EFFECT OF CONTRACEPTIVE ESTRADIOL ON HIPPOCAMPUS KINDLING SEIZURE ACTIVITY

An Undergraduate Research Scholars Thesis

by

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ABSTRACT

Effect of Contraceptive Estradiol on Hippocampus Kindling Seizure Activity. (May 2015)

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Contraceptive management is critical in women with epilepsy because of the potential for maternal and fetal risks if unexpected pregnancy develops while on AED treatment. Although oral contraceptives (OCs) are widely used by many women with epilepsy, there has been little investigation into how interactions among circulating hormones and antiepileptic drugs (AEDs) impact both epileptic seizures and contraception. Ethinyl estradiol (EE) is the primary component of combined OC pills which are used by several million women worldwide. The present study was undertaken to investigate the potential adverse effect of EE on epileptogenesis and seizure activity using the hippocampus kindling model in female mice. Animals were stimulated daily without or with EE, given daily for 14 days, at 125% afterdischarge (AD) threshold until a fully-kindled state where stage 5 seizures could be elicited consistently. EE impacted the development of kindling by acceleration of rate of epileptogenesis. Fully-kindled mice were treated with various doses of EE and seizure susceptibility was assessed by seizure stage, seizure severity, AD duration, and AD threshold. In acute studies, the AD current threshold was dose-dependently reduced by EE treatment. The highest dose tested caused a ~50% decrease in AD threshold and increased the incidence and severity of seizures in kindled mice. In chronic studies, EE treatment caused greater susceptibility to kindling seizures. These

results provide strong evidence that EE exacerbates seizures, lowers seizure threshold, and promotes epileptogenic responses, which are consistent with its proconvulsant effects. Such excitatory effects may underlie an increase in seizure risk in women with epilepsy using oral contraceptives.

ACKNOWLEDGEMENTS

I am grateful to my mentors who have guided me through uncharted waters this year and seen me grow through this process. I am especially grateful to Dr. Samba Reddy for trusting in me to carry out this project and offering valuable insight and expertise along the way. I am also grateful to Bryan Clossen for being genuinely interested in shaping me to become a stand-out undergraduate researcher at Texas A&M University. I am forever indebted to my mentors and the rest of the personnel at the Reddy Lab for their unending support. Thank you for everything along this journey.

CHAPTER I

INTRODUCTION

Epilepsy is one of the most common neurological disorders and is characterized by the unpredictable reoccurrence of seizures. Epilepsy incidence is gender-dependent with predisposed effect to sex-specific hormonal regulation. In approximately 39-60% of women with epilepsy (WWE), the condition of catamenial epilepsy occurs in which seizure periodicity is not completely random. Instead, seizure events tend to cluster around specific points in the menstrual cycle (Reddy, 2010). An increase in seizure activity in WWE occurs at times of high estrogen levels such as ovulation and when progesterone levels fall such as the start of menstruation. The cyclical changes of estrogen, progesterone, and neurosteroids are now widely believed to be important in the pathogenesis of catamenial epilepsy (Reddy, 2013). Although it is known that reproductive hormones have properties that alter sensitivity to anticonvulsant drugs and seizures, there has been little investigation into how interactions among circulating hormones and antiepileptic drugs (AEDs) impact both epileptic seizures and contraception.

Catamenial epilepsy is a multifaceted condition with numerous causes and underlying neuroendocrine mechanisms. The most common form of catamenial epilepsy is perimenstrual, in which cyclical seizure exacerbations occur around menstruation. Evidence from animal models suggests that this enhanced seizure susceptibility during menstrual periods is caused by the withdrawal paradigm of progesterone-derived neurosteroids. During menstruation, low serum levels of progesterone are present in the body (Reddy 2009). These observations in animal models are supported by clinical studies in women, who report seizure exacerbation after

neurosteroid synthesis is inhibited by finasteride (Herzog 2003). Neurosteroids are hormones that play a crucial role in regulating seizure susceptibility by rapidly altering neuronal excitability through GABA-A receptors. Endogenous neurosteroids such as allopregnanolone (AP) modulate seizure susceptibility and confer seizure protection in catamenial epilepsy. Due to the fluctuating level of hormones in women, current drug therapy for WWE may be significantly compromised.

Contraceptive management in WWE is critical owing to the potential maternal, fetal, and neurological risks if contraception or seizure management fails. A wide range of contraceptive methods are available for women. These can be grouped in two distinct categories: hormonal and non-hormonal. Hormonal methods of contraception widely used by women of child-bearing age include injectable progestogens, combined OC pills, and contraceptive patches. These methods, unlike barrier methods, are highly dependent on correct use and individual lifestyles. OCs are the most common hormonal contraceptives and are designed to stimulate the 28-day natural menstrual cycle. Combined OC pills are composed of low-dose synthetic estrogen and progestogen and are usually taken for 21 days with a 7 day gap.

Ethinyl estradiol (EE) is the major estrogen used in OCs in the form of synthetic EE or mestranol, a prodrug which is converted to EE in the body. Currently available combined OCs can be divided into three types: monophasic (only one dose of estrogen and progestogen during the 21 days), biphasic (varying doses of estrogen and progestogen) and triphasic (varying doses of estrogen and progestogen). Amounts of estrogen and progesterone are fixed in monophasic combinations and as a result, blood levels rise and fall together. In biphasic and triphasic combinations, altering doses of progesterone and estrogen are delivered to mimic physiological

levels as seen during the cycle. Non-hormonal contraceptive methods include intrauterine devices (IUDs), barrier methods such as condoms, and others. The non-hormonal methods have been regarded as highly effective and may be ideal for women with epilepsy since the contraceptive mechanisms of these methods are undeterred by hepatic enzymes and drug-interactions (Reddy, 2010).

It is essential to understand the impact of reproductive hormones on seizures and the pharmacological effect of OCs for effective contraception and seizure management in WWE. There is no strong evidence that the use of OCs increase the risk of seizures in many WWE. Although some reports suggest that OCs might exacerbate seizures, most studies show no effect of estrogen-based contraceptives on seizure frequency. However, there are many factors to consider in the choice of AED therapy and hormonal contraception, since some AEDs can reduce the efficacy of OCs owing to pharmacokinetic interactions. OCs are widely used because of their high effectiveness in stimulating the natural menstrual cycle but many AEDs can influence the effectiveness of hormonal contraception and pose a serious risk. These interactions between AEDs and OCs can influence drug efficacy, pregnancy prevention, and seizure exacerbation.

Progesterone is a powerful anticonvulsant hormone and reduces seizure frequency due to its metabolic conversion to allopregnanolone (AP) in the brain (Reddy, 2004). AP is an endogenous neurosteroid that exhibits neuroprotective affects through the positive modulation of $GABA_A$ receptors (Reddy, 2004). This supports emerging evidence that neurosteroids derived from adrenal steroid hormones substantially retard the development and occurrence of seizures.

In contrast, estrogens are generally found to have proconvulsant properties which cause periovulatory seizure exacerbation attributed to a surge of estrogen mid-cycle. The antagonistic role of estrogen to progesterone, in conjunction with the fluctuation of other reproductive hormone levels during the menstrual cycle, can substantially increase the onset of seizures. As a result, WWE have a much greater disposition to the risk of endocrine dysfunction, reduced reproduction, and altered function of the hypothalamic-pituitary axis due to the impact of seizures. Furthermore, WWE have much higher possibility of developing polycystic ovaries and approximately 30% of WWE experience irregular menstrual cycles (Crawford, 2005).

A rise in WWE showing inadequate seizure control with AED treatment can be attributed to changes in the circulating levels of estrogen and progesterone during the menstrual cycle (Reddy, 2010). AEDs are mainly metabolized by the Cytochrome P450 3A4 (CYP3A4) system (Crawford, 2002). As a result of CYP3A4 induction by AEDs, there is a sharp increase in the metabolism of the estrogen and progesterone components of OCs and their circulating serum levels are reduced by 50% (Crawford, 1990). Moreover, some OCs can increase the metabolism of AEDs by increased induction of hepatic enzymes which can have serious consequences and contribute to elevated seizure incidence. This antagonistic effect of AEDs and OCs lead to both a reduced efficacy of hormonal contraceptives and an increase in seizure susceptibility.

Despite wide-spread use of OCs, their impact on seizures has been less well studied. The pharmacokinetic interactions affecting enzyme-inducing AEDs and OCs pose a serious risk and studies indicate that OC failures are the cause of one in four unplanned pregnancies in WWE (Fairgrieve, 2000; Coulam, 1979). Patient-awareness programs on AED interactions with OCs

may also help to improve contraception efficacy in WWE (Reddy, 2010). EE has been shown to increase neuronal excitability through proconvulsant effects in clinical and animal seizure models (Backstrom, 1984; Saberi, 2003). EE (17α-ethinyl estradiol) is classified as one of the major orally bioactive estrogens and is the major component of combined OC pills. Forms of combined OCs which contain EE are used by several million women worldwide. The role of EE in the pathophysiology of epilepsy is unclear and there is a lack of clinically relevant studies that explore the effect of EE on seizure susceptibility. Further investigation may help reveal some of the effects of OCs, including their role on seizures in WWE. Therefore, this study was undertaken to investigate the potential adverse effect of EE on epileptogenesis and seizure activity using the hippocampus kindling model in female mice.

CHAPTER II

METHODS

Animals

Adult female C57BL/6 mice, with masses of 25 to 30g each were used in this study. All mice were housed four to a cage 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.

Hippocampus Kindling Model of Epilepsy

To study seizure modulating activity of EE, 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 (Albright and Burnham, 1980). 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 4/5 models generalized seizures. The ADs are first localized to the region of stimulation but propagate to other parts of the brain with repeated stimulation. As the stimulations generalize to other regions of the brain, the animal becomes epileptic leading to the onset of behavioral convulsions after any subsequent stimulation.

Electrode Implantation

Electrode implantation and stimulation procedures for mouse hippocampus kindling were performed as described previously (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 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.

AD Threshold (ADT) Determination and Kindling Stimulation

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 ADT was determined by stimulating at 5-min intervals beginning with an intensity of 25 µA and increasing in steps of 25 µA until an AD of at least 5s was obtained. Stimulation on consecutive days used a stimulation intensity of 125% threshold value. 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. The AD 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 (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 continued on a 5 days/week schedule each afternoon. Kindling stimulation was delivered daily until stage 5 seizures were elicited on 3 consecutive days. Mice were used for drug testing when they consistently exhibited stage 5 seizures after stimulation, which is considered the "fully kindled" state.

Test Drugs and Treatment Protocols

Stock solutions of EE (Sigma-Aldrich, St. Louis, MO) for injections were made in sorbital-based solubilizer in saline, and additional dilutions were made using sterile saline. Drug solutions were administered in a volume equaling 1% of the animal's body weight. To examine the ability of EE to modulate the expression of seizures in the acute study, fully kindled mice were injected subcutaneously (SC) with EE (10-100 mg/kg) fifteen minutes prior to stimulation. After each stimulation, the behavior seizure expression, electrographic AD activity and ADT were recorded.

Data Analysis

Group data are expressed as the mean ± standard error of the mean (SEM). To construct dose-response curves, EE was tested at several doses spanning the dose producing 10% change and more towards the ED_{50} dose. Differences in kindling seizure stage between groups were compared with appropriate nonparametric test for significance. Comparison of means of the AD duration between groups was made with one-way analysis of variance, followed by unpaired two-tailed Student's *t*-test. Comparison of the mean ADT for seizures stage and seizure duration in fully kindled animals was made by unpaired Student's *t*-test. In all statistical tests, the criterion for statistical significance was $p < 0.05$.

CHAPTER III

RESULTS

Acute Effects of EE on Hippocampus Kindling Seizure Activity

To investigate whether EE displays proconvulsant-like activity, we explored its acute effects on ADT, seizure expression, and electroencephalogram (EEG) AD activity fifteen minutes after SC injection in kindling model of epilepsy (Fig.1). Mice were kindled daily until they reach stage 5 seizures (Fig.2). Fully kindled female mice were treated with increasing doses of EE and the acute effects on ADT are displayed in Figure 3. The results show that the ADTs were reduced significantly 0.25-hour post-EE treatment in a dose dependent manner. The lowest dose tested (10 mg/kg) failed to produce any significant changes in ADT. However, treatment with 50 mg/kg EE caused a 25% decrease in ADT. The highest dose tested (100 mg/kg) caused an approximate 50% decrease in ADT. The significant decrease in ADTs required to elicit stage 5 seizure (SS5) response confirms that EE is an excitatory steroid that can elicit pro-convulsive effects in epileptic animals. Parameters of electrographic AD activity were compared by measuring AD duration (ADD) at 50% ADT. As indicated by EEG AD activity, seizure incidence is markedly higher in mice treated with increasing doses of EE (Fig.4). This significant difference in EEG AD activity between vehicle and treatment groups indicates that EE enhances neuronal excitability in female mice.

Chronic Effects of EE on Hippocampus Kindling Seizure Activity

To simulate the daily use of OCs by women, we tested chronic sub-effective doses of EE on seizure activity in mice that were treated daily with EE (25 mg/kg, sc) for 21 days. Then, we

assessed treatment impact on ADT, seizure expression, and AD activity on the 22nd day. Subsequently, we also tested the effect of EE on seizure parameters fifteen minutes after SC injection. The seizure activity data in fully-kindled female mice were treated daily with EE for 3 weeks is displayed in Figure 5. Mice displayed a reduced ADTs after the chronic treatment as compared to controls not exposed to any drug (Fig.5). In dose-response studies in chronic studies, mice displayed ADTs that were reduced significantly 0.25-hour post-EE treatment in a dose dependent manner (Fig.5). The low dose EE (10 mg/kg) failed to produce any significant changes in ADT. However, treatment with 25 mg/kg EE caused a significant decrease in ADT. The AD duration was increased to a greater extend in EE group compared to control. There was a significant decrease in ADTs required to elicit stage 4/5 seizure in EE-treated mice, which confirms that EE is proconvulsant steroid that can facilitate epileptic seizures.

CHAPTER IV

CONCLUSION

The results indicate that treatment with EE was associated with significant effects on seizure activity in epileptic female mice. Our results in the hippocampus kindling model provide strong evidence that EE affects seizure expression, seizure threshold and AD duration in fully kindled mice. These effects are evident from the ADTs required to elicit SS5 being significantly lowered post-EE treatment in a dose-dependent manner. Such proconvulsant-like facilitating effect was noted in both acute and chronic settings. This evidence suggests that brain regions close to the site of stimulation were sensitive to the pro-convulsive effects of EE. The lowered ADTs in the hippocampus kindling model are consistent with observations in previous studies showing that high EE levels are comparable to that observed in females at the proestrus stage of the estrous cycle (Edwards, 1999). The results demonstrate that EE increases neuronal excitability and facilitates seizure susceptibility via reduction of ADTs in the hippocampus kindling model.

It has been reported in WWE that a positive correlation exists between seizure susceptibility and the estrogen-to-progesterone ratio. This positive correlation is consistent with serum hormone levels during perimenstrual and periovulatory periods and reported seizure exacerbation in catamenial epilepsy. Therefore, the increase in the ratio of estrogen-to-progesterone levels during these periods may partly contribute to seizure exacerbation and may facilitate some forms of catamenial epilepsy (Herzog, 1997).

There are many underlying mechanisms influenced by EE, whereby it is difficult to predict its net effect in the brain. Rats chronically exposed to EE have shown increased number and density of dendritic spines and excitatory synapses on hippocampal neurons. This mechanism increases the synchronization of synaptically driven neuronal firing in the hippocampus and could be relevant to EE's proconvulsant effects in animal models. EE may also increase excitability through modulation of neuropeptides and increased levels of brain-derived neurotrophic factor (BDNF) in the hippocampus, shown to have both protective actions and increase hippocampus excitability (Scharfman and MacLusky, 2006). EE and BDNF have many effects that could influence seizures and epilepsy because they both influence neuropeptide Y (Veliskova, 2007).

The dose and time dependent properties of EE are well reported. The rapid onset of EE may be due to its direct interactions at the membrane level or through a post-membrane secondary messenger cascade (Saberi, 2001). Receptors for estrogens include various membrane-associated and cell nuclear receptors. The cell nuclear receptors can also localize to the plasma membrane and activate numerous signaling pathways (Reddy, 2014). The G-protein coupled membrane estrogen receptors may also be activated by EE, producing actions on many downstream signal transduction cascades (Scharfman and MacLusky, 2006). In addition to traditional receptor-mediated effects, EE affects neuronal excitability due to its organizational effects on synaptic structure and function. Through this mechanism, EE enhances glutamate receptor-mediated excitatory neurotransmission (Smith, 1988; Wong and Moss, 1994) and decreases GABAergic inhibition (Murphy, 1998). The direct effects on glutamate receptors and indirect effects through an increase in dendritic spine density of hippocampal NMDA receptor have been shown to be

involved in EE modulation of the NMDA receptor function (Rudick and Woolley, 2001; Woolley and McEwen, 1994; Woolley, 1997).

In conclusion, these studies confirm that EE is proconvulsant steroid that can elicit exacerbation of seizure activity in epileptic animals. The central effects of EE in the hippocampus kindling model provide evidence that EE may show similar pro-epileptic effects upon long-term administration, such as that present in oral contraceptive pills. Furthermore, our results are consistent with clinical reports of increased generalized seizure incidence during peak estrogen levels (Backstrom, 1984). It is likely that such effects may manifest an increase in seizure risk in WWE using EE-containing oral contraceptives.

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FIGURES

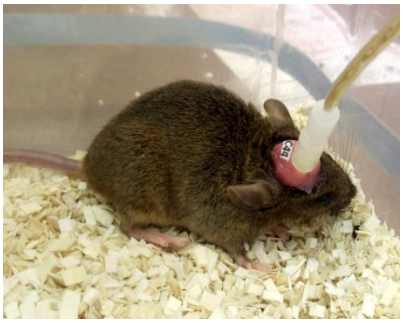


Figure 1. A mouse with an implanted bipolar kindling electrode in the hippocampus.

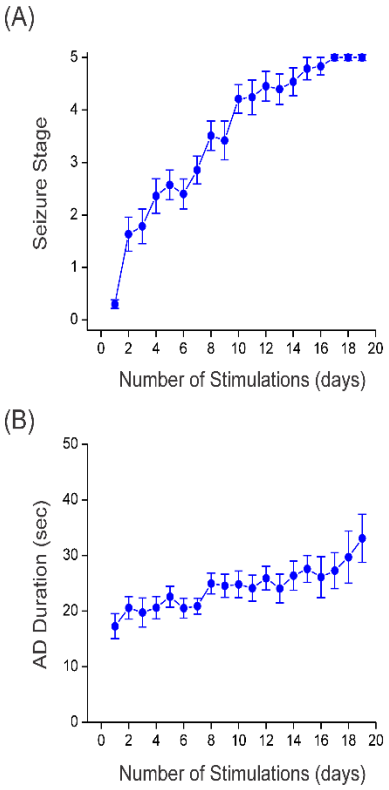


Figure 2. Progression of hippocampus kindling development in mice.

Panel A shows progression of behavioral seizure stage following each stimulation. Panel B shows progression of AD duration following each stimulation. Data was presented as mean ± SEM (N=10-14 mice).

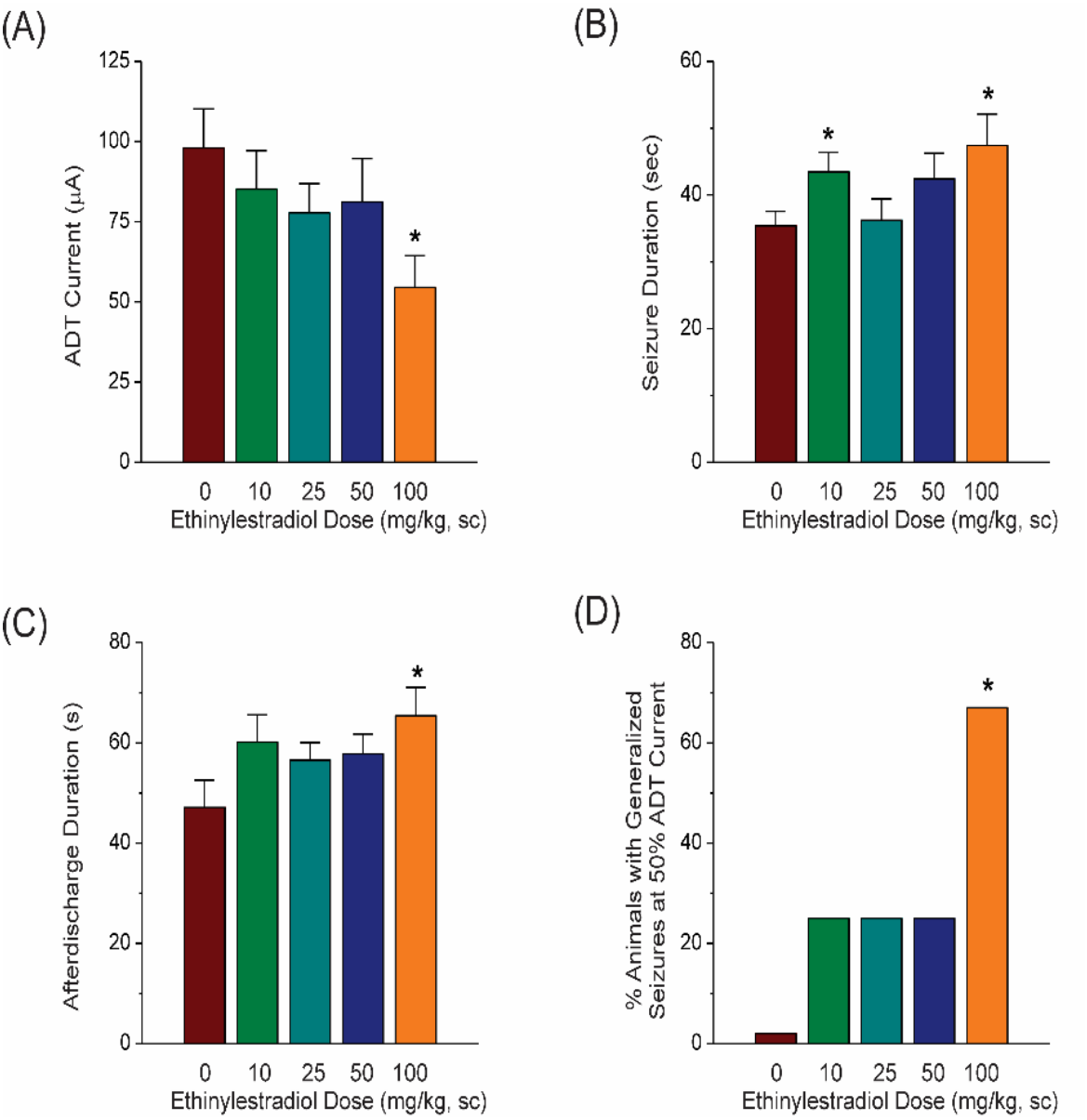
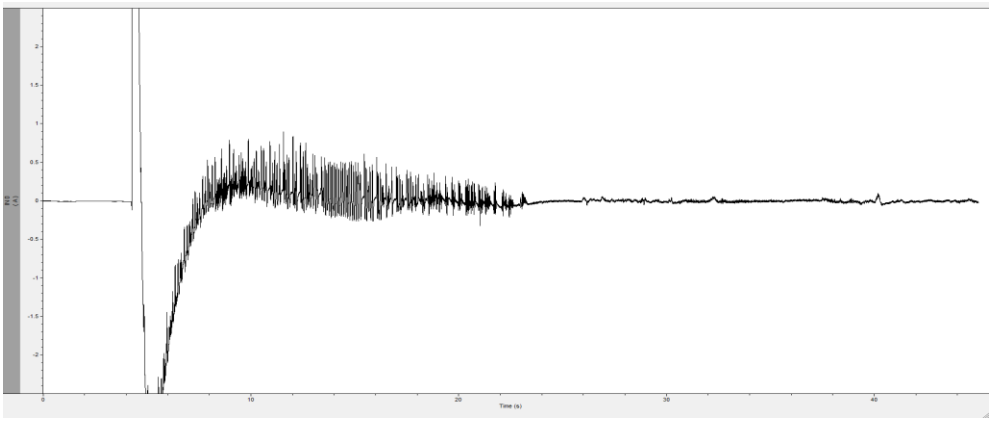


Figure 3. Dose-dependent effect of EE on seizure activity in fully-kindled mice.

Panel A shows intensity of ADT current for eliciting generalized (stage 4/5) behavioral seizures after EE treatment. Panel B shows duration of behavior (stage 4/5) seizures after EE treatment. Panel C shows the duration of AD after EE treatment. Panel D shows the dose-response of percent animals exhibiting generalized seizures.

Control (No EE)



EE treatment (100 mg/kg sc)

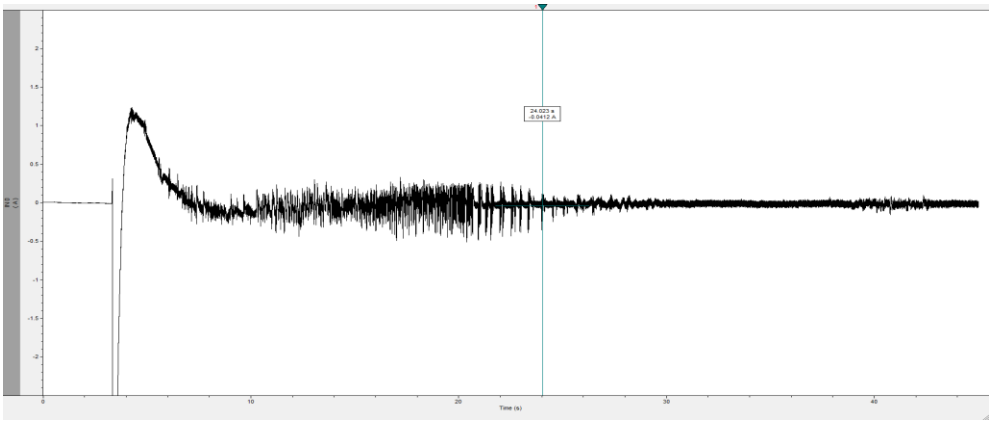


Figure 4. Sample traces of AD duration in control and EE-treated mice.

Representative traces illustrate EE exacerbation of electrographic seizure activity in a fully kindled mouse. Traces show depth recording from a right hippocampus bipolar (stimulating and recording) electrode. Arrows indicate onset of the 1-s kindling stimulus, which is followed by the stimulus artifact. Control trace was obtained without EE treatment in a fully kindled mouse.

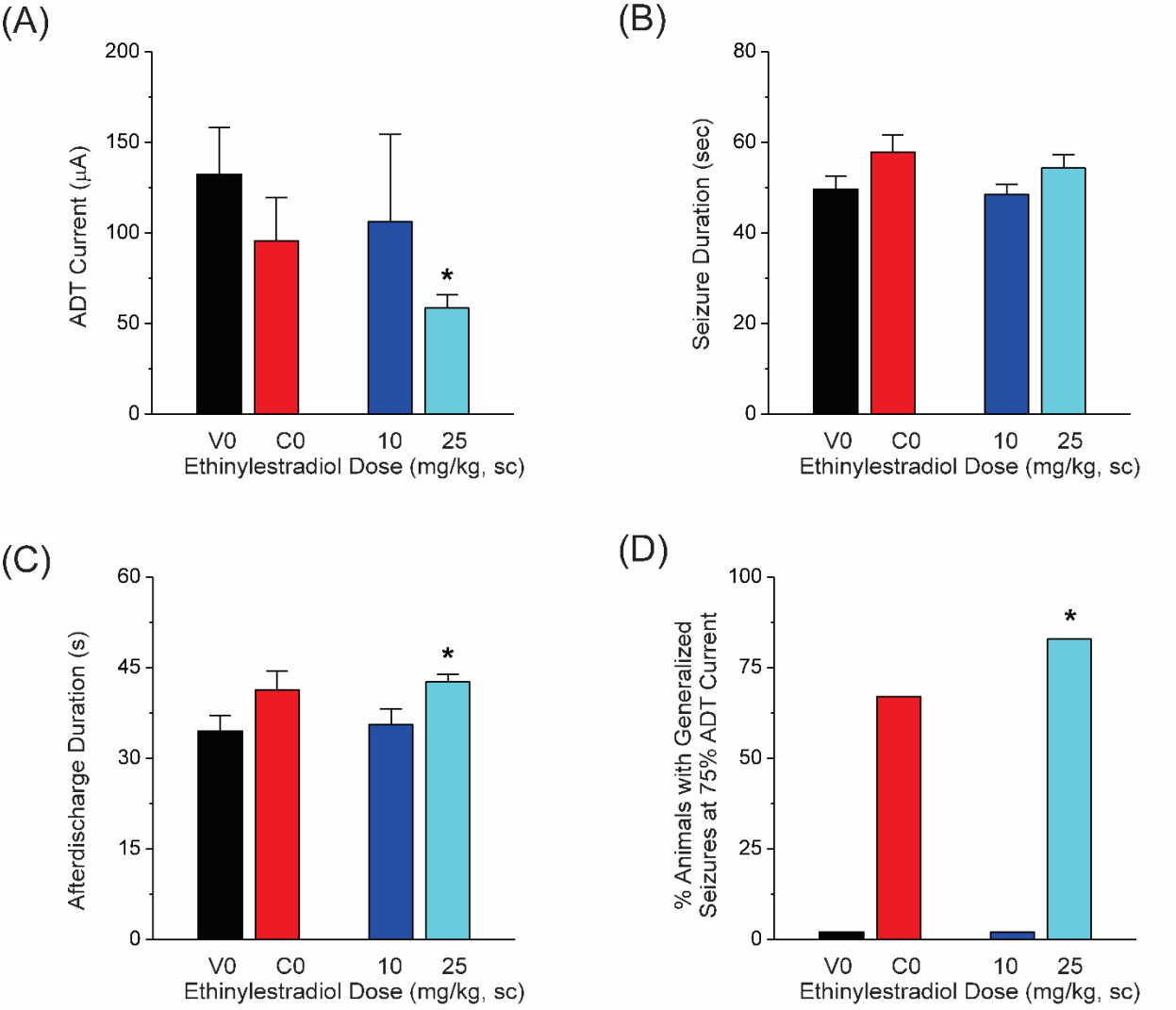


Figure 5. Dose-dependent effect of EE on seizure activity in mice treated chronically with EE (25 mg/kg, sc) for 21 days.

Panel A shows intensity of ADT current for eliciting generalized (stage 4/5) behavioral seizures after EE treatment. Panel B shows duration of behavior (stage 4/5) seizures after EE treatment. Panel C shows the duration of AD after EE treatment. Panel D shows the dose-response of percent animals exhibiting generalized seizures.