JPET Fast Forward. Published on November 3, 2017 as DOI: 10.1124/jpet.117.244939
This article has not been copyedited and formatted. The final version may differ from this version.

JPET #244939 (Final version)

Epigenetic Histone Deacetylation Inhibition Prevents the Development and Persistence of Temporal Lobe Epilepsy

Sandesh D. Reddy, Bryan L. Clossen and Doodipala Samba Reddy*

Department of Neuroscience and Experimental Therapeutics, Texas A&M University Health Science Center College of Medicine, Bryan, TX 77807.

JPET Fast Forward. Published on November 3, 2017 as DOI: 10.1124/jpet.117.244939
This article has not been copyedited and formatted. The final version may differ from this version.

Running Title: Epigenetic HDAC pathway in epileptogenesis

Address correspondence to:

Dr. Doodipala Samba Reddy
Department of Neuroscience and Experimental Therapeutics
College of Medicine, Texas A&M University Health Science Center
2008 Medical Research and Education Building
8447 Riverside Parkway, Bryan, TX 77807-3260
Phone: 979-436-0324
Email: reddy@medicine.tamhsc.edu

Manuscript statistics:

Total text pages = 41 (Figures = 10 & Tables= 1)

Abstract, **212** words

Significance, **121** words

Introduction, **690** words

Discussion, **1656** words

Conflict of Interest

The authors declare no competing financial interests.

ABBREVIATIONS:

AD, afterdischarge; ADT, afterdischarge threshold; DH, dentate hilus; FDA, food and drug administration; GABA, γ-aminobutyric acid; GX, ganaxolone (3β-methyl-3α-hydroxy-5α-pregnan-20-one); HDAC, histone deacetylase; miRNA, microRNA; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; TLE, temporal lobe epilepsy; SEM, standard error of the mean;

Recommended Section Assignment: **Neuropharmacology**

ABSTRACT

Epilepsy is a chronic brain disease characterized by repeated unprovoked seizures. Currently, no drug therapy exists for curing epilepsy or disease-modification in people at risk. Despite several emerging mechanisms, there have been few studies of epigenetic signaling in epileptogenesis, the process whereby a normal brain becomes progressively epileptic because of precipitating factors. Here, we report a novel role of histone deacetylation as critical epigenetic mechanism in epileptogenesis. Experiments were conducted using the histone deacetylase (HDAC) inhibitor sodium butyrate in the hippocampus kindling model of temporal lobe epilepsy (TLE), a classic model heavily used to approve drugs for treatment of epilepsy. Daily treatment with butyrate significantly inhibited HDAC activity and retarded the development of limbic epileptogenesis without affecting afterdischarge signal. HDAC inhibition markedly impaired the persistence of seizure expression many weeks after epilepsy development. Moreover, subchronic HDAC inhibition for 2 weeks resulted in a striking retardation of epileptogenesis. HDAC inhibition, unexpectedly, also showed erasal of epileptogenic state in epileptic animals. Finally, butyrate-treated animals exhibited a powerful reduction in mossy fiber sprouting, a morphological index of epileptogenesis. Together these results underscore that HDAC inhibition prevents the development of TLE, indicating HDAC's critical signaling in epileptogenesis. These findings, therefore, envisage a unique novel therapy for preventing or curing epilepsy by targeting the epigenetic HDAC pathway.

Introduction

Epilepsy, which affects about 3 million Americans and 65 million people worldwide, is a common brain disease characterized by repeated unprovoked seizures. Despite such wide incidence and the dozens of drugs available to combat seizures, there is currently no specific drug for preventing or curing epilepsy (Jacobs et al., 2009). About 150,000 new cases of epilepsy are diagnosed in the United States annually (Hesdorffer et al., 2013). The mechanisms underlying the development of acquired epilepsy are not very well understood. The term "epileptogenesis" is used to describe the complex plastic changes in the brain that, following a precipitating event, convert a normal brain into a brain debilitated by recurrent seizures (Pitkänen et al., 2009). Temporal lobe epilepsy (TLE) is caused by diverse precipitating factors such as brain injury, stroke, infections, or prolonged seizures. TLE progression can be described in three stages: (i) the initial epileptogenic event; (ii) the latent period with little or no seizure activity; and (iii) the chronic period characterized by recurrent seizures. The kindling model has provided a conceptual framework for epileptogenesis and for developing new molecular targets for preventing epilepsy (Goddard,1969, McNamara et al., 1992). Kindling is the classic model for epileptogenesis, in which repeated stimulation below the convulsion threshold leads to progressive intensification of behavioral and electrographic seizure activity (Goddard, 1969). It has become a widely-used model for human TLE with complex partial seizures (Löscher, 2002; He et al., 2004; Gorter et al., 2016). There is an unmet need for drugs that truly prevent the development of epilepsy, known as antiepileptogenic agents, or alter its natural course to limit the appearance or severity of epileptic seizures, known as disease-modifying agents, in people at risk. Several antiepileptic drugs such as levetiracetam, topiramate, lamotrigine, and lacosamide have been tested for disease-modifying activity but have exhibited little efficacy (Albertson et al., 1984; Silver et al., 1991; Loscher et al., 1998; Brandt et la., 2007; Pitkanen et al., 2013). A variety of potential treatments have been proposed, targeting mechanisms such as the neurotrophic, anti-inflammatory, neuroprotective, and epigenetic pathways (Pitkanen and Lukasiuk, 2011; Ravizza et al., 2011; Clossen and Reddy, 2017; Younus and Reddy, 2017a). However, there have been few studies on the potential role of HDAC signaling in epileptogenesis.

The potential epigenetic mechanisms, including DNA methylation, histone alterations, and miRNA-based transcriptional control, can alter the neuronal gene expression profile and thus affect epilepsy pathology (Fig. 1). Although epigenetic signaling alterations are evident in epileptogenesis (Qureshi and Mehler, 2010; Younus and Reddy, 2017b), there has been little effort for drug discovery to target specific epigenetic mechanisms. *This epigenetic approach, however, is broader than typical antiseizure treatments and represents a mechanistic strategy for preventing TLE.* Histone acetylation is catalyzed by histone acetyltransferases, and histone deacetylation is carried out by histone deacetylases (HDACs). Generally, acetylation of histone lysine residues is associated with transcriptional activation (Verdone et al., 2005; Garcia-Bassets et al., 2007). Histone modifications can play a role in epilepsy (Younus and Reddy, 2017b). Therefore, interruption of the HDAC pathway may provide a targeted strategy to prevent epilepsy progression. Sodium butyrate is a broad inhibitor of multiple HDACs from classes 1, 2, and 7 (Gurvich et al., 2004; Pajak et al., 2007). It acts similarly to, but has a wider range than, sodium valproate, which inhibits HDACs from classes 1 and 2 only. Sodium butyrate, a low molecular weight volatile fatty acid that was originally discovered for prevention of cancer, is a specific inhibitor of HDAC activity and can affect the expression of many mammalian genes (Davie, 2003). Thus, butyrate is commonly used as specific epigenetic tool for interrupting HDAC signaling. Sodium butyrate crosses the blood-brain barrier and inhibits HDAC activity, inducing hyperacetylation of core histones that allow DNA binding of transcription factors to inhibit induction or repression of gene expression in the brain (Minamiyama et al., 2004; Deutsch et al., 2008; Kim et al., 2013; Takuma et al., 2014).

In the present study, we investigated the functional role of the HDAC pathway in experimental models of limbic epileptogenesis and TLE. Our results suggest that HDAC inhibition leads to a striking interruption of the development and persistence of epileptogenesis in a classic kindling model of TLE.

Materials and Methods

Animals. Young male wild-type C57BL/6 mice of 25 to 30g each were used in this study. All mice were housed individually in cages with access to food and water *ad libitum*. The mice were housed in an

environmentally controlled animal facility with a 12 h light/dark cycle. The animals were cared for in strict compliance with the guidelines outlined in the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*. All animal procedures were performed in a protocol approved by the university's Institutional Animal Care and Use Committee.

HDAC Activity Assay. To determine HDAC activity and its inhibition by sodium butyrate treatment, brain tissue samples were extracted from control and butyrate-treated (600 mg/kg, i.p.) mice. HDAC activity was measured from nuclear lysates using the Fluor-de-Lys HDAC activity assay kit (Biomol, Plymouth Meeting, PA), as reported previously (Nian et al., 2009; Rajendran et al., 2011). Briefly, incubation was performed at 37°C for 30-min with either cortex or hippocampal tissue lysates (15 µg protein/ well), and the HDAC reaction was initiated by the addition of Boc-Lys(Ac)-AMC substrate. After a trypsin developer was added, the mixture was incubated for another 15-min at room temperature. In some experiments, the protein extracts from cortical or hippocampal tissues were treated with trichostatin A, a standard HDAC inhibitor, and then samples were assayed for HDAC activity. Fluorescence was measured using a BioTek Synergy 2 plate reader, with 360-nm excitation and 460-nm emission. The data was normalized to samples containing trichostatin A. HDAC enzymes convert the acetylated substrate, Boc-Lys(Ac)-AMC, to a deacetylated product. The addition of a trypsin-containing developer allows the product to be converted into a quantifiable fluorophore. HDAC inhibitors, such as sodium butyrate, can inhibit such a reaction.

Classic Hippocampus Kindling Model of Epileptogenesis. To study the antiepileptogenic activity of sodium butyrate, we used the hippocampus kindling model, which is the best model of human temporal lobe epilepsy, characterized by progressive complex partial seizures with secondary generalization (Goddard et al., 1969; Reddy et al., 2010). A mild focal, nonconvulsant electrical stimulus to the hippocampus on a daily basis leads to the development of a kindled state exhibiting electrographic and behavioral seizures. In mouse kindling, the focal electroencephalogram afterdischarge (AD) models complex partial seizures, whereas the behavioral motor seizure stages 4 and 5 model generalized seizures. Electrode implantation and stimulation procedures for

mouse hippocampus kindling were performed as described previously (Reddy and Mohan, 2011; Reddy et al., 2012). Mice were anesthetized by an intraperitoneal injection of a mixture of ketamine (100 mg/kg) and xylazine (10 mg/kg). A twisted bipolar stainless-steel wire electrode (model MS303/1; Plastic Products, Roanoke, VA) was stereotaxically implanted in the right hippocampus (2.9 mm posterior and 3.0 mm lateral to bregma and 3.0 mm below the dorsal surface of the skull) using the Franklin and Paxinos atlas (Franklin and Paxinos, 1997) and anchored with dental acrylic to four jeweler's screws placed in the skull. A period of 7 to 10 days was allowed for recovery. The stimulation paradigm consisted of 1-ms duration, bipolar, square current pulses delivered at 60 Hz for 1s using a kindling stimulator (A-M Systems, Sequim, WA). The afterdischarge threshold (ADT) was determined by stimulating at 15-min intervals beginning with an intensity of 25 μA and increasing in steps of 25 μA until an AD was obtained. Stimulation on subsequent days used a stimulation intensity of 125% threshold value. The afterdischarge was recorded from the hippocampus electrode with a Grass CP511 preamplifier (Astro-Med, West Warwick, RI) and stored in digital form using Axoscope 8.1 software (Axon Instruments, Foster City, CA). AD duration was the total duration of hippocampal electrographic spike activity (amplitude >2x baseline) occurring in a rhythmic pattern at a frequency of 1 Hz. The day of ADT determination was considered day 0 of kindling. Stimulation was performed daily, and seizure activity after each stimulation was rated according to the criterion of Racine (1972) as modified for the mouse: stage 0, no response or behavior arrest; stage 1, chewing or head nodding; stage 2, chewing and head nodding; stage 3, forelimb clonus; stage 4, bilateral forelimb clonus and rearing; and stage 5, falling. Kindling stimulation was continued until stage 5 seizures were elicited on 3 consecutive days. Mice were used for the study until they consistently exhibited stage 5 seizures after stimulation, which is considered the "fully kindled" state.

Rapid Hippocampus Kindling Model of Epileptogenesis. Rapid kindling is a model of epileptogenesis that allows accelerated evaluation of experimental manipulations during the progression of epilepsy induction (Lothman and Williamson, 1993; Sankar et al., 2010). The rapid kindling procedure was similar to the conventional kindling described above except that stimulations were applied every 30 min until mice exhibited

consistent stage 5 seizures. This procedure has been used extensively as a model of compressed epileptogenesis as an alternative paradigm to the conventional kindling models (Sankar et al., 2010; Reddy and Mohan, 2011).

Motor Toxicity Test. Sodium butyrate and other test drugs were evaluated for motor toxicity by using a modification of the horizontal screen test as described previously (Reddy and Jian, 2010). Mice were placed on a horizontally oriented grid (consisting of parallel 1.5-mm diameter rods situated 1 cm apart), and the grid was inverted. Mice failing to remain on the screen for 60 seconds were considered failures due to excessive sedation or motor ataxia.

Brain Histological Timm Staining. Timm staining of mossy fiber sprouting is a standard histological index of epileptogenesis. Timm staining was conducted as described previously (Cavazos et al., 1991; Rao at al., 2006). Mice were deeply anesthetized with ketamine/xylazine mix and transcardially perfused with 75 mL 0.9% saline solution followed by 100 mL 1% sodium sulfide solution. Perfusion of 100 mL of 4% paraformaldehyde in 0.1 M phosphate buffer (7.4 pH) followed, and a final perfusion with 50 mL 1% sodium sulfide was carried out. The brain was dissected and postfixed in 4% paraformaldehyde overnight at 4˚C. Brains were then processed with phosphate buffer and sucrose treatment and cut in 20 μm transverse sections with a cryostat. Slices were then dry-mounted onto slides and allowed to dry overnight. Gum arabic, citrate buffer, hydroquinone, and silver lactate reagents were mixed mechanically on the day of staining. Mounted slides were uniformly soaked in the Timm stain in dark for 3.5 hours. Slides were then washed with distilled water and counter-stained with 0.1% cresyl violet, if desired. Slides were permanently fixed with DPX mountant (Sigma) and allowed to dry before imaging and analysis. Staining intensity for Timm histology was quantitatively measured by densitometric analysis. Densitometry was completed in hippocampus regions of interest with Photoshop software. Mean density of gray-scale staining was normalized to area and white background. Density scores were then non-parametrically graded using a linear scale.

Test Drugs and Treatment Protocols. There are many HDAC inhibitors, such as vorinostat, trichostatin-A, valproic acid, and sodium butyrate (Younus and Reddy, 2017b). Sodium butyrate was the focus of this study for its broad-spectrum HDAC inhibition (Ferrante et al., 2003; Deutsch et al. 2008; Takuma et al., 2014). Valproic acid, a widely used antiepileptic drug, was not used because it is a limited-spectrum HDAC inhibitor and hence offers comparatively limited disease-modification of epileptogenesis (Silver et al., 1991; Gurvich et al., 2004; Takuma et al., 2014). The overall experimental protocol for drug treatment and kindling stimulations is illustrated in Fig. 2. In the first kindling development study (Protocol #1), sodium butyrate (600 mg/kg) was injected intraperitoneally twice daily (9 A.M. and 5 P.M.) to maintain chronic HDAC inhibition. The dose of butyrate used in this study has been shown previously to significantly inhibit multiple HDACs and reliably increase the acetylation of H3 and H4 histones in the brain and other tissues (Ferrante et al., 2003; Takuma et al., 2014). Sodium butyrate has been shown to effectively inhibit downstream H4 and H4 histone signaling in cells in vitro and after administration in animals (Davie, 2003; Minamiyama et al., 2004). Our design consists of treated animals (n = 11) alongside an untreated control group (n = 10). Animals were stimulated daily in the morning and received their respective treatments daily until becoming fully kindled. Kindling, however, is a permanent phenomenon and an intense seizure can be elicited weeks or months after kindling development. Therefore, eight weeks after inducing stage 5 kindling, both groups of mice were tested for persistence of the kindling state by restimulation at the baseline ADT. Additionally, mice were also tested for retention through additional stimulations at 20 minute intervals, increasing current in 25 μA increments until the occurrence of stage 4 or 5 seizures. This protocol is henceforth referred to as "chronic treatment." In the second kindling development study (Protocol #2), sodium butyrate (600 mg/kg) was injected intraperitoneally twice daily (9 A.M. and 5 P.M.) for exactly 14 days, and animals were stimulated daily even after the end of drug treatment until they became fully kindled. These animals (n = 8) were treated alongside animals (n = 9) receiving the synthetic neurosteroid ganaxolone (0.5 or 1.0 mg/kg) 15 minutes prior to stimulation for 14 days, as well as a combination group (n = 9) receiving butyrate twice daily and ganaxolone 15 minutes prior to stimulation. Ganaxolone (GX, 3β-methyl-3α-hydroxy-5α-pregnan-20-one) is an allosteric modulator of GABA-A receptor with antiseizure properties (Reddy

and Rogawski, 2010). This protocol is henceforth referred to as "subchronic treatment." The final kindling study format (Protocol #3), involves two groups of animals stimulated daily without any drug treatment until achieving complete kindling. To test the retroactive effect of epigenetic treatment on epilepsy-susceptible mice, sodium butyrate (600 mg/kg, i.p.) was injected twice daily in the experimental group (n=17) for two weeks, during which time no stimulations were conducted. A control group (n=12) received no treatment during this time. At the end of the treatment period, ADTs were redetermined for all animals and all mice were rekindled until they exhibited consistent stage 5 seizures. In another test, as a measure of the drugs' acute seizure suppression, butyrate and/or ganaxolone were also administered as one-time pretreatments to previously kindled mice. In parallel studies, we tested the effect of the synthetic neurosteroid ganaxolone on kindling progression. GX is an effective antiseizure agent in the kindling model (Reddy and Rogawski 2010). Age-matched male mice (n = 7-10) were given sodium butyrate (300 or 600 mg/kg, i.p.) approximately 2 hours before stimulation, GX (0.5 or 1.0 mg/kg, s.c.) 15 minutes before stimulation, or both butyrate (600 mg/kg) and GX (0.5 mg/kg) before stimulation.

Data Analysis. Group data are expressed as the mean ± standard error of the mean (SEM). Differences in kindling seizure stages between groups were compared with the nonparametric Kruskal-Wallis test followed by the Mann-Whitney *U*-test. Comparison of means of the AD duration between groups was made with a one-way analysis of variance, followed by an unpaired two-tailed Student's *t*-test. Comparison of the percent animals exhibiting seizures and percent difference in seizure duration in various groups was made by Wilcoxon signed ranks test. In all statistical tests, the criterion for statistical significance was $p < 0.05$.

Results

Sodium Butyrate Inhibits HDAC Activity in the Brain. To determine the ability of sodium butyrate to inhibit HDAC activity, we performed HDAC activity assay in hippocampus and cortex samples from untreated control and butyrate-treated (600 mg/kg, i.p.) mice. HDAC activity assay was carried out by the Fluor-de-Lys HDAC activity assay kit, as described previously (Nian et al., 2009; Rajendran et al., 2011). Our results show that

JPET Fast Forward. Published on November 3, 2017 as DOI: 10.1124/jpet.117.244939
This article has not been copyedited and formatted. The final version may differ from this version.

HDAC activity in the hippocampus and cortex of mice treated with sodium butyrate was significantly (20-40%, $p<0.05$) reduced compared to that of control mice. Hippocampal HDAC activity in the butyrate group (1.0 ± 0.02 HDAC % control after normalization per μg protein) was significantly lower than in the control group (1.2 ± 0.06). Similarly, sodium butyrate significantly reduced HDAC activity in the cortex (1.1 ± 0.01 HDAC % control after normalization per μg protein) compared to the control group (1.5 ± 0.16). These results confirm a significant inhibition of HDAC activity in mice following sodium butyrate treatment, as consistent with earlier reports (Ferrante et al., 2003; Takuma et al., 2014).

Acute HDAC Inhibition Produces Little Change in Seizure Expression in Fully-kindled Mice. To determine the acute effect of HDAC inhibition by sodium butyrate on kindled seizures, we tested butyrate in a dose-dependent fashion in fully-kindled mice expressing stage 5 (generalized) seizures. The acute effect of sodium butyrate is shown in Fig. 3. Sodium butyrate pretreatment 1-h before stimulation in fully-kindled animals provided little or no seizure protection, as evident from the lack of seizure suppression by either dose of butyrate (Fig. 3A). Butyrate also did not significantly affect the mean afterdischarge (AD) duration (Fig. 3B). To determine the comparative and combination efficacy, we also tested a neurosteroid antiseizure agent ganaxolone (Reddy and Rogawski, 2010). Our results show that while neither a low dose of ganaxolone (0.5 mg/kg) or a combination treatment of butyrate and ganaxolone provided acute protection, a high dose of ganaxolone (1.0 mg/kg) displayed modest seizure protection (Fig. 3D). None of these treatments, however, significantly influenced AD duration (Fig. 3E). An important criterion to take into consideration when determining dose selection, however, is the significant animal behavioral sedation resulting from high dosage, which is not as prevalent in the selected doses of butyrate or ganaxolone (Fig. 3CF). In addition, we tested the effect of sodium butyrate on acute seizures at longer intervals between treatment and stimulation. Similar seizure outcomes resulted even 4 h after treatment, as all animals exhibited stage 4/5 seizures (n = 9/group). These results indicate that acute treatment with butyrate has no effect on seizure expression and motor behavior.

Chronic HDAC Inhibition by Sodium Butyrate Inhibits the Development of Limbic Epileptogenesis.

To determine the chronic effect of HDAC inhibition by sodium butyrate on kindled epileptogenesis progression, we administered butyrate twice daily to several animals, which were concurrently kindled with a control group (Fig. 2). The development of kindled seizures in control mice and mice treated daily with sodium butyrate (600 mg/kg) is shown in Fig. 4. There was no significant difference in mean AD threshold between the control and butyrate groups (Fig. 4B). Sodium butyrate produced marked impairment in the development of kindling epileptogenesis, most evident in the significant suppression of seizures at corresponding stimulation sessions compared to the control (Fig. 4C). Mean AD duration, however, was not significantly reduced (Fig. 4D). Mice treated with butyrate also showed a drastically decreased rate of kindling, measured by the number of stimulations required to elicit three consecutive stage 5 (generalized) seizures, in comparison to the control group (Fig. 4E, Table 1). These results indicate the striking epileptogenesis-delaying effect of persistent HDAC inhibition by butyrate treatment.

Chronic HDAC Inhibition by Sodium Butyrate Impairs Lasting Retention of Limbic Epileptogenesis.

To examine the long-term antiepileptogenic inhibition of sodium butyrate, both groups of animals were restimulated at their baseline AD threshold eight weeks after the initial chronic kindling to determine the strength of epileptogenic retention (Fig. 5). While the majority of control animals still displayed severe generalized seizures (stages 4 or 5), only a slight minority of animals treated with butyrate in the initial study showed such seizures, demonstrating butyrate's prolonged disease-modifying effect even without drug administration (Fig. 5A). Furthermore, during AD threshold redeterminations (Fig. 5B), butyrate-treated animals displayed significant seizure resistance compared to control animals at corresponding stimulation currents, and this butyrate group required 50 μA more current (a 33% increase) than the control group to achieve stage 5 seizures. Butyrate did not significantly affect the mean AD duration in this retention study (Fig. 5C). These results indicate that HDAC inhibition by sodium butyrate carries a long-lasting antiepileptogenic or disease-modification effect.

Subchronic HDAC Inhibition by Sodium Butyrate Inhibits the Development of Limbic Epileptogenesis. To observe the effects of sodium butyrate treatment and discontinuation within the same kindling cycle, we administered sodium butyrate to various subgroups, alongside a control group, for two weeks, and then allowed kindling to continue without treatment (Fig. 2). The development of kindled seizures in control mice and mice treated for 14 days with sodium butyrate (600 mg/kg) is shown in Fig. 6. There was no significant difference in mean AD threshold between any of the four groups in the study (Fig. 6E). During the fourteen days of injection, sodium butyrate strikingly inhibited the development of kindling epileptogenesis (Fig.7), seen prominently in the significantly reduced seizure stages at corresponding stimulation sessions compared to the control (Fig. 6A). Butyrate, however, did not significantly affect the mean AD duration overall (Fig. 6B). As consistent with the chronic group (Fig. 4), subchronic butyrate treatment led to complete retardation of kindling epileptogenesis progression beyond stage 2/3 (partial) seizures despite daily stimulation for 35 days (Fig. 6AB, Table 1), which indicates a true antiepileptogenic effect. To determine whether inhibition of HDAC confers synergistic effects when used in combination with an antiseizure drug, we sought to study the effect of the synthetic neurosteroid ganaxolone alone or in combination with butyrate. Treatments of ganaxolone (0.5 mg/kg), as well as the combination treatment of ganaxolone (0.5 mg/kg) and butyrate (600 mg/kg), induced similar levels of epileptogenic retardation as compared to butyrate group alone (Fig. 4C). Similarly, these treatments also did not significantly affect the mean AD duration (Fig. 6D). Expectedly, the antiepileptogenic butyrate and/or ganaxolone treatments significantly retarded the development of kindled seizures compared to the control (Fig. 6F), indicating a modest synergistic protection offered by the antiseizure ganaxolone.

Acute HDAC Inhibition Fails to Affect Epileptogenic-like Response in Rapid Kindling Epileptogenesis. To further elucidate the effective window of HDAC mechanisms in susceptibility to epileptogenesis (acute vs. chronic inhibition), we utilized the “rapid kindling” model of acute epileptogenesis, the process of epilepsy induction by stimulation every 30 min (acute), rather than the once-daily (chronic) stimulation implemented in the classic kindling model. Acute treatment with butyrate did not modify the rapid kindling progression, as evident by

the essentially identical seizure stage and AD duration curves of control and butyrate groups (Fig. 8A,B,C,D). A representative EEG discharge is shown in Fig. 8E. There was no significant difference in mean AD threshold between the control and butyrate groups (Fig. 8C). Furthermore, butyrate did not significantly affect the rate of kindling compared to the control, indicating the inability of butyrate treatment for acute HDAC inhibition to inhibit rapid epileptogenic development (Fig. 8D). These results are consistent with the evidently delayed effects of epigenetic interruption of epileptogenesis.

HDAC Inhibition Reverses Epileptogenic State in Fully-kindled Epileptic Animals. To determine any disease-modification effects of HDAC inhibition in animals which have already developed advanced stages of epilepsy, we administered butyrate in previously fully-kindled animals with stage 5 (generalized) seizures for checking epileptogenic reversal or erasal of epileptic state. Animals were fully kindled without any injected treatment until they show consistent stage 5 seizures, followed by a two week period of butyrate treatment (600 mg/kg, i.p., twice daily) and hiatus from stimulation (Fig. 2). On the sixth week, treatment was discontinued and individual AD threshold were redetermined. Animals were subsequently rekindled to full without treatment, identical in procedure to the initial kindling. Butyrate-treated mice displayed a significant reduction in seizure severity when tested the week following termination of butyrate therapy (Fig. 9A). During rekindling, butyrate-treated mice required significantly more stimulations to rekindle fully (Fig. 9A,C). Furthermore, AD duration among treated mice progressively increased, while AD durations of control mice remained relatively constant across stimulations (Fig. 9B). Thus, these results indicate that HDAC inhibition by butyrate treatment can promote significant unwiring or erasal of even prior chronic epileptogenic state ("curing epilepsy").

HDAC Inhibition Prevents Epileptogenic Mossy Fiber Axonal Sprouting in the Hippocampus. To determine whether HDAC inhibition by sodium butyrate treatment is associated with morphological changes in neuronal circuits involved in epileptogenesis, we examined the extent of mossy fiber sprouting in hippocampus samples isolated from control and butyrate-treated animals. The pathological role of mossy fiber sprouting in temporal lobe epileptogenesis is controversial because inhibition of mossy fiber sprouting may not reflect an

antiepileptogenic process (Heng et al., 2013). Nevertheless, mossy fiber sprouting is the most commonly-used morphological marker of epileptogenesis in experimental models and it provides valuable information on the impact of therapeutic interventions on aberrant sprouting of granule cell axons in the dentate gyrus. A characteristic histological sign of TLE is the loss of hilar cells in the dentate gyrus and mossy fiber sprouting. Mossy cells in the hippocampus are highly excitable. Mossy cells are bi-directionally connected through a positive feedback loop to granule cells, the principal cells of the dentate gyrus. This recurrent circuit is strategically placed between the entorhinal cortex and the CA3 region and thus potentially pro-convulsive and may promote epileptogenesis (Ratzliff et al., 2002). We observed a significant increase in mossy fiber sprouting in control, fully-kindled animals (Fig. 10A). Quantification of axonal sprouting within the dentate gyrus region by densitometry and staining intensity scoring revealed complete reversal of seizure-induced increase in mossy fiber sprouting in animals treated with butyrate (Fig. 10BCDE), suggesting possible disease-modification from HDAC inhibition. Butyrate when given in combination with ganaxolone also resulted in significant reduction in mossy fiber sprouting (Fig. 10AE), indicating further disease-modifying potential of the antiseizure treatment with ganaxolone.

DISCUSSION

The present study shows that targeted subchronic and chronic inhibition of HDACs can inhibit or stop the progression of epileptogenesis in experimental models of TLE. The key observations of this study are: (i) Daily treatment with the HDAC inhibitor sodium butyrate significantly retarded the development of kindling epileptogenesis without affecting AD activity; (ii) The persistence of this epileptic condition was also impaired in butyrate-treated animals; (iii) Subchronic butyrate treatment for 2 weeks led to complete retardation of epileptogenesis progression; (iv) Butyrate treatment carried a long-term antiepileptogenic retention, whether administered during or after kindling; (v) Butyrate treatment has little or no behavioral motor toxicity; (vi) Acute butyrate treatment did not affect rapid kindling epileptogenesis; (vii) Butyrate treatment facilitated a strong reduction in mossy fiber sprouting; (viii) A combination treatment of butyrate and an antiseizure neurosteroid

showed further disease-modification of epileptogenesis. Collectively, these results indicate that the HDAC signaling pathway mediates the development and persistence of epileptogenesis in an experimental model of TLE.

Epigenetic HDACs Signaling in Epilepsy. There is emerging evidence that epigenetic signaling plays a crucial role in the pathophysiology of epileptogenesis and chronic epilepsy (Qureshi and Mehler, 2010; Kobow and Blumcke, 2014; Younus and Reddy, 2017b). The main epigenetic mechanisms include histone modifications, DNA methylation, and miRNA-based transcriptional control (Fig. 1). The primary histone modification associated with epilepsy and other neural disorders is lysine acetylation at the histone tail (Kobow and Blumcke, 2014). Histone acetylation is catalyzed by histone acetyltransferases and histone deacetylation is carried out by histone deacetylases. As a result of these two opposing epigenetic modifications, chromatin is known to exist in two higher-order structures. Euchromatin refers to the loosely packaged and open form that is transcriptionally active whereas heterochromatin refers to the highly compact and transcriptionally inactive form (Kouzarides, 2007). HDACs play a crucial role in repressing gene transcription by condensing chromatin structure. Removal of the acetyl group increases the positive charge of the histone and confers a more compact state, characteristic of gene repression. HDACs can also remove acetyl moieties from transcription factors, thereby further suppressing gene activity (Morris et al., 2010). There are four classes of HDAC superfamilies, with classes 1 and 2 being the most relevant to epilepsy because they have the highest expression seen in the brain (Gray and Ekstrom, 2001; Carey and La Thangue, 2006). Thus, HDAC inhibitors are important tools for unraveling the pathology of epilepsy. There are many HDAC inhibitors, such as vorinostat, trichostatin-A, entinostat and sodium butyrate (Ververis et al., 2013). Sodium butyrate is a widely used for broad-spectrum HDAC inhibition and consequent augmentation of H3 and H4 acetylation in the brain (Ferrante et al., 2003; Deutsch et al. 2008;Takuma et al., 2014). In the present study, we presented convincing evidence that chronic but not acute sodium butyrate administration inhibited development of seizures in the kindling model of TLE. These findings are very significant and show that the epigenetic HDAC pathway represents a novel target for the development of new drugs disease-modifying capacity for the treatment and prevention of epilepsy. There is one minor caveat with

sodium butyrate. It is likely that these effects of sodium butyrate are unrelated to HDAC inhibition. For example, sodium butyrate is a short chain fatty acid used as an energy source and directly stimulates NF-KB transcription (Borthakur et al., 2008). However, this is highly unlikely given the significant mechanistic data on HDAC inhibition as assessed by HDAC activity in brain samples. Moreover, there is little evidence of NF-KB involvement in seizure susceptibility.

Subchronic and Chronic HDAC Inhibition Retards Epileptogenesis. In the present study, sodium butyrate treatment inhibited HDAC activity but produced no acute antiseizure effect in epileptic animals. Similarly, butyrate treatment was ineffective in reducing the development of rapid kindling. These outcomes were expected given the longer time necessary for the HDAC inhibitor to affect enough gene networks and synaptic processes to manifest in significant protection. During chronic treatment, butyrate significantly hindered the rate of kindling epileptogenesis without affecting afterdischarge progression. The extent of protection, however, was similar to that of antiseizure ganaxolone treatment given fifteen minutes before each stimulation. Although both treatments produced similar protective effects against epileptogenesis, sodium butyrate shows exceptionally long-lasting protection (disease-modification), whereas ganaxolone only temporarily suppressed the seizure occurrence. To delineate the difference between the modes of action of HDAC inhibition, we also performed a subchronic study, wherein animals received butyrate for 2 weeks only, but continued to get stimulations until they reached the epileptogenic state. During the treatment window, both butyrate and ganaxolone provided significant hindrance of the kindling process. When treatment was discontinued, however, epileptogenic progression rapidly rose in ganaxolone-only groups, while all butyrate-treated groups saw chronic seizure protection even after treatment was discontinued. These results suggest that the induction and persistence of limbic epileptogenesis are markedly reduced by HDAC activity inhibition by butyrate. Focal electrographic AD is required for induction of epileptogenesis in the kindling model (Goddard et al., 1969; Albertson et al., 1984). The delayed kindling development after HDAC inhibition is not due to differences in focal electrographic seizures because the duration of evoked AD was similar among control and butyrate groups during the early stimulation session. However, the

JPET Fast Forward. Published on November 3, 2017 as DOI: 10.1124/jpet.117.244939
This article has not been copyedited and formatted. The final version may differ from this version.

progressive increase in duration of the AD activity with successive stimulations was significantly attenuated in the butyrate group. Since butyrate did not affect rapid kindling progression, the structural and functional circuits that are required to exhibit behavioral and electrographic seizures are not affected by acute HDAC inhibition. Thus, the complex plasticity that is required for transforming a normal brain to an epileptic brain in the kindling model is attenuated by chronic inhibition of HDAC activity.

HDAC Inhibition Intercepts and Erases the Epileptic State. The ultimate goal of epilepsy therapies is curing epilepsy, with complete erasal of the epileptogenic state from the brain, so that additional treatments are not needed. This goal has not been met with success so far, since there has been no drug that can truly prevent epilepsy. To our knowledge, no compounds have been identified that can erase or promote un-wiring of epileptogenic state. Unexpectedly, our studies uncovered that butyrate has the potential to reverse or erase the hyperexcitability state of epileptic animals. In fully-kindled epileptic animals, butyrate treatment and discontinuation caused seizure severity to actually decrease rather than remain at an epileptic steady-state. This dramatic outcome indicates that butyrate treatment modified the persistence of the epileptogenic state, a phenomenon called reversal or erasal of the disease. The potential mechanisms underlying such erasal or regression of epilepsy remain elusive. Epigenetic HDAC inhibition by butyrate could potentially limit the proliferation of genes involved in structural aspects of neuronal circuitry involved in seizure generation or propagation; this could reduce the severity of future epileptogenic response or facilitate “un-wiring” of hyperexcitable neuronal networks in epilepsy. Further studies are warranted to advance this novel phenomenon with other HDAC inhibitors.

HDAC Inhibition with Concurrent Antiseizure Drug Promotes Further Modification of Epileptogenesis. Neurosteroids are powerful allosteric modulators of tonic inhibition with strong antiseizure properties (Reddy, 2010; Reddy and Estes, 2016). A combination of both epigenetic and neurosteroid treatments could provide the “best of both worlds” with strong acute action and effective long-term antiepileptogenic management. Neurosteroids have potent seizure suppression when administered shortly before stimulation, while

an epigenetic agent such as sodium butyrate offers long-term inhibition of the kindling process. In the present study, co-administration of butyrate and ganaxolone drastically slowed progress of epileptogenesis when injected daily alongside stimulations. This result persisted even after withdrawal of both drugs, with the combination treatment displaying a lasting seizure protection more potent than that of butyrate or ganaxolone alone. A combination therapy of butyrate and ganaxolone facilitated effective disease-modification as reflected by nearly complete prevention of hippocampus mossy fiber sprouting, which is a hallmark morphological index of epileptogenesis.

Therapeutic Implications of HDAC Inhibition for Curing Epilepsy. Currently, there is no known treatment that can prevent epilepsy from developing in those individuals at risk. A variety of HDAC inhibitors are currently being used in clinical trials for cancer and neurological diseases. They hold promising potential as effective epileptogenic inhibitors. This targeted strategy has many advantages, including a wide availability of clinically-approved HDAC inhibitors, a concrete strategy based on the pathogenic mechanism involved in epileptogenesis, and the potential for preventative, long-term protection rather than symptomatic treatment. Epigenetic HDAC intervention appears a rational strategy for preventing epilepsy because it targets the primary pathway that initially triggers the numerous downstream neuronal events mediating epileptogenesis. The mechanisms underlying epigenetic regulation of epileptogenesis remain unclear, but appear to be complex and multifaceted (Walker et al., 2002; Reddy et al., 2010; Reddy and Mohan, 2011). Neuronal injury activates diverse signaling events, such as inflammation, apoptosis, neurogenesis and synaptic plasticity, which eventually lead to structural and functional changes in neuronal circuits (Löscher, 2002; Pitkänen et al., 2009). These changes are eventually manifested as abnormal network hyperexcitability and spontaneous seizures. HDACs, as upstream regulators of transcription of genes, can dynamically regulate a variety of downstream signaling pathways involved in epileptogenesis, including gene networks that govern the circuit excitability and synaptic function (Crosio et al., 2003; Dong et al., 2007; Jessberger et al., 2007; Younus and Reddy, 2017b). The target mRNA and protein abundance assays are being performed to uncover the antiepileptogenic mechanism underlying the HDAC inhibition. Specific HDAC components may serve as valid biomarkers for epileptogenesis monitoring. One of the

mechanisms of the ketogenic diet, which also has antiepileptogenic activity, is thought to be based on HDAC inhibition via β-hydroxybutyrate (Wang et al., 2017; Moreno and Mobbs, 2017). These findings are consistent with our findings on HDAC interruption of the antiepileptogenic process. Thus, the HDAC inhibitors may hold great potential as antiepileptogenic agents for curing epilepsy. Other epigenetic mechanisms, including DNA methylation, miRNA-based transcriptional control, and bromodomain activity, may alter epileptogenesis by exerting their regulatory effects on neuronal plasticity (Younus and Reddy, 2017b). Although such targets represent alternatives for antiepileptogenic interventions, but there are currently few approved drugs with acceptable safety profiles for experimental investigations.

In conclusion, the powerful inhibition of kindling epileptogenesis progression under both subchronic and chronic HDAC inhibition by sodium butyrate protocols suggests a critical role of the epigenetic HDAC pathway in the development and persistence of epilepsy. This was associated with reduced neuronal fiber sprouting in the hippocampus. The HDAC inhibition caused a long-lasting disease-modification and strikingly reversed the kindling epileptogenic state, which is a unique aspect of epigenetic targeting compared to traditional antiseizure treatments. These findings, therefore, envisage a novel therapy for preventing or curing epilepsy by targeting specific epigenetic HDAC pathways in the brain.

Acknowledgments

We thank Daniel Jones and Amir Bhimani for their outstanding kindling assistance and to Xin Wu and Victoria Golub for their help with HDAC assay. The authors declare no competing financial interests.

AUTHORSHIP CONTRIBUTIONS

Participated in research design: Reddy and Reddy

Conducted experiments: Reddy, Clossen and Reddy

Performed data analysis: Reddy and Reddy

Wrote or contributed to the writing of the manuscript: Reddy and Reddy

References

Albertson TE, Joy RM, and Stark LG (1984) A pharmacological study in the kindling model of epilepsy. *Neuropharmacology* **23**:1117−1123.

Borthakur A, Saksena S, Gill RK, Alrefai WA, Ramaswamy K, and Dudeja PK (2008) Regulation of monocarboxylate transporter 1 (MCT1) promoter by butyrate in human intestinal epithelial cells: involvement of NF-kappaB pathway. *J Cell Biochem* **103**:1452-1463.

Brandt C, Glien M, Gastens AM, Fedrowitz M, Bethmann K, Volk HA, Potschka H, and Löscher W (2007) Prophylactic treatment with levetiracetam after status epilepticus: lack of effect on epileptogenesis, neuronal damage, and behavioral alterations in rats. *Neuropharmacology* **53**: 207-221.

Carey N and La Thangue NB (2006) Histone deacetylase inhibitors: gathering pace. *Curr Opin Pharmacol* **6**: 369-75.

Cavazos JE, Golarai G, and Sutula TP (1991) Mossy fiber synaptic reorganization induced by kindling: time course of development, progression, and permanence. *J Neurosci* **11**:2795–2803.

Clossen BL and Reddy DS (2017) Novel therapeutic approaches for disease-modification of epileptogenesis for curing epilepsy. *Biochim Biophys Acta* **1863**: 1519-1538

Crosio C, Heitz E, Allis CD, Borrelli E, and Sassone-Corsi P (2003) Chromatin remodeling and neuronal response: multiple signaling pathways induce specific histone H3 modifications and early gene expression in hippocampal neurons. *J Cell Sci* **116**(Pt 24):4905-4914.

Davie JR (2003) Inhibition of histone deacetylase activity by butyrate. *J Nutr* **133**(7 Suppl):2485S-2493S.

Deutsch SI, Rosse RB, Long KD, Gaskins BL, Burket JA, and Mastropaolo J (2008) Sodium butyrate, an epigenetic interventional strategy, attenuates a stress-induced alteration of MK-801's pharmacologic action. *Eur Neuropsychopharmacol* **18**:565-568.

Dong E, Guidotti A, Grayson DR, and Costa E (2007) Histone hyperacetylation induces demethylation of reelin and 67-kDa glutamic acid decarboxylase promoters. *Proc Natl Acad Sci USA* **104**: 4676–4681.

Ferrante RJ, Kubilus JK, Lee J, Ryu H, Beesen A, Zucker B, Smith K, Kowall NW, Ratan RR, Luthi-Carter R, and Hersch SM (2003) Histone deacetylase inhibition by sodium butyrate chemotherapy ameliorates the neurodegenerative phenotype in Huntington's disease mice. *J Neurosci* **23**: 9418-9427.

Franklin KBJ and Paxinos G (1997) The Mouse Brain in Stereotaxic Coordinates, 1st ed, Academic Press, New York.

Garcia-Bassets I, Kwon YS, Telese F, Prefontaine GG, Hutt KR, Cheng CS, Ju BG, Ohgi KA, Wang J, Escoubet-Lozach L, Rose DW, Glass CK, Fu XD, and Rosenfeld MG. (2007) Histone methylation dependent mechanisms impose ligand dependency for gene activation by nuclear receptors. *Cell* **128**: 505–518.

Goddard GV, McIntyre DC, and Leech CK (1969) A permanent change in brain function resulting from daily electrical stimulation. *Exp Neurol* **25**:295−330.

Gorter JA, van Vliet EA, and Lopes da Silva FH (2016) Which insights have we gained from the kindling and post-status epilepticus models? *J Neurosci Methods* **260**: 96-108.

Gray SG and Ekström TJ (2001) The human histone deacetylase family. *Exper Cell Res* **262**: 75-83.

Gurvich N, Tsygankova OM, Meinkoth JL, and Klein PS (2004) Histone deacetylase is a target of valproic acid-mediated cellular differentiation. *Cancer Res* **64**:1079-1086.

He X-P, Kotloski R, Nef S, Luikart BW, Parada LF, and McNamara JO (2004) Conditional deletion of TrkB but not BDNF prevents epileptogenesis in the kindling model. *Neuron* **43**:31−42.

Heng K, Haney MM, and Buckmaster PS (2013) High-dose rapamycin blocks mossy fiber sprouting but not seizures in a mouse model of temporal lobe epilepsy. *Epilepsia* **54**:1535–1541.

Hesdorffer DC, Beck V, Begley CE, Bishop ML, CushnerWeinstein S, Holmes GL, Shafer PO, Sirven JI, and Austin JK (2013) Research implications of the Institute of Medicine Report, epilepsy across the spectrum: promoting health and understanding. *Epilepsia* **54**: 207-16.

Jacobs MP, Leblanc GG, Brooks-Kayal A, Jensen FE, Lowenstein DH, Noebels JL, Spencer DD, and Swann JW (2009) Curing epilepsy: progress and future directions. *Epilepsy Behav* **14**:438−445.

Jessberger S, Nakashima K, Clemenson GD Jr, Mejia E, Mathews E, Ure K, Ogawa S, Sinton CM, Gage FH, and Hsieh J (2007) Epigenetic modulation of seizure-induced neurogenesis and cognitive decline. *J Neurosci* 27: 5967–5975.

Kim SW, Hooker JM, Otto N, Win K, Muench L, Shea C, Carter P, King P, Reid AE, Volkow ND, and Fowler JS (2013) Whole-body pharmacokinetics of HDAC inhibitor drugs, butyric acid, valproic acid and 4-phenylbutyric acid measured with carbon-11 labeled analogs by PET. *Nucl Med Biol* **40**:912-918.

Kobow K and Blumcke I (2011) The methylation hypothesis: do epigenetic chromatin modifications play a role in epileptogenesis? *Epilepsia* **52**: 15-9.

Kouzarides T (2007) Chromatin modifications and their function. *Cell* **128**: 693-705.

Löscher W (2002) Animal models of epilepsy for the development of antiepileptogenic and disease-modifying drugs. A comparison of the pharmacology of kindling and post-status epilepticus models of temporal lobe epilepsy. *Epilepsy Res* **50**:105−123.

Löscher W, Hönack D, and Rundfeldt C (1998) Antiepileptogenic effects of the novel anticonvulsant levetiracetam (ucb L059) in the kindling model of temporal lobe epilepsy. *J Pharmacol Exp Ther* **284**: 474-479.

Lothman EW and Williamson JM (1993) Rapid kindling with recurrent hippocampal seizures. *Epilepsy Res* **14**:209−220.

Moreno CL and Mobbs CV (2017) Epigenetic mechanisms underlying lifespan and age-related effects of dietary restriction and the ketogenic diet. *Mol Cell Endocrinol* **455**:33-40.

McNamara JO, Morrisett R, and Nadler JV (1992) Recent advances in understanding mechanisms of the kindling model. *Adv Neurol* **57**:555−560.

Minamiyama M, Katsuno M, Adachi H, Waza M, Sang C, Kobayashi Y, Tanaka F, Doyu M, Inukai A, and Sobue G (2004) Sodium butyrate ameliorates phenotypic expression in a transgenic mouse model of spinal and bulbar muscular atrophy. *Hum Mol Genet* **13**:1183-1192.

Morris MJ, Karra AS, and Monteggia LM (2010) Histone deacetylases govern cellular mechanisms underlying behavioral and synaptic plasticity in the developing and adult brain. *Behav Pharmacol* **21**:409-419.

Nian H, Bisson WH, Dashwood WM, Pinto JT, and Dashwood RH (2009) Alpha-keto acid metabolites of organoselenium compounds inhibit histone deacetylase activity in human colon cancer cells. *Carcinogenesis* **30**:1416-1423.

Pajak B, Orzechowski A, and Gajkowska B (2007) Molecular basis of sodium butyrate-dependent proapoptotic activity in cancer cells. *Adv Med Sci* **52**:83-88.

Pitkanen A and Lukasiuk K (2011) Mechanisms of epileptogenesis and potential treatment targets. *Lancet Neurol* **10**: 173-186.

Pitkänen A, Nehlig A, Brooks-Kayal AR, Dudek FE, Friedman D, Galanopoulou AS, Jensen FE, Kaminski RM, Kapur J, Klitgaard H, Löscher W, Mody I, and Schmidt D (2013) Issues related to development of antiepileptogenic therapies. *Epilepsia* **54**(Suppl 4):35-43.

Qureshi IA and Mehler MF (2010) Epigenetic mechanisms underlying human epileptic disorders and the process of epileptogenesis. *Neurobiol Dis* **39**:53-60.

Qureshi IA and Mehler MF (2010) Genetic and epigenetic underpinnings of sex differences in the brain and in neurological and psychiatric disease susceptibility. *Prog Brain Res* **186**:77-95.

Racine RJ (1972) Modification of seizure activity by electrical stimulation. II. Motor seizure. *Electroencephalogr Clin Neurophysiol* **32**:281–294.

Rajendran P, Delage B, Dashwood WM, Yu TW, Wuth B, Williams DE, Ho E, and Dashwood RH (2011) Histone deacetylase turnover and recovery in sulforaphane-treated colon cancer cells: competing actions of 14-3-3 and Pin1 in HDAC3/SMRT corepressor complex dissociation/reassembly. *Mol Cancer* **10**(68): 1-18.

Rao MS, Hattiangady B, Reddy DS, and Shetty AK (2006) Hippocampal neurodegeneration, spontaneous seizures, and mossy fiber sprouting in the F344 rat model of temporal lobe epilepsy. *J Neurosci Res* **83**:1088-105.

Ratzliff AD, Santhakumar V, Howard A, and Soltesz I (2002) Mossy cells in epilepsy: rigor mortis or vigor mortis? *Trends Neurosci* **25**:140-144.

Ravizza T, Balosso S, and Vezzani A (2011) Inflammation and prevention of epileptogenesis. *Neurosci Lett* **497**: 223-230.

Reddy DS (2010) Neurosteroids: endogenous role in the human brain and therapeutic potentials. *Prog Brain Res* **186**: 113-137

Reddy DS and Mohan A (2011) Development and persistence of limbic epileptogenesis are impaired in mice lacking progesterone receptors. *J Neurosci* **31**:650–658.

Reddy DS and Rogawski MA (2010) Ganaxolone suppression of behavioral and electrographic seizures in the mouse amygdala kindling model. *Epilepsy Res* **89**: 254-260.

Reddy DS and Estes WA (2016) Clinical Potential of Neurosteroids for CNS Disorders. *Trends Pharmacol Sci* **37**:543-561.

Reddy DS, Gangisetty O, and Briyal S (2010) Disease-modifying activity of progesterone in the hippocampus kindling model of epileptogenesis. *Neuropharmacology* **59**: 573-581.

Reddy DS and Jian K (2010) The testosterone-derived neurosteroid androstanediol is a positive allosteric modulator of GABA-A receptors. *J Pharmacol Exp Ther* **334**:1031-1041.

Sankar R, Auvin S, Kwon YS, Pineda E, Shin D, and Mazarati A (2010) Evaluation of development-specific targets for antiepileptogenic therapy using rapid kindling. *Epilepsia* **51**(Suppl.3):39−42.

Silver JM, Shin C, and McNamara JO (1991) Antiepileptogenic effects of conventional anticonvulsants in the kindling model of epilepsy. *Ann Neurol* **29**:356−363.

Takuma K, Hara Y, Kataoka S, Kawanai T, Maeda Y, Watanabe R, Takano E, Hayata-Takano A, Hashimoto H, Ago Y, and Matsuda T (2014) Chronic treatment with valproic acid or sodium butyrate attenuates novel object recognition deficits and hippocampal dendritic spine loss in a mouse model of autism. *Pharmacol Biochem Behav* **126**:43-49.

Verdone L, Caserta M, and Di Mauro E (2005) Role of histone acetylation in the control of gene expression. *Biochem Cell Biol* **83**: 344–353.

Ververis K, Hiong A, Karagiannis TC, and Licciardi PV (2013) Histone deacetylase inhibitors (HDACIs): multitargeted anticancer agents. *Biologics* **7**: 47-60.

Walker MC, White HS, and Sander JW (2002) Disease modification in partial epilepsy. *Brain* **125**:1937-1950.

Wang X, Wu X, Liu Q, Kong G, Zhou J, Jiang J, Wu X, Huang Z, Su W and Zhu Q (2017) Ketogenic metabolism inhibits histone deacetylase (HDAC) and reduces oxidative stress after spinal cord injury in rat. *Neuroscience* (in press).

Younus I and Reddy DS (2017a) A resurging boom in new drugs for epilepsy and brain disorders. *Expert Rev Clin Pharmacol* **23**:1-19.

Younus I and Reddy DS (2017b) Epigenetic interventions for epileptogenesis: A new frontier for curing epilepsy. *Pharmacol Ther* **177**: 108–122.

FOOTNOTES

This work was partly supported by the Office of the Assistant Secretary of Defense for Health Affairs through the FY 2015 Epilepsy Research Program under Award #W81XWH-16-1-0660 (to D.S.R.). Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Department of Defense.

Figure Legends

Fig. 1. Epigenetic pathway and HDAC sites of sodium butyrate's action. Sodium butyrate inhibits the histone deacetylase 1, 2, and 7 enzymes, thus interfering with chromatin structure and reducing expression of genes involved in epileptogenesis by influencing several DNA transcription factors to regulate gene expression during transcription in the brain. Modified with permission from (Qureshi and Mehler, 2010).

Fig. 2. Overall experimental design. In protocol #1, animals were treated with butyrate (600 mg/kg, i.p., twice daily) until they achieved the fully kindled state, exhibiting consistent stage 5 seizures on three consecutive days. In protocol #2, animals were treated with butyrate (600 mg/kg, i.p., twice daily), ganaxolone (GX, 0.5 mg/kg, s.c., 15 minutes prior to stimulation), or a combination of both drugs for 2 weeks, followed by a drug-free withdrawal period until fully kindled. In protocol #3, animals were fully kindled without any injected treatment, followed by a two week period of butyrate treatment (600 mg/kg, i.p., twice daily) and hiatus from stimulation. On the sixth week, treatment was discontinued and individual ADTs were redetermined. Animals were subsequently rekindled to full without treatment, identical in procedure to the initial kindling.

Fig. 3. HDAC inhibition by butyrate produces little acute seizure suppression and motor activity in epileptic mice. (A) Sodium butyrate given one hour before stimulation in fully-kindled mice fails to reduce severity of acute seizure expression. (B) Afterdischarge (AD) duration was similar between treated and control groups. (C) Limited effect of sodium butyrate on motor ataxia. Mice were allowed to climb on an inverted screen, and time before falling was monitored. Animals failing to remain on the screen for at least 60 seconds were considered failures due to sedation or motor ataxia. (D) Slight acute effect of ganaxolone (0.5 & 1 mg/kg, sc) and/or butyrate on behavioral seizure expression in fully-kindled mice. (E) None of the treatment groups displayed a significant change in AD duration. (F) Mild effect of ganaxolone on motor ataxia in mice. All values represent the mean ± SEM (n = 9 to 15 mice/group).

Fig. 4. Chronic butyrate treatment inhibits the development of kindling epileptogenesis in mice. (A) A comparison of electrographic AD traces between one randomly selected mouse from both the butyrate and control groups on day 12 of daily kindling stimulations. The butyrate-treated animal exhibited an AD that was similar but smaller in amplitude than the control group. (B) The mean AD threshold was similar for both groups. (C) Chronic sodium butyrate (600 mg/kg, i.p.) treatment twice daily significantly retarded kindling development. (D) AD duration was similar in both groups. (E) Rate of kindling was significantly slowed in the butyrate group as

JPET Fast Forward. Published on November 3, 2017 as DOI: 10.1124/jpet.117.244939
This article has not been copyedited and formatted. The final version may differ from this version.

compared to the control group. All values represent the mean ± SEM (n = 8 mice/group). *p<0.05 vs. control group.

Fig. 5. Butyrate treatment shows prolonged disease- modification long after initial treatment in epileptic mice. (A) Eight weeks after the original study in Figure 2, animals of both groups were re-stimulated at their past AD threshold values. Animals from the sodium butyrate cohort displayed marked resistance for induction of generalized seizures. (B) Animals of both groups were subjected to increased stimulation currents until they elicited stage 5 seizures. Animals previously treated with butyrate required significantly greater currents to display the corresponding stage seizures as compared to the control. (C) Animals from the butyrate cohort displayed reduced AD durations during initial stimulations. All values represent the mean ± SEM (n = 6−8 mice/group). *p<0.05 vs. control group.

Fig. 6. Subchronic butyrate treatment inhibits the development of kindling epileptogenesis in mice. (A) Sodium butyrate (600 mg/kg, i.p.) treatment twice daily for 14 days (dotted line) greatly retarded kindling development. This antiepileptogenic effect persisted even during the drug-free period. (B) AD duration was similar in both groups for the first 17 days, after which butyrate-treated animals demonstrated notably shortened AD durations. (C) Subchronic ganaxolone (GX, 0.5 mg/kg, s.c.) 15 minutes before stimulation markedly slowed kindling development. At the beginning of the drug-free period, however, mice treated with ganaxolone displayed a sharp increase in seizure severity. (D) The GX group did not show significant reduction in mean AD duration in comparison to the control group for the first 18 days, but displayed significant AD duration reduction in the remaining days. (E) The mean AD threshold was similar for the control, butyrate only, GX only, and GX-butyrate combination groups. (F) Rate of kindling was significantly delayed in all treated groups as compared to the control group. For butyrate-treated animals that failed to show stage 4/5 seizures, a cut-off value of 35 was assigned for comparative analysis. All values represent the mean ± SEM (n = 9 mice/group). *p<0.05 vs. control group.

Fig. 7. Comparison of the rate of kindling developing during subchronic treatment and following withdrawal period in mice. Mean seizure stage values from stimulations 1 through 14 or stimulation 14 through stage 5 kindling (Fig. 6A and 6C) were fit to the linear function $S_n=R_n+A$, where S_n is the mean seizure stage value for the nth stimulation, R is the rate of kindling, and A is set equal to either 0 (stimulations 1 through 14) or S_{14} (stimulations 14 to stage 5 kindling). The rate of kindling R_n for each of the four groups is depicted above. The control group exhibited no significant kindling difference before and after the two-week cutoff, while all

treatment groups demonstrated drastically increased rate of kindling following withdrawal of treatment, though still slower in rate than that of the control group. Values represent the mean ± SEM. *p<0.05 vs. control group.

Fig. 8. Acute butyrate treatment fails to affect rapid kindling epileptogenesis in mice. (A) Butyrate (600 mg/kg, i.p.) was given 1 hr prior to stimulation sessions in the designated group. Butyrate-treated mice failed to display any retardation of rapid kindling development. (B) AD duration was also similar in both groups. (C) AD threshold was similar in both groups. (D) The rate of rapid kindling development, expressed as mean number of stimulations to achieve kindling stages 1 to 5 seizures, was similar in both groups. (E) A comparison of electrographic AD activity between one randomly selected mouse from both the butyrate and control groups on stimulation 8 showing similar duration and stage 2/3 seizures. Values represent the mean ± SEM (n = 6–9 mice per group).

Fig. 9. Fully-kindled epileptic animals treated with butyrate exhibit kindling reversal (curing epilepsy). Previously-kindled mice expressing stage 4/5 (generalized) seizures were given daily butyrate treatment for two weeks. At the end of the treatment period, animals were rekindled to full. (A) Butyrate-treated mice displayed significant reduction in seizure severity during rekindling, in addition to requiring significantly greater stimulations to fully kindle again. (B) In contrast to the relatively steady AD durations shown in control animals, butyrate-treated mice exhibited progressively longer AD durations with further stimulations. (C) Although both groups required a similar number of stimulations to reach stage 1 and 2 seizures, butyrate-treated animals required significantly more stimulations to reach seizures of stages 3, 4, and 5. *p<0.05 vs. control group.

Fig. 10. HDAC inhibition reduces epileptogenic mossy fiber sprouting in the hippocampus in epileptic mice. At the completion of chronic treatment experiments, animals from each kindling group were perfused and brain slices from the hippocampus were prepared. Slices were then Timm stained and fiber sprouting was quantified through densitometric analysis. (A) The top row of images is an expanded view of the entire hippocampus, while the bottom row is a 5X zoom onto the dentate hilus (DH) subfield. (B) Butyrate treatment significantly limited fiber sprouting in the hippocampus during kindling. (C) Butyrate treatments greatly reduced fiber sprouting in the DH subfield. (D) Butyrate and ganaxolone (GX) treatments reduced fiber sprouting in the CA3, but did not prevent the bulk of sprouting. (E) Butyrate and/or GX treatment during kindling significantly reduced Timm scores in the hippocampus, but butyrate treated groups showed greater reduction than the GX group. *p<0.05 vs. naïve (non-kindled) control group; #p<0.05 vs. kindling (untreated) group.

JPET Fast Forward. Published on November 3, 2017 as DOI: 10.1124/jpet.117.244939
This article has not been copyedited and formatted. The final version may differ from this version.

TABLE 1.

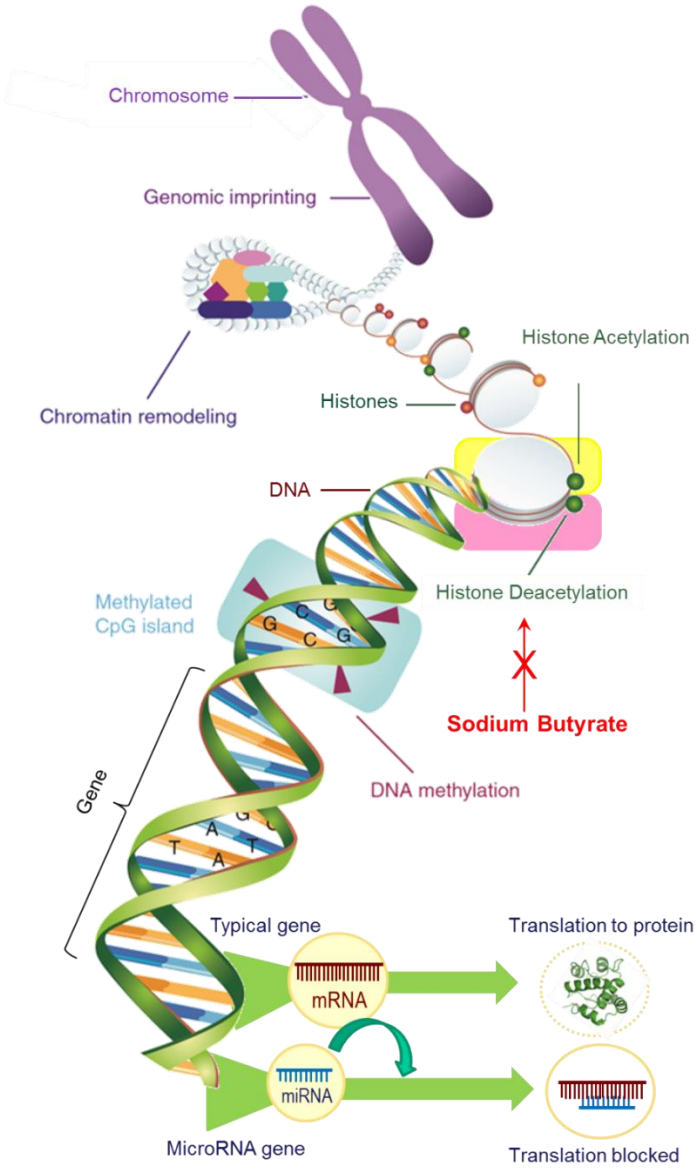
Mean number of stimulations to achieve kindling stages in vehicle-treated control and butyrate-treated mice.

	Vehicle	Butyrate Treatment	
		Chronic	*Subchronic*
Stage 1	1.9 ± 0.31	2.3 ± 0.19	1.4 ± 0.26
Stage 2	3.4 ± 0.51	14.2 ± 1.54*	15.3 ± 2.51*
Stage 3	7.5 ± 0.68	15.8 ± 1.71*	16.7 ± 2.43*
Stage 4	10.3 ± 0.89	18.4 ± 1.40*	22.5 ± 3.22*
Stage 5	12.3 ± 0.92	20.3 ± 1.47*	23.5 ± 3.30*

Values represent mean ± SEM of number of stimulation values derived from the experiments in Figs. 5C, 6A, and 8C. *$p<0.05$ vs. vehicle control.

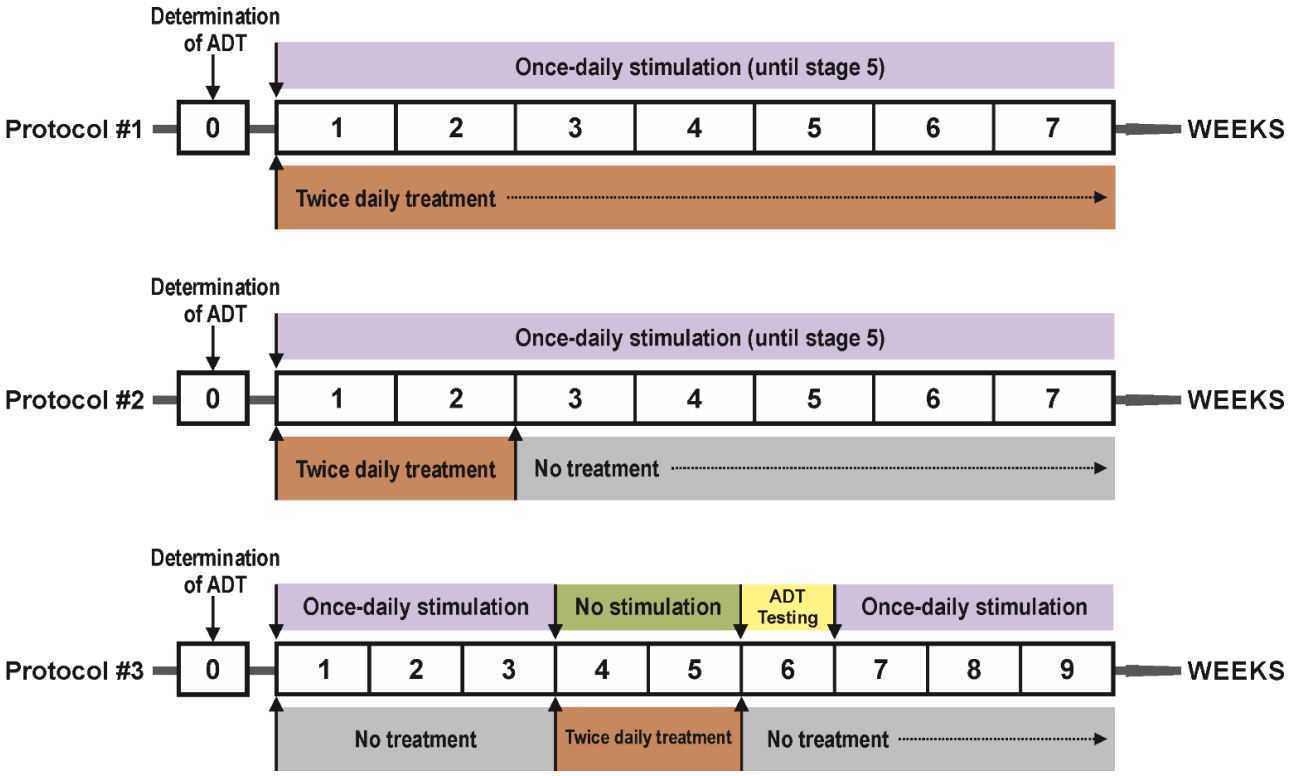
JPET Fast Forward. Published on November 3, 2017 as DOI: 10.1124/jpet.117.244939
This article has not been copyedited and formatted. The final version may differ from this version.

Figure-1



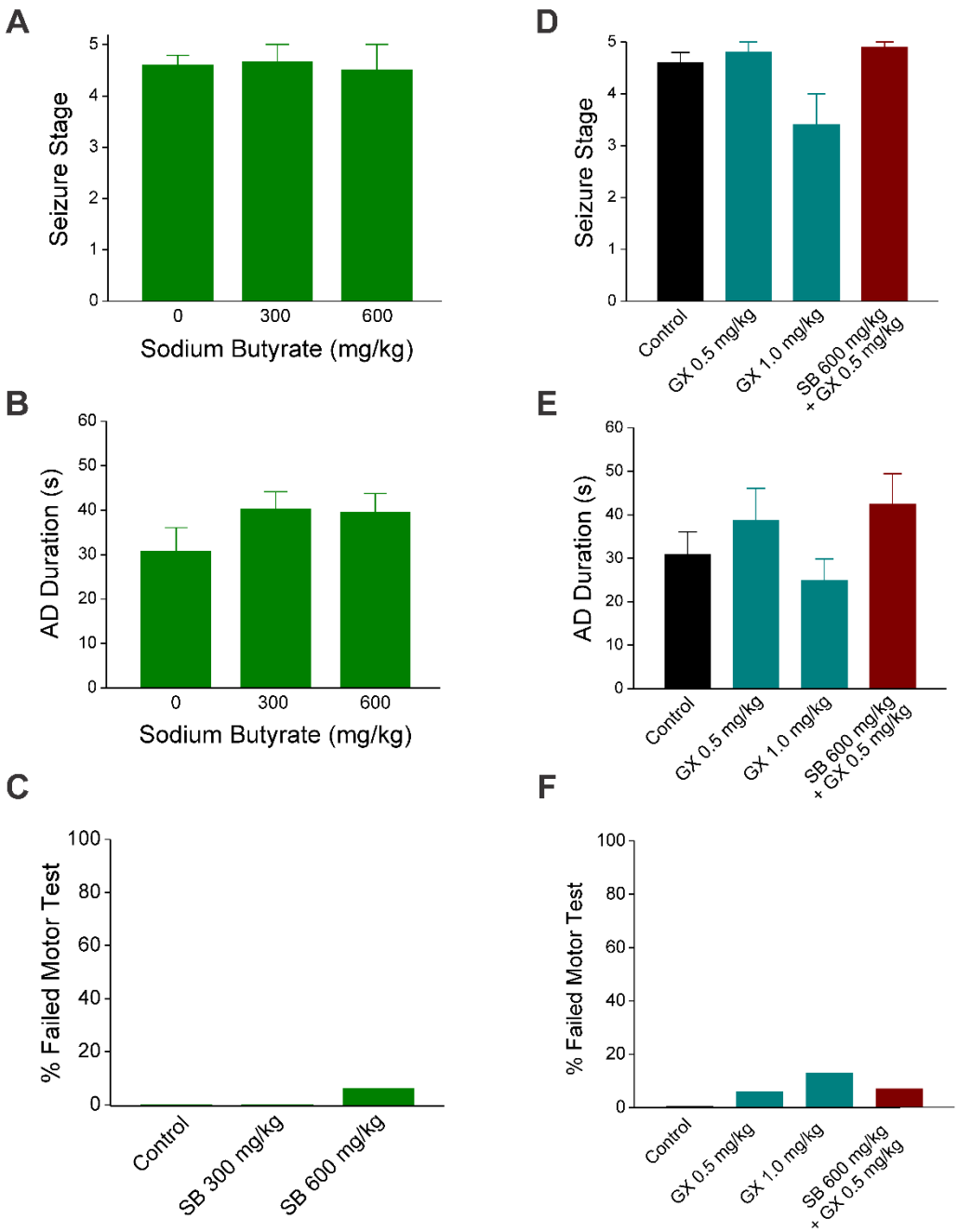
JPET Fast Forward. Published on November 3, 2017 as DOI: 10.1124/jpet.117.244939
This article has not been copyedited and formatted. The final version may differ from this version.

Figure-2



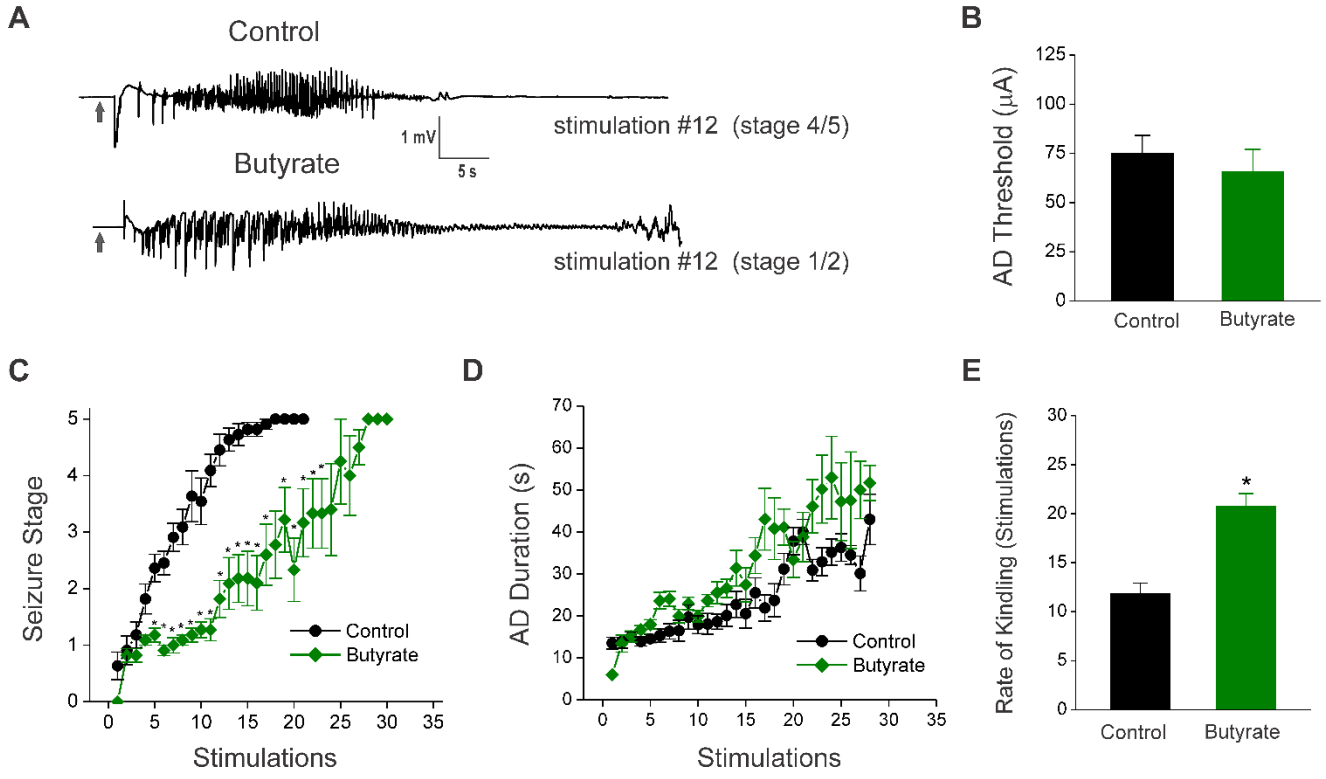
JPET Fast Forward. Published on November 3, 2017 as DOI: 10.1124/jpet.117.244939
This article has not been copyedited and formatted. The final version may differ from this version.

Figure-3



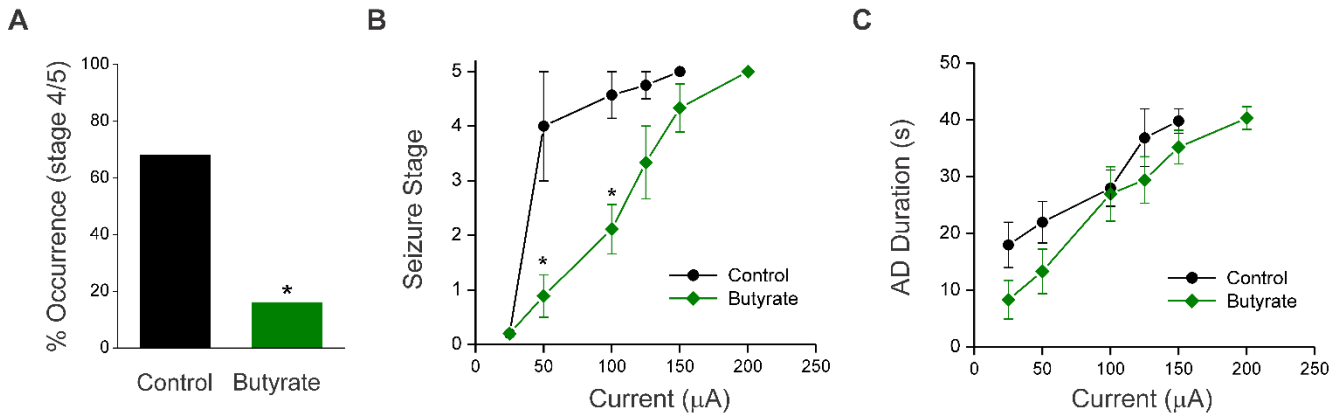
JPET Fast Forward. Published on November 3, 2017 as DOI: 10.1124/jpet.117.244939
This article has not been copyedited and formatted. The final version may differ from this version.

Figure-4



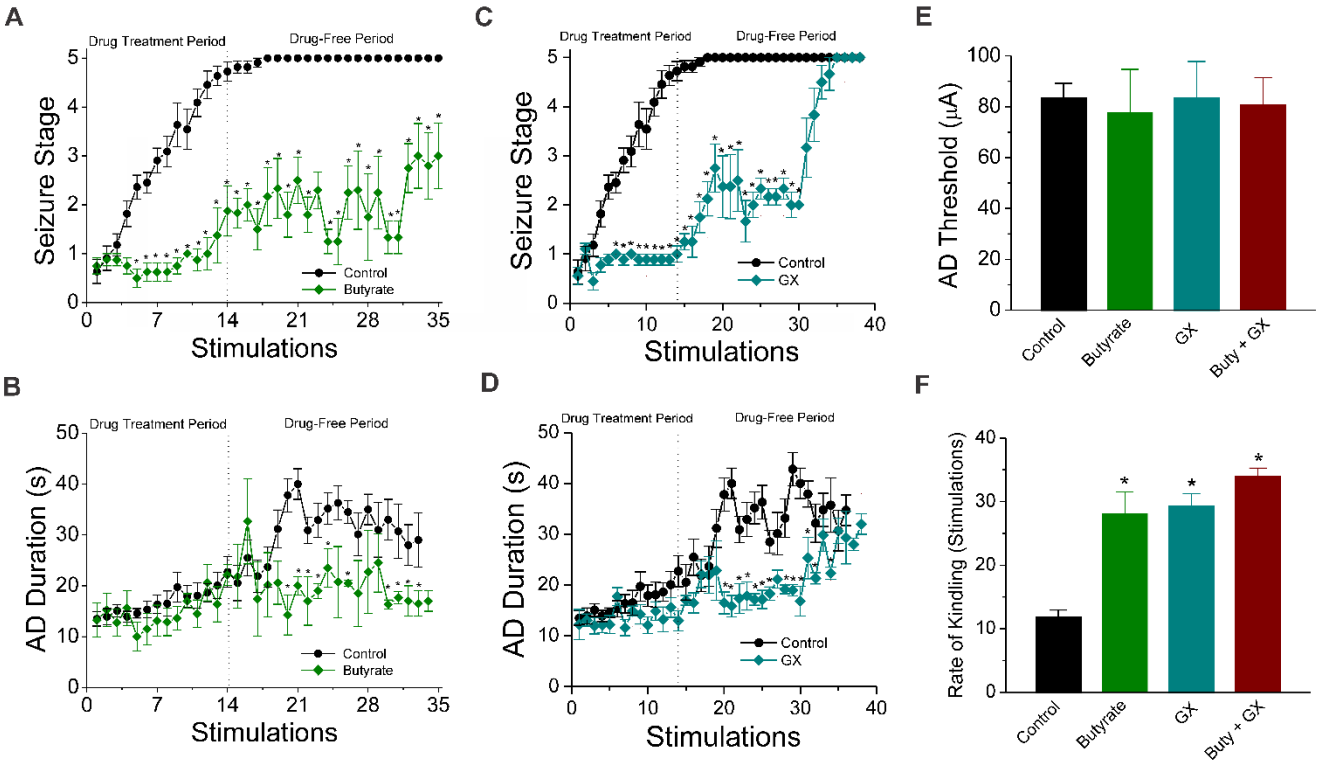
JPET Fast Forward. Published on November 3, 2017 as DOI: 10.1124/jpet.117.244939
This article has not been copyedited and formatted. The final version may differ from this version.

Figure-5



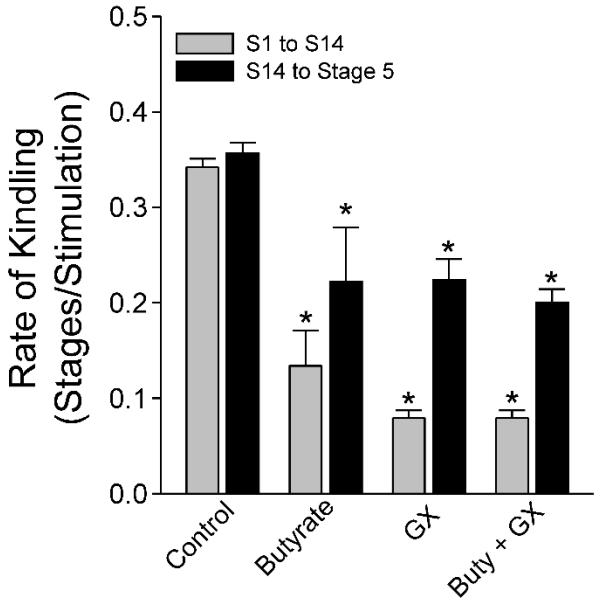
JPET Fast Forward. Published on November 3, 2017 as DOI: 10.1124/jpet.117.244939
This article has not been copyedited and formatted. The final version may differ from this version.

Figure-6



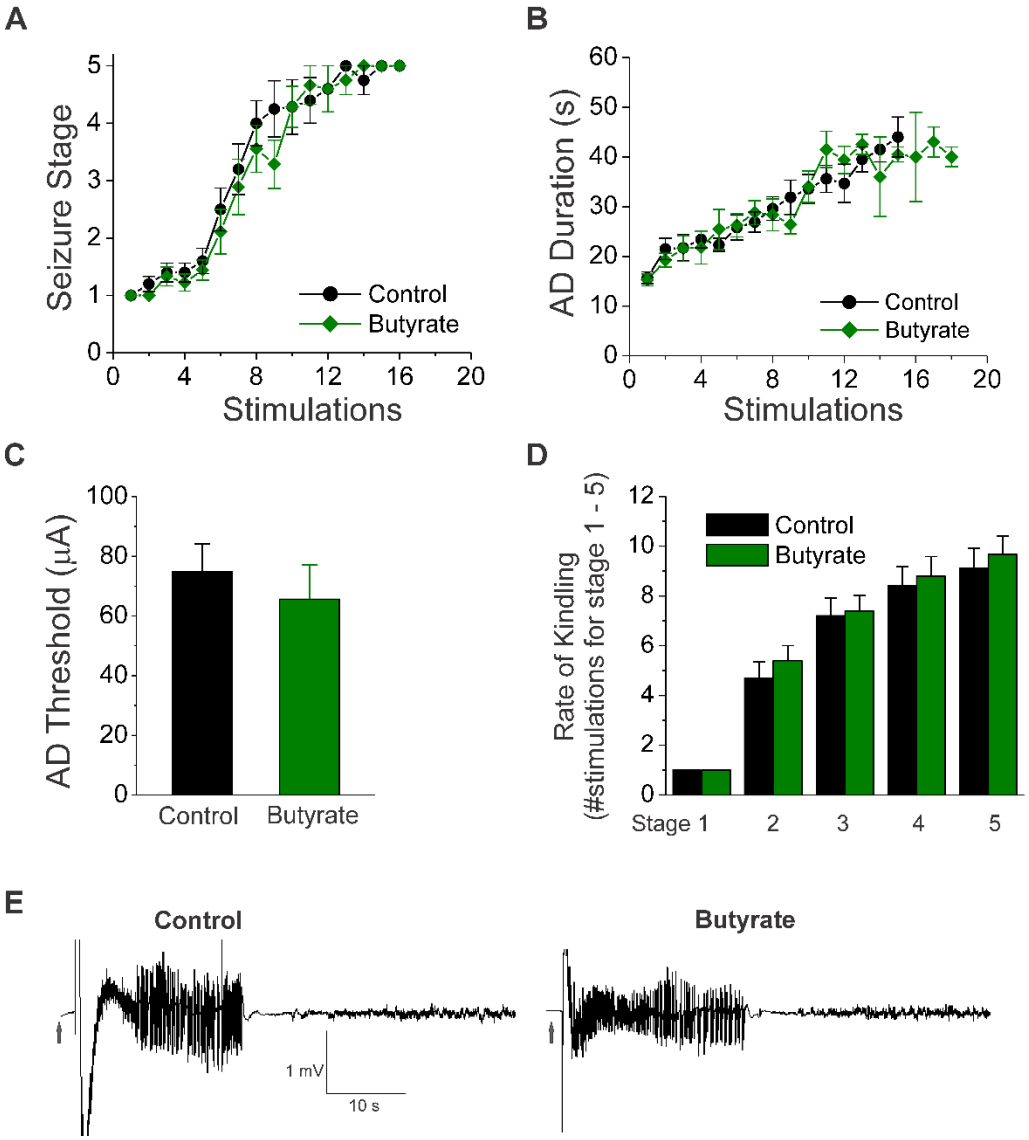
JPET Fast Forward. Published on November 3, 2017 as DOI: 10.1124/jpet.117.244939
This article has not been copyedited and formatted. The final version may differ from this version.

Figure-7



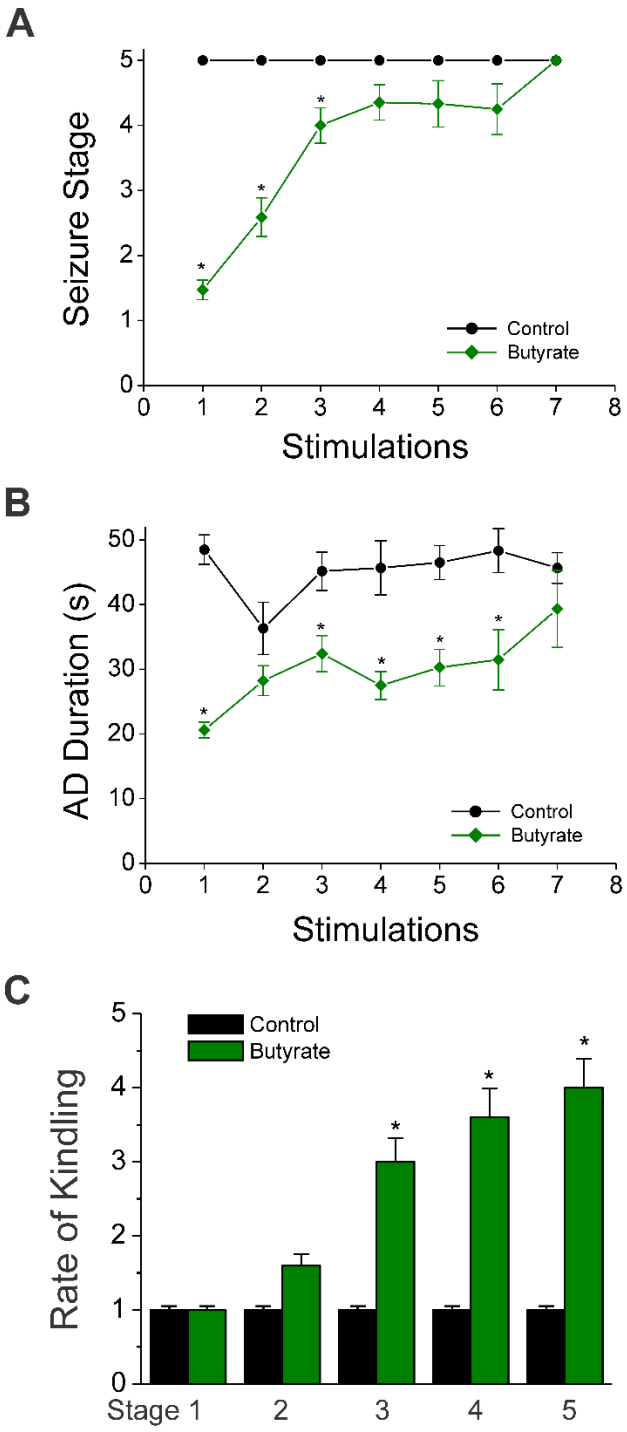
JPET Fast Forward. Published on November 3, 2017 as DOI: 10.1124/jpet.117.244939
This article has not been copyedited and formatted. The final version may differ from this version.

Figure-8



JPET Fast Forward. Published on November 3, 2017 as DOI: 10.1124/jpet.117.244939
This article has not been copyedited and formatted. The final version may differ from this version.

Figure-9



JPET Fast Forward. Published on November 3, 2017 as DOI: 10.1124/jpet.117.244939
This article has not been copyedited and formatted. The final version may differ from this version.

Figure-10