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Glial Source of Nitric Oxide in Epileptogenesis: A Target for Disease Modification in Epilepsy

Shaunik Sharma¹, Sreekanth Puttachary², Thimmasettappa Thippeswamy^{1,*}

¹Epilepsy Research Laboratory, Department of Biomedical Sciences, College of Veterinary Medicine, Iowa State University, Ames, IA 50011, USA

²College of Veterinary Medicine, Oregon State University, Corvallis, OR 97331, USA

Abstract

Epileptogenesis is the process of development of epileptic condition and/or its progression once it is established. The molecules that initiate, promote, and propagate the remarkable changes in the brain during epileptogenesis are emerging as targets for prevention/treatment of epilepsy. Epileptogenesis is a continuous process that follows immediately after *status epilepticus* (SE) in animal models of acquired temporal lobe epilepsy (TLE). Both SE and epileptogenesis are potential therapeutic targets for discovery of anticonvulsants and anti-epileptogenic or disease modifying agents. For translational studies, SE targets are appropriate for screening anticonvulsive drugs prior to their advancement as therapeutic agents, while targets of epileptogenesis are relevant for identification and development of therapeutic agents that can either prevent or modify the disease or its onset. The acute seizure models do not reveal anti-epileptogenic properties of anticonvulsive drugs. This review highlights the important components of epileptogenesis, and the long-term impact of intervening one of these components, the nitric oxide (NO), in the rat and mouse kainate model of TLE. The NO is a putative pleotropic gaseous neurotransmitter and an important contributor of nitro-oxidative stress that co-exists with neuroinflammation and epileptogenesis. The long-term impact of inhibiting the glial source of NO during early epileptogenesis in the rat model of TLE is reviewed. The importance of sex as a biological variable in disease modification strategies in epilepsy is also briefly discussed.

Keywords

Status epilepticus; kainate; temporal lobe epilepsy; anti-epileptogenic; nitro-oxidative stress; neuroinflammation

*Correspondence should be addressed to: Dr. T. Thippeswamy, Epilepsy Research Laboratory, Department of Biomedical Science, 2036 College of Veterinary Medicine, Iowa State University, Ames, Iowa 50011-1250. tswamy@iastate.edu, Phone: 515-294-2571.

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SS: performed the experiments, analyzed the data, reviewed the literature, and edited the manuscript, tables, and figures. SP: performed the experiments, analyzed the data, and edited the manuscript. TT: conceived the idea, designed the study, reviewed the literature and wrote the manuscript.

Introduction

Epilepsy is the fourth most common neurological disorder in humans and animals worldwide (Bernstein et al., 2010; Kirkley et al., 2014; Pakodzy et al., 2014; De Risio et al., 2015; Vos et al., 2015; WHO report, 2015; Beghi, 2016). It is a chronic and debilitating disorder that affects both men and women of all ages (Vos et al., 2015; WHO report, 2015). The lifetime prevalence of active epilepsy worldwide is ~2% (>65 million people) (WHO report, 2015). According to the Centers for Disease Control (CDC) report, it is estimated that ~10% of Americans experience a seizure during their lifetime and ~3% of these develop epilepsy by the time they are 80 years of age. In the US alone, about 150,000 new cases of epilepsy are reported annually (Hesdorffer et al., 2011; Austin et al., 2012; Hesdorffer and Begley, 2013; Koh et al., 2014). Epilepsy affects nearly 3.2 million Americans and its management, including caregiver expenses, costs an estimated \$15.5 billion annually (Bernstein et al., 2010). Apart from direct impact of epilepsy on the economy, the epilepsy disorder spectrum has a huge secondary impact on physical, psychological, and social issues (Austin et al., 2012; Krumholz et al., 2015). In spite of more than 30 anti-seizure or anti-epileptic drugs (ASD/AED) available to treat epilepsy, >50% of the people with epilepsy (PWE), presented with their first seizure, do not become seizure-free with the first ASD/AED therapy. Moreover, about 17% of the PWE requires a combination therapy (Kwan and Brodie, 2000; Krumholz et al., 2015). Whether the first seizure is appropriately treated or not, or the second seizure is treated, the AEDs do not reduce/prevent the long-term probability of seizure freedom and comorbidity. Furthermore, ~1/3 of the PWE are refractory to the current AEDs (Musicco et al., 1997; Kwan et al., 2011). Although AEDs control seizures in rest of the 2/3 of PWE, they do not cure or modify the disease process in temporal lobe epilepsy (TLE) (Kwan et al., 2011). The TLE is the most common type among all epilepsies with cognitive dysfunction as a prevailing comorbidity (Pearson et al., 2015; Rzezak et al., 2016; Thompson et al., 2016). The majority of the AEDs (including 47 failed drugs in human trials) that are not very effective, or do not cure the disease, are ion-channel targeted drugs (Temkin et al., 2001; Varvel et al., 2015). This suggests the need for the development of drugs that target alternate pathways to cure/modify the disease. To achieve this, a better understanding of the mechanisms of epileptogenesis in animal models is required.

Severity of *status epilepticus* determines epileptogenesis

Experimentally induced convulsive SE in rodents by neurotoxins such as kainate or pilocarpine or diisopropylfluorophosphate (DFP), an organophosphate, causes irreversible brain damage if not adequately treated immediately (Lemercier et al., 1983; Furtado et al., 2012; Iyer et al., 2015; Brandt et al., 2016; Puttachary et al., 2016a and b). The duration and severity of SE determines the outcome of epileptogenesis. The definition of SE depends on the context, and has been constantly changing (Manno, 2011; Seinfeld, 2016). According to the recommendation of the Commission on Classification and Terminology and the Commission on Epidemiology of the International League Against Epilepsy (ILAE), the SE is defined as “*a condition resulting either from the failure of the mechanisms responsible for seizure termination or from the initiation of mechanisms, which lead to abnormally, prolonged seizures (after time point t1). It is a condition, which can have long-term*

consequences (after time point t2), including neuronal death, neuronal injury, and alteration of neuronal networks, depending on the type and duration of seizures” (Trinka et al., 2015). Traditionally, it has been accepted that the duration of convulsive seizures during SE is sufficient to cause long-term brain injury/damage to enable the brain to generate spontaneous seizures i.e., “an enduring epilepticus” (Gastaut, 1983). The initial duration of SE for humans was 60 min which was then reduced to 30 min. This duration is now widely accepted for studies that investigate the long-term consequences of SE i.e., epileptogenesis and epilepsy (Commission on Epidemiology and Prognosis, 1993; Dodson et al., 1993). Seizures normally self-terminate by activating the inhibitory mechanism, however, if this mechanism fails it can lead to prolonged seizures (SE), which may then require administration of intervention drugs to terminate SE (Manno, 2011). Interestingly, the clinical trial guidelines recommend ~5 min to intervene in the case of continuous convulsive seizures (Dodson et al., 1993; Lowenstein et al., 1999; Shinnar et al., 2001; Seinfeld et al., 2016). In chemoconvulsant animal models, it has been known that a minimum of 10 min of convulsive SE is sufficient to cause brain injury and to induce temporal lobe epilepsy (Nairismagi et al., 2004; Puttachary et al., 2015b).

It is also important to note that in some mouse models even though the initial SE is severe and prolonged, the development of epilepsy may be compromised, and may not manifest the classical features of epileptogenesis such as progressive increase in frequency of spontaneous convulsive seizures (CS). For example, the SE induced by repeated low dose of kainate (i.p) in C57BL/6J mouse model produce severe SE, and continuous video-EEG confirmed that they develop epilepsy in less than 5 days (Puttachary et al., 2015b). A similar rapid epileptogenesis has been reported in the mouse pilocarpine model (Mazzuferi et al., 2012). The spontaneous CS persists for about 4–6 weeks, but they become infrequent thereafter. However, the electrographic non-convulsive seizures (NCS) persist for a longer period, i.e., up to 4 months post-SE (Puttachary et al., 2015b). Intra-hippocampal kainate administration in the mouse model also produced similar results with respect to infrequent CS, but frequent electrographic NCS (Klee et al., 2017) and widespread granule cell dispersion has been reported (Suzuki et al., 2005; Murphy and Danzer, 2011; Murphy et al., 2012; Hester and Danzer, 2014). This could be due to the direct impact of intra-hippocampal injection of kainate. It is also important to note that the kainate-induced SE via the intraperitoneal route in transgenic mice, bred on C57 genetic background (e.g., eGFP expressing mice), caused inconsistent epileptogenesis resulting in ~5 spontaneous CS in the first month and the CS were completely absent in the subsequent months (Fig. 1). However, as in the C57BL/6J mice, large numbers of electrographic NCS persisted in the transgenic mice in a three month continuous video-EEG study (data not shown). Therefore, the mouse kainate (i.p) models are not suitable for chronic studies if the experimental objective is to determine the effects of disease modifier on the frequency of convulsive seizures. However, they can be useful to assess the impact of drugs on epileptiform spikes and or electrographic NCS in the absence of CS paradigm. Therefore, in such scenarios, one could consider NCS and spike trains as variables, instead of CS, to compare between control and drug treated groups. To mimic human TLE for translational purpose, rat kainate model is more suitable in terms of progressive nature of the disease since the frequency of spontaneous CS increases consistently over time in rats in contrast to the mouse kainate models (Fig. 1).

In a recent review article by Löscher et al (2017), the impact of inter- and intra- strain differences in rats and mice for epilepsy research has been thoroughly discussed. According to this review, the following variables should be controlled to achieve reproducibility and rigor, and to minimize experimental bias in epilepsy research. These are environmental (housing, enrichment, food, water, and litter size); experimental (seizure frequency and duration, and seizure threshold); biological (age and sex); and genetic (genetic background and gene manipulation). Since the focus of our review is to address the targets of epileptogenesis for disease modification, we considered the initial SE severity as an important factor to initiate epileptogenesis. We largely overcame variability in severity of SE in inbred rats (Sprague Dawley) and mice (C57BL/6J) by: choosing the same vendor; selecting similar age group of animals (6–8 weeks old); appropriate training of experimenters to minimize stress during handling of animals; implementing methodological rigor, for example, 2–3 tiered blinded behavioral analyses by both direct observation and a secondary validation by analyses of recorded videos to distinguish between NCS and CS and to accurately determine the exact duration of CS during the SE. This type of methodological rigor is essential for selecting post-SE animals for unbiased grouping for vehicle control and test drug treatments in experiments. This will avoid confounding results in the long-term studies aimed at determining the disease modifying effect of the test drug (Puttachary et al., 2016a).

In rats, the route of kainate administration, use of anesthesia while administering the kainate, and the strain used in the experiments also impact SE and epileptogenesis. With respect to sensitivity and variability of response to a single dose of kainate by subcutaneous route, the Fischer-344 (F-344) rats are reported as a reliable strain (Golden et al., 1995; Sharma et al., 2008). In the adult male F-344 rats study, a single dose of kainate at 9 mg/kg (s.c.) induced SE in 93%, of which 95% survived and 80% developed epilepsy (Sharma et al., 2008). In another long-term study in F-344 rats, the kainate (3 mg/kg) was administered repeatedly at one hour intervals for four hours to induce SE (Rao et al., 2006). In this F-344 rat model, the duration and severity of seizures increased from third to fourth month, and the average number of seizures were 2.57 to 2.63 per hour. Therefore, F-344 rats may be appropriate to model the extreme severe spectrum of the disease. In our pilot studies in F-344 rats, the mortality was too high (>30%) in both RLD and SHD intraperitoneal method of kainate administration at 12 to 17.5 mg/kg (unpublished). Moreover, for expensive long-term telemetry experiments (continuous six month study), high mortality is neither economical nor justifiable from ethical perspective. Therefore, we chose the Sprague Dawley rats for our long term studies. However, these rats are less sensitive and showed more variable responses to a single dose of kainate by subcutaneous or intraperitoneal routes (Golden et al., 1995; Cosgrave et al., 2008). To overcome this advantage, we used RLD method of kainate administration (i.p.) as described in our publication (Puttachary et al., 2016a). It is also worth noting that the administration of kainate via intra-hippocampal route under isoflurane anesthesia impacts epileptogenesis in rats. Interestingly, isoflurane did not affect the SE severity, but it dampened the epileptogenesis in rats (Bar-Klein et al., 2016).

The diazepam is commonly used to terminate behavioral seizures. It is well documented that diazepam indeed controls behavioral seizures, but it has little or no impact on electrographic events if the SE is severe (Goodkin et al., 2008; Pibiri et al., 2008; Qashu et al., 2009;

Kadriu et al., 2010; Todorovic et al., 2012; Aplan et al., 2014). However, diazepam does suppress epileptiform activity in animals with mild SE (Fig. 2). Diazepam has been known to reduce neurodegeneration after prolonged SE, but not in the hippocampus during epileptogenesis (Qashu et al., 2009; Aplan et al., 2014). However, diazepam administration minimizes mortality to some extent and controls variability in the duration of behavioral SE between animals. Therefore, it is a common practice in our laboratory to administer diazepam at 2h after the onset of first CS (stage 3), and the exact duration of CS during this 2h period is calculated to determine the severity of SE as described in our previous publications (Tse et al., 2014; Puttachary et al., 2015b and 2016a).

The hallmarks of epileptogenesis

Traditionally, epileptogenesis was limited to “latent period”, the duration between the brain insult and the onset of spontaneous recurrent seizures. As per the new guidelines of ILAE, the epileptogenesis extends from the time of first brain insult, such as SE, with a consequence of structural (cellular and molecular) and functional changes in the brain, leading to a decreased seizure threshold for onset of spontaneous recurrent seizures, which continue to progress thereafter (Hellier et al. 1998; Williams et al. 2009; Kadam et al. 2010; Goldstein and Coulter 2013; Pitkanen and Engel, 2014; Pitkanen et al., 2015). The process of epileptogenesis follows immediately after SE and the brain changes continue to progress beyond the first couple of spontaneous seizures. Several studies have shown that epileptogenesis could start as soon as the SE begins (Bumanglag and Sloviter, 2008; Sloviter, 2008; Loscher and Brandt, 2010). The hyperexcitability of neurons, manifested by epileptiform spiking, occurring during SE triggers series of overlapping molecular and cellular changes in the brain (Aronica et al., 2017; Barker-Haliski et al., 2017; Klee et al., 2017). In the rat and mouse kainate models, though the behavioral seizures stopped after the diazepam treatment, the electrographic seizures persisted for several hours (Fig. 3) (Puttachary et al., 2016a and b). This could be due to the residual effects of kainate in the brain. In C57BL/6J mouse kainate model, we detected high concentrations of kainate in the hippocampal tissues at 4h post-injection (i.p. route). Interestingly, the kainate residues were also detected at 24h post-injection (Fig. 4). Therefore, the initial epileptiform activity during the post-SE could be due to the direct effects of kainate receptors’ activation. The hyper excited neurons modify the internal and external milieu due to altered membrane potential for exchange of ions, which ultimately sensitizes the glial cells (Vezzani et al., 2011 and 2017). The intrinsic changes that occur in neurons during SE, and thereafter, determine the fate of the neurons and the brain as a whole (Varvel et al., 2015). The altered neurons’ intrinsic properties and their subsequent communication with glial cells can trigger either a compensatory survival mechanism or neurodegeneration. In a recent *in vivo* two photon live imaging study, it was shown that the microglia, the resident macrophages of the brain, become activated as early as 30 minutes following an insult to the brain. The microglia migrate to the synaptic terminals and engage in dendritic pruning to limit the damage (Parkhurst et al., 2013; Szalay et al., 2016). In addition, microglia initially tend to support neuronal survival by producing trophic agents (Vezzani et al., 2011). The astrocytes, being close to the synaptic terminals and the blood vessels, tend to support neurons by up-taking the extra synaptic glutamate and potassium, and by transporting glucose from blood vessels

to the neurons (David et al., 2009; Vezzani et al., 2011; Puttachary et al., 2015a; Murphy et al., 2017). It is also suggested that astrocytes, in concurrence with endothelial cells and pericytes, regulate the blood-brain-barrier function, which is compromised during SE (Puttachary et al., 2016a). If these compensatory mechanisms fail, miscommunication between neurons and glia can turn the normal brain into an epileptic brain. Therefore, identification of molecules that play a critical role in such miscommunication could be a potential therapeutic target for epileptogenesis. One such molecule is a gaseous signaling agent, the nitric oxide (NO) (Thippeswamy et al., 2006).

The consequences of SE are reactive gliosis, excessive production of reactive oxygen/nitrogen species (ROS/RNS) and proinflammatory cytokines and chemokines, increased epileptiform spiking, neurodegeneration, excessive neurogenesis (with aberrant migration of neuroblasts and inappropriate integration), spontaneous recurrent CS with or without mossy fiber sprouting (Buckmaster, 2010; Vezzani et al., 2011; Bertram, 2013; Goldberg and Coulter, 2013; Ryan et al., 2014). These are some of the well-known hallmarks of epileptogenesis (Fig. 5). It is imperative to assume that intervening some or all of these will either prevent or modify epilepsy. The majority of AEDs do impact some of these components of epileptogenesis, especially by targeting the neuronal ion channels to control seizures, but they do not cure the disease completely. Therefore, there is a need to develop new drugs with a different mechanism of action that can act on multiple targets to prevent or modify the course of development of epilepsy.

AEDs, anti-epileptogenic agents, and animal models

The vast majority of currently available anticonvulsant drugs had been, presumably, thoroughly screened using a battery of preclinical high throughput tests. The most common methods used are acute seizure mouse/rat models and electrophysiology of brain slices, and in recent years, the zebra fish model has been proposed (White, 2002; Rowley and White, 2010; Baraban, 2013). The test drug of interest is administered either before inducing seizures or co-administered with chemoconvulsants to understand the therapeutic effect of the drug in controlling seizures. Indeed, this approach is useful for screening test compounds intended for the discovery of anticonvulsive drugs, but the outcome does not reveal whether the chosen compound would be useful as a potential anti-epileptogenic and/or anti-epileptic agent. Therefore it is important to screen potential anti-epileptic or anti-epileptogenic test drug in a relevant and highly reproducible preclinical/animal models of epilepsy to further advance it as a therapeutic agent. In our studies, we found that the rat kainate chronic model of TLE is the most appropriate model for testing disease modifying agents post-SE. Unlike certain mouse models, the rat kainate model has several advantages for studying epileptogenesis. The early epileptogenic hallmarks (the “disease promoters”) such as reactive gliosis, proinflammatory cytokines’ production, and neurodegeneration are consistent and the spontaneous CS are progressive in nature in the rat model, in contrast to the mouse model, especially with respect to frequency of CS (Bertram et al., 1990; Jorgensen et al., 1993; Rao et al., 2006; Williams et al., 2007 and 2009; Vezzani et al., 2011; Puttachary et al., 2016a and b).

Nitric oxide and epilepsy

NO is a diffusible gaseous molecule that cannot be stored in the cells as such, therefore, its role as a therapeutic target remains debatable. NO effects are largely mediated either through the activation of soluble guanylyl cyclase and/or nitrosylation of cytosolic and membrane proteins. For example, S-nitrosylation modulates NMDA receptor activity (Lei et al., 1992; Manzoni et al., 1992) and limits excessive Ca^{2+} influx to protect neurons. However, the role of NO as a protective or toxic molecule depends on the time and the source of NO production following an insult, and most importantly, the isoform of NOS involved. The substrate for NO production is one of the essential amino acids, arginine which is also a limiting factor for NO production/regulation. Three major isoforms of NO synthases (NOS) catalyze the formation of NO. Based on the abundance of NOS in various cell types, NOS are classified as neuronal NOS (nNOS), inducible NOS (iNOS), and endothelial NOS (eNOS). The pharmacological inhibitors have been used to target these enzymes to regulate the levels NO in various *in vitro* and *in vivo* models with conflicting outcomes (Thippeswamy et al., 2006). Several studies have also highlighted the controversial pathophysiological roles of NO in peripheral and central nervous systems (Dawson and Snyder, 1994; Dawson and Dawson, 1998; Hobbs et al., 1999; Moncada and Erusalimsky, 2002; Chung et al., 2005; Thippeswamy et al., 2006; Cosgrave et al., 2008; Puttachary et al., 2015a). In epilepsy models, the controversies could be due to inappropriate use of NOS inhibitors with respect to selectivity, dose and time of treatment (pre- or post- insult), solvents used as vehicle and method of reconstitution, and route/method of administration (Takei et al., 2001; Hagioka et al., 2005; Kato et al., 2005; Cosgrave et al., 2008; Kovacs et al., 2009; Beamer et al., 2012). The NO has been shown to have both anticonvulsive (Penix et al., 1994; Kendrick et al., 1996; Sardo and Ferraro, 2007; Royes et al., 2007) and proconvulsive actions (De Sarro et al., 1993; Tutka et al., 1996), which seems to depend on the species and the types of chemoconvulsants employed in the study (Cosgrave et al., 2008). All three NOS isoforms are expressed during epilepsy, but at different stages of seizures or epileptogenesis. For example, eNOS is upregulated in a rodent model of SE within 3–24 hours of intracranial injection of KA (Chuang et al., 2007), while nNOS and iNOS are upregulated in the mouse model of electrically-induced SE (Catania et al., 2003). In the rat kainate model, nNOS and iNOS are upregulated in less than three days of post-SE and persisted for longer duration (Cosgrave et al., 2008; Vezzani et al., 2011). In this review, anticonvulsive and/or disease modifying effects of two NOS inhibitors [nNOS specific inhibitor, N^{ω} -propyl-L-arginine (L-NPA); iNOS specific inhibitor, 1400W] in the mouse and rat model of TLE are discussed.

Neuronal nitric oxide synthase in *status epilepticus* and epileptogenesis

It is challenging to develop a single drug to act upon multiple targets without having any adverse effects. All drugs have off-targets to a variable proportion, which cause side effects accordingly. It is customary to weigh benefits of a drug against side effects in a given situation. A drug that targets an event or molecules during SE may not be effective during the post-SE period if the target molecule of interest is not expressed in a significant amount during epileptogenesis. For example, the nNOS mediated NO production was thought to play an important role during SE. The brain slice experiments have demonstrated the

neuronal source of endogenous NO as a key promoter for initiating seizure-like events in the hippocampal formation and entorhinal cortex (Kovacs et al., 2009). We tested the hypothesis in the mouse kainate model with nNOS specific inhibitor, L-NPA, which has 149- and 3158-folds greater selectivity over endothelial and inducible NOS (eNOS/iNOS), respectively (Zhang et al., 1997; Beamer et al., 2012). Initially we tested the anticonvulsive effect of the nNOS inhibition in mice by treating with the L-NPA (20 mg/kg, i.p.) 30 min prior to the induction of SE with kainate. Kainate is a glutamate analogue, which causes seizures with a hippocampal focus and is widely used to develop models of TLE in rodents (Ben-Ari and Cossart, 2000). L-NPA pre-treatment significantly reduced the severity and duration of convulsive seizures, the gamma EEG power, and the epileptiform spike rate during SE and also during the first 7 days of post-SE (Beamer et al., 2012). Moreover, the histology of brain sections revealed a significant reduction of c-Fos, a cellular marker of neuronal hyperexcitability, in dentate granule cells at 2h post-SE (Beamer et al., 2012). These results suggest that nNOS also facilitates seizure generation during SE.

The reactive gliosis, neurodegeneration, and neurogenesis are the most commonly recognized features of epileptogenesis (Parent and Lowenstein, 2002; Vezzani et al., 2011; Puttachary et al., 2016a and b). To get a better understanding of these, we did immunohistochemistry (IHC) on brain sections collected at 3 day post-SE from the L-NPA experiments. As expected, we observed a significant reduction in hippocampal microgliosis and astrogliosis in the L-NPA pretreated group, as compared to the vehicle control group, since the initial SE severity was compromised in the L-NPA pre-treated animals (Beamer et al., 2012). However, neurodegeneration and neurogenesis were not significantly changed at 3 day post SE. It was suggested that C57BL/6J mice are resistant to kainate-induced neurodegeneration (Schauwecker and Steward, 1997; Schauwecker, 2012). However, we have demonstrated neurodegeneration in C57BL/6J mice using fluoro-jade-B and NeuN co-staining in the hippocampal formation, entorhinal cortex, and amygdala at 7 day post-SE (Puttachary et al., 2016a). Likewise, gliosis (both astrogliosis and microgliosis) was maximum at 7 day post-SE, but decreased at later time points, in contrast to the rat kainate model of TLE (Puttachary et al., 2016a, 2016b). These are important differences between C57BL/6J mouse kainate model and the rat kainate model. In crossbred mice and rats, we observed reactive gliosis and neurodegeneration as early as 24h post-SE (personal observation, unpublished work), and in the rat they persisted throughout the period of epileptogenesis and also during the chronic phase (Puttachary et al., 2016b). Interestingly, neurogenesis persistently increased in C57BL/6J mice during epileptogenesis (at 7day post-SE), perhaps to compensate the ongoing neurodegeneration (Puttachary et al., 2016a). It is also interesting to note that SE increases activity-dependent synaptogenesis in the outer and middle molecular layers of the dentate gyrus in the early stage of epileptogenesis, observed at 72 h post-SE in C57BL/6J mice, which was suppressed in the L-NPA treated group (Beamer et al., 2012). The suppressed gliosis and neo-synaptogenesis at 3 day post-SE in the L-NPA treated group were not due to the direct effects of nNOS inhibition, but due to compromised initial severity of SE owing to pretreatment approach. The results from pre-treatment experiments of test drugs provide proof-of-concept for their anti-seizure efficacy and confirms that the decreased severity of seizures during SE dampens the events of epileptogenesis. A similar conclusion can be drawn from gene knockout or transgenic mice

experiments. Therefore to confirm anti-epileptogenic or anti-epileptic effects of a pharmacological agent or gene of interest from translational aspect, intervention strategies during the post-SE period are appropriate for investigating disease modifying agents in epilepsy. An example for intervention of epileptogenesis soon after SE, targeting the NO signaling, and its long-term impact on epilepsy is further discussed.

Inducible NOS inhibitor and epileptogenesis

The complex role of NO in epilepsy owes to cell-specific expression of three different isoforms of NOS expressed at various stages of epileptogenesis. As mentioned earlier, the neuronal source of NO produced by nNOS-mediated mechanism promotes seizures. Both *in vitro* and *in vivo* studies have confirmed proconvulsive role of NO in acute models (Kovacs et al., 2009; Beamer et al., 2012). IHC of the brain sections at 3 day post-SE in the rat kainate model of TLE showed a significant increase in nNOS levels in neurons and inducible NOS (iNOS/NOS2) in microglia (Cosgrave et al., 2008). Since we knew that L-NPA pretreatment reduced SE severity and epileptiform spikes, we further tested if post-treatment will have a similar beneficial effect. Surprisingly, we observed a marginal increase, rather than anticipated significant decrease, in epileptiform spike rate and gliosis at 24h and 72h post-SE between the vehicle and L-NPA treated groups. This may suggest a beneficial role of nNOS during early stages of epileptogenesis in contrast to its proconvulsive role during acute seizures onset (SE). It has been shown that a transient increase in nNOS, following insult to the brain, protect neurons by S-nitrosylation of NR2B subunit of NMDAR to control excessive calcium influx (Gidday et al., 1999; Gonzale-Zulueta et al., 2000; Campelo et al., 2012). Since we did not observe expected modifications in early epileptogenesis by inhibiting nNOS with L-NPA, we focused our investigation on the role of iNOS in epileptogenesis. We first tested a highly potent iNOS inhibitor, 1400W [N-(3-(Aminomethyl) benzyl) acetamidine] on the brain slices to understand whether it suppresses epileptiform spiking activity before it was tested in animal models. The 1400W indeed significantly suppressed kainate induced epileptiform spikes in the brain slices (Puttachary et al., 2016b). 1400W is a slow, tight binding and highly selective pharmacological inhibitor of iNOS with a K_d value of 7 nM. It is >5,000 and >200 fold selective for iNOS than eNOS and nNOS, respectively (Garvey et al., 1997; Parmentier et al., 1999; Alderton et al., 2001; Jafarian-Tehrani et al., 2005; Perez-Asensio et al., 2005). It is biologically active *in vivo* and it has no pulmonary or cardiovascular side effects, and the physiological activities mediated by eNOS and nNOS are not compromised at the optimum dose of 20 mg/kg (Garvey et al., 1997; Eissa, 2003). 1400W is BBB permeable and has shown to suppress abnormal levels of NO metabolites in rodents (Garvey et al., 1997; Parmentier et al., 1999; Crowell et al., 2003; Perez-Asensio et al., 2005). It is 100-fold potent than other iNOS inhibitors (ED₅₀= ~0.3 mg) in reducing delayed vascular injury in the rat LPS model (Garvey et al., 1997). Importantly, studies have shown that rats tolerated a dose of 120 mg/day for a 7-day period when 1400W was administered as intravenous infusion, however it was lethal at 50 mg/kg when given as single intravenous bolus (Garvey et al., 1997). As discussed earlier, this is an important finding considering the reported controversial roles of NO in brain pathology (Dawson and Snyder, 1994; Dawson and Dawson, 1998; Hobbs et al., 1999; Moncada and Erusalimsky, 2002; Chung et al., 2005; Thippeswamy et al., 2006).

The 1400W, at 20 mg/kg, effectively ameliorated brain pathology and suppressed seizures by >90% in the rat kainate model of chronic epilepsy in six months continuous video-EEG study (Puttachary et al., 2016b). In the TBI and stroke models, it reduced brain injury by decreasing glutamate release (Parmetier et al., 1999; Jafarian-Tehrani et al., 2005; Perez-Asensio et al., 2005). The 1400W also protected the BBB integrity, which is compromised soon after the SE (Boje, 1996; Puttachary et al., 2016b). The serum albumin (SA) is considered as a biomarker for BBB leakage (Frigerio et al., 2012). Increased SA and glutamate levels induce reactive astrogliosis that causes hyperexcitability of neurons and hence seizures (Boje, 1996; Frigerio et al., 2012; Liu et al., 2012; Salar et al., 2014; Weissberg et al., 2015). Our previous studies from the rat and mouse epilepsy models have shown an increased SA and GFAP levels in the hippocampus at 7 day post-SE, and 1400W treatment reduced their levels (Puttachary et al., 2016a and b). The 1400W also reversed SE-induced suppression of K_{ir} 4.1 and GLT1 levels, perhaps by reducing reactive gliosis, which could also be due to decreased levels of glutamate and SA (Puttachary et al., 2016b). Overall these changes attenuated neuronal excitability which was evident from a significant reduction in the epileptiform spike rate and seizures in the rat kainate model (Puttachary et al., 2016b). The 1400W reduced neurodegeneration, as indicated by reduction in the FJB+ve neurons, both at 7day and at 6 months post-SE (Puttachary et al., 2016b). These findings provide evidence for the long-term neuroprotective role of 1400W and its disease modifying properties in epilepsy.

1400W is commercially available as a pure (>99%) water soluble compound. It has been tested in healthy human volunteers, patients with heart failure (Dover et al., 2006), and liver cirrhosis (Ferguson et al., 2006). There were no adverse effects of 1400W in humans and no toxicity are reported. It effectively suppressed inflammatory mediators in human cartilage derived from osteoarthritic patients and in the rat neuropathic pain model (Jarvinen et al., 2008; Makuch et al., 2013). Other iNOS inhibitors such as L-NIL-TA (referred to as SC-51 in the literature), VAS203 (Vasopharm, 2013) and KD7040 are in clinical trials for asthmatic humans (and healthy volunteers), TBI patients, and for neuropathic pain, respectively (Hansel et al., 2003; Vasopharm, 2013). Furthermore, in our ongoing studies on organophosphate neurotoxicity rat model, we observed similar effects of neuroprotection by 1400W and its long-term seizure suppression as observed in the rat kainate model of TLE. Collectively, these studies strongly support therapeutic potential of iNOS inhibitors, and 1400W in particular, as a promising drug for disease modification not only in epilepsy, but also in various other neurodegenerative disorders.

Sex as a biological variable in epilepsy research

Over dependence on male animals in preclinical research obscures the key sex differences that could create a huge gap in the knowledge base, and it undermines the quality of data (Clayton and Collins, 2014). For translational studies, this is unjustified since women experience higher rates of adverse drug reactions than men do (Franconi et al., 2007; Zucker and Beery, 2010; Clayton and Collins, 2014). A population-based study on epilepsy in women in the US revealed that out of a million women of childbearing age treated with AED every year, about 33% did not respond to the drugs and some even had severe side effects (Katz et al., 2006). The males responded 75% better than the females to the same

class of drugs with least side effects (French et al., 2003; Arroyo et al., 2004; Beydoun et al., 2005; Elger et al., 2006; Whitley and Lindsey, 2009; Tomson and Battino, 2011; Pulman et al., 2014). The bias towards the choice of male animals versus female in preclinical studies is due to undisputed influence of sex hormones, during female reproductive cycle, on experimental results (Backstrom et al., 2003; Herzog and Frye, 2003; Herzog et al., 2004 and 2011). Indeed this is an important variable that has been left out of experimental designs in most preclinical studies so far. This negligence seems to have had a huge impact on success/failure of certain drugs in females during clinical trials and withdrawal of FDA approved drugs from the market (Parekh et al., 2011). In 1993, the National Institutes of Health (NIH) introduced Revitalization Act requiring the inclusion of women in all NIH-funded clinical research (Clayton and Collins, 2014). The National Institute of Mental Health (NIMH) recently conducted a workshop to highlight the impact of sex differences in brain on behavior and neurological disorders. The NIMH has emphasized the need for more neuroscientists to incorporate sex as a variable in experimental designs (NIH Mental Health report, 2011). The NIH new policy requires applicants to report their plans for the balance of male and female animals in preclinical studies in all future applications (Clayton and Collins, 2014). The challenge for epilepsy researchers, using female animals as models, is the influence of sex hormones on seizure threshold during various stages of reproductive cycle (Backstrom et al., 2003; Herzog and Frye, 2003; Herzog et al., 2004 and 2011).

The differences in male and female responses to a drug or an insult to the brain are mainly due to the effects of neurosteroids and sex hormones on neurons and glial cells. The neurosteroids alter neuron-glial communication in normal brain development and function, and also during neuropathological conditions (Backstrom et al., 2003; Herzog and Frye, 2003; Herzog et al., 2004 and 2011). It is plausible that this communication is compromised if the levels of hormones in females are altered during different phases of reproductive cycle (Bellefontaine et al., 2011; Wu et al., 2013; Carver et al., 2014). It has been known for some time that the neurons primed by estrogen are protected against neurodegeneration and females live longer and experience less pain than males do (Tenenbaum et al., 2007; Arevalo et al., 2014). On the flip side, in some women higher estrogen and lower levels of progesterone excite neurons and trigger seizures, and their response to AEDs is compromised (Backstrom et al., 2003; Herzog and Frye, 2003; Herzog et al., 2004 and 2011), which has been demonstrated in the rat models (Wu et al., 2013; Carver et al., 2014).

During the follicular phase (the first half of the estrus cycle), the brain is under the influence of estradiol, while during the luteal phase (the second half of the cycle) progesterone influences the brain function (Herzog et al., 2011; Wu et al., 2013). Estradiol peaks at midcycle and progesterone declines before menstruation begins (Backstrom et al., 2003; Herzog and Frye, 2003; Herzog et al., 2004 and 2011). These alterations influence the receptor plasticity in the brain, and cause premenstrual syndrome, migraine and “catamenial” epilepsy in humans (Backstrom et al., 2003; Herzog et al., 2011; Amour et al., 2015). Low progesterone levels during perimenstrual or before ovulation trigger frequent seizures and they could become refractory to the conventional AEDs (Herzog and Frye, 2003; Herzog et al., 2004). Until recently, these factors were not adequately considered in the vast majority of preclinical drug discovery experiments in epilepsy research. The female rats attain puberty by 4 weeks of age and maintain regular estrous cycles thereafter. Each

cycle is 4–5 days long and occurs throughout the year (Ojeda et al., 1990; Maguire et al., 2005; Westwood, 2008). There are four stages of estrous cycle in rats as in humans; diestrus, proestrus, estrus and metestrus. Progesterone levels are high during diestrus and pregnancy but low during estrus, which are comparable to humans (Westwood, 2008; Wu et al., 2013). Progesterone have anticonvulsant, anxiolytic, and neuroprotective properties (Reddy, 2003, 2004, 2009 and 2010a; Scharfman and MacLusky, 2006) and, therefore, it plays a significant role in epilepsy, anxiety and depression (Smith et al., 1998; Van Broekhoven and Verkes, 2002; Reddy, 2003; Reddy and Jian, 2010b). In the brain, progesterone is converted into allopregnanolone, a neurosteroid which rapidly alters excitability of neurons through direct interaction with the inhibitory pathway via the gamma amino butyric acid (GABA)-A receptors (Scharfman et al., 2005; Scharfman and MacLusky, 2006; Hosie et al., 2007; Reddy and Mohan, 2011). Several reports suggest the critical role of NO in modulating neurosteroids-mediated signaling between neurons and glial cells in the brain (Cheepsunthorn et al., 2006; Chamniansawat and Chongthammakun, 2014; Chakraborti et al., 2014; Lima et al., 2014; Priyanka et al., 2014; Del-Bianco-Borges and Franci, 2015; Pandey and Deshpande, 2015). However, this is not yet tested in our models using either L-NPA or 1400W. Moreover, a detailed study on estrus-stage specific expression of different isoforms of NOS is required prior testing the drugs to target them for epileptogenesis in female animal models.

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Significance Statement

Neuroinflammatory mediators are emerging as therapeutic targets for epilepsy. *Status epilepticus* induced neuroinflammation in epileptogenesis is characterized by reactive gliosis and production of reactive oxygen and nitrogen species. Glial-mediated nitric oxide (NO) production in the central nervous system, in contrast to the peripheral nervous system, is cytotoxic. Therefore, the agents that target the glial source of NO could be neuroprotective and may restore brain function. This approach can be beneficial to modify/prevent epileptogenesis and the disease. In this review, we discuss the potential disease modifying role of the iNOS inhibitor, in contrast to nNOS inhibitors, in epilepsy.

Figure 1.

Comparison of the spontaneous CS frequencies between the kainate models of rat, C57BL/6J and crossbred wildtype mice. The seizures were quantified from three months of continuous video-EEG recordings. The behavioral spontaneous CS were verified against EEG pattern and the power spectrum as described previously for the rat and mouse kainate model of TLE (Puttachary et al., 2015b and 2016b). The CS were progressive in the rat, while they decreased over time in the mouse models. Mann-Whitney test, $*p < 0.05$, $n = 6-8$. CS, convulsive seizures.

Figure 2.

SHD and RLD methods of kainate administration in C57BL/6J mice and their impact on SE, and diazepam treatment on spiking activity (**A–D**). In the severe group, diazepam treatment was effective on behavioral seizures, but not on the electrographic seizures (**A, B**). In the mild group, it was effective on both behavioral and electrographic seizures (**C, D**). Each vertical bar within the box represents spike train that could contain epileptiform spikes, spike clusters (<12s) and/or a seizure (>12s) at a given time point. The spike trains could be continuous for several minutes or intermittent. Upward red arrow represents the onset of first convulsive seizure, i.e. stage 3 and grey arrow represents the time when diazepam was administered after 2 hours of the onset of first CS. SHD, single high dose; RLD, repeated low dose; SE. *status epilepticus*.

Figure 3.

Examples of 30 min EEG traces, after the DZP treatment, from the mouse (A) and rat (B) that had severe SE after administering the kainate. The DZP administration did not control epileptiform spiking in the animals that had severe SE.

Figure 4.

The ion chromatogram showing the relative abundance of kainate in the hippocampus. The LC-MS analysis confirmed that the kainate was present in the hippocampus at higher levels at 4h, but persisted even at 24h post-administration. n=4.

Figure 5.

A) The common features of epileptogenesis that occur soon after SE is illustrated. **B)** An example of epileptiform spikes on EEG from an epileptic brain is compared with a normal brain. **C)** IHC images from the hippocampus (i to vi, and ix to x) and the dentate gyrus (vii, viii). The astrocytes [green in (i) and (ii)] and microglia [red in (iii) and (iv), and green in (ix) and (x)] become reactive (ii, iv, x) in an epileptic brain. Reactive gliosis causes production of proinflammatory cytokines, chemokines, and ROS/RNS to induce neurodegeneration [FJB+NeuN (vi)]. Red labelled cells are NeuN positive in the panel (v), (vi), (ix), and (x). Yellow in (v) and (vi) represents FJB positive cells. Epileptic brain showed increased production of neuroblasts (pink labelled cells) in the subgranular zone of the dentate gyrus (white arrows in viii) in contrast to the control brain (vii). Further details on these parameters and the quantified data can be found in Puttachary et al., 2016a and b. Scale bar, all 100 μ m.