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Ethosuximide reduces electrographical and behavioral correlates of alcohol withdrawal seizure in DBA/2J mice

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Abstract

Chronic alcohol abuse depresses the nervous system and, upon cessation, rebound hyperexcitability can result in withdrawal seizure. Withdrawal symptoms, including seizures, may drive individuals to relapse, thus representing a significant barrier to recovery. Our lab previously identified an upregulation of the thalamic T-type calcium (T channel) isoform Ca_v3.2 as a potential contributor to the generation and propagation of seizures in a model of withdrawal. In the present study, we examined whether ethosuximide (ETX), a T-channel antagonist, could decrease the severity of ethanol withdrawal seizures by evaluating electrographical and behavioral correlates of seizure activity. DBA/2J mice were exposed to an intermittent ethanol exposure paradigm. Mice were treated with saline or ETX in each withdrawal period, and cortical EEG activity was recorded to determine seizure severity. We observed a progression in seizure activity with each successive withdrawal period. Treatment with ETX reduced ethanol withdrawal-induced spike and wave discharges (SWDs), in terms of absolute number, duration of events, and contribution to EEG power reduction in the 6–10 Hz frequency range. We also evaluated the effects of ETX on handling-induced convulsions. Overall, we observed a decrease in handling-induced convulsion severity in mice treated with ETX. Our findings suggest that ETX may be a useful pharmacological agent for studies of alcohol withdrawal and treatment of resulting seizures.

Keywords

alcohol; withdrawal; seizure; ethosuximide; T-type calcium channel

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Introduction

Approximately 18 million Americans abuse or are dependent on alcohol, which results in significant economic and societal burdens (Grant et al., 2004; Thavorncharoensap, Teerawattananon, Yothasamut, Lertpitakpong, & Chaikledkaew, 2009). Individuals who abuse alcohol frequently cycle between periods of drinking and withdrawal. The depressant effects of alcohol on the central nervous system during chronic drinking lead to compensatory excitatory mechanisms that become apparent upon withdrawal from alcohol. Symptoms of alcohol withdrawal include anxiety, delirium tremens, insomnia, and seizures (Saitz, 1998). Alcohol withdrawal seizure is particularly dangerous and stems from a progressive neuronal excitation with concurrent disruption of brain rhythms. Few of the mechanisms underlying these changes are understood. Both clinical and animal studies have demonstrated that with increasing numbers of withdrawals there is a decrease in the threshold for seizures and a worsening of withdrawal symptoms (Becker, 1998; Brown, Anton, Malcolm, & Ballenger, 1988), suggesting it is critical to reduce the risk of seizures when an individual undergoes alcohol withdrawal. Early interventions that target the mechanisms responsible for hyperexcitability may prevent an individual from relapsing and may be protective from kindling-like progression of these seizures and other withdrawal symptoms.

Benzodiazepines (BZDs) are the first-line therapy used during alcohol withdrawal (Ait-Daoud, Malcolm, & Johnson, 2006). However, BZDs such as lorazepam produce rebound effects during untreated withdrawal periods (Veatch & Becker, 2005), suggesting that BZDs may not be an optimal treatment option. Unfortunately, there are few alternative treatments, mainly because the molecular mechanisms of withdrawal are poorly understood. This gap must be bridged in order to find innovative therapeutic approaches to reduce the number of, and risk for, alcohol withdrawal seizures.

T-type calcium channels (T channel) may be an important target of ethanol. Graef et al. (Graef, Huitt, Nordskog, Hammarback, & Godwin, 2011) identified an upregulation of T-channel expression and function, including a persistent upregulation of $\text{Ca}_v3.2$ mRNA in midline thalamic nuclei during ethanol withdrawal. Increased burst-firing and a depolarizing shift in the T-channel steady state inactivation curve was also observed. This enhanced T-channel activity was shown to contribute to hyperexcitability during withdrawal, suggesting that T channels might be a novel target for reducing the effects of withdrawal. If T channels are an essential feature of withdrawal seizures, drugs that block T channels should reduce withdrawal seizure. One clinically important T-channel blocker is the antiepileptic drug ethosuximide (ETX) (Gören & Onat, 2007; Huguenard, 2002).

We used a vapor chamber to expose DBA/2J mice to an intermittent schedule of ethanol. We treated mice acutely with ETX to determine if it would reduce electrographical and behavioral correlates of ethanol withdrawal-induced seizure activity. Our findings demonstrate that ETX can inhibit withdrawal-induced seizure activity, suggesting that ETX or other pharmacological agents that target T channels may warrant further consideration as a therapeutic treatment option for alcohol withdrawal.

Materials and Methods

All experiments were approved by the Institutional Animal Care and Use Committee of Wake Forest University. Experiments were conducted in agreement with the National Institutes of Health and United States Department of Agriculture guidelines, including procedures that reduce animal use and mitigate suffering.

Surgical Procedure

Group-housed 8–10-week-old male DBA/2J mice (Jackson Laboratory; Bar Harbor, ME) were surgically implanted with a tethered electroencephalography/electromyography (EEG/EMG) acquisition system (Pinnacle Technologies Inc., Lawrence, KS). Briefly, mice were anesthetized with ketamine and xylazine (100 mg/kg and 10 mg/kg, respectively). Ketamine was supplemented until areflexia was apparent. A 1-cm incision was made at midline. The skin was reflected back to expose the surface of the skull. Four holes were drilled through the skull, with two anterior to bregma (~1 mm) and two anterior to lambda (~1 mm) holes on each side of the midline (~1.25 mm) for placement of four stainless-steel screw electrodes. The screws were secured to the prefabricated headmount, and a silver epoxy was applied to maintain electrical continuity. Dental acrylic was used to secure the headmount to the skull, allowed to cure, and the incision was sutured.

Intermittent Ethanol Exposure Paradigm

Following at least one week of surgical recovery, mice were exposed to a modified intermittent ethanol inhalation paradigm characterized by Becker & Hale (1993). Surgically implanted mice were placed in a Plexiglas® vapor chamber in the same room in which the mice were housed. The room was on a 12-h light/dark cycle with lights off at 6:00 PM and on at 6:00 AM. Mice were exposed to one cycle consisting of four ethanol exposures and four withdrawal periods (Fig. 1A). For each exposure, ethanol (95%) was volatilized and delivered to the chamber by an air pump for 16 hours (5:00 PM to 9:00 AM). Following each exposure, mice underwent a withdrawal period lasting 8 h (9:00 AM to 5:00 PM). Mice were subcutaneously treated with pyrazole (100 mg/kg, Sigma Aldrich; St. Louis, MO), an alcohol dehydrogenase inhibitor, at the beginning of each ethanol exposure (5:00 PM) to maintain blood ethanol concentration (BEC) levels. Pyrazole was dissolved in saline and prepared daily. Over the four withdrawal periods, BECs were 262.6 ± 8.6 mg/dL. To measure BEC levels, 5- μ L samples of blood were collected from the tail. Samples were immediately placed in trichloroacetic acid (6.25%) and analyzed using a NAD-ADH enzyme assay (Sekisui; Kent, ME).

Mice were injected with ETX (250 mg/kg, Sigma Aldrich; intra-peritoneal [i.p.]) or saline during each withdrawal period at 1:30 PM (Fig. 1A). Control mice did not receive ethanol exposure (only air exposure), but underwent identical handling procedures, including pyrazole injections. Control mice were treated with ETX (250 mg/kg) or saline at the same time points as the experimental mice. ETX was dissolved in saline and prepared daily. The dose was selected based on a previous investigation evaluating ETX in mice undergoing withdrawal (Kaneto, Kawatani, & Kaneda, 1986) and its use in studies of absence seizures

(Aizawa, Ito, & Fukuda, 1997; Dezsi et al., 2013; Frankel et al., 2005; Marrosu et al., 2007; Nissinen & Pitkänen, 2007).

EEG Analysis

During each withdrawal period (9:00 AM to 5:00 PM) or equivalent period for air-exposed mice, EEG signals were recorded with the acquisition system. EEG was sampled at a rate of 200 Hz and band-pass filtered from 0.5 to 40 Hz. The digitized EEG signals were analyzed using NeuroScore software (NeuroScore 2.1, Data Sciences International; St. Paul, MN). A dynamic threshold protocol was applied to each recording to identify spike and wave discharge (SWD) events (Fig. 1B) occurring between 6–14 Hz. Briefly, a band-pass filter from 6–14 Hz was applied to each signal. The root mean square was derived from the filtered signal to determine the minimum threshold value. The amplitude threshold ratio was set at 2 with a maximum ratio set to 20. Using the defined algorithm, the software detected the SWD events and generated a seizure report for each recording. The report identified the total number of seizures, the time when each seizure was detected, and the duration of each event. We chose this method of scoring to reduce any bias, because an experimenter was not manually scoring the recordings. The signals were manually validated by an experimenter to ensure that the protocol was accurately identifying the seizure events; however, there were no adjustments to the scoring. High amplitude events that did not meet criteria were not included in SWD counts, which reflects the conservative nature of our protocol. The number of SWDs and duration of events were collected for each withdrawal period (or the equivalent period for air-exposed mice) following treatment from 2:00–5:00 PM. An additional analysis was performed within the ethanol-exposed group of mice treated with ETX with SWD counts measured prior to treatment (10:30 AM to 1:30 PM) during each withdrawal to determine how many seizures the mice were having prior to ETX treatment and whether ETX treatment could rescue the mice from withdrawal-induced seizures. In a subset of mice within this same group, the fourth withdrawal period was extended to determine if the SWDs returned (5:00–8:00 PM). Baseline EEG recordings were analyzed for all mice prior to the first ethanol or air exposure. Mice were not treated with saline or ETX during baseline recordings. We performed a power spectral analysis on the recordings in the 6–10 Hz frequency range after identifying that this is the range in which the SWDs were occurring in the recordings (2:00–5:00 PM during each withdrawal; NeuroScore software 2.1, Data Sciences International). This range includes the frequencies associated with SWDs in DBA/2J mice (Marrosu et al., 2007; Reid, Kim, Berkovic, & Petrou, 2011). Values were normalized to control for amplitude differences across EEG recording rigs.

Handling-Induced Convulsion Experiment

A separate group of 8-week-old male DBA/2J mice ($n = 16$) was used to assess ETX treatment on behavioral seizure activity during ethanol withdrawal. All 16 mice underwent the intermittent exposure paradigm previously described; however, these mice were not surgically implanted. Mice were randomly assigned to the saline and ETX treatment groups (8 per group). BEC levels did not differ between the two groups during each withdrawal, thus the levels were combined to determine an average BEC for each withdrawal period. The mean BEC levels for each withdrawal period were as follows: 1st, 271.7 ± 9.4 ; 2nd, 260.7 ± 11.2 ; 3rd, 228.0 ± 11.6 ; 4th, 154.6 ± 13.12 (mg/dL). The levels in the 4th

withdrawal, compared to the 1st and 2nd withdrawal periods, were significantly lower in both groups. As determined in our first experiment and by previous investigations (Becker, 1994; Becker, Myrick, & Veatch, 2006; Veatch & Becker, 2002), withdrawal severity progressively increases over time and occurs 6–8 h into withdrawal.

To assess the acute effects of ETX, mice were treated with saline or 250 mg/kg ETX at 6 h into the 4th withdrawal period (3:00 PM). We evaluated handling-induced convulsions (HIC) as a measure of behavioral withdrawal severity 45 min following injection of saline or ETX. The mice were then tested again for handling-induced convulsions one hour later following the first testing. One experimenter blinded to the treatment conducted all testing. Briefly, each mouse was placed in an open-field chamber with a video camera affixed to the side. Each mouse was free to roam the chamber for 30 sec. The experimenter lifted the mouse by the tail, and after a pause, spun the mouse clockwise and then counterclockwise. Two reviewers (one blinded) scored the videos as well as the documented observations during testing. We assessed overall HIC score as well as whether or not each mouse had a tonic/clonic seizure regardless of when it occurred (before or after the spin). The scale (Table 1) used to determine HIC score was previously described by Becker (1994). It was adapted from Goldstein's original description (1972) and Crabbe & Kosobud (1990).

Statistical Analyses

Groups assessed in the EEG study were as follows: ethanol-exposed with saline treatment ($n = 8$ animals); ethanol-exposed with ETX treatment ($n = 10$ animals); air-exposed with saline treatment ($n = 3$ animals); air-exposed with ETX treatment ($n = 9$ animals). For the HIC study, the groups were as follows: ethanol-exposed with saline treatment ($n = 8$ animals) and ethanol-exposed with ETX treatment ($n = 8$ animals). Due to differences in group size, a nonparametric ANOVA was performed to determine whether there were group differences in SWD events at baseline prior to any other assessment. A two-way repeated-measures ANOVA was used to determine differences between saline- and ETX-treated mice over the course of four withdrawal periods and for within-group comparisons evaluating prior to and after ETX treatment. Follow-up *post hoc* tests included linear regression analyses to determine if there was a significant increase in SWDs with progressive withdrawal periods and if ETX treatment decreased seizure events significantly below baseline. Bonferroni multiple-comparison analyses were used as *post hoc* tests to determine if ETX treatment was significantly different from saline treatment and if SWD events differed before ETX treatment compared to after ETX treatment. A nonparametric ANOVA was used to determine if SWD events returned once ETX had been metabolized in an extended fourth withdrawal period. A nonparametric repeated-measures ANOVA was used to determine if saline treatment affected SWD events in mice exposed to air only. A nonparametric *t* test was used to determine differences in power between saline- and ETX-treated animals during withdrawal. The data from the four withdrawal periods were pooled for this analysis. Lastly, we used a nonparametric *t* test to evaluate overall HIC score. The overall HIC score data did not meet the criteria for the chi-square test. We did use a chi-square test to determine if there was a significant difference in the amount of tonic/clonic seizures in the different treatment groups. All data are presented as mean $\pm$ SEM with statistical analyses for significance provided within the results section.

Results

SWDs in DBA/2J mice progress with the number of withdrawals

We evaluated the number of SWDs in DBA/2J mice over the course of four intermittent ethanol exposures and withdrawals during the peak withdrawal time, 2:00–5:00 PM. In saline-treated mice undergoing withdrawal, SWDs increased with each successive withdrawal period (Fig. 2A; Linear regression, $R^2 = 0.6903$, $F = 84.69$, $p < 0.0001$, $n = 8$). The mean $\pm$ SEM SWDs for each withdrawal period are presented in Table 2 (the number of SWDs did not differ between groups during baseline measurements) (Table 2; Kruskal-Wallis ANOVA, $p = 0.964$, $n = 3–10$). For this predetermined analysis, there was a total of four different groups assessed, thus we used a nonparametric ANOVA to determine if there were any differences between the mice that would be in ethanol- or air-exposed groups. Mice were not treated with saline or ETX during baseline recordings.

ETX decreased ethanol withdrawal-induced SWDs

To determine whether ETX decreased the number of ethanol withdrawal-induced seizures, we compared the number of SWDs in saline-treated mice to ETX-treated (250 mg/kg) mice during each withdrawal period from 2:00–5:00 PM (saline group, $n = 8$; ETX group, $n = 10$). Two-way ANOVA repeated-measures analysis revealed a significant difference in time (Fig. 2A; df: 4, $F = 25.21$, $p < 0.0001$), treatment (df: 1, $F = 126.4$, $p < 0.0001$), and interaction (df: 4, $F = 46.28$, $p < 0.0001$). A Bonferroni multiple-comparison analysis revealed a significant difference between saline and ETX treatment in each withdrawal period (Fig. 2A, $p < 0.0001$). The mean $\pm$ SEM SWDs for each withdrawal period are presented in Table 2 for the treatment groups. Thus, the progressive increase in withdrawal seizure was blocked by administration of ETX.

Duration of SWDs increases during ethanol withdrawal; ETX restores the increase to baseline

We evaluated the duration of SWD activity during ethanol withdrawal and determined the effects of ETX on this variable. Two-way ANOVA repeated-measures analysis revealed a significant interaction (Fig. 2B; df: 4, $F = 3.877$, $p = 0.0070$) and treatment effect (df: 1, $F = 45.09$, $p < 0.0001$). A Bonferroni multiple-comparison analysis revealed a significant difference between saline and ETX treatment in the second, third, and fourth withdrawal periods (Fig. 2B, $p < 0.0001$). In saline-treated mice undergoing withdrawal, SWD duration was significantly increased (Fig. 2B; Linear regression, $R^2 = 0.1129$, $F = 4.835$, $p = 0.034$). The number of animals was 8 and 10 for the saline and ETX groups, respectively. The mean duration (seconds) for the SWD events in saline-treated mice were as follows: baseline, 0.887 ± 0.04 ; 1st withdrawal, 0.938 ± 0.02 ; 2nd withdrawal, 0.975 ± 0.04 ; 3rd withdrawal, 0.975 ± 0.04 ; 4th withdrawal, 0.988 ± 0.04 . The mean duration (seconds) for the SWD events in ETX-treated mice were as follows: baseline, 0.840 ± 0.02 ; 1st withdrawal, 0.830 ± 0.03 ; 2nd withdrawal, 0.760 ± 0.03 ; 3rd withdrawal, 0.777 ± 0.03 ; 4th withdrawal, 0.760 ± 0.02 . The effect of withdrawal was not as robust on the duration of the events as it was on the number of events; however, these data support our findings that repeated withdrawal episodes increase seizure risk and severity. ETX inhibited the observed increase in both number and duration of events.

Saline treatment did not affect SWDs in air-exposed mice

To determine whether stress from the injection affected the number of SWD events, we counted the number of events from 2:00–5:00 PM in saline-treated animals exposed to air. Saline injections alone did not affect the number of SWDs (Table 2; Friedman test, $p = 0.1723$, $n = 3$). Based on these findings, significant increases in SWDs observed in saline-treated mice undergoing ethanol withdrawal appear to be due to the effects of repeated ethanol withdrawal, and not to stress from injections or placement in the recording chambers.

ETX treatment decreased SWDs below baseline levels

After observing that ETX treatment reduced SWDs compared to saline-treated animals during ethanol withdrawal, we sought to further evaluate whether the ETX treatment reduced the number of SWDs below baseline levels. Analysis confirmed that ETX treatment reduced SWDs below baseline values (Fig. 2A; Linear regression, $R^2 = 0.3196$, $F = 22.55$, $p < 0.0001$, $n = 10$). These results are supported by a previous study demonstrating that ETX reduces SWD events in naïve DBA/2J mice (Marrosu et al., 2007). Baseline recording values represent naïve mice prior to exposure (air or ethanol) or treatment (saline or ETX).

ETX treatment rescues mice from SWDs prior to withdrawal treatment

We evaluated the number of SWDs within the group of mice exposed to ethanol and treated with ETX (250 mg/kg) before (pretreatment, 10:30 AM to 1:30 PM) and after treatment (posttreatment, 2:00–5:00 PM). This within-group comparison allowed us to evaluate the effects of ETX further by determining whether SWD activity increased in mice within this group prior to treatment. Our data suggest that ETX rescued mice from withdrawal-induced SWDs that occurred prior to treatment (Fig. 2C, $n = 10$). Two-way repeated-measures ANOVA revealed a significant difference in time (df: 4, $F = 7.072$, $p < 0.0001$), treatment (df: 1, $F = 35.05$, $p < 0.0001$), and interaction (df: 4, $F = 13.16$, $p < 0.0001$). *Post hoc* analyses revealed a significant difference between pretreatment and posttreatment in the second withdrawal period (Bonferroni multiple comparisons, $p < 0.05$) and in the third and fourth withdrawal periods ($p < 0.0001$). We also observed a kindling-like effect in the pretreatment period with SWDs increasing with each successive withdrawal (Linear regression, $R^2 = 0.3559$, $F = 26.52$, $p < 0.0001$). To confirm that this finding was not the result of these animals having an abundance of events at 9:00 AM when they are removed from the ethanol chamber, we analyzed the number of events from 9:00–10:00 AM. If the number of events were high and continued to drop, our findings would be difficult to interpret. We observed that mice exhibit the least amount of withdrawal signs (only observations made by experimenter), and the number of SWD events were not increased compared to what was scored from the pretreatment time point (10:30 AM to 1:30 PM). The mean number of events in this first hour for the ETX-treated group was 75.5 ± 12.6 ($n = 10$). This value was not significantly different from the saline-treated group, 56.4 ± 8.5 ($n = 8$), (Mann-Whitney test, $p = 0.3981$).

SWDs return as ETX is metabolized

We evaluated whether SWDs returned in the ethanol-exposed mice treated with ETX, due to the rapid metabolism of ETX in mice (el Sayed, Löscher, & Frey, 1978), with anticipation that ETX effects would be transient. In a subset of animals, we prolonged the fourth withdrawal EEG recording to evaluate the number of events from 5:00–8:00 PM (“washout” period). We compared the number of events prior to treatment (pretreatment, 10:30 AM to 1:30 PM), immediately following treatment (posttreatment, 2:00–5:00 PM), and at the point when ETX should be metabolized (washout, 5:00–8:00 PM) during the fourth withdrawal of mice treated with ETX. SWDs returned to levels that were intermediate between those observed prior to treatment and immediately following treatment. During the washout period, SWDs were significantly higher compared to the number of events immediately following treatment (Fig. 2D; Kruskal-Wallis, $p < 0.0001$; Dunn’s Multiple Comparison Test, $p < 0.05$, $n = 10$ in pretreatment and posttreatment, $n = 6$ in “washout”).

ETX Reduces Power during Ethanol Withdrawal

To further characterize our findings showing an increase in SWDs during withdrawal, we performed a power spectral analysis during each withdrawal period to evaluate the effects of ETX on power in the SWD activity range 6–10 Hz. If ETX decreases the number of SWD events in the 2:00–5:00 PM period of withdrawal, we would expect to observe a power reduction in this range during this time frame. However, due to the highly variable nature of EEG power measurements, we pooled the normalized power for all four withdrawal periods for saline and ETX ethanol-exposed groups. ETX treatment decreased power in the SWD activity range of 6–10 Hz compared to saline-treated mice (Fig. 2E; Mann-Whitney test, $p = 0.0038$, saline $n = 36$, ETX $n = 40$). The mean normalized power for mice treated with saline during withdrawal was 42.02 ± 5.47 and 19.72 ± 3.60 for ETX-treated mice.

ETX reduces handling-induced convulsions during withdrawal

To measure the effects of ETX on behavioral correlates of ethanol withdrawal-induced seizure activity, we used the handling-induced convulsion model. Mice underwent the intermittent ethanol exposure paradigm and were tested for HICs during the fourth withdrawal after being treated with either saline or ETX. Mice were tested at 7 h into withdrawal and the test was repeated 1 h later (8 h into withdrawal). Mice treated with ETX had a significantly lower HIC score compared to mice treated with saline during the first test (Fig. 3A; Mann-Whitney test, $p = 0.0479$, $n = 8$ per group). At this time point, mice treated with ETX had significantly fewer tonic/clonic seizures than mice treated with saline (Fig. 3A; chi-square test, $p = 0.0209$, $n = 8$ per group). All mice treated with saline had a tonic/clonic seizure either on the tail lift or following the 360° spin, whereas only 4 out of 8 mice treated with ETX had tonic/clonic seizures. One hour later, the test was repeated and we observed similar findings. Mice treated with ETX had a significantly lower HIC score compared to mice treated with saline (Fig. 3B; Mann-Whitney test, $p = 0.0081$, $n = 8$ per group). At this time point, mice treated with ETX also had significantly fewer tonic/clonic seizures than mice treated with saline (Fig. 3B; chi-square test, $p = 0.0209$, $n = 8$ per group). The scores for the ETX treated mice were overall less severe one hour later, and the mice treated with saline had higher scores one hour later (mean HIC scores: ETX, 2.313 and

2.063; Saline, 3.688 and 4.250). Both experiments indicate that ETX reduces ethanol withdrawal seizure severity with results demonstrating a reduction in behavioral and electrographical correlates of seizure activity.

Discussion

Our prior studies have shown significant effects of ethanol exposure and withdrawal on the physiology of T-type channels (Carden et al., 2006; Graef et al., 2011; Mu, Carden, Kurukulasuriya, Alexander, & Godwin, 2003; Shan, Hammarback, & Godwin, 2013; Wiggins, Graef, Huitt, & Godwin, 2013). Our current study extends these findings with the following observations: 1) ethanol withdrawal increased the incidence of seizure in DBA/2J mice; 2) ETX decreased ethanol withdrawal-induced seizures evident by reduced electrographical and behavioral measures of seizure; 3) ETX treatment rescued mice from seizures prior to withdrawal treatment. The effects of ETX persisted for the anticipated half-life of the drug. We consider each of these findings in turn.

Increased SWDs during Ethanol Withdrawal

We observed a progressive increase in SWDs with each successive ethanol withdrawal period (Fig. 2A). We also demonstrated a modest increase in duration of events (Fig. 2B). This suggests kindling-like effects similar to those previously reported in other rodent models (Becker, 1998). Studies demonstrated that as the number of ethanol withdrawals increased, there was a progressive increase in the severity of handling-induced convulsions (Becker, 1994; Becker, Diaz-Granados, & Hale, 1997; Becker, Diaz-Granados, & Weathersby, 1997). Consistent with our investigation, Veatch & Becker also demonstrated that brief spindle episodes increased in tandem with the number of withdrawal cycles in C3H/He mice (Veatch & Becker, 2002, 2005). Different strains show differences in sensitivity to withdrawal (Metten & Crabbe, 2005; Metten, Sorensen, Cameron, Yu, & Crabbe, 2010), and our study demonstrates a similar kindling-like phenomenon in DBA/2J mice. The electrophysiological signature of brief spindle episodes and SWDs are similar between our studies, but our study has not ruled out the possibility of independent mechanisms. However, thalamic involvement in spindle wave phenomena (Fuentelba & Steriade, 2005) suggests common underlying circuit mechanisms.

Clinical investigations of alcoholics have demonstrated an increased risk of seizure with an increased number of detoxifications, suggesting a possible kindling-like effect based on prior history (Brown et al., 1988; Lechtenberg & Worner, 1990). However, other reports suggest that the kindling phenomenon is only one potential mechanism for the development of alcohol withdrawal symptoms and is relevant only in certain situations (Wojnar, Bizo, & Wasilewski, 1999). T channels are one of many factors that may contribute to hyperexcitability and regional specific effects of ethanol withdrawal (Chen, Reilly, Kozell, Hitzemann, & Buck, 2009; Reilly, Milner, Shirley, Crabbe, & Buck, 2008). The observation of “kindling-like” effects, while they may be due to multiple targets of ethanol, nevertheless underscores the importance of early intervention. If this is borne out experimentally, treatment against early changes in withdrawal may prevent the progressive reduction in seizure threshold and the ultimate development of convulsive seizures observed in the clinic.

Reduction of WD Seizure through ETX Treatment

In this study, we demonstrated that acute administration of ETX inhibits ethanol withdrawal-induced seizures both electrographically and behaviorally. ETX is a first-line antiepileptic drug in the treatment of absence epilepsy (Hughes, 2009). It inhibits T-channel currents in the thalamus (Coulter, Huguenard, & Prince, 1989; Huguenard & Prince, 1994) and decreases seizures by reducing burst discharges that are consistent for both normal spindles and SWDs. Our prior results showed enhanced T-channel function during ethanol withdrawal, suggesting that ETX might be effective in reducing alcohol-related seizures.

ETX reduced the prevalence of SWDs compared to mice treated with saline in each withdrawal period and restored the duration of events to baseline values (Figs. 2A and B). We performed a within-group analysis among the mice treated with ETX to characterize seizure activity prior to treatment during each withdrawal. Interestingly, this comparison revealed that activity was increased prior to treatment and that ETX essentially rescued the mice from SWDs occurring before treatment (Fig. 2C). The SWD events returned to levels below what was observed during baseline activity. This interpretation was further supported by the fact that these mice did not have increased SWD events at 9:00 AM when they were removed from the chamber.

ETX is metabolized in mice very quickly with a biological half-life of approximately one hour (el Sayed et al., 1978). In a subset of mice within the ETX-treated ethanol-exposed group, we extended the fourth withdrawal period for an additional 3 h from 5:00–8:00 PM to determine if the SWDs would return as ETX was metabolized. Our results indicate that the SWDs return to levels intermediate between the levels observed prior to treatment and immediately following treatment (Fig. 2D), suggesting that ETX effects are dependent on metabolism of the drug.

We further validated our findings by performing a spectral power analysis to determine if ETX reduced the activity in the 6–10 Hz frequency range of the SWD events. We found that ETX treatment reduced power in the same frequency range as the SWD activity (Fig. 2E), thus reduction in this frequency band may be seen as a surrogate for detailed SWD counts.

Finally, we also demonstrated that ETX reduced the behavioral correlate of withdrawal seizure activity by measuring its effects on handling-induced convulsions. ETX treatment reduced the overall HIC score, and mice treated with ETX had fewer tonic/clonic seizures (Figs. 3A and B). These findings support the observations with the effects of ETX on electrographical correlates of withdrawal seizure. Our findings with ETX are similar to observations from a previous study demonstrating the effects of anticonvulsants, including ETX, on withdrawal symptoms (Kaneto et al., 1986). This study reported a decrease in withdrawal symptoms after ETX treatment; however, Kaneto et al. had a different focus and used a different model of alcohol exposure.

Our previous investigation demonstrated changes in T-channel gene expression and function as early as the third withdrawal period (Graef et al., 2011). These results suggest that it takes only a few exposures and withdrawals to initiate changes that can cause progressive withdrawal-induced seizures with each successive withdrawal, and we were able to alleviate

this increase in SWDs with ETX treatment. In this investigation we evaluated the acute effects of ETX. Further investigation will be necessary to determine if chronic treatment with ETX can prevent the kindling effects that occur with multiple alcohol exposures and periods of withdrawal. It should be noted that T channels contribute to the seizure activity present at baseline in DBA/2J mice as evidenced by the reduction in SWDs in naïve mice. Thus, our data suggest that seizures that are dependent upon T-channel activity are enhanced after ethanol exposure. The effects of ETX on the reduction of SWDs in naïve mice have been reported previously (Marrosu et al., 2007). This supports our previous findings that ethanol exposure and withdrawal dysregulate T-channel function and the hypothesis that this dysregulation contributes to withdrawal excitability. Our findings demonstrating an abnormal increase in SWDs during withdrawal, which did not occur in air-exposed mice, provide further support that T channels may play a significant role in withdrawal hyperexcitability. Further testing in different models, including nonhuman primates, will be necessary for determining the exact role of T-channel activity.

While it is widely accepted that ETX is a non-selective inhibitor of T channels, off-target effects have been proposed. Studies have demonstrated indirect effects on a persistent Na^+ current and Ca^{2+} -dependent K^+ current (Crunelli & Leresche, 2002; Leresche et al., 1998). Evidence from a recent study also suggested that ETX may increase GABA transmission (Greenhill, Morgan, Massey, Woodhall, & Jones, 2012). Thus, while a parsimonious explanation is that ETX exerts its effects solely through T channels, ETX could be acting at least partly through these other mechanisms. Another limitation is that T channels are distributed throughout the brain (Perez-Reyes, 2003), and while the form of SWDs recorded in our study are a characteristic of thalamic circuitry, follow-up studies will be necessary to verify direct thalamic T-channel involvement.

The DBA/2J Model of Alcohol Withdrawal Seizure

DBA/2J mice are advantageous because of their sensitivity to ethanol and ability to develop withdrawal-related symptoms. A pitfall of this model is that the rodents possess a background seizure phenotype, thus the relationship to human alcoholism may be indirect. C3H/HeJ mice, another model of severe HICs (Metten & Crabbe, 2005), also possess a seizure-prone phenotype (Beyer et al., 2008). Evidence from previous studies supports our interpretations with the electrographical experiments. Veatch & Becker (2002) indirectly demonstrated that increased electrographical activity in the form of brief spindle episodes correlates with increased severity in handling-induced convulsions during withdrawal. Mice had increased brief spindle activity by 6–8 h into the withdrawal period, which is also the time when the mice had increased handling-induced convulsions. This finding was also repeated in later studies testing different drugs (Becker et al., 2006; Veatch & Becker, 2005). We demonstrated a reduction in HIC severity in DBA/2J mice treated with ETX, which further validates the use of electrographical activity as a seizure indication. Thus, DBA/2J mice may prove useful as a model of withdrawal-elicited seizure progression.

A few reports have indicated nonconvulsive seizure-like activity in EEG recordings of patients undergoing withdrawal (Fernández-Torre & Martínez-Martínez, 2007; LaRoche & Shivdat-Nanhoe, 2011) and although inconsistencies exist in these findings, several

abnormalities in EEG of alcoholics have been identified (Cohen, Porjesz, Begleiter, & Wang, 1997; Coutin-Churchman, Moreno, Añez, & Vergara, 2006; Feige, Scaal, Hornyak, Gann, & Riemann, 2007; Porjesz & Begleiter, 2003; Rangaswamy et al., 2003; Rodriguez Holguin, Porjesz, Chorlian, Polich, & Begleiter, 1999; Salety-Zyhlarz et al., 2004). Individuals with multiple relapses have a lower seizure threshold; however, more work is needed to determine whether the lowering of seizure threshold in one seizure type may influence the progression to generalized convulsions and the physiological consequences of abnormal EEG activity.

Conclusions

Our studies emphasize three key points: 1) DBA/2J mice may constitute a useful model of ethanol withdrawal in that this strain shows a marked, progressive increase in SWDs that scale with the number of chronic, intermittent withdrawals; 2) pharmacological treatment with a current first line antiepileptic drug blocks both electrographical and behavioral ethanol withdrawal-induced seizures (and because ETX targets T channels, these channels may play a role in ethanol withdrawal symptoms); and 3) we have characterized the temporal dynamics of ethanol withdrawal seizure and early changes that may contribute to longer-term consequences.

Altered ion channel function is an important element of alcohol abuse and withdrawal. Our study implicates a contribution of T channels and thalamocortical circuitry to hyperexcitability in response to multiple, intermittent withdrawals, and highlights a novel model in which to explore detailed mechanisms and the potential of T channels as targets for pharmacotherapeutic intervention during withdrawal. Further investigation is necessary to determine the effect of ETX on other aspects of alcoholism and withdrawal such as consumption and withdrawal-induced anxiety. ETX decreases ethanol withdrawal-induced seizures, and further study will determine whether ETX or compounds that are more selective may hold promise as a novel treatment for alcohol withdrawal.

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Figure 1.

A. Schematic of intermittent ethanol exposure paradigm. Saline or ethosuximide (ETX, 250 mg/kg) treatments were administered at 1:30 PM during each withdrawal period in the EEG experiment (represented by star in schematic). Treatments (saline or ETX) were not administered during the baseline recording. For the HIC study, treatment was only administered in the fourth withdrawal at 3:00 PM. **B.** The unfiltered, raw EEG trace represents a spike and wave discharge (SWD) event in a DBA/2J mouse. See methods for SWD inclusion criteria.

Figure 2.

A. Ethosuximide (ETX, 250 mg/kg) decreased alcohol withdrawal-induced spike and wave discharges (SWDs). We analyzed the number of SWDs following saline or ETX treatment from 2:00–5:00 PM. There is a significant difference in time, treatment, and interaction. *Post hoc* analyses revealed a significant difference between saline and ETX treatment in each withdrawal period. We also observed a kindling-like effect in the saline-treated animals with SWDs increasing with each successive withdrawal. ETX treatment reduced SWD events below baseline values. Baseline bars represent naïve baseline EEG activity prior to exposure or treatment. These indicate the mice that will be receiving treatment. **B.** ETX (250 mg/kg) rescued mice from SWDs prior to treatment. Within the group of mice exposed to ethanol and treated with ETX, we compared the number of SWDs prior to each treatment from 10:30 AM to 1:30 PM to the number of events that occurred following treatment from 2:00–5:00 PM. There is a significant difference in time, treatment, and interaction. *Post hoc* analyses revealed a significant difference between pretreatment and posttreatment in the second, third, and fourth withdrawal periods. We also observed a kindling-like effect in the

pretreatment time period with SWDs increasing with each successive withdrawal. **C.** SWD events return as ETX is metabolized. We compared the number of events prior to treatment (pretreatment, 10:30 AM–1:30 PM), immediately following treatment (posttreatment, 2:00–5:00 PM), and at the point when ETX should be metabolized (washout, 5:00–8:00 PM) during the fourth withdrawal of mice treated with ETX. During the washout period (5:00–8:00 PM), SWD events were significantly higher compared to the number of events immediately following treatment. As previously shown, the number of events immediately following treatment was significantly decreased compared to the number of events prior to treatment. **D.** We analyzed the power in the 6–10 Hz frequency range representing the range of SWD activity. Data for each withdrawal period were collapsed and normalized. ETX treatment (250 mg/kg) decreased power in the 6–10 Hz frequency range to a level indistinguishable from baseline. $*p < 0.05$; $**p < 0.01$; $***p < 0.0001$

Figure 3.

A. We measured the effect of ETX treatment on handling-induced convulsions. Mice treated with 250 mg/kg ETX had a significantly lower HIC score compared to mice treated with saline. Mice treated with ETX also had fewer tonic/clonic seizures compared to mice treated with saline. **B.** We repeated this test one hour later and observed the same results. Mice treated with ETX still had lower HIC scores and fewer tonic/clonic seizures. * $p < 0.05$; ** $p = 0.02$

Table 1**Handling-induced convulsion (HIC) Scale**

Score	Description of behavior
0	No activity on tail lift, or after gentle 360° spin
1	No activity on tail lift, but a facial grimace after 360° spin
1.5	Facial grimace on tail lift
2	Tonic convulsion after 360° spin
3	Tonic/clonic convulsion after 360° spin
4	Tonic convulsion on tail lift
5	Tonic/clonic convulsion on tail lift, onset delayed by 1–2 s
6	Severe tonic/clonic convulsion on tail lift, no delay in onset
7	Severe tonic/clonic convulsion prior to tail lift

Scale used to score handling-induced convulsions (HIC). The scale was described by Becker (1994) and had been adapted from the original description by Goldstein (1972) and Crabbe & Kosobud (1990). We did not make any modifications to the scale described by Becker.

Table 2

Summary of SWD Events

Baseline	49.67±8.8 (n=3)	55.78±7.8 (n=9)	57.38±8.3 (n=8)	52.90±7.7 (n=10)
	<u>Air-Exposed Mice</u>		<u>Ethanol-Exposed Mice</u>	
2:00–5:00PM	Saline (n=3)	250 mg/kg ETX (n=9)	Saline (n=8)	250 mg/kg ETX (n=10)
1st WD	55.67±13.5	38.00±6.8	87.25±12.7	19.30±2.9
2nd WD	56.00±2.5	31.22±5.2	129.00±9.7	19.80±2.7
3rd WD	60.00±7.6	34.00±4.8	175.80±19.1	20.80±3.4
4th WD	103.30±33.9	39.56±5.7	237±23.6	14.00±2.8

The mean ± SEM values of SWD events during baseline and each withdrawal period (or equivalent time frame for air-exposed mice). Baseline values indicate naïve activity prior to any exposure (air or ethanol) and treatment (saline or ETX). The number of animals in each group: air-exposed, saline (3); air-exposed, ETX (9); ethanol-exposed, saline (8); ethanol-exposed, ETX (10); SWD, spike and wave discharge; ETX, ethosuximide