarboxylase 65/67. These findings suggest that NBP is a potential therapeutic agent to control epileptic seizures and related psychological and cognitive comorbidities.

Song et al., developed a new rodent model of extended hippocampal kindling to investigate the effects of three AEDs with different mode of action on spontaneous recurrent seizures. The authors showed that phenytoin and levetiracetam mitigated the spontaneous recurrent seizures whereas lorazepam reduced the severity of motor seizures, but had little effect on duration of hippocampal discharges. Although the model needs to be further characterized, it may prove valuable for assessment of novel drugs in the future.

The mini review by Kasahara et al. provides an overview of the salient features of animal models of neonatal seizures, namely hypoxia-ischemia-induced seizures and febrile seizures. Furthermore, pharmacological strategies to address epileptogenic changes have also been addressed.

The research article by Amini et al. provides interesting insights regarding the link between neuroinflammatory changes and pathophysiology of seizures. The authors studied the effects of lipopolysaccharide (LPS) preconditioning in a rat model of electroconvulsive shock (ECS) induced seizures. Their data indicate LPS preconditioning exerts a protective effect on seizure duration, which was associated with enhanced expression of interferon regulatory factor (IRF3), and other genes associated to Toll like Receptor 4 (TLR4) signaling pathway as well as down-regulation of pro-inflammatory mediators in the hippocampus.

The studies by Meng Choo et al., explore the effects of ethanolic leaf extract of *Orthosiphon stamineus* in seizures using a zebrafish model. The authors report that treatment with the extract reduces Pentylenetetrazol (PTZ) induced seizures and ameliorates seizure-induced elevation of NF-kB, neuropeptide Y and TNF-α in the brain. Although he active constituent of *O stamineus* was not identified as yet, it is noteworthy that the anti-seizure effect of the extract was comparable to standard AED like Diazepam. This highlights that this herbal product may be a promising candidate for drug development in epilepsy.

We are very grateful to the basic research and clinical investigators who have contributed to this research topic. We hope that these diverse and thoughtful papers will be useful to researchers to inspire their work and stimulate new discussions in this exciting research area. These studies also highlight the avenues for advancing the field for improving treatment and quality of life of patients. Further characterization of neural circuits and signaling mechanisms that contribute to the

pathogenesis of these conditions may lead to the development of safer and more effective therapeutic approaches to tackle epilepsy as well as the associated comorbidities.

AUTHOR CONTRIBUTIONS

AC took the initiative for the editorial write-up. MS, JA, and AV also contributed in writing, revising, and proofreading. All the authors approved the editorial.

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SYSTEMATIC REVIEW
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A Systematic Review on Non-mammalian Models in Epilepsy Research

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Epilepsy is a common neurological disorder characterized by seizures which result in distinctive neurobiological and behavioral impairments. Not much is known about the causes of epilepsy, making it difficult to devise an effective cure for epilepsy. Moreover, clinical studies involving epileptogenesis and ictogenesis cannot be conducted in humans due to ethical reasons. As a result, animal models play a crucial role in the replication of epileptic seizures. In recent years, non-mammalian models have been given a primary focus in epilepsy research due to their advantages. This systematic review aims to summarize the importance of non-mammalian models in epilepsy research, such as in the screening of anti-convulsive compounds. The reason for this review is to integrate currently available information on the use and importance of non-mammalian models in epilepsy testing to aid in the planning of future studies as well as to provide an overview of the current state of this field. A PRISMA model was utilized and PubMed, Springer, ScienceDirect and SCOPUS were searched for articles published between January 2007 and November 2017. Fifty-one articles were finalized based on the inclusion/exclusion criteria and were discussed in this review. The results of this review demonstrated the current use of non-mammalian models in epilepsy research and reaffirmed their potential to supplement the typical rodent models of epilepsy in future research into both epileptogenesis and the treatment of epilepsy. This review also revealed a preference for zebrafish and fruit flies in lieu of other non-mammalian models, which is a shortcoming that should be corrected in future studies due to the great potential of these underutilized animal models.

Keywords: epilepsy, fruit fly, drosophila, leech, planaria, roundworm, tadpole, zebrafish

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INTRODUCTION

Epilepsy is a type of central nervous system (CNS) disorder that affects neuronal activity in the brain, causing unprovoked seizures and other behavioral changes (Mussulini et al., 2013). An abnormality in neuronal activity relates to excessive electrical discharges which results in seizure episodes that can vary in frequency from less than one per year to a few times per day. Seizures can be classified into two main types, partial seizures and generalized primary seizures. Furthermore,

a seizure is divided into three stages: aura, ictus and postictal. The aura stage is experienced by the victim when he or she begins to display abnormal sensations such as nausea, headaches, abnormal senses and sudden emotions (fear, panic, etc.). The victim then enters the ictus stage in which the symptoms can be convulsive (vigorous jerking of the body and loss of consciousness) or non-convulsive (inability to respond and muscle spasms), depending on the type of seizure. The postictal stage is also known as the recovery stage and is when the victim begins to experience post-seizure symptoms such as drowsiness, confusion and partial paralysis. The onset of this disorder can occur in all ages, although it mostly occurs in childhood or later adulthood and is the fourth most common CNS disorder (England et al., 2012).

Currently around 70 million of the world's population is affected by epilepsy (Cunliffe et al., 2015). Epilepsies with known causes (secondary epilepsies) may be the result of prenatal brain damage, genetic abnormalities which lead to brain deformities, severe brain trauma, severe stroke, infection of the brain and brain tumors. Sufferers tend to have problems both physical (bruising and fractures from seizure) (Baker et al., 1997) and psychological (anxiety and depression) (Ettinger et al., 1998) in nature. The abnormal epileptic brain waves typical of epilepsy patients can be detected using the magnetoencephalography (MEG) and electroencephalogram (EEG) techniques (Karis, 2008). Unfortunately, epilepsy cannot be cured and can only be symptomatically treated. Treatment typically involves the usage of anti-epileptic drugs (AEDs) (White et al., 2007), surgery (Jette et al., 2014), vagus nerve stimulation (Uthman et al., 1993) or the ketogenic diet (Neal et al., 2008). However, about a third of the epileptic population do not respond to current epilepsy treatments (White et al., 2007). Moreover, epileptic individuals face a lower quality of life as they are burdened by the limitations placed on their physical activities and can be subjected to prejudice due to their disruptive seizures (Baker et al., 1997).

The usage of animal models is essential for the study of epilepsy as the process of epileptogenesis and ictogenesis cannot be induced in human trials due to ethical reasons. Rats and mice have previously been used for animal testing due to their similar morphological structure to humans. However, in recent years, non-mammalian epilepsy models have been the primary focus in animal testing due to multiple factors. These factors including increased cost-effectiveness, high genetic correlation with humans and rapid breeding, all of which improves the efficiency of epilepsy research (Cunliffe et al., 2015). In non-mammalian epilepsy models, Pentylenetetrazole (PTZ) (Kundap et al., 2017), kainic acid (KA) (Kandratavicius et al., 2014) and pilocarpine (PILO) (Kandratavicius et al., 2014) are among the proconvulsants used for stimulating seizures. In addition, electrical stimulation may also be used to induce seizures in animal models and differs from proconvulsants in that its effects can be studied without the continued presence of the epileptogenic cause (Kandratavicius et al., 2014). Thus, a comprehensive literature research was commenced to establish a systematic review which discusses the importance and usage of non-mammalian models in studies concerning epilepsy. This review aims to consolidate current information on the use and importance of non-mammalian models in epilepsy testing to better aid the planning of future research and screening of potential anticonvulsive compounds.

MATERIALS AND METHODS

Search Method

Studies between January 2007 and November 2017 were considered for evaluation. This restriction is to ensure that the most recent publications are covered in this review while minimizing the possibility of inadvertently excluding older studies. An initial search of relevant studies was performed using Google Scholar to grasp the general scope of the topic. After that, a final search of relevant studies was conducted using several databases which were: PubMed, SCOPUS, SpringerLink and ScienceDirect. A search using the keywords "epilepsy," "seizures," "animal model," and "convulsions" was first done to generate a list of relevant articles for each database. After that, the keywords "non-mammalian," "drosophila," "leech," "planaria," "roundworm," "tadpole," and "zebrafish" were searched for in each of the generated lists and used to select the final articles. This was done by first screening through the abstracts of the generated results, before proceeding to full text screening of potentially highly relevant articles.

Study Selection and Inclusion Criteria

Studies that were searched and considered for the systematic review were limited to original research articles as other publications (symposiums, conferences, editorials, book chapters, reviews and systematic reviews) would not provide sufficient information for evaluation and comparison. Any duplicated articles from the different databases were removed and articles that have no relevance to the importance of non-mammalian models for epilepsy research were also excluded. The selection of studies was conducted as per PRISMA guidelines (Moher et al., 2015).

RESULTS

The initial search based on the keywords mentioned in the methodology yielded a total of 11,206 records. After applying exclusion criteria, total articles removed were 11,155, which includes; (a) 4,154 non-original research articles (b) 2,120 articles not related to animal models used in epilepsy research, (c) 306 duplicates, (d) 4,155 articles not related to non-mammalian models used in epilepsy research and (e) 420 full text articles not relevant to the aim of the review (**Figure 1**). Fifty-one eligible articles were included, compiled in **Table 1** and discussed in the present systematic review. Based on the inclusion criteria, the final articles selected for evaluation consist of 19 articles relating to fruit flies, one article relating to leeches, three articles relating to planaria, two articles relating

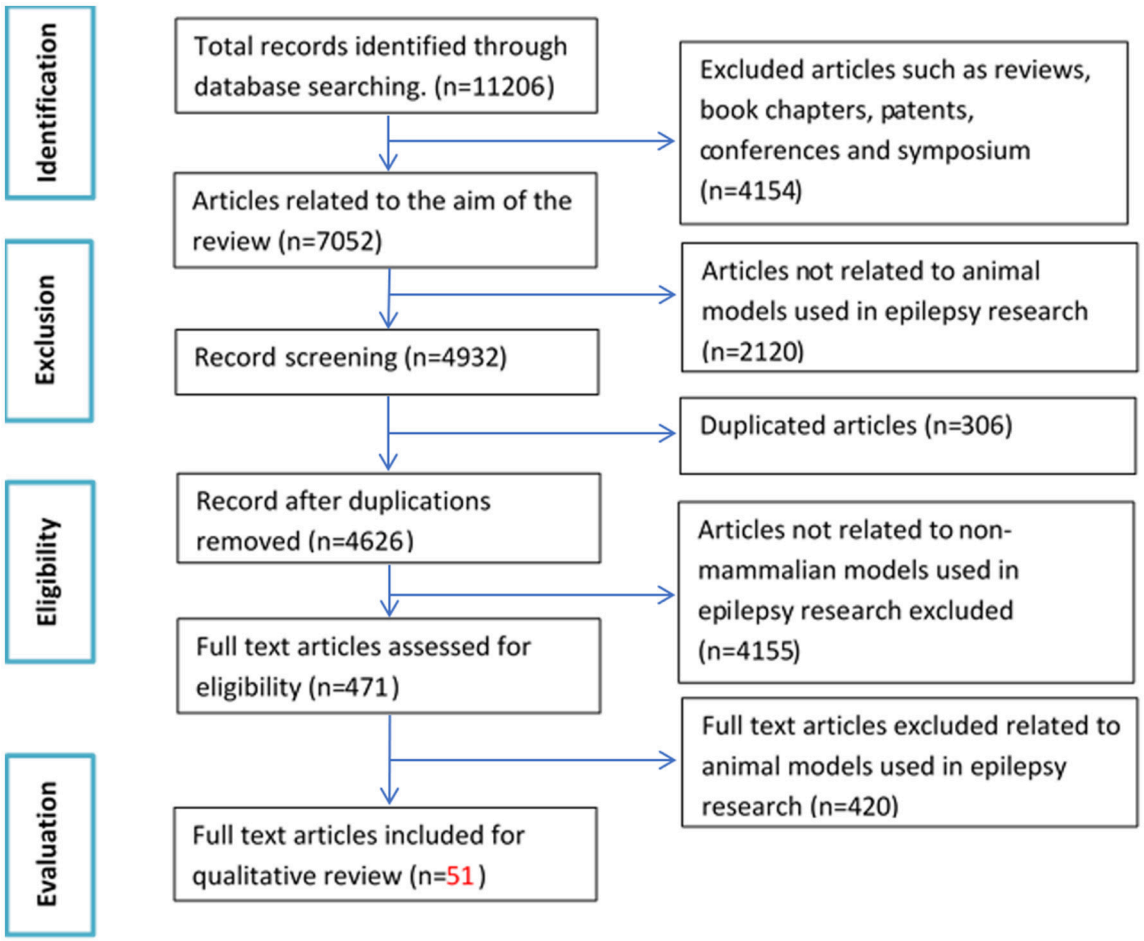

FIGURE 1 | Flow chart of study selection criteria based on Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines.

to roundworms, two articles relating to tadpoles and 24 articles relating to zebrafish, for a grand total of 51 articles. A brief overview of all the non-mammalian models for epilepsy research in our review is summarized in graphical form in **Figure 2**.

Fruit Fly (*Drosophila melanogaster*)

Drosophila have been widely used as a genetically tractable animal model for epilepsy research, especially the transgenic and mutant types. Seizures in *Drosophila* are defined as a period of brief leg twitches and is followed by a failure to maintain standing posture with leg shaking, abdominal muscle contractions, wing flapping and scissoring as well as proboscis extensions (Sun et al., 2012).

The usage of fruit fly models has been observed in 19 studies relating to epilepsy that were covered in this review.

Sleep Deprivation-Induced Seizures

Sleep and seizures have already been associated in humans as sleep deprivation is known to cause paroxysmal epileptiform activity (Lucey et al., 2015), though the exact mechanisms which relate the two together are currently unknown. *Drosophila melanogaster* is a powerful model organism that has been successfully used to study human physiologic processes such as sleep and disease states such as epilepsy (Shaw et al., 2000). Research into sleep and the fruit fly has shown that not only do flies sleep, but that data obtained in flies can be applied directly to humans (Lucey et al., 2015) as biomarkers of sleepiness first identified in the fruit fly have also been found to be elevated in healthy humans after a prolonged period of wakefulness (Seugnet et al., 2006). *Drosophila* mutants that are prone to mechanical and temperature induced seizures are a validated model to investigate the molecular and cellular networks responsible for seizure phenotypes (Burg and Wu, 2012) as mechanical and temperature stress-induced seizures exhibit several similar features to seizures in humans, such as a stereotyped behavioral sequence of spasm-and-paralysis, followed by a refractory period when the mutant flies are no longer sensitive to their respective stress disturbance (Lucey et al., 2015). The effects of sleep deprivation on seizure activity are not confined to a particular class of seizure mutant and can be observed using mechanical

stimuli or changes in temperature to induce seizures (Lucey et al., 2015).

Effect of Cryptochrome-Dependent Magnetic Field on Seizure Response in *Drosophila* Larvae

Drosophila embryos exposed to pulsed blue light (470 nm) develop a heightened seizure-phenotype when tested post-embryonically at the third instar larvae stage, with the effect significantly potentiated when a magnetic field is also present (Marley et al., 2014). This is because the flavin adenine dinucleotide (FAD)-containing, circadian clock photoreceptor protein, cryptochrome (DmCRY) present in *Drosophila* central neurons, will render those neurons photosensitive and result in the increase in action potentials generated when illuminated with blue light (Fogle et al., 2011). The reason that magnetic fields potentiate the effect of blue light is that DmCRY plays a role in the magnetic sense of *Drosophila* (Gegear et al., 2010) and thus would also modulate the ability of light activated DmCRY to influence level of synaptic excitation in the *Drosophila* CNS.

Modeling Epilepsy Using *Drosophila* Mutants

Recent technical advances have now made it practical to readily target and replace endogenous sequences in the fly genome using homologous recombination (Rong and Golic, 2000; Rong et al., 2002; Staber et al., 2011). Knocking-in of specific disease-causing mutations into the fly genome could provide a rapid and low-cost platform for studying the cellular mechanisms of human diseases. Knock-in flies can be used in combination with forward genetic screens to identify suppressor and/or enhancer mutations, a strategy that is challenging in humans and rodent models but well established in *Drosophila* (Song et al., 2007, 2008). One example is the human *SCN1A* sodium channel gene, which has more than 600 possible mutations which can confer a wide spectrum of epilepsies (Claes et al., 2009; Lossin, 2009; Catterall et al., 2010). *SCN1A* has more than 40 missense mutations that are known to be linked to an epilepsy syndrome termed "genetic epilepsy with febrile seizures plus" (GEFS+) (Sun et al., 2012).

While undoubtedly there are many mutant strains of *Drosophila* useful for the modeling of epilepsy that have been and will be discovered, the bang-sensitive (BS) group of *Drosophila* mutants are perhaps more popular than others. BS flies are useful for modeling epilepsy as they are significantly more prone to seizure like activity from a variety of insults as compared to their wild type counterparts (Burns et al., 2004; Stone et al., 2013). In addition, the seizure like activity displayed by BS flies such as violent bursts of uncoordinated leg, wings and abdomen contractions that are interspersed with paralysis, have parallels to human epilepsy (Lee and Wu, 2002; Stone et al., 2013). This bodes well for the validity of the BS model of epilepsy and any subsequent translation of results to human studies.

Prickle proteins, which were first discovered in *Drosophila*, are highly conserved (Spencer, 1945). pk^{sple1} mutants can be produced by crossing pk^{sple1} homozygous mutants with yw^{67} control flies. pk^{sple1} heterozygotes display none of the morphological abnormalities of the homozygotes but are significantly more bang sensitive than control flies of the same age. An epilepsy phenotype that is found in disparate species and crosses the invertebrate-vertebrate transition strongly suggests that the prickle proteins are part of a highly conserved evolutionary pathway for regulating seizures (Tao et al., 2011).

Brain Electrical Stimulation-Induced Seizures

As commonly seen in other models of epilepsy, electrical stimulation can also induce seizures in *Drosophila* (Song et al., 2007). This method essentially involves inserting electrodes into the *Drosophila* brain and delivering a certain amount of high frequency stimulation depending on their genotype, to induce seizures. The lowest possible intensity whereby seizures are elicited is known as the seizure threshold and if the flies fail to display seizures at the lowest possible intensity, the intensity is increased at 1V intervals until seizures are seen, with the fly being allowed to rest for several minutes in between each round of stimulation. Seizure activity in *Drosophila* is characterized by uncontrolled high frequency ($>$ 100 Hz) neuronal firing and this can be most conveniently seen indirectly in the motor neurons that extensively innervate *Drosophila* thoracic muscle groups (Song et al., 2007; Kroll et al., 2015).

Drosophila Model of Refractory Epilepsy

The multidrug resistance-associated protein 1 (MRP1) is a member of the Adenosine Triphosphate (ATP) binding cassette (ABC) superfamily and is a transporter of organic anions and drugs. As MRP1 is typically found in the blood brain barrier of humans, it likely functions as a protective mechanism to prevent the build-up of xenobiotics and drugs in the brain. However, an overexpression of MRP1 would also lead to a decrease in the extracellular concentration of AEDs in the brain and thus lead to refractory epilepsy. Unsurprisingly, MRP1 has been found to be overexpressed in the neurons and astrocytes of patients with refractory epilepsy (Sun et al., 2016).

Drosophila mutants with sodium ion channelopathies, such as those with *bss* phenotypes, have a lower seizure threshold as compared to normal flies and are thus more prone to seizure episodes (Parker et al., 2011; Howlett et al., 2013). Seizures in *bss* flies are typically very difficult to supress using either suppressor mutations or AEDs, making them potential models of refractory epilepsy (Howlett et al., 2013). It is also possible to model human refractory epilepsy in *Drosophila* by causing an overexpression of human MRP1 in bss mutant flies. Such flies are resistant to the seizure attenuation effects of phenytoin (acute and chronic application) as well as valproic acid (chronic application) (Bao et al., 2011). Thus, bss mutant flies which overexpress human MRP1 have the potential to become a model of human refractory epilepsy.

Medicinal Leech (*Hirudo verbena*)

With the emergence of non-mammalian models for use in research, the potential benefits of the medicinal leech for epilepsy research was evaluated. Despite its uncommon usage in epilepsy studies, medicinal leeches possess a relatively simple CNS in terms of neuron count. This is further supported by the similar physiological processes found in both leech and mammalian nervous systems in terms of neural circuitry and development. The maintenance and handling of leeches is also simpler and

TABLE 1 | Summary of the selected articles, together with high throughput screening feasibility.

Animal Model ***Scientific name*** **(*n* = Sample size)**	**Strain**	**Epileptogenic insult type (parameters)**	**Experimental findings**	**Possibility of high-throughput screening (HTS)**	**References**
Fruitfly *Drosophila melanogaster* ($n = 5$–100)	GEFS+ *SCN1A* mutation	• High temperature (40°C) • PTZ (0–0.4 mM)	• GEFS+ flies exhibit a heat-induced seizure phenotype • Blockade of GABA receptors increases sensitivity to heat-induced seizures in GEFS+ flies	Yes (Stilwell et al., 2006)	Sun et al., 2012
	Canton Special (CS)	High frequency stimulation (0.5 ms pulses at 200 Hz for 300 ms)	• Acute feeding of potassium bromide ameliorates *bss* phenotypes • Acute feeding of Top1 inhibitors does not eliminate seizure-like behaviors, but did reduce the mean recovery time (MRT) of BS mutants		Song et al., 2008
	$para^{bss1}$	High-frequency electrical brain stimulation (0.5-ms pulses at 300 Hz for 400 ms)	• Recovery time from BS paralysis for $para^{bss1}$ varies with genetic background, age, and other factors • Recovery time appears to be primarily dependent on the number of bouts of tonic–clonic like activity		Howlett et al., 2013
	• Easily shocked *(eas)* • Bang senseless *(bss)* • Technical knockout *(tko)*	• 70 ml fly vials • VWR VortexGenie (10 s)	• The *atu* mutation reduces SLA and shortens the recovery time in BS mutants		Stone et al., 2013
	Drosophila melanogaster	• Fly vials (10 s) • 39°C water bath • C4162 High Power Strobe, Chaney Electronics (10 Hz for 10 s)	• Glia endogenously express both Dube3a and ATPα proteins • Overexpression of Dube3a in glia using repo-GAL4 results in seizure susceptibility • Glial Dube3a overexpression alters neuronal architecture • Glial-specific overexpression of Dube3a causes reduced intercellular K^+ in glial cells		Hope et al., 2017
	Wild type Oregon-R	PTZ (3.48, 0.33, and 5 mg/ml)	• Convulsions were not observed, visual examination found hyperkinetic behavior in PTZ treated flies • NaVP and LEV alleviates PTZ induced climbing speed deficit in flies • LEV exerted a long-term effect		Mohammad et al., 2009
	pk^{sple1}/pk^{sple1}	Fly vials (20 s followed by 10 s)	• Drosophila with homozygous prickle mutations display seizures		Tao et al., 2011
	• Easily shocked*(eas)* • Bang senseless *(bss)* • Technical knockout *(tko)*	70 ml fly vials (10 s)	• Acute exposure (10 s) to 100% CO^2 caused SLA in all three BS mutants • Refractory period following acute gas exposure • Susceptibility to SLA and anaesthetization following hypercapnia • Ability of hypoxia to trigger SLA • Prolonged exposure to anesthetic gases delayed SLA		Whelan et al., 2010

(Continued)

TABLE 1 | Continued

Animal Model *Scientific name* (*n* = Sample size)	Strain	Epileptogenic insult type (parameters)	Experimental findings	Possibility of high-throughput screening (HTS)	References
	• Canton-S (wild-type) • Technical knockout *(tko)* • sesB • eas^{alaE13} BS mutants	Enclosed square recording arena with Vortex Genie2 (10 s)	• The ketone body β-hydroxybutyrate (β-HB) reduces SLA in the *eas* BS strain • The anticonvulsant effects of β-HB on SLA are partially mediated by both K_{ATP} channels and $GABA_B$ signaling		Li et al., 2017
	Wild type Canton S	PTX (5 μM) 4-aminopyridine (3 mM)	• Exposure to proconsulvants heightens stimulation of neuronal activity • Exposure to Gbp reduces synchronicity		Streit et al., 2016
	• Bang senseless • W^{1118} • elav-Gal4 • *UAS-MRP1* • *UAS-MRP1: elav-Gal4*	Fly vials that were vortexed at VORTEX-5 for 10 s	• PHT reduces mean recovery time of three control groups containing the *bss* mutation • PHT and VPA caused negative effects on eclosion rate but positive effect on seizure behavior		Bao et al., 2011
	Wild-type	High-frequency stimulation 0.5 ms pulses at 200 Hz for 300 ms using oscilloscope Minimal voltage: 2 V	• During induction of seizure, oscilloscope shows seizure-like activity • Presence of 7 min refractory period in flies after high frequency stimulation • If seizure is still induced, refractory period increases to 17 min		Howlett and Tanouye, 2009
	• Wild-type • $para^{bss1}$ • *eas* • *sda*	High-frequency stimulation 0.5-ms pulses at 200 Hz for 400 ms using electrodes	• Injection of saline control caused a slight increase in seizure threshold whereas injection of 25 mM valproate saline solution caused a significant increase • Seizure sensitivity of $para^{bss1}$ is the most difficult to suppress		Howlett and Tanouye, 2013
	• eas^{PC80} • $sda^{iso70.8}$	High-frequency electrical brain stimulation (0.4-msec pulses at 200 Hz for 300 ms using electrodes)	• sh^{its1} mutation in flies contributes to suppressing seizures • Seizure-like activity is related to the synaptic depletion • Disruption of synaptic transmission in excitatory neurons results in action potentials that are linked with seizures		Kroll et al., 2015

(Continued)

TABLE 1 | Continued

Animal Model *Scientific name* (*n* = Sample size)	Strain	Epileptogenic insult type (parameters)	Experimental findings	Possibility of high-throughput screening (HTS)	References
	• *Stress sensitive B* (*sesB9*$^{Ed-4}$) • bss paralytic mutant with loss of ANT • *Seizure* (*sei*^{ts}) • Temperature-sensitive paralytic mutant • UAS-*sesB*^{31320} • UAS-*sesB*^{36661}	Sleep deprivation Fly vials (10 s) and vortex machine	• Sleep deprivation can further influence seizure activity when *sesB* is disrupted • VPA can decrease susceptibility to sleep deprivation-enhanced seizure in *sesB9*$^{Ed-4}$ • The effect of sleep deprivation on seizure activity can be observed using mechanical stimuli • In early development, sleep deprivation can increase susceptibility to sleep deprivation-enhanced seizure		Lucey et al., 2015
	• Canton-S wild-type • Cry^{03}homozygotes^{29} • Larvae	Electroshock (10–30 V) Direct current pulse, created	• Exposure to pulsed blue light increased seizure duration • 100 mT magnetic field increased the effect of blue light on seizure severity and is light dependent • Antiepileptic drugs can prevent prolongation of seizure due to their effect on neuronal activity		Marley et al., 2014
	• Canton-S wild-type • Bang senseless • eas	High-frequency electrical brain stimulation (0.5-ms pulses at 200 Hz for 300 ms)	• Mechanical shock results in six specific seizure phases in flies • Genetic background can influence seizure duration • bss flies have lower seizure threshold • The behavior of *bss* mutants is similar to other BS mutants such as *eas*^{PC80}, *sda*$^{iso7.8}$, and *tko*^{25t}		Parker et al., 2011
	• *para*^{bss1} • *eas*^{PC80} • *sda* $^{iso7.8}$	High frequency brain stimulation (0.5 ms-pulses at 200 Hz for 300 ms using tungsten electrodes)	• *cac*^{TS2} acts as a general seizure-suppressor mutation and can revert the effects of mutations such as sda, eas and para^{bss1} • *cac*^{TS2} is a seizure-resistant mutant at room temperature but a seizure-sensitive mutant at high temperature		Saras and Tanouye, 2016
	• *eas*^{PC80} • *sda* $^{iso7.8}$ • *topo I*	High frequency brain stimulation (0.5 ms-pulses at 200 Hz for 300 ms using tungsten electrodes)	• *top1*^{JS} mutation is a general seizure-suppressor • *top1*^{JS} mutation increases seizure thresholds and reduces the recovery time of *eas* flies		Song et al., 2007
Medicinal leech *Hirudo verbana*	Stage IV larvae (*cca-1 mutant*)	PTZ (4–10 mM)	No seizures in T-type Ca^{2+} channel (*cca-1*) mutant worms	No data found	Hahn and Burrell, 2015
Planaria (*n* = 8–20)	Brown *D. tigrina*	NMDA (1, 3, 10 mM) PTX (0.01–5.0 mM) Nicotine (0.1–10 μM)	Planaria exhibit an increasing number of sudden asynchronous convulsive movements in a dose dependent manner when exposed to proconvulsants	No data found	Ramakrishnan and DeSaer, 2011

(Continued)

TABLE 1 | Continued

Animal Model *Scientific name* (*n* = Sample size)	Strain	Epileptogenic insult type (parameters)	Experimental findings	Possibility of high-throughput screening (HTS)	References
	Brown *D. tigrina*	Glutamate (0.6 mM) NMDA(1.4 mM) Semicarbazide (4.5 mM).	• Riluzole reverses l-glutamate, NMDA and semicarbazide induced PSLA • (+)-MK-801 reverses l-glutamate and NMDA induced PSLA		Ramakrishnan et al., 2013
	Dugesia dorotocephala	NMDA (0.01, 0.1, 1, 3, 10 mM) or water	• NMDA produced PSLA • MK-801 or DNQX antagonizes NMDA-induced PSLA • Topiramate antagonizes PSLA induced by NMDA or AMPA		Rawls et al., 2009
Roundworm *Caenorhabditis elegans* (*n* = 4–6)	Stage IV larvae (wild type N2)	High temperature (26°C-28 ± 1°C)	Seizure frequency was significantly decreased by Baccoside A	Yes (O'reilly et al., 2014)	Pandey et al., 2010
	Stage IV larvae (*cca-1 mutant*)		No seizures in T-type Ca^{2+} channel (*cca-1*) mutants		
	• Bristol N2 • CB156 *unc-25(e156)* • CB382 *unc-49 (e382)*	Electrical shock (200 Hz, 3.5 ms, 47 V) PTZ (72.0 mM)	Electric shock induces paralysis and convulsions in *C. elegans*		Risley et al., 2016
Tadpole *Xenopus laevis*	Albino *Xenopus laevis* (*n* = 20)	PTZ (15 mM) Bicuculline Picrotoxin Kainic acid (0.25 mM) Pilocarpine (75 mM) 4-ami-nopyridine, 4-AP (1 mM)	• All proconvulsants caused seizures which are divided into classes; (I) Rapid swimming, (II) Behavior arrest, (III) Loss of posture, (IV) Repetitive side-to-side lateral movement of head, (V) Fast, alternating contractions of axial musculature • A low number of TUNEL-positive and PI-positive cells were present, indicating progressive cell-loss within normal brain growth	No data found	Hewapathirane et al., 2008
	Wild type	PTZ (10 mM or 15 mM)	• PTZ induces seizures in tadpoles, causing uncontrolled tail bends and excessive turning • Seizure susceptibility decreases when the level of putrescine increases • Endogenous protective mechanisms prevent long-term cell damage due to the presence of polyamine		Bell et al., 2011
Zebrafish *Danio rerio*	Larvae, 7 d.p.f, Ekkwill strain	PTZ (40 mM)	• PTZ induces agitation (Stage I) before degrading into occasional body-stiffening and loss of posture (Stages II and III) • TPR, VPA, LTG • reduces PTZ-induced movement as to compare with VHC+PTZ, within 30 min. • GBP increases PTZ-induced movement • Other AEDs were inactive at their MTC	Yes - embryo (Liu et al., 2012)	Afrikanova et al., 2013

(Continued)

TABLE 1 | Continued

Animal Model *Scientific name* (*n* = Sample size)	Strain	Epileptogenic insult type (parameters)	Experimental findings	Possibility of high-throughput screening (HTS)	References
	• Adult, wild type (*n* = 6)	PTZ (220 mg/kg)	• The PTZ-treated group had an increase in distance, velocity, mobility, and circular rotations • GBP prevented PTZ-induced increases in zebrafish • cephalic field potential		Banote et al., 2013
	Larvae (*n* = 5)	PTZ (15 mM)	• SC-560 reduces *c-fos* mRNA expression compared to PTZ group whereas SC-236 has no effect • SC-560 prevents PTZ-induced increase of locomotor activity whereas SC-236 had no effect		Barbalho et al., 2016
	Larvae, WIK wild-type (*n* = 12)	PTZ (20 mM)	Oxcarbazepine, zonisamide and diazepam caused a decrease in locomotor activity		Berghmans et al., 2007
	• Adult, (AB), wild-type strain • Male (*n* = 8; 10 months) • Female (*n* = 9; 8 months)	PTZ (15 mM)	• Zebrafish experienced 4.93 seizure events on average • Epileptiform discharges last for 85 s on average		Cho et al., 2017
	Adult, WIK strain	PTZ (15 mM)	• Reactive gliosis manifests after PTZ-induced seizures • Total number of leukocytes increases after seizure • Increased cellular proliferation in ventricular zone and parenchyma		Duy et al., 2017
	Adult, wild-type (4–5 months)	PTZ (2, 4, 6, and 8 mM) Caffeine (1, 10, or 30 μM)	• Lower concentration (2 mM) of PTZ evokes stage I (increased swim activity) • Higher concentrations (4, 6 and 8 mM) of PTZ evokes stages II and III (II—rapid whirlpool-like circling swim, III—clonus-like seizures) • Valproic acid, gabapentin, lacosamide and carbamazepine increases latency to all stages • Pregabalin was ineffective		Gupta et al., 2014
	Larvae, 7 d.p.f	PTZ (15 mM) Picrotoxin Biculline	s334/Colbert mutant demonstrates inability to generate long duration epileptiform discharges in response to PTZ		Hortopan et al., 2010b
	• Wild type larvae, 3 d.p.f • Wild-type (*n* = 22), mib mutant (*n* = 28)	PTZ (15 mM)	• Stage 1 and 3 seizures were noted in mib^{hi904} mutants • 93% of mib^{hi904} mutants acquire recurrent spontaneous multi-spike bursts > 1,000 ms in duration		Hortopan et al., 2010a

(Continued)

TABLE 1 | Continued

Animal Model *Scientific name* (*n* = Sample size)	Strain	Epileptogenic insult type (parameters)	Experimental findings	Possibility of high-throughput screening (HTS)	References
	• 7 d.p.f., Larvae, wild-type (AB) • 5 d.p.f morpholino-injected larvae (WT) • 7 d.p.f scn1La mutant larvae (HO)	PTZ (40 mM)	• PTZ-treated larvae suffered more seizures than VHC-treated larvae • PTZ-treated larvae had a longer seizure duration than VHC-treated larvae • Seizures per larvae in the HO group is higher than the WT group • Chemical models produced frequent and long seizures as to compared to genetic models		Hunyadi et al., 2017
	Adult (3–4 months old) heterogenous WT stock	PTZ (170 mg/kg)	• PTZ group had seizures characterized by abnormal circular movements • PHY, RSV, OXC, GBP, and DZP provided resistance against PTZ		Kundap et al., 2017
	• Larvae (*n* = 10) • Embryo	Ginkgotoxin (40 mM)	Ginkgotoxin exposure induces seizure-like behavior within embryos		Lee et al., 2012
	Larvae (7, 15, and 30 d.p.f.)	Kainic acid (100, 300, and 500 μM)	• No locomotor or seizure activity recorded in 7 d.p.f. larvae • Locomotor activity detected in 15 d.p.f larvae • No locomotor or seizure activity recorded in 30 d.p.f. larvae		Menezes et al., 2014
	Adult, 4–6 months (*n* = 12)	PTZ (5, 7.5, 10, and 15 mM)	• Zebrafish immersed in PTZ solution experienced seizures • Seizure scores of zebrafish increases as PTZ concentration increases		Mussulini et al., 2013
	• Adult, wild-type *TupLF* strain • Adult, transgenic Tg[*HuC:GFP*]	Loss of cln3 protein using a knockout model	• Using *in situ* hybridization, cln3 gene expression was shown in WT zebrafish • EEG reveals increased frequency activity and higher amplitude in cln3 ATG MO morphants, which reflects epileptiform activity • Loss of cln3 protein in zebrafish causes motor abnormalities		Packer et al., 2016
	• Larvae, wild-type 7–14 d.p.f. (*n* = 5) • *Danio rerio* larvae, aldh7a1 mutants 7–14 d.p.f. (*n* = 5)	Transgenic model aldh7a1	• Light induces rapid "whirlpool-like" swimming (stage 2) and body convulsions (stage 3) when exposed to aldh7a1 mutants at 10/11 d.p.f. • High number of bursts of abnormal electrical discharge with long duration and high amplitude was observed in the tested mutants • Pyridoxine treatment prevents seizure-like behavior		Pena et al., 2017

(Continued)

TABLE 1 | Continued

Animal Model *Scientific name* (*n* = Sample size)	Strain	Epileptogenic insult type (parameters)	Experimental findings	Possibility of high-throughput screening (HTS)	References
	• Adult, laboratory strain WIK • (9–14 months)	PTZ (15 mM)	• PTZ induces seizures within zebrafish • Eugenol at high concentration prolongs seizure latency • Visual appearance of seizure tracings was observed and can be differentiated into baseline, pre-seizure, seizure and post-seizure		Pineda et al., 2011
	• Adult, Female laboratory strain WIK • (9–14 months)	PTZ (15 mM)	• Hindbrain stimulation in • the locus coerulus region promotes seizure resistance • Higher PTZ concentration causes higher stimulation rate		Pineda et al., 2013
	Adult, wild-type strain	PTZ (7.5 mM)	• PTZ induces behavioral changes in zebrafish (Stage I, II, and III seizures) • Increased latency to clonus-like convulsions are due to pretreatments PHT, GBP, and VPA • PTZ has no effect on ATP, ADP, and AMP hydrolysis but increases ecto-ADA and soluble-ADA activities		Siebel et al., 2013
	Adult, AB strain (6–9 months)	Kainic acid (6 mg/kg)	• KA induces behavioral changes in zebrafish, followed by dose-dependent seizures • DHA supplemented groups showed an increase in latency at time of seizure onset		Sierra et al., 2012
	• Larvae, wild-type strain (*n* = 6) • Larvae, Scn1a mutant (*n* = 6)	Transgenic model with Scn1a mutation	• FA reduces epileptiform brain activity • σ1-agonist, 5-HT1D–or 5-HT2C-antagonist can prevent FA's effect on locomotor activity • 66% decrease in monoamines due to FA treatment		Sourbron et al., 2017
	Larvae, AB wild-type	PTZ (1–25 mM)	• Stage II seizures were evoked in larvae • DA treatment reduces time in seizure latency • The DA cohort travel 5 times more than another other PTZ groups • DA treatment increases mobility of larvae		Tiedeken and Ramsdell, 2007
	Larvae, Tg (elavl3:GCamP6s), 4 d.p.f	PTZ (1–15 mM)	• Tetrodotoxin suppressed neuronal seizure activity caused by PTZ • GCaMP measurements can monitor basal activity and dynamics due to drug induced seizures • Valproate reduced larva motility and fluorescence		Turrini et al., 2017
	Larvae, AB wild-type	PTZ (1–16 mM) Picrotoxin, PTX (1–625 micrometer)	PTX-treated group has a higher maximum and lower minimum locomotor activity as compared with PTZ-treated group in dark conditions		Yang et al., 2017

PTZ, Pentylenetetrazol; GEFS+, Generalized epilepsy with febrile seizures plus; GABA, γ-Aminobutyric acid; BS, Bang Sensitive; LEV, Levetiracetam; SLA, Seizure Like Activity; PTX, Picrotoxin; PHT, Phenytoin; VPA, Valproic Acid; Eas, Easily Shocked; Bss, Bang Sensless; Tko, Technical Knockout; Sda, Slam Dance; NMDA, N-Methyl-D-aspartic acid; PLSA, Planarian Seizure Like Activity; TPR, Topiramate; LTG, Lamotrigine; VHC, Vehicle Control; AED, Anti-Epileptic Drugs; MTC, Maximum Tolerated Concentration; GBP, Gabapentin; OXC, Oxcarbazepin; RSV, Rivastigmine; DZP, Diazepam; D.p.f, Days Post Fertilization; WT, Wild Type; ADA, Adenosine deaminase; KA, Kainic Acid; DHA, Docosahexaenoic acid; FA, Fenfluramine; DA, Domoic Acid.

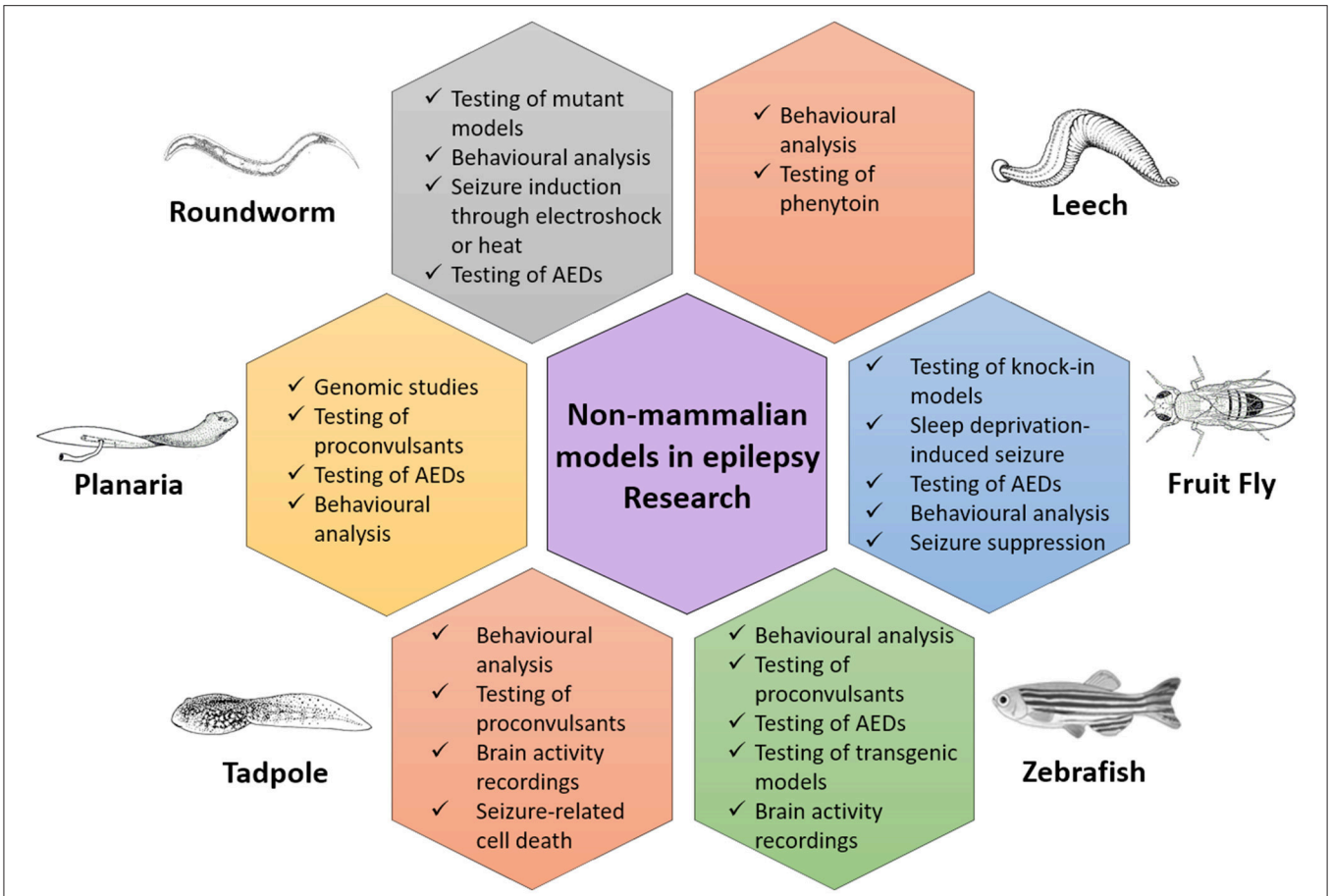

FIGURE 2 | A brief overview of all the non-mammalian models for epilepsy research in this review.

inexpensive as compared to mammalian models (Hahn and Burrell, 2015).

Only one study by Hahn and Burrell (2015) was conducted using the medicinal leech as an animal model in this review. They did this by first placing a single leech in a 100 ml beaker filled with 50 ml of pond water. PTZ (10 mM) was then added and the resulting motor behaviors observed in the medicinal leeches were categorized into normal (1 and 2) and abnormal (3 and 4). The level of motor behavior was further categorized into different classes: Type 1 – no activity, Type 2 – normal exploratory behavior with posterior sucker attached to beaker, Type 3 – abnormal exploratory behavior coupled with an inability to attach to the beaker, Type 4 – spontaneous twisting and tumbling behavior. The results reveal that spontaneous behavioral movement occurred during PTZ bath application as PTZ induced a concentration-dependent increase in motor activity within 5 min of treatment. The heightened motor activity was maintained for the period of PTZ treatment before reducing to baseline levels during washout. The PTZ treatment induced almost entirely abnormal behavior but did not cause any lethal effects. Pretreating the leeches with 1 mM of the AED phenytoin for 30 min prior to the PTZ treatment reduced the level of abnormal behavior by causing a reduction in motor activity, as well as affecting the behavior score.

Planaria

Planaria are free-living non-parasitic flatworms of the phylum Platyhelminthes and possess a bilaterally symmetrical CNS composed of neurons similar to those of humans, a body plan common to all vertebrates and many invertebrates, as well as mammalian-like neurotransmitters (Rawls et al., 2009). Comparative genomic studies of invertebrate genomes such as the planarian *Schmidtea mediterranea*, with the human genome have identified gene and protein homologs, which portrays the fundamental principle that biochemical processes are comparable in simple and complex organisms (Ramakrishnan et al., 2013). Planarians have systems which correspond to all the major neurotransmission systems found in vertebrate brains, such as glutamate (excitatory) and GABA (inhibitory) (Ramakrishnan et al., 2013). Planarians also express the genes for at least two ionotropic glutamate receptor types, both of which have a high sequence similarity to neural specific genes isolated from both mice and humans. The effects of drugs acting on glutamatergic, serotoninergic, dopaminergic and cholinergic CNS neural transmission can also be examined in behavioral

pharmacological studies involving planaria (Ramakrishnan and DeSaer, 2011). Invertebrate planaria also have the genes and neurotransmitters which correspond to the major inhibitory (GABA) and excitatory (glutamate) neurotransmission systems (Eriksson and Panula, 1994; Agata et al., 1998; Rawls et al., 2006). Even though these results suggest that glutamate-like receptors are important factors in planarian physiology, they are clearly not identical to mammalian glutamate receptors (Rawls et al., 2009). These differences in receptor homology and function may result in pharmacological effects that are not entirely the same across planarians and mammals. Nevertheless, planarian seizure models are easier to maintain and handle under laboratory conditions and are rather inexpensive in comparison with the other animal seizure models (Ramakrishnan and DeSaer, 2011), making planaria a fairly promising model for future research.

The usage of planaria models has been observed in three studies relating to epilepsy that were covered in this review.

Chemically Induced Seizures

Planaria exhibit sudden asynchronous convulsive movements which are very distinct from their normal locomotor activity, in proportion to the concentration of proconvulsant given (Ramakrishnan and DeSaer, 2011). *D. tigrina* exhibits screw-like hyperkinesis when exposed to 5 mM of picrotoxin. Planaria in the presence of low concentrations of (–)-nicotine (0.1 to 10 μM) display C-like and screw-like hyperkinesises whereas a more than 50 μM (–) of nicotine causes an increasing tendency to undergo longitudinal contraction, resulting in a walnut-like position. Once a planarian takes this walnut-like position, it essentially remains frozen in that position for a period of time (Ramakrishnan and DeSaer, 2011).

Nicotine, which is a cholinergic agonist, has also been found to induce hyperkinesia in planaria as neuronal nicotinic acetylcholine receptors (nAChR) play an excitatory role in the brain (Buttarelli et al., 2008). In addition, the molecular target for nicotine-induced seizures (α7 nAChR) was reported to be present in flatworms (Ribeiro et al., 2005). Planaria also exhibit seizure-like movements in nitrate and ammonia free tap water (Rawls et al., 2009). *Dugesia dorotocephala* has also been reported to display dose-dependent, seizure-like paroxysms when exposed to the excitatory neurotransmitters NMDA, α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) and L-glutamate (Rawls et al., 2009).

Roundworms (*Caenorhabditis elegans*)

Caenorhabditis elegans (*C. elegans*) is emerging as an important model for furthering insight into the cellular and molecular basis of neurological disorders (Dexter et al., 2012; Alexander et al., 2014). This microscopic nematode has several physiological similarities to mammals such as possessing ion channels, neurotransmitters and a conserved neuron morphology. *C. elegans* is small, inexpensive to maintain and has a relatively short 3-day generation time, all of which make large scale screening feasible (Risley et al., 2016). Seizures in *C. elegans* are typically induced via electrical shock or by increasing the ambient temperature (Pandey et al., 2010; Risley et al., 2016). Alternatively, convulsions in *C. elegans* can also be induced by mutations in the lis-1-allele (pnm-1), a defect in γ-aminobutyric acid (GABA) transmission (unc-25 and unc-49 mutants) or by PTZ and RNAi treatments in roundworms with depleted LIS1 pathway compounds (NUD-1, NUD-2 and DHC-1, CDK-5, CDKA-1) (Pandey et al., 2010). In the case of genetic changes, the resulting seizures which are induced in *C. elegans* are taken to be repeated contractions in either the dorsal or ventral directions (Pandey et al., 2010).

The usage of roundworm models has been observed in two studies relating to epilepsy that were covered in this review.

Electroshock Assay for *C. elegans*

An electroshock assay similar to previously established convulsion models in fruit flies, was developed to quantitatively monitor paralysis duration and convulsions in *C. elegans* following electroshock. This method relies on the fact that young adult roundworms immediately exhibit paralysis with body stiffness and elongation following a brief three-second electric shock, though shocked animals resume normal movement after a short recovery period (Risley et al., 2016). As in mammals, the Maximal Electroshock Seizure Test (MEST) is the gold standard test for anticonvulsant activity (Risley et al., 2016).

High Temperature Assay for *C. elegans*

On the other hand, an increase in the surrounding temperature can cause abnormal bursts of neuronal cells which may be linked to seizures or convulsions (Pandey et al., 2010). Seizure index parameters were created by Pandey et al. (2010) as a way of quantify the convulsions in *C. elegans*. The seizure index is determined by placing the roundworms in a seizure promoting buffer (100 mM NaCl, 50 mM $MgCl_2$) and then gradually increasing the temperature (26–28 ± 1°C) of the buffer using a variable intensity incandescent light source to generate heat. The seizures are ranked from 0 to 3, with 0 = no seizure or convulsion, 1(+) = two twitches in 10 s, 2(++) = two to five twitches in 10 s, 3(+ + +) = more than five twitches in 10 s or continuous twitching.

Tadpole (*Xenopus laevis*)

Tadpoles have been utilized as an animal model in a small number of epilepsy studies. They possess many traits that allow for the detailed analysis of their developing nervous systems to study disorders and neural development (Pratt and Khakhalin, 2013). Similar to zebrafish, tadpoles have a similar neural circuitry as compared to other vertebrates and is homologous to other mammals, yet simpler in design. The epileptiform discharges recorded from the optic tectum of tadpoles show similarity to those recorded in zebrafish and rodents (Bell et al., 2011; Pratt and Khakhalin, 2013). Their transparency also conveniently allows for *in vivo* imaging of neuronal activity and development as well as synaptogenesis (Hewapathirane et al., 2008) Their small size increases cost effectiveness as tadpoles are low maintenance due to their basic requirements. Thus, their neurological traits coupled with simpler maintenance, reinforces their role as an animal model in epilepsy research and an effective alternative to other animal models.

TABLE 2 | Characteristics, advantages, disadvantages, proconvulsants used with and the seizure behavior of the different non-mammalian models of epilepsy in this study.

Models	Features	Proconvulsant	Behavior	Uses/advantage	Limitations
Fruit fly (*Drosophila melanogaster*)	• High breeding rate • Low maintenance cost • Large chromosomes that are easy to manipulate • Quick and inexpensive genetic manipulation is possible • Completely sequenced genome • Relatively high genetic similarity to humans	• Pentylenetetrazole • Valproic acid • Picrotoxin • 4-aminopyridine • Electrical stimulation • Heat-induced seizure • Light stimulus (Transgenic models) • Mechanical shock	Loss of posture with leg shaking, abdominal muscle contractions and wing flapping	• Growth can be accelerated by heat • Short generation times • Assessment of seizure-induced behavior • Testing of proconvulsants • Testing of anti-epileptic drugs • Screening of potential anti-epileptic compounds	• Anatomy of flies and humans are very different • Complex behavior is difficult to measure • Effect of drugs is less predictable due to the differences in body systems
Medicinal Leech (*Hirudo verbana*)	• Simple central nervous system in terms of number of neurons • Similar physiological • processes of nervous system to mammalian nervous system • Seizure-like activity in leeches are applicable to human CNS through physiological processes • Low maintenance cost and easy handling	Pentylenetetrazole	Spontaneous twisting and tumbling behavior with inability to attach to beaker	• Assessment of seizure-induced behavior • Testing of proconvulsants • Testing of anti-epileptic drugs	Epilepsy studies involving medicinal leeches are lacking
Planaria	• Many planarian proteins are significantly similar to human proteins • RNA interference can be efficiently carried out by feeding, injecting or soaking planaria with solutions of double-stranded RNA • Planarians possess genes and neurotransmitters which correspond to all the major neurotransmission systems found in vertebrate brains • Low maintenance cost and easy handling	• N-methyl-D-aspartate • Picrotoxin • Nicotine • Semicarbazide • Glutamate	Increasing number of sudden asynchronous convulsive movements	• Assessment of seizure-induced behavior • Testing of proconvulsants • Testing of anti-epileptic drugs • Planaria can regenerate lost tissue or limbs • Modeling addiction behaviors	Epilepsy studies involving planaria are lacking The glutamate-like receptors in planaria are not identical to those in mammals

(Continued)

TABLE 2 | Continued

Models	Features	Proconvulsant	Behavior	Uses/advantage	Limitations
Roundworm (*Caenorhabditis elegans*)	• The hermaphrodite roundworms can self-fertilize • Complete gene sequenced • Can be grown cheaply and in large numbers on plates containing bacteria • Roundworms are small in size and produce over 1000 eggs per day, with a short life cycle of just 2 weeks • Many roundworm genes have human counterparts • Low maintenance cost and easy handling	• Pentylenetetrazole • Electrical stimulation • Heat-induced seizure	Multiple contractions while moving in the same direction	• Assessment of seizure-induced behavior • Testing of proconvulsants • Testing of anti-epileptic drugs • Screening of potential anti-epileptic compounds • Producing genetic mutants to model certain human diseases	Epilepsy studies involving roundworms are lacking Lacks many defined organs/tissues
Tadpole (*Xenopus laevis*)	• Similar neural circuitry to other vertebrates • Genetically homologous to other mammals • Similar to zebrafish in terms of recording epileptiform discharges • Low maintenance cost and easy handling	• Pentylenetetrazole • Kainic acid • Picrotoxin • Pilocarpine • 4-aminopyridine	Directional loss, immobility and C-shaped contractions.	• Assessment of behavior of seizure-induced tadpole • Measurement of neural activity in brain during seizures • Testing of proconvulsants • Application of electrophysiological recordings • Examination of seizure-related cell death	Epilepsy studies involving tadpoles are lacking
Zebrafish (*Danio rerio*)	• Both larvae and adult zebrafish can be used as models • Mass-breeding capabilities up to hundreds of eggs weekly • Rapid growth and development rate • 70% genetic similarity with human • 84% of human diseases can manifest in zebrafish • Low maintenance cost (<0.01$ per day) and easy handling • Can be genetically modified in early embryo development	• Pentylenetetrazole • Kainic acid • Ginkgotoxin • Picrotoxin • Caffeine • Light stimulus (Transgenic models)	**Larvae** – Thigmotaxis, rapid and "whirlpool" swimming followed by loss of posture **Adults** – Short-burst rapid swimming followed by distinctive "whirlpool" swimming and loss of posture	• High-throughput screening for drugs, specifically antiepileptic drugs • Assessment of behavior of seizure-induced fish • Analysis of epileptogenesis process • Measurement of neural activity in brain during seizures • Testing of various proconvulsants and antiepileptic drugs • Producing genetic mutants to model certain human diseases	High mortality Sensitivity to proconvulsants differ in each fish tested Lack of reliable EEG equivalents to record brain electrical activity in adult fish

The usage of tadpole models has been observed in two studies relating to epilepsy that were covered in this review.

Behavioral Changes and Testing of Proconvulsants

A study relating to the *in vivo* imaging of seizure activity was conducted using a tadpole model. The tadpoles that were used in the study are transparent, which allows for the examination of seizure-induced effects on the developing brain. Five specific proconvulsants which include PTZ, picrotoxin (PTX), KA, pilocarpine and 4-aminopyridine (4-AP), were used to induce seizure behavior in the tadpoles (Hewapathirane et al., 2008; Bell et al., 2011). The first study discovered that all the proconvulsants tested can induce seizure behavior in tadpoles at different levels of severity. The following proconvulsants doses induced seizures within 20 min: PTZ (10–50 mM), PTX (1 mM), KA (0.25–1 mM), PILO (75 mM), 4-AP (0.5–2.5 mM). PTZ-induced seizures were given primary focus in ensuing experiments due to PTZ's dose range (10–50 mM) that rapidly causes severe seizures in the absence of toxic effects (Hewapathirane et al., 2008). Another study discusses the use of *Xenopus* tadpoles in examining the roles of polyamines in maintaining neural excitability. Bath application of 10 and 15 mM PTZ was used to induce seizures in tadpoles (Bell et al., 2011). Seizure behavior was observed after a few minutes exposed to PTZ and gradually increased, leading to loss of motor control (Bell et al., 2011). The seizure activity in both studies was characterized by directional control loss, immobility and C-shaped contractions.

In Vivo Electrophysiological Recordings

Xenopus tadpole models can also be used for electrophysiological recordings. In the first study, agar-immobilized tadpoles in a recording chamber were used to examine neuronal activity during proconvulsant-induced seizures, via extracellular field recordings (Hewapathirane et al., 2008). The recordings showed distinctive epileptiform discharges after exposure to different proconvulsants such as PTZ, PILO, 4-AP, and KA. The magnitude of epileptiform discharges gradually increases, eventually growing into high-amplitude spikes, in contrast to baseline recordings that are only comprised of sporadic low-amplitude spikes. The appearance of epileptiform discharges after proconvulsant washout varies based on the proconvulsant used. PILO (75 mM) induced spaced, sporadic high-amplitude epileptiform spiking (0.3 mV) whereas 4-AP (1 mM) caused sustained epileptiform discharges with a higher frequency. On the other hand, KA induced bursting in the form of fast-rhythmic spiking. Thus, it is possible to pinpoint the type of proconvulsant based on electrophysiological recordings using *Xenopus* tadpole models.

Analysis of Seizure-Related Cell Death

Two approaches were used to examine seizure-related cell death within the tadpole brain for the first study (Hewapathirane et al., 2008). One was the usage of fluorescein-based *in situ* terminal uridine deoxynucleotidyl transferase dUTP nick end labeling (TUNEL), which aimed to detect DNA fragmentation related to cell apoptosis. The second method utilized the *in vivo* incorporation of propidium iodide (PI). The results revealed that a low number of TUNEL-positive sand PI-positive cells (assays) were present throughout control brains, which indicated a reduction in continuous cell loss during normal development. Cellular apoptosis due to prolonged seizures (4 h) failed to increase when measured by TUNEL labeling over a span of 6 h−2 days post-seizure. PI labeled cells slightly increased, which is indicative of abnormal cell membrane permeability mostly linked with necrotic cell death and sometimes cell apoptosis (Hewapathirane et al., 2008).

Zebrafish (*Danio rerio*)

Zebrafish have been acknowledged as one of the most widely-used animal models and have gained a reputation as an alternative to rodents and other animal models in epilepsy research over the last decade (Kundap et al., 2017). From a physiological perspective, they possess systems that are highly homologous to other vertebrates. The zebrafish genome has a 70% similarity to humans and zebrafish also possess complex anatomy and behavior which allows for the modeling of diseases (Cho et al., 2017). Both adult zebrafish and larvae can be used as animal models for epilepsy research, with larvae being the primary model in the majority of studies. Modification of gene and hence protein expression can be performed in zebrafish to produce genetically-modified zebrafish models by means of genetic intervention such as the injection of RNA, DNA and protein constructs during the early stages of embryo development (Berghmans et al., 2007). In contrast with rival models, zebrafish have lower maintenance costs and are capable of laying hundreds of eggs per week. Their complex behaviors and physiology allows for the modeling of diseases and mass drug screening (Cho et al., 2017). As a result, the increased utilization of zebrafish models in epilepsy research would present less economic issues to researchers.

Zebrafish have been used in 24 studies relating to epilepsy research that were covered in this review and are subdivided into the larval, adult and transgenic zebrafish subsections below.

Larval Zebrafish

Behavioral Changes

The evaluation of behavioral changes in zebrafish larvae treated with proconvulsants plays a huge role in the understanding of epilepsy and seizures. As zebrafish larvae only become free-swimming at 3 days post-fertilization (dpf), they can only then be clearly evaluated for behavioral changes (Hortopan et al., 2010b). The observed behavioral changes are evaluated from the aspects of locomotor activity and thigmotaxis (Berghmans et al., 2007; Yang et al., 2017). Seizure score systems have been devised to categorize seizures based on the criteria above (Berghmans et al., 2007; Hortopan et al., 2010b; Afrikanova et al., 2013), to provide a more objective way of evaluating behavioral changes. All zebrafish larvae tend to display a similar pattern of seizure progression, with signs of increased agitation within seconds of seizure induction, followed by increased locomotor activity and thigmotaxis soon thereafter. This behavior soon progresses into "whirlpool-like" swimming behavior and rapid swimming. Brief convulsions and loss of posture soon follows, as the larvae enters the advanced stages of a seizure. The final stage of a

seizure is marked by a decrease in the distance traveled by the larvae as they experienced brief convulsions and loss of posture (Berghmans et al., 2007; Tiedeken and Ramsdell, 2007; Hortopan et al., 2010b; Lee et al., 2012; Afrikanova et al., 2013). At higher concentrations of proconvulsant, zebrafish larvae expire due to neural damage (Berghmans et al., 2007). Zebrafish larvae can also be tracked on multi-well plates for high-throughput screening of behavioral changes (Yang et al., 2017). These behavioral changes are similar to that in adult zebrafish, with slight differences. Some notable differences include increased seizure onset times, possibly due to relatively immature respiratory and nervous systems in larvae resulting in a slower proconvulsant absorption rate. The degree of seizure activity is also largely dependent on the concentration and type of proconvulsant (Berghmans et al., 2007; Yang et al., 2017). As in adults, behavioral changes in proconvulsant treated zebrafish larvae can also be influenced by dark-light environments (Yang et al., 2017).

Utilization of Proconvulsants

Zebrafish larvae provide a robust model that can be studied to understand the process of epileptogenesis through seizure induction using proconvulsants. In many of the studies, PTZ was primary proconvulsant used to induce seizures in zebrafish larvae. Other proconvulsants which have been used with zebrafish larvae include kainic acid, ginkgotoxin and picrotoxin (PTX) (Lee et al., 2012; Menezes et al., 2014; Yang et al., 2017). Proconvulsants are typically administered via bath application in multi-well plates, at different concentrations depending on the type of convulsant used. In majority of the studies covered, a 15–20 mM dose of PTZ was administered as these doses clearly produce all seizure scores in zebrafish larvae. Domoic acid (DA) can also be given to increase the seizure susceptibility of PTZ-treated zebrafish larvae by reducing the seizure threshold to produce an increase in locomotor activity (Lee et al., 2012).

PTX has also been used to induce seizures in other animal models (rodents and adult zebrafish) and causes significant increases in the locomotor activity of 5 dpf old zebrafish (Yang et al., 2017). Besides that, KA's efficacy in inducing seizure activity is dependent on the age of the zebrafish larvae as discovered by Menezes et al. (2014). These different results could be linked with state of the neural development in which the maturation of neurotransmitter systems influences the response to KA (Menezes et al., 2014). Similarly, ginkgotoxin at various concentrations (0.2–1 mM) causes hyperactive swimming in zebrafish larvae and also shows age dependent effects (Lee et al., 2012).

Screening of Anti-convulsive Compounds

Zebrafish larvae can also be used for the screening of anti-convulsive compounds by first pretreating them with the compound to be screened and then challenging them with a proconvulsant after a given habituation time. This screening method relies on the idea that if a compound is anti-convulsive, it should prevent or reduce the seizure behavior changes seen in proconvulsant treated zebrafish. Only microgram amounts of compound is required and can be quickly absorbed by zebrafish larvae through their gills, skin or gastrointestinal tract (Afrikanova et al., 2013). The small size of zebrafish larvae also allows it to thrive in small volumes, such as in the wells of a 96-well plate, which allows for the use of medium to high-throughput screening with an automated locomotor tracking system for the analysis of larval movement (Berghmans et al., 2007; Afrikanova et al., 2013). Prior to pretreatment, a toxicology evaluation may be performed to determine the maximum-tolerated concentrations (MTCs) of the drugs utilized, to avoid toxicity. This can be performed by using test compounds and observing the effects of AEDs on affected zebrafish for a period of 24 h (Barbalho et al., 2016).

Recording of Brain Electrical Activity

Another aspect of epilepsy research in zebrafish larvae is the tracking of brain electrical activity using EEG and by the evaluation of field potential. By tracking brain electrical activity, the epileptiform discharges that occur in proconvulsant treated zebrafish larvae and hence the number of seizure events that occur, can also be monitored (Afrikanova et al., 2013; Hunyadi et al., 2017; Turrini et al., 2017). The measurement of local field potential (LFP) in zebrafish larvae can also be performed by placing glass electrodes into their optic tectum (Afrikanova et al., 2013). Besides that, genetically-encoded calcium indicators (GCaMP6) have also been utilized as an optical method to track neuronal activity in zebrafish larvae (Turrini et al., 2017). This method is based on fluorescent calcium indicators and relies on the fact that there is a correlation between concentration of intracellular calcium and spiking frequency. Thus, it can be used to analyse seizure activity and to map zebrafish larvae brains. The peak amplitude of any recorded spikes is directly proportional to the concentration of proconvulsant used to induce seizures in the zebrafish larvae (Turrini et al., 2017). In another study, both invasive LFP and non-invasive LFP were utilized in zebrafish larvae to record local field potential and to determine the number of seizure events by interpreting the number of epileptic discharges (Hunyadi et al., 2017).

Adult Zebrafish

Behavioral Changes

The study of behavioral changes in adult zebrafish treated with proconvulsants is vital to epilepsy research. Behavior changes in adult zebrafish primarily involve its locomotor behavior. Aspects that are quantified in the evaluation of locomotor behavior include distance traveled, velocity, mobility, number of rotations and movement pattern of the zebrafish (Banote et al., 2013). Based on the severity of seizure activity, qualitative scoring systems were established to categorize zebrafish seizure activity into different scores (Pineda et al., 2011, 2013; Banote et al., 2013; Mussulini et al., 2013; Siebel et al., 2013; Gupta et al., 2014; Kundap et al., 2017; Choo et al., 2018). Initially, seizure activity begins with an increase in an adult zebrafish's swimming activity and is followed by short-burst, rapid swimming coupled with body jerking. Later stages of seizure activity are characterized by distinctive "whirl-pool" swimming behavior, clonic seizure-like behavior and loss of posture (Pineda et al., 2011; Mussulini et al., 2013). At a higher concentration of proconvulsant, zebrafish have a higher mortality

rate due to the neural damage caused by epileptic seizures (Mussulini et al., 2013; Gupta et al., 2014). Severity of seizure activity and seizure latency are also largely dependent on the period of exposure to proconvulsant and the concentration of proconvulsant (Pineda et al., 2011; Mussulini et al., 2013; Siebel et al., 2013; Gupta et al., 2014). Other factors include gender differences, as demonstrated by one study which revealed that male zebrafish suffer more seizures than female zebrafish (Cho et al., 2017). In addition, the route of proconvulsant administration is also a factor as oral administration of proconvulsant elicits a seizure in adult zebrafish as quickly as bath application, but more weakly in terms of severity (Cho et al., 2017).

Utilization of Proconvulsants

When challenged with proconvulsants, adult zebrafish express seizure behavior which can be observed and analyzed. PTZ was utilized as a proconvulsant for many adult zebrafish studies in this review (Pineda et al., 2011, 2013; Banote et al., 2013; Mussulini et al., 2013; Siebel et al., 2013; Gupta et al., 2014; Cho et al., 2017; Duy et al., 2017; Kundap et al., 2017). Other types of proconvulsants such as KA (Sierra et al., 2012) were used to a lesser extent. Proconvulsants are typically administered through the oral, intraperitoneal (Sierra et al., 2012; Banote et al., 2013; Kundap et al., 2017) and bath application (Pineda et al., 2011, 2013; Mussulini et al., 2013; Siebel et al., 2013; Gupta et al., 2014; Cho et al., 2017) routes. Each route of administration produces a similar behavioral response, with different seizure onset times at different concentrations. In the studies covered, PTZ injections were done using concentrations ranging from 170 to 220 mg/kg, while PTZ bath applications used concentrations ranging from 2 to 15 mM (Sierra et al., 2012). The results obtained from multiple studies showed that PTZ at a lower concentration (<7.5 mM) induced minimal seizure activity in the first 5 min and more severe seizure activity in the last 15 min, whereas PTZ at a higher concentration (>7.5 mM) induced severe seizures in the first 5 min (Pineda et al., 2011; Mussulini et al., 2013; Gupta et al., 2014). On the other hand, KA at a concentration of 6 mg/kg induces seizures in adult zebrafish, 2.5 min post-intraperitoneal injection, which is entirely different that the typical elicitation of seizures using PTZ in other studies (Sierra et al., 2012). Another study utilized caffeine as a proconvulsant with concentrations ranging from 1–30 μM. The results of the study revealed that the higher the concentration of caffeine applied, the lower the latency of seizure onset in zebrafish (Gupta et al., 2014).

Screening of Anti-convulsive Compounds

Much like their larval counterparts, adult zebrafish can and have been utilized for the screening of anti-epileptic compounds. The method of doing so is essentially the same as in zebrafish larvae, but a key difference is that adult zebrafish are larger in size and thus would be more difficult to screen at high throughputs. Another difference is a larger ranger of administration routes such as intraperitoneal injections (Kundap et al., 2017), which may be impossible or very challenging to perform in larvae.

Recording of Brain Electrical Activity

The study of epilepsy in adult zebrafish also extends to the recording and evaluation of electrical activity within the brain. To track electrical activity within the brain of a zebrafish, an EEG may be used (Mussulini et al., 2013; Cho et al., 2017). Alternatively, an EEG equivalent for zebrafish would be the measurement of their cerebral field potential (Pineda et al., 2011, 2013; Banote et al., 2013). The long-term multichannel EEG utilizes a multichannel EEG electrode array for data acquisition. The two forms of seizure activity that can be recorded by an EEG are high-amplitude theta activities (5–7 Hz) which represent full-blown seizures similar as to TLE and low-frequency spike-wave activities which represent a an epilepsy discharge (2–3 Hz) (Cho et al., 2017). Similarly, other studies revealed high-amplitude sharp complexes in the measurement of the cephalic field potential in zebrafish, which is the result of seizure activity within the fish (Pineda et al., 2011, 2013; Banote et al., 2013). In addition, pre-treatment with AEDs resulted in a reduction in high-amplitude sharp complexes in PTZ-treated zebrafish, in a dose-dependent manner (Banote et al., 2013). It is further noted that continuous exposure to PTZ at 15 mM for 90 min resulted in a flat-line EEG, which is indicative of brain damage (Pineda et al., 2011; Mussulini et al., 2013).

Transgenic Zebrafish

Some studies have revealed that transgenic zebrafish models provide an efficient alternative to proconvulsants as they are more susceptible to seizures (Packer et al., 2016; Pena et al., 2017; Sourbron et al., 2017). Both transgenic adult and larvae models have been utilized in the studies covered.

Behavioral Changes

Behavioral changes in transgenic zebrafish models have been analyzed in certain studies, usually those relating to seizure activity and motor behavior (Packer et al., 2016; Pena et al., 2017; Sourbron et al., 2017). A zebrafish model of *CLN3* disease or Batten's Disease was evaluated for seizure susceptibility, which revealed the first sign of motor abnormality at 36 h post-fertilization (hpf) whereby the *CLN3* ATG morphants displayed higher movement activity and a higher number of tail flicks, both of which are indicative of seizure activity (Packer et al., 2016). Another study discussed the prospects of having a mutant *scn1a* gene be expressed in zebrafish as high levels of locomotor activity were observed in zebrafish scn1a mutants (Sourbron et al., 2017). The last study conducted presented *aldh7a1* mutant zebrafish, with pyridoxine-dependent epilepsy (PDE) that showed signs of hyperactivity characterized by "whirlpool" swimming and loss of posture by 10 dpf when stimulated with light (Pena et al., 2017).

Seizure Susceptibility of Transgenic Models

The seizure susceptibility of transgenic models differs from each other to a certain degree and is influenced by external factors such as the presence of light and the age of the animal. For example, *cln3* ATG mutants experience motor abnormalities at 36 hpf while aldh7a1 mutants experience motor abnormalities at 10 dpf. The *aldh7a1* mutant is also affected by light stimuli, which appears to worsen the motor abnormality, causing "whirlpool"

swimming activity and eventually loss of posture as it increases seizure susceptibility (Packer et al., 2016; Pena et al., 2017). Another study discusses the accumulation of piperideine 6-carboxylate (P6C), which can eventually lead to an increased seizure occurrence in affected zebrafish (Pena et al., 2017). An *aldh7a1* mutation refers to PDE in which the seizure activity is dependent on levels of pyridoxine (Pyr). Aldh7a1 mutant larvae that were treated with Pyr have significantly suppressed seizure activity and daily treatment successfully prolonged their lives. The immediate withdrawal of Pyr treatment resulted in seizure activity and eventual death (Pena et al., 2017).

Electrographic Component of Induced Seizures Within Transgenic Models

The study of transgenic zebrafish models also extends to the tracking and evaluation of epileptiform electrographic activity. Once again, EEG was used for the recording of brain electrical activity. The field potential recordings acquired from both *aldh7a1* mutants when stimulated with light, showed that the *aldh7a1* mutant was suffering from spontaneous seizure activity which was marked by sudden bursts of high amplitude, long duration waves that were similar in appearance to the spontaneous seizure activity in other animal models. In comparison, wild type zebrafish had normal electric activity characterized by the absence of high amplitude waves (Pena et al., 2017). Similar results were also observed in a surface EEG conducted in using *cln3* ATG MO morphants injected with p53 MO and ng *cln3* ATG MO. Significantly higher amplitude waves with a frequency of 2–4 Hz was recorded, which reflects epileptiform activity (Packer et al., 2016). Sporadic multi-spike bursts which were similar to ictal-like waveforms were also recorded in 93% of mib^{hi904} zebrafish following exposure to 15 mM of PTZ (Hortopan et al., 2010a).

DISCUSSION

Research using non-mammalian models of epilepsy offers substantial advantages for identifying and studying several seizure parameters including behavior, different types of cellular activities, electrophysiological changes and the genes involved in regulating seizures (**Table 2**). Though there are many insults that may be used to trigger seizure like behavior in the non-mammalian models of epilepsy, the behavioral changes that result are typically heightened motor activity. While these seizure behaviors can be measured qualitatively (the absence or presence of seizures), seizure scoring systems for the quantitative measurement of seizure behavior have also been designed for certain non-mammalian models such as zebrafish (Kundap et al., 2017) and roundworms (Pandey et al., 2010). Having a more finely grained method of scoring seizures could enable anti-epileptic compound screens to also detect compounds with less than ideal anti-epileptic activity in its unmodified form but could potentially become more potent after chemical modification. This is useful as even the first line AEDs being used today may not completely or permanently supress seizures at the more conservative doses usually preferred (Xia et al., 2017). However, care should be taken as the method of inducing seizures and the route of administration could potentially affect the animal in ways that may be directly observable (type and onset of behavioral changes) or otherwise (changes in neuronal activity).

Although the rodent seizure models continue to serve as the foundation for basic and translational epilepsy research, unconventional vertebrate (zebrafish) and invertebrate (fruit fly and roundworm) models are proving to have greater potential for analyzing the epilepsy phenotype as they are genetically tractable organisms (Baraban, 2007). As much regarding epilepsy is yet unknown, the ease of genetically modifying such organisms coupled with short generation times would greatly aid in identifying additional molecules, gene or signaling mechanisms which affect epileptogensis and epileptic behavior to better understand epilepsy. One possible use of genetically alterations is to produce mutant non-mammalian models of epilepsy which have different seizure thresholds. This would also aid understanding of the epilepsy phenotype by allowing researchers to mimic the clinical conditions of a wide variety of different epilepsy types. One day, it could even become possible to insert genes which confer seizure resistance into humans in the form of gene therapy to help ameliorate epilepsy, though there are many hurdles to overcome before that day arrives.

While the similarity of certain non-mammalian seizure like behaviors to human seizures (Lucey et al., 2015) is a great boon, the dissimilarity of these non-mammalian models to humans also ironically makes them ideal models. As an example, the non-mammalian models covered in this review typically have a relatively simpler CNS, yet retain much of the functionality and characteristics of their more complicated mammalian counterparts such as the major neurotransmission systems (Ramakrishnan et al., 2013) as well as neural circuitry and development (Hahn and Burrell, 2015). As the non-mammalian models covered in this review are relatively small, they can be easily and cheaply maintained in large numbers with little logistical and financial difficulties. Their small body size would also reduce the amount of chemicals such as proconvulsants and novel compounds that are needed for experimentation or screening, if they are available in limited quantities or are prohibitively expensive. In addition to their small size, the high breeding rate of some non-mammalian models also facilitates the use of high-throughput screening (Yang et al., 2017). When taken individually, it becomes clear that each non-mammalian model in this review has different characteristics, advantages, limitations and seizure behavior in response to different methods of inducing seizures.

The seizures induced in animal models typically mimic either human generalized or partial seizures. As mentioned previously, different seizure induction methods can be used to induced different seizures types. For example, MES can induce generalized tonic-clonic seizures whereas PTZ can induce non-convulsive generalized seizures (Löscher, 2011). That being said, it is worth noting that these seizure induction methods actually have poor face validity as the defining feature of epilepsy are the spontaneous recurrent seizures (Mussulini et al., 2013). However, the seizures induced by certain chemoconvulsants such as PTZ only cause transient acute seizures rather than true epilepsy.

Nevertheless, the National Institute of Neurological Diseases and Stroke (NIH) actually recommends the use of PTZ and MES to induce acute seizures during the initial screening process (National Institute of Neurological Disorders and Stroke, 2013). This is likely an attempt to save both time and resources as although chronic seizure models using the proconvulsants kainic acid and pilocarpine which more closely mimic true human epilepsy exist, they require a significant investment in time and resources to carry out. That being said, the construct and predictive validity of acute seizure models is not entirely zero as compounds which show great anticonvulsive potential in such models would be more likely to also display that potential in true chronic models of epilepsy. The articles found in this review seem to support this belief, such as that by Kundap et al. (2017) who found that currently available anti-epileptic drugs which work in humans, also counteract PTZ induced seizures. It is also worth noting that although anti-epileptic drugs are so termed, they are actually anti-convulsants which only provide symptomatic treatment rather than treat the underlying cause of epilepsy.

With all the advantages of non-mammalian models over rodent models, one might wonder why rodent models remain so prolific? One of the reasons could be that rodents have been an established model since the days of old for their similarities to humans both anatomically and genetically as well as having a short reproductive cycle (Butterweck and Nahrstedt, 2012). Another reason for the widespread use of rodents, especially in the case of pharmacological safety and pharmacokinetic studies of potential novel drugs, is simple regulatory in nature. This is because drug regulatory agencies typically require the use of at least two mammalian species, including one non-rodent species, prior to the authorization of human trials (Atanasov et al., 2015). While the practice of using rodent models for epilepsy research no doubt arose from the cumulative work of our predecessors, perhaps it is time for a paradigm shift in the form of non-mammalian models. Rather than conducting preliminary studies using non-mammalian models before committing to rodent models for promising compounds, perhaps future pre-clinical epilepsy studies could be done solely in non-mammalian models and proceed directly to human trials if successful. This is supported by the findings that the disease-related genes in non-mammals such as zebrafish (84%) and drosophila (75%) have human orthologs (Pandey and Nichols, 2011; Kalueff et al., 2014). This coupled together with the human seizure like behavior and a higher effectiveness of non-mammalian models of epilepsy could 1 day allow them to replace rodent models of epilepsy instead of merely supplanting them. It is hoped that the information on the importance and usage of non-mammalian models in studies concerning epilepsy provided by this review will serve as a stepping stone for future research into the field of epilepsy and also the screening of anti-convulsive compounds.

CONCLUSION

In conclusion, non-mammalian models of epilepsy covered in this review have already shown great promise in the field of epilepsy research and the animals themselves offer many advantages over the typical mammalian rodent models of epilepsy. It is hoped that future research will take advantage of all these non-mammalian models of epilepsy as the tadpole, planarian, roundworms and medicinal leech models appear to be underutilized as compared to zebrafish and fruit flies, in terms of publication numbers. These models should not be discounted as researchers will need every possible tool at their disposal in the difficult quest to unravel the enigma that is epilepsy and possibly even discover a treatment for it 1 day.

AUTHOR CONTRIBUTIONS

MJ and BC have joint first authorship for this publication. MJ and YL were responsible for the searching and screening of articles for this review. MJ, BC, and YL were responsible for the writing of the manuscript. MS was responsible for conceptualizing and revising the manuscript. YK was also involved in conceptualizing, proofreading and diagram preparation. All authors gave their final approval for the submission of the manuscript.

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frontiers in Pharmacology

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Neuroprotective Effect of Coumarin Nasal Formulation: Kindling Model Assessment of Epilepsy

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Epilepsy is a brain disorder characterized by sudden recurrent seizures. Considering the fact that epileptogenesis is a process that affects the quality of life, our goal is to delay the process of epileptogenesis and to increase the latency of epileptic attacks, offering better quality of life to patients. Traditional system of medicines has a promise in some of the medicines, which have been used for the treatment of epilepsy. One such medicinal plant is *Eclipta alba* (EA). According to Ayurvedic philosophy, the juice of leaves of EA is pounded with garlic and pepper for the treatment of epilepsy. Taking clue from the Ayurvedic system of medicines, we formulated coumarin fraction of EA, namely, coumarin nasal formulation (CNF) for its nasal delivery. CNF was analyzed by using high performance liquid chromatography (HPLC) and ultraviolet absorption spectroscopy for its drug content determination. *In vitro* drug release studies were performed in simulated nasal electrolyte solution (SNES) maintaining constant pH of 5.5 at 37°C. Irritation by CNF was evaluated using hen's egg test chorioallantoic membrane (HET-CAM) assay. Formulation was found to be non-irritant in HET-CAM assay. CNF was further assessed *in vivo* by measuring the progress and attainment of pentylenetetrazole (PTZ) kindling in mice. Neuronal changes were assessed by hematoxylin and eosin (H&E) and Nissl staining technique. Glial fibrillary acidic protein (GFAP) a neuroinflammatory marker and tumor necrosis factor alpha (TNF-α) an inflammatory marker were also measured. CNF (10 mg/kg, nasal route) when given as a pretreatment lowered seizure score and delayed the progression of seizure similar to diazepam. CNF decreased the PTZ induced oxidative damage, TNF-α as well as GFAP levels in the midbrain tissue particularly in hippocampus region. The results suggest that CNF may be a promising therapeutic approach to offer protection from sudden recurrent seizures alone or in combination with current drugs in management of epilepsy.

Keywords: coumarin fraction, coumarin nasal formulation, epilepsy, kindling, pentylenetetrazole, neuroinflammatory marker

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INTRODUCTION

Epilepsy is a neurological disorder characterized by transient occurrence of abnormal excessive synchronous neuronal firing in the brain. The predominant cognitive, psychological, and behavioral manifestations in epilepsy further aggravate the vulnerability of seizures incidence, which eventually leads to plaintive quality of life of patients suffering from it

(Chang and Lowenstein, 2003). According to World Health Organization, the proportion of general population with active epilepsy at a given time is about 4 to 10 per 1000 people. The epilepsy scenario is far more alarming in the developing nations with a proportion of about 7–14 per 1000. This disorder has been found to prevail since ages and presently affects approximately 50 million population worldwide (Epilepsy, 2012). The signs and symptoms include warnings, such as visual or sensory auras, tingling fingers, altered awareness, and convulsive or abnormal movements. The pathophysiology underlying the epileptic process includes mechanisms involved in instigation of seizures (ictogenesis), in addition to those involved in transforming the normal brain into a seizure-prone brain (epileptogenesis) (Fisher et al., 2005). Currently, no antiepileptogenic treatments are available in the market that prevents the progression of epileptogenesis. The epilepsy plight suggests the imperative need for the development of novel therapeutic strategy. The rational approach in designing the new strategy should consider molecules which block both ictogenesis and retard the progression of epileptogenesis. The ideal anticonvulsant drug should constrain seizures resulting from the rapid and excessive firing of the neurons. Additionally, the drug therapy currently available as antiepileptic drugs (AEDs) is associated with side effects and dose related chronic toxicity involving vital organ system. Furthermore, all the currently available AED have potential for adverse effects like cognitive and behavioral impairment. The cost of new AED, namely, oxcarbamazepine, lamotrigine, felbamate, gabapentin, and vigabatrin is three to six times higher than the conventional AEDs like phenytoin, carbamazepine, and valproic acid which is a major concern and important factor for a physician to prescribe (Britton and So, 1996). In Ayurveda system of medicine, EA is considered a *rasayana* for longevity and rejuvenation. The leaves of EA are used to treat epilepsy in India (Pakrashi and Chatarji, 1994). EA has been used as a traditional healer and medicine especially in the southern regions of India for the treatment of epilepsy since ancient times (Reddy et al., 1989). EA extract showed significant decrease in the locomotor activity at the dose of 50 mg/kg and dose dependent protection from seizures in maximal electroshock model (Bikjdaouene et al., 2003). Above study confirmed the antiepileptic potential of the extract possibly due to presence of phytoconstituents like wedelolactone, luteolin, and β-amyrin in it (Shaikh et al., 2012). There are studies indicating that EA phytoconstituents also have affinity toward benzodiazepine (BZD) binding site on the GABA receptor (Coleta et al., 2008). EA contains wide range of active phytoconstituents including coumestans, alkaloids, flavonoids, glycosides, sterols, and triterpenoids. These EA fractions were screened in various models of acute and chronic epilepsy (unpublished data from our lab). In preliminary study, we found that coumarin fraction (100 mg/kg) exhibited an excellent antiepileptic activity in acute PTZ induced seizure model in mice. Considering these literature reports and results, we designed the present study to formulate the active fraction for its anti epileptic potential in kindling model of epilepsy.

Abbreviations: AED, antiepileptic drug; ANOVA, analysis of variance; BBB, blood–brain barrier; CA1, Region 1 Cornu Ammonis; CAT, catalase; CNF, coumarin nasal formulation; CT, coagulation time; EA, *Eclipta alba*; FITC, fluorescein; FTIR, Fourier transform-infrared; $GABA_A$, gamma-aminobutyric acid (GABA) type A receptor; GFAP, glial fibrillary acidic protein; GRP, group; GSH, glutathione; H&E, hematoxylin and eosin; HET-CAM, hen's egg test choroiallantoic membrane; HPLC, high performance liquid chromatography; HT, hemorrhage time; ICH, International Council for Harmonization of Technical Requirement; i.p., intraperitoneal; i.v., intravenous; LT, lysis time; MDA, malondialdehyde; MES, maximum electroshock; NFκB, nuclear factor kappa-light-chain-enhancer of activated B-cells; PBS, phosphate buffer saline; PDA, photo diode array; PTZ, pentylenetetrazole; SD, standard deviation; SEM, standard error of mean; SNES, simulated nasal electrolyte solution; SOD, superoxide dismutase; TNF-α, tumor necrosis factor-alpha; UV, ultraviolet; WL, wedelolactone.

MATERIALS AND METHODS

Plant Material

The leaves of the EA plant were procured from the local market in Mumbai. Professor Ganesh Iyer, Department of Botany, Ruia College, Mumbai, India, authenticated the specimen. The voucher specimen (No. SSS/1313/15) of the same was deposited at the Institute of Chemical Technology for future reference.

Drugs and Chemicals

Pentylenetetrazole (Sigma Chemical Co., St. Louis, MO, United States), diazepam (Mumbai, Roche Pharmaceuticals, India), and wedelolactone (Natural remedies Private Limited, Bangalore, India) were used in the present study. The drugs were dissolved in distilled water and subsequently used for administration. The solvents used for the extraction in the study were, petroleum ether, methanol, diethyl ether, and ethyl acetate (S.D. Fine-Chem, Mumbai, India). The surfactants like Tween 80, polyethylene glycol 300, benzalkonium chloride, citric acid, and sodium citrate (S.D. Fine-Chem, Mumbai, India) were used in formulation. All other chemicals and reagents used in the experiments were of analytical grade.

Preparation of the Extracts

The leaves of EA were dried in shade and stored at 30°C temperature. They were crushed further to obtain a coarse powder and passed through a sieve (no. 40). Shade dried and powdered leaves (500 g) were subjected to soxhlet extraction with petroleum ether and methanol (60–80°C) for 24 h. After completion of the extraction, the solvent was removed by distillation and the extract was concentrated under vacuum (40°C) to yield the petroleum ether extract of EA and methanolic extract of EA, respectively. Dried greenish crystals of methanolic extract were further partitioned with solvent diethyl ether to get coumarins from EA.

Isolation of Coumarins From EA

The methanolic extract of EA (30 g) soaked in distilled water containing 2% acetic acid (300 ml) and heated for 1 h. The extract was filtered using Whatman No. 42 paper. Filtrate was then evaporated to 100 ml under reduced pressure at a temperature of 40°C. Filtrate (100 ml) was added to the same amount of solvent diethyl ether. The whole (200 ml) was then partitioned using separating funnel by standing for 30 min at room temperature after vigorous shaking, which was repeated five times until diethyl

ether fraction was no longer fluorescent under long UV light (366 nm). Diethyl ether was evaporated to get concentrated solution (100 ml). On cooling at 0–4°C, a greenish yellow precipitate formed at the bottom of the flask was separated. It was dried and weighed (Prajapati and Patel, 2012). Greenish yellow dried powder of coumarin fraction was stored in fridge until further use.

Preparation of the Coumarin Nasal Formulation and Stability Studies

The formulation comprising of coumarin fraction, isolated and characterized, was formulated in solution form and denoted as CNF [1% coumarin fraction (w/v) total solids]. CNF comprised of 1gm of coumarin extract per 100 ml of formulation comprising Tween 80 (1% v/v) + polyethylene glycol 300 (1.5% v/v) + benzalkonium chloride (0.1% v/v) + 0.1 M citrate buffer pH 7.4 (1.05 g citric acid and 1.47 g sodium citrate in water for injection) to make up the volume to 100 ml. CNF was stored in an amber color vials until further analysis. Stability studies were carried out as per the ICH guidelines at 25 ± 2°C/60 ± 5% RH and 2–8°C. The average particle size (if turbidity occurred) and drug content were evaluated at 1, 2, and 3 months.

Phytoconstituents Analysis of Coumarin Fraction and CNF by HPLC

A HPLC system of JASCO Corporation was used for the analysis. HPLC was equipped with auto sampler and PDA detector system. The chromatogram was analyzed by JASCO ChromNAV version 1.19.01 software. In house method was developed using stationary phase C18 column (4.6 mm × 250 mm; 5 μm) by Jasco corporation with mobile phase composition of acetonitrile and 0.1% formic acid and buffer pH adjusted to 2.5 with triethylamine, operated at 1 ml/min flow rate. Ethyl acetate was used for preparation of sample preparation. Dried powder of coumarin fraction as in 2.3.1 was dissolved in methanol (1.0 mg/mL), filtered by 0.22 μm filter, and directly injected into HPLC for the analysis of major phytoconstituents. Jasco Corporation HPLC system was used with auto sampler and PDA detector for the analysis. Wedelolactone, luteolin, and apigenin were separated and identified on Thermo BDS Hypersil-C18 (250 × 4.6, 5 μ) with Purospher® STAR RP-18e (5 μm) guard column. Wedelolactone was eluted isocratically and detected on UV PDA detector at wavelength of 352 nm. Quantitative estimation of wedelolactone in coumarin fraction and CNF was determined. Luteolin and apigenin were eluted isocratically with 50% acetonitrile and 50% formic acid buffer (0.1%, pH 2.5) at flow rate of 1.0 ml/min and detected at wavelength of 325 nm. Chromatogram for wedelolactone, apigenin, and luteolin in coumarin fraction are shown in **Figure 2**.

Wedelolactone Content of CNF

One milligram of CNF was diluted in 100 μl methanol and 900 μl PBS at pH 7.4 under continuous stirring for 10 min and further subjected to centrifugation at 2000 rpm for 10 min (Remi Centrifuge CPR 24 plus Centrifuge). Wedelolacone (WL) content was quantified in the supernatant by UV spectroscopy at 350 nm (UV Epoch microplate reader Biotek). Calibration curve of WL in PBS was linear (R^2 = 0.999) in the range of 10–50 μg/ml. All experiments were repeated in triplicate. Drug content was determined according to the following equation.

$$\% \text{ drug Content} = \text{Weight of WL in Formulation (mg)} / \text{Weight of Formulation (mg)} \times 100$$

Solubility Study of CNF

Solubility of coumarin extract, containing wedelolactone, was determined in various surfactants (Labrasol, Brij 58, Solutol HS 15, Tween 20, Tween 80) and co-surfactants (PEG 300, PEG 400, PEG 600, Transcutol P, and Glycerol). Briefly, excess coumarin extract was added to 1 ml surfactant or co-surfactant separately in eppendorf tubes. The tubes were sealed and vortex mixed intermittently followed by sonication and equilibration at room temperature for 24 h. Equilibrated samples were centrifuged at 20,000 rpm for 10 min. Aliquot of 0.1 ml was withdrawn from the supernatant and diluted appropriately with methanol. Diluted sample was analyzed for wedelolactone by UV spectrophotometry at 354 nm. All experiments were performed in triplicate.

Fourier Transform-Infrared (FTIR) Study

Fourier Transform-Infrared spectra of the crude fraction and formulation were recorded to confirm the functional groups and structural similarity between coumarin fraction and CNF. FTIR was recorded using an IR Prestige-21 spectrometer (Shimadzu, Kyoto, Japan) in the range between 4000 and 500 $cm^{-}1$ with a resolution of 1 cm^{-1} after 57 scans.

In vitro Release Studies

In vitro drug release studies for CNF and coumarin fraction for the determination of wedelolactone release were carried out in SNES (NaCl 7.45 g/L, Kcl 1.29 g/L, and Cacl2 0.32mg/L) pH 7.4 at 35 ± 0.2 °C. Dialysis bag method was used [dialysis membrane (HIMEDIA LA 395-5MT), avg. flat width 32.34 mm and avg. diameter 21.5 mm] in a beaker loaded with magnetic stirrer. Dialysis bags (previously soaked overnight in distilled water) were filled with 10 mg of formulation dispersed in 1 ml of phosphate buffer solution (PBS) pH 7.4 preheated at 35°C. Dialysis bags were then sank in small screw-top glass vials filled with 20 ml of SNES solution (35°C), sealed with Parafilm® and placed in the water bath under mild agitation. At predetermined time intervals (0, 5, 15, 30, 60, 120 min, 12 h, and 24 h), 200 μl samples were withdrawn and replaced with equal volumes of fresh and preheated SNES solution. Quantification of WL in the samples was performed spectrophotometrically at 350 nm (UV Epoch microplate reader Biotek). *In vitro* release experiments were repeated in triplicate.

Ex vivo Permeation Studies of CNF on Goat Nasal Mucosa

Ex vivo permeation study were carried out across goat nasal mucosa collected from the local (Deonar, Mumbai) slaughter house. Immediately after the animal sacrifice, the right and left nasal concha were isolated and nasal epithelium was carefully

excised, cleansed, and immersed in icecoldphosphate buffer pH 7.4. The nasal epithelium (mucosal thickness ca. 100 μm) was immediately mounted on a jacketed vertical Franz cell (4.91 cm^2 active surface area and receptor volume: 20 ml) under mild magnetic stirring (20 rpm), with the mucosal surface facing the donor compartment and the serosal side facing the receptor compartment. The membrane integrity and proper arrangement on the cell surface was assessed carefully. Tissue thermal equilibrium was achieved by filling both compartments with prewarmed (35 ± 0.2°C) SNES solution (pH 5.5). After 20 min, buffer solution was removed from the donor chamber and replaced by a dispersionof 1 ml of 1 mg/ml of CNF formulation, solution of coumarin fraction of the same concentration in PBS was used as control. The donor compartment was sealed with Parafilm® throughout the experimental procedure. At predetermined time intervals (15, 30, 60, 120, 240, 360, and 480 min), samples of 0.5 ml were withdrawn from the receptor compartment and replaced with equal volume of preheated SNES solution. All experiments were repeated at least three times. Samples were centrifuged at 10,000 rpm for 15 min (Eppendorf centrifuge Remi Centrifuge CPR 24 plus) and the supernatants (60 μl) were directly loaded into HPLC vials to quantify WL content, under the following chromatographic conditions: HPLC setup comprised of a PU-2089 plus quaternary gradient pump, AS-2055 plus intelligent auto sampler, and a MD-2018 plus photodiode array detector (JASCO). The HPLC column used was Thermo Scientific syncronis C18, 150 mm × 4.6 mm, particle size 5 μm. Mobile phase consisted of ACN: 0.1% formic acid 60:40 (v/v), 10 mM, pH 5.5 (pH adjustment with triethylamine 1 M). Flow rate was regulated at 1 ml/min; injection volume at 50 μl. Retention time was approximately three minutes at ambient temperature conditions. Calibration curve was linear ($R^2 = 0.997$) over the concentration range of 10–1000 μg/ml. Steady state flux (Jss) is determined by calculating the slope of the linear portion of the curve obtained after plotting the cumulative amount of drug permeating nasal epithelium per unit area (μg/cm^2) against time (Fisher et al., 2005). Consequently, apparent permeability coefficient, P (in cm/s) is calculated as follows:

$$P = \text{Jss/Cd, where Cd is the initial concentration in the donor compartment } (\mu g/cm^3).$$

Subsequently, for both CNF and coumarin fraction, the apparent permeability coefficient was calculated and compared on the basis of enhancement ratio (*R*).

$$R = P_{(\text{CNF})}/P_{(\text{Coumarin Fraction})}$$

Steady state flux (Jss) was calculated according to method developed by Karavasili et al. (2016).

Evaluation of CNF Irritancy Potential Using Hen's Egg Test Chorioallantoic Membrane (HET-CAM)

The HET-CAM assay was performed following "The Interagency Coordinating Committee on the Validation of Alternative Method" recommendations. Some modification in method were adopted as per (Dehelean et al., 2011) to suit our laboratory conditions.The purpose of this protocol was to describe the components and procedures used to assess the potential nasal irritancy of a formulation as measured by its ability to induce toxicity in the chorioallantoic membrane of a chicken. Fertilized eggs were procured from Central Poultry Development Organization (Western Region) Mumbai. Study was divided into four groups each group containing six eggs, group I: normal control (saline 0.9%), group II: vehicle control [Tween 80 (1% v/v), polyethylene glycol 300 (1.5% v/v), benzalkonium chloride (0.1% v/v) in 0.1 M citrate buffer pH 7.4 (1.05 g citric acid and 1.47 g sodium citrate in water for injection)], group III: negative control (1% sodium dodecyl sulfate in 1 M potassium hydroxide solution), group IV: CNF. Parameters observed were the onset of (1) hemorrhage; (2) coagulation; and (3) vessel lysis. These results were considered individually and then combined to derive irritancy score (Fisher et al., 2005), which is used to classify the irritancy level of the formulation. Results were analyzed by using method described by some modification to available literature (Kishore et al., 2008) with reference to irritation severity score. After the treatment time of 5 min of a particular component or formulation, irritation severity was determined by either hemorrhage or lysis, or coagulation. The following scheme: 0–1 = non-irritant; 1–5 = slight reaction; 5–9 = moderate reaction; 9–21 = severe reaction. Mean scores were determined further (Luepke, 1985; Kalweit et al., 1987, 1990).

In vivo Evaluation of Coumarin Fraction and CNF

Experimental Animals: Rodents

Swiss male albino mice (20–25 g) were procured from Bombay Veterinary College, Parel, Mumbai, India, and were acclimatized in the animal house of the Institute of Chemical Technology (ICT), Matunga, Mumbai. Young healthy male mice were housed eight per cage and maintained at a temperature of 23 ± 2°C, at a humidity of 51 ± 10% and in a 12:12-h light/dark cycle with free access to rodent chow by Pranava Agro Industries Ltd., Sangli, India, and tap water *ad libitum*. The experiments were carried out between 9 AM and 6 PM. The animal study was approved by the Institutional Animal Ethics Committee (ICT/IAEC/2017/P30), Mumbai.

Evaluation of Coumarin Fraction in Acute PTZ-Induced Seizure in Mice

Male albino mice with a body weight between 18 and 22 g comprising of six mice in each experimental group were used. Experimental groups taken were Group I: normal control (saline only), Group II: positive control (diazepam 2 mg/kg i.p. dose), Group III: negative control (PTZ 100 mg/kg i.p. dose), Group IV: treatment (PTZ+ coumarin fraction 50 mg/kg i.p. dose), Group V: treatment (PTZ+ coumarin fraction 75 mg/kg i.p. dose), and Group VI: treatment (PTZ+ coumarin fraction 100 mg/kg i.p. dose). Pre-treatment was given 30 min prior to i.p. injection PTZ 100 mg/kg. Each animal was placed into an individual plastic cage for observation lasting 30 min. Seizures and tonic–clonic convulsions were recorded and score was assigned as follows,

onset of myclonic jerks; onset of clonic seizures; onset of tonic seizures; status of animal (recovery or death; Smith et al., 2007).

Pharmacokinetic and Brain Distribution of CNF

Male wistar rats (240–270 g) were initially subjected to the jugular vein cannulation using polyethylene cannula tubing (0.5 mm ID × 1.0 mm OD). The rats were anesthetized using ketamine (40 mg/kg) and xylazine (4 mg/kg) intraperitoneally (*i.p.*) and the jugular vein was cannulated for the purpose of blood collection. The entire PK study was broadly divided into four groups, Group I: single intravenous bolus dose of wedelolactone (0.4 mg/kg i.v.), Group II: CNF (0.4 mg/kg i.v.), Group III: CNF (0.4 mg/kg nasal), and Group IV: CNF (0.4 mg/kg i.p). Serial blood samples (0.250 ml) were collected via the jugular vein at 0.083, 0.25, 0.5, 1, 2, 4, 8, 12, and 24 h after wedelolactone and CNF administration. At each time point, the amount of withdrawn blood volume was replaced by heparinized saline via the jugular cannula (Mandlekar et al., 2007). The plasma samples were obtained after centrifugation of blood samples at 10,000 × *g* and stored at −80°C until HPLC analysis. Brains of rats were isolated at 12 and 24 h (n = 4) for brain distribution study. Brain was quickly removed and rinsed with 0.9% saline. The brain tissues were homogenized using ice-cold 0.1 M PBS (pH 7.4). The homogenate was centrifuged at 4°C (3000 rpm; R-248M of CPR-223 24 plus Instrument, Remi, India) for 15 min. The aliquots obtained were used for the estimation of wedelolactone content (Omura et al., 1965; Walawalkar et al., 2006). Linearity for wedelolactone was done using reaction mixture (300 μl) consisting of 0.1 M sodium phosphate buffer pH 7.4, plasma matrix or brain homogenate, and wedelolactone (0, 1, 3, 10, 30, and 100 μg/ml). All reactions were carried out in duplicate and mean value obtained from each set of duplicate incubations were used for further calculations.

Evaluation of CNF in PTZ Induced Kindling Model of Epilepsy

According to Tambe et al. (2016), repetitive administration of PTZ (35 mg/kg, i.p.) as a subconvulsant dose on every alternate day (Monday, Wednesday, and Friday) induces kindling in mice. After each PTZ injection, the convulsive behavior was observed for 30 min. The intensity of seizure response was scored according to (Shaikh et al., 2013): Score 0 (no response); Score 1 (myclonic jerk); Score 2 (straub tail); Score 3 (clonic jerk without loss of righting reflex); Score 4 (clonic seizure with loss of righting reflex); Score 5 (tonic seizure), and Score 6 (death).The maximum seizure response was recorded in each animal. Kindling occurs when animal gets Stage 4 or Stage 5 for consecutive 3 days after PTZ administration (**Figure 1**). The experimental groups taken were as follows, Group I: positive control (diazepam 1 mg/kg i.p. dose), Group II: negative control (PTZ 35 mg/kg i.p. dose), Group III: normal control (saline only), Group IV: vehicle control (PTZ + vehicle for nasal dose), Group V: treatment (PTZ+ CNF 5 mg/kg nasal dose), and Group VI: treatment (PTZ+ CNF 10 mg/kg nasal dose).

Evaluation of superoxide dismutase, catalase, reduced glutathione, and malondialdehyde levels

All groups were sacrificed at the end of the study; brain was quickly removed and rinsed with 0.9% saline. The brain tissues were homogenized using ice-cold 0.1 M PBS (pH 7.4). The homogenate was centrifuged at 4°C (3000 rpm; R-248M of CPR-223 24 plus Instrument, Remi, India) for 15 min. The aliquots obtained were used for the estimation of SOD, CAT, GSH, and malondialdehyde (MDA) using methods described by Sinha (1972); Nandi and Chatterjee (1988), Ellman (1959), and Ohkawa et al. (1979), respectively.

Histopathology

After fixation with 4% paraformaldehyde, the brain samples were routinely processed and subjected to paraffin embedding. The coronal sections of 10 μm passing through hippocampus were sliced, mounted and stained by H&E and observed under microscopes at different magnifications. The sections were assessed for the alterations pertaining to neuronal damage like pyknotic nuclei, distorted morphology of cell, etc.

Immunohistochemistry

Immunohistochemistry was performed according to (Tambe et al., 2016)on a previously fixed, 5-μm-thick brain sections (consisting 10–12 sections) passing through hippocampus. Sections were dried and fixed on poly-L-lysine coated glass slides at 37°C for 24 h. Xylene was used for de-waxing. The sections were rehydrated with decreasing concentrations of alcohol, namely, 95, 70, 50% for 5 min in each concentration. Peroxidase activity was blocked by incubating with 3% hydrogen peroxide in methanol for 30 min. Sections were washed with distilled water for 5 min. Antigen recovery was initiated by heat mediated antigen recovery technique using citrate buffer pH 6 for 15 min. followed by washing with distilled water for several times. Subsequently, it was treated with PBST (0.3% Triton X-100) for creating pores in sections to absorb antibody. Primary monoclonal anti-GFAP antibody (ab10062) was added and incubated for overnight at 4°C. After washing with PBS, secondary antibody Alexa fluor @ 488 (goat anti-mouse) was added to it and incubated for 1.5 h. After giving a secondary wash in PBS, it was incubated with DAPI (1.5% in PBS). Number of positive cells to GFAP in the slides were counted under a light microscope at a intensification of 40x (Matsuda et al., 2009).

Determination of TNF-α by ELISA

Tumor necrosis factor alpha, a pro-inflammatory cytokine, plays a major role in initiating and regulating the cytokine cascade during an inflammatory response. Brain homogenates prepared were used for the determination of TNF-α. ELISA plate was incubated overnight with Capture antibody in coating buffer at 4°C. After washing with the wash buffer, the wells were blocked with assay diluents and incubated at room temperature for an hour. Standard curve was plotted against rat standard TNF-α (eBioscience, United States). Subsequently, the plate was incubated with the samples for 2 h at 4°C. Brain TNF-α was estimated by using ELISA kit (eBioscience, United States) as per the methodology provided by manufacturer in the kit. Finally, readings were taken at 450 and 570 nm and analyzed.

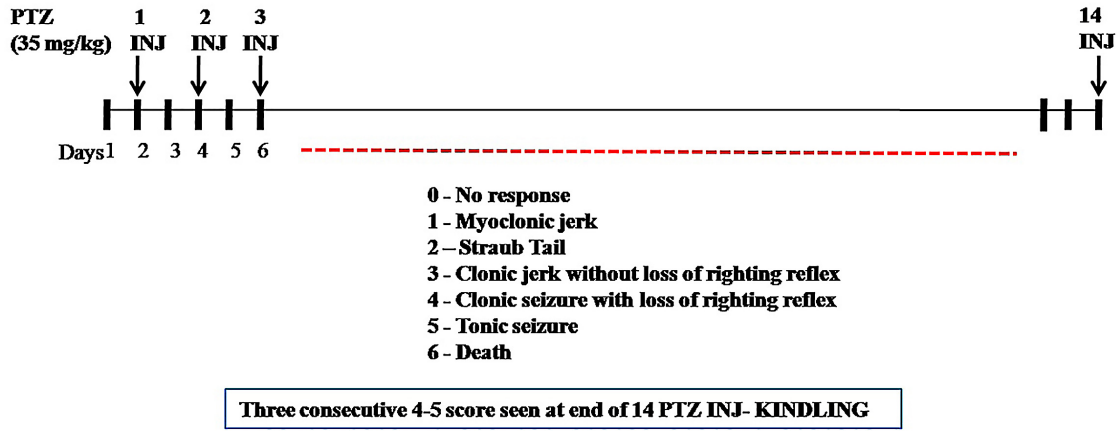

FIGURE 1 | Protocol for PTZ induced kindling.

Statistical Analysis

Data of all the results were presented as mean ± SEM. The analyses of all the studies were done with the help of ANOVA followed by Dunnett's test. The difference in the results of PTZ and MES were considered statistically significant when $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ compared to negative control.

For *in vitro* and *ex vivo* permeation study, statistics were performed by paired *t*-test. Differences were considered statistically significant at $P < 0.05$.

RESULTS

Extraction of Different Phytochemical Fractions From EA

The coumarin fraction was successfully separated from methanolic extract of EA with % yield of 7.6% w/w.

HPLC Determination of Coumarin Fraction and CNF

Coumarin fraction and CNF both revealed similar phytoconstituents, i.e., wedelolactone, apigenin, and luteolin with different mobile phase combination. As evident from **Table 1**, wedelolactone as a major component was eluted isocratically with 45% acetonitrile and 55% formic acid buffer (0.1%, pH-2.5) at flow rate of 1.0 ml/min and detected on UV PDA detector at wavelength of 352 nm; 81.18 and 10.97% of wedelolactone is present in coumarin fraction and CNF, respectively. Quantitative estimation was calculated on the basis of peak area with reference to the respective standards. Luteolin and apigenin present in the coumarin fraction and CNF were isocratically eluted with 50% acetonitrile and 50% formic acid buffer (0.1%, pH 2.5) at flow rate of 1.0 ml/min and detected at wavelength of 325 nm (**Figure 2**).

TABLE 1 | HPLC analysis of coumarin fraction and CNF.

Compound name	Drug % in coumarin fraction	Drug % in CNF
Wedelolactone	81.18	10.97
Apigenin	4.37	0.95
Luteolin	1.24	0.56

CNF Stability Studies

Formulated CNF was slightly yellowish in color with pleasant odor. CNF shows turbidity after 3 months at normal storage condition so the particle size was carried out by multimodal size distribution on a Nanobrook®. CNF was diluted with water with 1: 10 ratio (v/v). The effective diameter found was 953.81 nm with poly-dispersity index of 0.404 which indicates that the formulation (CNF) should be stored in refrigerated condition.

Drug Content Determination of CNF

Coumarin nasal formulation revealed high values of drug content. On the basis of linearity of WL, the CNF was reported to contain 1.70% w/v of WL and recovery yield was 34.79641% of WL as evident from **Table 2**.

Solubility Study of CNF

Figure 3 shows the equilibrium solubility of coumarin extract in various surfactants and co-surfactants. Coumarin extract exhibited maximum solubility in Tween 80 and PEG 300. Thus, Tween 80 was chosen as the surfactant and PEG 300 as the co-surfactant.

FTIR Analysis

Fourier transform-infrared spectra of the coumarin fraction and CNF are as shown in **Figure 4**. The FTIR spectrum exhibited peaks at 3448 cm^{-1}due the OH groups and a strong band at 2924 cm^{-1} (aliphatic C–H stretching) indicating the presence of –CH2– and –C–H in addition to peaks at 1606, 1523, and 1477 cm^{-1} which indicated the presence of an aromatic ring system. Broad and strong peak in the range of 1665–1685 cm^{-1} confirms the presence of α,β-unsaturated ketone or aromatic ketone. Several medium-weak multiple bands ranging from 1400 to 1600 cm^{-1} indicate the presence of C = C bending in aromatic

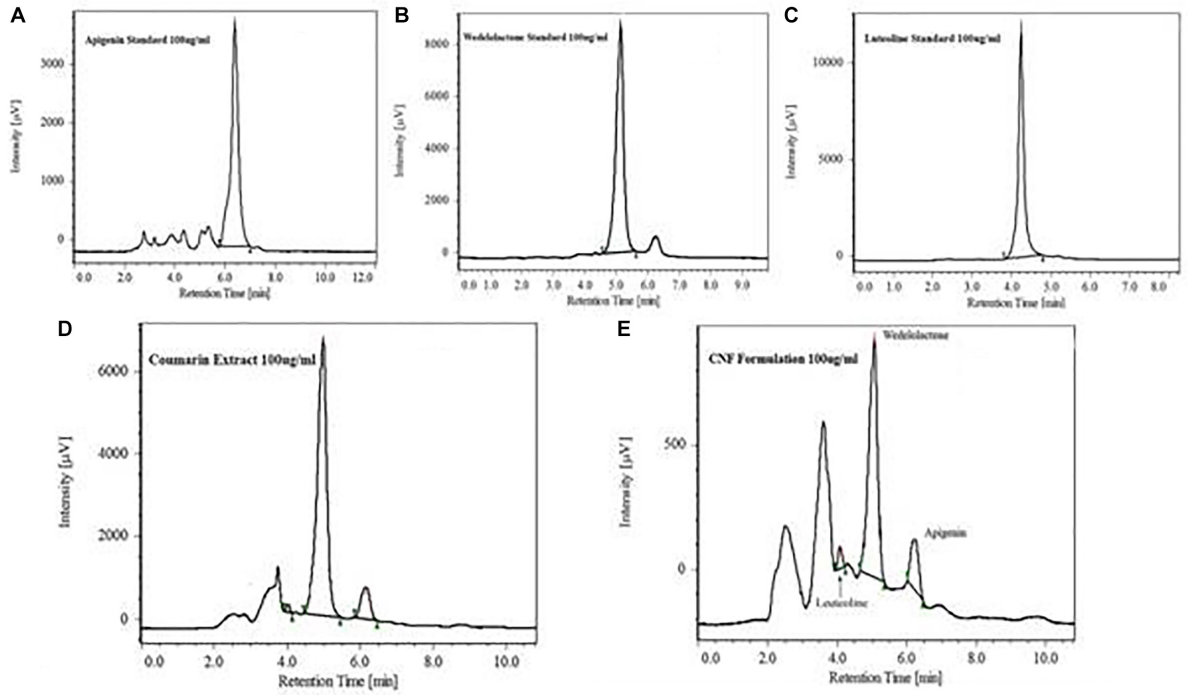

FIGURE 2 | HPLC chromatogram showing major peak of standard apigenin **(A)**, standard wedelolactone **(B)**, standard luteolin **(C)**, wedelolactone in coumarin fraction **(D)**, and CNF **(E)**.

TABLE 2 | Drug content over accelerated stability of CNF.

Formulation	Drug content % (w/v)	Yield %
CNF (1 mg/ml)	0.98 ± 0.02	100
CNF (1 mg/ml) at 2 months	0.97 ± 0.03	98.83
CNF (1 mg/ml) at 3 months	0.96 ± 0.03	98.07

Results are the mean values ± SD (n = 3) at all interval of the study (0, 2, and 3 months).

ring while the strong peaks belonging to 1000–1300 cm^1 are the identities of cyclic ethers. Strong, broad peaks within 3200–3600 cm^{-1} are due to phenolic OH– groups.

In vitro Release Studies

In vitro release of WL from CNF and coumarin fraction was assessed in SNES solution prepared in PBS (pH 7.4) at 37 ± 0.2°C for a time period of 24 h. Cumulative drug release profiles are illustrated in **Figure 5**. Calibration curve of wedelolactone in SNES solution revealed linearity ($R^2 = 0.999$) in the concentration range of 10–50 µg/ml.

Coumarin nasal formulation release patterns achieved a total 19% WL release within the first 30 min. Thereafter, it followed a slower release profile, reaching to a total drug release of 55.04% over the time period of 24 h, contrary to coumarin fraction which show 12% of total release of WL within first 30 min and total drug release of 23.05% over the time period of 24 h.

% Cumulative release of WL is plotted against time (h) and the slope of the linear section of the curve is calculated. Consequently, the enhanced release is calculated by taking the ratio of the two slope values. The enhanced release ratio is found to be 1.62. Thus, CNF releases 1.62 times more WL than the coumarin fraction over total period of time. In first 15 min, CNF released 1.59 times more WL compared to the coumarin fraction. This may help enhancement of the nose-to-brain delivery.

Ex vivo Permeation Study

Figure 6 represents *ex vivo* permeation profile of coumarin fraction and CNF across goat nasal mucosa. This is the first time we are reporting the permeability studies for wedelolactone release from fraction and a particular formulation. The overall enhancement ratio of CNF over coumarin fraction solution was found to be 2.29. At the start, CNF permeates faster than coumarin fraction. It showed 1.36 times faster permeation compared to the coumarin fraction. CNF resulted in a twofold higher permeation (*t*-test, $p < 0.05$) of wedelolactone compared to coumarin fraction solution over a period of 8 h.

Evaluation of CNF Irritancy Potential on HET-CAM

Prior to the irritation scoring, two different concentrations of the coumarin extract (i.e., 1 and 5%) were subjected to irritation severity scoring. The mean irritation severity score for 1%

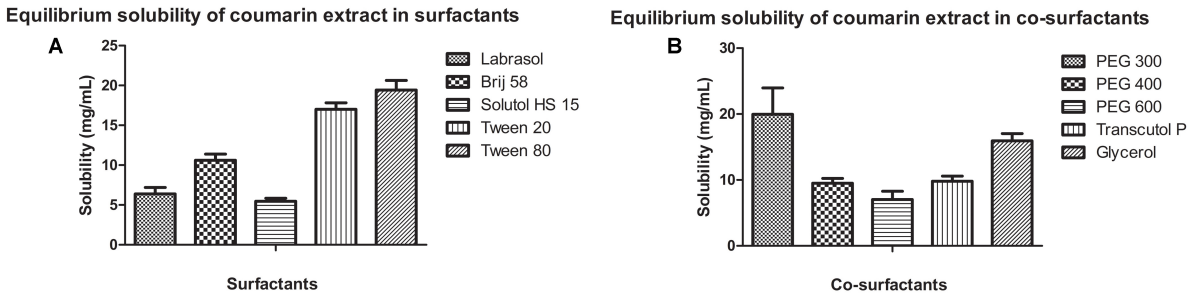

FIGURE 3 | Equilibrium solubility of coumarin extract in **(A)** surfactants and **(B)** co-surfactants.

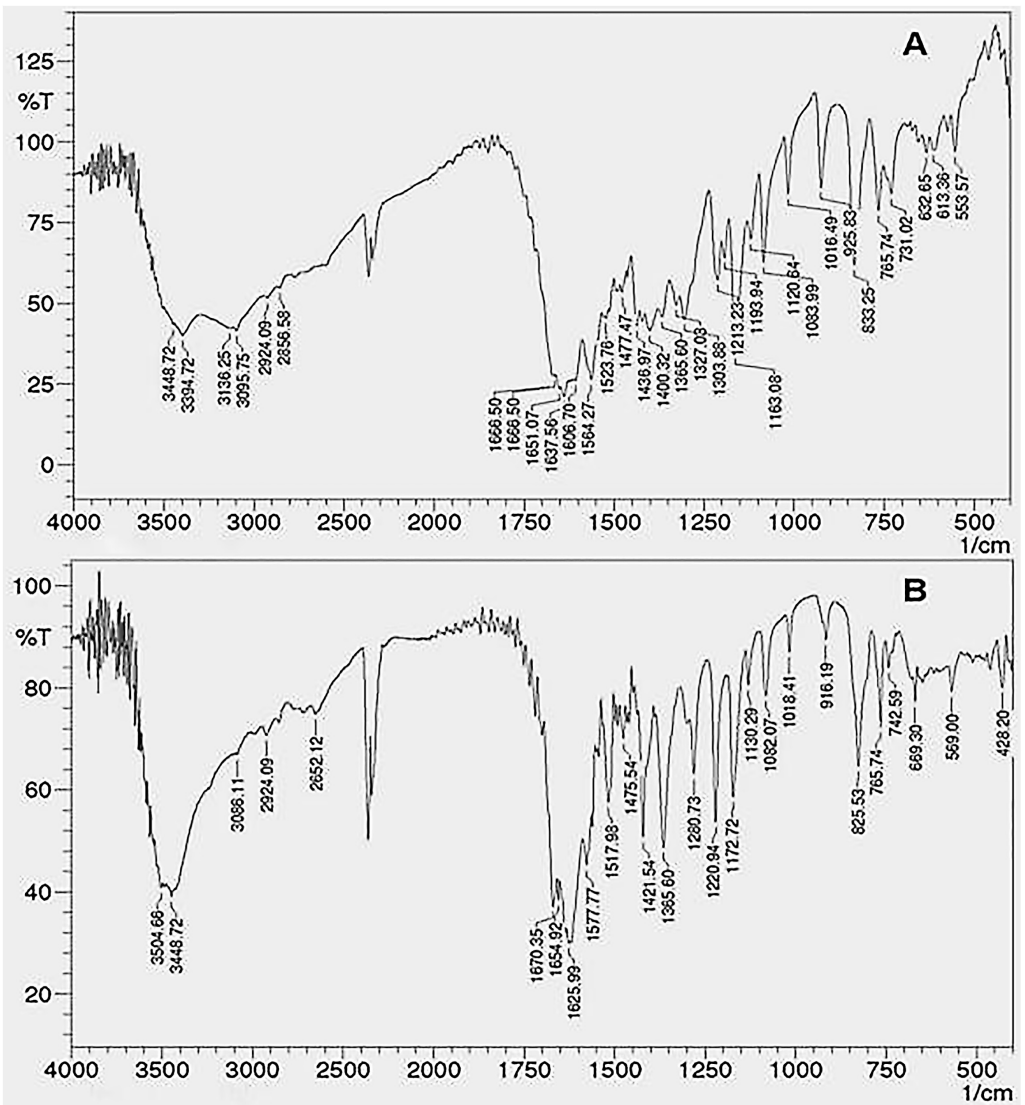

FIGURE 4 | Fourier transform-infrared of **(A)** coumarin fraction and **(B)** CNF.

coumarin extract in CNF was found to be 0.07 and that of 5% was 0.57. So, 1% coumarin extract was used further to formulate CNF.

The irritancy score as evident from **Table 3** of the negative control group was found to be 11.59 while that of normal saline, vehicle control and CNF was 0. The CNF treated group

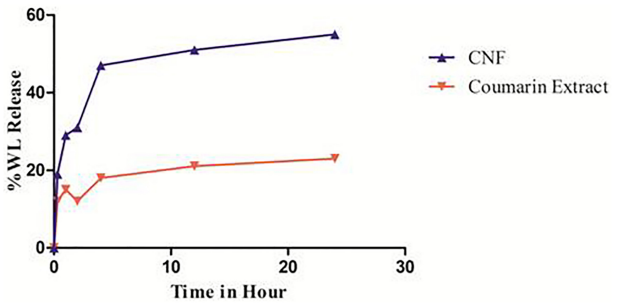

FIGURE 5 | *In vitro* release pattern of CNF and coumarin fraction. Data presented as mean values ± SD ($n \geq 3$) by paired *t*-test. Differences were considered statistically significant at $p < 0.05$.

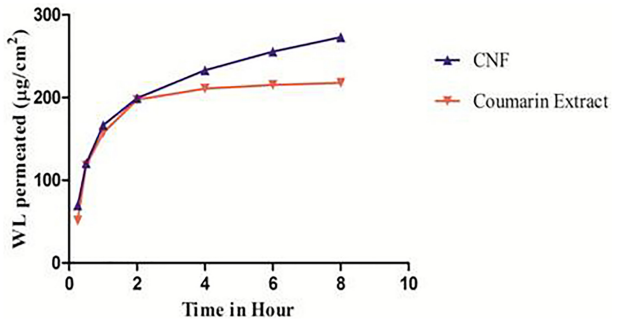

FIGURE 6 | *Ex vivo* permeation pattern of CNF and coumarin fraction. Data presented as mean values ± SD ($n \geq 3$) by paired *t*-test. Differences were considered statistically significant at $p < 0.05$.

(i.e., Group IV) as well as vehicle control did not exhibit any irritancy (**Figure 7**). Thus, the mean irritation severity score was zero.

TABLE 3 | Irritancy score on HET-CAM for given formulations.

Groups/image no	HT	LT	CT	Irritancy score
Group 1 (normal)	300	300	300	0.07
Group 2 (vehicle control)	300	300	300	0.07
Group 3 (negative control)	7.67	15	300	11.59
Group 4 (CNF)	300	300	300	0.07

Results are the mean values of n = 6.

Screening of Coumarin Fraction in Acute Model of PTZ-Induced Seizures in Mice

Coumarin fraction treatment at the dose of 100 mg/kg i.p. dose ($P < 0.001$) and diazepam 2 mg/kg i.p. Dose produced a significant ($P < 0.001$) increase in onset of myoclonic seizure and clonic seizure in the PTZ induced seizures in mice (**Table 4**). Coumarin fraction significantly delayed the onset of HLE and time taken for death ($p < 0.05$ and $P < 0.001$) at 75 mg/kg. Coumarin fraction at 100 mg/kg treatment showed 100% protection from death.

The treatment with coumarin fraction (100 mg/kg) and diazepam (2 mg/kg) produced a significant ($P < 0.001$) increase in onset of myoclonic and clonic seizures (**Table 4**). Coumarin fraction significantly delayed the onset of HLE and time taken for death at 75 mg/kg ($p < 0.05$ and $P < 0.001$). Coumarin fraction at 100 mg/kg treatment showed 100% protection from death.

Evaluation of Pharmacokinetic and Brain Distribution Parameter of CNF

Based on the encouraging results of the *in vitro* data, pharmacokinetic study was designed further to verify the brain to plasma distribution potential of CNF in rats. The intravenous plasma concentration-time profiles of wedelolactone, CNF (i.v.), CNF (nasal), and CNF (i.p.) (0.4 mg/kg) in rats are shown

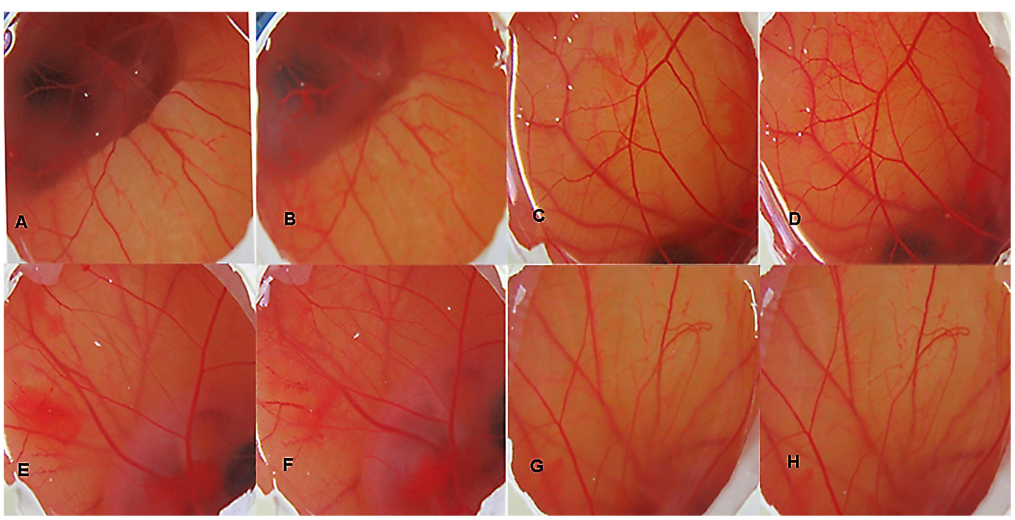

FIGURE 7 | HET CAM bioassay, images of CAM before and after 5 min of contact with the different formulations, DSLR camera images x20: **(A)** Group I (30 s); **(B)** Group I (5 min); **(C)** Group II (30 s); **(D)** Group II (5 min); **(E)** Group III (30 s); **(F)** Group III (5 min); **(G)** Group IV (30 s); **(H)** Group IV (5 min).

TABLE 4 | Screening of coumarins in PTZ induced seizure model in mice. **(A)** Onset of myoclonic jerks. **(B)** Onset of clonic seizures. **(C)** Onset of hind limb extensor (HLE). **(D)** Onset of death.

Treatments	Time in seconds				
	Onset of myoclonic jerks	Onset of clonic seizures	Onset of HLE	Onset of death	% Protection from death
PTZ control (100 mg/kg)	49.5 ± 6.69	71.83 ± 7.23	411.33 ± 12.96	411.33 ± 12.96	0
Diazepam 2 mg/kg + PTZ (100 mg/kg)	0 ± 0.00***	0 ± 0.00***	0 ± 0.00***	0 ± 0.00***	100***
Coumarin 50 mg/kg + PTZ (100 mg/kg)	49.17 ± 2.15	72 ± 3.3	573.40 ± 89.15	613 ± 105.42*	83.33***
Coumarin 75 mg/kg + PTZ (100 mg/kg)	57.33 ± 3.56***	74.5 ± 3.97	686.25 ± 137.94*	820 ± 104.13***	50.00
Coumarin 100 mg/kg + PTZ (100 mg/kg)	61.83 ± 5.96	104.66 ± 17.29*	0 ± 0.00***	0 ± 0.00***	100***

*Data are expressed as mean ± SEM (N = 6). *p < 0.05, **p < 0.01, ***p < 0.001 as compared with the PTZ control group; one-way ANOVA followed by Dunnett test.*

in **Figure 8**. Brain levels of wedelolactone with CNF (nasal) displayed a 2.43- and 4.01-fold increase in the area under curve as compared to CNF (i.v) and CNF (i.p.), respectively. There was no significant alteration found in pharmacokinetic parameter in plasma matrix of CNF (i.v.), CNF (nasal), and CNF (i.p.) as compared to wedelolactone (i.v.) data. As shown in **Tables 5**, **6**.

Screening of CNF in PTZ-Induced Kindling Model in Mice

Repeated administration of sub-convulsant dose of PTZ (35 mg/kg) on every alternate day produced kindling in negative control (vehicle + PTZ) group which required 14 injections (30 days) resulting in increased convulsive activity that can be termed as epileptogenesis, leading to generalized clonic–tonic seizure. Diazepam 1 mg/kg i.p. and CNF 10 mg/kg nasal dose showed similar protection, as none of the animals exhibited seizure score of 4–5 during the kindling period. CNF at dose 10 mg/kg nasal dose significantly ($P < 0.001$) reduced both the incidence as well as severity of seizures occurring during the repeated treatments with PTZ during the course of kindling period (**Figure 9**). CNF at 5 mg/kg nasal dose did not protect the animals from seizures and there was no significant reduction in the seizure score. However, the lower dose (5 mg/kg) protected from mortality as well as prevented development of kindling (No 4–5 seizure score).

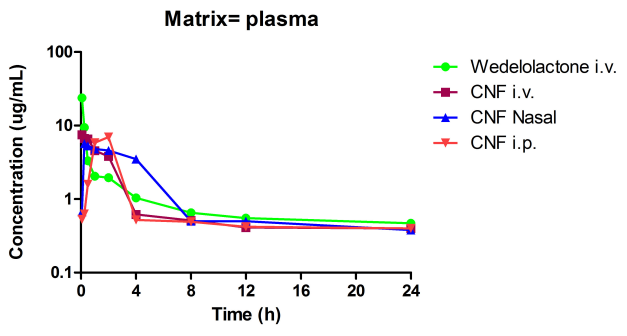

FIGURE 8 | Pharmacokinetic and brain distribution parameter of wedelolactone and CNF.

Screening of CNF on Cellular Antioxidants Namely SOD, CAT, GSH, and LPO Levels in PTZ Kindling Model

Pentylenetetrazole induced kindling caused a remarkable decrease in the activities of the major antioxidant enzymes, i.e., SOD and CAT ($P < 0.001$, $P < 0.05$). CNF at 5 and 10 mg /kg significantly increased the activity of SOD and CAT ($P < 0.05$, $P < 0.01$) whereas at vehicle control did not exhibit any effect on these antioxidant enzymes. Moreover, diazepam significantly increased the SOD and CAT activity ($P < 0.01$, $P < 0.05$; **Figure 10**). GSH level in PTZ control group was significantly ($p < 0.001$) lower than the normal control group. CNF at 5, 10 mg/kg ($P < 0.01$, $P < 0.001$) and diazepam ($P < 0.01$) showed significant increase in the levels of GSH as compared to PTZ control. The lipid peroxidation (MDA level) was markedly increased in the negative control group which was significantly ($P < 0.001$) higher than that seen in the vehicle control group. CNF and diazepam ($P < 0.01$) treated groups showed significant

TABLE 5 | Pharmacokinetic parameter of wedelolactone and CNF.

Formulation	Route	Tmax (h)	Cmax (μg/ml)	C0 (μg/ml)	AUClast (h*μg/ml)	AUC in (h*μg/ml)	Cl (ml/min/kg)	Vss (l/kg)	T1/2 (h)	Clast (μg/ml)	Tlast (h)
CNF	IP	2.00	6.99		24.93	28.01			8.51	0.40	24.00
CNF	IV	0.08	7.54	7.99	23.88	27.71	0.24	0.14	6.64	0.40	24.00
CNF	Nasal	0.25	5.67		32.46	34.76			5.89	0.38	24.00
Wedelolactone	IV	0.08	23.84	37.82	25.18	31.11	0.21	0.16	10.84	0.47	24.00

TABLE 6 | Brain distribution parameters of wedelolactone and CNF.

Formulations	Brain (AUC)		Plasma (AUC)		Brain/plasma ratio (AUC)	
	12 h	24 h	12 h	24 h	12 h	24 h
Wedelolactone i.v.	3863.6	977.625	1153	635	3	2
CNF i.v.	1192.125	878.15	237	204	5	4
CNF Nasal	2908.325	380.4	803	67	4	6
CNF i.p.	724.775	791.075	283	177	3	4

reductions in the levels of MDA as compared to negative control group.

Histopathology

Hematoxylin and eosin staining as per Fujikawa scaling system were applied. Group II showed dead neurons with pyknotic nuclei [Score 2.5 (55–75% damage)] which were clearly distinguishable from surviving cells that showed round-shaped, cytoplasmic membrane-intact cells, without any nuclear condensation or distorted aspect (**Figure 11**). Group I (standard) V and VI (treatment groups) (Score 0 – no damage) improved the seizure induced neuronal damage in the treatment groups in kindled mice. Coumarin at 10 mg/kg [Score 1.75 (25–45% damage)] did not protect from seizure mediated neuronal damage. Moreover, diazepam 1 mg/kg prevented the neuronal damage as a result of kindling.

Immunohistochemistry

In our study, GFAP-fluorescein (FITC) exhibited fluorescent astroglial cells particularly in CA1 region of hippocampus. This is the first time we are reporting immunohistochemistry where astroglial cells show fluorescence after taking GFAP-FITC. Astroglial cells produce GFAP during neuronal injury. These damaged neurons contain GFAP which when bound to anti-GFAP antibody (which in turn is bound to the secondary antibody alexafluor 488) gives fluorescence. Group III (normal control) and Group VI (CNF 10 mg/kg) did not show any fluorescence indicating the absence of GFAP in the astroglial cells of CA1 region of hippocampus. Group V (CNF 5 mg/kg) and Group I (diazepam 1 mg/kg) showed little fluorescence with no distortion in the morphologies of the neurons in CA1 region but, on the other hand, Group II (PTZ-controlled) and Group IV (vehicle-controlled) showed strong fluorescence indicative of inflammations in the damaged neurons of CA1 region (**Figure 12**). Thus, Group II and Group IV showed significantly higher levels of GFAP ($P < 0.001$) compared to the other groups.

Determination of TNF α by ELISA

TNF-α, a pro-inflammatory marker, forms during injury and is estimated using an ELISA technique in the brain homogenate. As depicted in **Figure 13**, PTZ causes significant elevation of level of TNF-α in negative control group ($P < 0.001$) as compared to the normal control group. Treatment with CNF 5, 10 mg/kg ($P < 0.001$) and diazepam ($P < 0.001$) significantly attenuated the levels of TNF-α in comparison to the negative controlled group.

DISCUSSION

Epilepsy is a complex neurological disorder which has plagued mankind since ages and continues to afflict more than 50 million people across the globe. It is a spectrum disorder characterized

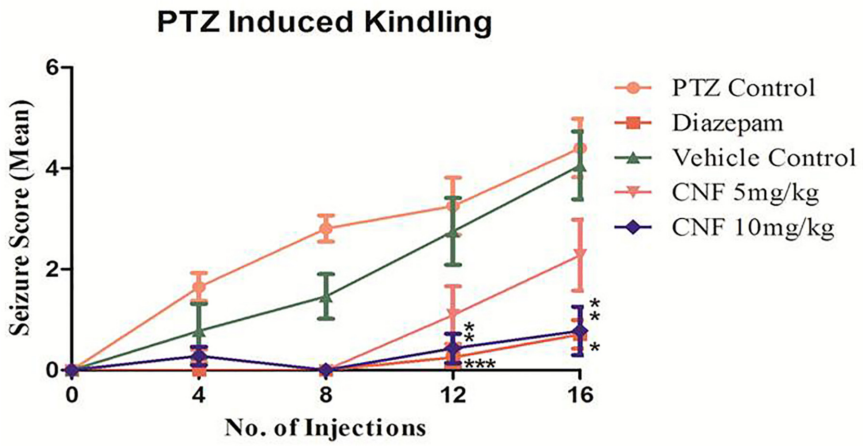

FIGURE 9 | Screening of CNF on seizure score in PTZ kindling model. $N = 8$, data are expressed as mean ± SEM, statistical analysis by one-way ANOVA followed by Dunnett's test. Significance at $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, and ns, not significant vs. PTZ control.

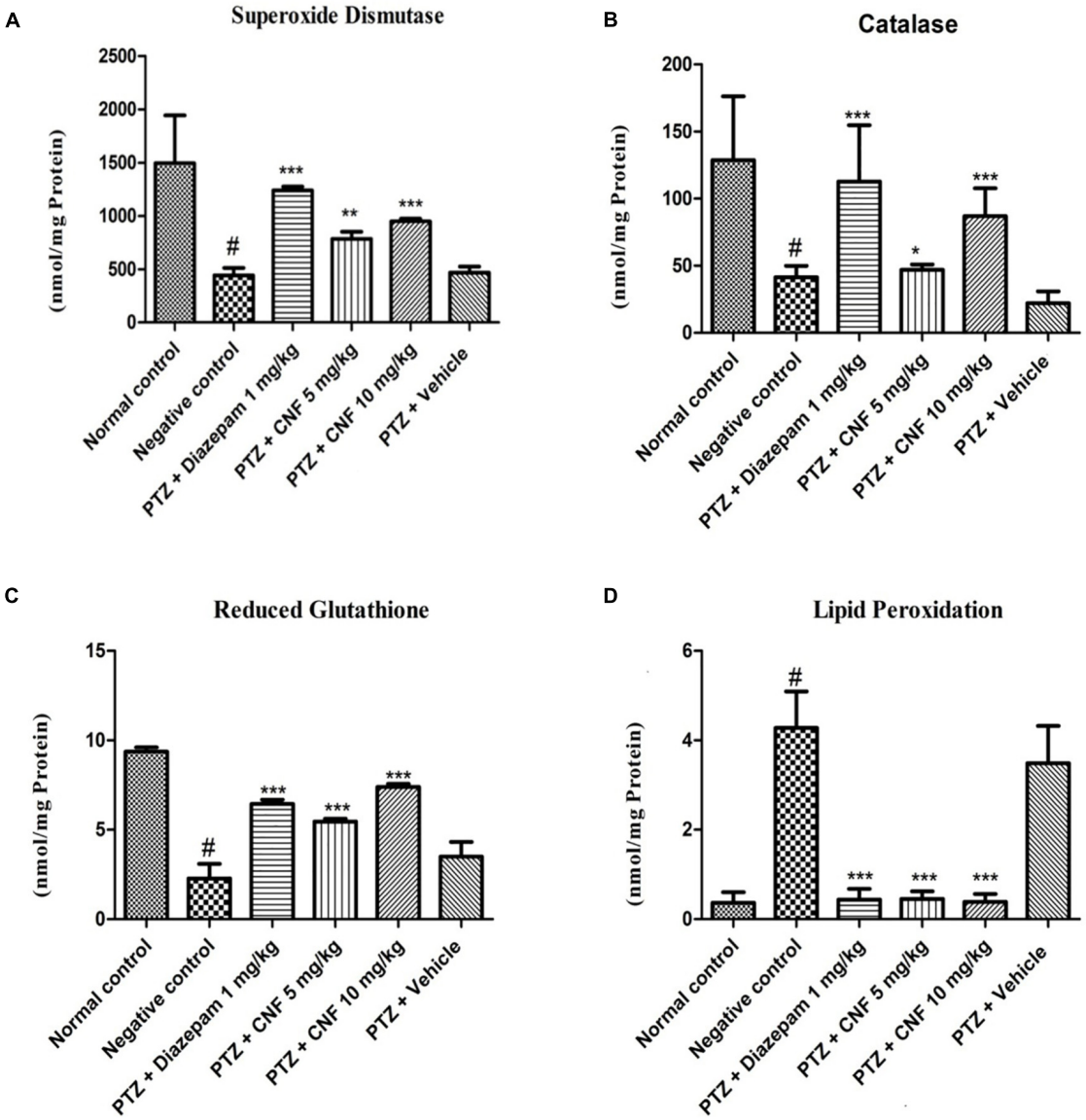

FIGURE 10 | Effect of CNF on **(A)** SOD activity (Fisher et al., 2005), **(B)** CAT activity, and **(C)** GSH and **(D)** LPO levels in midbrain ofPTZ induced kindling model in mice **(A)** SOD (Fisher et al., 2005), **(B)** catalase, **(C)** GSH, and **(D)** LPO. Data are expressed as mean ± SEM ($N = 6$). $^{*}p < 0.05$, $^{**}p < 0.01$,$^{***}p < 0.001$ as compared with the PTZ control group and $^{\#}p < 0.05$ compared with normal group; one-way ANOVA followed by Dunnett test.

by recurrent seizures due to abnormal excessive and synchronous neuronal activity in the brain. The current medications primarily act to symptomatically suppress seizures rather than correcting the underlying abnormalities causing epilepsy or altering its natural histopathology. Furthermore, trepidations about the safety (narrow therapeutic window) and costs of the available drugs is a major concern. There are no direct reports in literature indicating the anticonvulsant effect of coumarin fraction of EA, however the activity of methanolic extract of EA in PTZ and MES models is reported. The study was undertaken to investigate the neuroprotective activity of CNF and to identify the active principle responsible for the antiepileptic activity. Primarily coumarins isolated from the crude extract ofEA were screened in the acute PTZ induced seizure model in mice. This was on the basis of previous study carried by (Shaikh et al., 2012) at ICT, which confirmed the antiepileptic activity of crude extract of EA. FTIR and HPLC analysis of CNF confirms the presence of wedelolactone, luteolin, and apigenin in the coumarin fraction. *In vitro* analysis and *ex vivo* permeation results confirmed the immediate release, enhanced permeation, and nose to brain delivery of CNF for its possible effect (Westin et al., 2006).

Coumarin fraction (100 mg/kg) exhibited an excellent anticonvulsant activity in PTZ test. PTZ induced seizure model is preliminary screening model to assess the anticonvulsant potential of a substance as it gives predictive relevance regarding the clinical spectrum of the investigational compound

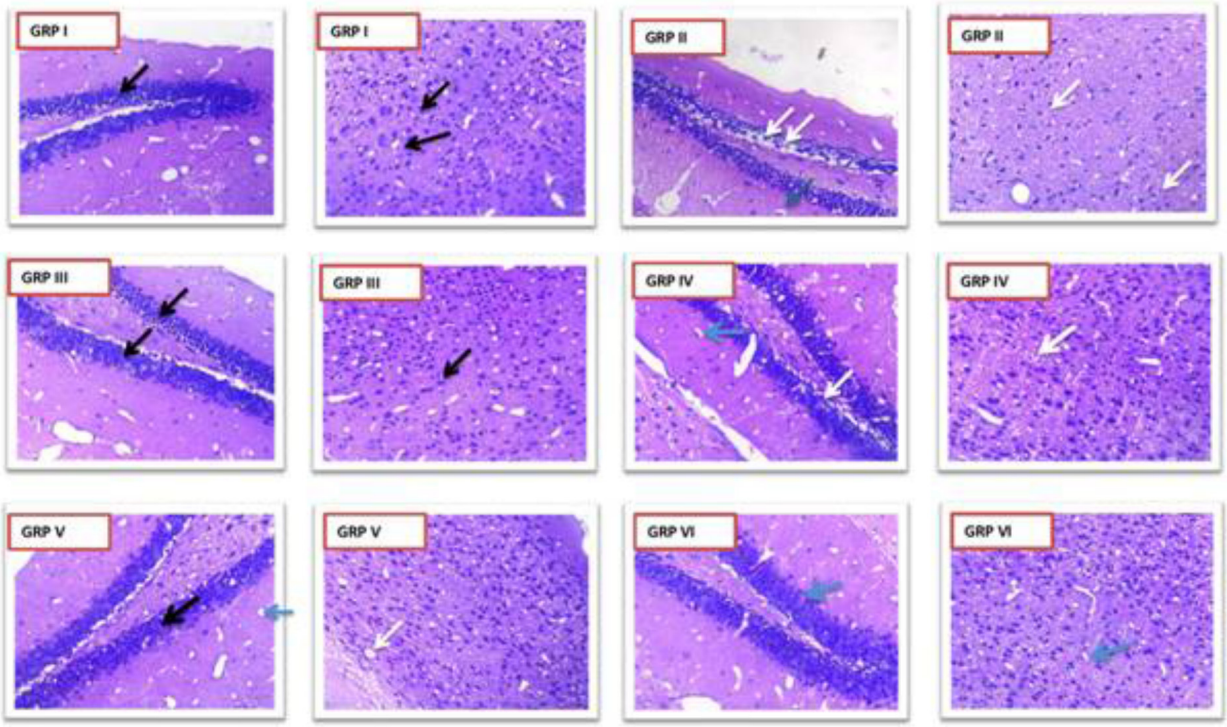

FIGURE 11 | Photomicrographs showing changes in histopathology of neuron. GRP-I standard control (PTZ + diazepam), GRP-II negative control (PTZ), GRP-III normal control, GRP-IV PTZ + vehicle control, GRP-V PTZ + CNF 5 mg/kg, GRP VI- PTZ + CNF 10 mg/kg Black arrows: intact neurons with basophilic cytoplasm and prominent nucleus. White arrows: degenerative neurons with pyknotic nucleus. Blue arrows: Hirani bodies.

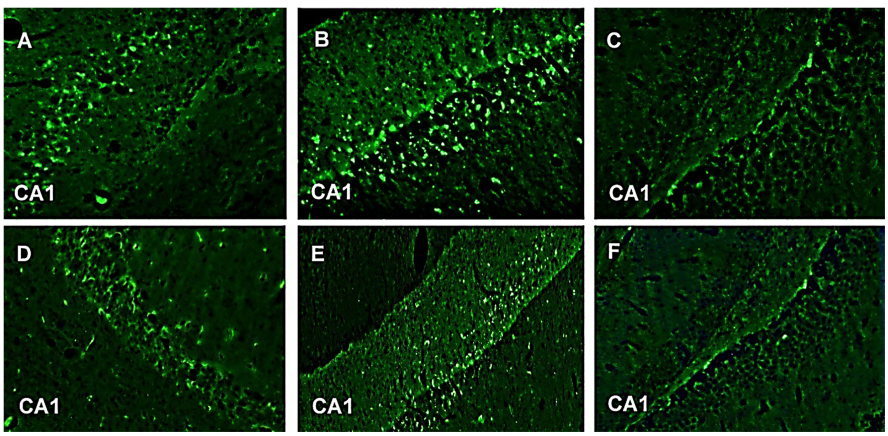

FIGURE 12 | GFAP-FITC immunohistochemistry. Representive photomicrographs of GFAP-immunoreactive neurons in CA1 region of mice hippocampus. **(A)** PTZ + diazepam, **(B)** PTZ, **(C)** normal, **(D)** PTZ + vehicle, **(E)** PTZ + CNF 5 mg/kg, and **(F)** PTZ + CNF 10 mg/kg.

(Jain et al., 2015). PTZ test is assumed to identify drugs effective against human generalized absence and tonic–clonic seizures (Sayyah et al., 2005). To further confirm the anticonvulsant activity of coumarins and on the basis of previous results; CNF was formulated using coumarin fraction from methanolic extract of EA. It was further evaluated and studied in PTZ induced kindling model of mice. Kindling is a very widely accepted and a suitable model for studying the process of epileptogenesis. Kindling is also a model of brain plasticity in which recurrent activation of neural pathways results in an increased vulnerability to evoked seizures that ultimately progresses to impulsive seizures (de Almeida Rabello Oliveira et al., 2008). This model is characterized by an amplified susceptibility to seizures after recurring brain stimulation with subcunvulsive stimuli. It has been shown that an effect comparable to electric kindling can be induced by the repeated administration of subconvulsant

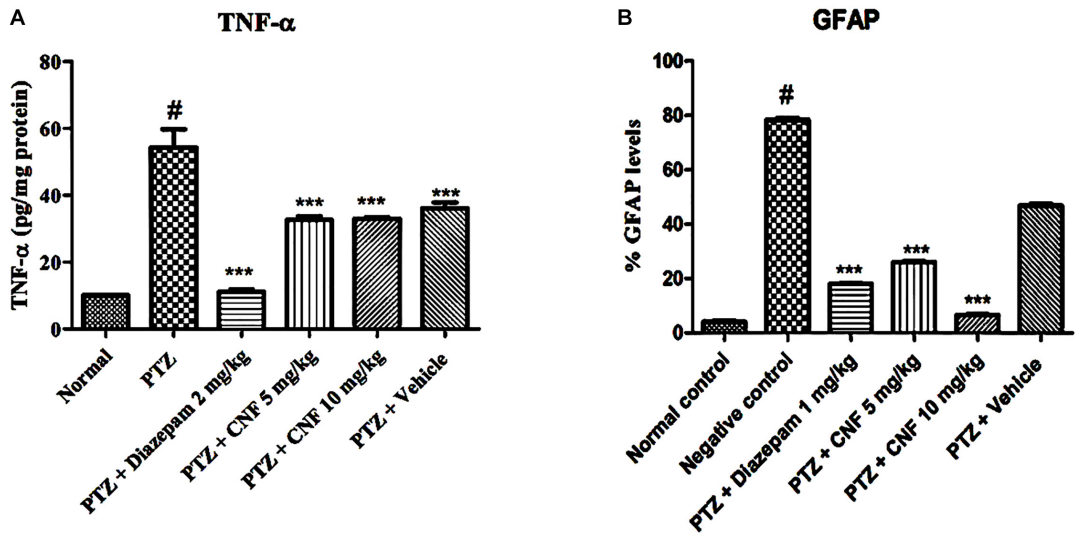

FIGURE 13 | Effect of CNF onTNF-α **(A)** and GFAP **(B)** in hippocampus. Data expressed as mean ± SEM. N-6. ***$P < 0.001$ compared with PTZ group and #$P < 0.01$, compared with normal group using one-way ANOVA followed by Dunnett's test as a post-ANOVA test.

doses of central nervous system stimulants (Corda et al., 1991). Thus, a progressive development of seizures, i.e., chemical kindling, is observed after the repetitive (on every alternate day) administration of PTZ (35 mg/kg; Tambe et al., 2016). Data from this study showed that the convulsion produced by PTZ was significantly delayed by CNF. In the various kindling experiments carried out, subconvulsive dose of PTZ (35 mg/kg) when given *i.p.* on alternate days induced kindling in PTZ treated mice on 14th injection of PTZ. During the course of kindling, following the subconvulsive doses of PTZ, there is a gradual increase in susceptibility to seizures ultimately leading to generalized tonic–clonic seizures. Administration of CNF (5 and 10 mg/kg) significantly decreased the seizure score as compared to PTZ treated animals which produced maximum seizure score. It was also observed that administration of CNF (5 and 10 mg/kg) did not produce kindling till 24 PTZ injections similar to diazepam 1 mg/kg i.p. (in a different set of animals, data not presented). This indicated increased latency to seizure onset implying neuroprotection offered by the formulation when administered by intranasal route. Oxidative stress causes an imbalance which leads to higher cellular building of reactive oxygen species (ROS) like superoxide radical (O_2^-, hydrogen peroxide (H_2O_2) etc.). This built up of reactive radicals reduces cellular antioxidant defense (SOD, CAT, and reduced GSH). Brain tissue is highly susceptible to the oxidative damage due to its high demand and consumption of oxygen. Seizures cause alterations in the membrane lipid composition, perturbing the membrane fluidity and permeability resulting in the disturbances in functioning of membrane bound enzymes which have deleterious consequences on neuronal functioning (Corda et al., 1992). In the current studies, PTZ induced kindling significantly decreased the ROS scavenging activity of normal cellular antioxidants, namely, SOD, CAT, and GSH in the brain which was in line with Ginkgo biloba extract (Ilhan et al., 2006). CNF (5 and 10 mg/kg) ameliorated the SOD and CAT enzyme activities and restored the levels of GSH. The increase in levels of MDA (an end product of free radical generation) and simultaneous decrease in the levels of GSH (free radical scavenger) in vehicle-PTZ treated mice indicated free radical generation on administration of subconvulsive dose of PTZ. The treatments, CNF 5, and 10 mg/kg caused a significant decrease in MDA levels in comparison to PTZ treated mice offering protection to neuronal cells. This might be attributed to the free radical scavenging potential consequently attenuating seizures (Bikjdaouene et al., 2003). The data also indicated that diazepam antagonized PTZ convulsion. PTZ may be exerting its convulsant effect by inhibiting the activity of gamma amino butyric acid (GABA) at $GABA_A$ receptors. GABA is the major inhibitory neurotransmitter implicated in epilepsy (De Sarro et al., 1999). The enhancement and inhibition of the neurotransmission of GABA will attenuate and enhance convulsions, respectively (Meldrum, 1981; Gale, 1992). Diazepam, a standard AED, has been shown to exert its antiepileptic effects by enhancing GABA-mediated inhibition in the brain (Porter and Meldrum, 2001). It is possible that diazepam antagonized PTZ convulsion in this study by enhancing GABA neurotransmission (Amabeoku et al., 2007). PTZ is reported to interact with the GABA neurotransmission and the GABA receptor complex antagonism by PTZ leads to PTZ-induced seizures (Bum et al., 2001). Since CNF was found to be active against PTZ convulsions, it is probable that it may be interfering with GABA mechanism by enhancing the activation of $GABA_A$ receptors owing to the phytoconstituents present in it (Kundaikar et al., 2015). This would facilitate the GABA-mediated opening of chloride channels offering symptomatic relief. HPLC results confirmed presence of wedelolactone, a naturally occurring coumarin as major phytoconstituent in coumarin fraction and CNF. It also confirmed the presence of flavonoids, namely, luteolin and apigenin in the coumarin

fraction. Reports suggest that wedelolactone has selectivity and affinity toward benodiazepine binding site which is an allosteric site on GABA receptor (Po^ças et al., 2006).

Astrogliosis is characterized by the hypertrophy of the cell bodies and processes of the astrocytes (a major neuralglia) along with increased expression of GFAP in response to various threats (Sherafat et al., 2013). Astrogliosis is an important feature of the epileptic foci and presence of GFAP is considered as a marker of neuroinflammation. The transformation of astrocytes to reactive astrocytesin epileptic disorder (especially in the kindling models) could be associated with the adaptive changes during disease progression and most likely precedes neuronal damage (Franke and Kittner, 2001). Series of repeated seizures in the kindled animals in the present study, shows increased GFAP immunoreactivity. The kindled animals treated with CNF showed significantly lower GFAP levels, which may be due to the anti-inflammatory activity of wedelolactone through inhibition of LPS-induced inflammation via NF-kappa B pathway (Yuan et al., 2013).

CONCLUSION

In conclusion, the report exemplifies the neuroprotective antiepileptogenic effect of CNF along with its anticonvulsant effect. In the present study, coumarin fraction was formulated for nasal delivery and evaluation of antiepileptic activity of the same.

The safety and non-irritant potential of CNF as a nasal delivery was confirmed in *in vitro* HET-CAM analysis. *Ex vivo* permeation data demonstrated considerable enhanced permeation of wedelolactone across the goat nasal mucosa from CNF compared to the coumarin fraction. *In vivo* experimental findings substantiate the neuro-protective, anti-oxidant, anti-inflammatory, and disease modifying effects of CNF in mitigating the sequel of events implicated in the progression of epileptic disorders/seizures. This study rationalizes CNF as a promising newer formulation in forestalling the process of epileptogenesis and associated co-morbidities.

AUTHOR CONTRIBUTIONS

SM performed extraction, formulation, development, and characterization. SM, VP, AK, and SB performed animal studies, biochemical, histological, and TNF-α analysis and analyzed the data. SM and VD performed the immunohistochemistry. SS designed the research, formulation, and supervised the project. SM, AK, and SS contributed to writing and editing of the manuscript.

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REVIEW
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Sleep Related Epilepsy and Pharmacotherapy: An Insight

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In the last several decades, sleep-related epilepsy has drawn considerable attention among epileptologists and neuroscientists in the interest of new paradigms of the disease etiology, pathogenesis and management. Sleep-related epilepsy is nocturnal seizures that manifest solely during the sleep state. Sleep comprises two distinct stages i.e., non-rapid eye movement (NREM) and rapid eye movement (REM) that alternate every 90 min with NREM preceding REM. Current findings indicate that the sleep-related epilepsy manifests predominantly during the synchronized stages of sleep; NREM over REM stage. Sleep related hypermotor epilepsy (SHE), benign partial epilepsy with centrotemporal spikes or benign rolandic epilepsy (BECTS), and Panayiotopoulos Syndrome (PS) are three of the most frequently implicated epilepsies occurring during the sleep state. Although some familial types are described, others are seemingly sporadic occurrences. In the present review, we aim to discuss the predominance of sleep-related epilepsy during NREM, established familial links to the pathogenesis of SHE, BECTS and PS, and highlight the present available pharmacotherapy options.

Keywords: epilepsy, sleep, seizure, SHE, BECTS, PS, rolandic, panayiotopoulos

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INTRODUCTION

Epilepsy is characterized by frequent and unpredictable disruptions of brain functions resulting in "epileptic seizures." Epilepsy has a great impact on the quality of life through increased incidence of injury and death, unemployment rates, lower monthly incomes, higher household costs and high absenteeism at work and schools (Jennum et al., 2017; Trinka et al., 2018; Wibecan et al., 2018). An epileptic seizure is considered as a transient episode of signs or symptoms, including transitory confusion, staring speech, irrepressible jerking movements, loss of consciousness, psychic symptoms such as fear and anxiety, due to the abnormal synchronous neuronal activity of the brain. The International League Against Epilepsy (ILAE) published a recent clinical definition of epilepsy in which a patient with any of the following conditions is considered to be an epileptic i.e., (i) two or more unprovoked seizures within more than 24 h apart; (ii) one unprovoked seizure and a probability of further seizures similar to the general recurrence risk, occurring over the next 10 years; (iii) definite diagnosis of an epilepsy syndrome (Fisher et al., 2014). Genesis of epilepsy is attributed to various predispositions that include neurological, perceptive, psychological, and social factors, which could either stimulate or worsen the syndrome. In early 2017, the point prevalence of active epilepsy was found to be 6.38/1,000 individuals, while the lifetime prevalence was 7.60/1,000

persons. Meanwhile, the annual cumulative incidence of epilepsy was 67.77/100,000 persons and the incidence rate was 61.44/100,000 person-years. The active annual prevalence, prevalence during lifetime and the incidence of epilepsy were found to be higher in the developing countries (Fiest et al., 2017).

A systematic review revealed that epilepsies of unknown etiology had the highest prevalence compared to the epilepsies of known origin (Fiest et al., 2017). These were due to known underlying factors that cause seizures such as brain damage (Sizemore et al., 2018), metabolic diseases (Tumiene et al., 2018), infections (Bartolini et al., 2018), hemorrhagic stroke (Zhao et al., 2018), and gene mutations (Leonardi et al., 2018). These precipitating factors tilt the balance between excitatory and inhibitory neurotransmissions which has been established in different types of epilepsy. Physical and psychological comorbidities are usually accompanied with epilepsy, such as depression (Jamal-Omidi et al., 2018), sleep disorders (Castro et al., 2018), and body injuries (Mahler et al., 2018). Advanced cases may suffer from memory loss (Reyes et al., 2018), behavioral disorders (Jalihal et al., 2018), and disturbance of autonomic functions (Fialho et al., 2018). The rate of sudden death in epileptic patients was reported to be three times higher than non-epileptic individuals (Kothare and Trevathan, 2018; Pati et al., 2018).

Sleep deprivation is very common among the epileptic patients and lack of sleep could worsen the seizure expressions (Neto et al., 2016). In animal models, sleep deprivation was shown to heighten the propensity to seizures (McDermott et al., 2003). Sleep deprivation has been correlated with decline in various aspects of brain functional connectivity (Nilsonne et al., 2017). Generally, sleep deprivation is secondary to other factors such as illness, emotional or psychological stress, and alcohol use. Hence, lack of sleep alone may not be sufficient to cause seizures (Razavi and Fisher, 2017). A large body of literature on the effects of epilepsy on sleep and/or sleep-deprivation on the epileptic state has been collated (St Louis, 2011; Unterberger et al., 2015).

Sleep-related epilepsy represents nocturnal seizures that manifest solely during the sleep state (Tchopev et al., 2018). Approximately 12% epileptic patients are affected by sleep-related epilepsy with the majority suffering from focal epilepsy (Derry and Duncan, 2013; Losurdo et al., 2014). In a recent case report, focal epilepsies were anatomically linked to epileptogenic origins at the right frontal lobe, using white matter tractography MRI (Tchopev et al., 2018). In a separate study, ambulatory electroencephalogram (EEG) measurement in outpatient setting reported frontal lobe seizures to manifest more readily between 12 a.m. and 12 p.m., particularly around 6:30 a.m., whereas temporal lobe seizures expressed more frequently between 12 p.m. and 12 a.m., specifically around 8:50 p.m. (Pavlova et al., 2012). In addition to seizure onset, few seizures seem to propagate more readily during sleep, based on anatomical locus. Medial temporal lobe regions were shown more likely to manifest spike production or propagation during NREM sleep stage compared to other brain regions (Lambert et al., 2018). Sleep-related epilepsy is often misdiagnosed as sleep disorders (Tinuper and Bisulli, 2017), especially in cases where the seizures manifest exclusively during sleep. Over the past decade, the discovery of numerous pre-disposing genes and availability of advanced diagnostic tools have shed more light in understanding the nature of sleep-related epilepsy.

In the present review, we discuss sleep-related epilepsy with particular emphasis on three of the most frequently implicated epilepsies during the sleep state which include sleep related hypermotor epilepsy (SHE), benign partial epilepsy with centrotemporal spikes (BECTS), and Panayiotopoulos Syndrome (PS).

Abbreviations: ILAE, International League Against Epilepsy; SHE, sleep related hypermotor epilepsy; BECTS, benign partial epilepsy with centrotemporal spikes; PS, Panayiotopoulos Syndrome; REM, rapid eye movement; NREM, non-rapid eye movement; Ca^{2+}, calcium; K^{+}, potassium; NFLE, Nocturnal Frontal Lobe Epilepsy; nAChRs, nicotinic acetylcholine receptors; CHRNA2, cholinergic receptor nicotinic alpha 2 subunit; CHRNA4, cholinergic receptor nicotinic alpha 4 subunit; CHRNB2, cholinergic receptor nicotinic beta 2 subunit; GABA, gamma-Aminobutyric acid; Ach, acetylcholine; PRIMA1, proline rich membrane anchor 1; Cl−, chloride; NKCC1, Na+/K+/Cl− co-transporter-1; KCC2, K+/Cl− co-transporter-2; PFC, prefrontal cortex; KCNT1, potassium-sodium activated channel subfamily T member 1; KCNT2, potassium-sodium activated channel subfamily T member 1; DEPDC5, Disheveled, Eg-10 and Pleckstrin Domain containing proteins; GATOR1, Gap Activity Toward Rags 1; mTOR, mammalian target of Rapamycin; mTORC1, mTOR Complex 1; NPRL2, nitrogen permease regulator-like 2; NPRL3, nitrogen permease regulator-like 3; RE, rolandic epilepsy; PRRT2, proline-rich transmembrane protein gene; ELP4, elongator acetyltransferase complex subunit 4; SRPX2, sushi-repeat containing protein X-linked 2; ARE, atypical RE; NMDARs, N-methyl-D-aspartate receptors; GRIN2A, α2 subunit of N-methyl-D-aspartate receptors; GABRG2, gamma-aminobutyric acid receptor subunit gamma-2; RBFOX1, RNA Binding Fox-1 Homolog 1; RBFOX3, RNA Binding Fox-1 Homolog 3; SCN1A, sodium voltage-gated channel alpha subunit 1; RTN4R, reticulon four receptor, NOGO RECEPTOR; SNAP29, synaptosomal-associated protein 29; DGCR8, microprocessor complex subunit 8; ATG16L1, Autophagy 16-like 1; SAG, S-antigen; retina and pineal gland; DGKD, diacylglycerol kinase delta; STM, sulthiamine; CBZ, carbamazepine; OXZ, oxcarbazepine; VPA, valproic acid; LEV, levetiracetam; PPARα, peroxisome proliferator-activated receptor alpha.

EXPRESSION OF SEIZURES IN NREM VS. REM SLEEP STAGES

In comparison with rapid eye movement (REM) sleep, the expression of focal seizure was 87 times more common in N1, 68 times more likely in N2, and 51 times more likely in N3. For generalized seizures, the seizure rate was 3.1 times higher in N1, 3.13 times higher in N2 and 6.59 times higher in N3 compared to the REM stage. Sleep-related epilepsies such as Benign Epilepsy of Childhood with rolandic spikes were common during the non-rapid eye movement (NREM) stages, especially during N3 (Ng and Pavlova, 2013), SHE was expressed more readily during N1/N2 (Nobili et al., 2014; Yeh and Schenck, 2014), and PS during N1 (Demirbilek and Dervent, 2004). Taken together, the existing literature suggests that sleep-related seizures are more likely to occur during the NREM stages of sleep.

NREM sleep is known as the state of neuronal synchronization, whereas REM as the most desynchronized sleep state. EEG findings suggest synchronization changes are more likely to take place during the transitions between the sleep states, rather than during the particular sleep states (Baghbani et al., 2018). In general, two types of synchronization

exist; long-range (involves numerous brain regions) and local synchronization (involves adjacent neurons). During seizures, the long-range synchronization is impaired and local synchronization is enhanced as a result of the altered extracellular content of calcium (Ca^{2+}) and potassium (K^{+}) ions. In the pathogenesis of paroxysmal discharge, various predisposing factors (familial vs. sporadic) could alter the electrophysiological properties of numerous receptors, which may potentially decrease the extracellular level of Ca^{2+} and increase the extracellular content of K^{+}, simultaneously (Amzica et al., 2002). Such changes inhibit synaptic transmission and propagation of action potential (Seigneur and Timofeev, 2011), which subsequently impair long-range synchronization and promote electrical coupling between cortical interneurons (Galarreta and Hestrin, 2001) and glial cells (Giaume and McCarthy, 1996). Long-range synchronization is also impaired during the slow-wave sleep (N3) (Ng and Pavlova, 2013). A preponderance of cortical slow oscillations takes place at this stage that results in a significant drop in the extracellular content of Ca^{2+}, leading to high rates of synaptic failures (Steriade et al., 1993; Crochet et al., 2005). For instance, during the N3 stage, the mesenphalic reticular formation cholinergic neurons that allow transmission of impulses from the thalamus to the cortex are least active (less active during NREM stages) (Ng and Pavlova, 2013). As hypothesized by Timofeev et al. (2012) the significant drop in extracellular levels of Ca^{2+} during slow-wave sleep can promote the opening of hemichannels (Thimm et al., 2005) and electrical coupling between neighboring neurons (local synchrony) (Timofeev et al., 2012). Taken together, neuronal synchronization (local) along with pre-existing pro-epileptic conditions (such as channellopathies) seem to reinforce the predominance of seizure expressions during sleep state.

The seizure expressions during NREM stages are simplified in **Table 1**.

SLEEP RELATED HYPERMOTOR EPILEPSY

SHE, or previously known as the Nocturnal Frontal Lobe Epilepsy (NFLE) is a type of sleep-related epilepsy with frontal

TABLE 1 | Table showing the expression of seizures during NREM sleep.

NREM sleep stage	Focal seizure[a]	Generalized seizure[a]	Sleep related epilepsy[a]
N1	87	3.1	SHE, PS
N2	68	3.13	SHE
N3	51	6.59	BECTS

[a] *number of times focal seizure taking place during NREM compared to REM stage.*
[a] *number of times generalized seizure taking place during NREM compared to REM stage.*
[a] *predominance of specific sleep related epilepsy during the NREM stages.*

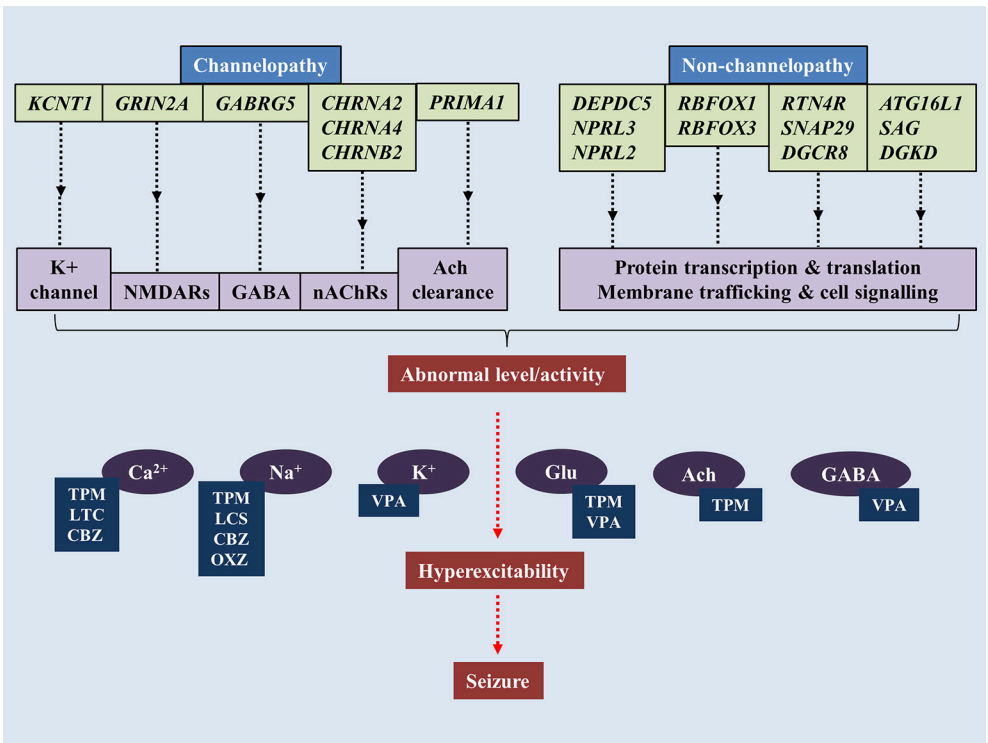

FIGURE 1 | Pathogenesis of sleep-related epilepsy. Mutations of genes associated with channelopathy and non-channelopathy origin of sleep related epilepsy could disrupt the balance between inhibitory and excitatory neurotransmissions in central nervous system, leading to manifestation of seizure. Various anti-epileptic drugs alleviate seizure by restoring chemical balance in brain. TPM, topiramate; VPA, valproic acid; CBZ, carbamazepine; OXC, oxcarbazepine; LTC, levetiracetam; LCS, lacosamide.

and extrafrontal regions as seizure onset zones that characterized by peculiar motor aspects of seizures (Tinuper et al., 2016; Vignatelli et al., 2017). The hallmark feature of this rare partial epilepsy is motor seizures that manifest almost exclusively during the NREM stage of sleep. The familial form of this epilepsy, also widely known as the autosomal dominant NFLE manifests between the age 8 and 12 years (Picard and Scheffer, 2012). SHE is the first of its kind to be associated with a causative agent, which are mutations of 3 subunit genes of nicotinic acetylcholine receptors (nAChRs) (Steinlein et al., 1995; Marini and Guerrini, 2007); reported in approximately 10% of the affected families (Heron et al., 2007).

The notion that SHE is a channelopathy is derived from early findings on mutations of nAChRs genes such as cholinergic receptor nicotinic alpha 2 subunit (*CHRNA2),* cholinergic receptor nicotinic alpha 4 subunit (*CHRNA4)* and cholinergic receptor nicotinic beta 2 subunit *(CHRNB2)* encoding for nAChRs containing subunits of α4β2 or heteromers with subunits of α2/β2/β4 (**Figure 1**) (Di Resta et al., 2010; Wallace and Bertrand, 2013; Becchetti et al., 2015). The anatomical projections of cholinergic neurons from pons and basal forebrain toward thalamus and cortex have been implicated in the regulation of sleep-wake cycle (Saper et al., 2010). Existing literature suggests increased acetylcholine release during wakefulness and REM sleep, whereas marked decrease during NREM sleep (Jones, 2008). Findings from mutant murine models of SHE (expressing B2-V287L) showed altered sleep pattern and development of spontaneous seizures during the slow wave sleep state (O'Neill et al., 2013). Molecular results show that mutated nAChRs could be hyperfunctional, and thus maintaining abnormal gamma-Aminobutyric acid-(GABA)ergic and glutamatergic neurotransmission, even with very minimal acetylcholine (Ach) available to bind to (Aracri et al., 2010). Hyperfunction of nAChRs also could be due to longer duration of Ach remaining in the synapse. In a recent study, a novel autosomal recessive phenotype of SHE was identified in a two-generation Australian family of Italian origins. Whole genome sequencing revealed mutations of proline rich membrane anchor 1 (*PRIMA1)* on chromosome 14 that encodes for PRIMA1 transmembrane protein, which anchors acetylcholinesterase at synapses for hydrolysis of acetylcholine. The authors went on to point out that perturbations in the cholinergic responses attributed to dysfunctional acetylcholinesterase could alter central and peripheral process of seizure expressions and likely to transform to SHE (Hildebrand et al., 2015). More recently, the occurrence of sleep-related epilepsy in SHE has been directly related to dysregulation of GABAergic neurotransmission. The reversal potential of $GABA_A$-mediated inhibitory post-synaptic potential requires the movement of chloride ions (Cl^-) in and out of the cells, which are mediated by co-transporters such as $Na^+/K^+/Cl^-$ co-transporter-1 (NKCC1) and K^+/Cl^- co-transporter-2 (KCC2) (Kaila et al., 2014). In a murine model of SHE (expressing B2-V287L), delayed surface expression of KCC2 was reported in layer V of prefrontal cortex (PFC), which is the most susceptible part of PFC to epileptiform activities. The delay was noticed during the first postnatal weeks, which led the authors to suggest that PFC is more prone to neuronal network-related pathologies such as epilepsy (Amadeo et al., 2018). Neural network in layer V of PFC is predominantly regulated by nAChRs expressing β2 subunit (Poorthuis et al., 2012). Despite mutation at B2-V287L, the cell surface expression of nAChRs remained the same (Manfredi et al., 2009). Thus, functional changes were thought to be attributable to expression of B2-V287L (Amadeo et al., 2018). Altered Ca^{2+} signals following hyperactivity of nAChRs could upregulate the surface expression of KCC2 as a compensatory mechanism to counterbalance the overactive neuronal network by increasing the Cl^- turnover. Collectively, these lead to retardation of GABAergic switch in PFC and ultimately hyperexcitability (Amadeo et al., 2018).

In addition to nAChRs, mutations in *KCNT1* (gene encoding for potassium-sodium activated channel subfamily T member 1) were also linked to a rather severe form of SHE and sporadic SHE. The patients found with these mutations are also presented with various psychiatric features and intellectual disabilities, which was dissimilar to the usual form of SHE. The onset age for *KCNT1* mutation-related SHE was below the onset age of classical SHE and the penetrance of KCNT1 mutations are 100% (Heron et al., 2012) which is higher than classical SHE. *KCNT1* encodes for KCNT1 channel subunit which binds with potassium-sodium activated subfamily T member 2 (KCNT2) to form the heterotetrameric complex of the channel. The *KCNT1* mutations mostly affect the nicotinamide adenine dinucleotide interaction with C-terminal of the channel, which could disrupt the modulation of the channels' functions (Tamsett et al., 2009).

More recent findings have focused on the non-channelopathy-based pathogenesis of the SHE (Dibbens et al., 2013; Picard et al., 2014; Korenke et al., 2016). *DEPDC5* (Disheveled, Eg-10 and Pleckstrin Domain containing proteins) is a gene that encodes a protein structurally-related to Gap Activity Toward Rags 1 (GATOR1), which is an important negative modulator of mammalian target of Rapamycin (mTOR) Complex 1 (mTORC1) that regulates various cell functions (Bar-Peled et al., 2013). *DEPDC5* mutations have been implicated in familial temporal lobe epilepsy, SHE and familial focal epilepsy with variable loci (Dibbens et al., 2013; Ishida et al., 2013; Martin et al., 2014). Other genes expressing GATOR1 such as nitrogen permease regulator-like 2 (*NPRL2)* and nitrogen permease regulator-like 3 (*NPRL3)* were also linked to sporadic and familial form of epilepsy (Ricos et al., 2016). It was thought that mal-interaction between mTORC1 and GATOR1 may alter cortical neuroarchitecture as epileptic patients with *NPRL3* mutations were presented with dysplastic brain lesions (Sim et al., 2016). Nevertheless, few patients despite experiencing seizures showed no anomalies in brain imaging, suggesting the structural changes could be microscopic or other unknown pathway could mediate the pathogenesis (Korenke et al., 2016). From a functional perspective, reduced negative modulation of mTORC1 leads to overactivity of the protein complex, which has been demonstrated in epileptic brains (Sha et al., 2012; Sosunov et al., 2012). In addition, inhibition of mTORC1 has been shown to block epileptogenesis (Huang et al., 2010). Thus, it was hypothesized that hyperactivated mTORC1 signaling leads to rhythmic increase in neuronal excitability (Cho, 2012).

BENIGN CHILDHOOD EPILEPSY WITH CENTROTEMPORAL SPIKES

BECTS, also known as Rolandic Epilepsy (RE) is the most common type of epilepsy syndrome in children. The typical onset age of BECTS is between 3 and 13 years, with spontaneous remission of seizures upon puberty (Berg et al., 2010). The hallmark feature of BECTS' EEG is high voltage spike and wave, mainly centrotemporal spikes. The seizures predominantly occur during NREM sleep and last for 1–3 min (Stephani, 2000).

Although initially described as idiopathic (Panayiotopoulos et al., 2008), several studies postulated a hereditary link to the disease (Vears et al., 2012; Shi et al., 2018). Numerous potential genes predisposing to BECTS were investigated to no avail (Neubauer et al., 1998; Strug et al., 2009; Pal et al., 2010). Proline-rich transmembrane protein gene (PRRT2) that was associated with paroxysmal kinesigenic dyskinesias (Chen W. J. et al., 2011) and RE (Dimassi et al., 2014), were screened in 9 cohorts of 53 sporadic patients and 250 controls in a mainland Chinese population. Genomic sequencing revealed no association between PRRT2 mutations and BECTS (Che et al., 2017). In a separate study, genes associated with epilepsy-aphasia spectrum, that encode for elongator acetyltransferase complex subunit 4 (ELP4) and sushi-repeat containing protein X-linked 2 (SRPX2) (Roll et al., 2006; Strug et al., 2009), were studied for their potential interactions in BECTS. The investigators utilized whole genome sequencing on 290 patients with European ancestry diagnosed with RE or atypical RE (ARE) in Germany, Canada and Austria and no pathological link between RE/ARE and ELP4 and SRPX2 genes were found (Reinthaler et al., 2014). The role of ELP4 in pathogenesis of BECTS was also downplayed by another study conducted in a Greek population (Gkampeta et al., 2014). The genetic risk factor in BECTS was identified in the gene that encodes for α2 subunit of N-methyl-D-aspartate receptors (NMDARs) (*GRIN2A)*. A mutational analysis carried out in 2 independent cohorts of 359 patients identified heterozygous mutations in 27 of 359 subjects and described exon-disrupting microdeletions in 3 of 286 individuals (Lemke et al., 2013). More recently, an unbiased gene-burden analysis of 194 patients against 567 in-house and 33370 online ExAC controls showed only GRIN2A rare CSDD15, CADD15 + LOF, and LOF variants were more frequent in BECTS (Bobbili et al., 2018). NMDARs are glutamate-bound excitatory receptors with important roles in synaptic transmission and plasticity (Paoletti, 2011). Numerous animal models have implicated altered NMDARs functions in development of epilepsy (Frasca et al., 2011; Di Maio et al., 2012). Thus, mutations in *GRIN2A* are thought to affect the electrophysiological property of GluN2A subunit containing NMDARs (Lemke et al., 2013). In addition to *GRIN2A*, genes *DEPDC5* (Lal et al., 2014), gamma-aminobutyric acid receptor subunit gamma-2 (*GABRG2)* (Reinthaler et al., 2015), RNA Binding Fox-1 Homolog 1 (*RBFOX1)*, RNA Binding Fox-1 Homolog 3 (*RBFOX3)* (Lal et al., 2013) and *KCNT1* (Shi et al., 2018) were also implicated in BECTS.

Persistent neuropsychiatric deficits in executive functions, intelligence and attention have been reported in BECTS patients despite spontaneous remission during the adolescence (Currie et al., 2018; Ofer et al., 2018). Myriad of neuroimaging studies associated the cognitive decline with abnormal cortical changes (Overvliet et al., 2013; Pardoe et al., 2013). Generally, cortical gray matter decreases over time from childhood to young adulthood (Shaw et al., 2006). However, in the BECTS population, the changes in cortical thickness were greater compared to the control group and there was also delay in reaching the normative values. This may explain the persistence of language problems in BECTS patients even after the remission (Pardoe et al., 2013). On the contrary, a more recent study precluded any direct relationship between centrotemporal spikes frequency and morphological changes in cortex of BECTS patients. However, one particular region, R pars opercularis showed thinner cortex in BECTS children relating the atypical cortical features with poor processing speed (Fujiwara et al., 2018). Several studies have shown BECTS to predominantly affect the left hemisphere of the brain, linking BECTS to language dysfunction seen in the children (Overvliet et al., 2013). This was further corroborated by the discovery of widespread white matter abnormalities confining to the left hemisphere of BECTS children with intellectual disabilities, particularly verbal IQ (Kim et al., 2014). In addition to intellectual disabilities, left hemisphere anomaly was also correlated with greater social fear among BECTS patients (Potegal et al., 2018). More recently, the network re-organization leading to cognitive dysfunctions in BECTS was thought to originate from right homologous brain areas. In treatment naïve, early-onset BECTS patients, escalated brain activity was seen in the right Broca's area during the early stage of the disease, suggesting the compensatory change to take place in the right hemisphere of the brain (Chen et al., 2018). Subcortical structures such as the basal ganglia, sensorimotor networks, and striato-cortical circuitry were also shown to have epilepsy-related functional connectivity with BECTS (Li et al., 2017).

PANAYIOTOPOULOS SYNDROME

PS is a common idiopathic childhood epilepsy syndrome with predominant features of autonomic signs. The majority of the children were found to be in the age group of 1–14 years, with 4–5 years being more predominantly affected by PS (Caraballo et al., 2000). The most common symptoms exhibited by PS patients are full emetic triad (nausea, retching, vomiting), pallor, deviation in head and eyes, generalized seizures, ictal syncope (Yalçin and Toydemir, 2017), changes in thermoregulatory (Panayiotopoulos, 2005) and cardiorespiratory functions (Yamamoto et al., 2018). A very recent investigation on the incidence of PS recorded over 16 months in a population aged below 16 years old, reported 0.8/100,000 new cases. Similar study also recorded 6.1/100,000 new cases of BECTS within the same population, reporting 13 times higher prevalence of BECTS than PS (Weir et al., 2018).

The transient focal EEG abnormalities that are usually seen in the epilepsy, or also known as the "functional spikes" were initially thought to be confined to the occipital lobe in PS patients (Tsai et al., 2001). However, follow-up studies documented the functional spikes to shift to extra-occipital regions such as

prefrontal and frontal (Kokkinos et al., 2010; Yoshinaga et al., 2010), parietal and lateral temporal lobe (Leal et al., 2008). This multifocal hyperexcitability nature of PS has led the researchers to correlate the affected cortical areas to the hypothalamic autonomic centers and the limbic system to the manifestation of transient hyperactive central autonomic network, which is the cardinal feature of PS (Ten Donkelaar and Horim, 2011). Generally, seizures in PS patients are sleep-related. Some studies have reported nearly all PS-associated seizures to take place during sleep (Caraballo et al., 2007), whereas some reported the 69.9% seizures to occur during sleep and 12.9% during awake (Specchio et al., 2010). More recent findings suggest the seziures are more likely to take place during awakenings (66.7%) (Yalçin and Toydemir, 2017). In sum, the constellation of findings suggests the PS-associated seizures to take place either during early awakening or sleeping hours.

The etiology of PS has been the subject of intense research, yet much of its pathophysiology has remained elusive. In 2007, a 12-year-old girl who presented with PS, was reported to carry a sporadic missense mutation in sodium voltage-gated channel alpha subunit 1 (*SCN1A)*, the gene encodes a voltage-gated sodium channel that was implicated in the pathology of Dravet Syndrome (Grosso et al., 2007). Two years later, another study reported two siblings with PS to have the *SCN1A* mutation. However, the father of the children also shared the *SCN1A* mutation, and surprisingly never experienced a seizure. This suggests that *SCN1A* mutation may merely increase the susceptibility to an idiopathic focal epilepsy phenotype (Livingston et al., 2009). In another study, 2 monozygotic twins presented with PS were found to have no mutations in the *SCN1A* gene or the *GABRG2* gene (another gene associated with Dravet Syndrome). Based on the early onset of PS in these patients and the severity of their symptoms, the authors concluded that mutations in the *SCN1A* gene may regulate the severity of the syndrome rather than the genesis of the disorder (Martín Del Valle et al., 2011). In a very recent study, a 6-year old girl diagnosed with PS was presented with a de novo 2.6 Mb deletion in 22q11.2 and an additional 172 kb duplication in 2q37.1 (Bertini et al., 2017). Deletion in 22q11.2 was associated with loss of genes involved in brain function and development, such as *RTN4R* (reticulon four receptor, NOGO RECEPTOR) (Pan et al., 2005; Ramasamy et al., 2014), *SNAP29* (synaptosomal-associated protein 29) and gene responsible for biogenesis of micro-mRNA, especially in mammalian brain such as *DGCR8* (microprocessor complex subunit 8) (Cheng et al., 2014). In addition to this, the 172 kb duplication in 2q37.1 was related to three genes, which include *ATG16L1* (Autophagy 16-like 1), *SAG* (S-antigen; retina and pineal gland), and *DGKD* (diacylglycerol kinase delta). Among these genes, *DGKD* coding for a cytoplasmic enzyme that phosphorylates diacylglycerol to produce phosphatidic acid has been implicated in epilepsy (Leach et al., 2007).

Similar to BECTS, children suffering from PS also demonstrate cognitive deficits, particularly in global visual-motor integration, writing, reading, arithmetic skills, verbal and visual-spatial memory (Germanò et al., 2005). The cognitive abnormalities reported in some of the PS patients was a result of the propagation of the interictal activity to various brain regions, including the frontal (Germanò et al., 2005) and parietal lobes (Lopes et al., 2014). In concordance with this finding, a more recent study reported changes in volume of prefrontal lobe and prefrontal-to-frontal lobe volume ratio in 3 PS patients who presented with status epilepticus. Conversely, the non- status epilepticus PS patients possess cortical growth pattern similar to that of healthy controls, suggesting the manifestation of SE in PS may impair the cognitive behavior of some PS patients (Kanemura et al., 2015).

PHARMACOTHERAPY FOR BECTS

The spontaneous remission of BECTS in adolescence has cast controversy over its treatment; in particular, as to whether or not to use anti-epileptic drugs (AEDs). A review of 110 recommendations from 96 published materials on BECTS revealed two-third of the findings to favor and one-thirds not to favor the use of AEDs. Most of those in favor with AEDs use, advocate for pharmacotherapy only in cases with early onset and multiple seizure expressions and also to limit the treatment to 1 year (Hughes, 2010). The need for AEDs was evident in some cases owing to the severity of the seizures, cognitive impairments and behavioral abnormalities that accompany the seizures in a large population of young children with BECTS (Kavros et al., 2008; Sarco et al., 2011; Samaitiene et al., 2012). In addition, there seem to be geographic differences in pharmacological management of BECTS. Sulthiamine (STM) was the most commonly prescribed AED in Austria and Germany (Gross-Selbeck, 1995). In the United States, the preferred AED for BECTS was carbamazepine (CBZ)/oxcarbazepine (OXZ) (Arzimanoglou and Wheless, 2007). European epileptologists prefer valproic acid (VPA) as the drug of choice for BECTS (Wheless et al., 2007).

There is a considerable amount of literature on VPA use in reducing electroclinical abnormalities (Gelisse et al., 1999; Xiao et al., 2014), increasing the threshold of motor evoked potentials (Nezu et al., 1997) and controlling epileptic negative myoclonus induced by other AEDs (Yang et al., 2008). In 2013, the ILAE recommended monotherapy with VPA for BECTS (level C evidence) (Glauser et al., 2013). Prolonged exposure to VPA was associated with weight gain in 40% of children (Corman et al., 1997). CBZ was shown to successfully treat BECTS populations in Japan (Oka et al., 2004), China (Ma and Chan, 2003), Greek (Gkampeta et al., 2015), and the United States (Wheless et al., 2005). Apart from reducing seizure incidences, CBZ also improved cognitive functions in BECTS patients by altering the epilepsy-induced changes in P300 event-related potential (Naganuma et al., 1994). One of the most undesirable side effects of CBZ was the drug-induced non-epileptic myoclonus and tic-like movements (Magaudda and Di Rosa, 2012). OXZ, a chemical twin of CBZ, was reported to normalize EEG, improved cognition and effectively controlled seizure expressions (Tzitiridou et al., 2005). It has a mild adverse effect profile such as headache and sedation (Coppola et al., 2007).

STM, a carbonic anhydrase inhibitor reduced spike and seizure frequencies (Wirrell et al., 2008), normalized EEG

TABLE 2 | Table showing pharmacotherapy options for BECTS.

Authors	Study[a]	Dose	Effects	Adverse effects	Adjunctive/ Monotherapy
VALPROIC ACID					
Gelisse et al., 1999	Case study	750 mg/day for 15 months	Reduce electrochemical abnormalities and seizures	None reported	Monotherapy
Xiao et al., 2014	Retrospective, uncontrolled, case-comparison cohort study	(9.3–27 mg/kg/day) followed at 6, 12, and 18 months	57.6% were seizure free at 6 months, 73.9% at 12 months, 100% at 18 months; 73.8% showed EEG normalization at 12 months, 95.7% at 18 months	Mild drowsiness (17.4%), mild weight gain (4.3%)	Monotherapy
CARBAMAZEPINE					
Ma and Chan, 2003	Retrospective observational study	(5–22.5) mg/kg for 3.35 years (mean)	Reduced seizure frequency	None Specific to CBZ was reported	Monotherapy
Kang et al., 2007	Multicenter, randomized, open-label, observer-blinded, parallel-group clinical trial	Started at 10 mg/kg/day and titrated to 20 mg/kg/day for over 22 weeks	Reduced seizure in 70% of patients, improved cognitive functions	Rashes, Weight gain (8.6% from initial weight)	Monotherapy
TOPIRAMATE					
Kang et al., 2007	Multicenter, randomized, open-label, observer-blinded, parallel-group clinical trial	Started at 12.5 mg per day and titrated to at least 50 mg per day in patients <30 kg and 75 mg or 100 mg per day in patients >30 kg over 4 weeks.	Reduced seizure in 69.6% of patients	Memory dysfunction and somnolence	Monotherapy
Liu et al., 2016	Randomized control trial	Group A: started with 0.1–1 mg/kg/day to 2 mg/kg/day Group B: TPM given twice a day with final dose 4 mg/kg/day	Group A: overall seizure reduction efficacy was at 90.9% Group B: overall seizure reduction efficacy was at 92.5%	Group A: 8 (anorexia and nausea), 6 (headache and dizziness), 2 (weight loss), 2 (hypohidrosis / adiaphoresis) 1 (difficulty in finding words), 1 (long term fever and enuresis) Group B: 4 (light anorexia and nausea), 2 (dizziness), 1 (weight loss)	Monotherapy
OXCARBAZEPINE					
Tzitiridou et al., 2005	Open label, long term study	10 mg/kg/day (first week) increased to 20–25 mg/kg/day (second and third week), dosage was increased to 30 mg/kg/day (if the patients were unresponsive)	64% were seizure free; 21% experienced >50% improvement; 5% no improvement	None reported	Monotherapy
Coppola et al., 2007	Prospective, open label, pilot trial	5 mg/kg, followed by a 3-day titration at increments of 5 mg/kg, up to a maximum daily dose of 20 mg/kg	72.2% were seizure free	1 (headache), 1 (sedation) had to be withdrawn due to excessive sedation	Monotherapy
LEVETIRACETAM					
Coppola et al., 2007	Prospective, open label, pilot trial	5 mg/kg, followed by a 3-day titration at increments of 5 mg/kg, up to a maximum daily dose of 20 mg/kg	90.5% were seizure free	2 (decreased appetite), 1 (decreased appetite combined with daily frontal cephalalgia)	Monotherapy
Verrotti et al., 2007	Prospective, multicenter trial	Started at 250 mg/daily and titrated to 1,000–2,000 mg/daily. Followed for 12 months	42.8% patients were seizure free (started with levetiracetam); 30.1% patients were seizure free (patients unresponsive to other drugs, then followed up with levetiracetam)	Drowsiness and irritability in 9.5% of the patients	Monotherapy

(Continued)

TABLE 2 | Continued

Authors	Study[a]	Dose	Effects	Adverse effects	Adjunctive/ Monotherapy
Borggraefe et al., 2013	Randomized, double-blinded, controlled trial	Started at 10 mg/kg bodyweight and was further increased weekly by increments of 10 mg/kg weekly to a final dosage of 30 mg/kg bodyweight	81% of the patients were seizure free for 6 months; seizure recurrence in 19% patients	23.8% of the patients were dropped out due to suicidal ideation, headache, sleep disturbance, nausea, abdominal pain	Monotherapy
SULTHIAME					
Borggraefe et al., 2013	Randomized, double-blinded, controlled trial	Started at 2 mg/kg bodyweight and was further increased weekly by increments of 2 mg/kg bodyweight weekly to a final dosage of 6 mg/kg bodyweight	91% of the patients were seizure free for 6 months; seizure recurrence in 9.1% patients	4.1% (1) of the patients were dropped out due to adverse events related to airways.	Monotherapy

CBZ carbamazepine.
[a]Study type or design.

TABLE 3 | Table showing pharmacotherapy options for Panayiotopoulos Syndrome.

Authors	Study	Dose	Effects	Adverse effects	Adjunctive/ Monotherapy
LEVETIRACETAM					
García and Rubio, 2009	Case studies (3)	(1) Started with LEV (250 mg twice a day), increased to 500 mg every 12 h after 2 weeks, dose was increased to 1,000 mg (3 months after an attack) (2) Started with 250 mg twice a day of LEV and increased up to 1,000 mg for 2 weeks. (3) Started with LEV 250 mg, and increased up to 1,000 mg in 2 weeks. After two brief episodes, LEV 1,000 mg was continued twice/day for a year.	(1 and 2) The patient has been seizure free for 3 years. (3) The patient has not experienced any seizure from 13 to 16 years old.	None was reported	LEV was given as adjunctive with VPA. VPA was discontinued after 6 months' treatment with LEV [case 1 and 2] Monotherapy [case 3]
VALPROIC ACID					
Martín Del Valle et al., 2011	Case study	Treatment was started with valproic acid and followed for 2 years (dose not mentioned in the study)	The patient was seizure free for 2 years; Partial improvement in the patient's EEG was reported	None was reported	Monotherapy

LEV, levetiracetam; EEG, electroencephalogram.
[a]Study type or design.

(Bast et al., 2003) with low seizure remission rate (91% success for 24 weeks of observation) (Borggraefe et al., 2013). Adverse effects such as impaired cognitive functions (Wirrell et al., 2008) and metabolic acidosis (Borggraefe et al., 2013) were associated with STM. On the other hand, LEV was shown to control seizures without (Bello-Espinosa and Roberts, 2003) and with minimum side effects (Verrotti et al., 2007). Few researchers reported that LEV improves BECTS-related impairments in auditory verbal memory and baseline auditory comprehension (Kossoff et al., 2007). The success rate of LEV on seizure remission was 81% as reported by Borggraefe et al. (2013). Adverse effects such as suicidal ideations (Borggraefe et al., 2013) and psychosis (Kossoff et al., 2001) were related to LEV use. Topiramate, a novel AED was shown to reduce epileptiform frequency and inhibit epileptiform discharges in BECTS patients. Its side effect includes anorexia, nausea, headache, and hypohidrosis / adiaphoresis (Liu et al., 2016).

TABLE 4 | Table showing pharmacotherapy options for sleep related hypermotor epilepsy.

Authors	Study[a]	Dose	Effects	Adverse effects	Adjunctive/ Monotherapy
POLYTHERAPY					
Yeh and Schenck, 2017	Case studies (10)	2 cases: Carbamazepine from 200 to 800 mg/day in monotherapy; 3 cases: polytherapy (combination of topiramate or lamotrigine). 4 cases: Oxcarbamazepine from 300 to 1,200 mg/day in polytherapy (2 with topiramate, 1 with topiramate and acetazolamide) One case: acetazolamide and clonazepam; One case: sodium valproate 300 mg bid and levetiracetam, 500 mg	75% reduction in nocturnal seizures and abolishment of occasional diurnal attack by more than 90%	None was reported	Adjunctive
LACOSAMIDE					
Samarasekera et al., 2018	Case study	Lacosamide was initiated at dosage ranging from 300 to 600 mg/day for 6–37 months.	Five patients showed more than 50% reduction in seizure expressions; One patient showed 25% response; Two patients withdrawn from lacosamide after 2 and 24 months	2 patients: Transient fatigue within the first 6 months 1 patient: was feeling "spaced out" 1 patient: experienced diplopia at 500 mg/day and symptoms resolved after the dosage reduced	Adjunctive lacosamide was given in adjunct to 6 other drugs such as carbamazepine, topiramate, Oxcarbazepine, Phenytoin, valproate and zonisamide
Liguori et al., 2016	Case study (2)	Lacosamide 200 mg/day was given along carbazepine, topiramate (case 1) and oxcarbazepine, clonazepam (case 2); After seizure reduction, lacosamide was maintained on monotherapy and followed up for 1 year	Both patients were seizure free at 12 months' follow up	None was reported	Adjunctive/ Monotherapy
OXCARBAZEPINE					
Raju et al., 2007	Case study (8)	Started oxcarbazepine at 10 mg/kg/day twice/daily and the dose was increased to 15–45 mg/kg/day	Six patients: seizure reduced within 4 days and under control in 2 weeks; Two patients: seizure under control at higher dose	Dizziness, somnolence, and diplopia in 2 patients	Adjunctive/ Monotherapy
Romigi et al., 2008	Case study	Oxcarbazepine was started at 10 mg/day/kg and increased to 20 mg/kg/day in 10 days and followed up for 4 months	Nocturnal seizures were completely disappeared	None was reported	Monotherapy
TOPIRAMATE					
Oldani et al., 2006		Topiramate was given as monotherapy in 21 patients: dosage ranging from 50 to 300 mg daily at bedtime and followed up from 6 months to 6 years. Topiramate was administered as add on to carmazepine in 3 patients.	6 patients were seizure free; 15 responders and 3 non-responders.	Weight loss (6), paresthesias (3), speech dysfunction in phonematic verbal fluency (2). All adverse events disappeared within 3 months	Adjunctive/ Monotherapy

[a] *Study type or design.*

TABLE 5 | Table showing the mechanism of actions for anti-epileptic drugs.

Anti-epileptic Drug	Mechanism of action(s)
Sulthiamine	Acidification of brain tissue via inhibition of carbonic anhydrase (Tanimukai et al., 1964) Reduction in intracellular pH level, affects the opening of ion channels such as Na^+/H^+ exchange and Cl^-/HCO_3^- exchanger (Bonnet and Wiemann, 1999; Bonnet et al., 2000)
Topiramate	Affects voltage gated sodium conductance (Zona et al., 1997) Block AMPA receptor activity (glutamate) (Coulter et al., 1993) Inhibits the release of glutamate (Hanaya et al., 1998) Anti-cholinergic effects (Avoli et al., 1998) Affects high-voltage N and L-type Ca^{2+} currents (Zhang et al., 1998)
Valproic acid	Stimulates glutamine synthetase (Phelan et al., 1985) Reduces the activity of phosphatidylinositol (3,4,5)-trisphosphate (Chang et al., 2014) Prevents hyperexcitability by acting on Kv7.2 channel and A-kinase anchor protein 5 (Kay et al., 2015) Blockade of NMDAR mediated current (Gean et al., 1994) Increases the brain level of GABA (Bertelsen et al., 2018)
Carbamazepine	Inhibits firing of cortical neurons by blocking the voltage gated sodium channel (Panayiotopoulos, 2005) Inhibits L-type calcium channels (Panayiotopoulos, 2005)
Oxcarbamazepine	Blocks voltage sensitive sodium channels (Panayiotopoulos, 2005)
Levetiracetam	Modulates synaptic vesicle glycoprotein 2A (Lynch et al., 2004) Inhibits presynaptic calcium channels (Vogl et al., 2012)
Lacosamide	Enhances slow sodium channel inactivation (Rogawski et al., 2015)

AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; NMDAR, N-methyl-D-aspartate; GABA, gamma-Aminobutyric acid.
Kv7.2 was referring to principal molecular components of the slow voltage-gated M-channel.

PHARMACOTHERAPY FOR SLEEP RELATED HYPERMOTOR EPILEPSY

CBZ has been documented as the most commonly prescribed drug to manage SHE (Provini et al., 1999; Gambardella et al., 2000). Almost two-thirds of the SHE patients responded well to bedtime low doses (200–1,000 mg/kg) of CBZ. However, one third of the patients remained resistant to the drug (Provini et al., 2000). High blood brain barrier penetrance of CBZ (Shorvon, 2000) indicates the propensity of the AED to react with a variety of neuronal receptors. In agreement with this, CBZ was shown to reduce the action potential frequency of voltage-gated sodium channels (McLean and MacDonald, 1986; Schwarz and Grigat, 1989) which in turn could alter the neuronal excitability by impairing the glutamate release (Sitges et al., 2007) or potentiating (gamma-Aminobutyric acid) $GABA_A$ receptors (Zheng et al., 2009). More importantly, CBZ inhibits α4β2 and α2β4 subunits of nicotinic receptors (Di Resta et al., 2010) which have often been implicated in SHE. Therefore, it seems that inhibition of the nicotinic receptors by CBZ may suppress glutamate excitability and potentiate GABA activity in the thalamocortical system and hippocampus to attenuate hyperexcitability (Albuquerque et al., 2009; Aracri et al., 2010).

Many epileptologists reported better outcomes with OXZ in controlling nocturnal seizures (Raju et al., 2007; Romigi et al., 2008), even in patients unresponsive to CBZ and other AEDs (Raju et al., 2007). Similar to CBZ, OXZ block voltage-gated sodium channels (MacDonald and Rogawski, 2008), potentiate $GABA_A$ receptors (Zheng et al., 2009) and inhibits α2β4 subunits of nicotinic receptors (Di Resta et al., 2010). In addition to OXZ, Oldani and co-researchers found administration of topiramate (50–300 mg daily at bedtime) as add-on (3 patients) or monotheraphy (21 patients) to reduce nocturnal seizures in 62.5% of patients and six of the total patients were seizure free in the follow-up that ranged from 6 months to 6 years. The authors reported mild adverse events include weight loss (6 patients), paresthesias (3 patients), and speech dysfunction (2 patients) which disappeared within 3 months (Oldani et al., 2006). More recently, in a Taiwanese series of 10 case studies, CBZ, OXZ, and topiramate along with other AEDs effectively reduced nocturnal seizures by 75% and abolished diurnal attack by more than 90% without producing any adverse effects (Yeh and Schenck, 2017).

Claudio Liguori and colleagues administered lacosamide as add-on therapy in 2 SHE patients that were unresponsive to other AEDs. Addition of lacosomide (200 mg/day) to polytherapy (CBZ+TPM) and (OXZ+clonazepam) dramatically abolished the nocturnal seizure expressions and both patients were then continued on lacosomide monotherapy for 12 months and remained seizures free (Liguori et al., 2016). In a separate study, administration of LCM (300–600 mg/kg) to eight patients with refractory-SHE reduced the seizure frequency in 5 patients for more than 50 and 25% in one patient. The authors also reported mild and reversible adverse events in most of the patients, such as transient fatigue and diplopia. However, one patient was withdrawn from lacosomide for feeling continuously "spaced out" (Samarasekera et al., 2018).

PHARMACOTHERAPY FOR PANAYIOTOPOULOS SYNDROME

Most clinicians believe children with PS may not require prophylaxis therapy with AED (Panayiotopoulos, 2002, 2004). Nevertheless, 10–20% of PS patients face persistent autonomic status epilepticus that could last for days (García and Rubio, 2009) which place them at great risk of developing life threatening severe cardiorespiratory dysfunctions (Camfield and Camfield, 2005; Verrotti et al., 2005). In addition, there is a consensus among the epileptologists that AED therapy should be reserved for the patients with unusually frequent and severe form of seizures that could affect the quality of their lives (Ferrie et al., 2006). To date, there is no single monotherapy of any AED has been shown to be superior to the rest.

Garcia and Rubio have reported recurrence of seizures in some PS patients after 6 months of treatment with VPA. The same authors also showed LEV (1,000–2,000 mg/kg) that initially introduced as add-on therapy to these patients, and then as

monotherapy, successfully reduced the occurrences of seizures and all the patients remained seizure free for 2–3 years (García and Rubio, 2009). In parallel to this finding, numerous studies have documented the potential advantages of LEV in pediatric epileptic cases owing to the AED's lack of interaction with other drugs, favorable elimination kinetics and significant protein binding ability (Leppik, 2001). In addition, LEV also lacks adverse effects such as weight gain, polycystic ovarian syndrome, hair loss, and rash that have been most frequently implicated in the use of VPA, CBZ and lamotrigine (Konishi et al., 1993; Barron et al., 2000). As mentioned earlier, long term use of LEV was associated with behavior-related adverse events (Kossoff et al., 2001; Borggraefe et al., 2013).

Rectal, buccal or intravenous (IV) benzodiazepines were commonly used to manage autonomic status epilepticus manifestations in PS (Ferrie et al., 2006). However, Lacroix et al. (2011) urged the clinicians to practice great caution over the use of BDZ to treat autonomic seizures. The authors reported severe respiratory depression following the benzodiazepines administration (diazepam 0.5–0.6 mg/kg, IR; lorazepam 0.05–0.06 mg/kg, IV) for seizures with autonomic manifestations in five patients. The authors went on to suggest that the use of other BDZ such as buccal midazalom, or a more autonomic tolerant AEDs such as VPA or LE for the acute management of autonomic seizures (Lacroix et al., 2011) should be considered.

FUTURE RECOMMENDATIONS

The past decade has witnessed the birth of various AEDs. Despite their efficacy, they are not without severe adverse effects, especially in prolonged exposure to refractory epileptic patients. This calls for discovery of novel, more specific molecular-targeting pharmacotherapies. Therapeutic diets such as ketogenic diet and low-glycemic index diet were shown to be effective in treating drug-resistant epileptic patients (Pfeifer and Thiele, 2005; Neal et al., 2008). As well, adjunctive therapy with fenofibrate was shown to markedly reduce the seizure frequency in human and animal models of SHE (Puligheddu et al., 2017). Fenofibrates are agonists of peroxisome proliferator-activated receptor alpha (PPARα), which inhibits β2-containing nicotinic receptors by phosphorylating β2 (Melis et al., 2010; Puligheddu et al., 2013). In animal models of SHE, chronic diet with fenofibrates reduced the nicotine-induced spontaneous inhibitory postsynaptic current in pyramidal neurons of the frontal lobe. This subsequently attenuated the cholinergic overactivation and expressions of seizures (Puligheddu et al., 2017). Taken together, these findings warrant further investigation of the role of fenofibrate and PPARα in the pathogenesis of sleep-related epilepsy.

Identification of *GRIN2A* mutations in BECTS and other childhood epilepsies has thrown light on the role of GluN2A subunit-containing NMDARs in epilepsy (Lemke et al., 2013; Gao et al., 2017; Von Stülpnagel et al., 2017). Recent findings indicate that *GRIN2A* mutations prolong NMDARs' deactivation time, decrease the amplitude of current responses, reduce glutamate potency, reduce channel open probability and accentuate the sensitivity of NMDARs toward negative allosteric modulators (Gao et al., 2017; Sibarov et al., 2017). It is postulated that reduced NMDARs function may impair the inhibitory effects of GABAergic interneurons in the prefrontal cortex (Xi et al., 2009) and cerebral cortex (Bagasrawala et al., 2016); leading to epilepsy (Gao et al., 2017). In addition, NMDARs are also modulated by metabotropic glutamate receptor subtype 5 (mGlu5) (Chen H. H. et al., 2011). Negative modulation of mGlu5 has been promising in attenuating hyperexcitability of central nervous system (Kumar et al., 2013, 2016, 2017) and even reducing the spike-wave discharges in numerous animal models of epilepsy (McCool et al., 1998; Chapman et al., 2000). More studies are needed to further elucidate the functional link between mGlu5, GABA, and NMDARs in epilepsy. Discovery of mutations in *DEPDC5*, *NPRL2*, and *NPRL3* that encode for GATOR1 (negative modulator of mTORC1) have pioneered the channelopathy-independent approach in understanding the pathological process of NFLE. To date, mTORC1 inhibitors have only been proven successful in treating epilepsy in tuberous sclerosis (Curatolo, 2015) and polyhydramnios megalencephaly symptomatic epilepsy (Parker et al., 2013). Nevertheless, the potential functional link between mutations of mTORC1-related genes and non-lesional focal epilepsy (Myers and Scheffer, 2017) merits future studies.

We summarized the pharmacotherapy of options for BECTS, PS and SHE in **Tables 2–4**, respectively. The mechanism of actions for the AEDs is listed in **Table 5**.

AUTHOR CONTRIBUTIONS

JK, AS, PM, and SD conceived the idea. JK, AS, PM, SD, and RM wrote the manuscript. JK and SD prepared the tables. JK, PM, and SD edited the manuscript.

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The Effects of Ginsenoside Compound K Against Epilepsy by Enhancing the γ-Aminobutyric Acid Signaling Pathway

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The imbalance between the GABA-mediated inhibition and the glutamate-mediated excitation is the primary pathological mechanism of epilepsy. GABAergic and glutamatergic neurotransmission have become the most important targets for controlling epilepsy. Ginsenoside compound K (GCK) is a main metabolic production of the ginsenoside Rb1, Rb2, and Rc in the intestinal microbiota. Previous studies show that GCK promoted the release of GABA from the hippocampal neurons and enhanced the activity of $GABA_A$ receptors. GCK is shown to reduce the expression of NMDAR and to attenuate the function of the NMDA receptors in the brain. The anti-seizure effects of GCK have not been reported so far. Therefore, this study aimed to investigate the effects of GCK on epilepsy and its potential mechanism. The rat model of seizure or status epilepticus (SE) was established with either Pentylenetetrazole or Lithium chloride-pilocarpine. The Racine's scale was used to evaluate seizure activity. The levels of the amino acid neurotransmitters were detected in the pilocarpine-induced epileptic rats. The expression levels of $GABA_AR\alpha1$, NMDAR1, KCC2, and NKCC1 protein in the hippocampus were determined via western blot or immunohistochemistry after SE. We found that GCK had deceased seizure intensity and prolonged the latency of seizures. GCK increased the contents of GABA, while the contents of glutamate remained unchanged. GCK enhanced the expression of $GABA_AR\alpha1$ in the brain and exhibited a tendency to decrease the expression of NMDAR1 protein in the hippocampus. The expression of KCC2 protein was elevated by the treatment of GCK after SE, while the expression of NKCC1 protein was reversely down-regulated. These findings suggested that GCK exerted anti-epileptic effects by promoting the hippocampal GABA release and enhancing the $GABA_A$R-mediated inhibitory synaptic transmission.

Keywords: ginsenoside compound K, epilepsy, neurotransmitters, $GABA_AR\alpha1$, KCC2, NKCC1

Abbreviations: GABA, γ-aminobutyric acid; $GABA_AR\alpha1$, $GABA_A$ receptor subunit α1; GCK, ginsenoside compound K; KCC2, K-Cl cotransporter isoform 2; NKCC1, Na-K-2Cl cotransporter isoform 1; NMDAR, *N*-methyl-D-aspartate receptor; SE, status epilepticus; TLE, temporal lobe epilepsy.

INTRODUCTION

Epilepsy is a common chronic neurological disease, characterized by the presence of spontaneous unprovoked recurrent seizures (Hauser et al., 2017). It affects over 70 million people around the world and approximately 2.4 million persons are diagnosed with epilepsy each year (Yemadje et al., 2011; van Vliet et al., 2017). Epilepsy appears from a variety of complex causes, such as febrile seizures, head trauma, birth injuries, stroke, brain tumor, infections, and genetics (Singh and Trevick, 2016). Epilepsy comprises numerous seizure types and syndromes, where it easily coexists with psychiatric and neurological comorbidities (Kanner, 2016). A number of patients diagnosed with new onset epilepsy obtain symptomatic remission with the use of anti-epileptic drugs (Brodie et al., 2012). However, 20–30% of patients are ineffective in the currently available anti-epileptic drugs (Sillanpaa and Schmidt, 2006). Nearly half of patients will suffer mild, moderate, or severe adverse reactions (Schmidt and Schachter, 2014). There is an urgent need to develop a novel high efficiency and low toxic anti-epileptic drug for the treatment of intractable epilepsy.

The imbalance between excitatory and inhibitory neurotransmission is known to be one of the most important causes of seizures (Margineanu and Klitgaard, 2009; Amtul and Aziz, 2017). Glutamate is the primary excitatory neurotransmitter in the brain. Glutamate acts on its postsynaptic receptors to mediate excitatory neurotransmission, which is involved in neural development and synaptic plasticity (Guerriero et al., 2015). However, large amounts of glutamate are released from presynaptic neurons under pro-epileptogenic stimuli, including status epilepticus (SE), stroke, and traumatic brain injury (Wang et al., 2012). Excessive glutamate in the synaptic cleft induces excitotoxicity by activating the NMDAR, which promotes calcium influx that leads to neuronal death (Wang and Qin, 2010). NMDAR activation also promotes limbic epileptogenesis by enhancing the synaptic excitation (McNamara et al., 2006). MK-801, a NMDA receptor antagonist, inhibits seizure activity in the amygdala kindling model of epilepsy (Sato et al., 1988). Suppressing the glutamate-NMDAR pathway could inhibit the occurrence of epilepsy.

γ-aminobutyric acid (GABA) is the main inhibitory neurotransmitter in the mammalian central nervous system. GABA acts on the GABA-A receptor after being released from the presynaptic vesicles, which promotes the opening of the Cl^- channels and causes hyperpolarization of the postsynaptic cell (Guerriero et al., 2015). Emerging evidence shows that $GABA_AR\alpha1$ was reduced in pilocarpine-induced epileptic rats (Raol et al., 2006b). The enhanced $GABA_AR\alpha1$ expression could increase the seizure threshold and inhibit the development of recurrent spontaneous seizures after SE, suggesting that $GABA_AR\alpha1$ plays a major role in inhibitory function (Raol et al., 2006a). The $GABA_AR$ agonist diazepam is a classical anti-seizure drug that is used for treating SE (Levi et al., 2015), suggesting that the $GABA_AR$-mediated inhibitory neurotransmission is a therapeutic target for epilepsy. The $GABA_A$ receptors subunit composition affects the function of GABAergic inhibition, the intracellular Cl^- concentration is a critical determinant of postsynaptic inhibition. The expression patterns, membrane trafficking, and protein degradation of cation-chloride cotransporters control the Cl^- levels in neurons (Loscher et al., 2013). The NKCC1 promotes the Cl^- influx while the KCC2 extrudes Cl^-. The dysfunction of NKCC1 and KCC2 enhances neuronal excitability and promotes susceptibility to seizures. Restoring Cl^- homeostasis could reduce the seizure severity by the NKCC1 inhibitor bumetanide or optogenetic removal of Cl^- (Moore et al., 2017), suggesting that NKCC1 and KCC2 are the key regulators of GABAergic inhibition which are potential targets for the treatment of epilepsy.

Ginsenoside compound K (GCK), also known as M1 or IH901, is a main metabolic product from Ginsenoside Rb1, Rb2, and Rc in intestinal bacteria following oral administration of ginseng (Lee et al., 2009). GCK has attracted a wide attention because of its good bioavailability (Paek et al., 2006). Several studies demonstrated that GCK exhibited multiple pharmacological activities including anti-tumor, anti-diabetic, anti-inflammation and hepatoprotective effects (Yang et al., 2015). GCK has not been reported as a new drug that is approved to enter the market. GCK has become a candidate drug for rheumatoid arthritis therapy due to its strong anti-inflammatory effect. At present, the GCK tablet was produced by Hisun Pharmaceutical Co., Ltd., which is being tested as an anti-rheumatoid arthritis drug in China. Our previous research has shown that GCK is safe and well-tolerated for healthy Chinese volunteers, where it exhibits a good pharmacokinetic profiles at a moderate dose (Chen et al., 2018). Its pharmacokinetic properties are affected by food and gender in humans (Chen et al., 2017). These findings indicated that GCK has a promising druggability that provides guidance for the development of CK. The research regarding the role of GCK in the nervous system has gradually increased. A previous study using quantitative autoradiography found that GCK decreased the binding of $[^3H]$MK-801 with the NMDA receptor in the frontal cortex and hippocampus and enhanced the binding of $[^3H]$muscimol and the $GABA_A$ receptor in the frontal cortex and cerebellum. This suggested that GCK may suppress the activity of NMDA receptor and increase the effect of GABA receptor agonist in brain which plays an important role in neurological disorders (Jang et al., 2004). GCK could also enhance the spontaneous GABA release into the CA3 pyramidal neurons to induce inhibitory transmission (Bae et al., 2010). A study reported that GCK inhibits glutamate-induced cytotoxicity in hippocampal HT22 cells by regulating the Nrf2-mediated induction of antioxidant enzymes (Seo et al., 2016). GCK could also decrease the morphine-induced NMDAR1 activation in cultured cortical neurons (Yayeh et al., 2016). These findings suggest that GCK might reduce neuronal hyperexcitability by correcting the neuronal excitation-inhibition imbalance.

Since GCK may regulate the GABA receptor activity and the NMDA receptor expression, we hypothesized that GCK could have an anti-epileptic effect. Therefore, the purpose of this study is to investigate the effects of GCK in epilepsy and its potential mechanisms. Two classical epileptic animal models were established to evaluate the anti-seizure activity of GCK. We found that GCK exhibited a good anti-epileptic effect via

enhancing the GABA-mediated inhibition in the hippocampus, which may possess a promising future for development of a novel anti-epileptic drug.

MATERIALS AND METHODS

Reagents

Ginsenoside compound K was provided from Zhejiang Hisun Pharmaceutical Company Limited (China). Sodium valproate was obtained from Hunan Xiangzhong Pharmaceutical Company Limited (China). Pentylenetetrazole, Lithium chloride, Pilocarpine and paraformaldehyde were purchased from Sigma (United States).

Animal Allocation and Drug Administration

Adult male Sprague–Dawley rats (6–8 weeks old, 180–200 g body weight) were purchased from Hunan SJA Laboratory Animal Co. Ltd. (China). Rats were housed in clear cages, 3 per cage. The experimental room was maintained in 22–23°C with humidity of 10–55%, it was kept on 12 h light or dark cycles. All rats access to food and water throughout the experiment. All experimental protocols were approved by the Ethics Committee of Drug safety evaluation research center of Hunan province and performed in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals. All efforts were made to minimize the animal's suffering.

After a week of acclimatization, 60 rats were randomly grouped into control, model, the positive control (Sodium valproate, 400 mg/kg), the GCK low (80 mg/kg), the middle (160 mg/kg), or the high dose (320 mg/kg) with 10 rats per group. The GCK suspension was prepared with the solution of sodium carboxymethyl cellulose (0.5% CMC-Na solution) prior to administration. Rats in the experimental group were given the corresponding drug via gavage twice a day in dosing intervals every 12 h for 5 days while the other rats were treated with the same dose of physicological saline. Rats were treated with pentylenetetrazole or lithium chloride-pilocarpine to initiate an epilepsy model 1 h after the last administration of GCK or Sodium valproate.

Pentylenetetrazole-Induced Seizures

Pentylenetetrazole (PTZ) is a $GABA_A$ receptor antagonist that is commonly used to establish tonic-clonic seizures and screen anti-seizure drugs. We choose this animal model to examine the anti-seizure activity of GCK. The seizure rat model was induced by injecting 60 mg/kg of PTZ. The seizure activity was immediately evaluated within 30 min after PTZ administration according to the modified Racine scale (Sadek et al., 2016): stage 0, inactive; stage 1, ear and facial twitching; stage 2, convulsive wave through the body; stage 3, myoclonic jerks and rearing; stage 4, turn over into side position and stage 5, turn over into back position, generalized clonic-tonic seizures. The time from injecting PTZ to the first appearance of convulsive wave through the body was measured for each animal and was referred to as the seizure latency. The total duration of the behavioral seizure activity was measured for each animal.

Lithium Chloride-Pilocarpine-Induced Status Epilepticus (SE)

Pilocarpine is an M-receptor agonist, used as a convulsant to induce SE or TLE in animal models. Lithium chloride potentiates the epileptogenic action of pilocarpine and reduces mortality rates. Here we built a rat model of SE with Lithium chloride-pilocarpine. Rats were treated with intraperitoneal injection of pilocarpine (30 mg/kg, i.p.) 18–20 h after the lithium chloride (127 mg/kg, i.p.) injection. Scopolamine methyl bromide (1 mg/kg, i.p.) was administered to reduce peripheral adverse reactions. Rats were continuously observed 2 h following the injection of pilocarpine. The evoked seizures were assessed according to Racine scale (Inoue et al., 2009): 0, no abnormality; 1, mouth and facial movements; 2, head nodding; 3, forelimb clonus; 4, rearing and bilateral forelimb clonus; 5, rearing, falling and jumping. The time from the pilocarpine injection to the first appearance of the forelimb clonus was measured for each animal and was referred to as the seizure latency. Rats were euthanized by being anesthetized with 3 ml/kg of chloral hydrate. The entire brain post-SE 24 h was collected for immunohistochemical analysis. The dissociated hippocampus post-SE 3 h was used to determine the neurotransmitters. The hippocampus post-SE 24 h was obtained for western-blot analysis.

Quantitative Analysis for Glutamate and GABA

To determine glutamate and GABA in the hippocampus, the hippocampus tissue was homogenized in an ice-cold PBS buffer and centrifuged at 10,000 rpm for 10 min at 37°C. The supernatant was used in the following assays.

The concentration of glutamate in the hippocampus was tested via the ultraviolet colorimetry method according to the instructions in the Glutamic acid measurement kit (Nanjing Jiancheng Bioengineering Institute, China).

The content of GABA was measured with the ELISA Kit for Gamma-Aminobutyric Acid (Cloud-Clone Corp., United States) in accordance with the manufacturer's instructions. The sample was added into the prepared Detection Reagent A and incubated for 1 h at 37°C. The unbound conjugate was washed off, each microplate well was added to the prepared Detection Reagent B, and subsequently incubated for 30 min at 37°C. The substrate solution and the stop solution were used for color development reaction and termination. The absorbance was measured at 450 nm with a microplate reader (Beckman Coulter, United States). Each experiment was repeated in triplicate.

Western-Blot Analysis

The frozen hippocampus was homogenized in the RIPA lysis buffer prepared with Phenylmethylsulfonyl fluoride (PMSF). The mixture was centrifuged at 12,000 rpm for 15 min at 4°C and the supernatant was collected. The content of total protein was measured with a BCA protein assay kit (Beyotime

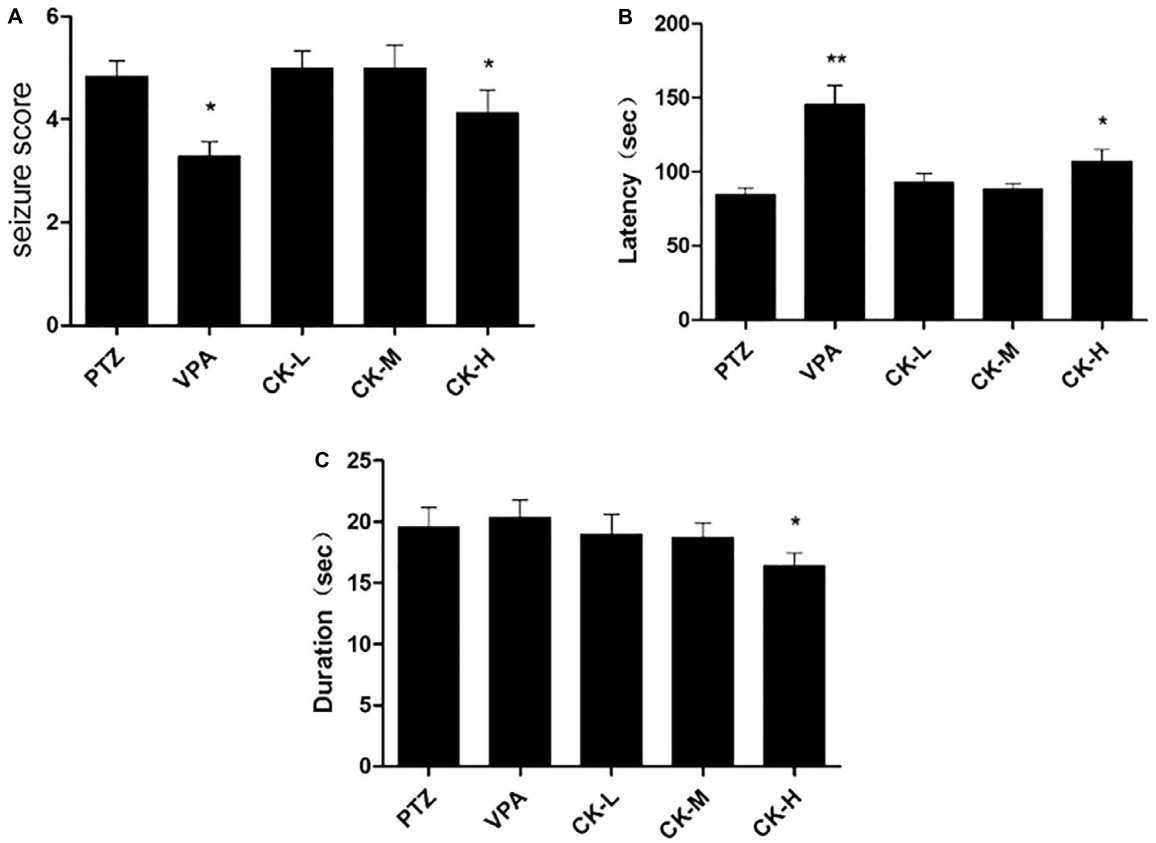

FIGURE 1 | Effects of GCK on behavioral seizures induced by pentylenetetrazole (PTZ). **(A)** The seizure scores of the PTZ, VPA, and GCK-treated groups. **(B)** The latency to clonic seizures in the PTZ, VPA, and GCK-treated groups. **(C)** The duration of the PTZ, VPA, and GCK-treated groups. Values are mean ± SEM (n = 10). PTZ 60 mg/kg, VPA 400 mg/kg, GCK 80 mg/kg, 160 mg/kg, and 320 mg/kg. Values were compared with the PTZ, $^{*}P < 0.05$, $^{**}P < 0.01$.

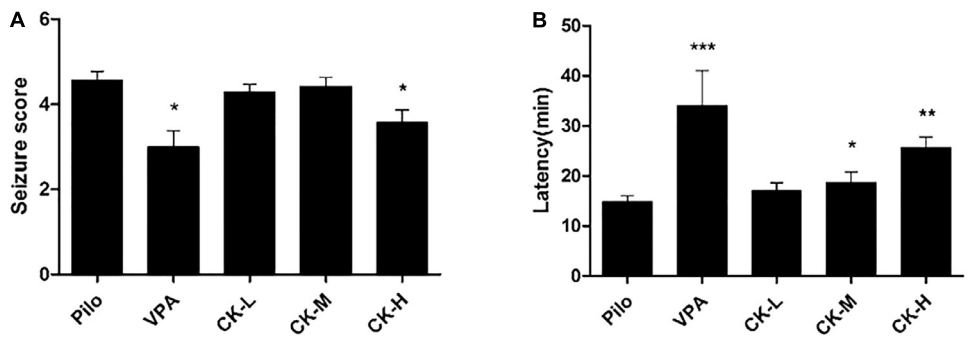

FIGURE 2 | Effects of GCK on behavioral seizures induced by lithium chloride-pilocarpine. **(A)** The seizure scores of the Pilo, VPA, and GCK-treated groups. **(B)** The latency to onset SE in the Pilo, VPA, and GCK-treated groups. Values are mean ± SEM (n = 10). Pilo 30 mg/kg, VPA 400 mg/kg, GCK 80 mg/kg, 160 mg/kg, and 320 mg/kg. Compared with the Pilo, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

Biotechnology, China). 50 μg of the protein sample was added and separated via SDS-polyacrylamide gel electrophoresis. The blots were blocked after being transferred onto the PVDF membrane. The membrane was incubated overnight at 4°C with the primary antibodies: the mouse anti-$GABA_AR\alpha1$ monoclonal antibody (1:500, Abcam, United Kingdom), the rabbit anti-NMDAR1 polyclonal antibody (1:500, Sigma, United States), the goat anti-NKCC1 polyclonal antibody (1:200, Santa Cruz, United States), goat anti-KCC2 polyclonal antibody (1:200, Santa Cruz, United States), and the rabbit anti-β-actin polyclonal antibody (1:1000, CST, United States). The blots were then incubated in the secondary antibodies: the HRP-labeled goat anti-mouse IgG, the goat anti-rabbit IgG, or the mouse anti-goat IgG (Beyotime Biotechnology, China) for 1 h at room temperature.

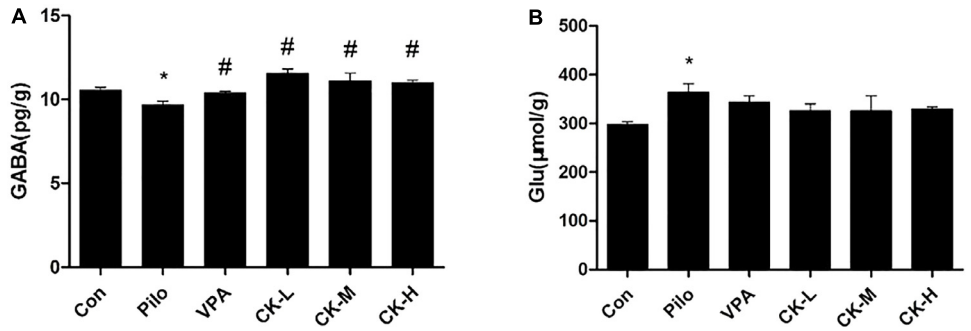

FIGURE 3 | Effects of GCK on the hippocampal neurotransmitters glutamate and γ-amino butyric acid (GABA) contents in pilocarpine-induced epileptic rats. **(A)** The contents of GABA in the Con, Pilo, VPA, and GCK-treated groups. **(B)** The contents of glutamate in the Con, Pilo, VPA, and GCK-treated groups. Values are mean ± SEM (n = 5). Pilo 30 mg/kg, VPA 400 mg/kg, GCK 80 mg/kg, 160 mg/kg, and 320 mg/kg. Compared with the Con $^{*}P < 0.05$; compared with the Pilo, $^{\#}P < 0.05$.

The bands were visualized with an ECL chemiluminescence substrate kit (Beyotime Biotechnology, China) and scanned. The OD value was analyzed with ImageJ 1.50i software (United States).

Immunohistochemical Staining

Rats were anesthetized and perfused transcardially with an ice-cold phosphate buffer, followed by an ice-cold 4% paraformaldehyde solution. The entire brain was removed immediately and immersed in 4% paraformaldehyde solution for 24 h at 4°C. The coronal sections were obtained through the dorsal hippocampus and used for immunohistochemical analysis.

The sections dewaxed and hydrated, then incubated in 3% hydrogen peroxide solution for 30 min. Antigen retrieval was performed under boiling conditions. Sections were incubated in goat serum for 2 h to block the antigens. Sections were incubated overnight at 4°C with the primary antibodies: the mouse anti-$GABA_AR\alpha1$ monoclonal antibody (1:100, Abcam, United Kingdom), the goat anti-NKCC1 polyclonal antibody (1:50, Santa Cruz, United States), and the goat anti-KCC2 polyclonal antibody (1:50, Santa Cruz, United States). The sections were then incubated in the secondary antibodies: the biotinylated-goat anti-mouse IgG, or the mouse anti-goat IgG (Beyotime Biotechnology, China) for 1h at room temperature. After being rinsed three times with PBS, a DAB kit was used to visualize the sites of antibody binding. The sections were observed under a microscope. The positive cells in the hippocampal CA1, CA3, DG, and H region were captured. Three high-power images were randomly selected for each animal. The immunoreactivity was evaluated with the staining intensity and the ratio of positive area to the total area.

Statistical Analysis

All experimental data was expressed as Mean ± Standard Error of the Mean (SEM), SPSS19.0 software was used for statistical analysis. The seizure score was analyzed by the Mann–Whitney U test. Seizure latency, duration, Glutamate, GABA and OD values were subjected to one-way ANOVA and *post hoc* comparisons were performed with an LSD test. $P < 0.05$ was considered statistically significant.

RESULTS

Effects of GCK on Behavioral Seizures Induced by Pentylenetetrazole

To investigate the protective effect of GCK in an acute seizure animal model, 60 mg/kg of PTZ was administered to establish a seizure rat model that could replicate generalized tonic-clonic and myoclonic seizures. Rats in the model group showed an obvious epileptic behavioral feature after the PTZ injection. Rats pre-treated with VPA had a lower seizure score (**Figure 1A**, $P < 0.05$) and a longer latency than the model group (**Figure 1B**, $P < 0.01$). Unfortunately, GCK did not display a protective effect in the low-dose group and the middle-dose group. Interestingly, high doses of GCK not only reduced the seizure intensity (**Figure 1A**, $P < 0.05$) but also prolonged the latency for the onset of seizures (**Figure 1B**, $P < 0.05$). Moreover, high doses of GCK could shorten seizure duration (**Figure 1C**, $P < 0.05$). These findings suggest that high doses of GCK demonstrate anti-epileptic activity.

Effects of GCK on Behavioral Seizures Induced by Lithium Chloride-Pilocarpine

To further identify GCK's antiepileptic activity, the lithium chloride-pilocarpine-induced SE rat model was selected. This model resembled human SE and complex partial seizures. All rats in the model group exhibited high seizure scores (forelimb clonus, rearing, falling and jumping, even death). Seizure score was decreased in the VPA group (**Figure 2A**, $P < 0.05$) while the latency to the first seizure was extended in the VPA group (**Figure 2B**, $P < 0.001$). High doses of GCK significantly reduced the seizure score (**Figure 2A**, $P < 0.05$). Intriguingly, both middle and high doses of GCK significantly lengthened the latency to the initial seizure (**Figure 2B**, $P < 0.05$, 0.01). These results further confirmed that GCK has an anti-epileptic effect.

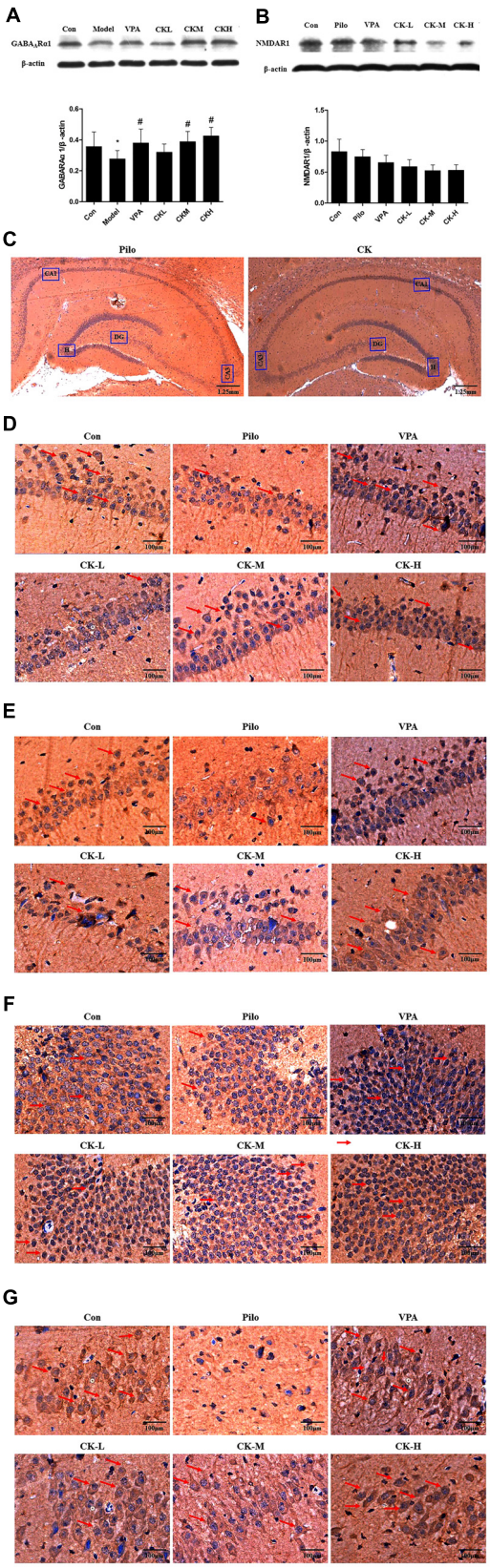

FIGURE 4 | Continued

FIGURE 4 | Effects of GCK on the expression of GABA$_A$Rα1 and NMDAR1 protein in the hippocampus of the pilocarpine-induced epileptic rats. **(A)** The expression of GABA$_A$Rα1 in the Con, Pilo, VPA, and GCK-treated groups. **(B)** The expression of NMDAR1 in the Con, Pilo, VPA, and GCK-treated groups. **(C–G)** The GABA$_A$Rα1 immunoreactivity in the hippocampal CA1, CA3, DG, and H regions. Scale bar: 100 μm. Values are mean ± SEM (n = 5). Pilo 30 mg/kg, VPA 400 mg/kg, GCK 80 mg/kg, 160 mg/kg, and 320 mg/kg. Compared with the Con $^*P < 0.05$; Compared with the Pilo, $^\#P < 0.05$.

Effects of GCK on the Contents of Amino Acid Neurotransmitter in Pilocarpine-Induced Epileptic Rats

To determine if GCK regulates the levels of amino acid neurotransmitter, glutamate and GABA were detected with either colorimetry or ELISA. Pilocarpine increased the content of glutamate (**Figure 3B**, $P < 0.05$), whereas GABA was declined in the hippocampus of the pilocarpine-induced epileptic rats (**Figure 3A**, $P < 0.05$). Pre-treatment with various doses of GCK (80, 160, and 320 mg/kg) and VPA (400 mg/kg) eliminated the pilocarpine-induced decreases in GABA levels (**Figure 3A**, $P < 0.05$). However, the hippocampal glutamate was not significantly affected by GCK and VPA. These observations revealed that the augmentation of GABA in the hippocampus could contribute to the anti-seizure effect of GCK.

Effects of GCK on the Expression of GABA$_A$Rα1 and NMDAR1 Protein in Pilocarpine-Induced Epileptic Rats

Glutamate acts on NMDAR to induce postsynaptic depolarization which is essential for neuronal excitability. GABA$_A$R is crucially involved in GABA-mediated inhibition. The effect of GCK in the expression of receptors was also investigated. As showed in **Figure 4A**, pilocarpine injection resulted in the downregulation of GABA$_A$Rα1 protein ($P < 0.05$). GCK displayed a significantly increased expression of GABA$_A$Rα1 in the hippocampus (**Figure 4A**, $P < 0.05$). GABA$_A$Rα1 immunohistochemical staining was distributed in all regions of the hippocampus. GABA$_A$Rα1 was located in the membrane of neurons. In the pilocarpine treatment group, the staining in the sections displayed pale yellow and the positive area of GABA$_A$Rα1 was smaller than those in the control group, suggesting that the GABA$_A$Rα1 immunoreactivity was reduced in the hippocampus in pilocarpine-induced epileptic rats. The sections showed brown and the positive area was larger in the VPA treatment group. Furthermore, the middle and high doses of GCK could also increase the staining intensity and enlarge the positive area of GABA$_A$Rα1 (**Figures 4C–G**), the results suggest that GCK also increased the GABA$_A$Rα1 immunoreactivity following SE. The expression changes of NMDAR1 has a tendency to decline in a dose-dependent manner within the GCK pre-treated group although there was no significant change in NMDAR1 protein's expression (**Figure 4B**). GCK may regulate GABA$_A$Rα1's expression to demonstrate its antiepileptic effect.

Effects of GCK on the Expression of KCC2 and NKCC1 Protein in Pilocarpine-Induced Epileptic Rats

$GABA_A$R-mediated action depends on the concentration of intracellular Cl^-. The expression of the cation chloride cotransporters, including NKCC1 and KCC2, were determined to further explain the mechanism of GCK against seizure. Western-blot analysis showed that GCK enhanced markedly the levels of the KCC2 protein in the hippocampus of pilocarpine-induced epileptic rats (**Figure 5A**, $P < 0.05$). Immunohistochemical staining showed that the KCC2 was mainly located in the membrane of neurons throughout the entire hippocampus. The KCC2 immunoreactivity was decreased in the pilocarpine-induced epileptic rats, suggesting that epileptic seizures could reduce the contents of KCC2 protein. The middle and high doses of GCK thickened the positive area of KCC2 around the surface of neurons in the hippocampus, it means that GCK increased the KCC2 immunoreactivity throughout the hippocampus (**Figures 5C–G**). GCK obviously reduced the pilocarpine-induced increased expression of NKCC1 protein in the hippocampus (**Figure 5B**, $P < 0.05$). We also observed that NKCC1 was distributed in the membrane of neurons. The positive expression of NKCC1 was very low in the hippocampus in the control group, while its staining intensity was stronger and the positive area was increased after pilocarpine induction. Interestingly, GCK significantly reduced the NKCC1 immunoreactivity in the hippocampus (**Figures 5H–L**). GCK regulates the cation chloride cotransporters to obtain an anti-seizure effect.

DISCUSSION

Ginsenoside compound K is a primary metabolite of ginsenoside Rb1, Rb2, and Rc in intestinal bacteria in the organisms following oral administration. Previous studies has revealed that GCK greatly improves memory impairment by inducing Nrf2-mediated antioxidant enzymes (Seo et al., 2016), attenuating cyclophosphamide-induced the deletion of hippocampal neurogenesis, and ameliorating Aβ (25–35) induced axonal atrophy and synaptic loss (Tohda et al., 2004; Hou et al., 2013). GCK could also suppress microglial activation to prevent brain impairment following cerebral ischemia (Park et al., 2012). These findings demonstrate that GCK has a beneficial neuroprotective effect in the treatment of neurological disorders (Oh and Kim, 2016). However, the anti-epileptic effect of GCK remains unknown. We found that GCK could reduce the seizure activity by increasing the content of GABA and enhancing the $GABA_A$R-mediated inhibitory neurotransmission in the hippocampus.

Epileptic seizures are caused by the disruption of the balance between excitatory and inhibitory neurotransmitters (Clynen et al., 2014). GABA is a key inhibitory neurotransmitter in the brain that was decreased after seizure. The deficiency of GABA would induce neuronal hyperexcitability, which contributed to the occurrence of seizures. PTZ is a $GABA_A$ receptor antagonist that acts as a chemical convulsant to build an animal model of generalized tonic-clonic seizures, myoclonic seizures, or absence seizures (Zhao et al., 2011). The rodent PTZ model is commonly used for the antiepileptic drug screening and studying the mechanism of seizures. Ethosuximide, trimethadione and valproate were discovered using this seizure model, so the PTZ test is recognized as a primary antiepileptic drug screening model by The Anticonvulsant Screening Program (ASP) of the United States National Institute of Neurological Disorders and Stroke (NINDS) (Loscher, 2017). Here we chose the PTZ-induced seizures model to test the anticonvulsant actions of GCK. We found that GCK reduced the intensity of seizures, prolonged the latency of seizures, and shortened the seizure duration in the PTZ-induced seizure rat model. Previous studies demonstrated that the Rb extract, the Mix1 (Rb1 plus Rb3) or the Mix2 (Rb1 plus Rb3 plus Rd) increases the latency to seizure onset and shortened the seizure duration in the PTZ-induced seizures rat model (Lian et al., 2005, 2006). Another study found that Rb1 dose-dependently reduces PTZ-induced seizure duration and prolongs seizure latency (Shi et al., 2018). Although these studies yielded positive results, the route of administration and the unspecified active ingredient limits its development and application. In this study, an oral administration of the gavage was performed to confirm that GCK inhibited PTZ-induced seizures. There were several causes of the beneficial effects of high doses of GCK. The intraperitoneally injection of higher doses of PTZ were used to induce a rat model of seizures, where GCK had poor water solubility and low bioavailability. These results implied that GCK could suppress myoclonic seizures and absence seizures.

Pilocarpine is an M muscarinic receptor agonist that can establish SE and partial seizures with secondary generalization (Kandratavicius et al., 2014). It is the most commonly used model of TLE, due to the appearance of spontaneous recurrent seizures, extensive brain damage, and resistance to the current antiepileptic drugs in this model (Curia et al., 2008). Lithium pre-treatment not only lowers the mortality rates, but also reinforces the epileptogenic action of pilocarpine (Martin and Pozo, 2006). In order to determinate the antiepileptic effect of GCK, we applied the lithium-pilocarpine model of epilepsy with GCK administration, where it was observed that GCK reduced the intensity of seizures and lengthened the latency to the onset of SE. These findings indicated that GCK might inhibit SE or complex partial seizures.

Alterations in the balance between Glutamate and GABA in brain could cause the occurrence and progression of seizures. Elevation of extracellular glutamate mediated neuronal excitation is generally considered as a critical factor in the pathological process of epilepsy. A growing body of evidence demonstrates a marked increase in glutamate concentration in patients with TLE (During and Spencer, 1993). Increased extracellular glutamate is strongly associated with decreased epileptogenic hippocampal volume in patients with drug resistant epilepsy (Cavus et al., 2008). The raised glutamate was found in various animal epilepsy models (Soukupova et al., 2015). In the present study, we also showed a high concentration of hippocampal glutamate. Previous studies have demonstrated

that GCK attenuated glutamate-induced cytotoxicity in HT22 cells by inducing Nrf2-mediated antioxidant enzymes (Seo et al., 2016). Our experimental results showed that GCK had no significant effect on pilocarpine induced glutamate levels. Glutamate regulates brain excitability by activating the two main ionotropic receptors including NMDA and AMPA receptors. Glutamate regulates postsynaptic depolarization and action potential by binding with the NMDA receptor. Nevertheless, excess glutamate released in the synaptic cleft or the over-activated NMDA receptor promotes calcium entering, which leads to neuronal death and neurodegeneration (Deshpande et al., 2008). GCK inhibits the expression and the activity of NMDAR (Jang et al., 2004). Therefore, we explored the role of GCK in NMDAR1 expression in the epileptic rat model, which revealed that GCK exhibited a tendency to decrease the expression of NMDAR1 protein in the hippocampus. There was no obvious difference in the results, which could be caused by the small sample size. A study has showed that GCK attenuated morphine-induced dependence by decreasing the NMDAR1 expression in the frontal cortical regions of the rat brain (Yayeh et al., 2016). The cause for the inconsistency of the results could be explained by the differences between brain regions, animal models and body conditions. Another study showed that ginseng total saponins and ginsenoside Rg3 decreases the intracellular Ca^{2+} level and the hippocampal neurons death by suppressing NMDAR-induced spontaneous recurrent epileptiform discharges (Kim and Rhim, 2004). We have not assessed the function of NMDAR after GCK treatment. Thus, the effects of CK on the glutamate-mediated neuronal excitability in epilepsy requires further study.

Loss of GABA release or abnormal synthesis that impairs GABA-mediated inhibition could also facilitate neuronal hyperexcitability, which ultimately triggers seizures. Promotion of GABA release to enhance the GABA-mediated inhibitory action has become an important target of antiepileptic drugs (Kammerer et al., 2011). GABAergic interneurons and basal GABA outflow were lowered during the latent period and the initial spontaneous seizure in the pilocarpine-induced TLE (Soukupova et al., 2014). A study *in vitro* found that GCK enhances spontaneous GABA release by elevating intraterminal Ca^{2+} concentration (Bae et al., 2010). The current study demonstrated that GCK increased the level of GABA in the hippocampus. Ginsenosides could not only raise GABA levels but could also reduce glutamate levels in the hippocampus and cortex in the rat model of Alzheimer's disease (Zhang et al., 2016). Although the models were different and the active ingredients were unclear, the results confirmed that GCK is a main metabolite of ginsenosides that could promote GABA release. These findings demonstrated that GCK could increase GABA release to suppress epileptic seizures.

γ-aminobutyric acid exerts its neuronal inhibitory via activating $GABA_AR$s-mediated inhibitory postsynaptic currents. $GABA_AR$s are identified as heteropentameric ion channels formed by 19 subunits (Uusi-Oukari and Korpi, 2010). The physiological, pharmacological, and targeting properties of $GABA_AR$s were determined by the subunit composition of $GABA_AR$. The γ subunit at the synaptic sites mediates rapid phasic inhibition while the extrasynaptic δ subunit regulates the persistent tonic inhibition (Gonzalez et al., 2013). The α subunit is an important and common subunit of $GABA_AR$s. $GABA_AR$α consists of six isoforms including α1-α6. $GABA_AR$α1 is the most commonly expressed in the brain (Laverty et al., 2017). $GABA_AR$α1 is the target of benzodiazepines, which have anticonvulsant and sedative effects (Rudolph et al., 1999; McKernan et al., 2000). Multiple bodies of evidence have shown that the mutation of the α1 subunit is closely associated with several types of seizures, including early infantile epileptic encephalopathy, childhood absence epilepsy and juvenile myoclonic epilepsy (Braat and Kooy, 2015). Previous studies have shown that the expression of α1 subunit was

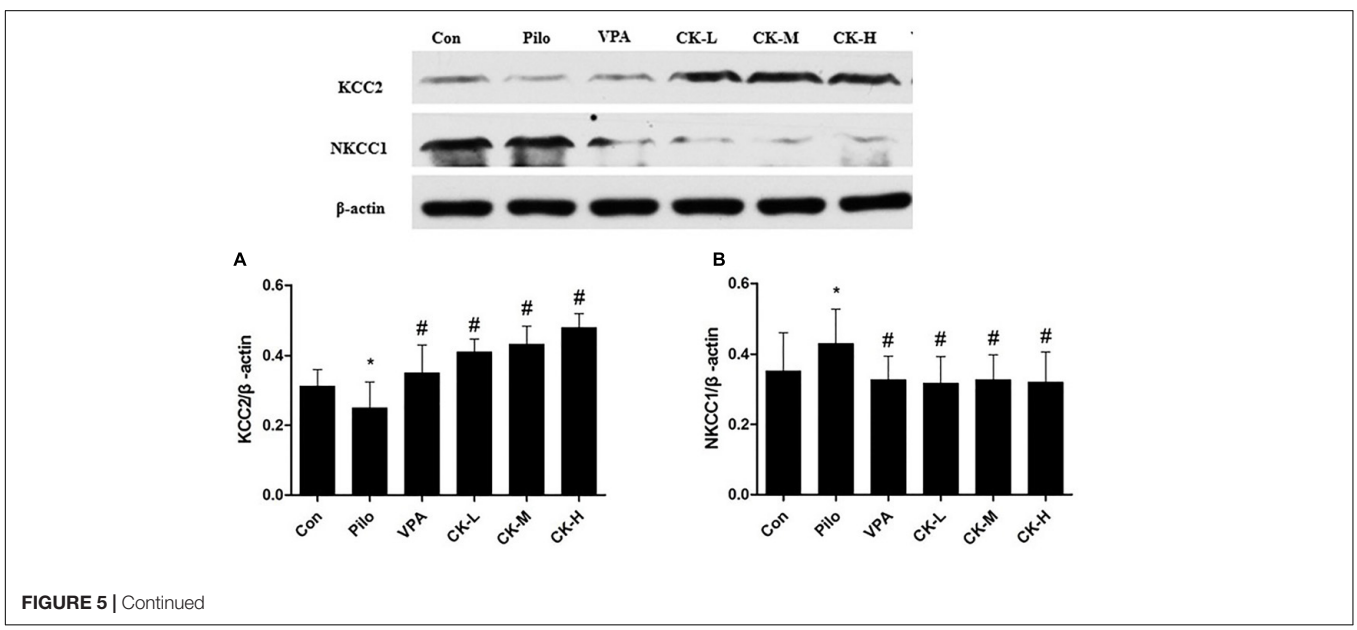

FIGURE 5 | Continued

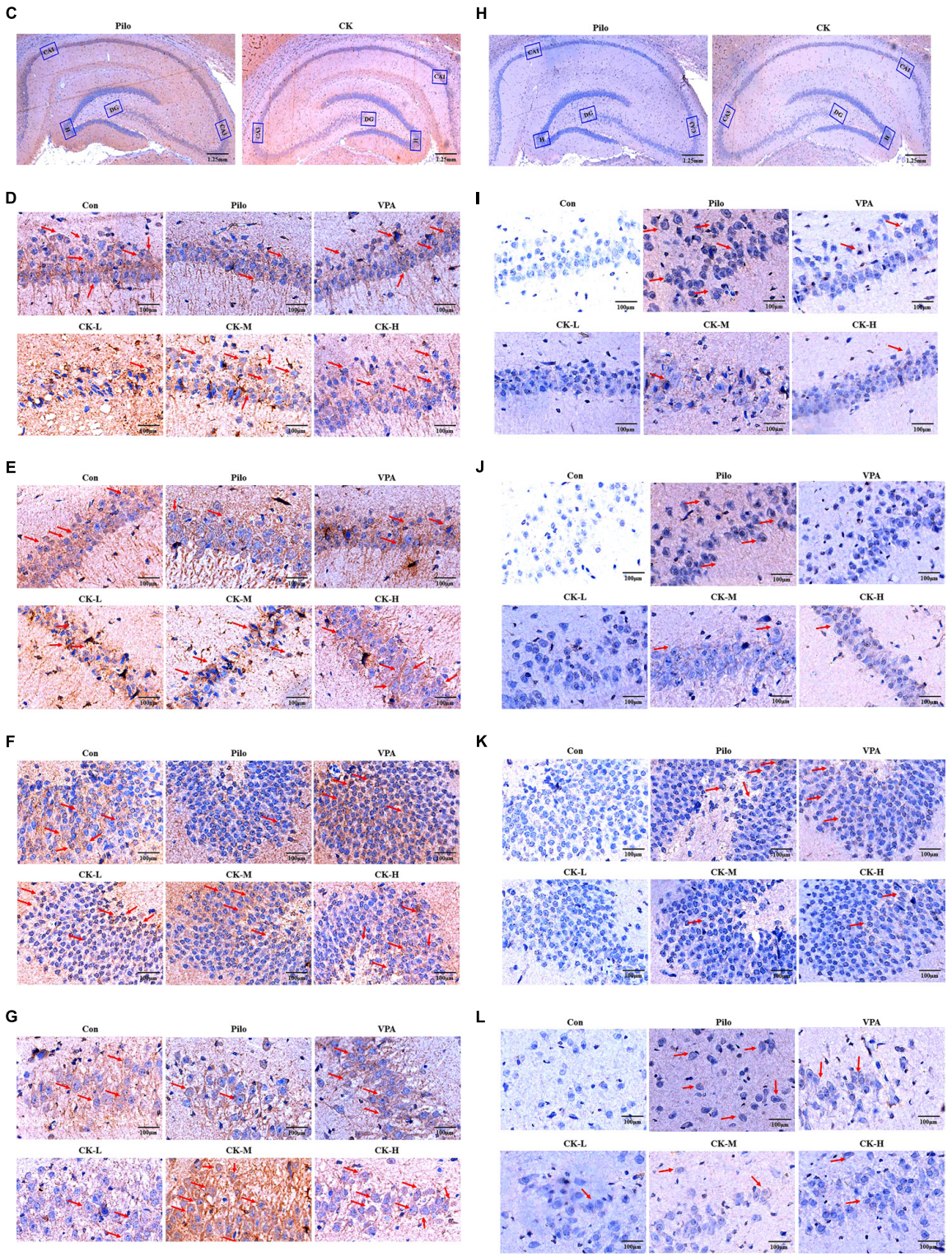

FIGURE 5 | Effects of GCK on the expression of KCC2 and NKCC1 protein in the hippocampus of the pilocarpine-induced epileptic rats. **(A)** The expression of KCC2 in the Con, Pilo, VPA, and GCK-treated groups. **(B)** The expression of NKCC1 in the Con, Pilo, VPA, and GCK-treated groups. **(C–G)** The KCC2 immunoreactivity in the hippocampal CA1, CA3, DG, and H regions. Scale bar: 100 μm. **(H–L)** The NKCC1 immunoreactivity in the hippocampal CA1, CA3, DG, and H regions. Scale bar: 100 μm. Values are mean ± SEM ($n = 5$). Pilo 30 mg/kg, VPA 400 mg/kg, GCK 80 mg/kg, 160 mg/kg, and 320 mg/kg. Compared with the Con $^{*}P < 0.05$; compared with the Pilo, $^{\#}P < 0.05$.

decreased in epileptic rats (Brooks-Kayal et al., 1998). The current study also observed reduced expression of $GABA_AR\alpha1$ in the hippocampus 1 day post-SE, suggesting $GABA_AR\alpha1$ changed during the development of epilepsy. An earlier study showed that GCK enhanced the action of GABA receptor agonist in the brain (Jang et al., 2004). The detailed mechanism of GCK's action remains unclear. The current study demonstrated that GCK up-regulated the expression of $GABA_AR\alpha1$ in the hippocampus after SE. Genetic conditional enhanced expression of $GABA_AR\alpha1$ in the DG prolonged the latency to the initial spontaneous seizure and inhibited the development of spontaneous seizures after SE (Raol et al., 2006a). GCK may inhibit the development of chronic epilepsy as a pharmacological regulator of $GABA_AR\alpha1$ expression. Although we did not explore the effect of GCK on other $GABA_AR$ subunits, the elevation of $GABA_AR\alpha1$ could offer a partial explanation of the neuroprotective effect of GCK against epilepsy.

$GABA_AR$-mediated synaptic transmission was determined by the intracellular Cl^- concentration. Cation-chloride cotransporters plays a crucial role for maintaining Cl^- homeostasis (Loscher et al., 2013). NKCC1 is a main driver that promotes Cl^- into cells while KCC2 is a key efflux pump that mediates Cl^- extrusion in neurons. NKCC1 regulates the depolarizing responses to $GABA_A$ receptor activation by elevating the intracellular Cl^- level. During the development of brain, NKCC1 expression decreases from postnatal week to maturity. KCC2 enhances fast hyperpolarizing $GABA_A$ receptor-mediated inhibition by lowering the intracellular Cl^- concentration. Its expression increases in brain with age and maturity (Puskarjov et al., 2014a). The balance between NKCC1 and KCC2 is destroyed in the patients with TLE (Eftekhari et al., 2014). The up-regulated NKCC1 expression has been found in the brain of epileptic patients with malformations of cortical development and hippocampal sclerosis (Sen et al., 2007). Its up-regulation is also reported in the amygdala kindling model and the pilocarpine-induced epileptic animal model (Okabe et al., 2002; Li et al., 2008). Previous literature found that NKCC1 accelerates neonatal seizures in the developing hippocampus (Dzhala et al., 2005). The NKCC1 inhibitor bumetanide represents anti-seizure activity and restores the anti-epileptic effects of diazepam and phenobarbital (Dzhala et al., 2008). Bumetanide has been found to reduce the development of pharmacoresistant epilepsy (Sivakumaran and Maguire, 2016). A previous study reported that seizure frequency and epileptiform discharges are reduced in patients with TLE following bumetanide administration (Eftekhari et al., 2013). NKCC1 is a potential target for the treatment of epilepsy. In the present study, we found that pilocarpine induced the upregulation of NKCC1 protein in epileptic rats. GCK could reduce NKCC1 expression in the hippocampus in the pilocarpine-induced epileptic rats. The results showed that GCK could lower the intracellular Cl^- concentration to suppress $GABA_AR$-mediated neuronal excitation.

KCC2 is a major impact factor of GABA-mediated hyperpolarizing postsynaptic inhibition. The expression level of KCC2 protein in the cell surface, as well as its phosphorylation state controls KCC2 function (Moore et al., 2017). Decreased KCC2 expression has been reported in human focal cortical dysplasia (Shimizu-Okabe et al., 2011). Its reduction was also observed in the animal model of pilocarpine-induced SE and brain injury-induced epilepsy (Bonislawski et al., 2007; Pathak et al., 2007). The KCC2-deficient mice exhibited spontaneous generalized seizures and were more vulnerable to seizures induced by PTZ (Tornberg et al., 2005). Two variants of KCC2, R952H, and R1049C, were discovered in human idiopathic generalized epilepsy and febrile seizures (Kahle et al., 2014; Puskarjov et al., 2014b). The loss of KCC2 impairs neuronal Cl^- extrusion leading to seizure-like ictal discharge (Buchin et al., 2016). CLP257 enhances KCC2 activity, which could mediate ictogenesis (Gagnon et al., 2013; Hamidi and Avoli, 2015), this suggests KCC2 is a viable therapeutic target for epilepsy. The current study found that GCK increased the pilocarpine-induced lowered KCC2 expression. These results suggested that GCK could correct the imbalance between NKCC1 and KCC2 expression to enhance GABA-mediated neuronal inhibition.

CONCLUSION

Ginsenoside compound K reduced the severity of epileptic seizures and prolonged the latency to the initial seizure. GCK increased the contents of GABA in the hippocampus. Moreover, GCK up-regulated the expression of $GABA_AR\alpha1$ and KCC2 protein while GCK reduced the pilocarpine-induced increased NKCC1 expression. Our study results suggested that GCK could inhibit the seizure activity by promoting GABA release from the hippocampal neurons and enhancing the GABAergic inhibition-related protein expression, which could demonstrate a promising future for the development of novel anti-epileptic drugs.

AUTHOR CONTRIBUTIONS

D-sO and KH conceived and designed the study. XZ, LC, LZ, WL, CL, WZ, SC, and QG performed the experiments. DJ and GZ provided an experimental support. XZ wrote the paper. KH helped in modifying the article. D-sO, KH, XL, and HZ reviewed and edited the manuscript. All authors gave their final approval for the submission of the manuscript.

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Anxiolytic and Antiepileptic Properties of the Aqueous Extract of *Cissus quadrangularis* (Vitaceae) in Mice Pilocarpine Model of Epilepsy

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Cissus quadrangularis (*C. quadrangularis*) is a plant of the Vitaceae family known for its anticonvulsant effects in traditional medicine. The objective of this study was to elucidate the anxiolytic and antiepileptic effects of aqueous extract of *C. quadrangularis*. The mice were divided into different groups and treated for seven consecutive days as follows: a negative control group that received distilled water, po, four test groups that received four doses of the plant (37.22, 93.05, 186.11, and 372.21 mg/kg, po), and a positive control group that received sodium valproate (300 mg/kg, ip). One hour after the first treatment (first day), epilepsy was induced by intraperitoneal administration of a single dose of pilocarpine (360 mg/kg). On the seventh day, the anxiolytic effects of the extract were evaluated in the epileptic mice using the elevated plus maze (EPM) and open field (OP) paradigms. Antioxidant activities and the involvement of gabaergic neurotransmission were determined by measuring the levels of malondialdehyde, reduced glutathione (GSH), GABA, and GABA-transaminase (GABA-T) in the hippocampus of sacrificed epileptic mice. The results show that the extract of *C. quadrangularis* significantly and dose-dependently increased the latency to clonic and generalized tonic–clonic seizures and decreased the number and duration of seizures. In the EPM, the extract of *C. quadrangularis* significantly increased the number of entries and the time spent into the open arms and reduced the number of entries and the time spent into the closed arms as well as the number of rearing. The extract of *C. quadrangularis* also increased the number of crossing, and the time spent in the center of the OP. The level of MDA and the activity of GABA-T were significantly decreased by the extract of *C. quadrangularis* while reduced GSH and GABA levels were increased. The results suggest that the anticonvulsant activities of *C. quadrangularis* are accompanied by its anxiolytics effects. These effects may be supported by its antioxidant properties and mediated at least in part by the GABA neurotransmission.

Keywords: *C. quadrangularis*, anticonvulsant, anxiolytic, pilocarpine, status epilepticus, epileptogenesis

INTRODUCTION

Status epilepticus (SE) is the first manifestation of epilepsy in approximately 50% of patients (Chapman et al., 2001). Generalized tonic–clonic SE is the most dangerous form (Chen and Wasterlain, 2006). It is characterized by continuous seizures or the succession of seizures without the improvement of consciousness over a period of 30 min (Gastaut, 1973; Proceedings of the American Epilepsy Society Course, 1993; Dupont and Crespel, 2009). Normal cerebral activity is based on both excitatory and inhibitory actions. But in the epileptic focus of the pilocarpine SE models, which are pharmaco-resistant models, 20% of the neurons would be excited by GABA (Cohen et al., 2002). The excitatory role of GABA is due to molecular alterations that affect the natural function of GABA in epileptic tissue (Huberfeld et al., 2007). It characterizes the silent phase: epileptogenesis groups together a series of events during which irreversible neuronal lesions appear. The excitation (glutamate + GABA) becomes much too strong and the inhibition much too weak and leads to the establishment of a chronic state characterized by the occurrence of abnormal discharges in the neurons (Cherubini et al., 2011) hence the recurrence of spontaneous crises (Cavalheiro et al., 2006). These pathophysiological changes lead to the development of comorbidities related to epilepsy. (Faure, 2016). It has been shown that during epileptogenesis, the occurrence of anxiety disorders may be the consequence of lesions located especially in the amygdala and other structures playing a role in emotions (Faure, 2016) in the same way reactive oxygen species (ROS) overproduction (Azam et al., 2010). The overproduction of ROS causes the oxidation of the polyunsaturated fatty acids at the origin of the formation of hydroperoxides and aldehydes. Their biological activities cause the alteration of the structure of cell membranes and the disruption of these membranes (Sies and Stahl, 1995; Bonnefont-Rousselot et al., 2003), hence the cellular apoptosis (Dröge, 2002). Oxidative stress can be assessed by the dosage of MDA, compound resulting from the peroxidation of lipids, by the measurement of non-enzymatic systems such as GSH. GSH is a cofactor of the ROS detoxification pathways formed, the regulation of the redox potential, and the reduction of oxidation of the thiols groups of the proteins (Sohal and Weindruch, 1996; Beckman and Ames, 1998).

The goal of the treatment of the SE is to obtain the fast and lasting stop of the crises. None of the antiepileptic drugs available today have the properties of the ideal drug in terms of efficacy and tolerance (Ichai et al., 1996). These limits have developed more interest for the use of medical plants. In Africa, herbal medicine still plays an important role in disease management, mainly in very low-income populations (Geoffrey and Kirby, 1996). In addition, certain diseases such as epilepsy, depression, and anxiety in Cameroon are considered as mystical diseases (personal communications). *Cissus quadrangularis* Linn (Vitaceae) (*C. quadrangularis*) is a medicinal plant used in traditional medicine in northern Cameroon for the treatment of epilepsy (Ngo Bum et al., 2008). *C. quadrangularis* is native to India and Malaysia and grows in savannah areas in Africa (Cameroon, Mali, Mauritania, Senegal, Somalia, and Chad; Dumas-Champion, 1997; Arbonier, 2000). In traditional medicine, the plant is used to treat epilepsy (Ngo Bum et al., 2008; Shah, 2011), convulsion (Shah, 2011), hemorrhoids, anorexia, indigestion, and asthma (Rajpal, 2002). Chemical studies have shown the presence of sterols, steroids, tannins, flavonoids (quercetin and kaempferols), carotenes, ascorbic acid, and linoleic acid in *C. quadrangularis* (Sen, 1966; Saburi et al., 1999; Murthy et al., 2003). Pharmacological studies on fresh leaves and roots have shown that *C. quadrangularis* has antioxidant, antibacterial, analgesic, and neurosedative (Amos et al., 2001; Murthy et al., 2003; Viswanatha et al., 2006). The studies on the stems have revealed anticonvulsant and sedative properties (Ngo Bum et al., 2008). A three-month subchronic toxicity study suggested that *C. quadrangularis* administered orally at a dose of 3 g/kg is non-toxic (Attawish et al., 2002). The purpose of this study was to investigate the antiepileptogenic and anxiolytic properties of *C. quadrangularis* following the study of Ngo Bum et al. (2008).

MATERIALS AND METHODS

Plant

The entire plant was harvested early in the morning in the Mogode village, Far-North Region, Cameroon, in June. A voucher specimen has been deposited at the Yaounde national herbarium on the number 36966 HNC/Cam.

Preparation of the Aqueous Extract of *C. quadrangularis* (AECQ)

The fresh and whole plant of *C. quadrangularis* was cut into pieces and air dried at room temperature.The powder was added to distilled water (10 g in 140 ml) and boiled for 20 min. Following cooling at room temperature, the solution obtained was filtered with Whatman No1 filter paper. The filtrate was considered as the stock solution. The amount of dry matter in the extract was determined by evaporating water in a drying oven (50°C). A solid residue (2.17 g) was obtained. The yield of extraction was 21.7%, and the stock solution dose was 372.21 mg/kg. The other doses used in the study (186.1, 93.05, and 37.22 mg/kg) were obtained by dissolving the stock solution in distilled water at ratios of 1/2, 1/4, and 1/10, respectively. The procedure for obtaining the dose used by traditional healers has been respected.

Animals and Experimental Design

White *Mus musculus* swiss mice of both sexes, weighting 18–28 g were used for this study. These mice came from the National Veterinary Laboratory (LANAVET, Garoua, Cameroon), they were raised under standard conditions at the University of Ngaoundere (Ngaoundere, Cameroon), and fed with water and food at will. Animals were maintained on a 12 h/12 h light/dark cycle. Animals were acclimated to laboratory conditions 72 h before starting the experiments. The study was carried out in accordance with the Cameroon National Ethical Committee (Ref No. FW-IRB00001954, 22 October 1987). The authorization

number (1244 CEI-UDO/12/2017/A) was given and the study was done also in conformation with the international regulation, minimizing the number of mice used and their suffering.

The mice were organized into nine lots of six mice each. The first day of the test, seven lots of six mice received the following treatments:

- lot 1, normal control group (DW + DW) receiving only distilled water (10 ml/kg, p.o.);
- lot 2, negative control group (DW + Pilo) receiving distilled water (10 ml/kg, p.o.);
- lots 3–6, test lots receiving the different doses of EACQ (37.22, 93.05, 186.11, and 372.21 mg/kg, p.o.);
- lot 7, positive control group (VS) receiving sodium valproate (300 mg/kg, i.p., Sigma-Aldrich).

The females used were non-pregnant since male mice were isolated from females before and during experimental testing. Males and females were distributed equally in the different lots.

The procedure for induction of SE by pilocarpine is based on previous studies by Turski et al. (1983), Curia et al. (2008), and Magnin (2014). Forty minutes after the first treatment, to reduce the peripheral effects of pilocarpine, each lot received *N*-methyl-scopolamine (1 mg/kg, i.p., Sigma-Aldrich), except lot 1. Twenty minutes after administration of *N*-methyl-scopolamine, lot 1 still received distilled water; all the other lots were treated with a single dose of pilocarpine hydrochloride (360 mg/kg, i.p., Sigma-Aldrich). Each mouse was returned to its initial cage and observed individually for 6 h to determine the severity and duration of acute epileptic seizures compared to the Racine scale (Racine, 1972). After about 20 min, some animals became hypoactive and then displayed successively oro-facial movements, salivation, eye blinking, twitching of vibrissae, yawning, and generalized convulsions. Only mice that developed stage 5 attacks of the Racine scale (tonic–clonic seizures with loss of righting reflex) for two consecutive periods were retained for this study.

Study of Anticonvulsant Effects of *C. quadrangularis* Extract During Epileptogenesis

Twenty three hours after the injection of pilocarpine, the mice received once again their different treatments (mice were treated with distilled water for lots 1 and 2, the respective doses of the plant for lots 3–6, and sodium valproate for lot 7). After 1 h, the behavioral parameters were evaluated for each mouse for a period of 30 min. The parameters observed were the latency time to the first tonic–clonic seizures, the duration of tonic–clonic seizures, and the number of tonic–clonic seizures. From the third to the seventh day, the mice were treated as on the second day.

Behavioral Assessment

Seven days the administration of pilocarpine and 1 h after the administration of the different daily treatments, the mice were observed for 5 min in the elevated plus maze (EPM; Pellow et al., 1985; Rodgers and And Johnson, 1995) and after in the open field (OP) paradigm (Lister, 1990).

Elevated Plus Maze Test

The test in the EPM has taken place in a quiet room. The method used in this study was that described by Rodgers and Dalvi (1997). It consisted of placing a mouse in the center of the EPM with two open arms and two closed arms. The mice were free to explore the maze during 5 min (Anderson et al., 2000). A group of mice receiving diazepam (3 mg/kg) was added to this test. The number of entries and the time spent in the center and in the different arms were recorded. The number of rearing and head dipping were recorded too. After observing the behavior of a mouse, the labyrinth was cleaned with alcohol.

Open Field Test

The method used in our experiment was that described by Belzung (1999). The OP test is commonly used to evaluate the level of locomotor activity, exploration, and emotional reactivity in rodents (Blizard, 1989; Van der Staay et al., 1990; Bronikowski et al., 2001). A group of mice receiving diazepam (0.3 mg/kg) was added to this test. The mice were placed one after the other in the center of the experimental device. The behavior of each mouse was observed for a period of 5 min. Several behavioral parameters were evaluated: the number of crossing (number of lines crossed), the number of grooming (when the animal cleans its body), the frequency of rearing (when the animal stands up on its hind legs and bears on the edges of the experimental device), the time spent in the center of the experimental device, and the fecal boli produced.

Biochemical Tests

At the exit of the OP, the animals were immediately sacrificed. Their brain was dissected and cleaned with ice-cold saline solution (0.9%, w/v) to remove the hippocampus. Then, the hippocampi were weighed. To perform biochemical analyzes, 10% (w/v) homogenates prepared with ice-cold 0.1 M phosphate buffer (pH 7.4) were centrifuged (10,000 × *g*, 15 min). The aliquots of the supernatants were collected and used to measure, according to different protocols, the level of reduced glutathione (GSH), malondiadéhyde (MDA), GABA, and GABA-transaminase (GABA-T).

GSH Level

Glutathione was measured using the method of Ellman (1959). Briefly, 1500 µl of DNTB and 500 µl of Tris-HCl (Sigma-Aldrich) buffer (50 mM, pH 7.4) were added to a blank tube containing 100 µl of Tris-HCl buffer (50 mM, pH 7.4) or to test tubes containing tissue homogenates (100 µl). The mixture solution was incubated for 1 h, and the absorbance was read against the blank at 412 nm. The GSH concentration was calculated using an extinction coefficient of 13,600 $mol^{-1}cm^{-1}$. The concentration of GSH was expressed as µmol/g of protein in the tissue.

MDA Level

The method of Wilbur et al. (1949) for MDA determination was used. Briefly, distilled water (250 µl) and homogenate (20 µl) were introduced in the control tube and in the test tubes, respectively. Then, 250 µl of Tris-HCl buffer (50 mM, pH 7.4), 500 µl of trichloroacetic acid (20%, Sigma-Aldrich),

and 1000 µl of thiobarbituric acid (0.67%, Sigma-Aldrich) were added. The mixture solution was heated in a water-bath (90°C, 10 min). After cooling at room temperature, the tubes were centrifuged (3000 rpm, 15 min). The absorbance of the pink-colored supernatant was measured against the blank at 530 nm. The MDA concentration was calculated using an extinction coefficient of $1.56 \times 105\ mmol^{-1}cm^{-1}$. MDA level was expressed in µmol/g of protein in the tissue.

GABA Level

The level of hippocampal GABA was determined by the colorimetric assay of mouse brain homogenates as described by Lowe et al. (1958). The working reagent consisted of a mixture of 0.2 ml of 0.14 M ninhydrin solution prepared in a solution of carbonate–bicarbonate buffer (0.5 M, pH 9.9), and 0.1 ml glacial trichloroacetic acid (10%, Sigma-Aldrich). A homogenate sample of 100 µl was taken and introduced into the working reagent; the mixture was incubated at 60°C in a water bath for 30 min. After cooling, the mixture was introduced into a solution of copper tartrate (5 ml, Sigma-Aldrich). The mixture was kept at a temperature of 25°C for 10 min. The fluorescence resulting from the reaction between ninhydrin and GABA in the basic medium was measured using a spectrofluorometer (377/451 nm). The measured absorbance was proportional to the concentration of GABA in the homogenates. A standard GABA solution was prepared in parallel from different masses of GABA (100, 150, 200, 250, 300, 350, and 400 µg) each mixed with 1.5 mg of glutamate dissolved in 0.1 ml of ice-cold solution TCA (10% Sigma-Aldrich). The concentration of GABA in the hippocampal homogenate samples was determined by the measurement of the formed fluorescent product resulting from the reaction of GABA with ninhydrin an alkaline medium, in the presence of glutamate (Sutton and Simmonds, 1974). The GABA content in brain was expressed in mg/g of brain tissue.

GABA-T Activity

The activity of GABA-T was evaluated by the colorimetric assay method of Nayak and Chatterjee (2003). Graduated 10 ml tubes were used for this assay; 15 µmol of Ɩ-oxoglutarate, 15 µmol of GABA, 10 µg of pyridoxal phosphate, 0.1 ml of homogenate supernatant (test tubes), and 0.1 ml of methanol 5% were introduced into these tubes (white tube). The final volume of the mixture was made up to 3 ml with Tris-HCl buffer (50 mM, pH 7.4). These tubes were then incubated at 37°C for 30 min in a 96-well microplate. The reaction was finalized by adding 0.5 ml of glacial TCA (20%, Sigma-Aldrich). The semialdehyde succinic acid produced during the incubation of the mixture was estimated spectrophotometrically and the absorbance was read at 610 nm after 30 and 90 s against the blank. Staining of the semialdehyde succinic acid complex and 3-methyl-2-benzothiazole-2-hydrazone in the presence of 12% $FeCl_3$ was measured and was proportional to the concentration of GABA-T in the homogenates. The activity of GABA-T was estimated in pg/min/mg of tissue.

Statistical Analysis

Statistical analysis of the values obtained and the construction of the graphs was performed using XL Stat software: GraphPad Prism version 5.03, Microsoft Office Excel 2013 version 15.0.4420.1017. The results were expressed as mean ± standard error of means (SEM) or as percentages. The different values were compared using one-way analysis of variance (ANOVA) and when differences existed, the Tukey (HSD) multiple comparison tests were used as the *post hoc* test. $P < 0.05$ was considered significant.

RESULTS

Effects of *C. quadrangularis* on Seizures Induced by Pilocarpine

Latency to Status Epilepticus

The latency time increased significantly and dose-dependently by 31.76% ($p < 0.01$), 71.37, and 87.92% ($p < 0.001$) in the mice treated with EACQ 93; 186 and 372 mg/kg, respectively, compared to the DW + Pilo group. Latency increased to 126.41% ($p < 0.001$) with sodium valproate relative to the DW + Pilo group (**Figure 1**).

Latency to First Clonic and Tonic–Clonic Seizures 24 h After Status Epilepticus

It appears that the time of onset of the first clonic and tonic–clonic seizures in mice in the DW + Pilo groups (4 and 43 s, respectively) are not significant compared to the DW + DW group. The onset of seizure time increased significantly and dose-dependently in mice treated with EACQ. The latency times of the first tonic–clonic seizures were 393, 904, and 1422 ($p < 0.001$) at the doses 93, 186, and 372 mg/kg. Sodium valproate increased the latency to 1567 s ($p < 0.001$; **Figure 1**).

Clonic Seizures Number and Duration

Cissus quadrangularis decreased the number and duration of generalized clonic seizures from 25.8 ± 2.7 in the DW + Pilo group to 8.5 ± 0.8 ($p < 0.01$) and 4.8 ± 0.9 ($p < 0.001$) in the groups of mice administered with 186 and 372 mg/kg of *C. quadrangularis*, respectively (**Figure 3**). The number of seizure continued to decrease to 3.3 ± 0.5 ($p < 0.001$) in the group of mice administered with sodium valproate (**Figure 2**). The duration of generalized clonic seizures was reduced from 49.6 ± 4.4 s in the DW + Pilo group to 13.8 ± 1.6 and 12.8 ± 1.4 s ($p < 0.001$) in the groups of mice administered with *C. quadrangularis* at doses 186 and 372 mg/kg, respectively (**Figure 3**). Sodium valproate reduced the generalized clonic seizures to 6.3 ± 1.5 s ($p < 0.001$) compared to the DW + Pilo group of mice.

Tonic–Clonic Seizures Number and Duration

Cissus quadrangularis decreased the number and duration of generalized tonic–clonic seizures from 7.6 ± 1.2 in the DW + Pilo group to 4.1 ± 0.7 ($p < 0.01$) in the group of mice administered with 372 mg/kg of *C. quadrangularis* (**Figure 3**). The number of

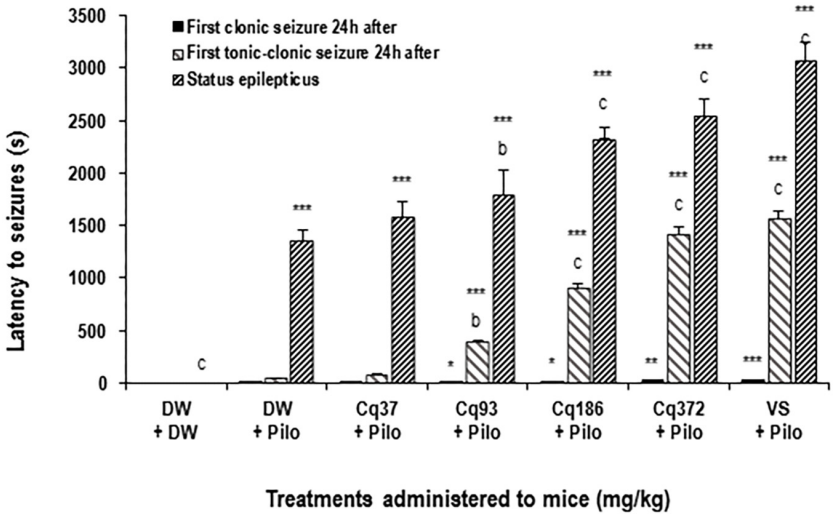

FIGURE 1 | Effects of *C. quadrangularis* extract on the latency to status epilepticus, to first clonic and tonic–clonic seizures 24 h after status epilepticus. Results are expressed as mean ± SEM for six animals. Data were analyzed by one-way ANOVA, followed by Tukey (HSD). ***$p < 0.001$, vs control animals (DW + DW group) receiving only distilled water; $^{b}p < 0.01$ and $^{c}p < 0.001$ vs disease control animals (DW + Pilo group) receiving distilled water and pilocarpine (360 mg/kg). DW, distilled water; Cq, *Cissus quadrangularis*; Pilo, pilocarpine; SV, sodium valproate (300 mg/kg). *$p < 0.05$, **$p < 0.01$.

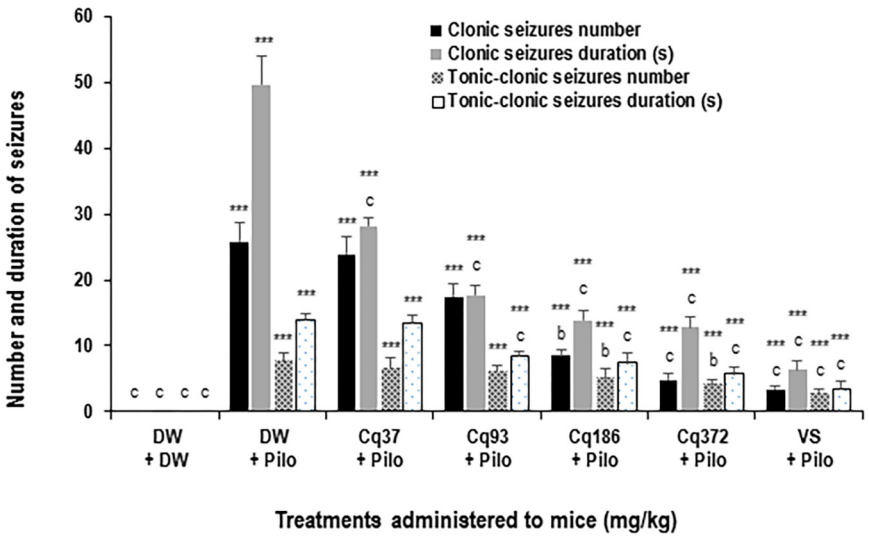

FIGURE 2 | Effects of *C. quadrangularis* extract on the number and duration of clonic and tonic–clonic seizures. Results are expressed as mean ± SEM for six animals. Data were analyzed by one-way ANOVA, followed by Tukey (HSD). ***$p < 0.001$, vs control animals (DW + DW group) receiving only distilled water; $^{b}p < 0.01$ and $^{c}p < 0.001$ vs disease control animals (DW + Pilo group) receiving distilled water and pilocarpine (360 mg/kg). DW, distilled water; Cq, *Cissus quadrangularis*; Pilo, pilocarpine; SV, sodium valproate (300 mg/kg).

seizure continued to decrease to 2.6 ± 0.8 ($p < 0.001$) in the group of mice administered with sodium valproate (**Figure 3**). The duration of generalized tonic–clonic seizures was reduced from 13.8 ± 0.9 s in the DW + Pilo group to 7.3 ± 1.5 s ($p < 0.001$) and 5.6 ± 1.2 s ($p < 0.001$) in the groups of mice administered with *C. quadrangularis* at doses 186 and 372 mg/kg, respectively (**Figure 2**). Sodium valproate reduced the generalized tonic–clonic seizures by 75.92% ($p < 0.001$) compared to the DW + Pilo group of mice.

Anxiolytic-Like Effects of *C. quadrangularis* on the EPM Test

Diazepam and *C. quadrangularis* at 372 mg/kg dose significantly increased the number of open-arm entries from 0.66 ± 0.44 in the DW + Pilo group to 3.66 ± 0.44 ($p < 0.001$) and 2.33 ± 0.44 ($p < 0.001$), respectively (**Figure 3**). The percentages of entries and time spent in the open arms increased from 10.25 and 4.27 in the DW + Pilo group up to 83.33, 83.33 for the group SV ($p < 0.001$) and 84.61, 79.94 for the DZP group ($p < 0.001$;

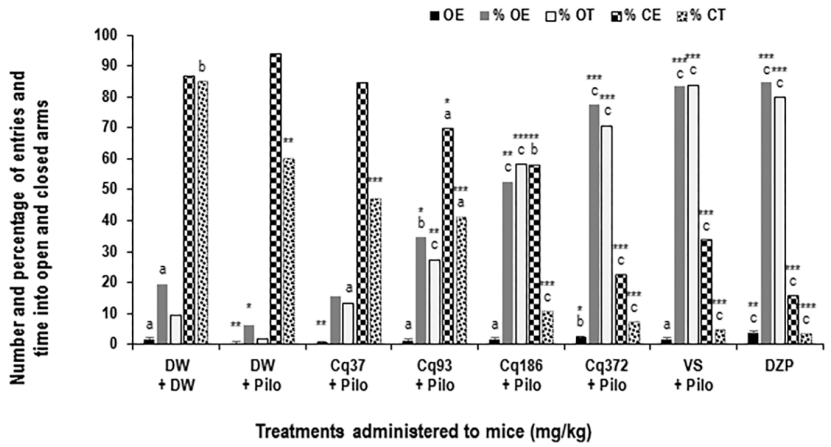

FIGURE 3 | Effects of *C. quadrangularis* extract on the number of open arm entries, on the percentages of entries and time in opened and closed arms. Results are expressed as mean ± SEM and as percentage for six animals. Data were analyzed by one-way ANOVA, followed by Tukey (HSD). *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$, vs control animals (DW + DW group) receiving only distilled water; $^{a}p < 0.05$, $^{b}p < 0.01$, and $^{c}p < 0.001$ vs disease control animals (DW + Pilo group) receiving distilled water and pilocarpine (360 mg/kg). DW, distilled water; Cq, *Cissus quadrangularis*; Pilo, pilocarpine; SV, sodium valproate (300 mg/kg); DZP, diazepam (3 mg/kg).

Figure 3). Mice treated with *C. quadrangularis* at doses 93, 186, and 372 mg/kg increased also significantly and dose dependently the percentages of entries and time spent in the open arms ($p < 0.001$; **Figure 3**). Like diazepam, *C. quadrangularis* reduced the number of closed arm entries (**Table 1**) and the percentage of entries and time in closed arms ($p < 0.001$; **Figure 3**). In addition, the numbers of rears and dips were reduced by both diazepam and *C. quadrangularis* (**Table 1**).

Anxiolytic-Like Effects of *C. quadrangularis* on the OF Test

The OF test revealed that *C. quadrangularis* increased in a dose-dependent manner, the number of crossing and grooming from 13.16 ± 1.16 and 0.66 ± 0.44 in the DW + Pilo groups to 39.83 ± 2.5 and 1.83 ± 0.55, respectively, at the dose of 372 mg/kg. The increase was also observed in the time spent by mice in the center from 2.50 ± 0.66 s in the DW + Pilo group to 32.83 ± 3.72 s at the dose of 372 mg/kg ($p < 0.001$). At the same dose of 372 mg/kg, *C. quadrangularis* decreased the number of rears and the mass of fecal boli ($p < 0.001$) and ($p < 0.001$), respectively. The effect was the same with diazepam (0.3 mg/kg; **Table 2**).

Levels of GHS, MDA, GABA, and GABA-T

GSH Level

The GSH rate decreased significantly in the DW + Pilo group (113.83 ± 1.22 mol/g tissue) compared to the DW + DW group (202.33 ± 1.33 mol/g tissue; $p < 0.001$). *C. quadrangularis* induced a dose-dependent increase of the GSH level, from 113.83 ± 1.22 mol/g of tissue to 160.16 ± 8.16 ($p < 0.001$) and 186.16 ± 4.55 mol/g of tissue ($p < 0.001$) at the doses of 186 and 372 mg/kg, respectively. Sodium valproate and diazepam groups increased GSH level to 189.66 ± 4.88 and 211.16 ± 22.27 ($p < 0.001$), respectively, relative to the DW + Pilo group (**Table 3**).

MDA Level

Malondiadéhyde level significantly increased from 129.83 ± 5.11 nmol/g tissue in the DW + DW group to 400.66 ± 4.44 nmol/g tissue in the DW + Pilo group. *C. quadrangularis* reversed the increase in MDA level from 400.66 ± 4.44 nmol/g in DW + Pilo group to 243.33 ± 4.44 ($p < 0.001$) and 141.16 ± 4.44 nmol/g of tissue ($p < 0.001$) at the respective doses of 186 and 372 mg/kg. Sodium valproate and diazepam also reversed the increase of MDA induced by pilocarpine ($p < 0.001$; **Table 3**).

GABA Level

The GABA level decreased from 397.50 ± 1.66 μg/g tissue of DW + DW to 257.16 ± 8.44 μg/g tissue ($p < 0.001$) in the DW + Pilo group. *C. quadrangularis* induced a dose-dependent increase in GABA level. The highest dose of *C. quadrangularis* induced an increase up to 393.66 + 5.77 μg/g tissue ($p < 0.001$) compared to the DW + Pilo group. The GABA level was also increased in sodium valproate and diazepam groups ($p < 0.001$), compared to the ED + Pilo group (**Table 3**).

GABA-T Activity Level

GABA-transaminase activity increased from 47.16 ± 2.11 pg/min/mg tissue in ED + ED group to 112.33 ± 4.88 pg/min/mg tissue ($p < 0.001$) in ED group + Pilo. *C. quadrangularis* reversed the increased GABA-T activity from 112.33 ± 4.88 pg/min/mg tissue in the ED + Pilo group to 63.16 ± 2.16 and 45.83 ± 2.83 pg/min/mg tissue ($p < 0.001$) at the respective doses of 186 and 372 mg/kg of the extract. Sodium valproate and diazepam also reduced GABA-T activity 52.66 + 5.11 and 50.83 + 2.83 pg/min/mg tissue ($p < 0.001$) compared to the ED + pilo group (**Table 3**).

TABLE 1 | Effect of *C. quadrangularis* in EPM test: closed arm entries, total arms entries, ratio OE/TE vs CE/TE, rearing, and head dipping.

Treatments	Doses (mg/kg)	Closed entries	Total entries	Ratio	Rearing	Head dipping
DW + DW	− + −	7.50 ± 0.83	8.66 ± 1.11	22.22	8.66 ± 0.38	2.66 ± 0.88
DW + Pilo	− + 360	5.16 ± 0.83*	5.50 ± 0.66*	06.45	14.33 ± 1.23***	5.83 ± 0.76**
Cq + Pilo	37 + 360	3.66 ± 0.44***	4.33 ± 0.66^{a}***	18.18**	7.83 ± 0.88^{c}	3.50 ± 1.16
Cq + Pilo	93 + 360	2.66 ± 0.66^{c}***	3.83 ± 0.27^{b}***	50.01^{c}*	5.83 ± 1.55^{c}	2.66 ± 2.00^{b}
Cq + Pilo	186 + 360	1.83 ± 0.61^{c}***	3.16 ± 0.88^{c}***	90.91^{c}***	3.50 ± 2.16^{c}***	2.33 ± 0.66^{c}
Cq + Pilo	372 + 360	0.66 ± 0.44^{c}***	1.66 ± 0.44^{c}***	350.01^{c}***	1.16 ± 0.94^{c}***	1.16 ± 0.55^{c}
SV + Pilo	300 + 360	0.66 ± 0.44^{c}***	2.00 ± 0.66^{c}***	250.01^{c}***	1.50 ± 0.66^{c}***	0.66 ± 0.66^{c}
DZP + DW	3 + −	0.66 ± 0.44^{c}***	4.33 ± 0.66^{a}***	550.01^{c}***	1.16 ± 0.27^{c}***	0.50 ± 0.66^{c}

*Results are expressed as mean ± SEM, N = 6. Data were analyzed by one-way ANOVA, followed by Tukey (HSD). *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$, vs control animals (DW + DW group) receiving only distilled water; $^{a}p < 0.05$, $^{b}p < 0.01$, and $^{c}p < 0.001$ vs disease control animals (DW + Pilo group) receiving distilled water and pilocarpine (360 mg/kg). DW, distilled water; Cq, Cissus quadrangularis; Pilo, pilocarpine; SV, sodium valproate (300 mg/kg); DZP, diazepam (3 mg/kg).*

TABLE 2 | Effect of *C. quadrangularis* in open field test: rearing, crossing, grooming, center time, and quantity of fecal boli.

Treatments	Doses (mg/kg)	Rearing	Crossing	Grooming	Center time (s)	Fecal boli (g)
DW + DW	− + −	6.33 ± 0.66	6.33 ± 0.83	1.50 ± 0.50	7.16 ± 0.83	0.29 ± 0.10
DW + Pilo	− + 360	10.83 ± 2.16***	13.16 ± 1.16	0.66 ± 0.44	2.50 ± 0.66	0.41 ± 0.32
Cq + Pilo	37 + 360	4.83 ± 2.16^{c}	18.66 ± 2.11***	1.33 ± 0.44	5.83 ± 0.83	0.08 ± 0.05^{a}
Cq + Pilo	93 + 360	4.33 ± 1.33^{c}	26.66 ± 2.11^{c}***	1.33 ± 0.55	11.83 ± 0.88^{c}	0.03 ± 0.04^{b}
Cq + Pilo	186 + 360	2.83 ± 0.61^{c}**	35.16 ± 2.55^{c}***	1.33 ± 0.44	23.16 ± 1.16^{c}***	0.03 ± 0.04^{b}
Cq + Pilo	372 + 360	2.50 ± 0.50^{c}**	39.83 ± 2.50^{c}***	1.83 ± 0.55	32.83 ± 3.72^{c}***	0.01 ± 0.03^{b}
SV + Pilo	300 + 360	1.83 ± 1.11^{c}***	40.83 ± 3.83^{c}***	2.33 ± 0.77^{b}	35.16 ± 4.16^{c}***	0.06 ± 0.08^{b}
DZP + DW	3 + −	1.50 ± 0.50^{c}***	49.33 ± 5.22^{c}***	2.16 ± 0.27^{b}	39.16 ± 1.88^{c}***	0.02 ± 0.03^{b}

*Results are expressed as mean ± SEM, N = 6. Data were analyzed by one-way ANOVA, followed by Tukey (HSD). **$p < 0.01$ and ***$p < 0.001$, vs control animals (DW + DW group) receiving only distilled water; $^{a}p < 0.05$, $^{b}p < 0.01$, and $^{c}p < 0.001$ vs disease control animals (DW + Pilo group) receiving distilled water and pilocarpine (360 mg/kg). DW, distilled water; Cq, Cissus quadrangularis; Pilo, pilocarpine; SV, sodium valproate (300 mg/kg); DZP, diazepam (0.3 mg/kg).*

TABLE 3 | Effects of *C. quadrangularis* on oxidative stress markers in hippocampi of pilocarpine-injected mice.

Treatments	Doses (mg/kg)	GHS (μmol/g)	MDA (μmol/g)	GABA (μg/g)	GABA-T (pg/min/mg)
DW + DW	− + −	202.33 ± 1.33	129.83 ± 5.11	397.50 ± 1.66	47.16 ± 2.11
DW + Pilo	− + 360	113.83 ± 1.22***	400.66 ± 4.44***	257.16 ± 8.44***	112.33 ± 4.88***
Cq + Pilo	37 + 360	126.16 ± 5.55***	385.16 ± 4.44^{c}***	277.16 ± 9.38^{b}***	89.83 ± 5.44^{a}***
Cq + Pilo	93 + 360	139.83 ± 3.77^{c}***	259.33 ± 9.55^{a}***	310.16 ± 2.88^{a}***	80.83 ± 6.22^{a}***
Cq + Pilo	186 + 360	160.16 ± 8.16^{a}***	243.33 ± 4.44^{a}***	369.83 ± 10.11^{a}***	63.16 ± 2.16^{a}***
Cq + Pilo	372 + 360	186.16 ± 4.55^{a}	141.16 ± 5.44^{a}	393.66 ± 5.77^{a}	45.83 ± 2.83^{a}
SV + Pilo	300 + 360	189.66 ± 4.88^{a}	130.66 ± 3.55^{a}	391.66 ± 3.33^{a}	52.66 ± 5.11^{a}
DZP + DW	3 + −	211.16 ± 22.27^{a}	133.83 ± 7.22^{a}	395.50 ± 2.16^{a}	50.83 ± 2.83^{a}

*Results are expressed as mean ± SEM, N = 6. Data were analyzed by one-way ANOVA, followed by Tukey (HSD). ***$p < 0.001$, vs control animals (DW + DW group) receiving only distilled water; $^{a}p < 0.05$, $^{b}p < 0.01$, and $^{c}p < 0.001$ vs disease control animals (DW + Pilo group) receiving distilled water and pilocarpine (360 mg/kg). DW, distilled water; Cq, Cissus quadrangularis; Pilo, pilocarpine; SV, sodium valproate (300 mg/kg); DZP, diazepam (0.3 mg/kg).*

DISCUSSION

This study aimed to show antiepileptogenic and anticonvulsant effects of *C. quadrangularis* accompanied by its anxiolytic effects since anxiety is also developed in epileptic patients (Robertson et al., 1987; Torta and Keller, 1999; Mondon et al., 2002). Administration of pilocarpine, an experimental seizures inducer, induced seizures in mice according to the Racine scale (Racine, 1972). *C. quadrangularis* administered to mice at different doses though was not able to stop the development of seizures, but it delayed the onset of epileptic status induced by pilocarpine (Kandeda et al., 2017). One hour after its administration, *C. quadrangularis* delayed the onset of SE. Also 24 h after pilocarpine administration, *C. quadrangularis* increased the latency of the first clonic and tonic–clonic seizures, and decreased the number and duration of clonic and tonic–clonic seizures. These effects similar to the effects of sodium valproate (which is an antiepileptic reference substance) show that *C. quadrangularis* possesses anticonvulsant effects. This result is in accordance with the results of Ngo Bum et al. (2008), who showed that *C. quadrangularis* has anticonvulsant or antiepileptic properties by antagonizing pentylene tetrazol, strychnine, and picrotoxine induced convulsions and inhibiting NMDA-induced behavioral excitation (Schmutz et al., 1990; Meldrum, 1992; Bum et al.,

2011). The antiepileptogenesis effect of *C. quadrangularis* was not clear since the induced epiloptogenesis was developed. Phytochemical studies of *C. quadrangularis* have revealed the presence of flavonoids (Shirley and Sen, 1966; Enechi and Odonwodo, 2003; Jainu et al., 2006), such as quercetins and kaempferols (Sen, 1966; Saburi et al., 1999; Amos et al., 2001), triterpenoids, and vitamin C. The presence of phenolic compounds and triterpenoids may partly explain the anticonvulsant activity of the plant as they are possessing actions against tonic–clonic seizures, respectively (Taviano et al., 2007).

In the EPM test, the number of entries, the percentages of entries, and time spent in the open arms increased in the presence of the extract, sodium valproate, and diazepam. In contrast, extract, valproate, and diazepam reduced the number of entries, the percentages of entries, and time spent in closed arms. An increase in the activity of mice in the open arms refers to a decrease in anxiety (Pitchaiah et al., 2008; Moto et al., 2013). And a decrease in these behavioral parameters in closed arms reflects a reduction in stress (Lister, 1990; Ngo Bum et al., 2009). This suggests that the aqueous extract of *C. quadrangularis* has anxiolytic properties. The decrease in the number of rearing and head dipping in the labyrinth, which indicates a decrease in anxiety (Rodgers et al., 1997; Ngo Bum et al., 2009), also contributes to the presence of anxiolytic effects of *C. quadrangularis*. Likewise, the effects of the extract are similar to those of diazepam (Löscher, 2012), which is a reference anxiolytic compound. The anxiolytic effects of *C. quadrangularis* would be mediated by GABAergic neurotransmission for EPM which is sensitive to benzodiazepines compounds.

The results obtained through the open arena test show that, like diazepam, the decoction increases the number of crossing, the number of grooming, and the time spent in the center of the device. The increase crossing, grooming, and time spent in the center of the open arena is a manifestation of the increase of locomotor and explorative activities in mice (Spruijt et al., 1992; Jenck et al., 1997; Kash et al., 1999; Augustsson, 2004; Ngo Bum et al., 2009; Moto et al., 2013). Because the total and closed arms entries and rearing in the EPM and rearing in OP were reduced (reduction of locomotion), the effect of the plant in this paradigm suggests mainly explorative activities which express the decrease in anxiety (File and Wardill, 1975; Jenck et al., 1997, Ngo Bum et al., 2009), so the decoction has anxiolytic properties. The significant decrease in stool production in mice treated with the decoction also suggests the presence of anxiolytic effects. Studies have shown that decreased stool mass is a result of lower stress (Rodgers et al., 1997) and any substance that reduces stress under stress conditions as imposed by the open arena is anxiolytic.

The aqueous extract of *C. quadrangularis* reduces the level of MDA and increases the level of GSH. Plasma MDA is a marker of lipid oxidation. It is known that MDA is one of the end products of the oxidation of polyunsaturated fatty acids. Therefore, a high level of MDA is an indicator of oxidative stress and cellular damage (Marnett, 1999). Similarly, a low or very high level of GSH is the manifestation of oxidative stress because GSH by reducing hydrogen peroxide prevents any deleterious effect such as lipid peroxidation (Lu, 2013). MDA and GSH are lipid peroxidation indices (lipid oxidation by ROS; Sivalokanathan et al., 2006). The drop in MDA and the increase in GSH reveal an antioxidant effect of the plant (Pryor and Stanley, 1975; Neve et al., 1989; Marnett, 1999; Sivalokanathan et al., 2006). Quercetin has been shown to have antioxidant activity. Quercetin decreases the production of ROS during moderate oxidative stress, improves the viability of H2O2-exposed neurons, and effectively inhibits cell apoptosis and hippocampal nerve cells (Suematsu et al., 2011; Jazvinscak Jembrek et al., 2012). Quercetin may partly explain the antioxidant effect of *C. quadrangularis*. The increase in GABA level and a decrease in GABA-T activity by the aqueous extract of *C. quadrangularis* were observed and suggest the interaction of the plant extract with the gabaergic neurotransmission, in the anticonvulsant effects of the plant. Sodium valproate induces the increase of cerebral GABA by the inhibition of semialdehyde succinyl dehydrogenase (a GABA degrading enzyme or GABA-T) or by the activation of GABA synthesis by glutamic acid decarboxylase (Hashimoto et al., 2006). This enzyme decreases the level of GABA in the brain and at the same time increases the level of the excitatory neurotransmitter glutamate, thereby causing the excitation of neurons (Johannessen, 2000). These results suggest that the extract would be able to restore and maintain the balance between neuronal excitation and inhibition and thus have anticonvulsant and anxiolytic activities.

CONCLUSION

In this work, the effects of AECQ on pre- and post-SE seizures, anxiety disorders, and oxidative activity of mice in epileptogenesis were shown. AECQ significantly increased pre- and post-SE latency and decreased the number and duration of tonic–clonic seizures. The EACQ increased the number of entries and the time spent in the open arm of the EPM. Decreases in GSH and GABA levels and increases in MDA and GABA-T levels were normalized in AECQ-treated mice. AECQ has been shown to be an anticonvulsant extract with anxiolytic effects in animal models of epilepsy.

AUTHOR CONTRIBUTIONS

FM, JN, AA, and GT performed all behavioral studies, accomplished the data analysis, and drafted the manuscript. AK, ENB, SP, and GT designed the study. ENB critically revised the manuscript for important intellectual content. FM, GwN, GiN, JN, JOO, SP, and NK helped in *in vivo* studies. All authors read and approved the final manuscript.

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Genetically Epilepsy-Prone Rats Display Anxiety-Like Behaviors and Neuropsychiatric Comorbidities of Epilepsy

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Epilepsy is associated with a variety of neuropsychiatric comorbidities, including both anxiety and depression. Despite high occurrences of depression and anxiety seen in human epilepsy populations, little is known about the etiology of these comorbidities. Experimental models of epilepsy provide a platform to disentangle the contribution of acute seizures, genetic predisposition, and underlying circuit pathologies to anxious and depressive phenotypes. Most studies to date have focused on comorbidities in acquired epilepsies; genetic models, however, allow for the assessment of affective phenotypes that occur prior to onset of recurrent seizures. Here, we tested male and female genetically epilepsy-prone rats (GEPR-3s) and Sprague-Dawley controls in a battery of tests sensitive to anxiety-like and depressive-like phenotypes. GEPR-3s showed increased anxiety-like behavior in the open field test, elevated plus maze, light-dark transition test, and looming threat test. Moreover, GEPR-3s showed impaired prepulse inhibition of the acoustic startle reflex, decreased sucrose preference index, and impaired novel object recognition memory. We also characterized defense behaviors in response to stimulation thresholds of deep and intermediate layers of the superior colliculus (DLSC), but found no difference between strains. In sum, GEPR-3s showed inherited anxiety, an effect that did not differ significantly between sexes. The anxiety phenotype in adult GEPR-3s suggests strong genetic influences that may underlie both the seizure disorder and the comorbidities seen in epilepsy.

Keywords: anxiety, comorbidity, seizures, audiogenic seizures, depression, rat models

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INTRODUCTION

Epilepsy is associated with a variety of neuropsychiatric comorbidities, including both anxiety and depression (1, 2). Despite the presence of both comorbidities in epilepsy, most studies have focused solely on the depression phenotype, resulting in reference to anxiety as the "forgotten psychiatric comorbidity" (3). In fact, persons with epilepsy have

an approximately two-fold increase in prevalence of generalized anxiety disorder (2), which may contribute to reduced quality of life (4). This underscores the need for further examination of anxiety in epilepsy and accentuates the importance of investigating common pathophysiology of both anxiety and epilepsy.

The etiology of anxiety in epilepsy remains unresolved. We suggest that at least two hypotheses can explain the high rate of comorbidity, (1) the pathology and circuit disruptions that lead to seizures also lead to the emergence of anxiety, and (2) recurrent seizures lead to emergence or exacerbation of anxiety [for further discussion of the "chicken or egg" problem with respect to epilepsy and comorbidities, see (5)]. In the clinic, the contributions of these factors are difficult to dissociate, necessitating preclinical models in which these features are separable. Here, rodent models of epilepsy provide a method for this assessment. In several strains of rats with inherited epilepsies, behavioral comorbidities (most notably depressive-like symptoms) have been reported; these include the genetic absence epilepsy rats from Strasbourg (GAERS) (6) and Wistar Albino Glaxo/Rijswijk (WAG/Rij) rats (7), both strains that display spontaneous absence seizures. However, in these studies, behavior was assessed after the onset of spontaneous seizures (i.e., on the background of repeated seizure history), making it impossible to dissociate the contribution of ongoing seizure activity and that of underlying genetics. Thus, while these data support the hypothesis that seizure activity *per se* can modulate the expression of anxiety-like and depression-like behaviors, they do not directly address the role of underlying pathology or genetic predisposition.

Acoustically evoked seizure (or audiogenic seizure, AGS) susceptible strains offer an opportunity to evaluate the contribution of genetic predisposition of seizures. In these models, behaviors can be assessed in animals that have no or minimal seizure history (8). One strain of interest is the genetically epilepsy-prone rat (GEPR-3) that exhibits inherited susceptibility to tonic-clonic seizures. The seizure susceptibility in GEPR-3s is associated with a deficit in both noradrenergic and serotonergic signaling, a profile similar to humans with depression (9, 10). Moreover, we have recently reported volumetric alterations in midbrain networks associated with defense behavior and anxiety in GEPR-3s (11). Finally, casual observations across independent laboratories using the GEPR-3s have reported increased aggression and anxiety-like responses to human handling. However, no experimental data have been published to confirm anxiety or depression-like comorbidities in this strain.

To address these gaps in knowledge, we evaluated the profile of anxiety- and depression-like behaviors in female and male adult GEPR-3s, as compared to female and male Sprague-Dawley (SD) rats. Despite the prevalence of sex-specific differences in neuropsychiatric disorders and the relationship they may have to incidence of epilepsy (3, 12, 13), sex has not been evaluated as a factor in prior studies of epilepsy comorbidity in animal models.

MATERIALS AND METHODS

Animals

Three-month-old male and female SD rats and GEPR-3s were used (10–12 per group). The same animals were tested on a within-subject basis on the tasks described below. SD rats were obtained from Harlan Labs and GEPR-3s were obtained from our animal colony maintained at Georgetown University. All animals were housed in a temperature/ humidity-controlled room on a 12 h/12 h light/dark cycle with free access to food and water. All efforts were made to minimize the number of animals used in these experiments. This study was carried out in accordance with the recommendations of the NIH Guide for the Care and Use of Laboratory Animals. The protocol was approved by the Georgetown University Animal Care and Use Committee.

Confirmation of Audiogenic Susceptibility

To ensure penetrance of AGS phenotype, GEPR-3s were tested once for seizures at postnatal day 21 (PND 21). GEPR-3s are exposed to an acoustic stimulus (100–110 decibels, sound pressure levels pure tones) that was presented until seizure was elicited (or 60 s if no seizure was observed) (14). All GEPR-3s exhibited wild running that evolved into bouncing tonic-clonic seizures. Although GEPR-3s have been exposed to one AGS episode as a required screening test for inherited seizure susceptibility, they are naïve to repetitive AGS. It is unlikely that a single AGS episode at PND 21 can account for all subsequent comorbidity phenotypes seen at PND 90, however, we acknowledge the limitation of this methodology. In future studies, examining seizure susceptibility *after* the completion of behavioral testing would allow address this possible concern.

Behavioral Testing

All behavioral tests were performed consecutively, on a within-subject basis, in the order described below. Twenty to forty eight hours elapsed between two tests. Prior to each test day, animals were transported from the vivarium to the testing room. Animals were allowed a minimum of 30 min to acclimate to the testing environment prior to initiating testing. The test apparatuses were sanitized (with 70% ethanol solution) between animals. Behavioral tests were conducted in the order specified below, i.e., Open Field test, Elevated Plus Maze (EPM), Light-Dark Transition test, Looming Threat test, Acoustic Startle response, Sucrose Preference test, Novel Object Recognition test, Electrical stimulation of DLSC. Ten to 15 days after the end of behavioral tests, male GEPR-3s ($n = 10$) and SD rats ($n = 10$) were implanted with a single electrode targeted to medial deep layers of the superior colliculus (DLSC) to evaluate defense responses caused by midbrain activation (see below for details).

Open Field Test

Open field testing was performed to measure spontaneous activity in rodents (15, 16). In this test, the desire to explore the novel arena is pitted against the species-typical response to avoid open spaces. Animals were placed into a Plexiglass enclosure ($16'' \times 16'' \times 16''$, TruScan Arena, Coulbourn Instruments,

Whitehall, PA) with 775 lux illumination over the center of the arena. A square (8.5″ × 8.5″) was drawn in the floor of the arena, forming the "inner" portion of the open field. Animals were allowed to explore for 10 min, during which total distance traveled and inner/outer entries were recorded using ANYmaze software (Stoelting Co., Wood Dale, IL), as previously described (17). Data for one animal (male, SD rat) were not recorded by the tracking software, thus this animal was excluded from open field analysis and subsequent correlation analyses.

Elevated Plus Maze

Elevated plus maze (EPM) testing was performed and scored as previously described (18, 19) using a standard gray rat EPM (50 cm arms, elevated 40 cm off the ground (Stoelting Co., Wood Dale, IL). This test pits the desire to explore the novel maze against the species-typical preference to avoid open, elevated spaces as compared to enclosed spaces, and has been established as a tool for assessment of anxiety in rats (20, 21). Testing was conducted under 20 lux red light. The test lasted 300 s. The number of arm entries, time spent in open and closed arms, head pokes from the closed to open arms, head dips off the maze from the open arms, and stretch-attend postures were recorded using ANYmaze software (Stoelting Co, Wood Dale, IL). Based on previous studies in epilepsy models, head pokes and dips were used as an ethological parameter (22, 23). Stretch-attend posture was categorized as either "protected" when occurring when the body was positioned in the closed arms, or "unprotected" when the body was positioned in the open arms.

Light-Dark Transition Test

Light-dark transition testing was conducted as we have previously described (24, 25). As with the EPM, this test pits rats' innate aversion to bright areas against their natural drive to explore in response to mild stressors such as a novel environment. While originally described as a test of anxiolytic sensitivity in mice, this task has also been validated in rats (26, 27). Animals were placed into a testing apparatus (San Diego Instruments) that was half open and half covered by a black box with an opening for animals to enter. Ambient illumination of the room was 775 lux. Animals are initially placed in the light side of the apparatus facing the door to the "dark" chamber and filmed for 5 min. Latency to initially cross into the dark side of the apparatus, total time spent in the dark part of the box, and total crossovers between the light and dark sides were scored. Video was recorded via ANY-maze and hand-scored by an observer blinded to treatment status of the animals.

Looming Threat Test

The looming threat test is modified from prior reports looking at the circuitry underlying unconditioned defense responses (28, 29). This test measures the species-typical reflex response to looming stimuli, i.e., freezing responses. In rodents, predators often strike from above, and an expanding visual stimulus thus triggers reflexive defense responses. Animals were placed into a transparent chamber (43.5 cm high × 18.5 cm diameter) with a computer screen placed above and a video camera placed beside to record changes in behavior. After a 2-min baseline period (solid gray screen, 23 lux) stimulus presentation was initiated. The stimulus consisted of a black dot which expanded from 2 to 20 degrees of visual angle over 250 ms. After reaching maximum size, the dot remained stable in size for 250 ms and then disappeared. This stimulus was repeated 15 times over a 22 s period with a 500 ms inter-stimulus interval. After the stimuli, the gray screen is presented until the experiment ends at 3 min; testing was conducted under 20 lux red light. Following behavioral testing, the videos were truncated into equivalent length periods (22 s each) and manually scored for freezing behavior by a blinded observer using the ANYMaze software. Freezing was defined as "ceasing" all activity, maintaining an attentive attitude at first, with head raised, eyes open, and body in the same position" (30).

Acoustic Startle Response, Startle Habituation, and Prepulse Inhibition

The acoustic startle (ASR), startle habituation, and prepulse inhibition (PPI) protocols were adapted from our previous studies (31). All testing occurred within three sound attenuated startle chambers (SR-Lab Startle Reflex System; San Diego Instruments, San Diego, CA). The 15 min sessions consisted of a 5-min acclimation period with background noise (70 dB), 5 habituating startling stimuli (105 dB; 40 ms pulse), 2 blocks startling stimuli (93–123 dB, 40 ms pulse), 6 blocks of pseudorandom trials containing pulse-alone (105 dB; 40 ms) and prepulse-pulse (prepulses: 4, 8, and 12 dB above background noise; 20 ms), and 5 min post-test startling stimuli (105 dB; 40 ms pulse). During the prepulse-pulse trials, an inter-stimulus interval of 50 ms (onset to onset) was used. The inter-trial interval ranged from 15–30 s, randomly selected for each trial. Startle amplitude was defined as the peak piezoelectric accelerometer output over a 175 ms period beginning at the onset of the pulse stimulus.

Sucrose Preference Test

The sucrose preference test used in this study was a modified version of the test previously described (15). Sucrose preference was measured over 5 days as followed: 4 consecutive days of 2 h exposure and 1 day of 2 h water restriction followed by 2 h exposure. Two bottles were available in each cage, one with 200 ml of 1% sucrose (w/v) and the other with 200 ml of tap water. At the end of the 2 h, the bottles were removed; consumption was noted, and the animals were returned to their previous housing conditions. Preference was measured as follows: total sucrose consumption (ml) / total sucrose consumption (ml) + total water consumption (ml).

Novel Object Recognition Test

The novel object recognition test (NORT) was a modified version of the test previously described by Bhardwaj et al. (32) and Ennaceur et al. (33). Novel object recognition test was performed in parallel in 4 enclosures (16″ × 16″ × 16″, TruScan Arena, Coulbourn Instruments, Whitehall, PA). In order to standardize the salience of the objects, 2 options were printed on a commercial 3D printer (TAZ 6, Lulzbot): red cylinder (3.8 cm diameter, 3.5 cm height, untextured) and blue square (3.5 cm

base length, 3.5 cm height, textured). Additionally, a set of gray objects were printed for test habituation: a half-egg (3.5 cm diameter, 3.5 cm height) and a pyramid (3.5 cm base length, 3.5 cm height).

The test consisted of habituation, training, and testing phases. For each test, objects were placed 20 cm apart, in the center of the cage. Rats were placed in the center of the box equidistant from both objects and preferences for objects were determined. The habituation and acquisition phases of the novel-object recognition test were each 5 min long, with a 5-min interval between the phases. The test phase was conducted 2 h after acquisition phase and also lasted 5 min. The three objects were randomly selected for each animal, and the cage placement of objects was randomized (left vs. right). After each run, objects and boxes were cleaned with 70% (v/v) ethanol to prevent odor cues. Automatic tracking of rats was performed with the detection of multiple body points (nose, middle, and tail) of the rat using the ANYMaze software. The time when the rat's nose was 2 cm from the object was defined as object exploration. The preference ratio (PR) for each animal was calculated from the time spent exploring the novel object (N) and the familiar object (F) during the test phase: $PR = 100 \times (N)/(N + F)$. Animals that failed to explore the objects for at least 10 s during the initial study phase were excluded from subsequent analyses (34).

Electrode Implantation

Twenty male animals (10 SD rats, 10 GEPR-3s) were implanted with a bipolar (twisted pair of stainless steel) electrodes (PlasticsOne) unilaterally targeting the DLSC 10–15 days after completion of all behavioral tests. SD rats and GEPR-3s were anesthetized with equithesin (a combination of sodium pentobarbital, chloral hydrate, magnesium sulfate, ethanol, and propylene glycol; 2.5 ml/kg, i.p.). Following anesthesia induction, animals were placed in a stereotaxic frame with animals positioned in the skull-flat plane. Electrodes were implanted in the DLSC using the coordinates (6.24 mm posterior to bregma, 1.0 mm lateral to the midline, and 3.7 mm ventral to the dura) from the atlas of Paxinos and Watson (35). Electrodes were fixed to the skull with three jeweler's screws using dental acrylic (Kooliner, GC America, Alsip, IL). Following recovery from anesthesia, rats were given caprofen (5 mg/kg, s.c.) as an analgesic and 1 ml warm normal saline (s.c.) to maintain hydration.

Electrical Stimulation of DLSC

One week after surgery, electrical stimulation of DLSC was conducted as described in Sahibzada et al. (36). Animals were placed into a circular acrylic chamber (30 cm diameter) and the implanted electrode was connected via a commutator (PlasticsOne) to a constant current stimulus isolation unit (AM Systems) triggered by a pulse generator (PulsePal). The testing session was comprised of a series of stimulations of ascending current amplitude, spaced a minimum of 15 s apart. The stimulation consisted of a 1 s train of negative monopolar square-wave pulses (0.2 ms) at a frequency of 100 Hz ranging in amplitude from 10 to 200 μA. After stimulation, the behavioral response was recorded, and the stimulating current was increased by 10 μA for the subsequent trial. A testing session was terminated either when the stimulating current reached 200 uA or when the animal displayed an escape behavior. Behaviors scored were binned into 3 categories: orienting, locomotion, and escape. Orienting was defined as a contralateral turning of the head, sometimes including turning of the body or circling behavior. Locomotion was defined as walking forward or "scooting," a behavior that appeared as a walk with a small jump included. Escape behavior was defined as cringing or flinching movements, running, and jumping.

Histology

Following the completion of all testing, rats were overdosed with deep equithesin (4 ml/kg) anesthesia and decapitated. Brains were fixed in 4% paraformaldehyde for a minimum of 72 h. After fixation, brains were cryoprotected in sucrose solution (30%) and frozen. Coronal brain sections (40 μm thick) were cut on a cryostat (Reichert Model 975C) and stained with cresyl violet acetate. Microscopic examination was performed to verify the location of electrode placement in DLSC according to the Swanson Brain Atlas (37). Electrode placement was performed blind to other data (behaviors evoked, stimulation thresholds).

Statistics and Data Analysis

Statistical analyses were performed in GraphPad Prism (GraphPad Software, Inc., La Jolla, CA) and SPSS (Ver 25, IBM Corp). Open field, EPM, light-dark transition test, and sucrose preference test data were analyzed by two-way analysis of variance (ANOVA) with sex and strain as between-subject factors. PPI and startle amplitude data were analyzed by three-way ANOVA with sex and strain as between-subject factors and prepulse (or pulse) intensity as a repeated measure. Looming threat data were analyzed by three-way ANOVA with sex and strain as between-subject factors and test phase as a repeated measure. Startle habituation was analyzed by two-way ANOVA and by one-sample *t*-test against a test value of 1 (indicating no habituation). NORT data were analyzed by unpaired *t*-test (comparing SD rats and GEPR-3s) and by a one-sample *t*-test comparing performance to chance levels (test value 0.5). The proportion of animals that failed to explore objects during the test were analyzed by Fisher's Exact Test. Behaviors evoked by DLSC-stimulation were analyzed by two-way ANOVA with strain as a between-subject variable and behavioral category as a repeated measure. Correlations were assessed using Spearman's correlation on ranks, followed by the Benjamini-Krieger-Yukutieli's correction for false discovery rate ($Q = 5\%$). Pairwise comparisons following all ANOVAs were analyzed using the Holm-Sidak correction for familywise error rate. *P*-values of < 0.05 were considered to be statistically significant.

RESULTS

Open Field Test

Total locomotor activity in the open field did not differ as a function of strain (no main effect of strain, $P = 0.97$; **Figure 1A**). However, in both SD rat and GEPR-3 strains, males explored the arena to a lesser degree than did females [$F_{(1, 39)} = 9.0$, $P = 0.005$; **Figure 1A** Holm-Sidak post-tests, $Ps < 0.05$]. As a measure of anxiety-like behavior, we also measured the time spent exploring

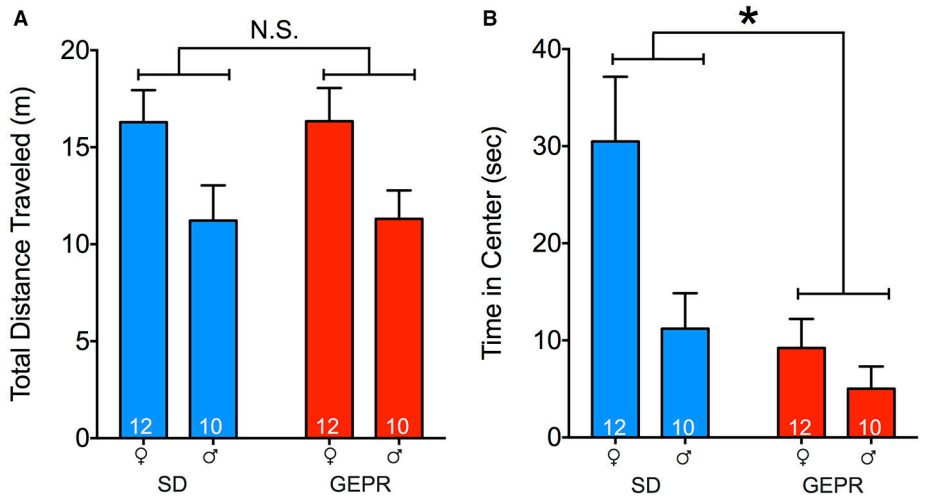

FIGURE 1 | Open field test. **(A)** Total distance traveled (meters) for the duration of the test (10 min); males of both strains explored the arena less than females [$F_{(1,39)} = 9.0$, $P = 0.005$; Holm-Sidak post-tests, $Ps < 0.05$]. **(B)** Total time spent exploring the center (8.5″ × 8.5″) of the open field. GEPR-3s explored less than SD rats [$^{*}F_{(1,39)} = 9.2$, $P = 0.004$], and males explored less than females [$F_{(1,39)} = 6.7$, $P = 0.01$]. Post-tests also showed a decrease in exploration of the center of the arena in female GEPR-3s relative to SD rats (Holm-Sidak corrected, $P < 0.05$). Figures show mean and standard error of the mean.

the center of the open field. There was a significant main effect of strain [$F_{(1,39)} = 9.2$, $P = 0.004$; **Figure 1B**], a significant main effect of sex [$F_{(1,39)} = 6.7$, $P = 0.01$; **Figure 1B**], but no sex-by-strain interaction ($P = 0.1$). Post-tests revealed a significant decrease in exploration of the center of the arena in female GEPR-3s as compared to female SD rats (Holm-Sidak corrected, $P < 0.05$). This did not reach the level of significance for males, likely due to a floor effect, as male SD rats explored the center of the arena for only a third of the time of females.

Elevated Plus Maze

In the EPM, we detected a borderline-significant main effect of strain on total maze exploration [$F_{(1,40)} = 3.9$, $P = 0.055$; **Figure 2A**], but neither a main effect of sex, nor a strain-by-sex interaction ($P > 0.4$ and $P > 0.8$, respectively). Time spent in the open arms of the EPM did not differ by either strain or sex ($Ps = 0.18$ and 0.23, respectively; **Figure 2B**). As a second measure of anxiety-like behavior, we examined the number of head pokes into the open arms and found a main effect of strain [$F_{(1,40)} = 5.2$, $P = 0.03$; **Figure 2C**] but neither a main effect of sex nor a strain-by-sex interaction ($Ps = 0.9$ and 0.8, respectively). Total arm entries did not differ by either strain or sex [$F_{(1,40)} = 0.6$ and 0.5; $Ps = 0.5$, respectively; **Figure 2D**]. Open arm entries relative to total arm entries (% open arm entries; **Figure 2E**) differed by sex [$F_{(1,40)} = 7.0$, $P = 0.01$] but showed no effects of strain or strain-by-sex interaction [$F_{(1,40)} = 0.3$ and 0.2; $Ps = 0.6$, respectively]. Average duration of open arm visit differed by strain [$F_{(1,24)} = 7.04$, $P = 0.01$] and showed a significant sex-by-strain [$F_{(1,24)} = 5.499$, $P = 0.03$] interaction, driven by males ($P = 0.009$; **Figure 2F**). Finally, we assessed head dips off the open arms (**Figure 1G**). We found a main effect of sex [$F_{(1,40)} = 7.04$, $P = 0.01$] and strain [$F_{(1,40)} = 13.7$, $P = 0.0006$], and a borderline significant interaction [$F_{(1,40)} = 3.6$, $P = 0.06$]. Strain differed significantly only in females ($P = 0.003$) but not males ($P = 0.24$), and sex differences were only evident in SD rats ($P = 0.0009$) but not GEPR-3s ($P = 0.29$). Accordingly, female GEPRs displayed fewer head dips than did female SD rats ($P = 0.008$), consistent with increased anxiety-like behavior. Finally, we examined stretch-attend postures, an ethological measure of risk-assessment. The number of stretch-attend posture counts were divided into "protected" (when the animal was in the closed arms) and "unprotected" (while the animal was in the open arms). More anxious animals would be expected to have a higher stretch-attend phenotype in the closed arms. Indeed, SD rats has a higher frequency of this behavior as compared to GEPR-3s [main effect of strain: $F_{(1,24)} = 6.5$, $P = 0.018$; **Figure 2H**]. There was a trend toward an effect of sex [$F_{(1,24)} = 3.3$, $P = 0.08$] but no strain-by-sex interaction ($P = 0.8$). An increased number of stretch-attend posture counts observed while the animal was in the "protected" portion of the maze (i.e., the closed arms) may heightened risk-assessment (38–40). There was a trend toward an effect of strain [$F_{(1,40)} = 2.9$, $P = 0.098$; **Figure 2I**] with GEPR-3s exhibiting a greater number of protected stretch-attend posture frequency than SD rats. There was neither an effect of sex, nor a strain by sex interaction ($Ps = 0.81$ and 0.67, respectively).

Light-Dark Transition Test

Figure 3 shows time spent in the light compartment in the light-dark transition test. Consistent with the plus maze and open field, GEPR-3s displayed increased anxiety-like behavior in this test, as is evident from reduced time spent in the light compartment [main effect of strain: $F_{(1,40)} = 10.1$, $P = 0.003$]. In addition to the main effect of strain, we

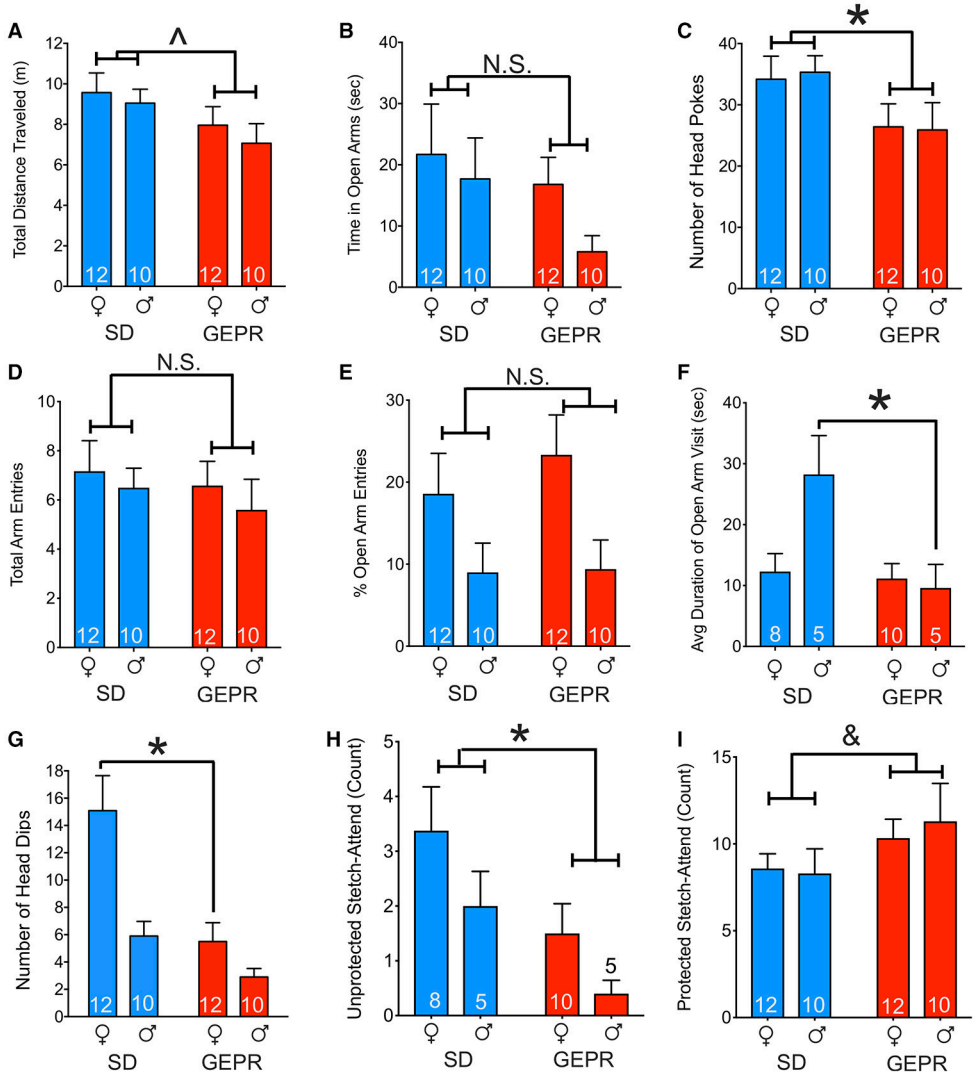

FIGURE 2 | Elevated plus maze. **(A)** Total distance traveled in the maze for the duration of the test (300 s); GEPR-3s trending toward traveling less [$\wedge F_{(1,40)} = 3.9$, $P = 0.055$]. **(B)** Time spent in the open arms of the EPM did not differ by either strain or sex (*Ps* = 0.18 and 0.23, respectively). **(C)** GEPR-3s displayed a decrease in number of head pokes into the open arms [$^{*}F_{(1,40)} = 5.2$, $P = 0.03$], but there was not an effect of sex. **(D)**, Total arm entries throughout the duration of the test did not differ significantly by either strain or sex (*Ps* = 0.50 and 0.46, respectively). **(E)** Percent open arm entries differed by sex [$F_{(1,40)} = 7.0$, $P = 0.01$] but showed no effects of strain or strain-by-sex interaction (*Ps* = 0.56 and 0.63, respectively). **(F)** Average duration of open arm visit differed by strain [$F_{(1,24)} = 7.04$, $P = 0.01$] and showed a significant interaction of sex-by-strain [$F_{(1,24)} = 5.499$, $P = 0.03$], driven by males ($^{*}P = 0.009$). **(G)** Number of head dips off the open arms differed by sex [$F_{(1,40)} = 7.04$, $P = 0.01$] and strain [$F_{(1,40)} = 13.7$, $P = 0.0006$]. The strain effect driven by females ($P = 0.003$). Accordingly, female GEPR-3s displayed fewer head dips than did female SDs ($^{*}P = 0.008$). **(H)** Number of stretch-attend posture counts observed while the animal was in the "unprotected" portion of the maze (i.e., the open arms). SD rats has a higher frequency of this behavior as compared to GEPR-3s [$^{*}F_{(1,24)} = 6.5$, $P = 0.018$]. There was a trend toward an effect of sex [$F_{(1,24)} = 3.3$, $P = 0.08$] but no strain-by-sex interaction ($P = 0.8$). **(I)** Number of stretch-attend posture counts observed while the animal was in the "protected" portion of the maze (i.e., the closed arms). There was a trend toward an effect of strain [$^{\&}F_{(1,40)} = 2.9$, $P = 0.098$] with GEPR-3s showing a greater number of this risk-assessing behavior than SD rats. There was neither an effect of sex, nor a strain by sex interaction (*Ps* = 0.81 and 0.67, respectively). Figures show mean and standard error of the mean.

found a main effect of sex [$F_{(1,40)} = 22.8$, $P < 0.0001$], with male animals spending less time in the light than female animals. We did not find a strain-by-sex interaction ($P = 0.3$). Pairwise comparisons indicated that this sex difference reached the level of significance for both strains (*Ps* < 0.05, Holm-Sidak corrected), and that the strain difference reached the level of significance for females, but not male animals ($P < 0.05$, Holm-Sidak corrected). As with the open field, the lack of strain effect in male animals may be due to a floor effect.

Looming Threat Test

In the looming threat test (**Figure 4**), we observed freezing as a measure of anxiety-like behavior during the: baseline period,

presentation of looming stimulus, and in the post-stimulus period. We found a main effect of test period [$F_{(1.6, 63.8)} = 113.1$, $P = 1 \times 10^{-19}$], a main effect of strain [$F_{(1, 40)} = 11.7$, $P = 0.001$], but no main effect of sex ($P = 0.12$). In addition, we observed significant stage-by-sex [$F_{(1.6, 63.8)} = 4.2$, $P = 0.03$] and stage-by-strain [$F_{(1.6, 63.8)} = 24.2$, $P = 0.00000003$] interactions, but no other significant two or three-way interactions ($Ps > 0.1$). Pairwise comparisons revealed no strain differences during the baseline and stimulus presentation period, but a significant increase in freezing in GEPR-3s as compared to SD rats during the post-stimulus period (Holm-Sidak Adjusted, Females: $P = 0.0004$ and Males: $P = 0.0007$).

Acoustic Startle Response, Startle Habituation, and Prepulse Inhibition

As shown in **Figure 5A**, with increasing intensity of white-noise pulse, startle amplitude increased. The amplitude of the startle response was normalized to the maximum startle response within each subject to control for chamber-to-chamber variability in startle amplitude. We found a main effect of pulse intensity [$F_{(1.6, 64.6)} = 23.4$, $P = 0.0000001$], but no effect of either strain or sex ($Ps = 0.5$ and 0.9, respectively), nor any two- or three-way interactions ($Ps > 0.2$). As a second measure, we examined habituation of startle within a session (**Figure 5B**). We found that all groups showed the normal profile of habituation to the startling stimulus, except for the female GEPR-3s ($P < 0.01$, one sample t-test when compared to theoretical mean of 1.0). The main effect of strain approached but did not reach statistical significance [$F_{(1, 40)} = 3.2$, $P = 0.085$]; there was not a main effect of sex [$F_{(1, 40)} = 2.513$, $P = 0.1208$], nor an interaction [$F_{(1, 40)} = 0.0089$, $P = 0.9$]. The magnitude of startle response did not differ as a function of either sex or strain [Sex: $F_{(1, 40)} = 0.05$, $P = 0.9$, Strain: $F_{(1, 40)} = 0.4$, $P = 0.5$].

We next assessed PPI (**Figure 6**), which measures a decrease in whole-body startle response when a startling stimulus is preceded by a low-intensity noise pulse. Analysis of PPI revealed the expected main effect of prepulse intensity [$F_{(2.3, 93.1)} = 82.2$, $P = 5 \times 10^{-23}$], as well as a main effect of strain [$F_{(1, 40)} = 43.5$, $P = 0.00000007$]. However, we found neither a main effect of sex, nor any significant two- or three-way interactions ($Ps > 0.08$). Collapsed across prepulse intensity, there were significant differences between the strains within each sex [Female: $F_{(1, 40)} = 13.0$, $P = 0.001$, Male: $F_{(1, 40)} = 31.7$, $P = 0.000002$]. Pairwise comparisons across strain for each sex revealed significant impairment in PPI at each prepulse intensity for male GEPR-3s as compared to male SD rats ($Ps < 0.002$, Holm-Sidak adjusted). For female GEPR-3s as compared to female SD rats, this effect was evident at lower prepulse intensities (PP3: $P = 0.005$; PP6 $P = 0.02$), but not higher prepulse intensities (PP9 and PP12 $Ps = 0.1$).

Sucrose Preference Test

To determine if comorbidities in the GEPR-3s extend beyond anxiety-like behavior and into symptoms related to depression, we next assessed hedonic response in the sucrose preference test. We calculated a sucrose preference ratio (vol sucrose consumed/vol water consumed) with total volume cumulated over the 5-day period of testing. We found a main effect of sex, with GEPR-3s displaying a significantly lower sucrose preference than SD rats [$F_{(1, 40)} = 29.3$, $P < 0.0001$; **Figure 7A**], but neither a main effect of sex, nor a sex-by-strain interaction ($Ps = 0.96$ and 0.5, respectively). Pairwise comparisons revealed that the decreased sucrose preference in GEPR-3s was significant in both sexes (female: $P = 0.001$, male: $P = 0.003$, Holm-Sidak corrected). Total volume consumed across days differed between strains [$F_{(1, 40)} = 9.768$, $P = 0.0033$; **Figure 7B**], with GEPR-3s consuming significantly less than SD rats, but not by sex [$F_{(1, 40)} = 0.4239$, $P = 0.5187$; **Figure 7B**].

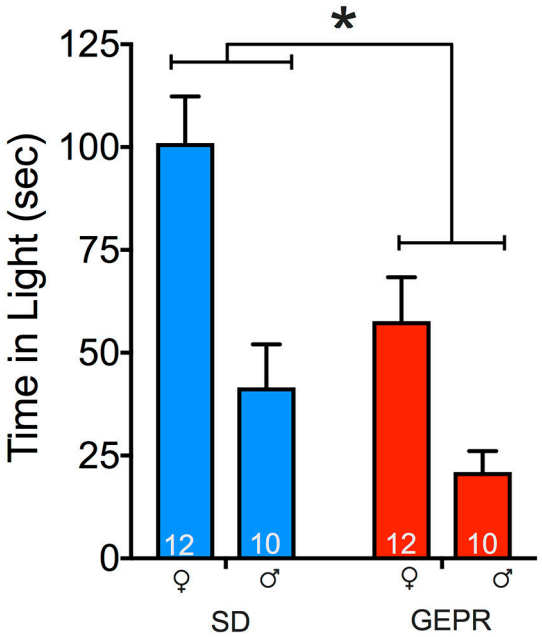

FIGURE 3 | Light-dark transition test. GEPR-3s displayed reduced time spent in the light compartment [$^*F_{(1, 40)} = 10.1$, $P = 0.003$] and males spend less time in the light than females [$F_{(1, 40)} = 22.8$, $P < 0.0001$]. Animals were initially placed in the light side of the apparatus; total test time was 5 min. Pairwise comparisons indicated a sex effect in both strains ($Ps < 0.05$, Holm-Sidak corrected), and the strain difference was significant for females, but not males ($P < 0.05$, Holm-Sidak corrected). Figures show mean and standard error of the mean.

Novel Object Recognition Test

Because cognitive impairment has also been reported as a comorbidity of epilepsy, we next examined the performance of GEPR-3s as compared to SD rats in the NORT (**Figure 8A**). To our surprise, only a small proportion of GEPR-3s explored the objects, perhaps due to high levels of anxiety (**Figures 8B,C**). The proportion of animals that failed to explore the objects was significantly greater in the GEPR-3 strain as compared to the SD rat strain ($P = 0.006$, Fisher's Exact Test). Because of this attrition, we collapsed across sex for the recognition memory trial (**Figure 8A**). While SD rats showed the expected preference for the novel objects (one sample t test, $t = 4.1$, $df = 17$, $P = 0.0008$), GEPR-3s did not (preference ratio did not differ significantly from chance, $P = 0.9$). Preference ratio trended toward but did not reach the level of statistical significance between these two

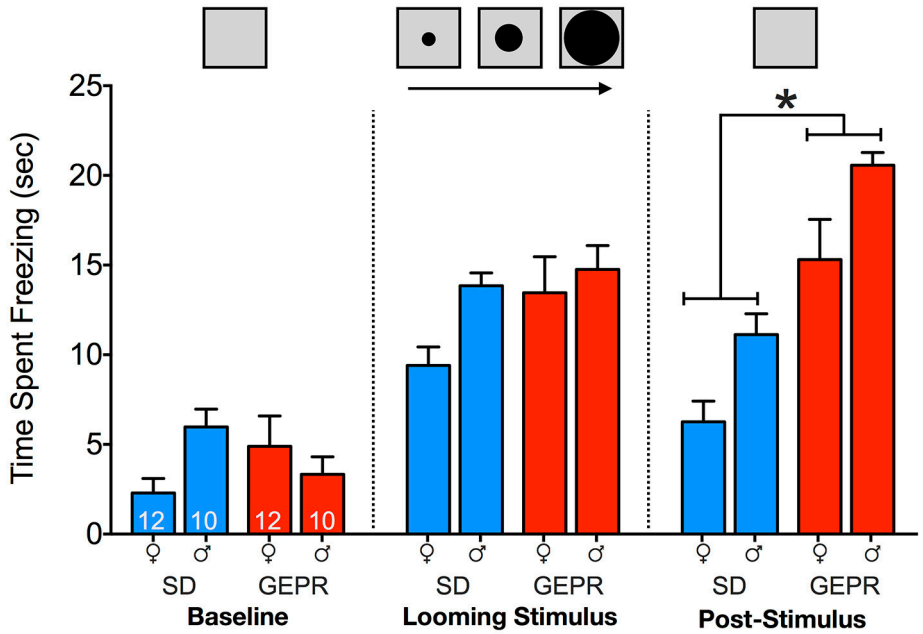

FIGURE 4 | Looming threat test. Time spent freezing during: baseline period (22 s prior to stimulus presentation), presentation of looming stimulus (22 s), and in the post-stimulus period (22 s immediately after stimulus presentation). Overall, GEPR-3s spent more time frozen [$F_{(1, 40)} = 11.7$, $P = 0.001$], but there was no effect of sex ($P = 0.12$). During the post-stimulus period, there was a significant increase in freezing in GEPR-3s as compared to SD rats (*Holm-Sidak Adjusted, Females: $P = 0.0004$ and Males: $P = 0.0007$). Figures show mean and standard error of the mean.

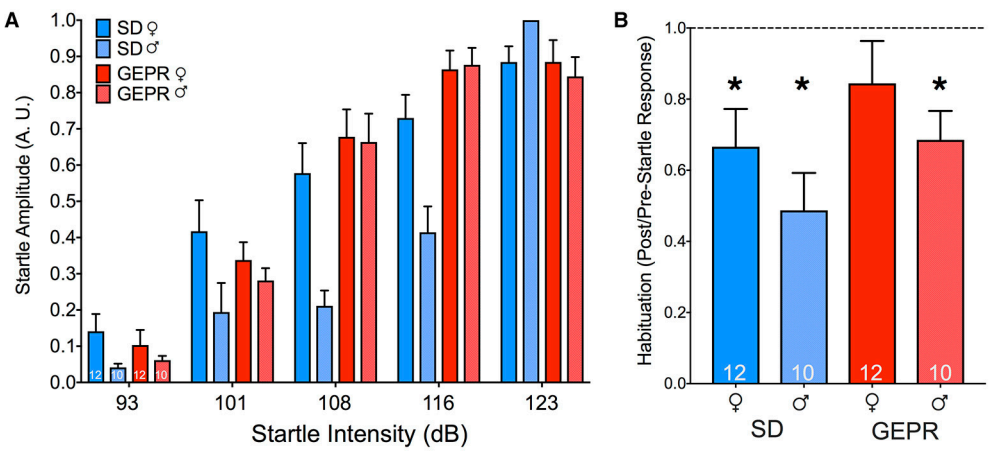

FIGURE 5 | Acoustic startle response and habituation. **(A)** Startle amplitude (A.U.) as a result of increasing noise burst intensity (dB). We found a main effect of noise intensity [$F_{(1.6, 64.6)} = 23.4$, $P = 0.0000001$], but no effect of either strain or sex ($Ps = 0.5$ and 0.9, respectively). **(B)** Habituation to the startling stimulus was present in all groups, except for the female GEPR-3s (*$P < 0.01$, one sample t-test when compared to the theoretical mean of 1.0). Figures show mean and standard error of the mean.

groups, likely due to the low statistical power due to attrition in the GEPR group ($t = 1.7$, $df = 23$, $P = 0.09$).

Electrical Stimulation of DLSC

Activation of components of the midbrain tectum result in species-specific defense responses (41–44). With increasing stimulation intensities, progressively more severe responses are evoked. For these studies, only male animals were available for use. Two SD rats and 2 GEPR-3s were excluded from analysis due to either: loss of electrode-containing head cap, lack of response to DLSC stimulation, postsurgical mortality, or inability to verify electrode placement. Electrode placement across groups is shown in **Figure 9A**; one GEPR-3 was excluded due to misplacement of the electrode in the superficial SC or the PAG (black "∧" in **Figure 9A**). A representative photomicrograph of electrode placement is shown in **Figure 9A**. When we measured

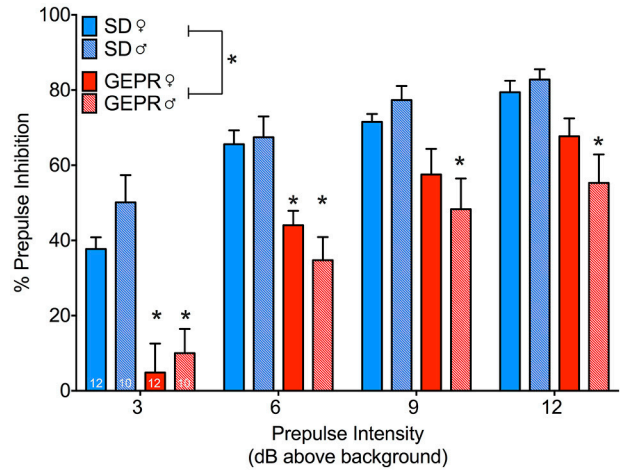

FIGURE 6 | Prepulse inhibition of the acoustic startle response. All groups showed the expected increase in inhibition as a function of increasing prepulse intensity [$F_{(2.3, 93.1)} = 82.2$, $P = 5 \times 10^{-23}$], however GEPR-3s displayed a significant PPI deficit relative to SD rats [$F_{(1, 40)} = 43.5$, $P = 0.00000007$]. Comparisons across strain for each sex revealed significant impairment in PPI at each prepulse intensity for male GEPR-3s as compared to male SD rats ($Ps < 0.002$, Holm-Sidak adjusted). For female GEPR-3s as compared to female SD rats, this effect was evident at lower prepulse intensities (PP3: $P = 0.005$; PP6 $P = 0.02$), but not higher prepulse intensities (PP9 and PP12 $Ps = 0.1$). Figures show mean and standard error of the mean. *significantl difference between strain, stratified by sex and prepulse.

the threshold current required to evoke orienting, locomotor responses, or escape behaviors, we found no differences between strain (**Figure 9B**; Orient: $t = 1.026$, $df = 10$, $P = 0.33$ Locomotion: $t = 0.488$, $df = 5$, $P = 0.65$ Escape: $t = 0.765$, $df = 13$, $P = 0.46$).

Correlation of Behaviors

To summarize our findings across tests, and to determine the degree to which behaviors may be considered "trait-anxious" (i.e., independent of the particular context), we correlated dependent measures across the tests in which we detected an effect of strain (45, 46). **Table 1** shows the Spearman's correlation coefficients for these comparisons. Correlations indicated in bold were considered discoveries using the Benjamini-Krieger-Yukutieli correction for false discovery rate ($Q = 5\%$). Of the measures we examined, the post-stimulus freezing time in the looming threat test was best correlated with other measures.

DISCUSSION

Here, we report that the GEPR-3 strain exhibits anxiety-like behaviors in both sexes and across an array of standard behavioral assays (open field, EPM, and light-dark transition test). Moreover, when tested in a novel implementation of a looming threat test, GEPR-3s demonstrated heightened anxiety-like responses. In addition, GEPR-3s displayed disrupted PPI of the acoustic startle response in the absence of changes in startle reactivity, reduced preference for sucrose, and impaired novel object recognition.

Altered behavioral responses in tasks thought to reflect affective state have been previously reported in animal models of epilepsy, although results have varied. For instance, following status epilepticus, both *increased* and *decreased* anxiety- and depression-like behaviors have been reported (47–50). Electrical and chemical kindling epileptogenesis are also associated with affective comorbidities including increased defensive, anxious-, or depressive-like phenotypes (51–56). However, similar to status epilepticus models, some kindling epileptogenesis studies have failed to find effects on comorbidities (57–59). Anxious- and depressive-like comorbidities have also been reported in GAERS (60); however, these comorbidities differ between sub-colonies of the strain (61), and are evident only when GAERS are compared to non-epileptic, inbred control rats but not when compared to the outbred strain from which they were derived, the Wistar rat (62). WAG/Rij rats, which also display absence-like seizures, have a co-morbid depressive-like phenotype, but not an anxiety-like phenotype (7, 63, 64).

While the above studies address the effects of acute seizures and/or a history of recurrent seizures on comorbidities, AGS models of inherited epilepsy allow for the assessment of phenotypes, i.e., those associated with underlying pathology or genetics in the absence of recurrent seizure activity. AGS models such as the GEPR, the Wistar audiogenic rat (WAR), and Kurshinsky-Molodkina (KM) rat display increased susceptibility to acoustically-evoked generalized seizures, which are common in models of inherited epilepsy across species (64–66). Of these strains, the WAR and KM strains have been evaluated for comorbidities (67, 68). While there has been some suggestion that the GEPR-3 strain may also display affective comorbidities (69, 70), our data clearly demonstrate that comorbidity in GEPR-3s is an anxiety-like phenotype. Across standard tests of conflict-exploratory activity, GEPR-3s consistently displayed reduced exploration of aversive maze components. In the open field test, this was manifest as a reduced exploration of the center of the arena, in contrast with the increased exploration reported in WARs (67). In the EPM test, anxiety-like behavior was evident in fewer exploratory headpokes into the open arms of the maze, which is consistent with reports in KM rats (68). In the light-dark test, GEPR-3s had reduced time spent in the light chamber of the apparatus; this phenotype extends beyond conflict-exploratory tests into unconditioned fear: when challenged with a looming visual stimulus, GEPR-3s displayed increased freezing in the post-stimulus period.

The anxiety-like phenotype observed in GEPR-3s may be in part explained by neurochemical abnormalities; these animals display deficits in brainstem serotonin, which is a well-known regulator of affective function (71–74). Consistent with the reduced serotonin levels reported in GEPR-3s, we have previously reported volumetric alterations in the region of the dorsal raphe nucleus (11). Serotonin is also a regulator of seizure susceptibility in GEPR-3s (69, 75), and accordingly, treatment with fluoxetine, a selective serotonin reuptake inhibitor that is primarily used for the treatment of depression and generalized anxiety, results in a decrease in AGS severity (9). In KM rats, fluoxetine, reduced

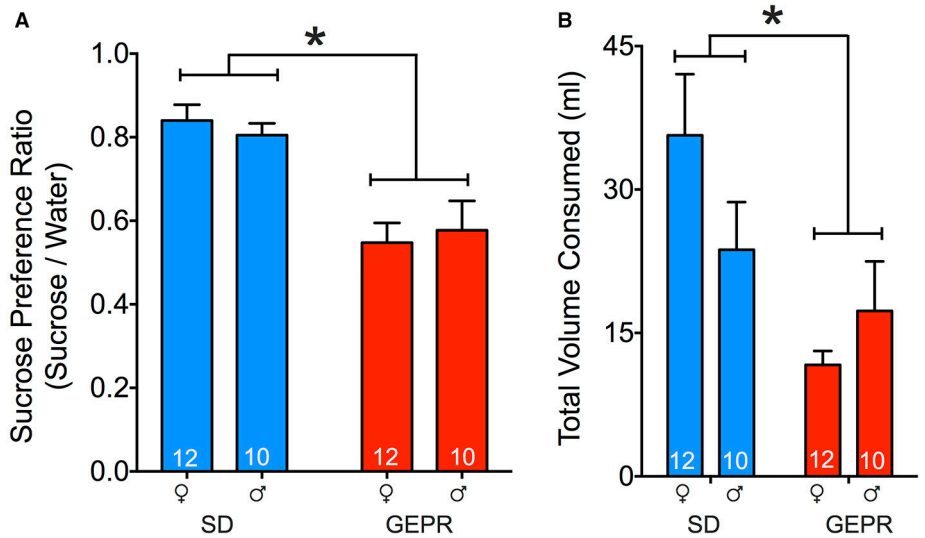

FIGURE 7 | Sucrose preference test. **(A)** GEPR-3s displayed a significantly lower sucrose preference ratio than SD rats [$*F_{(1,40)} = 29.3$, $P < 0.0001$]. Pairwise comparisons showed that decreased sucrose preference in GEPR-3s was significant in both sexes female: $P = 0.001$, male: $P = 0.003$, Holm-Sidak corrected. **(B)** Total volume consumed across days differed between strains [$F_{(1,40)} = 9.768$, $P = 0.0033$], with GEPR-3s consuming significantly less than SD rats. Figures show mean and standard error of the mean.

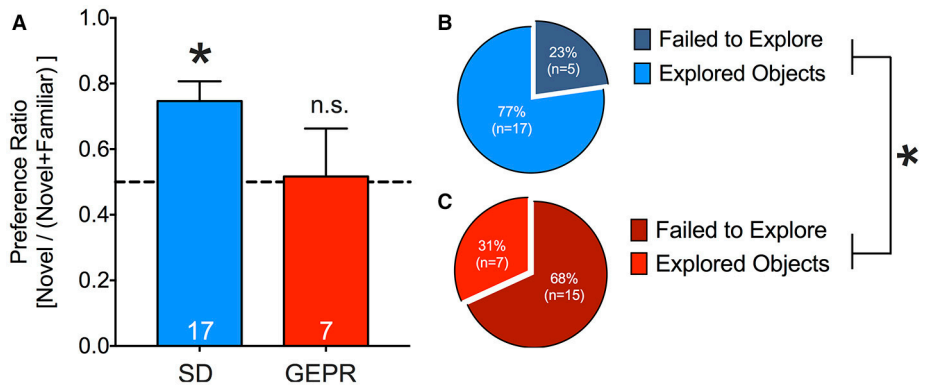

FIGURE 8 | Novel object recognition test. **(A)** SD rats showed the expected novel object preference during the retention probe conducted 2 h after the familiarization session (one sample *t*-test, $t = 4.1$, $df = 17$, $*P = 0.0008$), GEPR-3s did not (preference ratio did not differ significantly from chance, $P = 0.9$). Preference ratio trended toward but did not reach the level of statistical significance between these two groups ($t = 1.7$, $df = 23$, $P = 0.09$). **(B,C)** Proportion of animals that failed to explore the objects during the familiarization session; A greater proportion of GEPR-3s failed to explore the objects as compared to SD rats ($*P = 0.006$, Fisher's Exact Test). Figures show mean and standard error of the mean.

immobility in the forced swim task (68), although it had no effect on EPM behavior. The degree to which serotonin-based pharmacotherapy would normalize behavioral co-morbidities in the GEPR-3 remains to be explored.

Anxiety-like responses were also observed in the NORT, where a large proportion of GEPR-3s failed to explore novel objects. This phenotype was at least as striking as the memory impairment evident in the subset of GEPR-3s that did explore objects. While a subset of GEPR-3s explored the objects to an extent sufficient to perform the test, the impairment seen in object recognition memory should be interpreted with caution because the anxiety phenotype may have impaired memory consolidation in GEPR-3s. While the mechanism(s) underlying impaired learning/memory in GEPR-3s is unknown, it is worth noting that heightened levels of corticosterone have been associated with impaired learning/memory, including NORT performance (76). Interestingly, GEPR-3s and WARs have elevated corticosterone levels (82, 83). These same caveats must be considered when interpreting the decreased sucrose preference in GEPR-3s. While these data are consistent with a decreased hedonic drive, they may also have resulted from anxiety-induced suppression of feeding (84). In fact, a recent study shows that administration of anxiolytic drug fluoxetine results in a recovery of feeding behavior in a corticosterone-induced rodent model of anxiety (85). Future studies of GEPR-3s investigating changes in reward and learning behaviors under

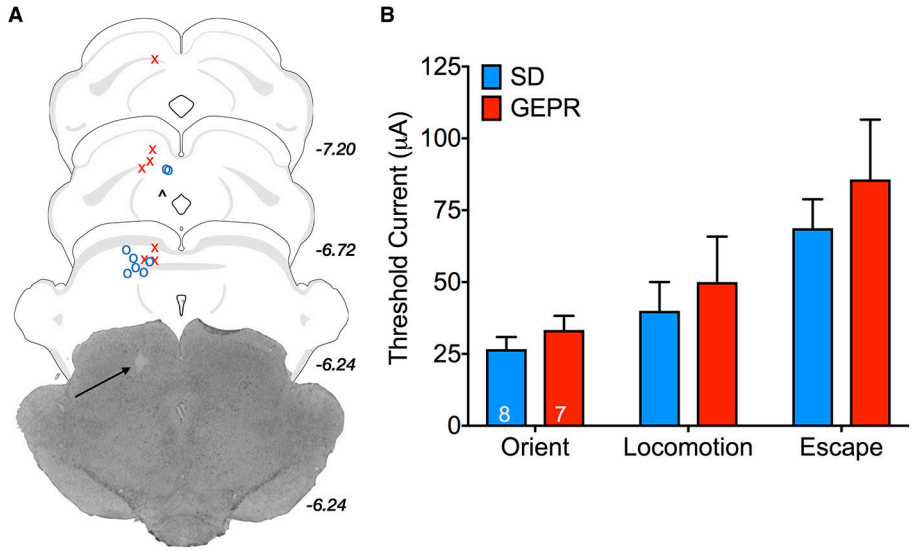

FIGURE 9 | Stimulation thresholds for DLSC-evoked behaviors in male rats. **(A)** Electrode placement; O = SD rats, X = GEPR-3s, ∧ = misplaced electrode. Black arrow points to electrode tip in the representative photomicrograph. Electrode tips are plotted on planes from the BrainMaps 4.0 Atlas (37). **(B)** Threshold current required to evoke orienting, locomotor responses, or escape behaviors. No differences between strain (Orient: $t = 1.026$, $df = 10$, $P = 0.33$ Locomotion: $t = 0.488$, $df = 5$, $P = 0.65$ Escape: $t = 0.765$, $df = 13$, $P = 0.46$). Figures show mean and standard error of the mean.

TABLE 1 | Spearman's correlation coefficients for characterizing behaviors consistent with "trait anxiety."

	EPM: head pokes	Looming threat: post-stimulus freeze time	Light/dark: light time	OF: inner time	Sucrose pref ratio	PPI: average
EPM: head pokes						
Loomer: post-stimulus freeze time	−0.288					
Light/Dark: light time	0.189	**−0.483**				
OF: inner time	0.150	**−0.482**	0.210			
Sucrose pref ratio	0.130	**−0.535**	**0.426**	0.175		
PPI: average	0.259	**−0.375**	0.127	**0.348**	**0.336**	

Correlations indicated in bold were considered discoveries using the Benjamini-Krieger-Yukutieli correction for false discovery rate (Q = 5%). The post-stimulus freezing time in the looming threat test was best correlated with other measures.

conditions of similarly reduced anxiety and/or in the presence of anxiolytic drugs may help to parse these effects.

The DLSC play a key role in the generation of the wild running component of AGS in the GEPR (86). Both functional and anatomical evidence suggests that the DLSC are abnormal in the GEPR; GEPR-3s display an increase in DLSC volume as compared to SD rats (11), and neurons within the DLSC of the GEPR-9 (a substrain of the GEPR that exhibit tonic seizures) display reduced sensitivity to acoustic stimulation relative to SD rats (86). On the basis of these findings, we hypothesized that GEPR-3s would display altered thresholds for DLSC-evoked behavioral responses; however, stimulation thresholds did not differ between strains. This was also surprising given the pronounced increase in freezing seen in the looming threat task, which critically relies upon the DLSC (29, 43, 87, 88). However, the DLSC is only one component of a midbrain network mediating both defensive responses and AGS; other loci include the inferior and superior colliculi, and periaqueductal gray (36, 89–92). Other nodes may be relevant for epilepsy-associated anxiety responses; for example, electrical kindling of the amygdala coincident with PAG stimulation exacerbates panic-like behaviors evoked from the PAG (55). Evaluation of thresholds for defense responses from these other sites may be merited in the GEPR.

The midbrain network mediating both defensive and AGS also play an important role in the control of acoustic startle and PPI. Lesions to either the inferior colliculus (IC) or superior colliculus (SC) disrupt PPI, whereas electrical or chemical stimulation of the IC or SC increases PPI (77–81). Importantly, the duration of tone burst intensity required to elicit AGS (10–60 s) is far longer than the noise burst used to induce auditory startle (40 ms pulse) (14), thus it seems unlikely that seizure activity could

account for the deficits in PPI seen in GEPR-3s. The PPI deficits in GEPR-3s may be related to the same underlying pathology as the anxiety-like behaviors as serotonin modulates PPI in rodents (93, 94), and human studies report disrupted PPI in patients with panic disorder (95, 96). However, while kindling epileptogenesis disrupts PPI (97, 98), GAERs display either normal or facilitated PPI depending on the age tested (99), suggesting that PPI deficits are not a universal comorbidity of seizures in animal models.

There were some notable cases in which GEPR-3s did not display heightened anxiety responses: open arm exploration in the EPM, acoustic startle, and electrical stimulation of the DLSC. Open arm exploration in the EPM is impacted by a variety of variables, including prior test experience (100) and history of handling (101). Prior exposure to novel environments may reduce subsequent exploratory drive in the EPM, diminishing ability to distinguish anxiety-like responses. However, it is worth noting that despite the lack of difference in open arm exploration, other measures of ethological activity in the EPM revealed a consistent anxiety-like phenotype in the EPM. Acoustic startle and electrical stimulation both differ from the conflict-based tasks (exploration vs. safety): these are unconditioned or evoked responses. In the cases in which anxiety-like responses were detected, animals were typically presented with a novel environment (or object) to explore, suggesting that at least part of the anxiety phenotype in GEPR-3s may be related to neophobia, and does not generalize to anxiety-like responses that may be more related to panic or acute fear.

There is an abundance of clinical evidence supporting sex differences in acquisition and expression of seizure disorders (102–104). Proposed etiologies of these sex-based differences include developmental mutations (as in the case of protocadherin 19 mutations, an X-linked gene), neuroendocrine fluctuations (e.g., perimenstrual catamenial epilepsy), and changes in neuroinflammatory response (103, 105). Some of these same mechanisms underlying sex differences in epilepsy may also contribute to divergent rates of comorbidities in males and females (13). Although women in the general population are more likely to show signs of anxiety and depression, sex appears to have a protective effect in patients with epilepsy—as they age, men become more susceptible to depression and women become less susceptible (106). The penetrance of the GEPR-3 seizure's phenotype is significantly greater in females as compared to males (107), although in the present study we found no notable sex-by-genotype interactions: anxiety-like responses were equally present in both female and male GEPR-3s. However, the effect of repeated seizures on these comorbidities remains unknown—future studies examining effects of repeated seizures on behavioral phenotype and potential influences of sex are clearly needed.

Here we demonstrate for the first time an anxiety phenotype in adult GEPR-3s; this phenotype was present in animals that experienced only a single AGS at PND 21. In the GEPR-3, anxiety-like responses were evident across a variety of tasks and conditions. Given the minimal seizure history, neuropsychiatric phenotypes in the GEPR-3 are likely *premorbid* rather than *comorbid*; this feature strongly suggests a genetic component of etiology of anxiety that provides a novel approach for future investigations of the pathogenesis of anxiety in epilepsy.

AUTHOR CONTRIBUTIONS

BA and PF: designed experiment; BA: conducted experiment; BA and PF: analyzed data; BA, LM, PN, and PF: interpreted findings; BA and PF: drafted manuscript; BA, LM, PN, and PF: edited and approved manuscript.

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REVIEW
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Preclinical Animal Models for Dravet Syndrome: Seizure Phenotypes, Comorbidities and Drug Screening

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Epilepsy is a common chronic neurological disease affecting almost 3 million people in the United States and 50 million people worldwide. Despite availability of more than two dozen FDA-approved anti-epileptic drugs (AEDs), one-third of patients fail to receive adequate seizure control. Specifically, pediatric genetic epilepsies are often the most severe, debilitating and pharmaco-resistant forms of epilepsy. Epileptic syndromes share a common symptom of unprovoked seizures. While some epilepsies/forms of epilepsy are the result of acquired insults such as head trauma, febrile seizure, or viral infection, others have a genetic basis. The discovery of epilepsy associated genes suggests varied underlying pathologies and opens the door for development of new "personalized" treatment options for each genetic epilepsy. Among these, Dravet syndrome (DS) has received substantial attention for both the pre-clinical and early clinical development of novel therapeutics. Despite these advances, there is no FDA-approved treatment for DS. Over 80% of patients diagnosed with DS carry a *de novo* mutation within the voltage-gated sodium channel gene *SCN1A* and these patients suffer with drug resistant and life-threatening seizures. Here we will review the preclinical animal models for DS featuring inactivation of *SCN1A* (including zebrafish and mice) with an emphasis on seizure phenotypes and behavioral comorbidities. Because many drugs fail somewhere between initial preclinical discovery and clinical trials, it is equally important that we understand how these models respond to known AEDs. As such, we will also review the available literature and recent drug screening efforts using these models with a focus on assay protocols and predictive pharmacological profiles. Validation of these preclinical models is a critical step in our efforts to efficiently discover new therapies for these patients. The behavioral and electrophysiological drug screening assays in zebrafish will be discussed in detail including specific examples from our laboratory using a zebrafish *scn1* mutant and a summary of the nearly 3000 drugs screened to date. As the discovery and development phase rapidly moves from the lab-to-the-clinic for DS, it is hoped that this preclinical strategy offers a platform for how to approach any genetic epilepsy.

Keywords: epilepsy, dravet syndrome, drug discovery, *in vivo*, precision medicine, antiepileptic drugs, animal models

INTRODUCTION

While many individuals with epilepsy achieve adequate seizure control with available antiepileptic drugs (AEDs), approximately one-third of patients remain refractory to treatment (Löscher and Schmidt, 2011). With epilepsy being one of the most common chronic neurological diseases worldwide and affecting almost 3 million people in the USA alone, there is a substantial unmet need to identify new AEDs for these refractory epilepsies (Thurman et al., 2011). Epilepsy is clinically defined by the International League Against Epilepsy (ILAE) as "at least two unprovoked (or reflex) seizures" (Scheffer et al., 2017). Although the clinical definition of epilepsy focuses on *unprovoked* seizure activity, the discovery and development of AEDs has traditionally relied, almost exclusively, on preclinical testing using *provoked* seizure models (Wilcox et al., 2013). Provoked seizure models are performed in rodents (wild-type rats or mice) and have led to the successful identification of many clinically useful anti-seizure treatments which often elicit broad-spectrum suppression against a range of different seizure types. However, a significant limitation of these methodologies is that they do not model epilepsy (spontaneous unprovoked seizures). Furthermore, provoked seizure models do not recapitulate the underlying pathology associated with genetic epilepsies. While techniques to genetically modify experimental animals have existed now for several decades, traditional AED discovery programs do not incorporate genetic epilepsy models. Despite the discovery of more than 20 AEDs since phenytoin (Dilantin®) was identified in 1936, the proportion of all patients with drug-resistant epilepsy has remained unchanged (Löscher and Schmidt, 2011), and this is a particularly acute problem for the subset of patients with genetic epilepsies. Therefore, it is worth considering that it may now be time for novel (genetic) models and methodologies for AED discovery.

Recent advances in genomics resulted in tremendous progress in identifying genes associated with epilepsy. Detection of genetic mutations in ion channels, synaptic vesicle proteins, neurotransmitter receptors and transporters, and proteins involved in various metabolic pathways is starting to improve our basic understanding of the varied and complex pathophysiology of epilepsy (Epi4K Consortium; Epilepsy Phenome/Genome Project, 2013; Howard and Baraban, 2017). Moreover, many of the refractory epilepsies previously classified as "idiopathic" can now be explained by single-gene mutations. Identifying the genetic causality of epilepsy also provides an opportunity for a "precision medicine" based approach to developing new treatments. In combination with recent breakthroughs in genome editing technology, there has emerged a unique opportunity for generating and characterizing genetically relevant *in vivo* epilepsy models for the identification and development of disease-specific treatments (Griffin et al., 2016). Particularly for highly intractable genetic epilepsies often seen in children, a disease-specific discovery approach could help identify effective treatments for these patients.

Dravet syndrome (DS) is one such genetic intractable epilepsy. Patients often present with persistent drug resistant seizures within the first year of life. The incidence of DS in the United States is 1 of 15,000–20,000 and almost 80% of patients have a loss-of-function mutation in a single copy of the *SCN1A* gene (Fukuma et al., 2004; Zuberi et al., 2011; Wu et al., 2015). This results in hapolinsuffieiency for the Nav1.1 sodium ion channel and is predicted to be the major pathophysiology resulting in DS (Bechi et al., 2012). As the Nav1.1 sodium channel plays a role in suppressing neuronal excitability, *SCN1A* loss-of-function mutations lead to neuronal hyperactivity and unprovoked seizures. While frequent unprovoked seizures are the main characteristic of epileptic encephalopathies like DS, patients also suffer from a range of comorbidities affecting the areas of locomotion, speech, and behavior. DS patients often have disrupted sleep and metabolic circadian rhythms, neurodevelopmental delay and intellectual disabilities, oculomotor deficits, and psychomotor regression (Nolan et al., 2006; Martin et al., 2010b; Dhamija et al., 2014). Sudden unexpected death associated with epilepsy (SUDEP) is also prevalent in this population (Shmuely et al., 2016). Due to the severity of these comorbidities, effective treatments which can address both seizures and the range of comorbidities associated with DS are urgently needed.

To date, there is no FDA-approved standard of care for DS. There has, at least recently, been an encouraging increase in drug candidates emerging from different preclinical pipelines and progressing into early clinical trials. Assessing the predictive validity of the available preclinical DS models and methodologies is of critical importance when it comes to determining which of these treatments offer the greatest chance of clinical success. This review will focus on the genetic mouse and zebrafish models of DS. Importantly, we will discuss the "construct" (causal mechanism), "face" (phenotypic features), and "predictive" (success in identifying treatments used clinically) validity of these models (and assays).

PRECLINICAL GENETIC MODELS OF DRAVET SYNDROME

Being able to replicate the "construct" or causal mechanism of Nav1.1 deficiency, relies on generating animal models with genetic mutations in the *SCN1A* orthologue gene. Similar to humans, in mice, the sodium ion channel Nav1.1 (*Scn1a*) is expressed throughout the central nervous system including the axon initial segment of parvalbumin-positive hippocampal interneurons and excitatory principal cells (Chen et al., 2004; Ogiwara et al., 2007; Kim et al., 2011). Currently, there are numerous genetic mouse models for DS which aim to replicate *SCN1A* loss-of-function observed in DS. These lines include targeted deletion of *Scn1a* exon 1 (*Scn1a*^{tm1Kea}) (Miller et al., 2014) and exon 26 (*Scn1a*^{tm1Wac}) (Yu et al., 2006), specific point mutation knock-ins; Scn1a R1407X (Ogiwara et al., 2007), Scn1a R1648H (Martin et al., 2010a), and Scn1a E1099X (Tsai et al., 2015), and a transgenic mouse model expressing a bacterial artificial chromosome (BAC) with the human SCN1A R1648H mutation (Tang et al., 2009). Additionally, several GABAergic neurons conditional knock-out lines have also been generated (Cheah et al., 2012; Dutton et al., 2013; Ogiwara et al., 2013). The development of these genetic mouse models has greatly advanced our understanding of the pathophysiology of DS. For example,

several mouse studies demonstrated that haploinsufficiency for Nav1.1 leads to decreased firing in the inhibitory GABAergic interneurons that results in reduced synaptic inhibition, and causing network hyperexcitability and seizures (Yu et al., 2006; Ogiwara et al., 2007; Hedrich et al., 2014).

The best characterized genetic zebrafish model of DS uses a Nav1.1 mutant (*scn1lab*^{s552}) first identified in a large-scale chemical mutagenesis screen for larvae with oculomotor deficits conducted by our colleague Herwig Baier (Schoonheim et al., 2010). Zebrafish larvae containing a single nucleotide substitution in the *scn1lab* gene were confirmed to have a loss-of-function in a sodium ion channel with 76% sequence identity to human *SCN1A*. In 2013, it was this *scn1lab*^{s552} mutant zebrafish which we first described as replicating many of the essential clinical phenotypes observed in DS patients (Baraban et al., 2013). Zebrafish have two *scn1a* genes which are both highly expressed within the central nervous system; *scn1lab* and *scn1laa* (Baraban et al., 2013). Genetically, homozygous *scn1lab*^{s552} mutants are haploinsufficient for the Nav1.1 sodium ion channel due to the expression of the duplicated paralogue gene *scn1laa* (67% protein identity to the human Nav1.1). As DS patients are also haploinsufficient for the Nav1.1 sodium ion channel, the zebrafish *scn1lab*^{s552} mutants therefore replicate the genetic etiology observed in the majority of DS patients.

Genomic editing methods such as the CRISPR/Cas9 system (clustered regularly interspaced short palindromic repeat/Cas9) allow for rapid and efficient modification of endogenous genes in a range of animal models (Hwang et al., 2013; Sander and Joung, 2014). Following identification of a potential disease causing allele in patients with epilepsy, it is now plausible to generate models with mutations in the homologous genes. Being able to precisely model patient variants will not only enhance our overall understanding of specific allele pathophysiology but also contribute to patient precision therapies. The development of *in vitro* neuronal models from DS patient derived induced pluripotent stem (iPS) cells or cerebral oganoids also allows for allele specific studies at the molecular and cellular levels (Higurashi et al., 2013; Jiao et al., 2013; Liu et al., 2013, 2016; Sun et al., 2016). The main advantage of patient-derived *in vitro* platforms is that they can precisely model the genetic construct, including any genetic modifiers which may influence disease severity and/or drug response. When evaluating how a given drug alters the function of a voltage-gated ion channel such as SCN1, these reduced systems have significant value. However, these primarily two-dimensional *in vitro* neuronal cultures are unable to recapitulate the full network where these disease causing alleles are embedded, making evaluation of antiepileptic actions for a network disorder like epilepsy difficult, and recapitulating behavioral comorbidities or SUDEP virtually impossible.

Another so-called "simple" species—*Drosophila melanogaster* (fruit flies)—which feature a single voltage-gated sodium channel gene, (*para*) has also been used to model SCN1 mutations. Several *para* mutants have emerged from forward genetic (Siddiqi and Benzer, 1976; Lindsay et al., 2008; Parker et al., 2011) screens or through knock-in of specific disease causing alleles identified in human DS patients (Schutte et al., 2014). Specifically, O'Dowd and colleagues introduced a homologous mutation in the *para* gene designed to replicate the *SCN1A* S1231R identified in a DS patient (Schutte et al., 2014). Similar to DS patients, this mutation resulted in loss-of-function of the sodium channel. While humans have nine different sodium channels, the *Drosphila para* gene produces a range of sodium channels with different functional properties through alternative splicing (Thackeray and Ganetzky, 1994). Many of the *para* mutations reside in an evolutionally conserved constitutively expressed exon and, therefore all the expressed sodium channels are affected. While it is possible to precisely model some patient variants in *Drosophila*, given the difference in genetic and brain architecture between *Drosophila* and humans, many of the molecular, cellular, behavioral and network changes associated with Nav1.1 haploinsufficiency are unable to be replicated in this non-vertebrate model system. Nonetheless, consistent with our discoveries in *scn1a* zebrafish models (discussed in detail below), serotonin pathways were recently implicated as a "potential therapeutic target for DS" based on studies using *SCN1A* S1231R *Drosophila* mutants (Schutte et al., 2014).

PHENOTYPIC VALIDITY OF DRAVET SYNDROME *IN VIVO* MODELS

Detecting Spontaneous Seizure Events in Dravet Syndrome Models

Evaluating epilepsy (spontaneous unprovoked seizures) in genetic models, rather than provoked seizures relies on continuous recording and monitoring efforts. Using video-electroencephalographic (vEEG) monitoring, some form of spontaneous seizure activity has been reported for many of the Nav1.1 haploinsufficient mouse models, though seizure phenotypes (and survival) in these mice is strongly dependent upon background strain owing to strain-specific genetic modifier genes. For example, on the 129S6/SvEvTac background strain *Scn1a*^{tm1Kea} heterozygotes exhibit no overt phenotype and have a normal lifespan. However, when crossed with the C57BL/6J background strain these mice exhibit spontaneous seizures and early lethality around 1 month of age (Miller et al., 2014; Mistry et al., 2014). Similarly, *Scn1a*^{tm1Wac} heterozygotes generated on the 129/SvJ background had no obvious phenotype, but when crossed with the C57BL/6 background strain they develop spontaneous seizures (Yu et al., 2006). These mice die prematurely and have a high incidence of short duration, generalized seizures in the hours preceding death, while prolonged status epilepticus is rare (Kalume et al., 2013). Hyperthermia-induced seizures and SUDEP, have also been extensively documented in *Scn1a* mouse lines (Ogiwara et al., 2007; Oakley et al., 2009; Cheah et al., 2012; Dutton et al., 2013; Kalume et al., 2013). Although febrile seizures are common in the early-life of DS patients, the association between febrile seizures and true epilepsy remains unknown. Furthermore, how seizure phenotypes in these mice respond to available AEDs—discussed in more detail below—has received less attention.

Local field potential (LFP) recording techniques in larval zebrafish offer a reliable method to monitor abnormal

electrographic seizure events associated with spontaneous unprovoked seizures. In its simplest form, using a micromanipulator and glass microelectrode patch pipettes with a diameter around 1.2 µm (approximately three times smaller than a single neuron), the larval skin can easily be punctured and a LFP recording can be obtained from any brain structure. In our hands, these recordings are extremely stable for many hours with no diminution of signal quality. To reduce potential twitch-like movement artifacts during LFP recordings, agarose-embedded larvae can be paralyzed with α-bungarotoxin or pancuronium. Importantly, movement artifacts are not multi-spike and have a waveform clearly distinguishable from seizure events. To increase throughput of the electrophysiological assay, we also developed a reliable non-invasive microfluidic platform-based recording approach that uses surface electrodes (thus, some attenuation of signal amplitude with no change in sensitivity) and parallel larvae trapped in individual wells. This integrated Zebrafish Activity Platform (iZAP), developed initially with a 12-well, 5-recording electrodes per fish format allowed us to record brain activity for several hours to days (Hong et al., 2016). An additional advantage of the iZAP system, as it relates to drug discovery, is the ability to wash drugs on, and off, in cross-over style pharmacology studies. Both recording approaches can be used to detect electrographic seizures in larval zebrafish up to 12 days post fertilization (dpf).

The *scn1lab*^{s552} homozygous mutants show spontaneous electrographic seizure activity from 3 dpf. Seizure frequency is highest between 4 and 5 dpf. LFP recordings from paralyzed and agar-immobilized larvae show an ictal-like pattern identified by large-amplitude (>5 times baseline), long-duration (>1,000 ms) events (Baraban et al., 2013). Often frequent, unprovoked small amplitude interictal-like short bursts are also observed at durations between 120 and 300 ms; small amplitude events between 50 and 100 ms in duration are not considered abnormal and can also be routinely observed in wild-type zebrafish between 3 and 10 dpf. Of note, the unprovoked abnormal ictal- and interictal-like electrographic events observed in *scn1lab*^{s552} homozygous mutants are similar in waveform to epileptiform events elicited in wild-type larvae upon exposure to a common convulsant drug, pentylenetetrazole (PTZ) (Baraban et al., 2005; Baraban, 2013) as well as other genetic zebrafish epilepsy models for *stxbp1* (Grone et al., 2017), *mindbomb*/Ube3a (Hortopan et al., 2010), and *aldh19a1* (Pena et al., 2017). Additionally, larvae with a homozygous loss-of-function mutation in the paralogue *scn1laa* gene (*scn1laa*^{sa1674}) exhibit spontaneous seizures (Griffin et al., 2017), as does a second N-ethyl-N-nitrosourea (ENU)-generated *scn1lab* mutant (*scn1lab*^{sa16474}) (Eimon et al., 2018) supporting the etiology underlying the spontaneous seizures observed in DS patients results from haploinsufficiency of the Nav1.1 sodium ion channel.

As an *in vivo* vertebrate model system, zebrafish larvae also exhibit spontaneous convulsive swim behaviors that are easily monitored using locomotion tracking software at video frame rates of 33 Hz. Based on our initial work with PTZ-induced seizures we established a classification scheme for these larval seizure behaviors: Stage 3 is the most severe, where larvae exhibit high speed (>20 mm/sec), full body convulsions followed by a brief loss of posture for a few seconds; Stage 2 behavior shows increased swim activity rate and a rapid "whirlpool-like" circling; and, slight increase in swim activity (Stage 1) or no swim activity (Stage 0) are considered as normal movement behaviors (Baraban et al., 2005). As an example of a potentially advantageous characteristic for high-throughput assays, PTZ (molecular weight, 367.8 g/mol) at concentrations as low as 2.5 mM elicits the first signs of larval seizure behavior within 5 min of acute exposure suggesting that drug penetrance is rapid and long-term exposures are not necessary. Moreover, high-speed, convulsive, swim behaviors are never observed in wild-type larvae, and at video acquisition rates of 200 Hz and above, these abnormal whole-body seizure behaviors are easily quantified. Based on the frequent high baseline occurrence of high-velocity Stage 3 seizure behaviors and intervening Stage 2 hyperactivity, locomotion plots measuring swim velocity offer a reliable surrogate measure of behavioral seizure activity. This automated locomotion approach serves as the first-stage of our high-throughput drug screening strategy, as described in more detail below.

Comorbidities and Characteristics of Dravet Syndrome Animal Models

DS is a multifaceted disease and thus there is a demand to understand the serious and complex comorbidities often experienced by patients. Most patients with DS have intellectual disability, and experience other neurodevelopmental disorders including autism from the second year of life. While it is expected that laboratory animals cannot recapitulate the full spectrum of human behaviors or cognition (and this is a limitation of all experimental model systems), it is encouraging that some of these comorbidities can be modeled at the preclinical level in *Scn1a* mutant mice. For example, mice with the *Scn1a*$^{tm1.1Kzy}$ loss-of-function allele (Scn1a R1407X), showed hyperactivity, altered anxiety-like behavior, lowered sociability, lack of social novelty preference, and spatial learning and memory impairment (Ito et al., 2013). Furthermore, many of these behavioral deficits were reported in mice with conditional deletion of *Scn1a* in parvalbumin-positive interneurons (Tatsukawa et al., 2018), suggesting some comorbid behaviors in DS patients are mediated specifically by this interneuron sub-population. Simmilarly, the Scn1a^{tm1Wac} heterozygote mice display hyperactivity, anxiety-like behavior, increased stereotypies, impaired social behavior, and impairment spatial learning and memory (Han et al., 2012). Additionally, conditional deletion of Scn1a in forebrain GABAergic neurons recapitulates many of the autism-related phenotypes and spatial learning deficits highlighting a putative role for interneuron dysfunction in these comorbid behaviors (Han et al., 2012). Importantly, treatment with low-dose clonazepam (Han et al., 2012), a benzodiazepine and GABA agonist, was shown to completely rescue many of the behavioral deficits indicating pharmacological treatment could improve not only seizures but also some of the comorbid behaviors observed. Subsequently, these mouse studies highlight the potential to assess the effectiveness of treatments against the comorbidities experienced by DS patients.

Sleep disturbance is often reported in DS patients and is more prevalent than levels reported in young children in the general population and general epilepsy cohorts (Licheni et al., 2018). Mimicking sleep disturbances reported in DS patients, abnormalities in sleep behavior and circadian rhythms have been observed in several Nav1.1 deficient mouse models. In mice, Nav1.1 is expressed in the regions of the brain known to regulate sleep and circadian rhythms (Han et al., 2012; Papale et al., 2013). The *Scn1a*^{tm1Wac} heterozygotes show longer circadian period, with delayed onset of activity during the dark phase (Han et al., 2012). Combined pharmacological treatment with tiagabine and clonazepam was able to rescue the impaired circadian behavior without inducing sedative effects, highlighting again the potential for *Scn1a* mouse models in assessing compounds against specific DS comorbidities (Han et al., 2012). Finally, several of the *Scn1a* mouse knockout and knockin lines exhibit premature death (Yu et al., 2006; Cheah et al., 2012; Auerbach et al., 2013; Kalume et al., 2013), which offer an important research tool for understanding mechanisms of SUDEP. The sodium channel blocker GS967, was shown to improve the survival of the *Scn1a*^{tm1Kea} x C57BL/6J F1 mouse model (Anderson et al., 2017) despite sodium channel blockers being contraindicated for most DS patients. A distinct advantage of rodent *Scn1a* knockout models lies in the range of complex bahaviorial phenotypes that can be assayed. Significant clinical benefit on one, or more, of these comorbid behaviors could help differentiate between novel antiepileptic drug candidates currently under evaluation for DS.

Although modeling complex neurological behaviors in larval zebrafish is also limited, some behavioral comorbid characteristics observed in DS patients can be recapitulated. Like mice haploinsufficient for Nav1.1, the *scn1lab*^{s552} homozygous mutants also exhibit premature death and fail to thrive past 12 dpf. Additionally, *scn1lab*^{s552} homozygous mutants have nighttime hyperactivity, suggesting disrupted sleep behaviors and diurnal locomotor activity deficit similar to what is observed in DS patients and mouse models (Grone et al., 2017). Furthermore, DS zebrafish larvae show "wall hugging" behavior (thigmotaxis) which is often considered a sign of increased anxiety in larvae (Schnörr et al., 2012; Baraban et al., 2013; Grone et al., 2017). Interestingly, the *scn1lab*^{s552} homozygous mutant zebrafish was first characterized as having saccadic eye movement abnormalities, a characteristic often found in pediatric epilepsies and DS patients (Lunn et al., 2016). Equally, altered glycolytic metabolism and oxygen consumption rates are a characteristic deficit in many pediatric epileptic syndromes and were shown in novel metabolic larval zebrafish assays first described by Kumar et al. (2016) to be altered in *scn1lab*^{s552} homozygous mutants. Importantly, the ketogenic diet, a treatment which has reported efficacy in some DS patients (Dressler et al., 2015), returned the altered metabolism of the larvae to normal levels and significantly suppressed seizures (Baraban et al., 2013; Kumar et al., 2016).

As the repertoire of complex zebrafish larvae behaviors become better understood, researchers can focus on clinically relevant characteristics and comorbidities as additional measurable outcomes for drug discovery. Pharmacological studies investigating the effect of diazepam, valproate, trazodone and clemizole on *scn1lab*^{s552} homozygous mutant behaviors was recently reported by our laboratory (Grone et al., 2017). Results showed that clemizole and diazepam reduce the nighttime hyperactivity and decrease the anxiety-like wall hugging-behavior observed in *scn1lab*^{s552} homozygous mutants to control levels. Consistent with these findings, diazepam is often prescribed for anxiety and short-term insomnia in humans. Conversely, the antidepressant trazodone which is frequently prescribed off-label for sleep issues (Wong et al., 2017) showed no effect on improving nighttime hyperactivity deficits in *scn1lab*^{s552} homozygous mutants (Grone et al., 2017). As the impact and severity of comorbidities of DS becomes better understood, animal models with good "face" validity, i.e., recapitulating the spontaneous seizures and comorbid characteristics, offer valuable preclinical tools to improve clinical treatments for patients.

ASSESSING THE PREDICTIVE VALIDITY OF DS ANIMAL MODELS

The ILAE definition of epilepsy includes a distinction for unprovoked seizures (Scheffer et al., 2017). However, traditional studies of epilepsy, including all of the legacy and current rodent models offered by the NIH Epilepsy Therapy Screening Program (ETSP) (https://www.ninds.nih.gov/Current-Research/Focus-Research/Focus-Epilepsy/ETSP) involve induced or provoked seizures: maximal electroshock test (MES), metrazol seizure threshold (MET), 6 Hz 44 mA seizure model, corneal kindled seizure model, lamotrigine-resistant amygdala kindled seizure model, and mesial temporal lobe epilepsy (mTLE) induced by focal chemoconvulsant injection. These are considered models of generalized tonic-clonic seizures, temporal lobe epilepsy or clonic seizures but *do not* model any genetic form of epilepsy. While this approach has successfully identified new anti-seizure drugs, the fact that 30–40% of all epilepsy patients remain resistant to available AEDs discovered using these ETSP models also suggests different pharmacological efficacy between spontaneous unprovoked seizures (epilepsy) and seizures that are induced/provoked. Furthermore, it suggests that these traditional seizure inducible rodent models are not appropriate as animal models for intractable genetic epilepsies. In fact, AED discovery guided by these ETSP preclinical models, does not incorporate any model of DS or other genetic human epilepsy in its repertoire. This may explain why DS remains refractory to drugs identified through this program and suggests limited predictive validity of these inducible seizure models when determining clinical efficacy for DS patients. In contrast, genetic mouse and/or zebrafish models exhibiting epilepsy (unprovoked seizures) offer a valid alternative, but currently under-appreciated (Galanopoulou et al., 2012; Simonato et al., 2014, 2017), approach for preclinical drug discovery and development. It is our strong opinion that genetic models which faithfully recapitulate clinical phenotypes (i.e., "construct" validity) and characterized by spontaneous unprovoked seizures (i.e., "face" validity) offer the most appropriate preclinical pathway to new drug discovery. To assess the "predictive" validity of each genetic model, or assay, two factors need to be

considered as a rigorous form of model validation: (i) whether the seizures are unprovoked and spontaneous in origin, and (ii) whether the pharmacological responses represent what is observed in DS patients.

Genetic models of epilepsy that show clinically relevant phenotypes should exhibit the same pharmacological profile as DS patients as a form of "predictive" validity. Although there is no FDA-approved treatment there is a clinical "standard of care" for DS which advises a recommended AED polytherapy for most patients. Retrospective studies of AED responses of DS patients rank benzodiazepines (clobazam, diazepam), valproate, or stiripentol as the most effective options and are considered as the standard of care "first line" treatment for most of this patient population (Hawkins et al., 2017; Villas et al., 2017). Additionally, topiramate, potassium bromide and the ketogenic diet have also shown some efficacy for DS (Caraballo, 2011; Wirrell, 2016; Villas et al., 2017; Lagae et al., 2018). At the same time, DS also meets the ILAE classification for a drug resistant epilepsy i.e., "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). Finally, a number of AEDs are contraindicated and known to exacerbate seizures in DS including, carbamazepine, oxcarbazepine, and lamotrigine. Given the wide range of *de novo* mutations associated with DS it is also not surprising that some patients exhibit atypical experiences with sodium channel blockers, levetiracetam or other AEDs not recommended as the standard of care (Genton et al., 2000; Snoeijen-Schouwenaars et al., 2015; Takaori et al., 2017). Based on these clinical observations, a validated animal model of DS should identify AEDs commonly used in a clinical setting, while at the same time demonstrating a failure to control seizures with at least two or more appropriate AEDs.

Currently, there is no rodent model of DS which has been pharmacologically validated against epilepsy i.e., spontaneous unprovoked seizures. While this is technically possible, the logistics of capturing a sufficient number of unprovoked seizure events to adequately power a statistical analysis of a given drug treatment would require hundreds of hours of labor-intensive continuous vEEG monitoring across many animals, potentially taking months (to years) for adequate analysis of even a handful of drug candidates. The incomplete penetrance, low seizure frequency and early fatality observed in *Scn1a* mutant mouse strains are additional confounds that render mouse models as a less than ideal for predicating efficacy of AEDs against spontaneous seizures. Recently, Kearney and colleagues attempted to pharmacologically validate the more severe *Scn1a*^{tm1Kea} x C57BL/6J F1 mouse line in this manner (Hawkins et al., 2017). In evaluating four AEDs including clobazam, valproate, and topiramate against spontaneous seizures, this model failed to show efficacy in suppressing seizures for any of these "first line" DS drugs. Lamotrigine produced a significant elevation of spontaneous seizure frequency in this model, consistent with what is observed in patients, and three additional AEDs failed to suppress seizures consistent with a drug resistant epilepsy classification (**Table 1**).

Due to the lower seizure frequency and variable phenotypes observed in Nav1.1 deficient mice, there is a growing trend to use traditional inducible seizure methods layered on the *Scn1a*$^{+/-}$ mouse background. Given the discrepancy between data on provoked versus unprovoked seizures models, simply inducing seizures in an animal with a Nav1.1 deficiency comes with the risk of identifying compounds which impact the seizure-induction mechanism and will ultimately not be effective against spontaneous seizures in DS patients. Oakley et al. (2013) reported the AED clonazepam was effective in suppressing hyperthermia-induced seizures in the *Scn1a*^{tm1Wac} C57Bl/6 line, but it also increased motor impairment at therapeutic doses. When evaluating the pharmacological validity of several AEDs to suppress hyperthermia-induced seizures, the *Scn1a*^{tm1Kea} × C57BL/6J F1 mouse mouse model succeeded in identification of valproate and clobazam but failed to identify topiramate and stiripentol (Hawkins et al., 2017). Additionally, phenobarbital, a drug with limited efficacy reported in DS patients was identified as a false positive. The limited accuracy of this pharmacological validation also makes it difficult to interpret recent reports on huperzine A or cannabidiol in *Scn1a* mice where only protection against provoked hyperthermia-induced seizures were described (Wong et al., 2016; Kaplan et al., 2017). For example, huperzine A suppressed seizure activity in a hyperthermia-induced assay in *Scn1a*$^{R1648H/+}$ mice and a PTZ assay in wild-type mice or zebrafish (Wong et al., 2016), but failed to suppress *scn1lab*^{s552} spontaneous seizures when tested directly (Dinday and Baraban, 2015), or screened blindly as part of a natural products drug library (see **Figure 3D**). As further evidence for a discrepancy between drugs that block hyperthermia-induced seizures versus those effective against spontaneous seizures, we reported a powerful suppression of hyperthermia-induced electrographic seizure events in a zebrafish model (Hunt et al., 2012) with NMDA receptor blockers (ifenprodil and MK-801) but neither drug inhibited spontaneous electrographic seizure events in *scn1lab*^{s552} mutant zebrafish; MK-801 actually increased spontaneous seizure frequency (Dinday and Baraban, 2015; Griffin et al., 2017). Taken together, these mixed results suggest that there is limited predictability in DS mouse models and therefore *Scn1a* mutant mice may not be the first (or even second) choice to screen existing or experimental AEDs.

Zebrafish larvae offer an alternate *in vivo* model for the assessment anti-seizure efficacy of AEDs for DS. While this model may not fully recapitulate complex cognitive and neurobehavioral abilities, as an epilepsy model it offers a valid alternative. In our initial description of *scn1lab*^{s552} mutant zebrafish, we not only characterized the epilepsy (unprovoked seizure) phenotype, but also established a reliable acute exposure assay to validate this model. Briefly, we successfully demonstrated the anti-seizure properties of available AEDs in a two-stage assay: (i) high-throughput automated video behavior tracking of individual larvae to identify drugs which reduce high speed swim behavior (Stage 2 and Stage 3) associated with an epilepsy phenotype to control (Stage 0 or 1) levels, and (ii) lower-throughput electrophysiological assessment to confirm drug suppression of spontaneously occurring electrographic seizure events within the brain. As abnormal electrical events in the

TABLE 1 | Predictive validity of DS models.

		Antiepileptic activity					
AED	**DS patients**	***scn1lab*^{s552} zebrafish**	***scn1lab*^{s552} zebrafish**	***scn1lab* MO zebrafish**	**DS mice**		
		Spontaneous (acute)	**Light induced**	**Spontaneous (long-term)**	**Hyperthermia-induced**	**Spontaneous**	**Spontaneous (primed)**
Valproate	Yes[a,b,c]	Yes[d,e]		Yes[j]	Yes[b]	No[b]	No[b]
Clobazam	Yes[a,b,c]		No[i]		Yes[b]	No[b]	Yes[b]
Stiripentol	Yes[a,b,c]	Yes[d]	No[i]	No[j]	No[b]		No[b]
Topiramate	Yes[a,b,c]	Yes[e]			No[b]	No[b]	
Clonazepam	Yes[a,b,c]				Yes[k]		
Bromides	Yes[a,c]	Yes[d]					
Diazepam	Yes[a]	Yes[d]	No[i]				
Levetiracetam	No[a,b]	No[f,h]			Yes[b]	No[b]	
Ethosuximide	No[a,c]	No[d,h]					
Zonisamide	No[a,b]	No[f]					
Carbamazepine	No (worse)[a,c]	No[d,f,g,h]	No[i]	No[j]	No[b]	No[b]	
Phenytoin	No (worse)[a,b,c]	No[f,g,h]			No[b]		
Lamotrigine	No (worse)[a,b,c]	No[f,g,h]			No[b]	No[b]	
Oxcarbazepine	No (worse)[a,b]	No[f,g,h]					
Phenobarbital	No[a,b,c]				Yes[b]	No[b]	
Gabapentin	No[a]	No[h]					
Rufinamide	No[a]	No[f,g]					
No. of AEDs predictive		14/14	1/4	2/3	5/9	4/7	1/3

[a] *(Villas et al., 2017),* [b] *(Hawkins et al., 2017),* [c] *(Chiron, 2011);* [d] *(Baraban et al., 2013);* [e] *Hong et al. (2016),* [f] *see* ***Figure 3****,* [g] *(Griffin et al., 2017),* [h] *(Dinday and Baraban, 2015),* [i] *(Eimon et al., 2018),* [j] *(Zhang et al., 2015),* [k] *(Oakley et al., 2013).*

brain are the hallmark of epilepsy, individual LFP recordings are essential to exclude false positive hits from the behavior assay (such as sedatives or muscle relaxants). More importantly, we do not advocate using the behavioral assay as a stand-alone screening tool in the absence of electrophysiological confirmation.

Using this two-stage assay, incorporating behavioral and electrophysiological techniques pioneered for zebrafish larvae over the past decade in our laboratory, we showed suppression of unprovoked seizure activity with AEDs (valproate, diazepam, stiripentol, potassium bromide and topiramate) prescribed for DS patients (**Table 1**). Furthermore, *scn1lab*^{s552} mutant zebrafish also fail to respond to eight different AEDs which are contraindicated for DS including ion channel inhibitors like carbamazepine or ethosuximide. Using *scn1lab*^{s552} homozygous mutant larvae at 5 dpf larvae ensures a high frequency of baseline spontaneous seizures, allowing us to use relatively short assay times (30 min), generate high statistical power and maintain high screening throughput. Moreover, each treated larva is normalized to its own baseline to account for inherent differences often observed during *in vivo* behavioral assessments. Using this two-stage approach, we successfully demonstrated that the pharmacological profile of the *scn1lab*^{s552} mutants resembles that of DS patients with 100% accuracy (i.e., 14 of 14 AEDs correctly classified; **Table 1**). The predictive validity accuracy of this model and assay confers confidence in our ability to identify novel drug candidates using *scn1lab*^{s552} zebrafish.

In contrast to our published studies (Baraban et al., 2013; Dinday and Baraban, 2015; Griffin et al., 2017), others have trialed long-term protocols with lower drug concentrations and longer drug exposures (Zhang et al., 2015; Sourbron et al., 2016, 2017a). Initial long-term exposure studies at 24- and 48-h utilized a morpholino-based *scn1lab* knock-down approach and screened five known AEDs against spontaneous behavioral seizures. While valproate, clobazam, topiramate and stiripentol were shown to be effective in the behavioral seizure assays, only valproate was established to suppress electrical seizure activity in the brain, confirming the successful predictive validation for only one AED typically used in DS (**Table 1**). Given the transient knockdown observed by morpholinos, the high variability in the swim behavior observed in control larvae (Zhang et al., 2015), concerns about off-target effects, particularly neuronal defects and differences observed in knockdown verse knockout approaches reported in the zebrafish community (Stainier et al., 2017), it is difficult to envision how this approach can be reliably used for large-scale screening efforts.

To investigate the predictive validity of long-term exposure protocols, we initiated a series of behavioral and electrophysiological experiments to test AEDs using *scn1lab*^{s552} zebrafish in the same low micromolar drug concentrations

described by de Witte and Lagae (Sourbron et al., 2016, 2017a). Using the iZAP multi-fluidic recording device for non-invasive continuous uninterrupted monitoring of EEG activity of zebrafish larvae (Hong et al., 2016), we screened known and putative AEDs as described (Zhang et al., 2015; Sourbron et al., 2016, 2017a). We previously demonstrated the efficiency of the iZAP device to continuously record EEG data from up to 12 larvae simultaneously during baseline, drug exposure and washout; valproate and topiramate were published as examples of the validity of this recording device to identify clinically relevant AEDs with the ability to suppress unprovoked electrographic seizure activity in *scn1lab*^{s552} zebrafish (Hong et al., 2016). Using the long-term exposure protocol, we consistently observed an approximately 60% decrease in electrographic seizure activity (compared to baseline) at the 22-h timepoint in vehicle-exposed zebrafish. This reduction in unprovoked seizure activity was identical to that seen at the 22-h timepoint with a control drug exposure that has no known antiepileptic activities (acetaminophen). This confirms that the seizure frequency of *scn1lab*^{s552} zebrafish larvae naturally decreases after 5 dpf (regardless of treatment) which is consistent with previously published electrophysiology recordings (Baraban et al., 2013; Hong et al., 2016). Most importantly, in our hands, this decline in spontaneous EEG seizure activity was noted with every drug tested, using concentrations and long-term drug periods identical to those described (Zhang et al., 2015; Sourbron et al., 2016, 2017a; **Figure 1**). Because we could not distinguish between the effects of acetaminophen (or vehicle) and AEDs known to inhibit electrographic seizures in *scn1lab*^{s552} DS zebrafish (valproate, stiripentol, diazepam), AEDs contra-indicated for DS (phenytoin, carbamazepine) or candidate drugs (fenfluramine and lorcaserin) using the low micromolar concentration, 22-h long-term exposure protocol we conclude that these assays are not valid predictors of antiepileptic drugs. In an additional attempt to validate these long-term protocols, we performed similar studies using our DanioVision locomotion tracking assays and again observed a natural decrease in spontaneous seizure behavior at 22-h, and a failure to distinguish between controls, AEDs or candidate drugs (**Figure 2**). Our inability to replicate the low micromolar concentration, 22-h exposure assay data using the same *scn1a* zebrafish model suggests that assay (and not necessarily model) differences can account for discrepancies in the literature. Taken together, our results indicate that (i) "positive hits" identified in long-term exposure assays (albeit using a validated *scn1lab*^{s552} DS zebrafish line) should be interpreted with caution and (ii) acute versus long duration incubations of larvae with test drugs can make a substantial difference in outcome.

PHENOTYPIC DRUG SCREENING FOR DRAVET SYNDROME

Whole organism phenotypic drug screening provides an unbiased approach to systematically identify molecules that can modify a specific disease phenotype. Although mice offer strengths for understanding the basic biology and pathophysiology of epilepsy, they are not well suited to higher throughput drug screening platforms. In contrast, many aspects of zebrafish biology make them amenable for moderate- to high-throughput drug screening (i) unlike rodents, zebrafish larvae are not fetal but are closer to a "juvenile" state in that the nervous system is mature, vital organs are functioning and tissue architecture is fully developed within the first few days post-fertilization; (ii) only milligrams of compound are needed for screening in 96-well plates as larvae; (iii) zebrafish are reasonably tolerant to dimethylsulphoxide (DMSO) concentrations generally used in drug libraries; and (iv) small molecule compounds dissolved in the swimming medium reach larval target tissues via rapid diffusion through the skin.

Using a whole-animal approach is also advantageous for identifying compounds targeting a network disorder such as epilepsy, as the complex neuronal interactions, vascular components, and neurotransmitter signaling pathways are difficult to recapitulate *in vitro*. Larval zebrafish enable parallel screening for toxicity and activity within the central nervous system, a critical requisite for targeting diseases that affect the brain. Given the evidence that the zebrafish *scn1lab*^{s552} model recapitulates salient genetic, behavioral, electrophysiological phenotypes observed in DS patients, and has been pharmacological validated against known AEDs, this model appears ideal for screening compound libraries for antiepileptic activity (with appropriate validated assays). Furthermore, because *scn1a* homozygous mutant zebrafish exhibit a very high baseline seizure frequency—approximately one ictal-like electrographic seizure event per minute with up to one interictal-like event per second—even relatively short recording epochs (10–20 min) are more than sufficient to monitor seizure activity and power statistical studies for drug discovery.

Using an acute exposure protocol and a two-stage screening platform the Baraban laboratory has screened seven commercially-available libraries consisting of almost 3000 compounds spanning multiple drug classes and targeting several suggested therapeutic mechanisms (Baraban et al., 2013; Dinday and Baraban, 2015; Griffin et al., 2017; **Figure 3**). After repeated locomotion testing, including assaying independently sourced compounds, only 13 compounds (<0.5%) have been identified by electrophysiology as "false positive" hits. These include the anesthetic lidocaine, the muscle relaxant pancuronium bromide, the N-methyl-D-aspartate antagonist MK-801 and the hallucinogen TCB-2 which was previously identified as an anti-seizure drug. (Sourbron et al., 2017a). On average, approximately 20% of compounds are classified as toxic when screened at 250 μM as they result in decreased or absent heart beat and/or an absent touch-evoked escape response after 90 min of exposure. Screening at higher (667 μM; Baraban et al., 2013) or lower (100 μM; Dinday and Baraban, 2015) concentrations as the assays were optimized, we observed toxicities of ∼50% and <10% respectively (**Figure 4**).

Probably the greatest advantage of zebrafish assays is that they facilitate blinded phenotypic screening of compound libraries and unbiased discovery of new AED candidates. For example, clemizole was identified to exert a powerful suppression of behavioral and electrographic seizures in the *scn1lab*^{s552} larvae (Baraban et al., 2013; Griffin et al., 2017).

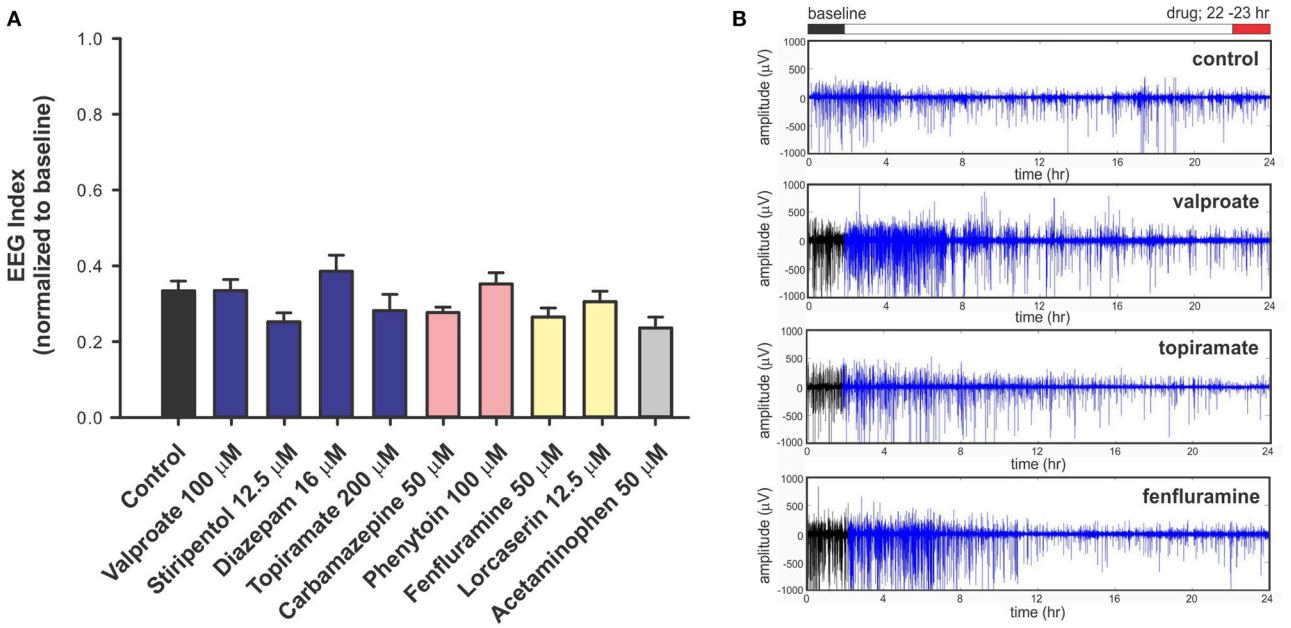

FIGURE 1 | Electrophysiology analysis of *scn1lab*^{s552} homozygous mutant larvae during long-term exposure of AEDs. For non-invasive long-term monitoring of electrographic seizure activity in larval zebrafish, we used an integrated zebrafish activity platform (iZAP) microfluidic recording system previously described by our laboratory (Hong et al., 2016). The iZAP system consists of three primary components: (i) microfluidic unit, (ii) multi-electrode array, and (iii) integrated electronic unit with multichannel amplifiers for simultaneous monitoring of surface EEG activity on five independent electrodes for up to 12 larvae at an acquisition rate of 1 kHz. For these experiment, *scn1lab*^{s552} homozygous mutants screened from larval clutches based on pigmentation are loaded into the iZAP at 5 dpf and continuously monitored for 24-h in embryo medium (or test drug) supplemented with 300 μM pancuronium. A custom algorithm based on distinct features of the zebrafish electrographic seizure signal and spatial correlation between the 5 surface electrodes was used to detect and score seizure events, as described (Hong et al., 2016). For each test compound, we first obtained a 2-h baseline recording, followed by a 22-h drug exposure; at least seven larvae were tested per drug/per experiment and all experiments included at least one biological replicate. Recordings were normalized to the baseline seizure activity and presented as an EEG index where a ratio of 1.0 would represent no change in activity at 22-h vs. baseline and 0.0 would represent a complete suppression of seizure activity. **(A)** Plot of all drug treatments and controls (embryo media) tested in the iZAP device using the low micromolar concentrations published in Sourbron et al. (2016), Sourbron et al. (2017b), or Zhang et al. (2015). Recommended treatments (blue), contra-indicated treatments (red), and recent experimental treatments (yellow) for DS are shown; acetaminophen is also shown (gray). Note that electrographic seizure activity diminishes by approximately 60% from baseline in all *scn1lab*^{s552} homozygous mutants larvae with no significant differences noted between putative antiepileptic drugs, control and acetaminophen exposures using these low micromolar, 22-h drug exposures (Sourbron et al., 2016, 2017a). Graph represents mean (±SD). Statistical significance was determined by one-way ANOVA followed by Dunnet's multiple comparison test. **(B)** Representative raw EEG recordings from one surface electrode channel (of five) are shown for the entire 24-h recording period. Baseline recordings are indicated in black; drug exposures are shown in blue. These raw traces highlight the reduction in electrographic seizure activity seen over time under any recording condition at this stage of *scn1lab*^{s552} larval development. In contrast to these data, but consistent with our acute LFP recording protocol, we previously used the iZAP recording device to demonstrate a significant and reversible suppression of electrographic activity monitored in *scn1lab*^{s552} homozygous mutants (2-h drug exposure epochs) for valproate (1 mM), topiramate (1 mM), lorcaserin (250 μM) and trazodone (250 μM) (Hong et al., 2016; Griffin et al., 2017) further highlighting the discrepancy between acute and chronic drug assays.

Although clemizole is a first-generation anti-histamine, 49 other antihistamines from the screening database failed to exhibit anti-seizure activity suggesting, not unexpectedly, that antagonizing the H1 receptor does not decrease seizures in DS. Through a series of binding studies, it was discovered that clemizole binds serotonin 2 receptors. From additional blinded screening of targeted libraries two additional serotonin 2 receptor modulating drugs, lorcaserin and trazodone, were identified as effective in suppressing seizure activity in the *scn1lab*^{s552} larvae. Taken together, from our blinded, unbiased, screening of ∼3,000 drugs, the three compounds which reduce seizures in *scn1lab*^{s552} larvae (clemizole, lorcaserin and trazodone) all bind serotonin 2 receptors. Furthermore, over 4% of all compounds tested are recognized to modulate serotonin signaling or bind serotonin receptors, suggesting this screening methodology can distinguish on-target effects of individual compounds (**Figure 3**). In addition, candidate screening of a selective serotonin reuptake inhibitor fenfluramine, which has shown some success as an add-on treatment for DS (Ceulemans et al., 2012) and is currently in Phase III clinical trials (https://clinicaltrials.gov/ct2/show/NCT02682927), also demonstrated efficacy against spontaneous seizures in the *scn1lab*^{s552} mutants (Dinday and Baraban, 2015; Sourbron et al., 2016). Although experimental evidence suggests that fenfluramine acts to modulate serotonergic signaling, Sourbron et al. (2017a) recently suggested that antagonism of the sigma-1 receptor is a putative mechanism of action for fenfluramine. Interestingly, retrospective analysis of our drug library database revealed eight sigma-1 receptor binding compounds that failed to suppress behavioral seizure activity in *scn1lab*^{s552} zebrafish using our blinded locomotion assay. As

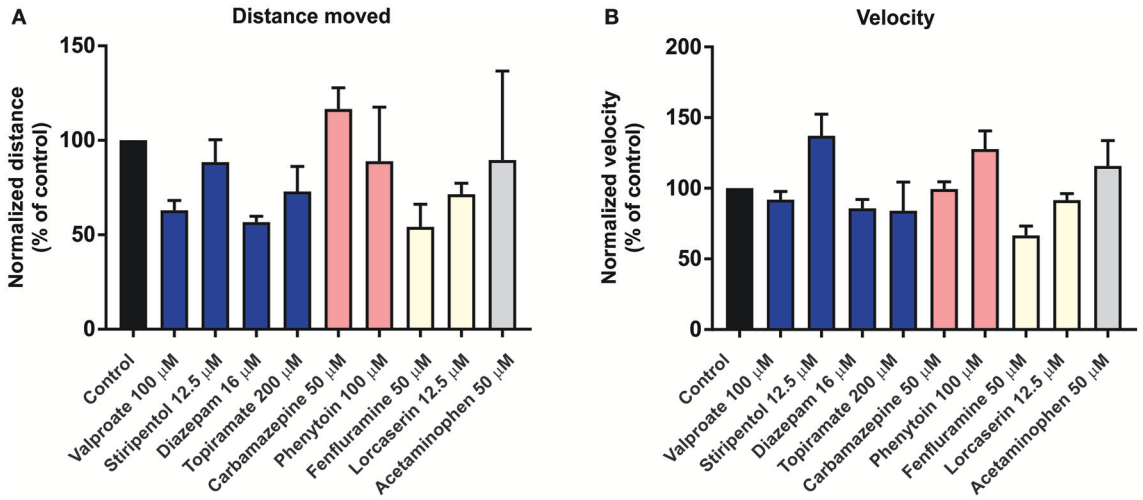

FIGURE 2 | Behavior analysis of *scn1lab*^{s552} homozygous mutant larvae during long-term exposure of AEDs. To examine the effect of long-term exposure of AEDs on *scn1lab*^{s552} swim behavior was analyzed using the DanioVision running EthoVision XT software (DanioVision, Noldus Information Technology). As previously described (Sourbron et al., 2017b), 6 dpf *scn1lab*^{s552} homozygous mutants were identified based on pigmentation and arrayed in a 96-well plate. Larvae were treated with 2% DMSO (control) or compound using drug concentrations previously published (Zhang et al., 2015; Sourbron et al., 2016, 2017a). After 22-h incubation at 28°C with a 14:10 h light/dark photoperiod, and 30 min of chamber habituation, the swim behavior of 7 dpf larvae was analyzed for 10 min under dark conditions. Both the **(A)** total distance moved and **(B)** velocity were analyzed by normalizing the activity of AED treated larvae to vehicle treated controls (previously described as method A, Sourbron et al., 2017b). Graphs represents mean (±SD) normalized to control treated larvae from three independent experiments using the average from 12 larvae per treatment each time. Statistical significance was determined by one-way ANOVA followed by Dunnet's multiple comparison test. No statistical significance changes in swim behavior of *scn1lab*^{s552} homozygous mutants when exposed to known AEDs or putative AEDs. Recommended treatments (blue), contraindicated treatments (red) and recent experimental treatments (yellow) for DS are shown. Acetaminophen is also shown (gray). These behavioral results, which also fail to distinguish between any of the experimental situations tested, are entirely consistent with the data independently obtained using the iZAP system and fail to support the validation of this low micromolar, 22-h exposure assay as an effective means to identify drugs with antiepileptic activity in *scn1lab*^{s552} homozygous mutants.

such, we could not independently confirm an antiepileptic action for sigma-1 antagonism.

Offering another alternative approach, Eimon et al. (2018) published a drug screening protocol using a multichannel local-field potential recording platform mimicking the invasive agarose-embedding procedure established in 2005 (Baraban et al., 2005). Here, *scn1lab*^{s552} zebrafish were tested at 7 dpf, but unlike previously discussed studies which focused on the naturally-occurring spontaneous unprovoked seizures, a 10 min light-provoked seizure protocol consisting of a dual-pulse light stimulus every 2 min was employed. Combined with an automated seizure detection algorithm to detect electrographic seizure-like events, 154 compounds were evaluated for their ability to restore provoked electrographic activity to a sibling level. Although swim behavior analysis identified stiripentol, diazepam, clonazepam and clobazam as decreasing light-provoked seizure-like activity, none of these drugs were successfully identified using the seizure algorithm or the behavioral analysis described by Eimon et al. (2018). For example, diazepam, clonazepam and clobazam (Onfi®), are the first line benzodiazepine AEDs used by DS patients, scored the same as the vehicle control using their LFP brain activity pattern algorithm combined with "deep behavioral phenotyping". This suggests poor predictive validity of this assay to identify AEDs suitable for DS patients. Likewise, stiripentol (Diacomit®), an AED approved in Europe for the treatment of DS was also not identified. Furthermore, five compounds (with entirely unrelated mechanisms of action) that previously failed to show any antiepileptic efficacy against spontaneous seizures in *scn1lab*^{s552} zebrafish (Baraban et al., 2013; Dinday and Baraban, 2015; Griffin et al., 2017)—pargyline, progesterone, promethazine, mifepristone and fluoxetine—were identified as the "highest-ranked compounds" in this publication suggesting, again, that these assay outcomes should be interpreted with caution. Finally, although the LFP complexity scoring failed to successfully predict clemizole at 10 μM (a concentration some 10-fold lower than previously reported) using a provoked seizure assay, it was stated that "retesting clemizole at higher concentrations reduces the number of spontaneous seizures", replicating our findings with *scn1lab*^{s552} zebrafish. Using an approach that does not first successfully identify AEDs clinically prescribed to DS patients makes it challenging to interpret the effectiveness of potential new therapies for this already difficult to treat patient cohort. Additionally, these studies highlight that drug discovery programs must consider the choice of model, as well as the predictability of the assay to have the best chance of identifying effective AEDs.

TRANSLATING FROM THE LABORATORY TO THE CLINIC

As alternative models like zebrafish emerge as valid preclinical models for drug discovery, understanding and translating

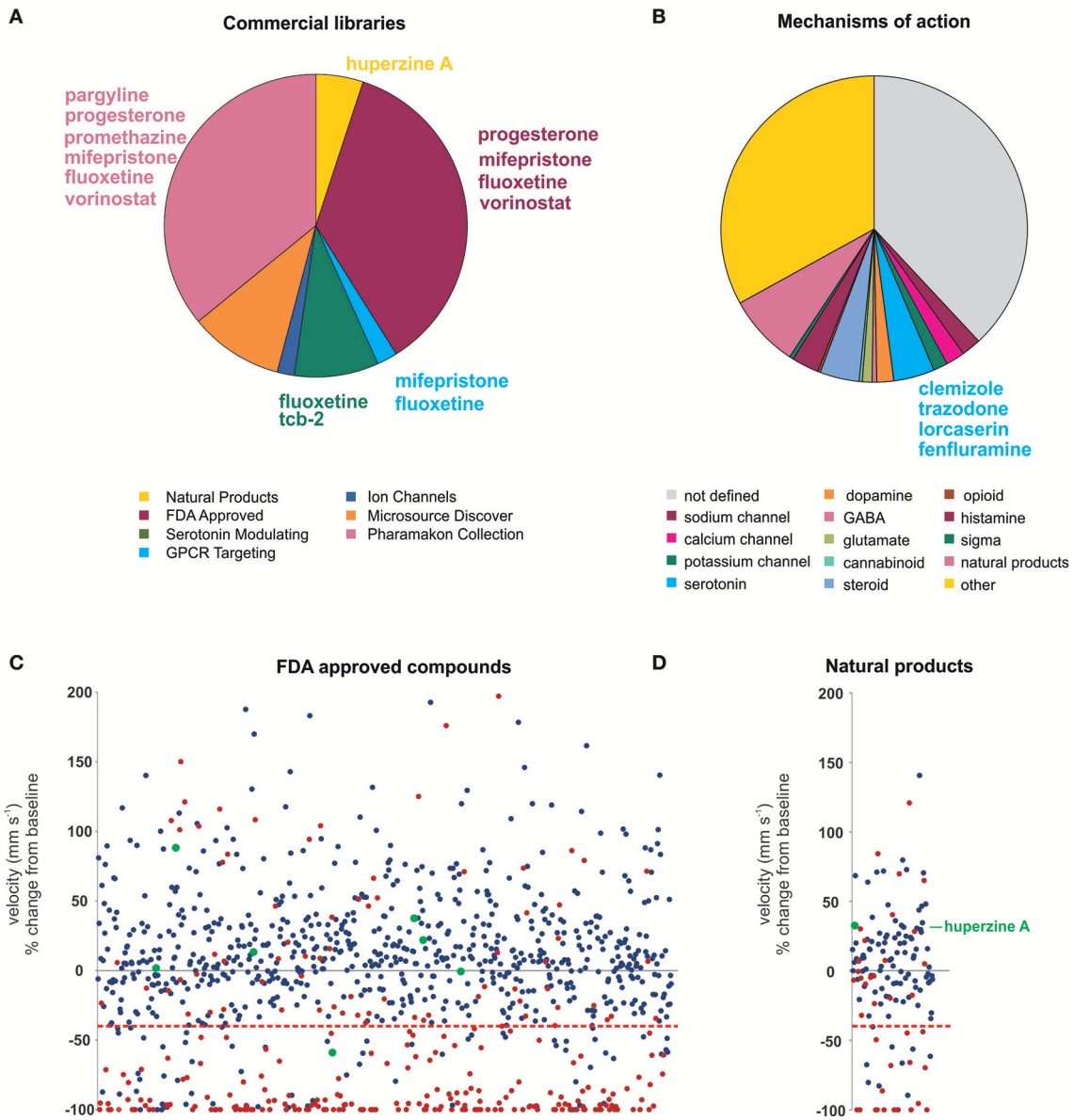

FIGURE 3 | Summary of the compound screening results using the *scn1lab*^{s552} homozygous mutant larvae to identify drugs for DS. Using our pharmacologically validated dual-stage assay screening protocol, 2,863 compounds have been blind tested for anti-seizure activity in the *scn1lab*^{s552} homozygous zebrafish larvae. **(A)** Seven commercially sourced drug libraries were screened including the MicroSource Discovery Systems' International Drug Collection (Baraban et al., 2013) and Pharmakon Collection (Dinday and Baraban, 2015) and Selleckchem's, ion channel library, GPCR compound library, a serotonin modulating compound library (Griffin et al., 2017), a natural product library and a FDA-approved compound library. Compounds highlighted in this plot were reported by other groups as being effective anti-seizure compounds, but could not be confirmed as such in our hands. Specifically, these compounds failed to induce an antiepileptic response when screened blinded as part of these commercial libraries. Blind screening of libraries allows for unbiased testing of compounds regardless of their mechanism of action. **(B)** A summary of identified mechanism of actions of all compounds screened highlighting the broad range of mechanisms covered by these libraries. Clemizole, trazodone and lorcaserin effectively suppressed seizure activity in the *scn1lab*^{s552} homozygous zebrafish larvae. These compounds have known activity at serotonin 2 receptors. 4.3% of all drugs tested are known to modulate serotonin signaling, however, only these drugs were effective. The serotonin reuptake inhibitor fenfluramine is also effective in suppressing *scn1lab*^{s552} homozygous larvae seizure activity and is currently in clinical trial for DS. **(C)** The FDA approved compound library, and **(D)** natural product library were also screened for compounds inhibiting seizure activity. Plots show the locomotor seizure behavior for 5 dpf *scn1lab*^{s552} mutants during the first stage screening. The threshold for inhibition of seizure activity (positive hits) was determined as a reduction in mean swim velocity of 40% (red line). Red data points represent compounds that were classified as toxic as treated larvae have no visible heartbeat or movement in response to touch after 90 min exposure. Green data points represent known AEDs. The natural product huperzine A which has been shown to be effective against hyperthermia induced seizures is labeled. No additional lead compounds were identified in these libraries.

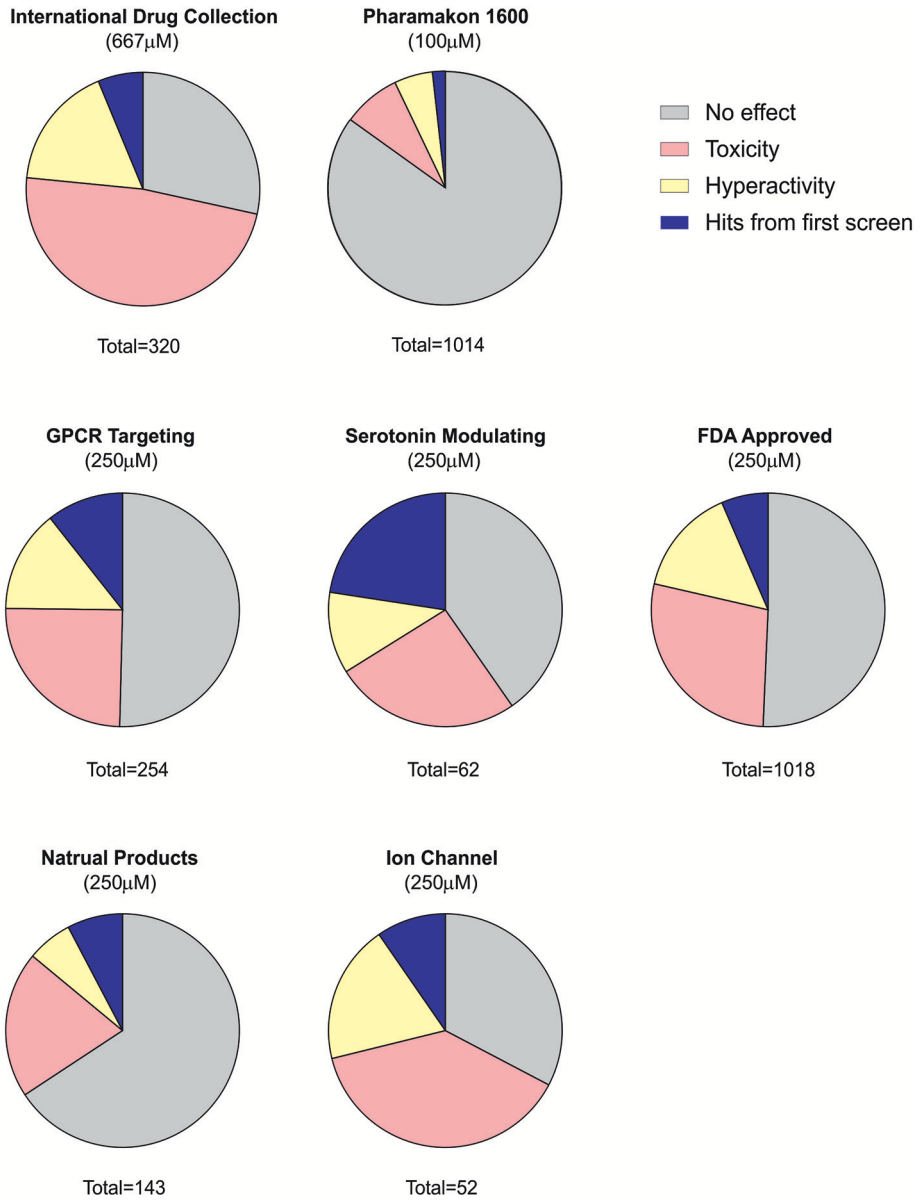

FIGURE 4 | Summary of behavioral screening results for anti-seizure compounds in DS zebrafish larvae. In total, seven commercially available libraries have undergone blind screening for compounds which suppress the seizure activity in *scn1lab*^{s552} homozygous larvae. These include the MicroSource Discovery Systems' International Drug Collection (Baraban et al., 2013) and Pharmakon Collection (Dinday and Baraban, 2015) and Selleckchem's, ion channel library, GPCR compound library, a serotonin modulating compound library (Griffin et al., 2017), a natural product library and a FDA-approved compound library. Plots represent the results from the first blinded screening of each library. A hit is recognized as a compound which reduces the high-speed seizure like swim behavior by more than 40% (>2 S.D.). Once a hit is retested it is then screened by an independent laboratory member. If identified as a hit again, the compound is unblinded and independently sourced for further testing including the second stage electrophysiology assay. The serotonin library exhibited the greatest percentage of positive hits in the first-pass behavioral assay consistent with subsequent identification of four serotonin modulating drugs for the potential treatment of DS e.g., clemizole, trazodone, lorcaserin and fenfluramine. It is also interesting to note that the percentage of toxic drugs is greatest in the ion channel library cohort. Approximately 20% of compounds are identified as toxic when screened at 250 μM as they result in decreased or absent heart beat and/or an absent touch-evoked escape response after 90 min of exposure. When screening at 667 μM, 48% of compounds were identified as toxic. The majority of compounds fail to elicit any significant change in the swim velocity of the larvae.

pharmacokinetics remains to be fully explored. Pharmacokinetic ADME (absorption, distribution, metabolism, and excretion) studies represent a crucial aspect of drug development. These studies are traditionally performed in rodents and are not well-suited to zebrafish. In traditional mammalian models, drug pharamacokinetics can be easily established from administration of single or repeated drug concentrations. In zebrafish larvae, exposure to drug remains constant as the larvae is immersed in bathing media containing the drug, which is rapidly absorbed through the skin and gills. Currently, quantifying drug uptake into zebrafish larvae remains a limitation of this model. Differences in drug absorption are unavoidable and

directly measuring drug concentrations in serum or tissues of microscopic larvae remains technically challenging particularly in a high-throughput drug screening environment. Currently, there are no zebrafish studies understanding how the effective concentrations in larvae can be related to appropriate effect levels in mammalian models, and we caution that these direct concentration comparisons may not be possible. As used, current zebrafish screening approaches can only assess whether a drug has anti-seizure properties (or not) and we would advise against over-interpreting concentrations used or ranking drugs based on effectiveness in these larval assays.

Nonetheless, with the current availability of accurate genetic models of DS and evaluating drugs against epilepsy, "personalized" treatment options are beginning to emerge. While this represents an exciting advance in the epilepsy field and an important alternative to traditional drug screening programs, the validity of drugs identified by these models will ultimately be determined in the clinic. Despite this potential, a major concern highlighted in several epilepsy community "white papers" has been the poor reproducibility of preclinical data for compounds progressing from academic laboratories to clinical trials (Galanopoulou et al., 2012, 2017; Simonato et al., 2014, 2017). As these reviews failed to adequately include or provide a rigorous evaluation of any preclinical zebrafish drug discovery research, we believe these types of concerns are irresponsibly premature and negligently misguided. In less than 5 years, using a well-characterized *scn1lab*^{s552} zebrafish model and pharmacologically validated methodologies described here, compounds effective against spontaneous seizures have already shown exciting early promise in clinical studies. As one small example, a serotonin receptor agonist (lorcaserin, Belviq®) identified only in a DS zebrafish model was used to treat five medically intractable DS patients and showed promising results in terms of reductions in seizure frequency and/or severity i.e., a 65% reduction in seizure frequency during the first 3-month treatment period (Griffin et al., 2017). Although this is the first "aquarium-to-bedside" example, and will ultimately require more rigorous clinical testing on larger patient cohorts, it hints at the tremendous potential a zebrafish-based platform holds for achieving true and effective personalized medicine. Furthermore, preclinical strategies that show "construct," "face," and "predictive" validity offer the best chance of success for identifying clinically effective treatments for genetic intractable epilepsy.

AUTHOR CONTRIBUTIONS

AG and SB: manuscript preparation; AG, KH, and MA: locomotion screening and analysis; SH and SB: electrophysiology and analysis; LL and SB: experimental oversight and supervision.

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frontiers in Pharmacology

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Protective Role of L-3-*n*-Butylphthalide in Cognitive Function and Dysthymic Disorders in Mouse With Chronic Epilepsy

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Epilepsy is a common neurological disease with recurrent seizures and neurobehavioral comorbidities, including cognitive impairment and psychiatric disorders. Recent studies suggest that L-3-*n*-butylphthalide (NBP), an extract from the seeds of *Apium graveolens* Linn. (Chinese celery), ameliorates cognitive dysfunction in ischemia and/or Alzheimer's disease animal models. However, little is known about the role of NBP in epilepsy and the associated comorbidities. Here, using a pilocarpine-induced chronic epileptic mouse model, we found that NBP supplement not only alleviated seizure severity and abnormal electroencephalogram, but also rescued cognitive and emotional impairments in these epileptic mice. The possible underlying mechanisms may be associated with the protective role of NBP in reducing neuronal loss and in restoring the expression of neural synaptic proteins such as postsynaptic density protein 95 (PSD95) and glutamic acid decarboxylase 65/67 (GAD65/67). In addition, NBP treatment increased the transcription of neuroprotective factors, brain-derived neurotrophic factor and Klotho. These findings suggest that NBP treatment may be a potential strategy for ameliorating epileptogenesis and the comorbidities of cognitive and psychological impairments.

Keywords: l-3-*n*-butylphthalide, chronic epilepsy, learning and memory, anxiety and depression, protection

INTRODUCTION

Epilepsy is a common chronic neurological disease with recurrent seizures and abnormal electroencephalogram (EEG) discharge, of which the annual cumulative incidence was 67.77 per 100,000 persons worldwide (Fiest et al., 2017). According to a population-based study, one-third of the people with epilepsy have associated anxiety or depressive disorder (Rai et al., 2012). In chronic epilepsies, about 70–80% of patients have cognitive impairment (Helmstaedter and Witt, 2012). Moreover, recurrent seizures or uncontrolled convulsions aggravate cognitive deficits and affect the life quality of the epilepsy patients (You et al., 2017). Furthermore, it has been reported that about 39% of patients with epilepsy are drug-resistant (Kwan and Brodie, 2000). Studies have shown that treatment with some antiepileptic drugs (AEDs) is associated with psychiatric comorbidity deterioration in the patients (Mula and Sander, 2007; Brodie et al., 2016; Mula, 2017). Therefore,

attentions need to be paid in finding new effective drugs concerning epilepsy comorbidities.

L-3-*n*-butylphthalide (NBP), extracted from the seeds of *Apium graveolens* Linn. (Chinese celery), is widely applied to treat ischemic stroke (Peng and Cui, 2013). Although the specific target of NBP is unknown, accumulative studies suggest that NBP ameliorates cognitive dysfunction in ischemic animal models, as well as transgenic mouse models of Alzheimer's disease (AD) (3xTg mice and APP/PS1 mice), by inhibiting oxidative damage, rescuing synaptic dysfunction, reducing inflammatory, and alleviating neuron loss (Peng et al., 2007, 2010; Wang et al., 2016; Zhao et al., 2016). Epilepsy and dementia/AD pathology share impairments in brain networks associated with hippocampus, and hence have similar behavioral and cognitive disturbances (Pulliainen et al., 2000; Vrinda et al., 2018). Importantly, some findings also suggest that older patients with epilepsy are at a higher risk of developing cognitive impairment and ultimately dementia (Breteler et al., 1995; Sen et al., 2018). These reports infer the therapeutic potential of NBP in the treatment of epileptic comorbidities. However, little is known about the role of NBP in epilepsy and the neurobehavioral comorbidities of epilepsy.

Given that neural synaptic proteins such as postsynaptic density protein 95 (PSD95), a scaffold protein associated with synapse maturation and synaptic stability, strength, and plasticity (El-Husseini et al., 2000; Elias et al., 2006), and glutamic acid decarboxylase 65/67 (GAD65/67), an important enzyme in gamma-aminobutyric acid (GABA) synthesis, are critical for neural synapse in cognitive dysfunction and epilepsy (Fernandez et al., 2017), and that brain-derived neurotrophic factor (BDNF) and Klotho are neuroprotective factors known to improve cognition and/or psychiatric behavior (Kang and Schuman, 1995; Sairanen et al., 2007; Dubal et al., 2014, 2015), whether these proteins were affected by NBP in the cellular processes also needs to be clarified.

In the present study, we found that NBP supplement alleviated seizure severity and abnormal EEG in pilocarpine-induced chronic epileptic mice. We also demonstrated that NBP treatment ameliorated cognitive impairment and emotional disorder in these epileptic mice. The possible mechanisms underlying the efficacy of NBP may be associated with its role in reducing neuronal loss and increase the expression of PSD95 and GAD65/67. In addition, NBP treatment increased the transcription of neuroprotective factors, BDNF and Klotho. These findings suggest that NBP may be a potential drug for ameliorating epileptogenesis and the comorbidities of cognitive and psychological impairments.

MATERIALS AND METHODS

Drugs and Chemicals

NBP (purity >98%), offered by CSPC NBP Pharmaceutical Co., Ltd. (Shijiazhuang, China), was dissolved in 0.5% Tween-80 (Solarbio, Beijing, China) solution at a concentration of 10 mg/ml. Pilocarpine was purchased from BSZH Co., Ltd. (Beijing, China) and was dissolved in 0.9% sodium chloride.

Animals

Fifty-seven 10-week-old male C57BL/6 mice (weighing 22–25 g) were from the Laboratory Animal Centre of Xiamen University. Animals were housed under a 12/12-h light/dark cycle (lights on at 6:00 a.m.) with food and water *ad libitum*. The temperature and humidity of the breeding house was kept consistent (temperature: 23 ± 1°C; humidity: 50–60%) during the experiments. All efforts were aimed to lessen animal's suffering. All animal experiments were performed in accordance with the protocols of the Institutional Animal Care and Use Committee at Xiamen University.

Pilocarpine Model of Temporal Lobe Epilepsy in Mice and Treatment

According to the method in pilocarpine-induced status epilepticus (SE), mice were intraperitoneally (i.p.) injected with small-dose of pilocarpine (100 mg/kg, n = 45) or saline (n = 12) every 20 min until the onset of SE (Groticke et al., 2007). For blocking peripheral cholinergic effects, atropine sulfate (1 mg/kg i.p.) was administered 30 min before pilocarpine injection (Clifford et al., 1987; Morrisett et al., 1987). Epileptic behavior of the mice was observed as previously described (Racine, 1972) with the following stages: Stage I, Mouth and facial movements; Stage II, Head nodding; Stage III, Forelimb clonus; Stage IV, Rearing; Stage V, Rearing and falling. SE was referred to as a Stage IV–V motor seizure sustaining more than 30 min. All mice developed SE and were injected with diazepam (10 mg/kg) after 120 min of SE to decrease mortality. About 20/45 (44%) mice died during or after SE. Fifteen days following SE, the survival mice were randomly divided into two groups: pilocarpine + NBP group and pilocarpine + Tween-80 group. For pilocarpine + NBP group (n = 13), mice were administrated NBP by i.p. for 14 consecutive days at a dose of 80 mg/kg according to the reported literatures with minor modification (Peng et al., 2010; Wang et al., 2014). For pilocarpine + Tween-80 group (n = 12), mice received 0.5% Tween-80 i.p. injection without NBP. The 12 male mice with saline control treatment received another 0.5% Tween-80 i.p. injection for vehicle group (n = 12 saline + Tween-80 group). EEGs were recorded at 1 h, 15 days, and 30 days following saline or pilocarpine injection (**Figure 1A**). Eight mice of each group were then subjected to cognitive and psychological behavior test following the final injection of NBP or 0.5% Tween-80. Another four or five mice were sacrificed and the brains were dissected for Nissl staining, Western blotting or quantitative polymerase chain reaction (qPCR).

EEGs Recording

Electroencephalograms were recorded according to the method depicted previously (Zheng et al., 2016). Briefly, two polyamide-insulated stainless steel monopolar microelectrodes (0.1 mm diameter; Plastics One, Inc., Roanoke, VA, United States) were imbedded into the frontal area of bilateral hemispheres for 1 cm of depth. Insulated 50 μm-diameter stainless steel wire (California Fine Wire) was implanted into the skin above nasal

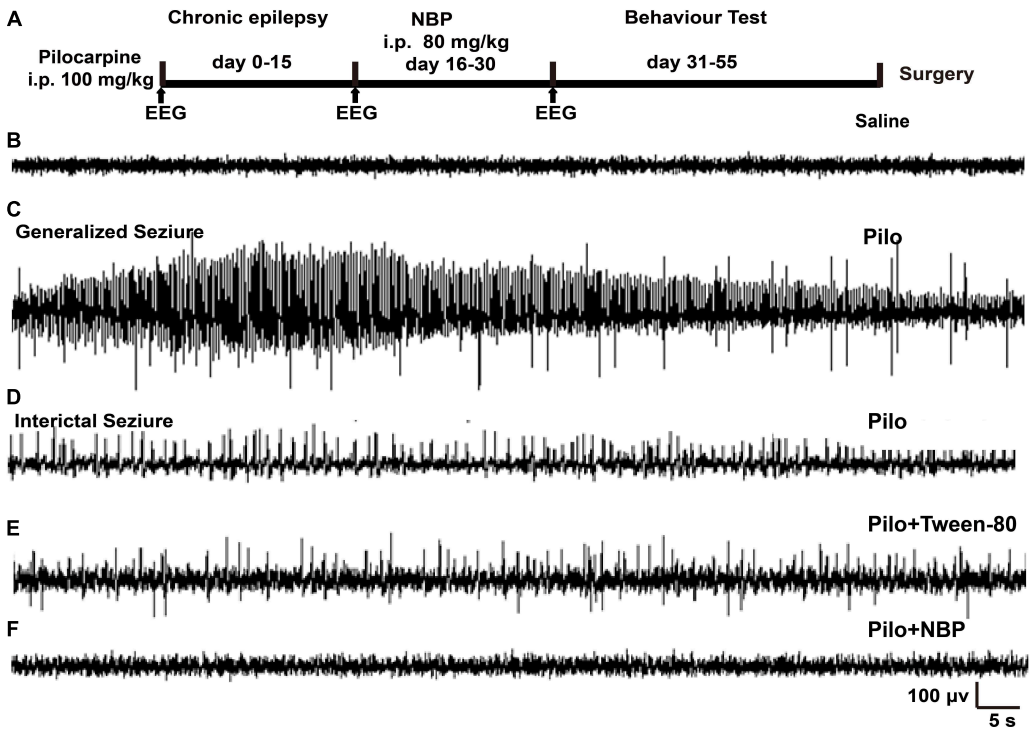

FIGURE 1 | NBP treatment reduced seizure incidence. **(A)** Schematic model of the experimental design for NBP treatment using pilocarpine-induced chronically epileptic mice. **(B,C)** Examples of EEG recordings in C57BL/6 mice at 1 h following saline **(B)** or pilocarpine **(C)** treatments. Note that pilocarpine generated high-frequency spikes. **(D)** In the epileptic animals 15 days post-SE, interictal EEG during a spontaneous behavioral seizure exhibited epileptiform discharges. **(E,F)** Fifteen days after SE, mice were treated with NBP or Tween-80 for 14 days. The EEGs on the 15th day post Tween-80 or NBP injection were recorded. The spontaneous epileptiform discharges and behavioral seizures were significantly decreased in NBP treated mice **(F)** whereas they were still exhibited in the Tween-80 treated mice **(E)**.

bone. The reference electrode was placed in the right musculi temporalis. Data were analyzed by Easy EEG II (Version 2.0.1).

Behavior Tests

Behavior tests were performed between 8:00 a.m. and 2:00 p.m., i.e., during the light period of the light–dark cycle on the 31st day after SE. All data were recorded and analyzed by SMART digital tracking system (Version 3.0).

Morris Water Maze

Morris water maze (MWM) was performed to evaluate the memory and learning capacity (Morris, 1984). The four quadrants pool of MWM, was 90 cm in diameter and 35 cm in height. A hidden platform was placed in the target quadrant 1.0–2.0 cm under the surface of the water (22–25°C). The MWM test was carried out within 48 h after last injection and the test was composed of spatial memory training and probe trial. In the spatial memory training, all mice were trained for four times per day for 7 days. Mice were put into the water facing the wall of the pool at four different directions in the four quadrants and were allowed to find the hidden platform within 1 min standing for 10 s. If the mouse failed to find the platform within 1 min, the mouse was then guided into the platform standing for 10 s. The time for finding the platform was recorded (escape latency). The probe trial was performed 24 h after the last training session. The platform was removed and the mice were released in the zone opposite to the area of the platform, allowing them to swim freely for 60 s. The mean speed in the zone and the percentage of time in each quadrant were recorded. After testing, all mice were dried with towel and were placed in a warming cage.

Black–White Box

The black–white box was carried out to assess anxiety-like behavior for mice's natural preference for dark spaces and exploring new environment spontaneously (Teixeira et al., 2011). Black–white box was made of wood, 40 cm long × 15 cm wide × 15 cm high, divided into two compartments (light and dark, accounting for 50% respectively) and connected by a small door by which the mice can pass. Each tested mouse was placed in the small door facing dark box and its behavior was recorded for a 10-min trial. The time the mice spent in the light one was analyzed to evaluate anxiety behavior.

Tail Suspension Test

Mice were suspended from an iron hoop by fastening a quarter of the tail with adhesive tape. The time of the animal's immobility was recorded during the final 6 min of the 7-min test. Immobility was defined as the deficiency of any limb or body movements, beside those caused by respiration.

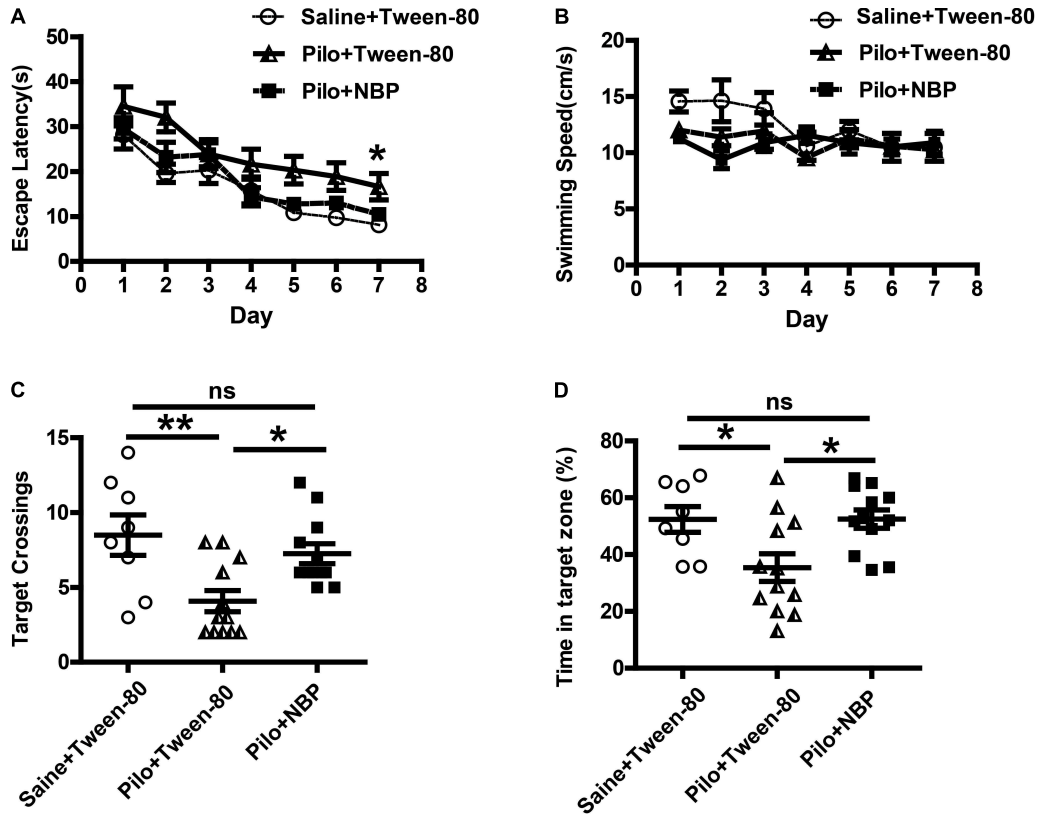

FIGURE 2 | NBP treatment improved spatial learning and reverted memory deficits in chronically epileptic C57BL/6 mice. **(A–D)** Morris water maze test. **(A)** Mean latency to reach platform with a 7-day training period. **(B)** Swimming speed. **(C)** Times of target platform crossing. **(D)** The percentage of time in target zone. $n = 8$ for each group. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$; ns, not significant.

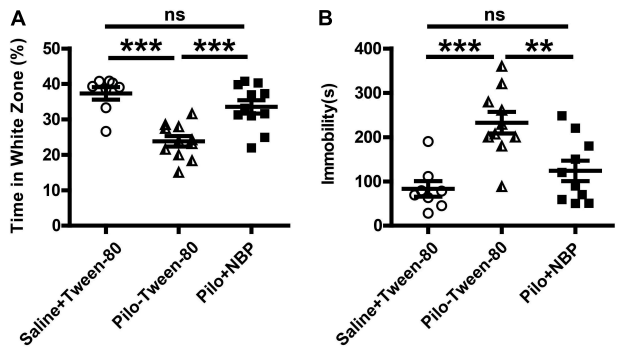

FIGURE 3 | NBP treatment ameliorated anxiety- and depression-like behavior in pilocarpine induced epileptic mice. **(A)** Black–white box: time spent in white compartment. **(B)** Total time of immobility in the tail suspension test during the last 6 min of the 7 min test session. $n = 8$ for each group. $^{**}p < 0.01$, $^{***}p < 0.001$; ns, not significant.

Western Blotting

All brain tissues were homogenized in RIPA buffer (Boster, Wuhan, China), with protease inhibitors and phosphatase inhibitors (Roche, Basel, Switzerland). The samples were centrifuged at 12,000 rpm for 15 min at 4°C and tissue debris was removed. Protein concentration was determined by BCA assay kit (Thermo Fisher Scientific, Waltham, United States). Protein samples (20∼40 μg per lane) were separated by SDS-PAGE and were then transferred into PVDF membranes (Millipore, Billerica, United States). Blocked for 60 min with 5% (w/v) non-fat milk in Tris-buffered solution-Tween 20, membranes were then incubated overnight at 4°C with PSD95 (1:500, Millipore, St. Charles, MO, United States), GAD65/67 (1:1000, Millipore, St. Charles, MO, United States), or β-actin (1:4000, Cell Signaling Technology, Boston, United States) primary antibody. Then they were incubated with HRP conjugated secondary anti-rabbit or anti-mouse IgG (Thermo Fisher Scientific, Waltham, United States) for 60 min at room temperature. The signals were detected using ECL kit (Millipore, St. Charles, MO, United States) and analyzed by Image J 1.46.

Quantitative Polymerase Chain Reaction

Total RNA was extracted using Trizol Reagent (Thermo Fisher Scientific, Waltham, United States), and was reverse transcripted to cDNA using ReverTra Ace qPCR RT Master Mix (TOYOBO, Osaka, Japan). Quantitative real-time PCR was performed using a Light Cycler 480 II (Roche, Basel, Switzerland) with SYBR Green PCR Master Mix (Roche, Basel, Switzerland) in a 10-μl reaction mixture with 250 nM primers. Relative mRNA level of examined

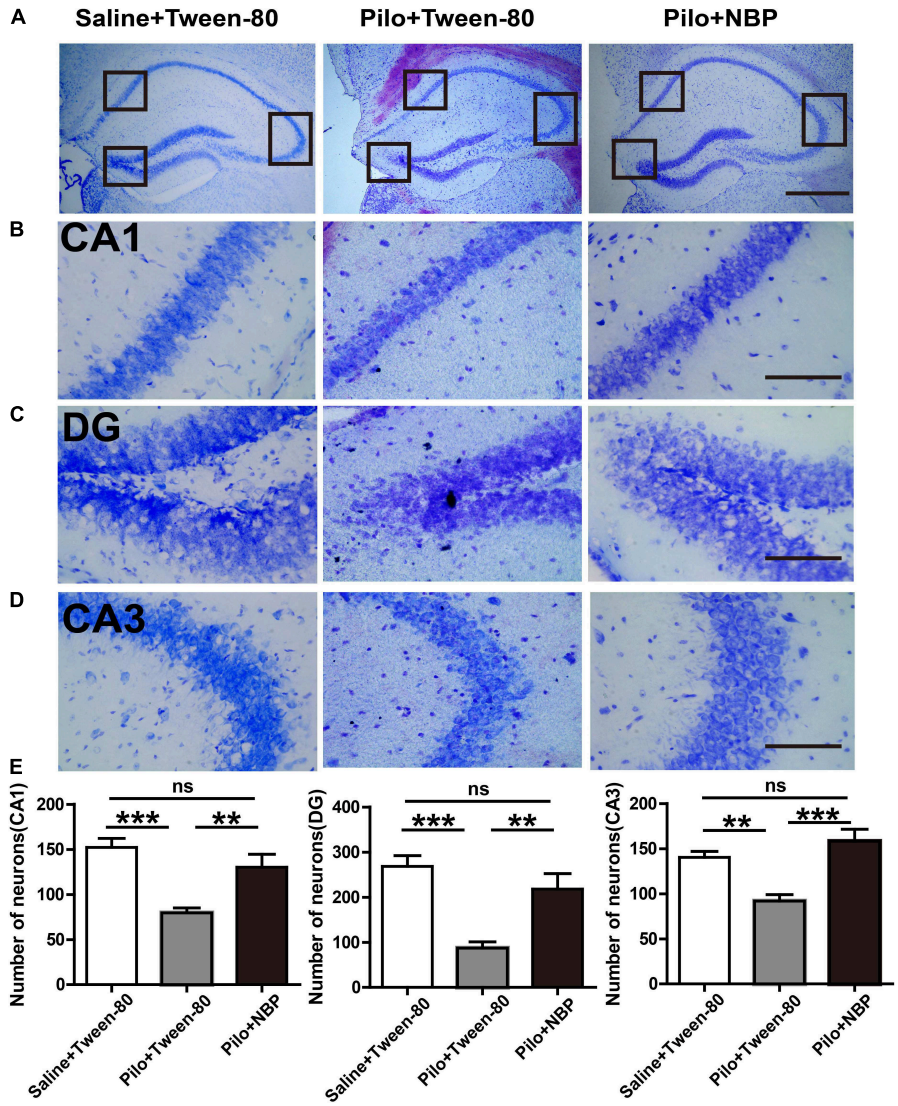

FIGURE 4 | NBP treatment decreased the hippocampal neuronal loss in chronically epileptic mice. **(A)** Representative Nissl staining in the hippocampus of the mouse brain. Scale bar = 50 μm. **(B–D)** Representative images of the Nissl staining in the DG, CA3, and CA1 areas with maximum projection were shown. **(E)** Average cell number in the areas of hippocampus from five sections per mouse was quantified. Scale bar = 10 μm. n = 4 or 5 for each group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; ns, not significant.

gene was estimated using the comparative Ct method. The real-time value for each sample was averaged and compared using the CT method, where the amount of target RNA ($2^{-\Delta\Delta CT}$) was normalized to the endogenous β-*actin* reference (ΔCT) and then normalized against those levels in vehicle group. The primer sequences were as follows:

Bdnf: Forward TCATACTTCGGTTGCATGAAGG
Reverse AGACCTCTCGAACCTGCCC
Klotho: Forward ACTACGTTCAAGTGGACACTACT
Reverse GATGGCAGAGAAATCAACACAGT
β-*actin*: Forward AGTGTGACGTTGACATCCGT
Reverse GCCAGAGCAGTAATCTCCTTC

Tissue Processing and Nissl Staining

Mice (n = 4 or 5 per group) were anesthetized and the brain was acquired, fixed in 4% paraformaldehyde for 24 h and then dehydrated consecutively with 20 and 30% sucrose at 4°C for 2 days. Brain tissues were then imbedded with OCT (Sakura, Culver City, CA, United States) at −80°C and were sectioned using a freezing microtome (Leica CM1950, Nussloch, Germany) to generate 30 μm sections. Brain sections were washed with phosphate buffer solution and were then incubated with Nissl Staining Solution (Beyotime, Shanghai, China) for 10 min. All sections were cover-slipped with mounting solution (Cwbio, Beijing, China) and inspected with a light microscope (Carl Zeiss, Göttingen, Germany). Photographs were taken with a Moticam HRC digital camera (Motic, Hong Kong, China) and the average

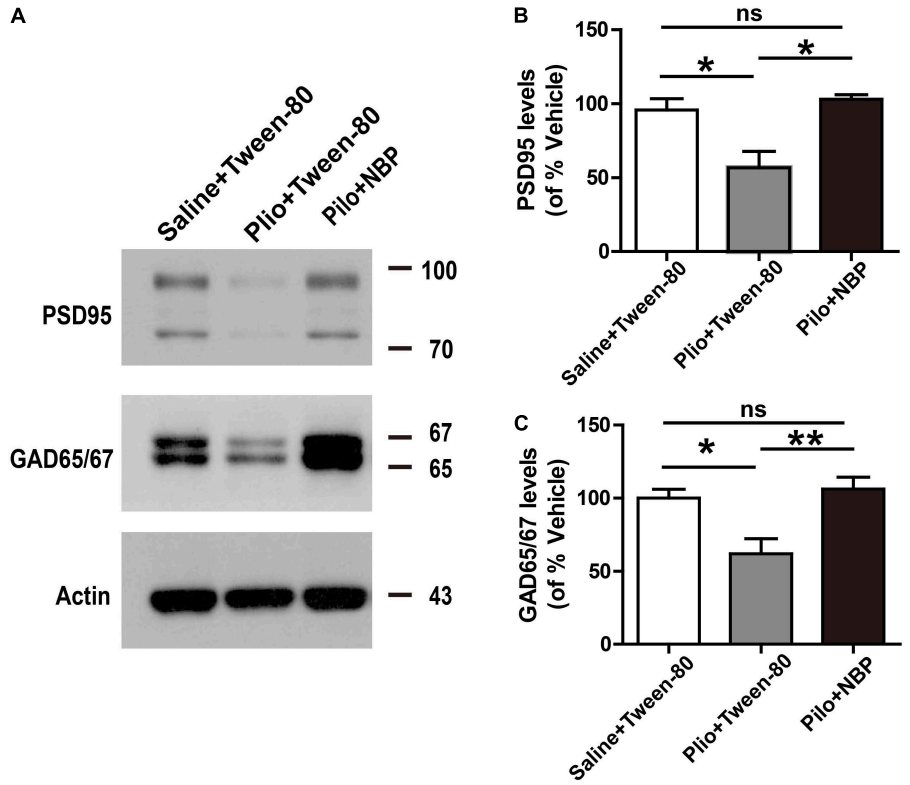

FIGURE 5 | Administration of NBP up-regulated the expression of PSD95 and GAD65. **(A)** Representative Western blotting images of brain extracts; **(B,C)** NBP treatment increased the protein level of PSD95 and GAD65 in epileptic mouse brain. $n = 4$ or 5 for each group. $^{*}p < 0.05$, $^{**}p < 0.01$; ns, not significant.

cell number in the areas of hippocampus from five sections per mouse was quantified and analyzed by Image J 1.46.

Statistical Analysis

Data were expressed as mean ± standard error of mean (SEM). Statistical significance was determined by one-way ANOVA and Bonferroni's test (GraphPad Prism 5.0). $p < 0.05$ was considered significant.

RESULTS

NBP Treatment Reduced Seizure Incidence

To determine the potential role of NBP in epilepsy, we first developed a chronically epileptic mouse model by i.p. injection of pilocarpine. An overview of the experiment design, which showed the timing of pilocarpine and NBP administration, was illustrated in **Figure 1A**. EEG recording was carried out at 1 h after treatment with saline control (**Figure 1B**) or pilocarpine (**Figure 1C**). In accordance with previous observation, sporadic pathological discharges and SE were observed in pilocarpine treated mice but not in the vehicle mice (saline + Tween-80 group). Fifteen days later, all the spontaneously epileptic mice confirmed by interictal seizure EEG (**Figure 1D**) were then divided into two groups and were injected with NBP or Tween-80 for another 14 days. EEG recordings on the 30th day showed that NBP significantly ameliorated the epileptiform activity in epileptic mice (pilocarpine + NBP group; **Figure 1F**) whereas there was no change in the pathological discharges in the Tween-80-treated mice (pilocarpine + Tween-80 group; **Figure 1E**), suggesting a potential protective role of NBP in epileptogenesis.

NBP Improved Spatial Learning and Memory Deficits in Pilocarpine-Induced Epileptic Mice

Epilepsy is complicated by neurobehavioral comorbidities, including cognitive impairment, psychiatric disorders, and social problems (Lin et al., 2012). Therefore, we examined whether NBP could improve behavioral phenotypes associated with the pilocarpine model. We first investigated the effects of NBP on mouse performance in MWM test, which requires the use of external visual cues to locate a hidden platform and to escape the water. The mean speed in the three groups animals showed no preference for each other, so that the possibility that speed might influence water maze performance in these animals can be excluded (**Figure 2B**). During a hidden platform test (acquisition), epileptic animals that treated with NBP showed significantly shorter escape latencies compared to pilocarpine treated epileptic mice (**Figure 2A**), similar to that

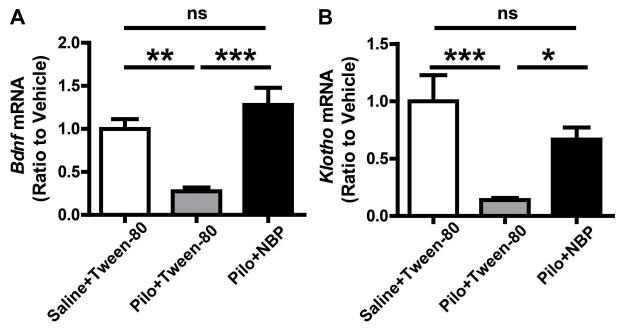

FIGURE 6 | NBP treatment enhanced *Bdnf* and *Klotho* mRNA level. **(A,B)** NBP up-regulated the mRNA levels of *Bdnf* **(A)** and *Klotho* **(B)** in epileptic mouse brain as quantified by qRT-PCR. $n = 4$ or 5 for each group. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$; ns, not significant.

in control group. This improvement was confirmed in a probe test 24 h following the final testing session (**Figures 2C,D**). The test showed that NBP treated mice made numerous platform crossings and spent significantly more time in the target quadrant (**Figures 2C,D**). NBP application rescued these spatial learning and memory deficits in pilocarpine-induced epileptic mice.

Anxiety- and depression-like behaviors in animals were assessed by the black–white box test or tail suspension test. During a 10-min test (locomotion), mice in vehicle group (saline + Tween-80 group) or pilocarpine + NBP group displayed hyperactivity and spent more time in the white box than those in pilocarpine + Tween-80 group (**Figure 3A**). The tail suspension test showed that NBP treated mice (pilocarpine + NBP group) had a profound increase in their reaction to suspension, similar to those in vehicle group, whereas the pilocarpine treated epilepsy mice (pilocarpine + NBP group) developed a characteristic immobile posture following the platform withdrawal (**Figure 3B**).

NBP Up-Regulated the Expression of PSD95 and GAD65/67 in Epileptic Mice

We next asked whether NBP affected neuronal loss in the hippocampus of epileptic mice. Nissl staining showed that Nissl positive neuron number in the hippocampal DG, CA3, or CA1 areas significantly decreased, and were restored in response to NBP treatment in epileptic mice (**Figures 4A–E**), suggesting that the dramatic protective effect of NBP treatment was due to a reversal of neuronal loss in the hippocampus. PSD95, a scaffold protein, is associated with synapse maturation and synaptic stability, strength, and plasticity (El-Husseini et al., 2000; Elias et al., 2006). Additionally, PSD95 expression is reduced in cognitive dysfunction and epilepsy (Zhang et al., 2014; Fernandez et al., 2017). GAD65/67, an important enzyme in GABA synthesis, is related to neurologic disorders, such as epilepsy, ataxia, cognitive impairment and emotion disorder (Dayalu and Teener, 2012; Muller et al., 2015; Qi et al., 2018). To clarify the cellular and synaptic mechanisms underlying the divergent effects of NBP application *in vivo*, the expression levels of PSD95 and GAD65/67 in animals were evaluated by Western blotting. We detected significantly decreased expression levels of PSD95 and GAD65/67 in the brain of mice treated with pilocarpine, and significantly increased levels of them in NBP treated mice (pilocarpine + Tween-80) (**Figure 5**).

Furthermore, BDNF is an important neurotrophic factor that enhances synapse formation and cognitive functions (Parkhurst et al., 2013; Karpova, 2014), and *Klotho* is already known to improve cognition (Dubal et al., 2014, 2015). When compared to epileptic mice without NBP treatment (pilocarpine + Tween-80), the mRNA levels of *Bdnf* and *Klotho* were strongly up-regulated in NBP treated epileptic mice (**Figure 6**) whereas NBP alone did not affect *Bdnf* expression (Supplementary Figure 1). These results provide direct biochemical evidence that systemic treatment with NBP can mitigate the epileptiform activity in epileptic mice.

DISCUSSION

Recurrent seizures lead to severe anxiety or depression, which is a major reason for cognitive decline (Lagae, 2006; Kimiskidis et al., 2007; Gaitatzis et al., 2012; de Toffol et al., 2017). Preventing seizures or mitigating symptoms of anxiety or depression are clinical need. Several studies have reported the protective role of NBP in central nervous system (Peng et al., 2010; Zhang et al., 2016), but few researches have discussed the effect of NBP on epilepsy. In this study, pilocarpine-induced chronic epilepsy model was used to investigate the effects of NBP on epilepsy associated anxiety, depression, and cognitive deficit (Inostroza et al., 2011; Pineda et al., 2014). Our findings suggest that NBP treatment reduced spontaneous spike-waves in chronic epilepsy. Furthermore, our results show that NBP plays a vital role in antidepressant, antianxiety and ameliorating learning and memory impairment by rescuing the chronic epilepsy-induced neuronal loss in CA1, CA3, and DG areas of hippocampus.

Here, the EEG recording showed that the occurrence of spontaneous discharge decreased in epileptic mice in response to NBP treatment. This is consistent with previous study that NBP could keep excitatory and inhibitory neuronal systems in balance in acute epileptic mice brain (Yu et al., 1988; Han et al., 2016). Additionally, imbalance between excitatory and inhibitory neurotransmitters, such as glutamic acid (Glu) and GABA, results in epileptogenesis (Svenningsen et al., 2006; Hao et al., 2016). GAD65/67, a GABA-synthesizing enzyme, decides GABA levels in postnatal synapse maturation (Horovitz et al., 2012; Fouka et al., 2015). Thus, our results revealed that the antiepileptic effect of NBP might be resulted from the up-regulation of GAD65/67 and the reversion of neuronal loss.

It has also been reported that NBP promotes neurogenesis and is neuroprotective against neuronal apoptosis, and improves synaptic plasticity (Chang and Wang, 2003; Yang et al., 2015). Furthermore, studies have shown that NBP improved cognitive deficit in a transgenic model of AD (Peng et al., 2010). What's more, recent studies indicate that AD and epilepsy had similar mechanism in the pathogenesis of cognitive impairment (Corbett et al., 2017; You et al., 2017). These results suggest that NBP might serve as a potential therapeutic drug in the treatment of epileptic

comorbidities. Here, we found that NBP treatment enhanced the learning and memory capacity in chronic pilocarpine-induced model by MWM test. In addition, we observed an up-regulated level of synapse-associated protein in response to NBP treatment. PSD95 plays an important role in synapse stabilization and plasticity (El-Husseini et al., 2000). The decrease in PSD95 levels are highly correlated with learning and memory impairments (Chen et al., 1998; Migaud et al., 1998), and PSD95 is down regulated in epileptic activity (Wyneken et al., 2001). Corroborating these researches, the up-regulation of PSD95 in response to NBP treatment in epileptic mice may contribute to the improvement of cognitive function.

In this study, we also found that NBP may have therapeutic effects on anxiety and depressive behavior by increasing *Bdnf* and *Klotho* mRNA level in pilocarpine-induced epileptic mice. There is now ample evidence that BDNF, a neurotrophic factor important in promoting immature neurons development, increasing the survival of adult neurons and synaptic plasticity (Kang and Schuman, 1995; Sairanen et al., 2007), is protective in antidepressant and antianxiety. Treatment with antidepressant or antianxiety drugs can restore *Bdnf* mRNA level in stress-induced model (Smith et al., 1995; Kozisek et al., 2008). Although it has been reported that BDNF expression was enhanced in pilocarpine-induced SE (Thomas et al., 2016), BDNF has also been found decreased in patients with chronic temporal lobe epilepsy (TLE) and in animal model of chronic cyclothiazide seizure (Kong and Cheng, 2014; Chen et al., 2016). Besides, Xiang et al. (2014) pointed out that NBP alleviated cognitive dysfunction in APP mice by BDNF/TrkB/PI3K/AKT pathway. Moreover, it has been reported that *Klotho* is reduced in TLE and its downregulation is involved in neurodegenerative disorders and inflammation (de Oliveira, 2006; Abraham et al., 2012). Consequently, we speculate the therapeutic function of NBP in chronic epileptic comorbidities associated with anxiety and depressive behavior by increasing the expression level of BDNF and Klotho.

Collectively, this study indicates that treatment with NBP could be a potential strategy to slow down or even to reverse chronic epilepsy and the epileptic comorbidities such as cognitive decline and psychological impairments. However, the molecular mechanisms for the application of NBP in epilepsy still need to be further investigated. In addition, the role of NBP in acute epilepsy was unclear. And there are also limitations for the use of atropine sulfate to block peripheral cholinergic effects in this study as it can cross the blood–brain barrier and in this way it might affect brain functions. NBP has already been approved by the State Food and Drug Administration (SFDA) of China for clinical use in stroke patients since 2002. This study suggests that therapeutic strategies of NBP for chronic epilepsy and the comorbidities may expand the applicative scope of this drug. However, additional experimental data are needed to prove the antiepileptic effects of NBP, as well as the dose-dependent responses of NBP in epilepsy.

AUTHOR CONTRIBUTIONS

WZ and HZ conceived and designed the study. XY, ZR, and YC performed the experiments and analyzed the data. XY, ZR, and HZ wrote the paper. HL, XW, XH, and BC coordinated the study and provided technical assistance. ZL, YL, and Y-wZ revised the paper. All authors reviewed the results and approved the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2018.00734/full#supplementary-material

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Effects of Antiepileptic Drugs on Spontaneous Recurrent Seizures in a Novel Model of Extended Hippocampal Kindling in Mice

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Epilepsy is a common neurological disorder characterized by naturally-occurring spontaneous recurrent seizures and comorbidities. Kindling has long been used to model epileptogenic mechanisms and to assess antiepileptic drugs. In particular, extended kindling can induce spontaneous recurrent seizures without gross brain lesions, as seen clinically. To date, the development of spontaneous recurrent seizures following extended kindling, and the effect of the antiepileptic drugs on these seizures are not well understood. In the present study we aim to develop a mouse model of extended hippocampal kindling for the first time. Once established, we plan to evaluate the effect of three different antiepileptic drugs on the development of the extended-hippocampal-kindled-induced spontaneous recurrent seizures. Male C57 black mice were used for chronic hippocampal stimulations or handling manipulations (twice daily for up to 70 days). Subsequently, animals underwent continuous video/EEG monitoring for seizure detection. Spontaneous recurrent seizures were consistently observed in extended kindled mice but no seizures were detected in the control animals. The aforementioned seizures were generalized events characterized by hippocampal ictal discharges and concurrent motor seizures. Incidence and severity of the seizures was relatively stable while monitored over a few months after termination of the hippocampal stimulation. Three antiepileptic drugs with distinct action mechanisms were tested: phenytoin, lorazepam and levetiracetam. They were applied via intra-peritoneal injections at anticonvulsive doses and their effects on the spontaneous recurrent seizures were analyzed 10–12 h post-injection. Phenytoin (25 mg/kg) and levetiracetam (400 mg/kg) abolished the spontaneous recurrent seizures. Lorazepam (1.5 mg/kg) decreased motor seizure severity but did not reduce the incidence and duration of corresponding hippocampal discharges, implicating its inhibitory effects on seizure spread. No gross brain lesions were observed in a set of extended hippocampal kindled mice submitted to histological evaluation. All these data suggests that our model could be considered as a novel mouse model of extended hippocampal kindling. Some limitations remain to be considered.

Keywords: antiepileptic drugs, convulsion, electroencephalograph (EEG), epilepsy, hippocampus, kindling, mice, spontaneous seizures

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INTRODUCTION

Epilepsy is a common neurological disorder characterized by naturally-occurring spontaneous recurrent seizures (SRS) and comorbidities. Temporal lobe epilepsy (TLE) is the most common type of drug-resistant epilepsy in the adult/aging population (Engel, 1996). Kindling via chronic electrical stimulation of limbic structures has long been used to model TLE, to assess the antiepileptic drugs (AEDs) efficacy and to explore drug-resistant epilepsy (Gorter et al., 2016; Löscher, 2017; Sutula and Kotloski, 2017). While classic kindling over a few weeks does not generally induce spontaneous seizures, extended kindling is able to induce SRS in several animal species. Specifically, extended kindling of the amygdala, hippocampus, perforant path or olfactory bulb induces SRS in monkeys (Wada and Osawa, 1976), dogs (Wauquier et al., 1979), cats (Wada et al., 1974; Gotman, 1984; Hiyoshi et al., 1993) and rats (Pinel and Rovner, 1978; Milgram et al., 1995; Michalakis et al., 1998; Sayin et al., 2003; Brandt et al., 2004). SRS development following extended amygdala kindling is generally associated with a loss of subgroups of hippocampal GABAergic interneurons in dentate gyrus-hilar areas (Sayin et al., 2003; Brandt et al., 2004) rather than gross brain lesions as seen in different status epilepticus models (Dudek and Staley, 2017; Gorter and van Vliet, 2017; Henshall, 2017; Kelly and Coulter, 2017). This way, extended kindling may help model the genesis of SRS without a major brain pathology as usually seen in TLE patients (Ferlazzo et al., 2016). To date, SRS following extended kindling and the effect of different AEDs on such seizures are still not well understood.

Mouse models have been largely used in epilepsy research because the genetic/molecular manipulations done in these animals offer great advantages in mechanistic investigation. However, although several studies have examined classic kindling seizures in mice, no extended kindling mouse model has been established yet. In this sense, we aim to develop such a model to facilitate future ictogenesis research and the possible effects that AEDs might have on SRS. To achieve success, we took into consideration several factors such as the strain-dependent susceptibility to have seizures/epilepsy (Löscher et al., 2017), the reliability of chronic electroencephalography (EEG) in mouse models (Bertram, 2017), and the pharmacokinetics of the AEDs in rodents (faster elimination than in humans) (Löscher, 2007; Markowitz et al., 2010). Based on these aspects, the experiments were conducted in middle-age/aging C57 black mice in an attempt to model new-onset TLE as seen in adult/aging populations (Ferlazzo et al., 2016). Moreover, the extended hippocampal kindling model and the continuous video/EEG monitoring method were chosen due to previous experience in our lab (Jeffrey et al., 2014; Bin et al., 2017; Stover et al., 2017). In the case of the AEDs, we had to apply the drugs via acute intra-peritoneal injections at anticonvulsive doses as a proof-of-principle test due to the lack of a method for chronic AED delivery in mice in our lab. The three AEDs tested in this study are: phenytoin (Na^+ channel blocker), lorazepam (benzodiazepine GABA enhancer), and levetiracetam (synaptic vesicle glycoprotein targeting agent). All with different action mechanisms and anti-seizure effects in classic kindling and other models (Löscher et al., 2016; Löscher, 2017).

Our present experiments were aimed to develop a mouse model of extended hippocampal kindling, monitor and assess the SRS stability in individual mice after termination of the kindling stimulation, and evaluate how the three AEDs chosen affect the SRS, the EEG, and the motor behavior.

METHODS

Animals

Six to 8 months old male C57 black mice were obtained from Charles River Laboratory (C57BL/6N; Saint-Constant, Quebec, Canada) and housed in a local vivarium for several months before experimentation. The vivarium was maintained between 22–23°C and with a 12-h light on/off cycle (light-on starting at 6:00 am). Mice were placed in standard cages with food and water *ad libitum*. We used male mice to avoid variable female sex hormones on kindling process. All experimentations conducted in the present study 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.

Drugs

Phenytoin and lorazepam were obtained in clinically injectable forms (Sandoz Canada Inc.; Boucherville, Quebec, Canada) while levetiracetam was obtained as a powder from Sigma-Aldrich (Oakville, Ontario, Canada). Phenytoin was stored at room temperature and lorazepam was kept in a fridge at 2–4°C prior to usage. Levetiracetam was dissolved in distilled water as a stock solution and stored in a freezer at −20°C before usage. All three AEDs were appropriately diluted with saline and applied via intra-peritoneal injections (∼0.25 ml per injection). Doses were as follows: 25 mg/kg for phenytoin, 1.5 mg/kg for lorazepam and 100 or 400 mg/kg for levetiracetam.

AED injections were made during the light-on period predominantly between 11 AM to 4 PM as SRS were more frequent in late afternoon to evening periods. Injections of different AEDs in each mouse were made $\geq$ 3 days apart to minimize overlapped drug effects. The AEDs protocol assessments were as follows:

1) Phenytoin. Applied at 25 mg/kg and analyzed its effects in a 10-h period post-injection. This protocol was based on the phenytoin doses used in other mouse models (20–50 mg/kg; Riban et al., 2002; Klein et al., 2015; Twele et al., 2015; Bankstahl et al., 2016) and the half-life of phenytoin plasma elimination in rodents (8–16 h; Löscher, 2007; Markowitz et al., 2010).
2) Lorazepam. Applied at 1.5 mg/kg, similar to the dose range used in rat models of status epilepticus (0.33–4 mg/kg; Walton and Treiman, 1990; Gersner et al., 2016; Kienzler-Norwood et al., 2017). In rats submitted to intravenous injections of this drug (0.9 or 3.2 mg/kg), the half-life of lorazepam plasma elimination is about 2 h. However, lorazepam's brain concentration keeps higher than plasma levels for at least 6 h post-injection (Greenblatt and Sethy,

1990). The latter phenomenon could be related to a more persistent anticonvulsive effect of lorazepam relative to diazepam (Walton and Treiman, 1990; Alldredge et al., 2001). Based on the above information, we used lorazepam instead of diazepam and analyzed its effects in a 10-h period post-injection.

3) Levetiracetam. Applied at 100 mg or 400 mg/kg, similar to the doses used in other rodent models (160–800 mg/kg; Löscher et al., 1993; Löscher and Hönack, 2000; Zhang et al., 2003; Ji-qun et al., 2005; Lee et al., 2013; Shetty, 2013; Twele et al., 2015; Duveau et al., 2016). In mice submitted to intra-peritoneal injections at 200 mg/kg, the half-life of levetiracetam plasma elimination is about 1.5 h and its brain-to-blood ratio is 0.8 at 4 h post-injection (Benedetti et al., 2004; Markowitz et al., 2010). In order to maintain the anti-seizure actions of levetiracetam over a period of time comparable to that of phenytoin or lorazepam, we adapted a three-injection protocol (Löscher and Hönack, 2000). This way, we applied levetiracetam every 4 h at 100 or 400 mg/kg per injection. The effects of levetiracetam were analyzed in a 12-h period following injections.
4) Data similarly analyzed following saline injections or in the next day post AED injection were used to control injection effects and determine recovered SRS.

The AEDs effects on SRS were examined in extended hippocampal kindled mice (15–17 months old), which might correspond to humans of $\geq$ 40 years-old (Flurkey et al., 2007). Therefore, we used this experimental paradigm in an attempt to model new-onset TLE seen in adult/aging populations (Ferlazzo et al., 2016). A schematic outline of our experimental procedures is shown in **Figure 1A**.

Electrode Implantation

Electrodes were constructed in our lab as previously described (Jeffrey et al., 2014; Bin et al., 2017). All electrodes were made of polyamide-insulated stainless steel wires (100 or 230 μm outer diameter; Plastics One, Ranoake, VA). The wires with 100 μm diameter were used in most of experiments to minimize electrode-related tissue injury/perturbations. Twisted bipolar electrodes were used for stimulation and recording (Jeffrey et al., 2014; Stover et al., 2017). For local differential recordings, the two open ends of each implanted bipolar electrode were connected to two inputs of an amplifier headstage (see below and **Figure 1** of Bin et al., 2017). For mono-polar recordings, one open end of the bipolar electrode was connected to the amplifier headstage and another open end was grounded as per the instruction of the amplifier manufacturer.

Twenty-six mice were used in the present experiments. These animals were operated for intracranial electrode implantations at ages 11–13 months and then randomly selected for kindling ($n = 18$) or control handling ($n = 8$) experiments. Electrode implantation surgeries were performed similarly as previously described (Jeffrey et al., 2014; Bin et al., 2017; Stover et al., 2017). Briefly, mice were anesthetized with isoflurane and placed on a stereotaxic frame. After skin incision and exposure of the skull, small holes were drilled through the skull, and electrodes were inserted in to the brain by micromanipulators. Electrodes were secured on the skull using a glue-based anchoring screw-free method (Jeffrey et al., 2014). Animals were treated with buprenorphine (0.1 mg/kg, s.c.; every 8 h for two days) to relieve post-surgery pain and allowed to recover for 1–2 weeks prior to further manipulation (**Figure 1A**). There were no noticeable differences in the animals' body weights and atypical behaviors post-electrode implantation in these two groups of mice. The locations of implanted electrodes were verified by hippocampal EEG activities (**Figure 1B**) and, in some cases, by later histological assessments (**Figures 1D,E**).

Bipolar electrodes were implanted in bilateral hippocampal CA3 areas, unilateral hippocampal CA3 and parietal cortical areas or unilateral hippocampal CA3 and piriform areas. The stereotaxic coordinates were 2.5 mm posterior, 3.0 mm lateral and 2.5 mm ventral to bregma for the hippocampal CA3; 0.5 mm anterior, 2.0 mm lateral and 0.5 mm ventral to bregma for the parietal cortical area; 0.5 mm posterior, 3.0 mm lateral and 5.0 mm ventral to bregma for the piriform area (Franklin and Paxinos, 1997). A reference electrode was positioned to a frontal area at 1.5 mm anterior, lateral 1.0 mm and ventral 0.5 mm to bregma. We used such electrode implantations in an attempt to monitor discharge spread from the stimulated CA3 to the contralateral CA3 area or to the ipsilateral parietal cortical or piriform area. However, EEG recordings from the parietal cortical or piriform area were often unstable or with poor signal-to-noise ratio likely due to contaminations of implanted electrodes. Due to these complications, only the EEG signals collected from the stimulated hippocampal area were analyzed in the present experiments.

Hippocampal Kindling

A train of stimuli at 60 Hz for 2 s was used for kindling stimulation (Jeffrey et al., 2014; Bin et al., 2017; Stover et al., 2017). Constant current pulses with monophasic square waveforms, 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 RI, USA). An ascending series was used to determine the threshold of evoked afterdischarges in individual mice. The afterdischarges were defined as repetitive spike waveforms with amplitudes of ∼2 times of background signals and durations of ≥5 s (Reddy and Rogawski, 2010; Jeffrey et al., 2014; Stover et al., 2017). In the ascending series, the stimulation train with incremental current intensities (10 μA per step) was applied every 30 min. The lowest current by which an afterdischarge event of ≥5 s was elicited 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 attempt was to keep constant stimulation intensity throughout the extended kindling period. However, the initial stimulation intensity often became inconsistent in evoking afterdischarges and motor seizures after ≥ 45 days of kindling experiments, which might be largely due to contaminations of implanted electrodes. Due to this this complication, stronger stimulation intensities (40–80 μA above

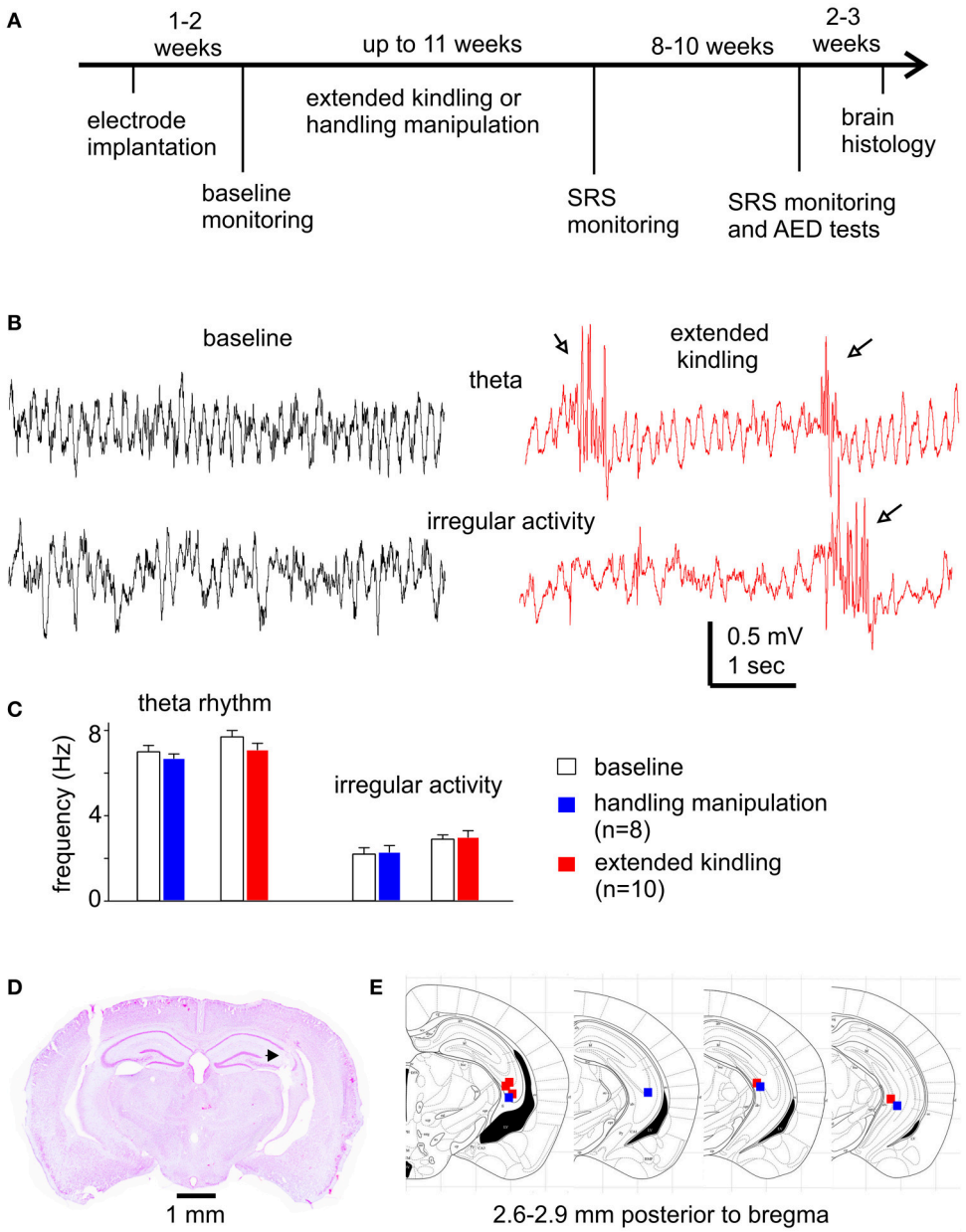

FIGURE 1 | Experimental outline and diverse assessments of the implanted hippocampal electrodes**. (A)** Schematic diagram of the experiments. See text for details. **(B)** Representative hippocampal EEG traces collected from a mouse during baseline monitoring and following extended hippocampal kindling. Arrows show interictal spikes. **(C)** Frequencies of theta rhythms and irregular activities from mice in the control and the extended hippocampal kindled groups. **(D)** Verification of deep electrode site. Image obtained from a mouse following extended hippocampal kindling. Track denoted by a filled arrow. **(E)** Schematic illustrations correspond to coronal brain sections between 2.6 and 2.9 mm posterior to bregma. The solid squares indicate the correct localization of the electrode tips (blue represents 4 control animals and red represents 5 extended hippocampal kindled animals).

the initial afterdischarge threshold) were used in subsequent experiments.

A protocol with twice daily stimulations and an inter-stimulation interval of $\geq$ 5 h, as used in a rat model of extended amygdala kindling (Sayin et al., 2003; Brandt et al., 2004), was adapted for extended hippocampal kindling in the present experiments. Kindling stimulations were conducted in the light-on period between 10 AM and 5 PM. Each stimulation episode lasted 3–5 min, during which the mouse was placed in a bowl-shaped glass container or a large glass beaker for video/EEG monitoring (Stover et al., 2017). All mice in the extended kindling cohort were stimulated for up to 70 consecutive days except 3–7

days for SRS detection (see below). Age-matched control mice received similar electrode implantations and experienced twice daily handlings for 60 consecutive days. During the handling manipulation, each mouse was held for about 1 min and then placed into a large glass beaker for 2–3 min to simulate the stress related to the electrode connection and kindling procedure.

Evoked motor seizures were scored as per the Racine scale (Racine, 1972) modified for mice (Reddy and Rogawski, 2010). Briefly, stage 0 - no response or behavioral arrest; stage 1 - chewing or head nodding; stage 2 - chewing and head nodding; stage 3 - single or bilateral forelimb clonus; stage 4 - bilateral forelimb clonus and rearing; stage 5 - rearing and falling with forelimb clonus.

EEG Recordings and Video Monitoring

Local differential recordings via twisted bipolar electrodes were used to sample local EEG signals as previously described (Jeffrey et al., 2014; Bin et al., 2017; Stover et al., 2017). Mono-polar EEG recordings were used if local differential recordings were unsuccessful due to electrode contaminations. EEG signals were collected using two-channel or one-channel microelectrode AC amplifiers with extended head-stages (model 1800 or 3000, A-M systems, Sequim, WA, USA). The afterdischarge ipsilateral to the stimulated hippocampus was captured using the model 3000 amplifier via TTL-gated switches between recording and stimulating modes. Signals were collected in a frequency band of 0.1–1,000 or 0.1–3,000 Hz and amplified 1,000 × before digitization (5,000 or 1,0000 Hz, Digidata 1440A or 1550, Molecular Devices; Sunnyvale, CA, USA). Data acquisition, storage and analyses were done using pClamp software (Version 10; Molecular Devices).

Continuous video/EEG monitoring (roughly 24 h daily for several consecutive days) was used in the morality of experiments to detect SRS (Bin et al., 2017). Mice were placed in a modified cage with food and water available *ad libitum*. A webcam was placed near the cage to capture the animals' motor behaviors. EEG and video data were saved every 2 h using a Mini Mouse Macro program. Dim lighting was used for webcam monitoring in the light-off period. Data collection was stopped for ∼30 min daily for animal care. Intermittent EEG recordings during the light-on period (4–8 h per session) were used in some of baseline monitoring experiments. For such recordings, each mouse was placed into the glass container and allowed to freely access hydrogel and food pellets provided on floor (Stover et al., 2017).

Baseline monitoring was conducted 1–2 weeks post-electrode implantation (**Figure 1A**). For each mouse following extended kindling, continuous video/EEG monitoring of 24–72 h per session was conducted after the 80, 100, 120, and/or 140th stimulation to assess SRS commencement. If ≥ 2 SRS events were observed in 24 h, no further stimulation was applied and additional video/EEG monitoring for up to 72 h was performed to assess initial SRS incidences. Video/EEG monitoring of 24–72 h was repeated 8–11 weeks later to assess SRS stability. The effects of AEDs were examined afterwards. For each mouse in the control group, continuous video/EEG recordings of 48 h were performed after 60 days of twice daily handling manipulations.

Brain Histological Assessments

Brain histological sections were prepared using a protocol modified from our previous studies (Jeffrey et al., 2014; Stover et al., 2017). Mice were anesthetized via sodium pentobarbital (100 mg/kg, intra-peritoneal injection) and perfused trans-cardiacally with saline and then with 10% neutral buffered formalin solution (Sigma-Aldrich; Oakville, Ontario, Canada). Removed brains were further fixed in a hypertonic (with 20% sucrose) formalin solution. Coronal sections of 50 μm thick were obtained using a Leica CM3050 research cryostat and placed onto glass slides (Superfrost plus microscope slides, Fisher Scientific, Canada). Brain sections were dried in room air for ≥1 week, processed sequentially with chloroform (24 h), 95% ethanol (24 h), 90% ethanol (12 h), and 70% ethanol (0.5 h), and then stained with cresyl violet (0.1%, Sigma Aldrich, Oakville, Ontario, Canada). Images of brain sections were obtained using a slide scanner (Aperio digital pathology slide scanner AT2, Leica) at 20 × magnification and analyzed using ImageScope (Leica) or Image J (National Institute of Health, USA) software.

Data Analysis

Spectral analysis was used to determine the main frequencies of hippocampal rhythms. Spectral plots (rectangular function, 50% window overlap and spectral resolution 0.3 Hz; PClamp software) were generated from 60- to 15-s data segments that encompassed theta rhythmic or irregular signals. Three spectral plots were averaged to assess baseline and post-kindling or post-handling EEG signals in each mouse.

Interictal EEG spikes were recognized by intermittent events with large peak amplitudes (≥6 times of standard deviation of background signals), simple or complex spike waveforms, and durations of 30–250 ms. Spike incidences were measured from the stimulated hippocampal area and in 10-min data segments collected during immobility/sleep as interictal spikes manifested in these "inactive" behavioral states. These data were collected ≥30 min after a preceding ictal event to avoid the influences of ictal discharges on subsequent interictal spikes. An event detection function (threshold search method) of pClamp software was used to detect spikes automatically, and detected events were then visually inspected and false events were rejected.

SRS were determined by EEG ictal discharges of ≥30 s in durations and concurrent motor seizures of stages 2–5 according to the Racine scale modified for mice (Racine, 1972; Reddy and Rogawski, 2010). In some experiments, SRS were determined by EEG ictal discharges alone due to errors in video acquisition/storage. In our present experiment, each mouse was monitored by a webcam from a side of its housing cage. This setting was inadequate to capture stage 2–4 motor seizures if the mouse's head and forelimbs were not faced toward the webcam. Due to this complication, we did not attempt to detect SRS by motor behaviors alone.

Statistical Analysis

A Student's *t*-test or Mann-Whitney Rank Sum Test was used for two-group comparisons (Sigmaplot; Systat Software Inc., San Jose, California, USA). A one-way ANOVA followed by a multiple comparison test (Dunn's or Holm-Sidak method) was

used for multiple group comparisons. Mean and standard error of the mean (SEM) were presented throughout the text and figures. Statistical significance was set at $p < 0.05$.

RESULTS

Twenty-six mice were used in the present experiments ($n = 18$ for kindling and $n = 8$ for control handling). Of the 18 mice in the kindling group, 14 were "kindled" following 25–35 stimulations as they exhibited 3 consecutively evoked stage-5 motor seizures (Reddy and Rogawski, 2010; Jeffrey et al., 2014; Stover et al., 2017); other 4 mice were excluded after several days of kindling experiments due to inconsistence/failure in evoking afterdischarges. The 14 "kindled" mice were used for extended kindling. SRS induction was successful in 12 mice following ≥ 100 stimulations (**Table 1**); the remaining 2 mice were euthanized after the 45 or 70th stimulation due to a loss of implanted electrodes or severe skin lesion. In the mice that exhibited SRS following extended kindling, the mean durations of evoked hippocampal afterdischarges were 17.5 ± 2.1, 22.4 ± 1.4, 25.4 ± 3.1, 27.9 ± 1.0, and 31.9 ± 3.1 s in response to 1–20, 21–40, 41–60, 61–80, and 81–100 stimuli, respectively. The mean stages of corresponding motor seizures were 3.7 ± 0.6, 4.5 ± 0.3, 4.1 ± 0.3, 4.1 ± 0.2, and 4.0 ± 0.3 respectively. The afterdischarges evoked by 61–80 and 81–100 stimuli were significantly longer than that evoked by the first 20 stimuli ($p \leq 0.05$); whereas there were no significant differences among corresponding motor seizure stages. Cumulative afterdischarge durations or motor seizure stages to SRS were variable in these mice, ranging from 2,311 to 3,009 s or from 343 to 449. In general, all these observations are in accordance with the previous study done in a rat model of extended amygdala kindling (Brandt et al., 2004). However, data from a larger cohort of mice are needed to delineate the relationship between evoked seizures and induced SRS in our model.

TABLE 1 | Mice examined in the present experiments.

Mouse number/total stimuli applied to achieve extended kindling	SRS events/monitoring times		AEDs tested in each mouse
	Initial[#] (h)	Later[$] (h)	
#1, 120	7/48	4/24	$PHE^{25mg/kg}$
#2, 140	13/72	16/144	$PHE^{25mg/kg}$
#3, 140	22/48	12/48	$PHE^{25mg/kg}$, $LEV^{100mg/kg}$
#4, 100	9/48	17/72	$PHE^{25mg/kg}$, $LEV^{100mg/kg}$
#5, 100	10/48	5/40	$PHE^{25mg/kg}$, $LEV^{100mg/kg}$
#6, 100	11/66	6/24	$PHE^{25mg/kg}$, $LEV^{100mg/kg}$
#7, 140	23/72	26/93	$LOZ^{1.5mg/kg}$, $LEV^{100,400mg/kg}$
#8, 120	20/72	20/72	$LOZ^{1.5mg/kg}$, $LEV^{100,400mg/kg}$
#9, 120	17/72	10/24	$LOZ^{1.5mg/kg}$, LEV^{400mg}
#10, 120	9/48	17/72	$LOZ^{1.5mg/kg}$, LEV^{400mg}
#11, 120	7/48	4/24	$LOZ^{1.5mg/kg}$, LEV^{400mg}
#12, 120	13/72	16/144	$LOZ^{1.5mg/kg}$, LEV^{400mg}
Mouse Number/totalandlings Applied			
#1-#8, 120	0/48 h		

PHE, phenytoin; LOZ, lorazepam; LEV, levetiracetam; # and $, individual mice were monitored initially within 3 days after termination of kindling stimulation; then, monitored 8–11 weeks later. Control mice were monitored after 120 handling manipulations.

Hippocampal EEG Rhythms and Brain Histological Observations

The mouse hippocampus is known to exhibit the theta rhythm (5–12 Hz) during movement/exploration and irregular activity (0.5–4 Hz) during immobility/sleep (Buzsáki et al., 2003). We examined these EEG signals to explore potential alterations by extended kindling and to electrographically verify the locations of implanted hippocampal electrodes. Hippocampal theta and irregular activities were consistently observed from mice in the kindling and control handling groups (**Figure 1B**). In the kindling group ($n = 10$), the frequencies of theta rhythm and irregular activity were 7.7 ± 0.3 and 2.9 ± 0.2 Hz during baseline monitoring and 7.1 ± 0.3 and 3.0 ± 0.3 Hz following 100–120 hippocampal stimulations. In the control group ($n = 8$), the frequencies of theta rhythm and irregular activity were 7.0 ± 0.3 and 2.2 ± 0.3 Hz during baseline monitoring and 6.7 ± 0.2 and 2.3 ± 0.3 Hz following 120 handling manipulations (**Figure 1C**). There were no significant differences between group measures or between baseline and post-stimulation or post-handling measures ($p \geq 0.207$). However, frequent interictal spikes were consistently observed in mice following extended kindling (**Figure 1B**). Further analysis are needed are needed to reveal whether these hippocampal rhythms are altered by extended kindling in other biophysical domains.

Brain histological sections were prepared from 10 mice that underwent extended kindling or handling manipulations ($n = 5$ in each group). These sections were stained with cresyl violet for general assessments of gross brain lesion and the tracks of implanted hippocampal electrodes. There were no evident gross brain lesions, such as structural deformation, cavity, or dark-stained scar tissues, in these kindled or control mice examined. However, detailed histological assessments are needed to reveal a potential loss of hippocampal GABAergic interneurons in our model (Sayin et al., 2003; Brandt et al., 2004). Of the 10 mice examined, the tracks of implanted hippocampal electrodes were evident in 5 kindled and 4 control mice. These tracks were recognizable in sections (2.6–2.9 mm posterior to bregma) appropriate to the stereotaxic coordinates of designated hippocampal CA3 area (**Figures 1D,E**). These histological observations together with the above EEG measures

suggest that targeting the mouse hippocampus for extended kindling was generally reliable in our present experiments.

Main Features of SRS

Continuous (≥24 h) video/EEG monitoring was used to detect SRS in our present experiments. Such monitoring was conducted after the 80, 100, 120, and/or 140th stimulation to assess SRS commencement. SRS were consistently observed following 100–140 hippocampal stimulations ($n = 12$; **Table 1**) but were undetectable or with very low incidences following the 80th stimulation. Neither SRS nor interictal spikes were observed in the age-matched controls ($n = 8$) when examined after 120 handling manipulations (**Table 1**). Together these observations suggest that SRS genesis may require sufficient accumulation of evoked seizures and that the stress imposed by chronic handlings per se is not a causal factor of SRS.

SRS were recognized by hippocampal ictal discharges and concurrent motor seizures. These discharges displayed a low-voltage fast onset (Lévesque et al., 2012), repetitive spike waveforms lasting tens of seconds and a postictal EEG suppression of a few seconds (**Figure 2A**). Such discharge waveforms were consistently observed in all 12 mice over a few months after termination of kindling stimulation. This phenomenon suggests that a similar epileptic network activity may underlie these discharges. EEG ictal discharges were also observed from the parietal cortical or piriform area in some experiments (data not shown). The motor seizures corresponding to the EEG discharges were featured predominantly with forelimb clonus, rearing and/or falling, which corresponded stage 3–5 seizures according to the Racine scale modified for mice (**Supplemental Videos 1-2**). Together these EEG and motor behavioral observations are indicative of SRS as generalized seizure events.

The incidence and severity of SRS were relatively stable in individual mice examined. Measured in 1–3 days after termination of kindling stimulation and 8–11 weeks later, SRS incidences were 6.1 ± 0.8 and 5.2 ± 0.8 events/day; corresponding hippocampal discharge durations were 53.9 ± 1.9 and 49.9 ± 1.7 s and motor seizure stages were 3.6 ± 0.1 and 4.1 ± 0.1 respectively ($n = 10$; **Figures 2B–D**). There were no significant time-dependent changes in the SRS incidences and discharge durations ($p = 0.43$ and 0.133), but a slight increase in motor seizure stages ($p = 0.002$). Examples of stable SRS are presented in **Figure 2A** and **Supplemental Videos 1–2**, where similar hippocampal discharges and corresponding stage-5 motor seizures were recorded from a mouse in 24 h after termination of kindling stimulation and about 8 weeks afterwards.

AED Effects on SRS

Phenytoin effectively suppressed SRS in 6 mice tested. SRS incidences were 3.5 ± 0.3 events/10 h following saline injections. No SRS were detected in 5/6 mice and only 1 SRS event was observed in another mouse following phenytoin injections ($p = 0.002$; **Figures 3A–C**). While hippocampal ictal discharges were nearly abolished by phenytoin, hippocampal interictal spikes persisted without evident changes in waveforms and incidences. Measured at 1 h post saline or phenytoin injection, the incidences of hippocampal interictal spikes were 199.2 ± 44.2 and 218.0 ± 55.2 spikes/10 min, respectively ($n = 6$ mice; $p = 0.789$; **Figure 3D**). Hippocampal EEG signals collected from a mouse are illustrated in **Supplementary Figure 1**, showing interictal spikes and a subsequent discharge event following a saline injection and interictal spikes alone after a phenytoin injection.

Levetiracetam at 400 mg/kg abolished SRS in 6/6 mice tested. SRS incidences were 3.8 ± 0.6 events/12 h following saline injections. Neither hippocampal discharges nor motor seizures were detected following levetiracetam injections (**Figures 3A–C**), whereas the incidences of hippocampal interictal spikes were not significantly different between post-saline and post-levetiracetam measures (212.3 ± 35.9 and 160.8 ± 23.2 events/10 min; $p = 0.123$; **Figure 3D**). Levetiracetam at 100 mg/kg was variable in suppressing SRS ($n = 5$ mice). SRS incidences were decreased in 4/5 mice tested but the overall SRS incidences were not significantly different between post-saline and post-levetiracetam measures (3.6 ± 1.0 vs. 1.6 ± 0.7 events/12 h; $p = 0.151$; **Figure 3A**). There were no significant differences between post-saline and post-levetiracetam measures about corresponding hippocampal discharge durations (55.0 ± 4.6 vs. 43.1 ± 5.7 s; $p = 0.134$), motor seizure stages (4.4 ± 0.2 vs. 3.3 ± 1.0; $p = 0.489$) and hippocampal interictal spikes (227.2 ± 51.6 vs. 171.8 ± 42.3 events/10 min; $p = 0.430$; **Figures 3B–D**).

Lorazepam at 1.5 mg/kg effectively suppressed motor seizures but spared corresponding hippocampal discharges in 6 mice examined. SRS incidences were not significantly different between post-saline and post-lorazepam measures (3.0 ± 0.5 and 2.8 ± 0.5 events/10 h, $p = 0.234$). Motor seizure stages were significantly decreased from 3.8 ± 0.1 following saline injections to 1.0 in all 6 mice following lorazepam injections ($p = 0.002$); whereas the durations of corresponding hippocampal discharges were not significantly different between post-saline and post-lorazepam measures (45.0 ± 2.5 and 50.0 ± 3.7 s; $p = 0.191$; **Figures 3A–C**). The incidences of hippocampal interictal spikes were also not different between post-saline and post-lorazepam measures (170.0 ± 16.2 and 203.8 ± 25.3 events/10 min; $p = 0.656$; **Figure 3D**). SRS sampled from a mouse are presented n in **Figure 4** and **Supplementary Videos 3-4**, showing two similar hippocampal discharges in correspondence to a stage-4 following a saline injection and a stage-1 motor seizure following a lorazepam injection.

DISCUSSION

We aimed to develop a mouse model of extended hippocampal kindling with a particular focus on AED effects in the present study. Three main observations emerged from our present study. First, extended hippocampal kindling and chronic SRS monitoring were reliable. Second, SRS manifested as generalized seizure events with relatively stable incidence and severity. Third, SRS responded differently to phenytoin, lorazepam,

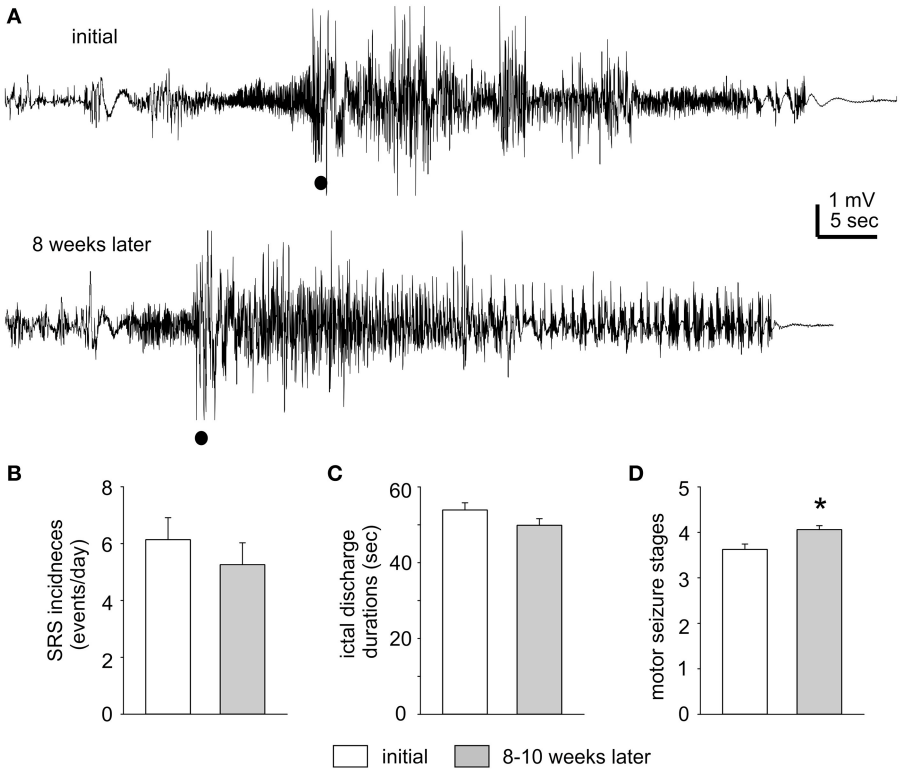

FIGURE 2 | Stable SRS observed after termination of kindling stimulation. **(A)** The first EEG trace corresponds to a mouse on Day 1 after termination of extended hippocampal kindling. The second trace shows the EEG of the same animal but 8 weeks later. Filled circles denote movement artifacts. **(B–D)** SRS incidences, corresponding hippocampal discharge duration, and motor seizure stages measured within 3 days after termination of extended hippocampal kindling stimulation and 8–11 weeks later ($n = 10$). The values represent the mean ± S.E.M. $^*p = 0.002$.

and levetiracetam according to EEG and motor behavioral measurements. These observations suggest that the extended hippocampal kindling could be considered as a mouse model of SRS.

Extended Hippocampal Kindling and SRS Induction

Reliable stimulation and recording of a desired brain structure are essential for extended kindling in mice. To address this issue, we used our group's previously developed methods in order to achieve stable implantations of intracranial electrodes and chronic EEG monitoring in mice (see Jeffrey et al., 2014 and Bin et al., 2017 for details). To verify the locations of the implanted hippocampal electrodes, we examined the theta rhythm and the irregular activity as they are intrinsic, behavioral state-dependent hippocampal activities (Buzsáki et al., 2003). Both hippocampal activities were consistently observed in all mice examined during baseline monitoring and following extended kindling or handling manipulation. We also performed brain histology to reveal the tracks of implanted hippocampal electrodes. This evaluation showed appropriate electrode placements and no gross brain lesions in the majority of the examined animals. These results support the idea that extended hippocampal kindling is in general a feasible and reliable model. However, extended kindling of other brain structures in mice remains to be tested.

SRS Following Extended Hippocampal Kindling

In our present experiments, SRS were recognized by hippocampal EEG ictal discharges and concurrent motor seizures. EEG ictal discharges were also observed from the parietal cortical or piriform area in some experiments. As the hippocampus is not directly involved in motor functions, we speculate that the hippocampal discharges may be a major component of focal seizures and that generalized motor seizures may be a result of seizure spread from the hippocampus to other brain areas. Regarding the EEG and the motor behavioral features of the SRS that had the animals, our observations are in line with previous studies of extended kindling in other animal species (Wada et al., 1974; Wada and Osawa, 1976; Pinel and Rovner, 1978; Wauquier et al., 1979; Gotman, 1984; Hiyoshi et al., 1993; Milgram et al., 1995; Michalakis et al., 1998; Sayin et al., 2003; Brandt et al., 2004). However, some important features such as the SRS development, the regional EEG discharges, the neuronal injury, and the hippocampal cellular and local circuitry activities (Sayin et al., 2003; Brandt et al., 2004) need to be characterized in our model.

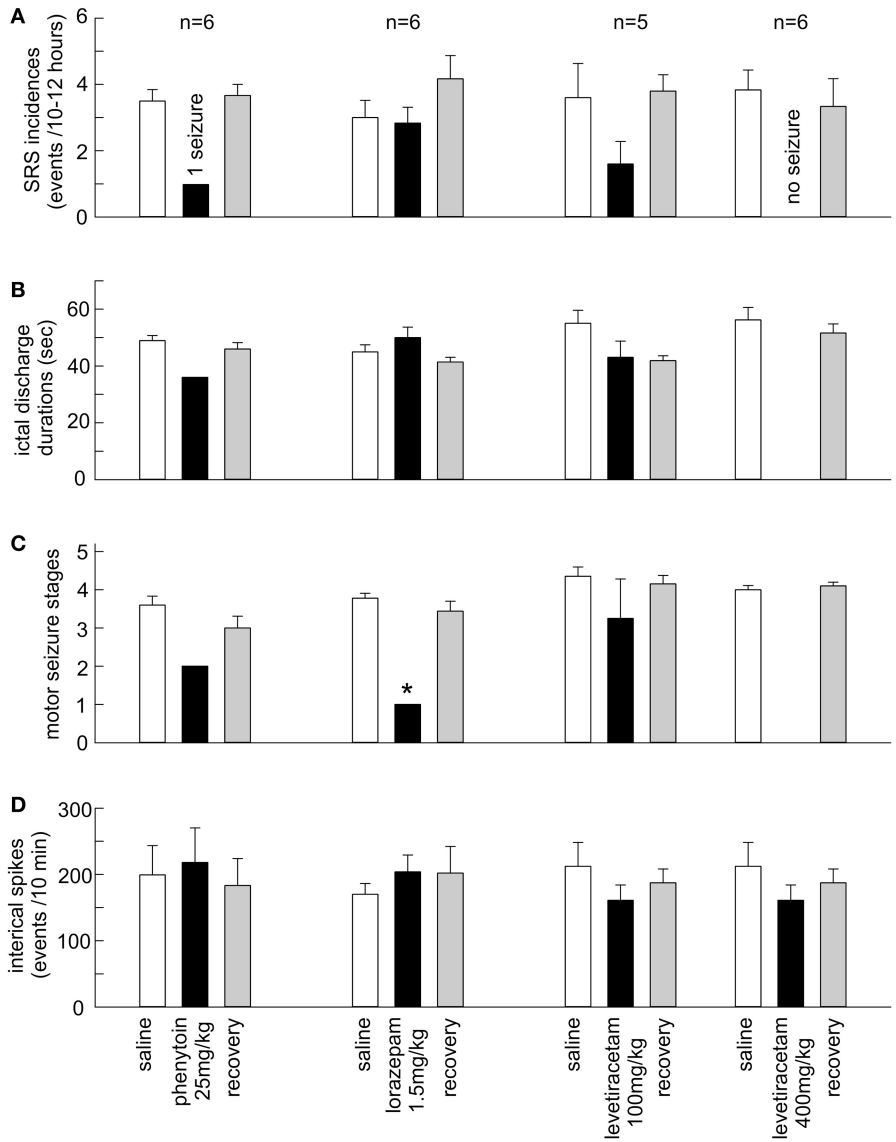

FIGURE 3 | Effects of the AEDs on SRS and hippocampal interictal spikes. **(A–D)** Phenytoin (25 mg/kg), lorazepam (1.5 mg/kg) and levetiracetam (100 and 400 mg/kg) were applied via intra-peritoneal injections. SRS were measured 10–12 h post saline or AED injection. Interictal spikes were measured ∼1 h post saline or AED injection. Recovered measures were done 24 h post AED injection. The values represent the mean ± S.E.M. $^*p = 0.002$.

To assess SRS stability, individual mice were monitored over a few months after termination of the kindling stimulation. These experiments revealed that SRS were relatively stable in incidence, hippocampal discharge durations and motor seizure stages. Overall, the mean SRS incidences in individual mice were in a range of 4–6 events/day, which appear to be higher than those monitored via continuous video/EEG in a rat model of extended amygdala kindling (1–2 SRS events/day; Brandt et al., 2004). While such a difference may be due to multiple experimental variables including animal species, ages and kindling sites, SRS with a relatively stable and high incidence are a significant outcome of our present experiments.

SRS Following Extended Hippocampal Kindling Are Suitable for AED Assessments

AED Effects and Potential Anti-seizure Mechanisms

Phenytoin and levetiracetam at 400 mg/kg abolished SRS in the majority of mice examined as neither hippocampal discharges nor motor seizures were detected in these mice following AED injections. Levetiracetam at 100 mg/kg was inconsistent in suppressing SRS, which might be due in part to a small tested sample size and to dose-dependent effects. In the case of lorazepam at 1.5 mg/kg, it differently affected the SRS when compared to phenytoin and levetiracetam (400 mg/kg); it decreased motor seizure stages while no significant reductions

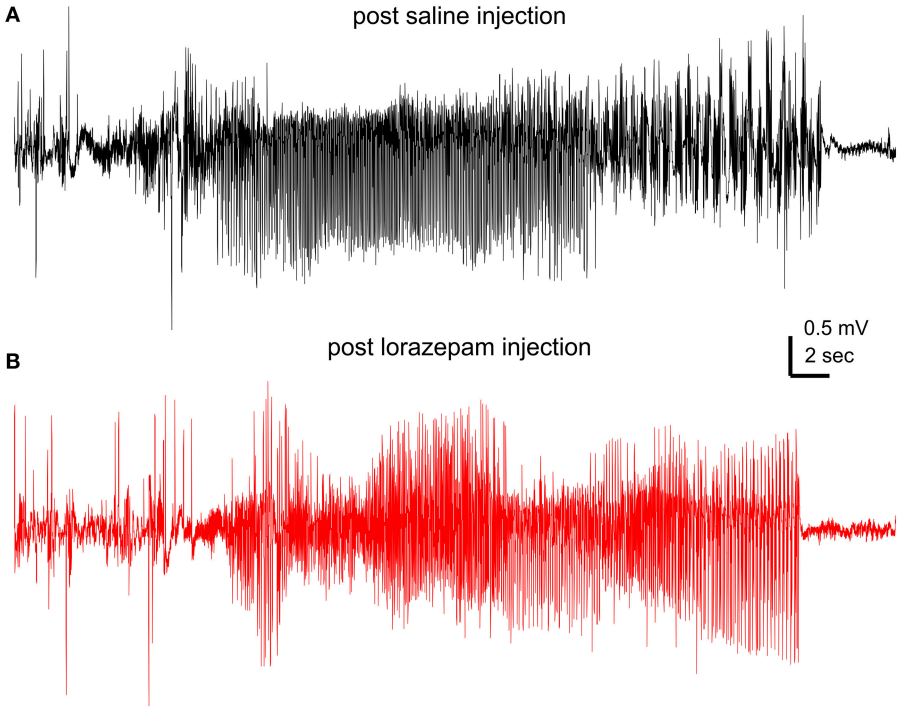

FIGURE 4 | Effects of lorazepam on hippocampal EEG discharges**.** EEG traces collected from a mouse following a saline **(A)** or a lorazepam (1.5 mg/kg) **(B)** injection. Corresponding motor seizures presented in **Supplementary Videos 3, 4**.

were observed in SRS incidence and hippocampal discharge durations.

Previous studies have examined anti-seizure effects of phenytoin, diazepam and levetiracetam in classic kindling models. The suppression of evoked focal seizures, by increased afterdischarge threshold and/or decreasing afterdischarge durations, is thought to be the primary target for phenytoin's anticonvulsant action (Ebert et al., 1997). Levetiracetam also increases afterdischarge thresholds or decreases afterdischarge durations (Löscher et al., 1998, 2016; Löscher and Hönack, 2000). Benzodiazepine GABA enhancers such as diazepam and clonazepam at low doses can decrease generalized motor seizures with or without weak effects on evoked focal afterdischarges (Löscher et al., 1986; Voits and Frey, 1994). Our present observations may be in line with these previous findings if spontaneous hippocampal discharges represent a major component of focal seizures and motor seizures result from seizure spread from hippocampus to other brain areas in our model.

Comparisons With Relevant Previous Studies

A previous study has tested phenytoin and diazepam in a rat model of extended amygdala kindling (Pinel, 1983). In this study, phenytoin at 100 mg/kg and diazepam at 1 mg/kg were administered repeatedly by daily intra-peritoneal injections, and AED effects on spontaneous motor seizures (mainly forelimb clonus) were examined in a 3-h period post-injection. Phenytoin effectively suppressed the spontaneous motor seizures whereas diazepam had litter effects. This previous study and our present experiments are in agreement regarding the effects of phenytoin, suggesting that this AED may be effective in controlling SRS in extended kindling models. The ineffectiveness of diazepam (1 mg/kg) seen in the rat model differs from the motor seizure suppression induced by lorazepam (1.5 mg/kg) in our model.

Several studies have examined phenytoin, diazepam and/or levetiracetam in a mouse model of intra-hippocampal kainate application (Riban et al., 2002; Klein et al., 2015; Twele et al., 2015; Duveau et al., 2016). In these studies, AEDs were applied by acute intra-peritoneal injections and their effects on hippocampal EEG discharges were examined in periods of 3-4 h post-injection. Diazepam at 2–5 mg/kg and levetiracetam at 600 or 800 mg/kg (but not at 200 or 400 mg/kg) were effective in suppressing hippocampal discharges; whereas phenytoin at 20–50 mg/kg was ineffective. Discrepancies appear to exist between these findings and our present observations on the effects of phenytoin and diazepam/lorazepam as well as on the anti-seizure doses of levetiracetam. However, mouse strains, ages, seizure types and AED administration and assessment protocols are also different between these studies and our experiments. Of these experimental dissimilarities, distinct seizure types and underlying epileptogenic processes are likely major influencing factors of model-specific AED effects (Löscher, 2017).

Significance and Other Limitations

Many patients with TLE do not present major structural abnormalities in standard brain imaging examinations

(Ferlazzo et al., 2016). In addition, status epilepticus is not usually recognized in these patients in the early phase of epileptogenic process. Therefore, it is important for experimental research to model these clinical scenarios. However, the commonly used status epilepticus models induce evident brain lesions (Dudek and Staley, 2017; Gorter and van Vliet, 2017; Henshall, 2017; Kelly and Coulter, 2017) which may be suboptimal for research along this direction. In this regard, extended kindling may represent an optimal candidate model since the SRS developed are not initiated and/or associated with status epilepticus and gross brain lesions (Pinel and Rovner, 1978; Milgram et al., 1995; Michalakis et al., 1998; Sayin et al., 2003; Brandt et al., 2004). To date, SRS genesis following extended kindling is not fully understood yet, and extended kindling is not widely used mainly due to its laborious nature and the required reliability of implanted intracranial electrodes in chronic experiments. Furthermore, no study involving a mouse model of extended kindling has been published yet. In this sense, our present work is pioneer in the development of a mouse model of extended hippocampal kindling and its validation for future chronic SRS examinations and AED assessments. This model, with further improvement and possible employment of genetically/molecularly manipulated mouse strains, may facilitate future research involving epileptogenesis and drug-resistant epilepsy in the absence of major brain lesions/pathology as seen clinically.

Our present experiments have some limitations. In particular, we did not test each AED at multiple doses nor examine all three AEDs in individual mice. The lack of these experiments disallows AED efficacy assessments in the present study. In addition, EEG recordings from the cortical or piriform area were inconsistent and/or poor in signal quality likely due to electrode contaminations and/or position errors. These drawbacks prevent analyses of regional discharges and their changes by AEDs. Furthermore, the methods/facility for chronic AED delivery (Grabenstatter et al., 2007; Ali et al., 2012) and for measuring AED levels in serum and brain (Löscher, 2007; Markowitz et al., 2010) need to be established for our model, as these approaches are critical for testing AEDs in a clinically relevant manner and for exploring drug-resistant epilepsy. Despite these limitations and weaknesses, our experiences may help future AED assessments in mouse models of extended kindling.

AUTHOR CONTRIBUTIONS

HS, UT, JC, NS, CC, SL, and CW: contributed to experimentation and data analysis; UT, JF, JE, and LZ: contributed to data analysis, data discussion, and manuscript writing. All authors contributed to experimental design.

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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.2018.00451/full#supplementary-material

Supplementary Figure 1 | Effects of phenytoin on hippocampal EEG signals. Continuous EEG signals presented separately for illustrative purpose. **(A,B)**, interictal spikes (top) and subsequent ictal discharge (bottom) observed following a saline injection. **(C,D)**, only interictal spikes observed following a phenytoin injection.

Supplementary Video 1-2 | Representative motor seizures**.** Video data collected from a mouse in the first day (initial) after termination of kindling stimulation and about 8 weeks later. Motor seizures corresponded to the EEG ictal discharges illustrated in **Figure 2A** top and bottom. Videos trimmed with motor seizures appearing around the 6 s time stamp.

Supplementary Video 3-4 | Motor seizure suppression by lorazepam**.** Motor seizures captured from a mouse following a saline injection (video 3) and a lorazepam injection (video 4). Videos arranged with the motor seizure activities appearing around the 7 s time stamp. Corresponding hippocampal discharges presented in **Figures 4A,B**.

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MINI REVIEW
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Neonatal Seizure Models to Study Epileptogenesis

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Current therapeutic strategies for epilepsy include anti-epileptic drugs and surgical treatments that are mainly focused on the suppression of existing seizures rather than the occurrence of the first spontaneous seizure. These symptomatic treatments help a certain proportion of patients, but these strategies are not intended to clarify the cellular and molecular mechanisms underlying the primary process of epilepsy development, i.e., epileptogenesis. Epileptogenic changes include reorganization of neural and glial circuits, resulting in the formation of an epileptogenic focus. To achieve the goal of developing "anti-epileptogenic" drugs, we need to clarify the step-by-step mechanisms underlying epileptogenesis for patients whose seizures are not controllable with existing "anti-epileptic" drugs. Epileptogenesis has been studied using animal models of neonatal seizures because such models are useful for studying the latent period before the occurrence of spontaneous seizures and the lowering of the seizure threshold. Further, neonatal seizure models are generally easy to handle and can be applied for *in vitro* studies because cells in the neonatal brain are suitable for culture. Here, we review two animal models of neonatal seizures for studying epileptogenesis and discuss their features, specifically focusing on hypoxia-ischemia (HI)-induced seizures and febrile seizures (FSs). Studying these models will contribute to identifying the potential therapeutic targets and biomarkers of epileptogenesis.

Keywords: neonatal seizures, epilepsy, hypoxia-ischemia, febrile seizures, bumetanide

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INTRODUCTION

The neonatal period is at higher risk of having seizures than other periods in life (Annegers et al., 1995). A population study indicated the incidence of seizures to be 1–5 per 1000 live births (Saliba, 2001; Volpe, 2008; Glass, 2014). Early life insults such as hypoxic-ischemic encephalopathy and fever are major causes of neonatal seizures (Rakhade and Jensen, 2009), and it has been reported that 16–56% of neonates that experience seizures develop epilepsy later in life (Mizrahi and Watanabe, 2005; Pisani et al., 2012). The primary process whereby principal neurons generate the first spontaneous and epileptiform discharges is referred to as epileptogenesis and is often accompanied by both structural and functional alteration of neuronal circuits (Goldberg and Coulter, 2013). Classical anticonvulsants, i.e., anti-epileptic drugs, are used to suppress ongoing and future seizures but not to prevent the onset of epilepsy (Temkin, 2009). Therapeutic strategies targeting the prevention of epileptogenesis are expected to prevent the onset of epilepsy, i.e., the occurrence of the first spontaneous epileptic seizures. To develop such "anti-epileptogenic" treatments, the cellular and molecular mechanisms underlying epileptogenesis have been studied using animal models of neonatal seizures (Dubé et al., 2006, 2010; Kadam et al., 2010). This mini-review will provide an overview of the representative animal models of neonatal seizures, especially

focusing on rodent models of hypoxia-ischemia (HI)-induced seizures and febrile seizures (FSs) (Dubé et al., 2010; Sun et al., 2016). We will also discuss potential pharmacological strategies that could target epileptogenic changes observed in neonatal seizure models.

HYPOXIA-ISCHEMIA (HI) MODELS

Neonatal seizures are often induced by perinatal asphyxia with a critical lack of oxygen during labor and delivery (Sun et al., 2016). To study this type of seizure and its sequelae, experimental HI-induced seizures have been extensively used. In this section, we will introduce rodent models of HI that are potentially useful to identify mechanisms that mediate epileptogenesis in the immature brain. Rice et al. (1981) first established a rat neonatal HI model known as the Rice–Vannucci model. The Rice–Vannucci model and its derivatives have been widely used to study neonatal HI. For preparing the HI model, rat pups of both sexes are typically used at postnatal day 7 (P7)-P10 because the brain development during this period roughly corresponds to that of humans in late embryonic to early postnatal periods (Talos et al., 2006; Rakhade and Jensen, 2009). It should be noted that sex differences in susceptibility to HI have been reported (Smith et al., 2014a). To induce HI, rat pups are exposed to a hypoxic environment (8% O_2 in N_2) for 30 min–2.5 h (sometimes up to 4 h) after ligation of the lateral common carotid artery. In this system, brain damage, including selective neuronal necrosis and infarction in the ipsilateral cerebral cortex, striatum, and hippocampus, was observed in no less than 90% of HI-induced animals and within 50 h after HI induction (Rice et al., 1981). In addition, the authors revealed that lower O_2 levels and longer exposure time to HI resulted in more-severe brain damage (Rice et al., 1981; Cuaycong et al., 2011). In HI models, cortical damage is mainly observed in columns at right angles to the pial surface, a pattern that is also observed in human infants who experienced repeated hypoxia-acidosis with hypotension (Norman, 1981; Rice et al., 1981). HI models also replicate many histological features, such as porencephalic cysts and cortical microgyri, observed in brains of infants with hypoxic-ischemic encephalopathy (Williams et al., 2004; Kadam and Dudek, 2007; Williams and Dudek, 2007). Further, 90 min of HI treatment evoked cortical electrographic seizures with correlated behavioral movements in 92% (11 of 12) and electrographic seizures in 83% (10 of 12) of the rats (Cuaycong et al., 2011). Additionally, simultaneous video monitoring and electroencephalogram (EEG) recording during and after HI induction revealed that all rats (12 of 12) showed electroclinical seizures, defined as EEG patterns abnormal in amplitude and frequency, and that the seizures persist for 24 h in 67% (8 of 12) and 48 h in 25% (3 of 12) (Sampath et al., 2014).

SEIZURE PHENOTYPES FOLLOWING HI INDUCTION

Kadam et al. (2010) examined whether HI insults initiate epileptogenic processes. Rat pups at P7 underwent unilateral carotid ligation followed by a hypoxic environment (8% O_2, 2 h), and continuous radio-telemetry and video recording of cortical electroencephalographic and behavioral seizures were performed from 2 to 12 months of age. It was reported that 56% (10 of 18) of HI-induced rats develop spontaneous epileptiform discharges and recurrent seizures. Furthermore, all rats with spontaneous electrographic seizures exhibited obvious cystic infarcts in the ipsilateral hemisphere, although the seizure rate per day did not always depend on the severity of infarct. Rats without spontaneous seizures exhibited no infarct and no associated neuronal death. It was suggested that the occurrence of cortical abnormalities is associated with the acquisition of epileptogenesis, i.e., the occurrence of first spontaneous seizures. Spontaneous electrographic seizures, which are often accompanied by behavioral seizures, occurred in distinct clusters with seizure-free periods as long as a few weeks and progressively became more severe and more frequent over time. In addition, 24-h behavioral monitoring for a week/month in HI-induced rats starting from P30 revealed that behavioral seizures were not detected until the second month of monitoring (Kadam and Dudek, 2007). These results indicate that the process of epileptogenesis requires a long and continuous latency period, which may allow therapeutic intervention to prevent subsequent progression of the epileptogenic process, especially by suppressing structural brain damage.

Some studies, however, reported few or no incidence of spontaneous seizures following HI (**Table 1**). When mouse pups were subjected to HI induction at P7 with a ligation of the lateral common carotid artery and a subsequent hypoxic challenge (8% O_2 for 45 min), evident epileptiform discharges were not observed at 2–3 months of age during hippocampal and cortical EEG recordings performed 5–8 h/day for 3–4 days (Peng et al., 2015). Chronic video monitoring for 10 consecutive days revealed that motor seizures occurred in only 2 of 23 post-HI mice. In addition, to evaluate seizure susceptibility of HI-induced mice, the authors stimulated the hippocampus with square current pulses to evoke motor seizures and found no evidence of increased seizure susceptibility in HI mice. These conflicting data in terms of reproducing HI-induced development of epilepsy may be attributable to the differences in animal species, sex (both sexes are often mixed), hypoxic environment (especially the duration of hypoxia), seizure criteria, and EEG recording time, as well as to the small numbers of animals in control groups in some cases. The establishment of stable HI models using mice is awaited because transgenic lines are more available in mice than in rats and will enable the study of cellular and molecular mechanisms underlying epileptogenesis.

FEBRILE SEIZURE (FS) MODELS

Fever (typically greater than 38°C)-induced FSs are the most common childhood seizures, with a prevalence of 2-14% worldwide (Verity and Golding, 1991; Vestergaard and Christensen, 2009; Koyama and Matsuki, 2010). FSs usually

TABLE 1 | Epileptogenesis in neonatal seizure models.

Model	Animal species/strain	Age	Behavioral seizures during induction	Electrographic seizures during induction	Spontaneous seizures	Reference
HI	Sprague–Dawley rats	P30	N/A	N/A	15% (3 of 20)	Williams and Dudek, 2007
HI	Sprague–Dawley rats	P7	N/A	N/A	30% (3 0f 10)	Kadam and Dudek, 2007
HI	Sprague–Dawley rats	P7	N/A	N/A	56% (10 of 18)	Kadam et al., 2010
Hypoxia	Long-Evans rats	P10	93% (58 of 61)	Yes	94% (48 of 51)	Rakhade et al., 2011
FS	Sprague–Dawley rats	P10-11	Yes	N/A	35% (6 of 17)	Dubé et al., 2006
FS	Sprague–Dawley rats	P11	Yes	N/A	50% (8 of 16)	Koyama et al., 2012
Kainic acid	c-Dawley rats	P5-60	Yes	Yes	N/A (KA at P5, P10) 14% (P20) 30% (P30) 44% (P60)	Stafstrom et al., 1992
Flurothyl	Sprague–Dawley rats	P0-9	Yes	N/A	Increased seizure susceptibility to flurothyl	Huang et al., 1999

HI, hypoxia-ischemia; FS, febrile seizure.

occur between 3 months and 5 years of age with the peak at 16–18 months. Although simple FSs are mostly benign, complex FSs with prolonged duration (>15 min), recurrent seizures or focal neurological features are associated with the development of temporal lobe epilepsy (Cendes et al., 1993; French et al., 1993; Theodore et al., 1999). To investigate the potential consequences of complex FSs and to screen potential anti-epileptogenic drugs, various highly reproducible animal models have been developed (Koyama, 2017). In these models, prolonged seizures are induced by exposing rodent pups to a hyperthermic environment to simulate fever-like conditions; the main such models are the hair dryer model (Dubé et al., 2000; Koyama et al., 2012; Tao et al., 2016), heated chamber model (Holtzman et al., 1981; Schuchmann et al., 2006), hot water model (Ullal et al., 1996; Jiang et al., 1999), microwave model (Hjeresen et al., 1983), and lipopolysaccharide model (Heida et al., 2004). Although there are limitations to completely mimicking clinical phenotypes of FSs in human neonates, experimental hyperthermia can induce the release of fever mediators that are necessary for the onset of FSs (Dubé et al., 2005).

Baram and his colleagues developed and refined the hair dryer model that is widely used (Toth et al., 1998; Dubé et al., 2010). In this model, P10–11 Sprague–Dawley rats or P14–15 129/Sv or C57BL/6 mice are often used because the developmental stage of hippocampus is roughly comparable to that in human infants, which are most susceptible to FSs. Significant sex differences were not observed, at least for the seizure duration (Lemmens et al., 2005). It has been reported that differences in mouse strain affect susceptibility to FS induction (van Gassen et al., 2008). Prolonged FSs are generated by maintaining hyperthermia (typically 38.5–42.5°C) for 30 min. During hyperthermia, the rectal temperature is measured every 2 min to maintain core temperature between 38.5 and 42.5°C, which corresponds to the threshold temperature to evoke complex FSs in human neonates. Hyperthermic controls can be conducted by administering barbiturates to suppress seizures for distinguishing whether the observed FS consequences result from the seizures or the hyperthermia. The onset of experimental FSs typically consists of an acute sudden arrest of hyperthermia-induced hyperactivity, such as oral automatism and running. Oral automatism is typically followed by forelimb clonus. In the later phase of FSs, tonic body flexion is often observed in rats, but it is rare in mice.

SEIZURE PHENOTYPES FOLLOWING FS INDUCTION

Dubé et al. (2000) showed that FSs during development decreased the seizure threshold in later life in rats. In brief, all the rats (11 of 11) at 10–11 weeks following early life FSs developed hippocampal seizures, and most (8 of 11) experienced status epilepticus after intraperitoneal administration of a subthreshold dose of the chemical convulsant kainate (5 mg/kg). In contrast, most normothermic (6 of 8) and hyperthermic controls without seizures (5 of 6) did not exhibit seizures or kainite-induced status epilepticus. The authors prepared hippocampal-entorhinal cortical slices 1 week after FS induction and then stimulated Schaeffer collaterals, observing more prolonged status epilepticus-like discharges than in control slices. The same research group examined whether experimental FSs induce spontaneous seizures by performing chronic monitoring of behaviors and EEGs from the hippocampus and cortex (Dubé et al., 2006). 2 months after FS induction, bipolar electrodes were implanted unilaterally into the dorsal hippocampus and frontoparietal cortex. After the surgery, long-term video-EEG recordings were performed on postnatal days 90, 105, 120, 135, 165, and 180 for 5 h at night. None of the normothermic or hyperthermic

controls developed spontaneous seizures or interictal events, whereas behavioral and hippocampal electrographic spontaneous seizures in 35% (6 of 17) and interictal epileptiform EEG abnormalities in 88% (15 of 17) of FS rats were observed. Moreover, longer duration of experimental FSs increased the probability of developing subsequent spontaneous seizures (Dubé et al., 2010). These results suggest that hyperthermia-induced experimental FSs lead to progression of the epileptogenic process.

PHARMACOLOGICAL STRATEGIES

Neonatal seizures should be treated adequately and rapidly because prolonged seizures would result in severe neurological morbidity (Ronen et al., 2007; Jensen, 2009). However, seizures in neonatal periods are sometimes resistant to conventional anti-epileptic drugs. For example, phenobarbital, which binds to an allosteric site on the $GABA_A$ receptor and thereby potentiates the action of endogenous GABA, has been used as the first-line treatment for neonatal seizures (WHO) (World Health Organization, 2011). However, studies have reported that fewer than 50% of neonates with seizures respond to phenobarbital in terms of the suppression of electrographic seizures (Painter et al., 1999; Boylan et al., 2002, Rennie and Boylan, 2007). In addition, it has been reported that phenobarbital could induce the apoptosis of neurons in gray and white matter and impair synaptic connectivity in the immature brain (Bittigau et al., 2003; Forcelli et al., 2012; Kaushal et al., 2016). Moreover, the administration of phenobarbital is associated with long-term alterations in behavioral phenotypes, including impaired cognition and depressive behaviors (Stefovska et al., 2008; Brodie and Kwan, 2012). Thus, more efficient pharmacological treatment than the use of phenobarbital alone should be considered to enhance the inhibitory effects of GABA in neonatal periods.

Because GABA can depolarize immature neurons that overwhelm mature neurons in the developing brain, GABA-mimetic and GABA-modulating anti-epileptic drugs are relatively ineffective in neonatal seizures. It depends on the intracellular Cl^- levels and the Cl^- equilibrium potential whether GABA provokes inhibitory or excitatory actions. The intracellular Cl^- levels are mainly controlled by cation-chloride cotransporter (CCC) family that involves $Na^+K^+2Cl^-$ co-transporter isoform 1 (NKCC1), which mediates Cl^- influx, and K^+2Cl^- cotransporter isoform 2 (KCC2), which mediates Cl^- efflux. The balance in the expression levels of NKCC1 and KCC2 shapes the developmental changes in the actions of GABA: early expression of NKCC1 and late expression of KCC2 underlie the excitatory action of GABA in immature neurons because of an elevated intracellular Cl^- level and a depolarized Cl^- equilibrium potential. It has been suggested that the diuretic bumetanide, a selective NKCC1 inhibitor, could be useful for epileptogenic treatment (Löscher et al., 2013a,b), although it should be noted that the efficacy of bumetanide is still controversial (Puskarjov et al., 2014; Ben-Ari et al., 2016; Hernan and Holmes, 2016). It has been reported that bumetanide blocked kainate-induced seizures in neonatal rats (Dzhala et al., 2005) and that the treatment of bumetanide alone or bumetanide with phenobarbital decreased seizure events and susceptibility after early life seizures in some animal models (Cleary et al., 2013; Holmes et al., 2015; Hu et al., 2017). Koyama et al. (2012) revealed that excitatory $GABA_A$ signaling mediates the emergence of ectopic granule cells that lead to hippocampal hyperexcitability and the development of epilepsy in FS rats. Continuous administration of bumetanide (0.1 mg/kg, i.p.) after FSs attenuated ectopic localization of granule cells, susceptibility to limbic seizures and development of epilepsy. In an HI model, bumetanide suppressed mossy fiber sprouting, an anatomical hallmark that may shape epileptogenic neural circuits (Koyama and Ikegaya, 2004), after hypoxia and prevented the onset of spontaneous electrographic seizures (Wang et al., 2015). In human patients, Pressler et al. (2015) conducted a trial of bumetanide for infants with hypoxic-ischemic encephalopathy, but this study was terminated due to the lack of clear efficacy and side effects of bumetanide. In clinical trials of human neonatal seizures, subjects are limited to infants with phenobarbital-resistant refractory seizures.

For the development of anti-epileptogenic drugs, future studies using various prodrugs of bumetanide or alternative NKCC1 blockers with enhanced penetration into the brain through the blood brain barrier, or drugs that enhance Cl^- extrusion via KCC2, are essential (Löscher et al., 2013b; Kaila et al., 2014; Puskarjov et al., 2014). The efficacy of bumetanide would depend on seizure phenotypes and the stage of epileptogenic process. Thus, it is important to clarify the changing roles of GABAergic signaling in the early and late phase of epileptogenesis. Some studies have reported the involvement of excitatory $GABA_A$ signaling in rather late phase of epileptogenic changes, i.e., structural changes, after neonatal seizures (Koyama et al., 2012; Wang et al., 2015). However, the roles of excitatory $GABA_A$ signaling in very early stage of epileptogenesis remain unclear. Thus, proper methods for recording the changes in neuronal activity in the onset of neonatal seizures to examine the effects of GABAergic signaling are necessary.

CONCLUSION

Proper treatment of neonatal seizures is essential to prevent the future development of epilepsy. However, evidence-based guidelines for pharmacological treatment are lacking (Slaughter et al., 2013; Glass, 2014). In this mini-review, we described two representative animal models of neonatal seizures with a relatively higher risk of epilepsy later in life. These animal models successfully replicate some of the structural abnormalities and cognitive dysfunctions that have been reported in human individuals who experienced neonatal seizures (Dubé et al., 2009; Smith et al., 2014b; Tao et al., 2016). However, the findings from the experimental neonatal seizures are not always consistent.

To avoid the inconsistency, it is of importance to consider the differences in experimental conditions such as animal species, sexes, developmental stages, and the criteria used to categorize seizures. It is also helpful to develop additional experimental models that mimic the initial triggers of neonatal seizures in humans, for example, exanthema subitum or influenza that lead to fever production. It should be also noted that the biomarkers to diagnose the development of epilepsy later in life need to be discovered, especially in terms of drug administration to infants and children. The proper adoption and use of animal models will allow investigators to clarify the cellular and molecular mechanisms of epileptogenesis following neonatal seizures and to identify pharmacological targets and biomarkers in human neonates.

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YK and RK wrote the manuscript. YK, RK, and YI discussed the manuscript.

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Brain Lipopolysaccharide Preconditioning-Induced Gene Reprogramming Mediates a Tolerance State in Electroconvulsive Shock Model of Epilepsy

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There is increasing evidence pointing toward the role of inflammatory processes in epileptic seizures, and reciprocally, prolonged seizures induce more inflammation in the brain. In this regard, effective strategies to control epilepsy resulting from neuroinflammation could be targeted. Based on the available data, preconditioning (PC) with low dose lipopolysaccharide (LPS) through the regulation of the TLR4 signaling pathway provides neuroprotection against subsequent challenge with injury in the brain. To test this, we examined the effects of a single and chronic brain LPS PC, which is expected to lead to reduction of inflammation against epileptic seizures induced by electroconvulsive shock (ECS). A total of 60 male Sprague Dawley rats were randomly assigned to five groups: control, vehicle (single and chronic), and LPS PC (single and chronic). We first recorded the data regarding the behavioral and histological changes. We further investigated the alterations of gene and protein expression of important mediators in relation to TLR4 and inflammatory signaling pathways. Interestingly, significant increased presence of NFκB inhibitors [Src homology 2-containing inositol phosphatase-1 (SHIP1) and Toll interacting protein (TOLLIP)] was observed in LPS-preconditioned animals. This result was also associated with over-expression of IRF3 activity and anti-inflammatory markers, along with down-regulation of pro-inflammatory mediators. Summarizing, the analysis revealed that PC with LPS prior to seizure induction may have a neuroprotective effect possibly by reprogramming the signaling response to injury.

Keywords: seizures, neuroprotection, preconditioning, tolerance, treatment, gene reprogramming, brain injury, signaling pathway

INTRODUCTION

A series of complex events are believed to trigger subsequent changes in the brain causing epilepsy. While the underlying cause of epilepsy remains largely unknown, increasing evidence supports links between neuroinflammation and epilepsy (Dupuis and Auvin, 2015; Vezzani et al., 2016). Several studies have highlighted that neuroinflammation is associated with an increased production of pro-inflammatory cytokines which normally contribute to neuronal damage and other events that make structural and functional changes in the hippocampus to initiate epileptogenesis (Iori et al., 2013; Amini et al., 2015; Kosonowska et al., 2015). Moreover, inflammatory processes in cell injury resulting from prolonged seizures also trigger molecular and cellular alterations, which leads to the production of vast numbers of different pro-inflammatory mediators (Amhaoul et al., 2015; Vezzani et al., 2016). So, in this regard, it is believed that neuroinflammation can be both a consequence and also a cause of epileptic seizures (Amhaoul et al., 2015; Gershen et al., 2015).

Previous experimental work, which has been conducted in animal models suggested that induction of seizures elicited by either various chemical agents (Kaur et al., 2014; Kołosowska et al., 2014) or repeated electrical stimulation such as ECS (Cardoso et al., 2011; Rothan et al., 2017) will ultimately lead to progressive development of seizures. Despite the development of new treatment approaches that have made great advances in the control of seizures, intolerable side effects of currently available treatments still pose a significant problem (Amhaoul et al., 2014; Moshé et al., 2015). Therefore, it is necessary to seek and explore novel and potential treatments for prevention of seizures in patients with epilepsy.

Recently, insights into neuroprotective mechanisms of the brain to modulate noxious stimuli and then recover itself from injury have opened up new prospects for the development of new and effective therapies. From this perspective, it is believed that PC by mild persisting inflammation may pave the way to novel treatments to modulate neurological disorders (Dmowska et al., 2010; Mirrione et al., 2010; Hickey et al., 2011; Vartanian et al., 2011). PC with a sub-lethal dose of LPS is one of the best characterized neuroprotective stimuli for inducing mild inflammation in the rodent brain (Hickey et al., 2011; Wang et al., 2015). Low doses of LPS can alter the genomic response by a process of reprogramming, which occurs through the TLR4 signaling pathway, mainly by activation of TRIF-dependent cascade and the inhibition of the MYD88-dependent pathway (Wang et al., 2015). This leads to a neuroprotective response through activation of anti-inflammatory markers (e.g., IFN-β and IL10) by increasing IRF3 and also inducing NFκB inhibitors (SHIP1 and TOLLIP) that are crucial to reduce NFκB production (Vartanian et al., 2011). Therefore, it ultimately reduces the generation of pro-inflammatory cytokines (e.g., TNF-α and IL-1β), modulates the inflammatory responses, and protects the brain against subsequent serious injury (Schaafsma et al., 2015).

Given the evidence above, we hypothesized that a single low dose and chronic ultra-low dose of brain LPS PC may have beneficial effects on neuronal survival following epileptic injuries by repeated seizures in the ECS model of epilepsy. Therefore, behavioral impairments related to epileptic seizures, lesions in hippocampal regions (CA1, CA3, and DG), and the molecular mechanisms underlying TLR4 and inflammatory signaling pathways were evaluated.

MATERIALS AND METHODS

Animals

Adult male SD rats (200–250 g) were used and these were obtained from the Universiti Kebangsaan Malaysia (UKM) Vendor (Kuala Lumpur, Malaysia). The handling of animals was carried out according to the National Institutes of Health guidelines and regulations. The animals were housed in a cage under controlled environmental conditions for temperature (∼24°C), humidity (∼50%), and light (a daily ratio of 1:1). The animals were allowed access to food and water *ad libitum* (free access to food and water). All efforts were made to minimize animal suffering and the number of animals required. The procedures involving animals were approved by the Animal Ethics Committee, Faculty of Medicine, UKM (Ethics No: FP/MED/NORLINAH/31-JAN-/493-FEB-2013-FEB-2016). The experimental designs, dosage, interval, and frequency of drugs administration are illustrated below (**Figure 1**).

Surgical Procedures

In this experiment, a total of 60 male SD rats were randomly assigned to five groups, namely, control (n = 12), single vehicle (n = 12), chronic vehicle (n = 12), single LPS PC (n = 12), and chronic LPS PC groups (n = 12). Animals allocated for LPS PC and matched vehicle groups were randomized and each rat was i.c.v injected with either LPS (*Escherichia coli 055:B5*) or an equivalent volume of NS. In the single LPS preconditioned group, animals received one injection of low dose LPS at 4 days prior to receiving shock, while in the chronic preconditioned group, animals received four injections of ultra-low doses LPS (one injection every 4 days), initiated from 16 days prior to receiving shock. Rats in the vehicles groups were pretreated with NS.

Abbreviations: β-Actin, beta actin; AP, anterior–posterior; BBB, blood–brain barrier; BSA, bovine serum albumin; CA1, Region 1 Cornu Ammonis; CA3, Region 3 Cornu Ammonis; cDNA, complementary DNA; CNS, central nervous system; DG, dentate gyrus; DV, dorso-ventral; ECS, electroconvulsive shock; $GABA_A$ R, gamma-aminobutyric acid (GABA) type A receptor; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; i.c.v, intracerebroventricular; i.p, intraperitoneal; IFC, integrated fluidic circuit; IFN-β, interferon-beta; IL10, interleukin 10; IL-1β, interleukin-1 beta; IRF3, interferon regulatory factor 3; LPS, lipopolysaccharide; ML, medio-lateral; mRNA, messenger RNA; MyD88, myeloid differentiation primary-response protein-88; n, number; NFκB, nuclear factor kappa-light-chain-enhancer of activated B cells; NMDAR, *N*-methyl D-aspartate receptor; NR_2B, subunit-containing *N*-methyl-D-aspartate (NMDA) receptor; NS, normal saline; PC, preconditioning; PCR, polymerase chain reaction; RNA, ribonucleic acid; RT-PCR, real-time polymerase chain reaction; SD, Sprague Dawley; SEM, standard error of mean; SHIP1, Src homology 2-containing inositol phosphatase-1; Taq, *Thermus aquaticus*; TBST, Tris-buffered saline-Tween 20; TLR, Toll-like receptor; TNF-α, tumor necrosis factor-alpha; TOLLIP, Toll interacting protein; TRIF, TIR-domain-containing adapter-inducing interferon β; vs., versus.

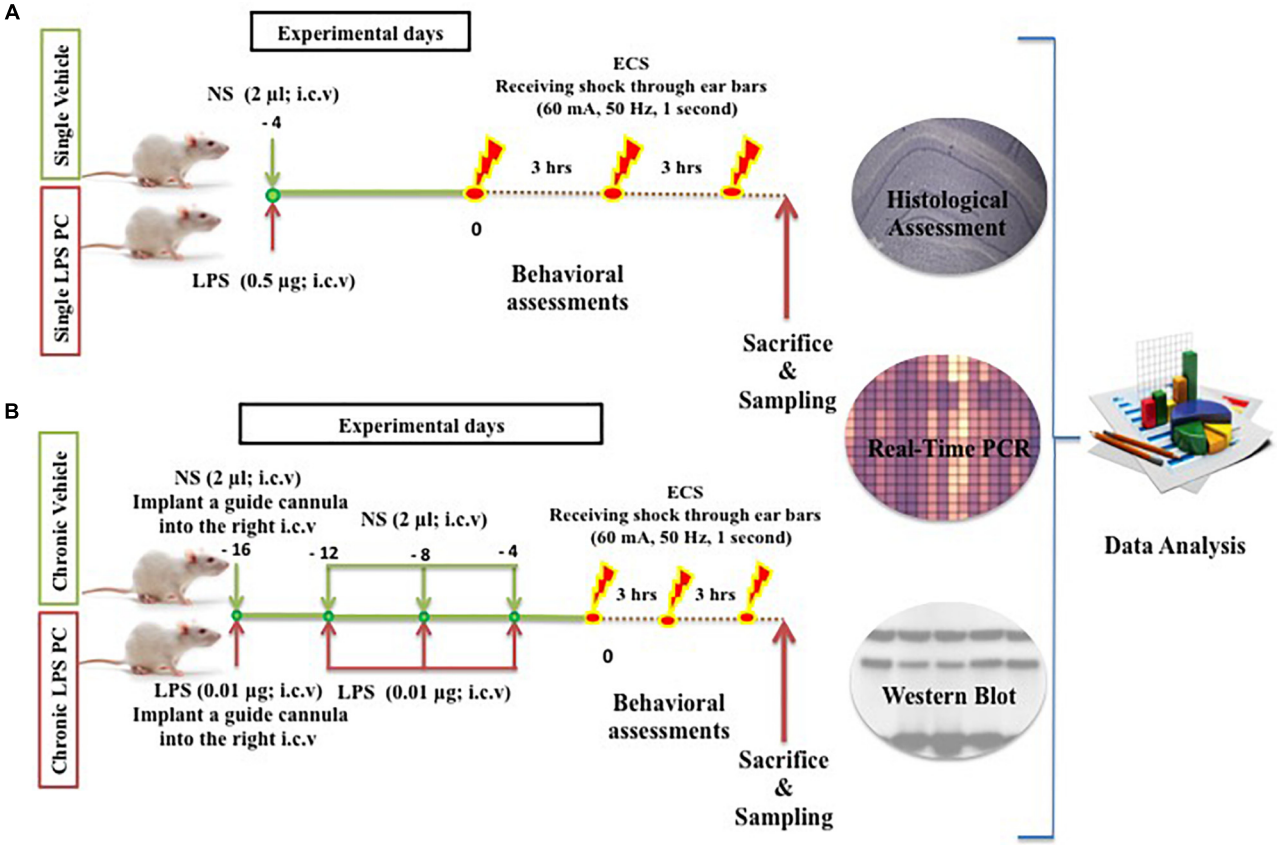

FIGURE 1 | Schematic diagram of experimental design and procedures in the **(A)** single and **(B)** chronic LPS preconditioning and vehicle groups in this study.

Rats were deeply anesthetized with ketamine, xylazine (0.1 ml/100 gm BW; i.p) and then placed on a stereotaxic surgery apparatus (David KOPF Instruments, United States). Injection of LPS or NS into the i.c.v of preconditioned and vehicle animals was performed under coordination of (relative to bregma): AP: −0.96 mm (posterior to bregma), ML: 2.00 mm (lateral to the midline of the skull), and DV: −3.8 mm (ventral to the dura surface), based on the atlas of Paxinos and Watson (1998). A Hamilton syringe needle was used for LPS injection at a dose of 0.5 µg (0.25 µg/µl) for single injection and 0.01 µg (0.005 µg/µl) for chronic injections (every 4 days, for four injections totally). The solution was delivered at a rate of 2 µl over 2 min. The needle remained in place for three more minutes post injection to avoid reflux through the path of the needle. Upon removal, a stainless steel guide cannula was implanted into the right i.c.v for microinjection. Finally, the guide cannula was fixed and the wound was closed with dental cement. Aseptic conditions prevailed for all surgical procedures performed. After surgery, rats were allowed to recover in new cages.

Induction of Seizures and Behavioral Assessments

In the ECS group, rats received a course of three ECS seizures in total, administered at 3-h intervals (i.e., at 0, 3, and 6 h). Each stimulus (50 Hz, 60 mA for 1 s) was delivered via ear-clip electrodes wired to a stimulus generator. Animals were behaviorally observed to ensure that full tonic–clonic seizures occurred. The seizure duration was recorded for all animals. In the control groups, animals were handled identically to the ECS-treated rats, and contact was made with the electrodes, except that no current was passed through the electrodes. All animals in this model were observed for 1 h after ECS administration. Animals were also excluded if they displayed any evidence of tonic–clonic seizures with hind limb involvement.

Tissue Preparation and Histology

Following the completion of the behavioral experiments, all animals were sacrificed by decapitation using a guillotine. Subsequently, the brains were quickly removed and dissected on ice. For the molecular assays such as gene and protein analysis, the right half of the hippocampus was dissected quickly, frozen in liquid nitrogen, and then stored at −80°C. In order to perform the histological assessment, the left half of the brains was immediately cut according to the atlas by Paxinos and Watson (1998). It was then fixed with a fixative solution containing 4% freshly prepared paraformaldehyde (Sigma, United States) in pH 7.4, at 4°C at least for 10 days.

Upon fixation, brain samples were routinely embedded in paraffin wax (Leica) and placed on embedding blocks. Three tissue blocks were selected randomly. The selected embedded tissue blocks were sectioned (5 μm thickness) using a microtome rotary (Leica), placed on glass slides, and stained with cresyl violet (Nissl Staining, Sigma). The CA1, CA3, and DG regions were evaluated under a light microscope (Nikon) and captured at fields of 400× magnification at the same locations within the hippocampal regions. The cells with a well-defined nucleolus and typical Nissl bodies were counted with the ImageJ 1.50 b software using the cell counting function. To avoid any bias in cell counting, this procedure was performed blind by an experienced independent observer.

RNA Isolation and Gene Expression

To evaluate the effective role of LPS PC as protection following tonic–clonic seizures, the expression level of important genes under TLR4 and inflammation pathways was examined. Frozen hippocampal tissues stored at −80°C were selected randomly by an investigator blinded to the treatment groups. Extraction of total RNA from the hippocampus was performed using RNeasy Plus Universal Mini kit (Qiagen Inc, Valencia, CA, United States) according to the instruction manual. Then, RNA quality and quantity were determined with nanodrop UV/V spectrophotometry, native agarose gel, and Agilent 2100 Bioanalyzer (Agilent Technologies, Inc., California), in accordance with the manufacturers' protocols. Following this, RNA was reverse transcribed to cDNA using high-capacity cDNA reverse transcription kits (Applied Biosystem). PCR reactions were finally carried out using a Biomark HD System (Fluidigm, South San Francisco, United States). The Fluidigm system with an IFC chip is a new platform for high-throughput RT-PCR. It is a reliable and sensitive method for measuring RNA transcript levels and performs quantification of multiple RNA targets simultaneously in nanoliter volumes. The expression level of 11 selected genes was evaluated using the IFC chips available for dynamic analysis (192 samples × 24 assays). In this assay, three replicates per each sample were tested. The expression of all genes was normalized to the housekeeping gene GAPDH relative to the expression level. The sequences of all primers are listed in **Table 1**.

Protein Assay

Brain samples were selected randomly by an investigator blinded to the treatment groups, homogenized in a Potter Elvehjem tissue grinder (Sigma, St Louis, MO, United States) using 1 ml chilled Tris buffer (20 mM Tris, pH 7.5; 0.75 M NaCl; 2 mM 2-mercaptoethanol) with 10 μl/ml protease inhibitor cocktail (lot no. P8340, Sigma, St Louis, MO, United States), and centrifuged at 23,000*g* for 45 min at 4°C. The protein concentrations of the supernatants were quantified using a bicinchoninic acid protein assay kit (BCA-1, B9643, Sigma–Aldrich, St Louis, MO, United States) with BSA as the standard. Total protein (25 μg) was loaded and separated on 10% polyacrylamide gels containing sodium dodecyl sulfate using a mini gel apparatus. Gels were electrophoresed at 120 V until the tracking dye reached the base of the gel. The fractionated proteins were transferred to polyvinylidene difluoride membranes (MSI, Westborough, MA, United States) using a semidry electrophoretic transfer cell. The non-specific binding sites were blocked by 10 ml cold blocking buffer TBST + BSA for 60 min. The membranes were incubated for 1 h with 5 ml blocking buffer containing antiserum monoclonal antibodies for SHIP1 (Santa Cruz), IRF3 (Santa Cruz), and NFκB (Abcam) and polyclonal antibodies for TOLLIP (Abcam) and β-actin (Sigma) in a 1:1000 dilution. Following 1 h of incubation, the blots were washed three times (5 min each) with 10 ml cold TBST. Blots were then incubated with horseradish-peroxidase-conjugated anti-mouse (Abcam) or antibody-rabbit (Abcam) secondary antibodies for 30 min in a 1:2000 dilution. After rinsing with cold TBST (three times, 5 min each), the blots were exposed to an enhanced chemiluminescent substrate (ChemiGlow; Alpha Innotech, San Leandro, CA, United States). Visualization of bands was performed using a chemiluminescent imaging system (FluorChem 5500; Alpha Innotech) followed by quantification of the band summation density by the ImageJ 1.50b software. Sizes of the immunodetected proteins were confirmed by molecular weight markers (Precision Plus Protein, Bio-Rad, Hercules, CA, United States). All solutions were made with Milli-Q water (Millipore, Bedford, MA, United States).

TABLE 1 | List of primer sequences used for RT-PCR analysis.

Primer	Forward	Reverse
GAPDH	GGGTGCCCAGTACAGTAGGA	ATTCCCAACCAACAGTGAGC
TLR4	ATGCCAGTGCTTGTGTGGTA	ACCATCCACCTATCCATCCA
SHIP1	AAGCCCGAGATGTTTGAGAA	CATGATGCTGGGTGAAGAGA
TOLLIP	AGGATGGAAGGAAGGAAGGA	CAAGTTGCCAAGCAATCTCA
NFκB	TCTCCCACACAGTGACAGGA	TCTCGCTGTGTGTGTTCCTC
IRF3	CCTGTATGTCAAGGGGCAAA	TGGAAAACTGTGGGGTAGGA
TNF-α	TGACCCCCATTACTCTGACC	TTCAGCGTCTCGTGTGTTTC
IL1-β	TGTTTGAGCAGCAAGGACAC	ACTAGGCGTACATGGCAACC
IFN-β	CAGCTACAGGACGGACTTCA	AGTCTCATTCCACCCAGTGC
IL10	CTTCCTTCTGCCTGTGAACC	TGCGTGTGTAGGCAGTCTTC
NR_2B	TCCTTTGCCAACAAGTCCTC	TGAAGCAAGCACTGGTCATC
$GABA_A$	CACATGGAGGAAGGGGACTA	GAGGTCCTCCACACTTCTGC

Statistical Analysis

All data were analyzed using the Student's *t*-test and one-way ANOVA procedure of GraphPad Prism and means were separated using Tukey *post hoc* test. In all analyses, $\rho < 0.05$ was considered statistically significant. All data were presented as mean ± SEM.

RESULTS

Effects of LPS Preconditioning on Behavioral Assessments

Animals in the ECS group (except control groups) received a series of three ECSs delivered via ear clip electrodes using a pulse generator in a day. Animals were behaviorally observed to ensure that tonic–clonic seizures occurred. Data analysis of behavioral assessments was done only on the vehicle groups

since there were no tonic–clonic seizures recorded for the control animals in this model. Our observations indicated significant reduction in the duration of seizures induced by ECS in single and chronic preconditioned groups compared to the vehicle animals (**Figure 2**). In our study, all behavioral changes including duration of tonic–clonic seizures, latency, and mortality induced by ECS were recorded. As all animals in the ECS model were evoked immediately after receiving shock, the seizure latency was not observed in this model. (The latency was defined as the time of onset of the first tonic–clonic seizures in ECS-induced animals.) Although, during our behavioral observation, administration of ECS exhibited high scores of tonic–clonic seizures, there was no mortality in the ECS-treated groups.

The Effect of LPS Preconditioning on the Cell Density in the Regions of the Hippocampus

We analyzed the severity of cell death in the regions of the hippocampus. The data presented in **Figures 3–5** demonstrate a remarkable decrease in cell number in the CA1, CA3, and DG in the vehicle groups compared with the control groups. The damaged neurons were characterized by nuclear fragments and shrunken cell bodies. In this part of the study, the cell percentage was expressed as mean% ± SEM of three animals (n = 3). However, animals pretreated with LPS demonstrated resistance to neurodegeneration and attenuation of neuronal loss.

Our results showed a significant increase in the percentage of cell survival in the CA1 region in both single and chronic preconditioned groups compared with the vehicle groups (**Figure 3**). Moreover, in the CA3 region, chronic LPS PC significantly reduced the neuronal degeneration compared with the vehicle groups as reflected by the significantly higher percentage of cell survival. In contrast, in the single LPS PC group, no significant change in the percentage of cell survival was observed (**Figure 4**). In the DG region, single and chronic LPS PC significantly reduced the neuronal damage compared with the vehicle groups with higher percentage of cell survival (**Figure 5**).

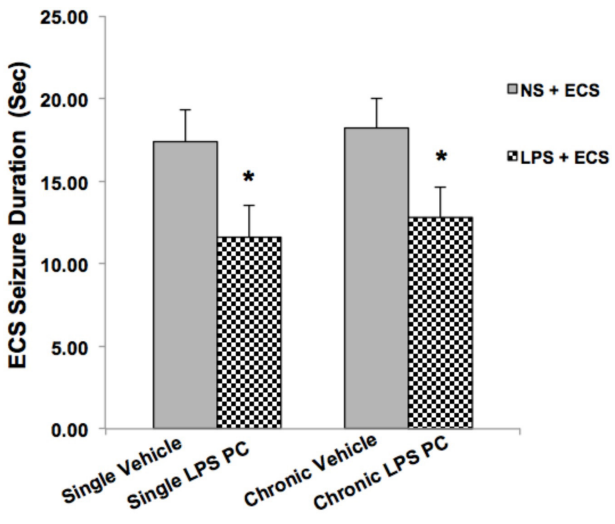

FIGURE 2 | Effect of preconditioning by single low dose and chronic ultra-low dose of LPS on seizure duration induced by ECS administration in a rat model. Asterisk (*) denotes a significant difference between the preconditioned groups and vehicle groups. Statistical analysis was done according to Student's t-test with significance levels of $^*\rho < 0.05$ vs. the vehicle groups. Data are shown as mean ± SEM of 12 animals (n = 12). ECS, electroconvulsive shock; NS, normal saline; LPS, lipopolysaccharide; PC, preconditioning.

Effect of LPS Preconditioning on TLR4 Signaling Pathway

To elucidate possible mechanisms of neuroprotection by low dose and ultra-low doses of LPS PC, we started by isolating RNA from the hippocampus of LPS-pretreated, vehicle, and control rats. Then, to examine the influence of LPS PC on specific genes expression, which may affect neuroprotection, we applied RT-PCR. Here, all results were normalized using the house-keeping gene GAPDH.

As shown in **Figure 6A**, we demonstrated that single and chronic PC showed significantly higher TLR4 gene expression in pretreated rats compared with the vehicle ones. Moreover, we also observed that PC with LPS resulted in a significant up-regulation in expression of SHIP1 (**Figure 6B**) and TOLLIP (**Figure 6C**) in single and chronic LPS-preconditioned groups compared with the vehicle animals. Following that, to determine the effect of LPS PC on inflammatory mediator production, NFκB expression level was also evaluated in preconditioned and vehicle groups. NFκB expression in the brain was elevated markedly in the vehicle groups; however, our study revealed that NFκB was significantly down-regulated in single and chronic preconditioned rats compared with the vehicle groups (**Figure 6D**). In addition, to determine the function of reprogrammed response after LPS PC, we compared the IRF3 gene expression level between preconditioned and vehicle groups. Our study demonstrated that IRF3 expression was significantly higher in the hippocampus of single and chronic LPS-preconditioned rats when compared to the vehicle animals (**Figure 6E**). Activation of IRF3 is associated with anti-inflammatory/type I IFN responses to enhance tolerance.

In order to confirm the results obtained from real time PCR, we isolated total protein from hippocampal tissue of all animals including control, vehicle, and LPS-preconditioned rats and analyzed the protein expression of five biomarkers including TLR4, SHIP1, TOLLIP, NFκB, and IRF3 (**Figure 7A**). In this study, all results were normalized by β-actin as the reference protein. Western blot analysis revealed significant up-regulation in total protein levels of TLR4 in single and chronic LPS preconditioned rats when compared with vehicle animals (**Figure 7B**). To confirm whether LPS PC had induced new gene regulation in response to epileptic injury, the protein expression level of NFκB inhibitors was determined. The results from our experiment showed that the protein levels of SHIP1 and TOLLIP were up-regulated significantly in single and chronic LPS-preconditioned rats when compared with the vehicle groups (**Figures 7C,D**). In addition, PC with a single dose of LPS

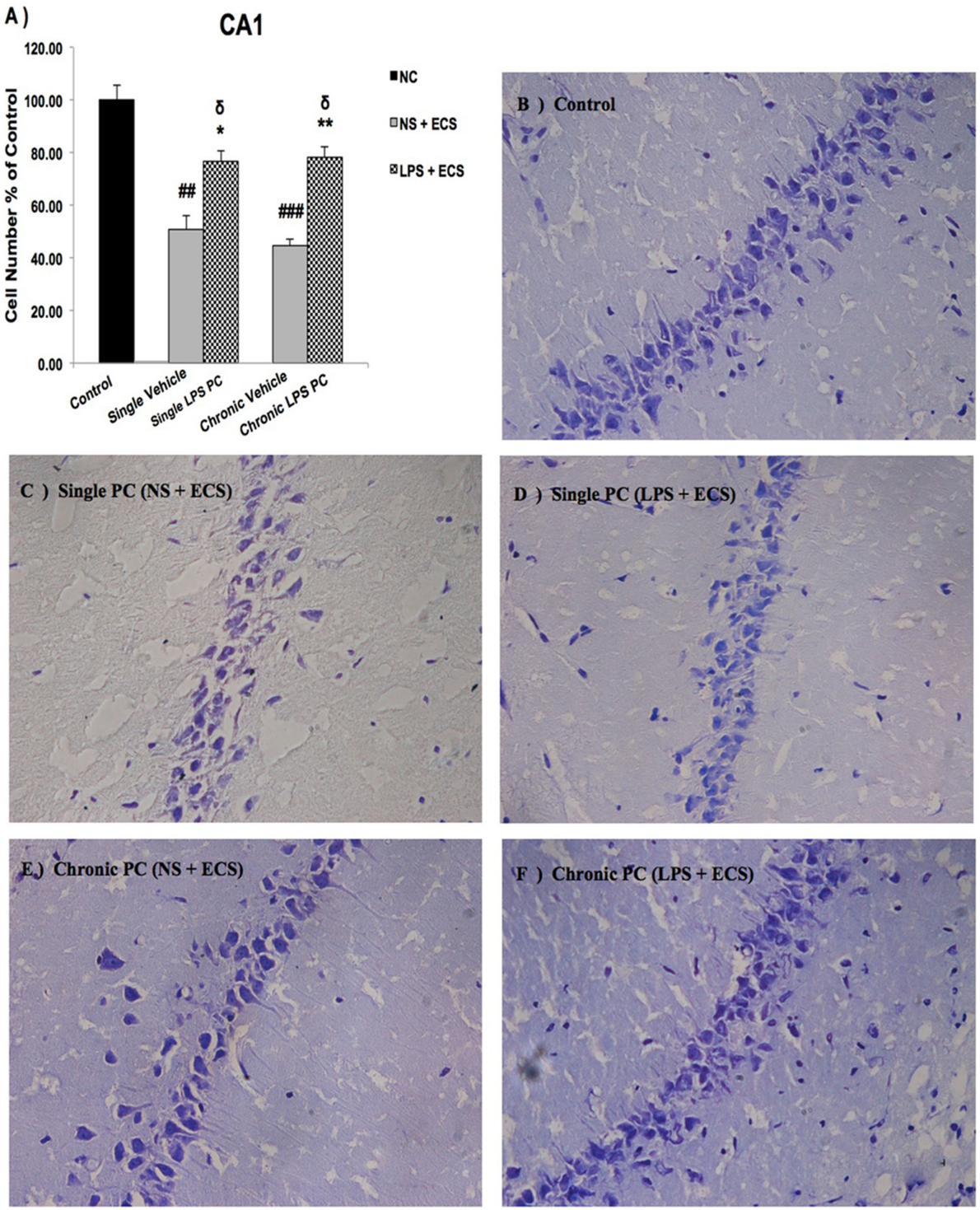

FIGURE 3 | Photomicrographs of Nissl-stained CA1 sub-region of rat brain hippocampus in the ECS model of epilepsy. **(A)** Effect of single low dose and chronic ultra-low dose of LPS preconditioning on the number of CA1 cells, **(B)** control, **(C)** vehicle (single NS), **(D)** single LPS preconditioned groups, **(E)** vehicle (chronic NS), and **(F)** chronic LPS preconditioned groups in the ECS model. Scale bars are equal to 50 μm. Asterisk (*) denotes a significant difference between the preconditioned groups and vehicle groups. Delta (δ) denotes a significant difference between the preconditioned groups and control groups. Hash (#) denotes a significant difference between the vehicle groups and control groups. Statistical analysis has been done according to the one-way ANOVA with significance levels of $^{*}\rho < 0.05$ and $^{**}\rho < 0.01$; $^{\delta}\rho < 0.05$; $^{\#\#}\rho < 0.01$, and $^{\#\#\#}\rho < 0.001$. Data are shown as mean% ± SEM of three animals ($n = 3$), which expressed as the cell number percentage with respect to the control. ECS, electroconvulsive shock; NS, normal saline; LPS, lipopolysaccharide; PC, preconditioning; NC, no current was passed through the electrodes.

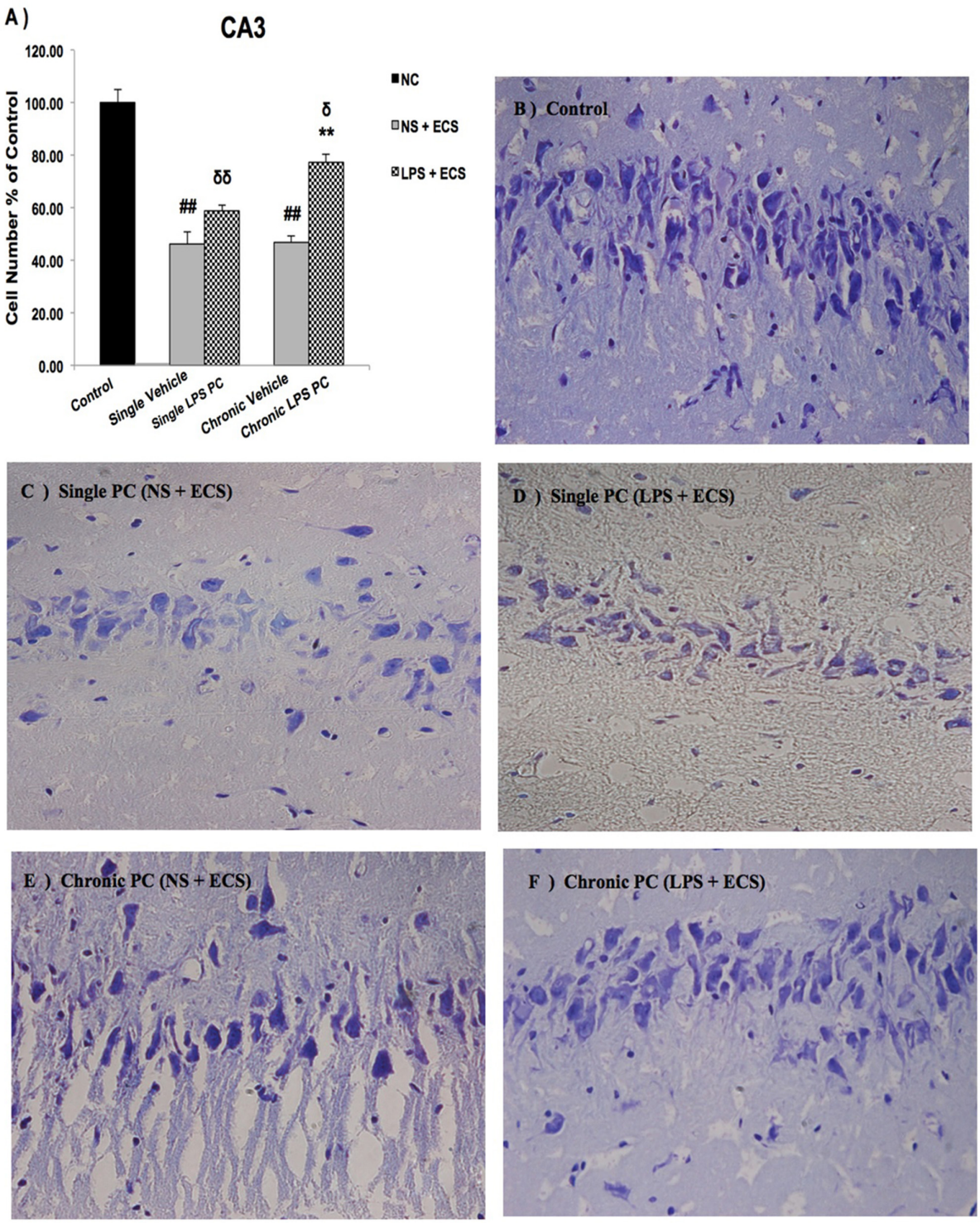

FIGURE 4 | Photomicrographs of Nissl-stained CA3 sub-region of rat brain hippocampus in the ECS model of epilepsy (n = 3). **(A)** Effect of single low dose and chronic ultra-low dose of LPS preconditioning on the number of CA3 cells, **(B)** control, **(C)** vehicle (single NS), **(D)** single LPS preconditioned groups, **(E)** vehicle (chronic NS), and **(F)** chronic LPS preconditioned groups in the ECS model. Scale bars are equal to 50 μm. Asterisk (*) denotes a significant difference between the preconditioned groups and vehicle groups. Delta (δ) denotes a significant difference between the preconditioned groups and control groups. Hash (#) denotes a significant difference between the vehicle groups and control groups. Statistical analysis has been done according to the one-way ANOVA with significance levels of $^{**}\rho < 0.01$; $^{\delta}\rho < 0.05$, $^{\delta\delta}\rho < 0.01$; and $^{\#\#}\rho < 0.01$. Data are shown as mean% ± SEM of three animals (n = 3), which expressed as the cell number percentage with respect to the control. ECS, electroconvulsive shock; NS, normal saline; LPS, lipopolysaccharide; PC, preconditioning; NC, no current was passed through the electrodes.

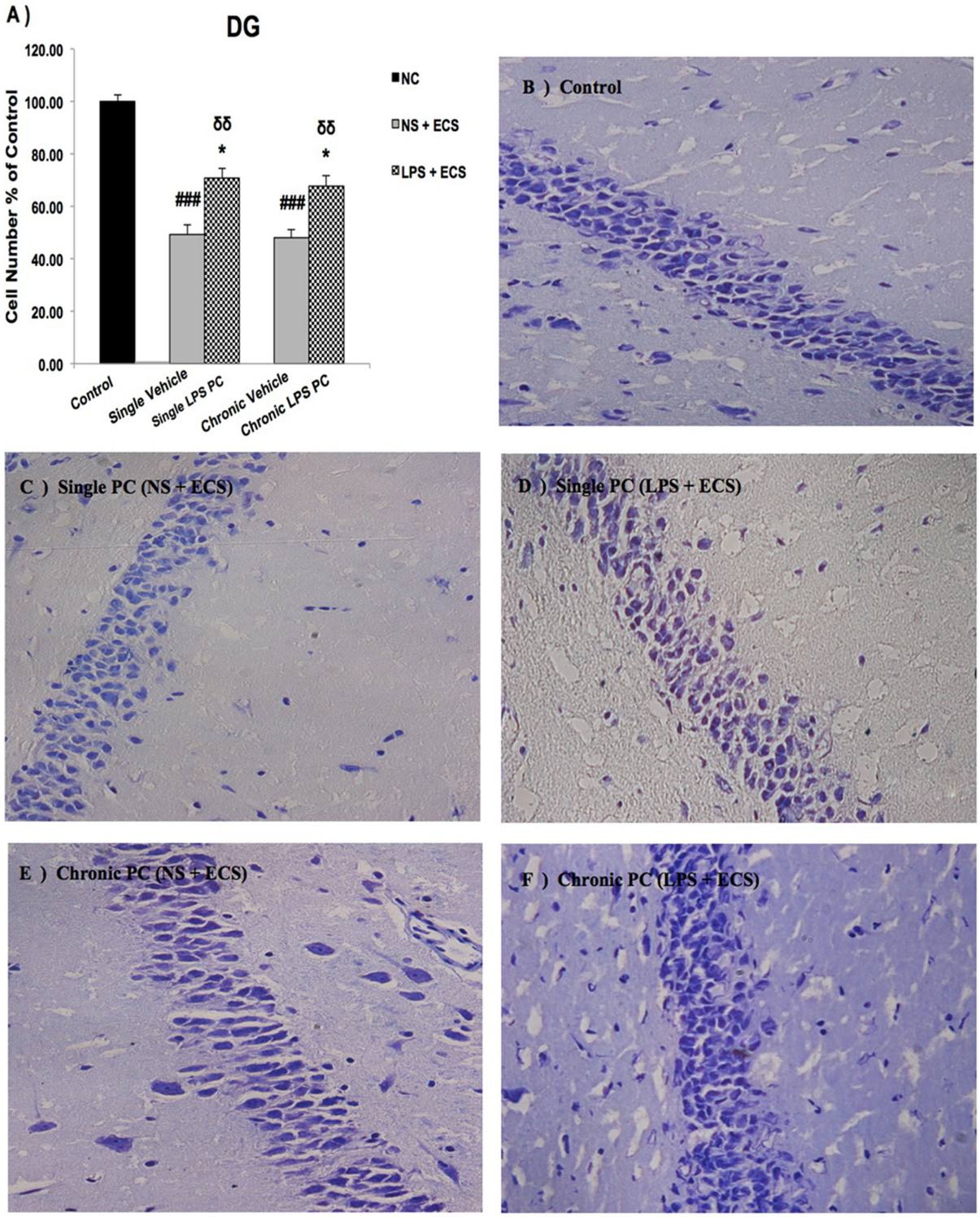

FIGURE 5 | Photomicrographs of Nissl-stained DG sub-region of rat brain hippocampus in the ECS model of epilepsy (n = 3). **(A)** Effect of single low dose and chronic ultra-low dose of LPS preconditioning on the number of DG cells, **(B)** control, **(C)** vehicle (single NS), **(D)** single LPS preconditioned groups, **(E)** vehicle (chronic NS), and **(F)** chronic LPS preconditioned groups in the ECS model. Scale bars are equal to 50 μm. Asterisk (*) denotes a significant difference between the preconditioned groups and vehicle groups. Delta (δ) denotes a significant difference between the preconditioned groups and control groups. Hash (#) denotes a significant difference between the vehicle groups and control groups. Statistical analysis has been done according to the one-way ANOVA with significance levels of $^{*}\rho < 0.05$; $^{\delta\delta}\rho < 0.01$; and $^{\#\#\#}\rho < 0.001$. Data are shown as mean% ± SEM of three animals (n = 3), which expressed as the cell number percentage with respect to the control. ECS, electroconvulsive shock; NS, normal saline; LPS, lipopolysaccharide; PC, preconditioning; NC, no current was passed through the electrodes.

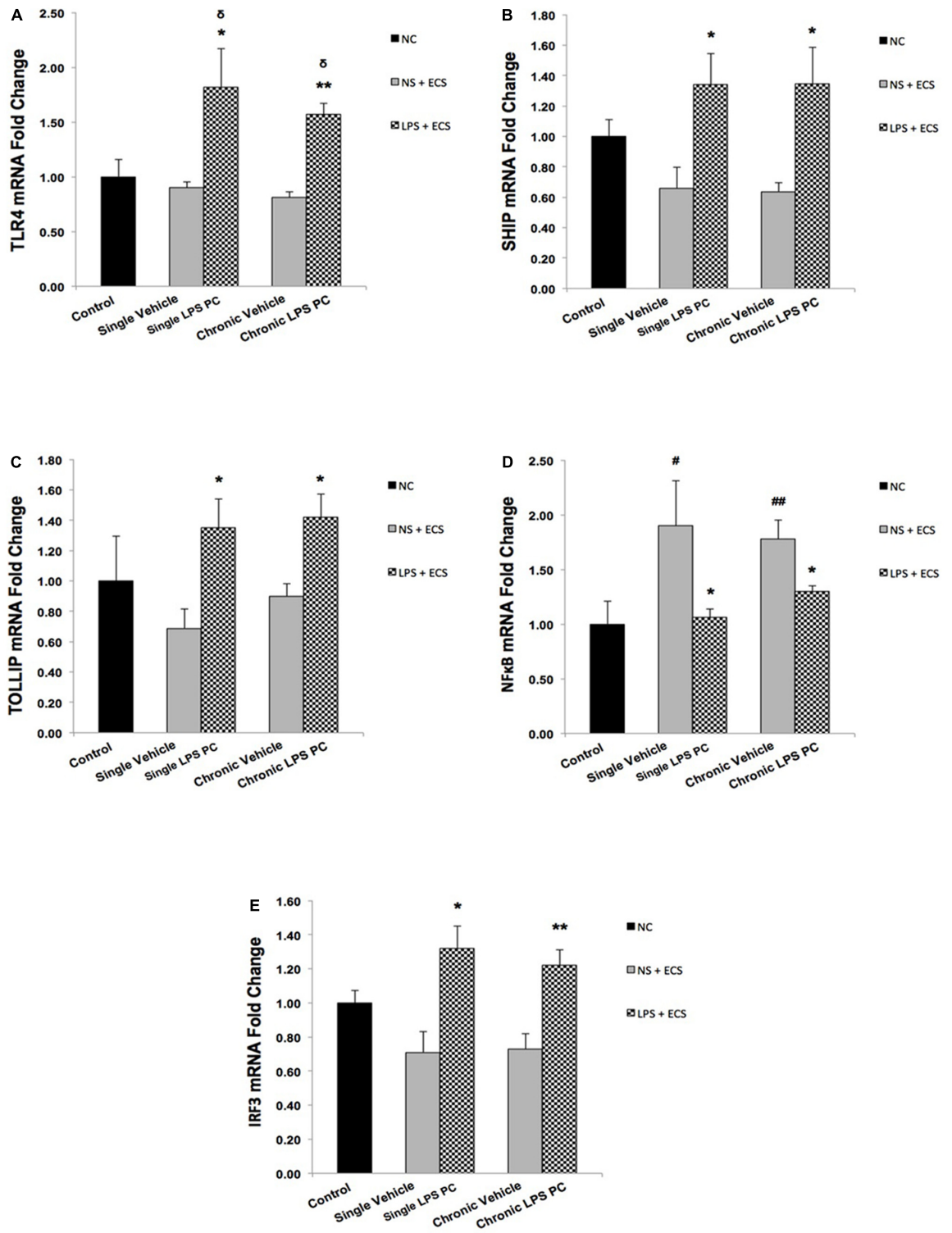

FIGURE 6 | Expression level of genes related to TLR4 signaling pathway in the hippocampus region of rat brain in the ECS model of epilepsy. The graphs above display the effect of PC by single low dose and chronic ultra-low dose of LPS on the gene expression level of **(A)** TLR4, **(B)** SHIP1, **(C)** TOLLIP, **(D)** NFκB, and **(E)** IRF3 in hippocampus in control, single preconditioned, and chronic preconditioned groups. Asterisk (*) denotes a significant difference between the preconditioned groups and vehicle groups. Delta (δ) denotes a significant difference between the preconditioned groups and control groups. Hash (#) denotes a significant difference between the vehicle groups and control groups. Statistical analysis has been done according to the one-way ANOVA with significance levels of $^{*}\rho < 0.05$; $^{**}\rho < 0.01$; $^{\delta}\rho < 0.05$; $^{\#}\rho < 0.05$; and $^{\#\#}\rho < 0.01$. Data are shown as mean ± SEM of three animals ($n = 3$), which expressed as fold change relative to control group. ECS, electroconvulsive shock; NS, normal saline; LPS, lipopolysaccharide; PC, preconditioning; NC, no current was passed through the electrodes.

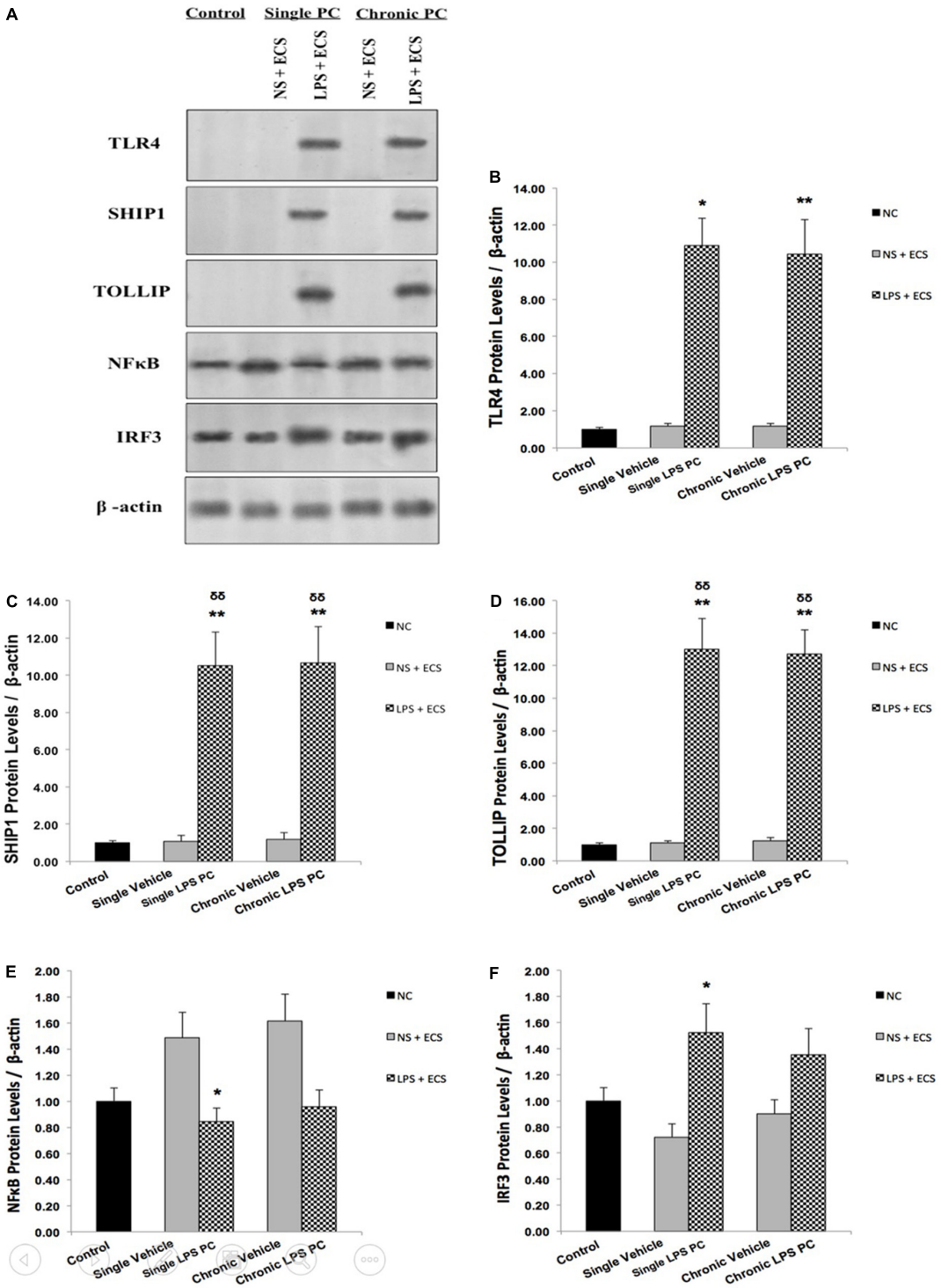

FIGURE 7 | The level of protein expression in the hippocampus of rat brain in ECS model of Epilepsy. **(A)** Representative western blots in the ECS model of epilepsy showing protein expression of TLR4, SHIP1, TOLLIP, NFκB, and IRF3 in the hippocampus in control, single and chronic LPS-preconditioned, and vehicle groups. The graphs above display the effect of preconditioning by single low dose and chronic ultra-low dose of LPS on the protein expression level of **(B)** TLR4, **(C)** SHIP1, **(D)** TOLLIP, **(E)** NFκB, and **(F)** IRF3 in the hippocampus in control, single and chronic LPS-preconditioned, and vehicle groups. Asterisk (*) denotes a significant difference between the preconditioned groups and vehicle groups. Delta (δ) denotes a significant difference between the preconditioned groups and control groups. Statistical analysis was done according to the one-way ANOVA with significance levels of $^{*}\rho < 0.05$, $^{**}\rho < 0.01$; and $^{\delta\delta}\rho < 0.01$. Data are shown as mean ± SEM of three ($n = 3$), which expressed as fold change relative to control group. ECS, electroconvulsive shock; NS, normal saline; LPS, lipopolysaccharide; PC, preconditioning; NC, no current was passed through the electrodes.

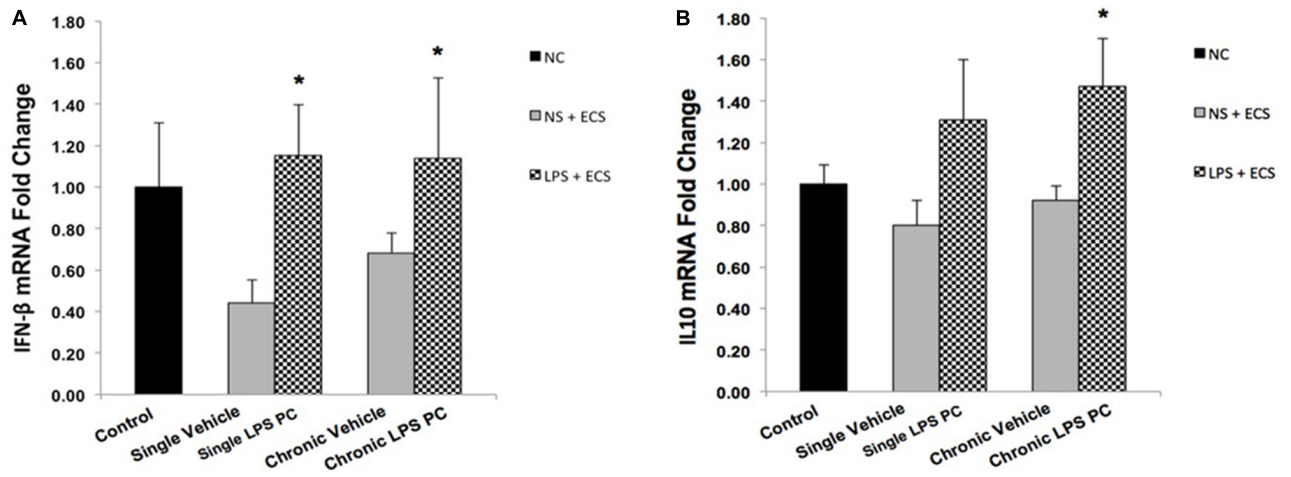

FIGURE 8 | Gene expression level of IFN-β and IL10 in the hippocampus region of rat brain in the ECS model of epilepsy. The graphs above display the effect of preconditioning by single low dose and chronic ultra-low dose of LPS on the gene expression level of **(A)** IFN-β and **(B)** IL10 in hippocampus in control, single preconditioned, and chronic preconditioned groups. Asterisk (*) denotes a significant difference between the preconditioned groups and vehicle groups. Statistical analysis has been done according to the one-way ANOVA with significance levels of $^{*}\rho < 0.05$. Data are shown as mean ± SEM of three animals ($n = 3$), which expressed as fold change relative to control group. ECS, electroconvulsive shock; NS, normal saline; LPS, lipopolysaccharide; PC, preconditioning; NC, no current was passed through the electrodes.

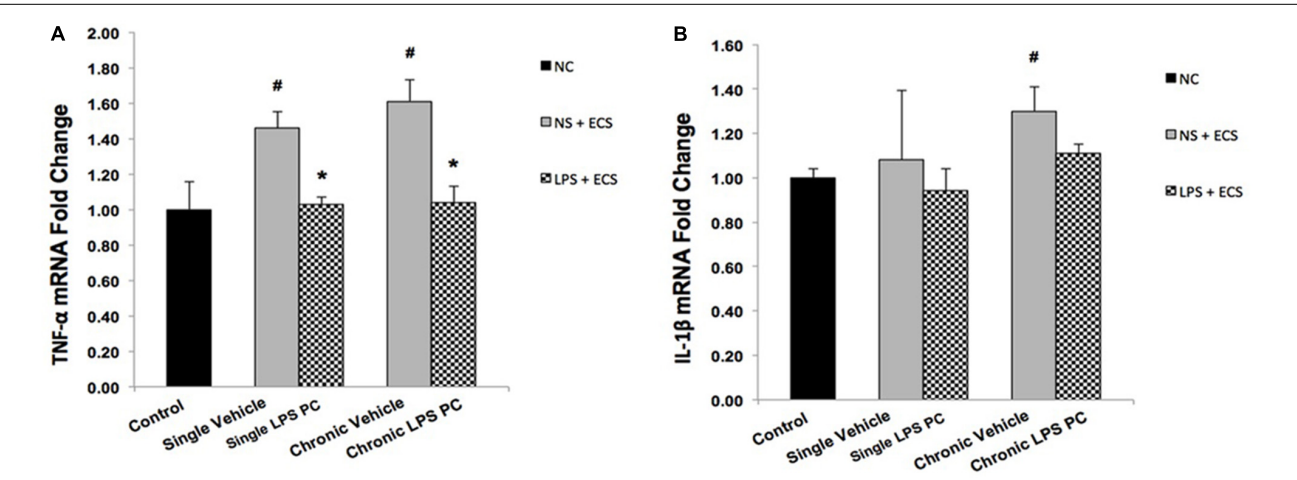

FIGURE 9 | Gene expression level of TNF-α and IL-1β in the hippocampus region of rat brain in the ECS model of epilepsy. The graphs above display the effect of preconditioning by single low dose and chronic ultra-low dose of LPS on the gene expression level of **(A)** TNF-α and **(B)** IL-1β in hippocampus in control, single preconditioned, and chronic preconditioned groups. Asterisk (*) denotes a significant difference between the preconditioned groups and vehicle groups. Hash (#) denotes a significant difference between the vehicle groups and control groups. Statistical analysis was done according to the one-way ANOVA with significance levels of $^{*}\rho < 0.05$ and $^{\#}\rho < 0.05$. Data are shown as mean ± SEM of three animals ($n = 3$), which expressed as fold change relative to control group. ECS, electroconvulsive shock; NS, normal saline; LPS, lipopolysaccharide; PC, preconditioning; NC, no current was passed through the electrodes.

resulted in a significant reduction in NFκB protein expression in response to the ECS stimulus as compared to the vehicle groups, while, no significant difference in NFκB protein expression was observed between chronic LPS-preconditioned and vehicle groups (**Figure 7E**). To further explore whether LPS-induced protection is expressed through the TRIF pathway, the protein expression level of IRF3, a critical effector of tolerance, was also tested. Our study showed that single preconditioned rats displayed significantly higher protein expression of IRF3 when compared to the vehicle rats. However, there was no significant change in IRF3 protein expression in chronic preconditioned rats vs. vehicle animals (**Figure 7F**). Original western blots presented in Supplementary Figure 1.

Effect of LPS Preconditioning on Anti-inflammatory Mediators

To determine the protective effect of TLR4 activation by LPS PC against epileptic injury through the TRIF signaling pathway, we examined the expression level of anti-inflammatory

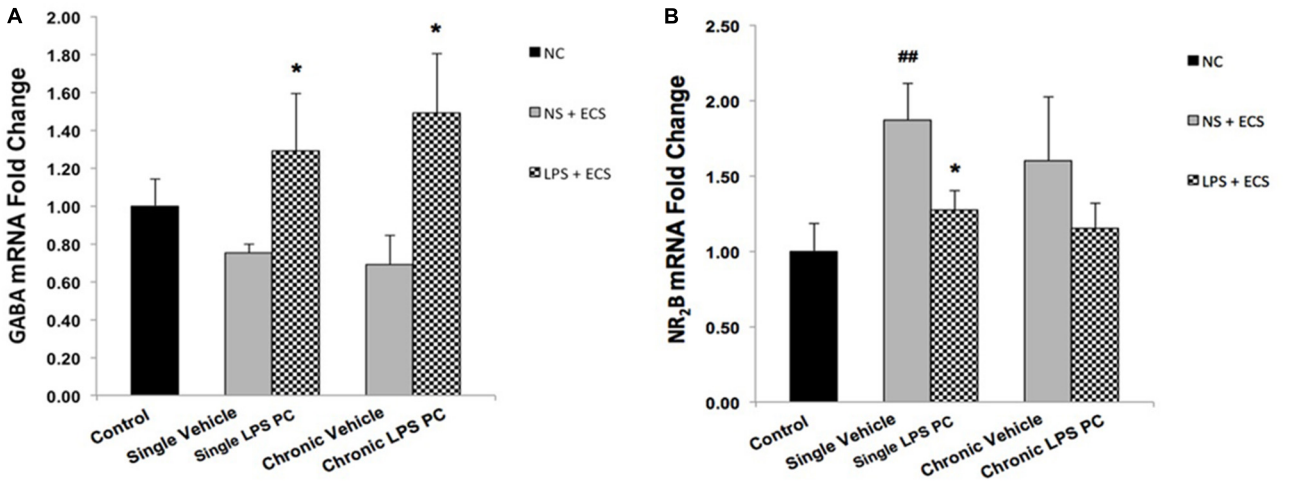

FIGURE 10 | Gene expression level of $GABA_A$ and NR_2B in the hippocampus region of rat brain in the ECS model of epilepsy. The graphs above display the effect of preconditioning by single low dose and chronic ultra-low dose of LPS on the gene expression level of **(A)** $GABA_A$ and **(B)** NR_2B in hippocampus in control, single preconditioned, and chronic preconditioned groups. Asterisk (*) denotes a significant difference between the preconditioned groups and vehicle groups. Hash (#) denotes a significant difference between the vehicle groups and control groups. Statistical analysis was done according to the one-way ANOVA with significance levels of $^{*}\rho < 0.05$ and $^{\#\#}\rho < 0.01$. Data are shown as mean ± SEM of three animals ($n = 3$), which expressed as fold change relative to control group. ECS, electroconvulsive shock; NS, normal saline; LPS, lipopolysaccharide; PC, preconditioning; NC, no current was passed through the electrodes.

mediators including IFN-β and IL10 in rat hippocampus. Rats preconditioned with single and chronic LPS stimulation resulted in a significant induction of RNA expression of IFN-β compared to the vehicle group (**Figure 8A**). Additionally, our study showed no significant change in the expression level of IL10 following single LPS PC vs. vehicle groups. However, significant increase in IL10 gene expression was observed in chronic LPS preconditioned rats (**Figure 8B**).

Effect of LPS Preconditioning on Inflammatory Mediators

Activity of NFκB is associated with inflammation in the brain, in response to damage by induction of pro-inflammatory mediators such as TNF-α and IL-1β. In this study, the contribution of LPS PC to alter expression levels of these pro-inflammatory markers after seizure induction has been demonstrated. Expression level of TNF-α in single and chronic LPS-pretreated rats were significantly reduced compared with vehicle groups (**Figure 9A**). As the results in **Figure 9B** revealed, no significant changes were observed in IL-1β gene expression level in single and chronic preconditioned rats compared with vehicle animals.

Effect of LPS Preconditioning on Expression of Genes Related to Receptor Function Following Seizures

To evaluate whether PC with LPS induced alterations in receptor function that are related to epilepsy, we examined gene expression levels of $GABA_A$ and NR_2B in preconditioned and vehicle groups. We found that preconditioned rats displayed a significant elevated expression in $GABA_A$ compared to the vehicle animals (**Figure 10A**). In addition, single preconditioned LPS pretreated animals showed significantly lower levels of NR_2B, compared to the vehicle group. However, the chronic LPS pretreated animals displayed no significant change in the level of NR_2B in LPS pretreated animals compared to the vehicle group (**Figure 10B**).

DISCUSSION

Neuroinflammation and changes in levels of related pro-inflammatory cytokines lead to varying degrees of long-term alterations in the brain. Accumulating evidence strongly supports the relevance of neuroinflammation in the pathophysiology of epilepsy (Vezzani et al., 2013, 2016). Thus, novel therapeutic approaches should be investigated to effectively prevent or attenuate neuroinflammation related to epileptic seizures. In the current study, we have demonstrated that single and chronic brain LPS PC has important implications in terms of neuroprotection against epileptic injuries. Since previous studies indicated that hippocampal damage could produce behavioral impairments in animal epilepsy models (Dmowska et al., 2010), we performed Nissl staining following cell counting on hippocampal tissues collected from all animals. Histological analysis demonstrated that repeated ECS-induced tonic–clonic seizures were capable of producing cell loss in the hippocampal regions and these pathologic changes in the brain could cause behavioral impairments in the vehicle rats compared to preconditioned animals (**Figures 3–5**). These results lend support to the notion that behavioral changes induced by the administration of repeated ECS can be ascribed to cell loss in the hippocampal regions. In contrast, with reference to the results obtained from preconditioned animals, a sub-lethal dose of LPS was capable of inducing tolerance to alleviate cell

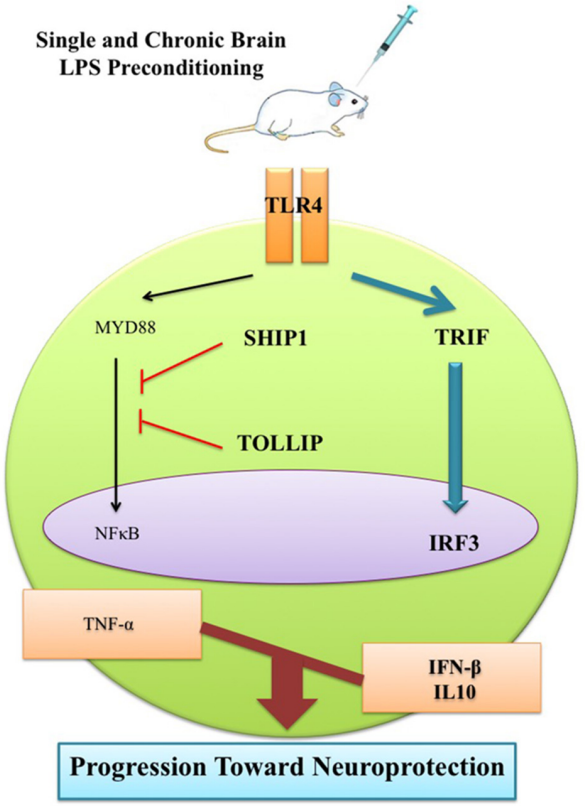

FIGURE 11 | Schematic representation of gene expression pattern related to TLR4 signaling pathway following epileptic seizure. Low dose of LPS reprograms TLR4 signaling in response to subsequent brain injury. This reprogrammed response that is reminiscent of neuroprotection comes with reduction of NFκB activity to reduce pro-inflammatory mediators, up-regulation of IRF3 activity, and enhancement in production of anti-inflammatory/type I IFN associated genes in the LPS-preconditioned animals following brain damage.

death in the hippocampal regions and consequently improving behavioral impairments related to seizures. In both single and chronic LPS-pretreated animals, a significant reduction in seizure duration was observed when compared to the vehicle groups (**Figure 2**). This difference in seizure duration and also the attenuation of cell loss due to administration of ECS in the hippocampus could be attributed to the neuroprotective effect of LPS PC. These findings are in agreement with a prior study which reported that LPS PC could be protective and resulted in improvement of behavioral impairments as well as cell death (Dmowska et al., 2010). Thus, it is possible to conclude that single and chronic LPS PC may activate an endogenous protective mechanism that could influence animal behavior and histopathological changes.

As previous evidence suggested that inflammation could play a central role in epileptic disorders (Kosonowska et al., 2015; Vezzani et al., 2016), our findings further revealed that the inflammatory response following tonic–clonic seizures could be modulated by LPS PC. As such, we proceeded with molecular investigations to determine the effective role of LPS-induced tolerance wherein TLR4 signaling pathway reprograms in response to epileptic injury and directs it toward neuroprotection. Administration of low doses of LPS induces a mild inflammation in the brain that alters the response to subsequent damage and induces a new pattern of gene regulation in preconditioned animals. This alteration in response to injury is manifested by the reduction of inflammatory cascades and activation of anti-inflammatory mediators. To explore the reprogramming response-induced protection by brain LPS PC, we examined the expression levels of gene sets in response to epileptic injury. With regard to TLR4 signaling cascade as one the notable pathways related to LPS PC, our study found a significant increase in the expression of multiple markers including TLR4 (**Figure 6A**), SHIP1 (**Figure 6B**), TOLLIP (**Figure 6C**), and IRF3 (**Figure 6E**) in preconditioned animals compared with the vehicle groups; however, NFκB expression was significantly down-regulated (**Figure 6D**). These data that were obtained from gene expression were also confirmed by results from protein expression.

Activation and reprogramming of several signaling pathways in the brain in response to sub-lethal doses of LPS had been previously reported in patients with stroke (Vartanian et al., 2011; Wang et al., 2015). Activation of a set of genes that are evident in the reprogrammed response to damage such as SHIP1 and TOLLIP could negatively regulate the induction of pro-inflammatory mediators via inhibition of NFκB (Wang et al., 2015). Activation of NFκB participates in a chain of events that ultimately leads to the expression of a large number of pro-inflammatory cytokines such as TNF-α and IL-1β (Aalbers et al., 2014; Kołosowska et al., 2014). As demonstrated in our study, ECS-induced seizures in rat brain elicited over-expression of pro-inflammatory cytokines, while LPS PC mediated a significant reduction of TNF-α (**Figure 9A**). To support this, Rosenzweig et al. (2007) also reported that pretreatment with low doses of LPS diminished the production of pro-inflammatory mediators such as TNF-α and provided neuroprotection against cerebral ischaemic injury.

The mechanisms that underlie the neuroprotection afforded by LPS PC may also be involved in the augmentation of anti-inflammatory markers (Vartanian et al., 2011). In this regard, activation of IRF3 is required for LPS-induced neuroprotection through the production of anti-inflammatory mediators such as IFN-β (Marsh et al., 2009; Wang et al., 2015). In the current study, the RNA level of IFN-β and IL10 was markedly enhanced in response to seizures in preconditioned rats compared to the vehicle groups (**Figure 8**). Enhancement in gene expression levels of IFN-β in the LPS-preconditioned animals mirrors the cascade of protective processes that reduce inflammation in the brain (Marsh et al., 2009; Schaafsma et al., 2015).

This observation can be ascribed to the LPS-induced window of protection through the reprogramming of the response to epileptic injury via activation of TRIF-mediated signaling at the time of PC. Therefore, this supports the notion that single low dose and chronic ultra-low doses of LPS could potentially redirect the TLR4 signaling pathway and alter the endogenous response to injury resulting in a neuroprotective state via enhancement of anti-inflammatory mediators, reduction of pro-inflammatory

cytokines, and finally modulation of inflammation in the brain (**Figure 11**). This shift from pro-inflammatory to anti-inflammatory response is considered a marker of reprogramming response in tolerance (Stevens et al., 2011; Ding et al., 2015).

Additionally, with respect to identifying the role of neurotransmitters in seizure mechanisms in the ECS model of epilepsy, the effect of LPS PC on gene regulation of two important receptors was investigated in all animal groups. Our study showed that $GABA_A$ was significantly increased in LPS-preconditioned groups (**Figure 10A**), whereas NR_2B gene expression was highly reduced in single preconditioned animals compared to the vehicle animals (**Figure 10B**). These results are in agreement with a previous study that demonstrated the capacity of PC to induce a neuroprotective response by decreasing glutamate release in neuron culture ischemia models (Tauskela et al., 2012). Therefore, our data and current medical literature support the role of LPS PC in neuroprotection. The role of neurotransmitters such as $GABA_A$ and NR_2B in the "neuroprotection cascade" provides insights to potential targets for new therapeutic approaches for epileptic seizures.

CONCLUSION

Despite different administration of LPS doses (single and chronic), a similar reduction in neuroinflammation was observed. However, it is not clearly understood that there are some possibilities to explain this observation. These similarities that were observed between single and chronic administration of LPS could be due to the fact that both treatments may be reached at a ceiling effect for their dose. It is also possible that both treatments have the same efficacy at the doses used. Another possible reason for this may be due to that neuronal cells in hippocampus may become tolerant to subsequent exposures of LPS applied in chronic PC. In agreement with this, Schwarz et al. (2002) already reported a similar finding between acute and chronic treatment. In conclusion, the present findings have shed some light on the possible neuroprotective mechanisms of single low dose and chronic ultra-low doses of brain LPS PC observed in the ECS model of epilepsy. This tolerance was accompanied by a change in gene expression suggesting stimulation of a fundamental genomic reprogramming in the brain that confers survival.

AUTHOR CONTRIBUTIONS

AA designed the research. MG and EA performed behavioral, histological, and gene expression assessments and analyzed the data. AF and BK performed the protein assay. NI, AR, ZM, and AA supervised the project. All authors contributed to writing and editing the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2018.00416/full#supplementary-material

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ORIGINAL RESEARCH
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Orthosiphon stamineus Leaf Extract Affects TNF-α and Seizures in a Zebrafish Model

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Epileptic seizures result from abnormal brain activity and can affect motor, autonomic and sensory function; as well as, memory, cognition, behavior, or emotional state. Effective anti-epileptic drugs (AEDs) are available but have tolerability issues due to their side effects. The Malaysian herb *Orthosiphon stamineus*, is a traditional epilepsy remedy and possesses anti-inflammatory, anti-oxidant and free-radical scavenging abilities, all of which are known to protect against seizures. This experiment thus aimed to explore if an ethanolic leaf extract of *O. stamineus* has the potential to be a novel symptomatic treatment for epileptic seizures in a zebrafish model; and the effects of the extract on the expression levels of several genes in the zebrafish brain which are associated with seizures. The results of this study indicate that *O. stamineus* has the potential to be a novel symptomatic treatment for epileptic seizures as it is pharmacologically active against seizures in a zebrafish model. The anti-convulsive effect of this extract is also comparable to that of diazepam at higher doses and can surpass diazepam in certain cases. Treatment with the extract also counteracts the upregulation of NF-κB, NPY and TNF-α as a result of a Pentylenetetrazol (PTZ) treated seizure. The anti-convulsive action for this extract could be at least partially due to its downregulation of TNF-α. Future work could include the discovery of the active anti-convulsive compound, as well as determine if the extract does not cause cognitive impairment in zebrafish.

Keywords: epilepsy, *Orthosiphon stamineus*, zebrafish model, TNF-α, anticonvulsant activity

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INTRODUCTION

Epileptic seizures are typically described as a short-term manifestation of numerous signs and/or symptoms because of unusually superfluous or concurrent activity in the brain. In contrast, epilepsy is a collection of neurological disorders characterized by the lasting tendency to spawn epileptic seizures (Fisher et al., 2014). Epilepsy is a serious disorder of the Central Nervous System (CNS) as the global epilepsy prevalence is approximately one in 100 people according to Holland (2014). Whilst the underlying cause of epilepsy is not always clear, anti-convulsant drugs or anti-epileptic drugs (AEDs) as they are commonly known, may be used for the symptomatic treatment of epilepsy. The older generation of AEDs have side effects which range from abdominal discomfort and anorexia to psychosis and aplastic anemia; together with an array of different idiosyncratic reactions. In comparison, AEDs from the newer generation can result in side effects which range from fatigue and drowsiness to vomiting and diplopia (French and Gazzola, 2011). Whilst the efficacy of the AEDs in use today has been demonstrated, a need for the discovery of new AEDs with fewer side effects remains.

Orthosiphon stamineus is a Malaysian herb also known locally as 'misai kucing' and is widely grown in tropical regions which have high temperatures and year-round rainfall (Akowuah et al., 2004). In the Southeast Asian region, *O. stamineus* leaves are harvested and dried to make tea leaves (Gan et al., 2017). The *O. stamineus* tea leaves can then be brewed into a herbal tea and used as a traditional medicine to treat epilepsy (Hossain and Mizanur Rahman, 2015). An extract of *O. stamineus* leaves has been found to possess anti-inflammatory, (Yam et al., 2008) anti-oxidant and free-radical scavenging abilities (Yam et al., 2007). Although the exact mechanism leading to the formation of seizures is unknown, there is evidence that pro-inflammatory mediators released by the brain and peripheral immune cells play a role (Vezzani et al., 2011). There has also been an indication that oxidative stress has a role in epilepsy, given the high degree of oxidative metabolism, limited antioxidant defense and the abundance of polyunsaturated fatty acids in the brain. It is possible that these conditions increase the vulnerability of the brain to free radical damage, leading to certain types of epilepsy (Devi et al., 2008). An experiment by Yam et al. (2010) suggested that the components of *O. stamineus* leaves which are responsible for its anti-inflammatory effect in a chloroform extract are the polymethoxylated flavones sinensetin, eupatorine and 30-hydroxy-5,6,7,40-tetramethoxyflavone; which possibly function by inhibiting the nitric oxide pathway and the synthesis of prostaglandin. Akowuah et al. (2005) also found that sinensetin, eupatorine, 30-hydroxy-5,6,7,40-tetramethoxyflavone, rosmarinic acid and quercetin form the major components in an *O. stamineus* extract which possess significant free radical scavenging and antioxidant ability. Thus, the properties of *O. stamineus* combined with its traditional usage for the treatment of epilepsy makes it an encouraging candidate for the development of novel AEDs.

One of the most frequently used approaches to inducing seizures in animals are chemoconvulsants. An example of a chemoconvulsant among many the different available is pentylenetetrazol (PTZ). PTZ is believed to induce seizures primarily by binding to the γ-Aminobutyric Acid ($GABA_A$) receptor and impeding the neuroinhibitory action of GABA (Berghmans et al., 2007). Although the majority of past research concerning epilepsy has been undertaken using rodents as the animal model, zebrafish are currently becoming increasingly popular as a model for epilepsy. One possible reason for this is that dissolving the compounds to be tested directly in the zebrafish tank water becomes an option, which eliminates the necessity of performing an invasive procedure such as an injection. Despite zebrafish being fish and hence more removed from humans in an evolutionary perspective in comparison to the mammalian rodents, their genes are nevertheless still around 75% homologous to human genes (Baraban et al., 2005; Berghmans et al., 2007). Among the other aspects in which zebrafish are superior to rodents as an animal model are their longer lifespan and robust phenotypes, as they display obvious and easily quantifiable behavioral endpoints (Stewart et al., 2012). The blood brain barrier in zebrafish is also tight-junction based, and highly permeable to macromolecules, meaning that zebrafish will be extremely responsive the compounds being tested (Eliceiri et al., 2011). It is for these reasons that this experiment utilized zebrafish as an animal model of epilepsy over rodents.

Once the animal model of epilepsy and the method of inducing seizures is ascertained, a technique for assessing compounds believed to be anti-convulsive is needed. One way of doing this is to test adult zebrafish inside a tank in which they can be observed so that their seizure behavior can be scored according to a predefined scoring system. Both the top and side points of view for the observation tank can be utilized for the neurophenotypic classification of the responses which result in chemoconvulsant treated adult zebrafish, as they are very similar to those observed during a seizure. Whilst the abnormal response displayed by the zebrafish varies based on the chemoconvulsant used, the conventional endpoints which are used include rapid twitching, loss of body posture, hyperactive, spiral or circular swimming, paralysis or immobility, body contractions similar to spasms and death (Stewart et al., 2012). An induced seizure has also been shown using rodent models of epilepsy, to also result in an upregulation of specific genes at the site where the seizure was initiated. The upregulated genes are known as immediate-early genes (Morgan et al., 1987) and are comprised of genes such as the early proto-oncogene c-Fos, which also functions as a neuronal activation marker. A similar pattern in the upregulation of seizure related genes during an induced seizure is also present in zebrafish brains (Stewart et al., 2012) and may be quantitively examined to possibly serve as biomarkers of brain disorders.

Thus, whilst the efficacy of the AEDs used today for the symptomatic treatment of epilepsy is proven, there is still a necessity for the discovery of new AEDs with comparable efficacies, but with fewer side effects. Given its beneficial properties and traditional usage, *O. stamineus* leaves have the potential to be a novel treatment for epilepsy. Thus, this study aimed to determine if an ethanolic leaf extract of *O. stamineus* is pharmacologically active against seizures. This was done by observing whether pre-treating zebrafish with varying doses of the extract has any effect on the progression of PTZ-induced seizures. This experiment involved the use of three different treatment doses of the *O. stamineus* ethanolic leaf extract; with the exact concentrations decided based on a prior toxicity study using adult zebrafish. The last part of this study involved harvesting the zebrafish brains for gene expression studies to help determine the mechanism of action by which an ethanolic leaf extract of *O. stamineus* exerts its anti-convulsive effect in the zebrafish brain as the expression level of certain genes changes characteristically after a seizure (Morgan et al., 1987).

MATERIALS AND METHODS

Materials

Chemicals

The standardized *O. stamineus* ethanolic leaf extract was purchased from Natureceuticals Sdn Bhd. According to the manufacturer, the extract was a 50% ethanolic extract prepared using Digimaz technology. Pentylenetetrazol (PTZ) and the standard AED diazepam (DZP) were purchased

from Sigma–Aldrich (United States). TRIzol® reagent was purchased from Invitrogen, Carlsbad, CA, United States. For the gene expression study, QuantiTect SYBR Green dye (Qiagen, Valencia, CA, United States) was used together with the following primer sets:

BDNF: Dr_bdnf_1_SG QuantiTect Primer Assay (Cat no. QT02125326);
NF-κB: Dr_nfkb1_2_SG QuantiTect Primer Assay (Cat no. QT02498762);
NPY: Dr_npy_1_SG QuantiTect Primer Assay (Cat no. QT02205763);
c-Fos: Dr_fos_1_SG QuantiTect Primer Assay (Cat no. QT02103243);
TNF-α: Dr_tnf_1_SG QuantiTect Primer Assay (Cat no. QT02097655);
IL-1: Dr_il1rapl1a_1_SG QuantiTect Primer Assay (Cat no. QT02131850);
eef1a1b: Dr_eef1a1b_2_SG QuantiTect Primer Assay (Cat no. QT02042684)

Software and Equipment

The Smart V3.0.05 tracking software (Pan Lab, Harvard apparatus) was used for the automated tracking of zebrafish swimming patterns. The Applied Biosystems StepOnePlus™ Real-Time PCR System was used for the gene expression study.

Animals

Adult zebrafish (*Danio rerio*) 3–4 months of age and of the heterogeneous wild-type strain with a typical short-fin phenotype were purchased at the aquarium shop 'Akarium Batu Karang Laut' (Subang Jaya, Malaysia). All zebrafish were held at the Monash University Malaysia animal facility under standard husbandry conditions. The zebrafish tanks were kept at a water temperature of between 26 and 30°C, a water pH of between pH 6.8 and pH 7.1 and under a 250-lux light intensity with a cycle of 14-h of light to 10 h of darkness. The lights were automatically turned on at 8 am and automatically turned off at 10 pm via a timer. The zebrafish were fed thrice a day with TetraMin® Tropical Flakes and their diet was supplemented with live brine shrimps (*Artemia*) purchased from Bio-Marine (Aquafauna, Inc. United States). Standard zebrafish tanks with a length of 36 cm, a width of 26 cm and a height of 22 cm were used to house the zebrafish. The tanks were equipped with a water circulation system to provide constant aeration. Group housing, whereby 10–12 fish were kept per tank, was practiced with the females and males being housed separately. All animal experimentation was authorized by the Monash Animal Research Platform (MARP), Australia.

Methods

Toxicity Study

A zebrafish toxicity study was carried out on adult zebrafish to determine the exact *O. stamineus* ethanolic leaf extract concentrations to be used with each of the three treatment groups. A limit test was first performed based on a modified version of the OECD Guidelines for the Testing of Chemicals No. 203 (OECD, 1992). An observation tank was first set up and filled with 13 L of the water normally used to fill the zebrafish tanks. One zebrafish from the untreated normal control group was then placed in the tank and its behavior was recorded for 10 min. After each recording, the zebrafish was transferred into individual 1 L tanks filled with the same water. The procedure then was repeated for each of the seven zebrafish in the control group. The recording and tank transfer procedure was then repeated with the seven zebrafish of the treatment group but with the extract added to the water to make up a concentration of 100 mg/L. All 14 zebrafish were then kept for 96 h in their respective one-liter tanks. All 14 zebrafish were checked on every 15 min for the first 2 h of exposure and every half an hour thereafter for the first day. On subsequent days, the zebrafish were checked on thrice daily during the morning, afternoon and evening. Any zebrafish found to exhibit severe symptoms of pain or suffering according to our predefined monitoring sheet at any checkpoint were humanely euthanized via an overdose of benzocaine. If no zebrafish require euthanasia at the limit concentration, the extract concentration will be raised by a factor of 2–200 mg/L and the test protocol repeated. If there is at least one zebrafish requiring euthanasia at the limit concentration, the concentration will be decreased by a factor of two, to 50 mg/L and the test protocol repeated. This protocol deviates from the OECD guidelines in that it does not use mortality as the criterion to determine toxicity due to the concerns of the MARP-Australia in using death as an endpoint. The highest dose which did not require euthanasia of any zebrafish was used as the 'High' dose in the following behavioral study with the 'Medium' dose and 'Low' dose being a factor of two and four lower than the 'High' dose, respectively.

Behavioral Study

Drug treatment and groups

Three-month-old adult zebrafish with weights ranging from 0.4 to 0.8 g were selected. The zebrafish were then divided into six groups, with 10 fish per group. PTZ was dissolved in distilled water whereas DZP and the *O. stamineus* extract was dissolved in the same water used to fill the zebrafish tanks.

Group I: Vehicle Control (CP), Tank Water Only;
Group II: Negative Control (CN), PTZ (170 mg/kg) Only;
Group III: Positive Control (CP), DZP (10 mg/L) + PTZ (170 mg/kg);
Group IV: Treatment Group 1, *O. stamineus* extract (Low dose) + PTZ (170 mg/kg);
Group V: Treatment Group 2, *O. stamineus* extract (Medium dose) + PTZ (170 mg/kg);
Group VI: Treatment Group 3, *O. stamineus* extract (High dose) + PTZ (170 mg/kg)

Procedure for a zebrafish intraperitoneal injection

All intraperitoneal injections were administered into the abdominal cavity at a location posterior to the pelvic girdle, using 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 hours prior

to experiment for the purpose of 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-gage needle. The zebrafish were restrained in a water saturated sponge under benzocaine 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., 2017) and did not cause any mortality throughout the experiment.

Each zebrafish was captured individually using a fish holding net, and then transferred into an anesthesia solution (30 mg/L Benzocaine). The zebrafish was taken out once anesthetized and then weighed to calculate the dose and hence the injection volume. A soft sponge approximately 20 mm in height was saturated with water and set inside a 60 mm Petri dish. A cut between 10 and 15 mm in depth was made in the sponge to restrain and hold the fish for the intraperitoneal injection. The intraperitoneal injection was given while using a dissecting microscope by inserting the needle into the midline between the pelvic fins. An appropriate volume was then injected into the zebrafish, after taking into account the body weight of the zebrafish. After the intraperitoneal injection, the zebrafish was immediately transferred to an observation tank.

PTZ-Induced Seizure Model

The zebrafish were habituated for 30 min in 1 L treatment tanks filled with 1 L of the water normally used to fill the zebrafish tanks, before administration of PTZ. Groups I and II were habituated in tanks only containing the water normally used to fill the zebrafish tanks. Groups III to VI had either diazepam (10 mg/L) or the extract added to the tank water. After the 30-min habituation time, the zebrafish from groups II to VI were injected with PTZ (170 mg/kg, IP). Group I zebrafish did not receive any injections. PTZ injected zebrafish present diverse seizure profiles, intensities and latency in reaching the different seizure scores. PTZ-induced seizures will persist for about 10 min after the PTZ injection and gradually decrease with time. The PTZ injected adult zebrafish were then moved to a 13-L observation tank filled three quarters of the way with water. The behavior of the zebrafish was then recorded for 10 min after recovery from anesthesia and the video was later viewed using computer to determine the highest seizure score every minute. The zebrafish seizure score was recorded as per the scoring system used by Kundap et al. (2017) and is given below.

Score 1 - Short swim mainly at the bottom of the tank
Score 2 - Increased swimming activity and high frequency of opercular movement
Score 3 - Burst swimming, left and right movements as well as erratic movements
Score 4 - Circular movements

Under the directives of MARP-Australia, the PTZ dose was set at 170 mg per kg of zebrafish body weight in order to limit the resulting seizure scores to a maximum of four. Time to score four seizure onset (seconds) and mean seizure score over 10 min were noted when viewing the recorded video. The mean seizure score over 10 min was calculated by first assigning the highest observed seizure score 1 min after the start of the video, as the seizure score for the first minute. This process was repeated until the end of the 10th min and all 10 seizure scores were averaged to obtain the mean seizure score over 10 min. The zebrafish swimming pattern was determined via analysis using the Smart tracking software. The dose of PTZ (170 mg/kg) and the duration of the behavior recording (10 min) represent the standard protocol of our laboratory for inducing seizures with PTZ, as determined previously by Kundap et al. (2017). The diazepam dose (10 mg/L) and the habituation time (30 min) were chosen based on the results of an unpublished preliminary trial using the same methodology. The diazepam dose and the habituation time were varied till a mean seizure score over 10 min of less than one was obtained.

Gene Expression Study

Brain harvesting

After the behavioral study, the zebrafish brains were harvested by removing the zebrafish skull and extracting the brain, before transferring it straight into 200 μl of ice-cold TRIzol®. The zebrafish brains were then immediately stored at −80°C till required.

RNA isolation and synthesis of first strand cDNA

The mRNA was isolated according to the protocol supplied by the kit's manufacturer, and was identical to the protocol used by Kundap et al. (2017). In short, the zebrafish brain was first homogenized whilst in TRIzol® before chloroform was mixed in. The resulting mixture was then centrifuged at a speed of 13,500 rpm (revolutions per minute) for a period 15 min and at a temperature of 4°C. After centrifugation, the resulting aqueous supernatant was then transferred into a new tube before the addition of isopropanol. After mixing, the new tube was incubated for 10 min at room temperature and subsequently centrifuged for a period of 10 min at a speed of 13,500 rpm and at a temperature of 4°C. The resulting supernatant was removed and the pellet was rinsed with 75% ethanol. The pellet was then allowed to air dry for between 5 and 10 min. Nuclease-free water was then added to the tube for the purpose of dissolving the mRNA pellet. The purity and concentration of the resulting isolated mRNA was then measured with a NanoDrop Spectrophotometer. Afterwards, the isolated mRNA was then converted to cDNA as per the instructions given in the Omniscript Reverse-transcription Kit from QIAGEN.

StepOne® real-time PCR

The gene expression level of Brain-Derived Neurotrophic Factor (BDNF), Nuclear Factor Kappa-light-chain-enhancer of activated B cells (NF-κB), Neuropeptide Y (NPY), c-Fos, Tumor Necrosis Factor alpha (TNF-α), Interleukin-1 (IL-1) and the housekeeping gene Elongation factor 1-alpha-1b (eef1a1b) were calculated via real-time quantitative RT-PCR (Applied Biosystems) together with QuantiTect SYBR Green dye and the appropriate Qiagen primer set for each gene; using a similar protocol to that used by Kundap et al. (2017). The samples were first incubated at 95°C for 2 min prior to thermal cycling. The thermal cycling settings used were 40 cycles of 95°C for 5 s, followed by 60°C for 15 s. The relative expression level (Fold Change) of the six genes of interest

was calculated by normalizing the threshold cycle (Ct) values obtained from the genes of interest, against the Ct value of the eef1a1b housekeeping gene using the formula: 2 ^ [Ct eef1a1b – Ct Gene of interest].

Statistical Analysis

All results were expressed as Mean ± Standard Error of the Mean (SEM). The data was analysed using one-way Analysis of Variance (ANOVA) and followed with Dunnett's test. The PTZ only negative control group (Group II/CN) was used as the control for Dunnett's test and all other groups were compared to it. The *P*-value, $^{***}P < 0.001$ was regarded as statistically significant for the behavioral study, whereas a *P*-value of $^{**}P < 0.01$ and $^{*}P < 0.05$ was regarded as statistically significant for the gene expression study.

RESULTS

Toxicity Study

The limit test performed using 100 mg/L of *O. stamineus* ethanolic leaf extract did not result in any mortality, morbidity or abnormal behavior in the zebrafish ($n = 7$). As per the protocol, the toxicity study was repeated using twice the concentration of the extract (200 mg/L) and again resulted in no mortality, morbidity or abnormal behavior in the zebrafish. Doubling the extract concentration once again to 400 mg/L produced no abnormal behavior in the zebrafish during the initial observation period, but later resulted in the death of all the treated zebrafish after an overnight exposure (less than 18 h after the last observation); during which the zebrafish were not monitored. From the results of the toxicity study, 200 mg/L was chosen to be the 'High' dose (T200) for the following behavioral study. The 'Medium' and 'Low' doses were thus chosen to be 100 mg/L (T100) and 50 mg/L (T50), respectively. From the software generated zebrafish swimming patterns (**Figure 1**), it was found that zebrafish treated with 100 mg/L of the extract spent more time at the bottom of the tank. This is in comparison to the vehicle control group (CV), which displayed a slight preference for the bottom of the tank but otherwise swam throughout the whole tank. In contrast, the zebrafish treated with 200 and 400 mg/L of the extract displayed no preference for any one location in the tank.

Behavioral Study

Seizure Onset Time and Seizure Score Analysis

Mean seizure onset time for the untreated CV group was taken to be 600 s, or the entire length of the video and a mean seizure score of 0 was assigned to the untreated CV group. This is because the vehicle control zebrafish were not injected with PTZ and thus did not develop seizures. Injecting PTZ into the zebrafish in the CV group resulted in a significant decrease in mean seizure onset time to 191 s and a significant increase in mean seizure score to 2.96 in comparison to the CV group. The results of the CN group were then used as a baseline for the positive control and treatment groups. Pre-treating the zebrafish with the positive control drug diazepam (CP) before challenging them with PTZ, significantly increased the mean seizure onset time to 453.4 s and significantly reduced the mean seizure score to 0.69. In contrast, pre-treating the zebrafish with 50 mg/L of *O. stamineus* ethanolic leaf extract (T50 Group) increased the mean seizure onset time to 314.4 s, although this was statistically insignificant ($P = 0.233$). However, the decrease in mean seizure score of the T50 group to 1.86 was considered statistically significant. Doubling the extract pre-treatment dose to 100 mg/L (T100 Group) produced a significant increase in the mean seizure onset time to 518.8 s and a significant decrease in the mean seizure score to 0.66. The final treatment group (T200 Group) was pre-treated with 200 mg/L of extract and did not reach seizure score 4 and thus the mean seizure onset time was recorded as 600 s or the full length of the recorded video. The T200 group also had a significant decrease in the mean seizure score to 0.47. All results were considered significant at the significance level of $^{***}\alpha = 0.001$. The mean seizure onset time (seconds) and seizure score for each zebrafish group are presented in a graphical format in **Figure 2**.

Representative Locomotion Patterns

Using the Smart tracking software for the automated tracking of zebrafish swimming patterns, one representative swimming pattern was chosen for each group from among the $n = 10$ zebrafish per group. The representative swimming patterns are given in **Figure 2**. The normal zebrafish swimming behavior demonstrated by the zebrafish in the CV group is to spend roughly an equal amount of time swimming throughout the entire tank. In contrast, the untreated negative control zebrafish had a more erratic swimming pattern after the PTZ challenge, with the zebrafish dwelling at bottom of the tank more frequently. Pre-treatment with the standard AED diazepam modified the post PTZ challenge swimming behavior into a zig-zag like swimming pattern, with a significant amount of time being spent at the top and bottom of the tank. Pre-treatment with all three *O. stamineus* ethanolic leaf extract doses produced a swimming pattern similar to that of the normal control, although the 50 and 200 mg/L doses produced more bottom dwelling in the zebrafish. In comparison, the 100 mg/L dose produced the most similar swimming pattern to the vehicle control, but showed an increase in time spent at the water surface.

Gene Expression Study

BDNF

The change in the gene expression level of BDNF was determined to be statistically insignificant in all groups in comparison to the negative control at a level of $^{*}\alpha = 0.05$. However, when graphically represented in **Figure 3**, an increase BDNF expression by the CN group as compared to the CV group is visible. The BDNF expression level was reduced in both the CP and T50 groups, whereas the T100 and T200 produced an increase in BDNF expression level in comparison to the CN group.

NF-κB

There was a significant rise in the gene expression level of NF-κB for the CN group in comparison to the vehicle control ($^{**}P < 0.01$). The CP, T100 and T200 groups had a significant reduction in NF-κB expression ($^{**}P < 0.01$) as compared to the

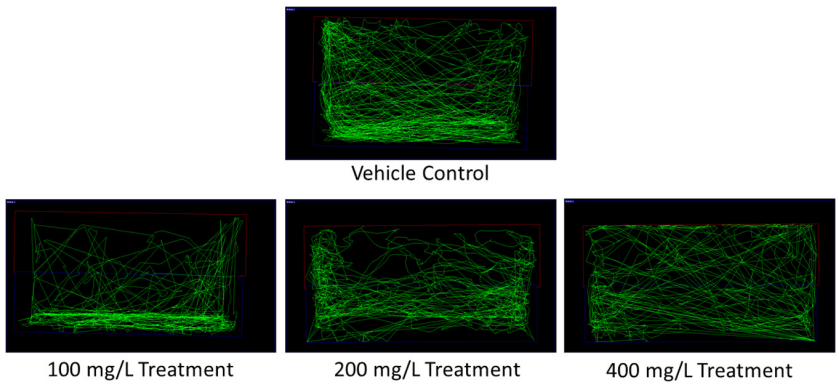

FIGURE 1 | Representative zebrafish swimming pattern of the vehicle control and extract treated groups for the toxicity study.

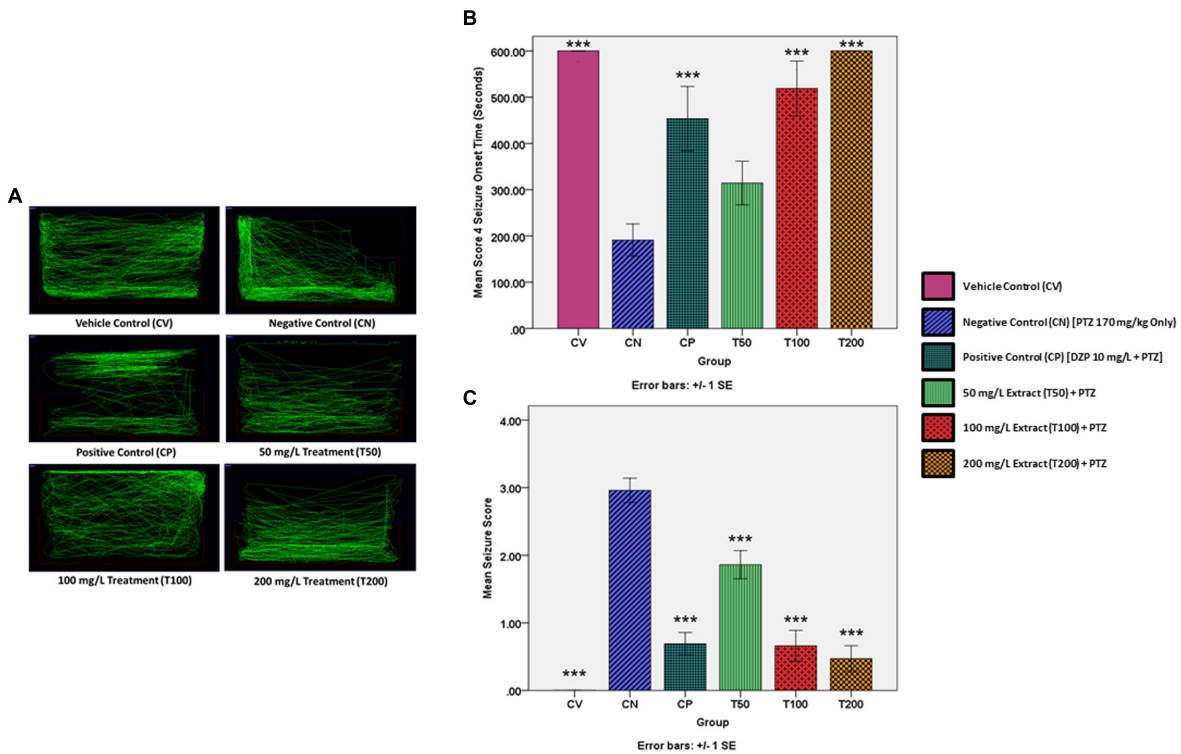

FIGURE 2 | PTZ-induced behavior and locomotion for each experimental group. **(A)** Representative zebrafish locomotion pattern for the vehicle, negative and positive control groups, as well as the extract treated groups. **(B)** Represents the score 4 seizure onset time (seconds) for the vehicle and positive controls as well as the extract treated groups, as compared to the negative control (CN, 170 mg/kg PTZ Only) **(C)** Represents the mean seizure score for the vehicle and positive controls as well as the extract treated groups, as compared to the negative control (CN, 170 mg/kg PTZ Only). The data is expressed as Mean ± SEM, $n = 10$ and was analyzed using one-way ANOVA, followed with Dunnett's test at significance level of $^{***}P < 0.001$.

CN group. The T50 group also showed a reduction in NF-κB expression, but this did not approach statistical significance ($P = 0.317$). The NF-κB expression level for each zebrafish group is graphically represented in **Figure 3**.

NPY

There was a significant rise in NPY expression for the CN group in comparison to the vehicle control. In comparison to the negative control, only the T100 group showed a significant decrease in NPY expression. The CP, T50, and T200 groups did show a decrease in NPY expression but this was not significant at the level of $^{*}\alpha = 0.05$. The NPY expression level for each zebrafish group is graphically represented in **Figure 3**.

c-Fos

The change in the gene expression level of c-Fos was determined to be statistically insignificant in all groups in comparison to the negative control. However, when graphically represented in

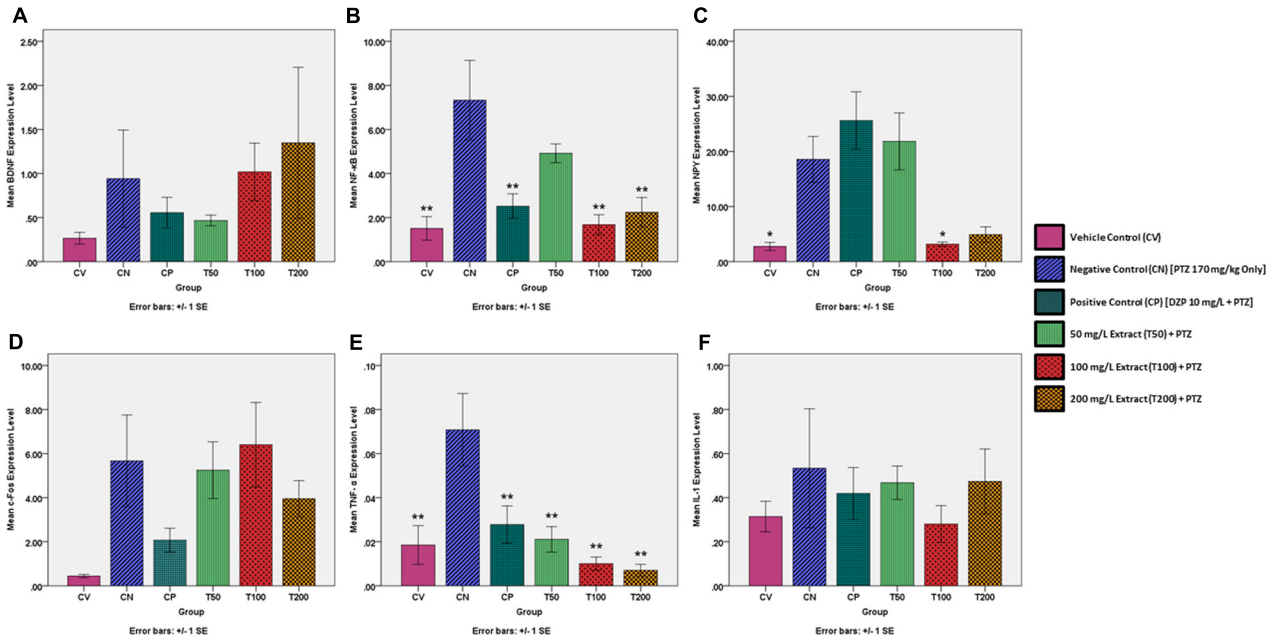

FIGURE 3 | Expression levels of several genes in the brains of each experimental group as determined by real time-PCR. All changes in expression levels were as compared to the negative control group (CN, 170 mg/kg PTZ Only), and included the genes: **(A)** BDNF **(B)** NF-κB **(C)** NPY **(D)** c-Fos **(E)** TNF-α and **(F)** IL-1. The data is expressed as Mean ± SEM, $n = 6$ and was analyzed using one-way ANOVA, followed with Dunnett's test at significance level of $^{**}P < 0.01$ and $^{*}P < 0.05$.

Figure 3, it can be seen that there is an increase c-Fos expression by the CN group as compared to the CV group. The level of c-Fos expression was decreased in the CP, T50, and T200 groups, whereas the T100 group had a decrease in c-Fos expression level when compared to the CN group.

TNF-α

There was a significant rise in the expression of TNF-α for the CN group as compared to the CV group. The CP, T50, T100, and T200 groups showed a significant reduction in the expression of TNF-α in comparison to the CN group. All changes in TNF-α expression were significant at the level of $^{**}\alpha = 0.01$. The TNF-α expression level for each zebrafish group is graphically represented in **Figure 3**.

IL-1

The change in the gene expression level of IL-1 was deemed to be statistically insignificant in all groups as compared to the negative control. However, when graphically represented in **Figure 3**, an increase IL-1 expression by the CN group as compared to the CV group is visible. The IL-1 expression level was reduced in the CP, T50, T100, and T200 groups as compared to the CN group. The IL-1 expression level for each zebrafish group is graphically represented in **Figure 3**.

DISCUSSION

This work aims to determine if an ethanolic leaf extract of *O. stamineus* has the potential to be a novel treatment for epileptic seizures. To that end, a toxicity study was carried out to determine if the extract is safe for use with zebrafish, as well as to determine the doses to be used for the following behavioral study. The toxicity study had to be conducted as no prior published study using this extract has been conducted on adult zebrafish before. A prior literature search only yielded *O. stamineus* toxicity studies on Sprague Dawley rats (Chin et al., 2008) and zebrafish embryos (Ismail et al., 2017), and thus this work represents the first of its kind. The reason that an *O. stamineus* ethanolic extract was used is because ethanolic extracts of *O. stamineus* tend to have the highest concentration of phenolic compounds, followed by methanolic and aqueous extracts (Saidan et al., 2015). Thus, as oxidative stress plays a role in epilepsy (Devi et al., 2008) and that the phenols in *O. stamineus* such as rosmarinic acid possess significant free radical scavenging, anti-inflammatory and antioxidant ability (Akowuah et al., 2005; Yam et al., 2010), an ethanolic extract of *O. stamineus* is the ideal choice for this experiment. The results of Saidan et al. (2015) support this idea as they found that an ethanolic leaf extract of *O. stamineus* possess the greatest anti-oxidant activity from among a combination of ethanolic, methanolic and aqueous extracts. The reason that a leaf extract of *O. stamineus* was used over another part of the plant was due to experimental evidence such as that by Saidan et al. (2015) showing that extracts of the leaves possess anti-oxidant activity and that the traditional remedy for epilepsy utilizes the leaves of the plant (Hossain and Mizanur Rahman, 2015). Given the uncertainty associated with any novel experiment, the toxicity study used in this experiment follows a modified version of the OECD Guidelines for the Testing of Chemicals No. 203, which concerns acute toxicity tests in fish. The test involves

the use of the test substance at a concentration of 100 mg/L of water, with a minimum of seven fish each for the treatment and control groups. The principle behind the test is that when there are no fish deaths after an exposure period of 96 h, the LC_{50} for the test substance can be said to be above 100 mg/L with a confidence of 99% or greater (OECD, 1992). As there have been no prior publications regarding the testing of the anti-convulsive potential of an ethanolic extract of *O. stamineus* in any animal species, this dose determination study was a necessity.

From the zebrafish swimming pattern after exposure to 200 and 400 mg/L of the *O. stamineus* ethanolic leaf extract, no bottom dwelling behavior was observed. Bottom dwelling in zebrafish in associated with anxiety and is initially seen in zebrafish which have just been transferred into a novel tank (Blaser and Rosemberg, 2012). As anxiolytics have been found to reduce bottom dwelling (Gebauer et al., 2011), the results of this study suggest that the extract has anxiolytic properties, at least at a concentration greater than 200 mg/L. The finding of this study that an overnight exposure to *O. stamineus* ethanolic leaf extract at a concentration of 400 mg/L is lethal to adult zebrafish is also noteworthy. This is because an acute oral toxicity study was performed using Sprague Dawley rats by Chin et al. (2008), by administering *O. stamineus* leaf extract up to a dose of 5.0 g/kg of rat body weight, daily for 14 days. The study by Chin et al. (2008) resulted in no rat deaths or any adverse effect on parameters such as body weight and they deemed that their methanolic *O. stamineus* whole plant extract seemingly lacked any toxic effects. Another toxicity experiment by Ismail et al. (2017) found that an aqueous extract of *O. stamineus* only significantly causes mortality in zebrafish embryos when the concentration reaches 5.0 g/L of water. However, both these experiments relied on a different manner of producing *O. stamineus* extracts compared to this study and thus may have a different proportion of constituents than the extract we used. In addition, a reliable correlation between zebrafish and rodent toxicities has not been established (Ducharme et al., 2015) and embryonic zebrafish toxicity may also not entirely correlate to adult zebrafish toxicity. Also, unlike dosing a rodent via the oral route, introducing a substance directly into the tank water makes it difficult to determine exactly how much of the substance has been taken up by the zebrafish (Kinkel et al., 2010). Thus, further work needs to be done to determine the mechanism behind the toxicity of *O. stamineus* ethanolic leaf extract in adult zebrafish to help reconcile the difference in toxicity results between this study and previous ones. It should also be noted that there is also no conversion factor for translating zebrafish toxicity to mammalian toxicity, although LC50 value zebrafish is generally lower than that for the corresponding rodent LC50 for certain chemicals such as polychlorinated biphenyls (Parng et al., 2002; Ducharme et al., 2015). However, given the popularity of *O. stamineus* as a traditional remedy for a plethora of illnesses, combined with multiple pharmacological studies demonstrating beneficial properties such as being hepatoprotective, antioxidant and antihypertensive (Ameer et al., 2012) as well as a relatively high toxic dose in rats (Chin et al., 2008), it is possible that *O. stamineus* derived AEDs would be relatively safe and non-toxic to humans.

Building on the toxicity study results, this experiment has also demonstrated that pre-treating zebrafish with an ethanolic leaf extract of *O. stamineus* for 30 min significantly increases the mean seizure onset time and decreases the mean seizure score of PTZ challenged zebrafish in a dose dependent manner. A 100 mg/L dose of the extract has been found in this study to rival the anti-convulsive effects of a 10 mg/L dose of the standard AED diazepam and a 200 mg/L dose of the extract has a stronger anti-convulsive effect than diazepam. The representative zebrafish swimming patterns also showed that diazepam reverses the bottom dwelling seen in PTZ challenge zebrafish, which is said to be comparable to the stupor like behavior and anxiety associated with an epileptic condition (Kundap et al., 2017). The swimming pattern produced by the zebrafish pre-treated with diazepam could be due to the sedative effect of diazepam, as it is a benzodiazepine (Gupta et al., 2014). In contrast to diazepam, zebrafish pre-treated with the extract produced a swimming pattern very similar to that of the vehicle control which was not challenged with PTZ. However, the 50 and 200 mg/L extract doses still produced some degree of bottom dwelling, although to a lesser degree than the negative control. This suggests that the 50 and 200 mg/L dose was insufficient to completely prevent the PTZ-induced seizures and this is supported by the mean seizure score for those doses being greater than zero. Interestingly, the 100 mg/L extract dose completely abolished bottom dwelling, although there was an increase in time spent on the water surface instead and the mean seizure score for 100 mg/L was also greater than zero. Taken together, the behavioral study results show that the *O. stamineus* ethanolic leaf extract does indeed possess dose dependent anti-convulsive properties but does not seem to produce the cognitive impairment associated with currently available AEDs such as diazepam.

Thus, our study shows that an *O. stamineus* ethanolic leaf extract derived novel AED has the potential to be comparable to diazepam, which is one of the standard AEDs available today. Undoubtedly, further work needs to be conducted to discover the active constituent/s of *O. stamineus* which contribute to its anti-convulsive properties. A follow up study similar to this one should then be conducted to test if a dose of the active constituent comparable to that of standard AEDs will still have similar or even better anti-convulsive efficacy. This is because our experiment shows that a dose of crude *O. stamineus* ethanolic leaf extract needs to be 10-fold that of diazepam to equal its effects. This is undesirable as high doses of substances in general tend to result in more side effects. Among the possible constituents responsible for the anti-convulsive effect an ethanolic leaf extract of *O. stamineus* are rosmarinic acid, sinensetin, eupatorine and 30-hydroxy-5,6,7,40-tetramethoxyflavone as they represent the major compounds in the extract which have anti-inflammatory action as well as substantial free radical scavenging and antioxidant ability (Pietta et al., 1991; Akowuah et al., 2005; Yam et al., 2010), all of which are factors that seem to protect against epilepsy (Devi et al., 2008; Vezzani et al., 2011). However, rosmarinic acid

seems to be a likely candidate as several studies have found that it possesses anti-convulsive properties, possibly due to its activation of the GABAergic system (Khamse et al., 2015; Grigoletto et al., 2016) and hence promotion of inhibitory neurotransmission. Rosmarinic acid is also neuroprotective as a result of its anti-oxidant and free radical scavenging abilities (Fallarini et al., 2009). Data provided by the manufacturer of our standardized extract also reiterates the importance of rosmarinic acid as they found that rosmarinic acid (5.02%) was the most abundant of the four marker compounds they tested, followed by sinensetin (0.21%), eupatorine (0.17%), and 30-hydroxy-5,6,7,40-tetramethoxyflavone (Trace Amounts). Interestingly, doubling the dose from 50 to 100 mg/L produced a much larger positive effect on both mean seizure onset time and seizure score as compared to doubling the dose from 100 to 200 mg/L. This suggests that some yet unknown factor could be limiting the bioavailability of the extract, at least for the given exposure period of 30 min. However, it is worth reiterating that the actual amount of substance taken up by the zebrafish is not known when the substance is dissolved in the tank water, unlike methods such as an intraperitoneal injection whereby the quantity delivered is defined based on the weight of the fish (Kinkel et al., 2010). Despite the limitations of dissolving the *O. stamineus* ethanolic leaf extract directly into the tank water, it is utilized by this study as the AEDs used today for the chronic symptomatic treatment of epilepsy are given orally (Anderson and Saneto, 2012). Thus, as we are aiming to develop a novel AED based on an ethanolic *O. stamineus* leaf extract, it must also work through the oral route. This is because if the AED must be injected into a patient to work, it will likely be underutilized due to the chronic nature of epilepsy; regardless of its efficacy.

Based on the results of the gene expression study, the downregulation of NF-κB by the *O. stamineus* ethanolic leaf extract is unusual as inhibition of the NF-κB pathway usually results in a decreased seizure threshold (Yu et al., 1999). This could be explained by the extract controlling the PTZ-induced seizures via another mechanism and hence there is minimal activation of the NF-κB pathway. This theory is supported by the fact that diazepam also reduces the NF-κB expression level in comparison to the negative control and that the CP, T100, and T200 groups displayed a NF-κB expression level very similar to that of the baseline expression level in the CV group. As NF-κB also regulates the expression level of BDNF during seizures (Lubin et al., 2007), the BDNF expression levels should also mirror that of NF-κB. However, we found no significant upregulation in the BDNF expression level after a PTZ-induced seizure for any pre-treated group as compared to the negative control. However, the role of BDNF in the development of seizures and epilepsy is somewhat controversial as although there is usually an upregulation of BDNF is associated with a seizure, it is unclear whether this promotes or inhibits seizure development (Lubin et al., 2007). In the case of NPY, our results are unusual, with diazepam and the 50 mg/L extract dose not having a significant effect on the NPY expression level as compared to the negative control whereas the 100 and 200 mg/L dose decreased it to around the same as the baseline vehicle control level. Although only the 100 mg/L group represented a significant change, the unusual results could be explained due to the anti-convulsive effect of NPY and also its regulation of learning and memory (Colmers and El Bahh, 2003). The 50 mg/L still produced an upregulation in NPY as it does not sufficiently control the PTZ-induced seizures on its own and thus requires the assistance of NPY. Whilst diazepam does control the PTZ-induced seizures, it also negatively affects cognitive abilities (Kundap et al., 2017) and hence an upregulation of NPY is needed to counteract the cognitive dysfunction which results. The explanation for the decrease in the expression level of NPY for the 100 and 200 mg/L treatment groups is similar to that of NF-κB, as the seizures are controlled via other mechanisms and thus the NPY expression level is similar to the baseline vehicle control.

In the case of c-Fos expression, we found no significant upregulation as a result of a PTZ-induced seizure and no significant difference in c-Fos expression levels as a result of any treatment. However, according to literature, a seizure usually results in an increase in c-Fos expression (Peng and Houser, 2005). This discrepancy could be explained by the time between the PTZ challenge and removal of the zebrafish brain, which was 10 min in our experiment. According to Barros et al. (2015), in the case of rodents at least, c-Fos takes around 30 min to become significantly elevated from baselines levels after challenging with a pro-convulsant. It is possible that in our experiment, there was not enough time for c-Fos expression to become significantly elevated. For TNF-α, we found that there was a significant increase in TNF-α expression as a result of a PTZ-induced seizure, which is consistent with the results found in literature (Wilcox and Vezzani, 2014). Although all pre-treatments significantly decreased the TNF-α expression level, the T100 and T200 groups had a slightly lower expression level than the baseline vehicle control. This suggests that the ethanolic *O. stamineus* leaf extract may at least partially exert its anti-convulsive effect by acting as an anti-inflammatory agent as TNF-α is involved in systemic inflammation. The anti-inflammatory action of the extract may in turn be due to the downregulation of TNF-α by the extract, along with IL-1, COX-1 and COX-2 as determined by Tabana et al. (2016). The last gene we tested was IL-1, which was found to have no significant upregulation in the expression level after a PTZ-induced seizure, nor any other significant change for any pre-treated group as compared to the negative control. Whilst this contrasts with reports in literature about an increase in IL-1 levels after a seizure and the ability of the extract to decrease IL-1 expression levels (Tabana et al., 2016), there are conflicting reports which describe a decrease in IL-1 levels after a seizure (Rijkers et al., 2009). The role of IL-1 in seizures also currently remains unknown and controversial (Rijkers et al., 2009).

FUTURE DIRECTIONS

Whilst this work represents a significant step in bridging the research gap, further research needs to be conducted on the

discovery of the active anti-convulsive compound in the extract. Once identified, dose comparison studies with currently available AEDs should be conducted for a true test of their relative efficacies. Another area of future research is the usage of zebrafish tests such as the T-maze, which is design to assess the cognitive ability of the zebrafish (Stewart and Kalueff, 2012). This would help to determine if the extract does not cause cognitive impairment in zebrafish as our zebrafish swimming pattern results suggest.

CONCLUSION

In conclusion, an ethanolic leaf extract of *O. stamineus* has the potential to be a novel symptomatic treatment for epileptic seizures as it is pharmacologically active against seizures in a zebrafish model. The anti-convulsive effect of this extract is also comparable to that of diazepam at higher doses and can surpass diazepam in certain cases. Treatment with the extract also counteracts the upregulation of NF-κB, NPY, and TNF-α as a result of a PTZ treated seizure. The anti-convulsive action for this extract could be at least partially due to its anti-inflammatory effects due to the downregulation of TNF-α.

ETHICS STATEMENT

The experimental protocol was approved by the Monash Animal Research Platform (MARP) Animal Ethics Committee, Monash University, Australia (MARP/2017/047).

AUTHOR CONTRIBUTIONS

BC performed all the experiments and was responsible for the writing of the manuscript in its entirety. UK performed the gene expression study in tandem with BC. MS was responsible for conceptualizing and revising the manuscript. YK, S-MH, and IO were also involved in conceptualizing and proofreading. All authors gave their final approval for the submission of the manuscript.

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