ETHOSUXIMIDE, A T-TYPE CALCIUM CHANNEL ANTAGONIST, REDUCES ALCOHOL WITHDRAWAL SEIZURE

BY:

MELISSA ANN RIEGLE

A Dissertation Submitted to the Graduate Faculty of
WAKE FOREST UNIVERSITY GRADUATE SCHOOL OF ARTS AND SCIENCES
in Partial Fulfillment of the Requirements
for the Degree of
DOCTOR OF PHILOSOPHY

Neuroscience

May 2014

Winston-Salem, North Carolina

Approved By:

Dwayne W. Godwin, Ph.D., Advisor
Emilio Salinas, Ph.D., Chair
Sara Jones, Ph.D.
Brian McCool, Ph.D.
Jeff Weiner, Ph.D.
ACKNOWLEDGEMENTS

In 2009, I embarked on the path of pursuing a doctorate degree in the Neuroscience Program at Wake Forest University. It is hard for me to describe the appreciation and gratitude I feel to so many people as this endeavor would not have been possible without help from an entire community.

First I would like to thank my mentor, Dr. Dwayne Godwin, and express sincere gratitude for all of his advice and support as the work detailed in my dissertation would not have been possible without it. Your willingness to guide and provide me feedback while allowing me to pursue interests of my own has helped me grow as a scientist, teacher, and as a person for which I am forever grateful. Not only did it help me succeed in the Neuroscience graduate program, but I will continue using what I have learned in my future endeavors. I received my own funding through an NRSA fellowship under his mentorship, which is just one of many examples illustrating how Dr. Godwin and his lab has provided a very successful training environment.

I would also like to thank my committee members, Drs. Sara Jones, Brian McCool, Emilio Salinas, and Jeff Weiner. Your advice and guidance have been extremely valuable. My project improved greatly because of your helpful critiques. Special thanks to Dr. Jeff Weiner and your lab for collaborating with us on our projects. Your enthusiasm and interest in our work is greatly appreciated as a big part of Chapter 3 of this thesis would not exist without your guidance. Special thanks to Dr. Brian McCool for your advice with our projects especially with the additional experiments in Chapters 2 and 4. Your support and the opportunity to be a trainee under your direction on the alcohol training grant is greatly valued. Thank you to Dr. David Roberts for support and guidance along the way; I hope retirement is treating you well!

Thank you to my current and former lab mates in the Godwin lab for all of your support and encouragement. I greatly appreciate the help given and more importantly the friendships I have made with each one of you. Special thanks to Melissa Massicampo and Hong Qu Shan for their help with projects described in Chapters 2 and 4. I am very thankful for your patience, hard work, and feedback, which have tremendously improved these projects. Thank you to David Klorig as his expertise in oscillatory activity and computer programming was very valuable. Special thank you to Dr. Erin Caulder, my
first friend in the lab and the one who initially trained me. Thank you for your patience, your willingness to help, and the lifelong friendship we share. Our collaboration on an epilepsy project described in the appendix of this dissertation solidified my interests and passion for understanding how the brain works especially in regards to the development of seizures. I am honored to have worked with each of these members of the Godwin lab and am forever grateful for all of their support.

Also, I would like to acknowledge the Neuroscience Program and Wake Forest Graduate School for the extensive training and for providing me with the scientific background necessary to pursue my research interests. I am sincerely thankful for all the help I have received from so many people that continuously devote their time and energy to helping graduate students. I also want to acknowledge our grant support as work in this dissertation would not have been feasible without it, F31AA021322-01, T32AA07565, F31AA017048, R01AA016852, R01AA015568, R21EY018159, Citizens United for Research on Epilepsy, and the Tab Williams Family Fund. I am grateful for the opportunities with the neuroscience training grant, the alcohol training grant, and my NRSA fellowship.

Thank you to my wonderful fiance, Dr. Philip Smaldino. You have been my rock, my support, my strength for which I am forever grateful. Your encouragement and endless optimism always amaze me, and I cannot thank you enough for your enthusiasm. Words cannot describe what I want to say but know that you mean the world to me and I would not be where I am without you. Of the many wonderful moments we have shared, building our Smal but Riegle Tiny Home while we were both finishing our doctorates was an incredible experience that will always live in my heart. You always believe in us and what we are capable of achieving. Your bravery, adventurous spirit, and warm heart are contagious, and I could not be more blessed to spend the rest of my life with you. I also want to thank the Smaldino Family, Jim, Camille, Emily, Eric, and Lindsey for all of their love and support.

Thank you to my Grandparents, Dr. Jack and Nancy Riegle. I have been so blessed to have such wonderful, supportive Grandparents in my life. They have always been such positive role models in my life and good examples of hard work and determination which has inspired me to set high goals. I have so many fond memories
with them and will cherish their love and kindness forever. Thank you to my Sister, Shantel Walker and her husband, Nathan Walker. Shantel, you are a beautiful person inside and out and have such a warm heart. Your kindness always inspires me to be a better person. I am also very grateful for my Nieces, Haley and Isabella Walker. Your youthful spirit and curiosity always keeps me on my toes.

Thank you to my Parents, David and Tina Riegle. Mom and Dad, words cannot describe how much you mean to me and how grateful I am for your endless love and support the past 29 years. I would not be where I am without you. Your encouragement through life challenges and work ethic are truly amazing. Your dedication, hard work, and kindness are the very essence of what I hope to be. I am forever grateful and have been blessed with a wonderful, supportive family.
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<td>antiepileptic drug</td>
</tr>
<tr>
<td>AW</td>
<td>alcohol withdrawal</td>
</tr>
<tr>
<td>BEC</td>
<td>blood ethanol concentration</td>
</tr>
<tr>
<td>BSE</td>
<td>brief spindle episode</td>
</tr>
<tr>
<td>BZD</td>
<td>benzodiazepine</td>
</tr>
<tr>
<td>C3H/HeCr</td>
<td>inbred mouse strain</td>
</tr>
<tr>
<td>C3H/HeJ</td>
<td>inbred mouse strain</td>
</tr>
<tr>
<td>C57Bl/6</td>
<td>inbred mouse strain (common name: C57 black; C57; black 6 mice)</td>
</tr>
<tr>
<td>Ca&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>calcium</td>
</tr>
<tr>
<td>Ca&lt;sub&gt;v&lt;/sub&gt;3.1</td>
<td>CACNA1G (T-type calcium channel)</td>
</tr>
<tr>
<td>Ca&lt;sub&gt;v&lt;/sub&gt;3.2</td>
<td>CACNA1H (T-type calcium channel)</td>
</tr>
<tr>
<td>Ca&lt;sub&gt;v&lt;/sub&gt;3.3</td>
<td>CACNA1I (T-type calcium channel)</td>
</tr>
<tr>
<td>cDNA</td>
<td>complimentary deoxyribonucleic acid</td>
</tr>
<tr>
<td>C/T</td>
<td>clonic/tonic</td>
</tr>
<tr>
<td>DBA/2J</td>
<td>inbred mouse strain</td>
</tr>
<tr>
<td>DRG</td>
<td>dorsal root ganglion</td>
</tr>
<tr>
<td>ED&lt;sub&gt;50&lt;/sub&gt;</td>
<td>effective dose in 50% of the population (median effective dose)</td>
</tr>
<tr>
<td>EEG</td>
<td>electroencephalography</td>
</tr>
<tr>
<td>EMG</td>
<td>electromyography</td>
</tr>
<tr>
<td>EGR1</td>
<td>early growth response protein 1</td>
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<tr>
<td>EtOH</td>
<td>ethanol</td>
</tr>
<tr>
<td>ETX</td>
<td>ethosuximide</td>
</tr>
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<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
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<tr>
<td>GABA</td>
<td>γ-aminobutyric acid</td>
</tr>
<tr>
<td>GAERS</td>
<td>Genetic Absence Epilepsy in Rats from Strasbourg model</td>
</tr>
<tr>
<td>GIRK</td>
<td>G-protein-activated inwardly-rectifying potassium channels</td>
</tr>
<tr>
<td>G-protein</td>
<td>guanosine nucleotide-binding proteins</td>
</tr>
<tr>
<td>HEK-293</td>
<td>human embryonic kidney cells</td>
</tr>
<tr>
<td>HIC</td>
<td>handling-induced convulsion</td>
</tr>
<tr>
<td>Hz</td>
<td>hertz</td>
</tr>
<tr>
<td>IC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>half maximal inhibitory concentration</td>
</tr>
<tr>
<td>IO</td>
<td>inferior olive</td>
</tr>
<tr>
<td>IP</td>
<td>intraperitoneal</td>
</tr>
<tr>
<td>K&lt;sup&gt;+&lt;/sup&gt;</td>
<td>potassium</td>
</tr>
<tr>
<td>LGN</td>
<td>lateral geniculate nucleus</td>
</tr>
<tr>
<td>L-type</td>
<td>L-type calcium channel</td>
</tr>
<tr>
<td>mGluR</td>
<td>metabotropic glutamate receptor</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger ribose nucleic acid</td>
</tr>
<tr>
<td>MSG</td>
<td>monosodium glutamate</td>
</tr>
<tr>
<td>N1E-115</td>
<td>neuroblastoma cell line from mouse</td>
</tr>
<tr>
<td>Na&lt;sup&gt;+&lt;/sup&gt;</td>
<td>sodium</td>
</tr>
<tr>
<td>NAD-ADH</td>
<td>nicotinamide adenine dinucleotide – alcohol dehydrogenase</td>
</tr>
<tr>
<td>NIH</td>
<td>National Institute of Health</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartate</td>
</tr>
<tr>
<td>NP078585</td>
<td>N- and T-type calcium channel antagonist</td>
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NRSE  neuron-restrictive silencer element
NRSF  neuron-restrictive silencer factor
N-type  N-type calcium channel
PAM  positive allosteric modulator
PC12  cell line derived from pheochromocytoma of the rat adrenal medulla
PKC  protein kinase C
PN200-110  isradipine
P/Q-type  P/Q-type calcium channel
PTZ  pentylenetetrazole
RT-PCR  reverse transcription polymerase chain reaction
R-type  R-type calcium channel
SE  status epilepticus
SRS  spontaneous recurrent seizures
SWD  spike and wave discharge
T/C  tonic/clonic
T channel(s)  T-type calcium channel(s)
T current  T-type calcium current
TLE  temporal lobe epilepsy
TRN  thalamic reticular nucleus
V₅₀  membrane potential at which 50% of the T-type calcium channels are available for activation
VGCC  voltage gated calcium channel
WD  withdrawal
ABSTRACT

Alcohol is the third highest risk factor for health related problems in the world and leads to over two million deaths per year. Individuals abusing alcohol frequently cycle between drinking and withdrawal states, which among other adverse side effects can induce withdrawal seizures. Alcohol withdrawal seizures are a major component of relapse and represent a significant barrier to recovery. Unfortunately, alcohol withdrawal seizures can cause more serious consequences such as status epilepticus and temporal lobe epilepsy, severe life-threatening conditions that often result in death.

Previous studies identified an upregulation in T-type calcium channels (T channel) during alcohol withdrawal. If enhanced T channel function is an essential feature of alcohol withdrawal seizures, ethosuximide (ETX), a T channel antagonist, should inhibit alcohol withdrawal seizures. Mice underwent an intermittent alcohol exposure paradigm with EEG activity recorded during each alcohol withdrawal period. ETX dose-dependently decreased the alcohol withdrawal seizures. In two additional models, ETX reduced the severity and number of convulsive-like seizures in mice undergoing alcohol withdrawal. Lastly, ETX significantly reduced the mortality rate caused by alcohol withdrawal seizures.

We expanded upon these findings and measured the effects of ETX on anxiety, locomotor activity, and alcohol intake. ETX reduced consumption in nondependent mice, which provided the first evidence for the potential role of targeting T channels with ETX in the treatment of alcohol abuse. Consistent with the T channel dependency of these effects, ETX did not affect motor function or anxiety in alcohol-naïve mice.
ETX has many advantages as a novel drug repurposed for treating alcohol withdrawal – it’s safe, it’s FDA-approved for childhood epilepsy, and it has little potential for abuse, which is a major limitation of current treatments. In summary, these investigations have resulted in a new preclinical target based on an existing drug that has promise to reduce potentially lethal alcohol withdrawal seizures.
CHAPTER 1

NEW INSIGHT ON OLD HYPOTHESES:
IMPLICATIONS FOR T-TYPE CALCIUM CHANNELS AND
ETHOSUXIMIDE IN ALCOHOL WITHDRAWAL

M. A. Riegle and D. W. Godwin

A portion of this chapter will be submitted for publication as a review article. The manuscript was written and prepared by M. A. Riegle. Dr. Dwayne Godwin provided guidance and editorial support.
Abstract

Alcohol abuse affects brain circuits in multiple functional dimensions, including synaptic and intrinsic membrane properties of neurons whose perturbation has significant consequences for the generation of brain rhythms. Multiple studies have demonstrated the importance of high threshold voltage-gated calcium channels in the development of hyperexcitable states including involvement in alcohol withdrawal symptoms such as seizure activity. Here we expand on these findings and review recent evidence that has now implicated disruption of low threshold voltage-gated calcium channels during ethanol exposure and withdrawal. Studies have demonstrated that T-type calcium channels are functionally inhibited by acute ethanol exposure. Chronic exposure, however, increases T-type calcium channel mRNA expression and produces functional changes that enhance calcium current mediated through these channels. Such changes persist during ethanol withdrawal. A gain of T-channel function contributed to increased burst firing in thalamic neurons, and this effect along with increased mRNA expression are hypothesized to contribute to withdrawal-related symptoms. In this review, we discuss the evidence that implicates T-type calcium channels and further evaluate their role in alcohol withdrawal seizure. To do this, we review circuitry involved in various models of alcohol withdrawal seizure. Early studies suggested that the thalamus acts as a hub in a much larger seizure network. We discuss these findings and propose that disrupted T-type calcium channels within the thalamus and its circuitry with other brain regions could facilitate its role in alcohol withdrawal seizure development. We also review studies that have targeted T-type calcium channels during withdrawal. Of particular interest, ethosuximide, a T-type calcium channel antagonist, reduced ethanol
withdrawal seizure activity and restored withdrawal-induced sleep disruptions. The evidence suggests that T-type calcium channel disruption early in alcohol withdrawal may presage and support abnormal epileptiform activity that further precipitates more severe, long-term consequences. Blocking this disruption with ethosuximide can serve as a potential treatment option for alcohol withdrawal seizures. Future investigations are necessary to test these hypotheses; however, preliminary evidence appears to be promising.
Alcohol Withdrawal

Cessation of drinking after a period of heavy or chronic use can result in the development of alcohol withdrawal symptoms (Ait-Daoud et al., 2006; Saitz, 1998). Unfortunately, as both the amount consumed and the duration of use increases, the risk for withdrawal symptoms significantly increases as well (Ait-Daoud et al., 2006). Withdrawal symptoms include: anxiety, insomnia, agitation, tremors, tachycardia, nausea, excessive perspiration, hallucinations, delirium tremens, and seizures (Ait-Daoud et al., 2006; Saitz, 1998), all of which can have detrimental effects on health and livelihood. These symptoms arise from physiological neuroadaptations that have developed in response to the decline in alcohol concentration. Due to the significant depressing effects of alcohol in the central nervous system, compensatory mechanisms of progressive neuronal excitation ensue, with concurrent development of disrupted brain rhythms. Withdrawal symptoms, including seizures, can represent a significant barrier to recovery. Understanding the neuroadaptations and mechanisms responsible for alcohol withdrawal is necessary for identifying novel targets and treatment options.

Calcium Channels

Calcium entry into cells is primarily mediated by voltage-gated calcium channels (VGCCs). VGCCs are distributed throughout the brain and other areas of the body, such as the heart, muscle, lung, pancreas, and kidney, and play a critical role in cell function (Simms and Zamponi, 2014; Walter and Messing, 1999). VGCCs have been implicated in a number of neurological disorders and thus, have been identified as potential pharmacological targets. Distinctive electrophysiological and pharmacological
characteristics differentiate the types of VGCCs (Perez-Reyes, 2003; Simms and Zamponi, 2014; Walter and Messing, 1999). L-, N-, P/Q, R-type calcium channels require a large depolarization for activation (classified as high voltage-gated channels), whereas T-type calcium channels (T channel; low voltage-gated channels) activate after small depolarizations. Other biophysical properties, including inactivation and deactivation, contribute to the distinguishing characteristics of these channels. Dihydropyridine blocks L-type channels (Randall and Tsien, 1995) and the other well distinguished high voltage gated channels (N-, P/Q-) are sensitive to neurotoxins (Adams et al., 1993; Dubel et al., 1992; McCleskey et al., 1987; Olivera et al., 1987; Williams et al., 1992). R-type channels can be blocked by nickel and cadmium (Zhang et al., 1993) or the venom, SNX-482 (Bourinet et al., 2001; Newcomb et al., 1998). T channels are differentiated from the other calcium channels by their sensitivity to nickel and ethosuximide (Coulter et al., 1989; Perez-Reyes, 2003). For a recent review on VGCCs, refer to Simms and Zamponi (2014).

Dysregulation of normal calcium channel function during ethanol exposure and withdrawal has been identified as a potential mechanism underlying withdrawal symptoms including hyperexcitability (Catlin et al., 1999; Walter and Messing, 1999). Ethanol can affect calcium homeostasis via a number of mechanisms including voltage-gated channels, receptor mediated channels, G-protein mediated effects, and intracellular calcium levels (for a review see Catlin et al, 1999). We first provide a brief overview of high threshold channels in this process but our specific focus is the low voltage-gated T channel and its role in ethanol exposure and withdrawal.
For a detailed review on the effects of ethanol and withdrawal on the following high voltage-gated calcium channels refer to Walter and Messing (1999). Briefly, acute ethanol exposure inhibited L-type channels in multiple systems including PC12 cells, DRG neurons, and isolated nerve terminals from rats (Huang and McArdle, 1994; Mullikin-Kilpatrick et al., 1995; Mullikin-Kilpatrick and Treistman, 1995; Walter and Messing, 1999; Wang et al., 1991; Wang et al., 1994). In contrast, chronic ethanol exposure increased L-type density and function in various models (Brennan et al., 1990; Dolin et al., 1987; Gerstein et al., 1997; Grant et al., 1993; Guppy et al., 1995; Messing et al., 1986; Messing et al., 1990; Skattebol and Robin, 1987; Walter and Messing, 1999; Whittington and Little, 1991). In PC12 cells, an increase in calcium uptake was observed after chronic ethanol exposure (Messing et al., 1986; Skattebol and Robin, 1987), and Grant et al. (1993) observed an increase in L-type current in these cells after an extended ethanol exposure had been removed. An increase in expression of L-type channels was suggested after chronic ethanol exposure enhanced the amount of dihydropyridine-binding sites in PC12 cells (Messing et al., 1986; Skattebol and Robin, 1987) and in ethanol dependent animals (Brennan et al., 1990; Dolin et al., 1987; Guppy et al., 1995).

It was hypothesized that the ethanol-dependent increase in L-type function contributed to withdrawal symptoms and enhanced ethanol consumption (Walter and Messing, 1999). Supporting this hypothesis, studies demonstrated that antagonizing L-type channels reduced withdrawal symptoms, withdrawal-induced mortality and ethanol consumption (Bone et al., 1989; Colombo et al., 1995; De Beun et al., 1996; Fadda et al., 1992; Gardell et al., 1997; Little et al., 1986; Littleton et al., 1990; Pucilowski et al., 1989; Pucilowski et al., 1992; Rezvani et al., 1991; Rezvani and Janowsky, 1990; Walter
and Messing, 1999). Less is known about the effects of ethanol on N- and P/Q-type calcium channels, but results have demonstrated that these channels are at least partially inhibited by ethanol (Solem et al., 1997; Walter and Messing, 1999; Wang et al., 1991; Woodward et al., 1990).

More recent studies following the Walter and Messing (1999) review further suggest an important role for calcium channels in alcohol dependence and withdrawal (Balino et al., 2010; Books et al., 2008; Katsura et al., 2005; McMahon et al., 2000; N’Gouemo and Morad, 2003; N’Gouemo et al., 2006; Newton et al., 2005; Rossetti et al., 1999). Also, L-type calcium channels have also been identified as important mediators for other physiological responses to alcohol exposure and withdrawal including cardiovascular symptoms (Kahkonen, 2004; Kahkonen and Bondarenko, 2004; Kahkonen et al., 2008).

Of interest to this review, L- and P-type currents have been implicated in ethanol withdrawal seizure development (N’Gouemo and Morad, 2003). N’Gouemo and Morad (2003) identified an increase in current density mediated by L- and P-type calcium channels in neurons isolated from the inferior colliculus of rats undergoing ethanol withdrawal. Also there was a greater amount of calcium current from channels that did not inactivate completely. The inferior colliculus is involved in audiogenic seizure development during ethanol withdrawal (Faingold et al., 1998; Faingold and Riaz, 1995; Frye et al., 1986); thus, this observed increase in calcium function was hypothesized to contribute to the development of audiogenic seizures as a separate cohort of rats undergoing withdrawal in this study had enhanced sensitivity for these seizures (N’Gouemo and Morad, 2003).
There have been mixed results with the effects of chronic ethanol exposure on N-type channels (McMahon et al., 2000; N'Gouemo and Morad, 2003; N'Gouemo et al., 2006). McMahon et al. (2000) observed an increase in binding sites for N-type calcium channels in chronically exposed PC12 cells and in the hippocampus and frontal cortex of dependent mice. N’Gouemo and Morad (N'Gouemo and Morad, 2003) did not observe an increase in N-type current in the inferior colliculus in rats undergoing withdrawal. Further, a downregulation in protein levels of N-type channels was observed in neurons isolated from the inferior colliculus of rats undergoing ethanol withdrawal (N'Gouemo et al., 2006). The exact role of N-type calcium channels and their dysregulation during chronic ethanol exposure and withdrawal remain elusive.

Several studies also have demonstrated the effectiveness of calcium channel antagonists on ethanol withdrawal seizures (Little et al., 1986). Specifically, the L-type antagonists, nitrendipine, nimodipine, and verapamil, and the nonselective calcium channel antagonist, flunarizine, reduced ethanol withdrawal seizure severity and mortality. These compounds were more effective than diazepam and, unlike diazepam, did not produce sedative effects. Nifedipine, an L-type antagonist, dose-dependently reduced audiogenic seizures in rats undergoing ethanol withdrawal (Pucilowski et al., 1989). Nitrendipine, nimodipine, and PN200-110 (L-type antagonists) reduced handling-induced convulsions in mice that were in ethanol withdrawal (Littleton et al., 1990), and levemopamil (L-type antagonist) reduced the severity and number of audiogenic seizures in rats undergoing withdrawal (Rezvani et al., 1993). Interestingly, L-type channels were implicated in seizure development during withdrawal, and treatment with nifedipine attenuated these alterations (Veatch and Gonzalez, 2000). This collection of
investigations provides strong evidence suggesting that ethanol exposure and withdrawal impacts calcium channel function. Further evidence indicates that calcium channels play a role in alcohol withdrawal symptoms including seizure activity and that targeting these channels may be a viable treatment strategy for alcohol withdrawal seizure reduction.

**T-type calcium channels**

Three functionally different isoforms of low-threshold (T-type) calcium channels (CaV3.1, CaV3.2, CaV3.3; T channels) are distributed heterogeneously throughout the brain and body (Perez-Reyes, 2003; Talley et al., 1999). In the brain, T channels primarily localize to dendrites (Christie et al., 1995; Gauck et al., 2001; Karst et al., 1993; Kavalali et al., 1997; Perez-Reyes, 2003; Pouille et al., 2000; Zhou et al., 1997); however, T channels can distribute on the soma as well (McKay et al., 2006; Zhou et al., 1997).

These channels are functionally unique in that they are primarily inactivated at resting membrane potentials meaning that these channels normally resist activation when depolarized from resting membrane potential (Perez-Reyes, 2003), though there are exceptions (Alonso and Llinas, 1992; Kang and Kitai, 1993) and specific isoform-dependent variations in electrophysiological properties exist (Huguenard and Prince, 1992; Klockner et al., 1999). To remove the inactivation (a process also called deinactivation), hyperpolarization of the membrane potential is required, and then the channels can be activated with a small membrane depolarization (Perez-Reyes, 2003). Activation of these channels permits calcium entry into the cell as the channel generates
T current. Ultimately, the low-threshold calcium spike can subsequently lead to a burst of Na\(^+\)-dependent action potentials.

Thus, T channels are critical to the neuronal output as their activity can switch the neuron from tonic firing to burst firing mode (Crunelli et al., 1989; Huguenard and Prince, 1992; Jeanmonod et al., 1996; Perez-Reyes, 2003; Steriade and Llinas, 1988; Suzuki and Rogawski, 1989). This activity is important to both normal and abnormal oscillatory behavior, such as sleep and seizure, respectively (Huguenard, 1999; Huguenard and Prince, 1994; Jeanmonod et al., 1996; Llinas and Steriade, 2006; McCormick and Bal, 1997; Steriade, 2005; Steriade et al., 1993; von Krosigk et al., 1993). Calcium entry via T channels can also lead to other voltage-gated channel activation as it causes the membrane to depolarize, and furthermore, can serve as a second messenger signaling molecule important for downstream intracellular mechanisms (Munsch et al., 1997; Perez-Reyes, 2003; Zhou et al., 1997).

The amount of available T current within neurons in particular nuclei is dependent upon the voltage dependency of the various isoforms, and which ones are present in a given nucleus. These differences in voltage dependency lead to differences in the tendency of neurons to burst. One resultant feature of this voltage dependency is their ability to generate window current through the interaction of the T current voltage-dependent properties (Crunelli et al., 2006; Gomora et al., 2001; Hughes et al., 1999; Lee et al., 1999; Perez-Reyes, 2003; Williams et al., 1997). Essentially, near resting membrane potentials, activation and inactivation overlap (in a criss-cross pattern) generating window current, which is not typical for high-voltage activated calcium channels. However, it is dependent on the membrane potential. Window current allows
for persistent T channel activity near physiological resting membrane potentials in
proportion to the amount of T channels that fail to inactivate completely near this range
of membrane potentials. Essentially, T-type window current can impact excitability as
well as intracellular calcium levels (Assandri et al., 1999; Chemin et al., 2000; Crunelli et
al., 2006; Crunelli et al., 2005; Graef et al., 2011; Mariot et al., 2002; Perez-Reyes, 2003;
Williams et al., 1997). For T channel physiology, see the detailed review by Perez-Reyes
(Perez-Reyes, 2003).

**Ethanol effects on mixed calcium current: first implications for T channels**

Few initial investigations suggested that ethanol may effect T current (Knott et al.,
2002; Twombly et al., 1990). These investigations, however, did not distinguish between
low voltage activated (T type) and transient high voltage activated (N-, P/Q-, R-type)
current; thus it is difficult to draw conclusions regarding the direct effects of ethanol on T
current. Twombly et al. (1990) demonstrated that acute ethanol (100mM and 300mM)
inhibited a mixture of calcium current (including T current) by 15% and 40%,
respectively, in the N1E-115 neuroblastoma cell line. The inhibition was attributed to
calcium current amplitude, rather than voltage-dependent gating properties. In ethanol-
naïve rat neurohypophysial terminals, a dose-dependent reduction in calcium current by
acute ethanol application was observed (Knott et al., 2002). For a summary, refer to
Table 1.
### Table 1. Ethanol Exposure and Withdrawal Effects on T-type Calcium Channels

<table>
<thead>
<tr>
<th>References</th>
<th>Methods</th>
<th>Findings</th>
</tr>
</thead>
</table>
| Twombly et al. (1990) | Neuroblastoma cell line  
100 mM and 300 mM EtOH  
whole-cell patch recordings | ↓ mixture of transient Ca\(^{2+}\) current amplitude                                             |
| Knott et al. (2002)  | Rat neurohypophysial terminals  
25mM – 100mM EtOH  
perforated patch recordings | Dose-dependent inhibition of transient Ca\(^{2+}\) current                                        |
| Mu et al. (2003)     | Ferret or rat thalamic slice (LGN)  
2.5mM – 50mM EtOH  
whole-cell patch recordings | Bidirectional effects: 5 and 10mM enhanced T current; >10mM, T current was dose-dependently inhibited |
| Joksovic et al. (2003) | HEK-293 cells, rat thalamic slice (TRN)  
50mM – 200mM EtOH  
whole-cell patch recordings | ↓ T current, IC50 ~100mM; suggested inhibition was mediated through Ca\(_V\)3.2 isoform           |
| Mah et al. (2011)    | Rat hippocampal cultures  
22mM – 87mM EtOH  
Whole-cell patch recordings | Dose-dependent inhibition of T current                                                            |
| Shan et al. (2013)   | HEK-293 cells, rat DRG neurons  
10mM – 200mM EtOH  
whole-cell patch recordings | Dose-dependent inhibition of Ca\(_V\)3.2 isoform; inhibition mediated by PKC                     |

### Chronic Ethanol Exposure

<table>
<thead>
<tr>
<th>References</th>
<th>Methods</th>
<th>Findings</th>
</tr>
</thead>
</table>
3-4 week liquid diet; 25mM – 100mM perforated patch recordings | Less inhibition of transient Ca\(^{2+}\) current compared to acute study, suggested compensatory mechanism |
Nordskog et al. (2006)
C57Bl/6 mice
4 week liquid diet
RT-PCR and Western blotting
↑ CaV3.2 and 3.3 gene expression; ↑ CaV3.3 protein expression
(CaV3.2 protein levels were not evaluated)

Carden et al. (2006)
Cynomolgus macaque thalamic slice (LGN)
consecutive 18 mo self-administration
whole-cell patch recording
↓ T current amplitude
↓ burst firing

Welsh et al. (2011)
Cynomolgus macaque inferior olive neurons
consecutive 12 mo self-administration
whole-cell patch recording
↑ T current; suggested that the increase contributed to withdrawal symptoms such as tremor

<table>
<thead>
<tr>
<th>Reference</th>
<th>Methods</th>
<th>Findings</th>
</tr>
</thead>
<tbody>
<tr>
<td>Graef et al. (2011)</td>
<td>C57Bl/6 mice intermittent vapor chamber</td>
<td>↑ CaV3.2 and 3.3 gene expression</td>
</tr>
<tr>
<td></td>
<td>RT-PCR and whole-cell patch recording</td>
<td>↑ CaV3.2 T current, ↑ burst firing</td>
</tr>
</tbody>
</table>

Abbreviations: EtOH, ethanol; Ca²⁺, calcium; LGN, lateral geniculate nucleus; T current, T-type calcium current; TRN, thalamic reticular nucleus; IC₅₀, half maximal inhibitory concentration; DRG, dorsal root ganglion; PKC, protein kinase C; Mo, month; IO, inferior olive; ↑, increase; ↓ decrease
Effects of acute ethanol exposure on T channels

More recent investigations have begun using specific cellular expression systems and voltage clamp protocols that can isolate T current allowing for direct assessments of ethanol-mediated effects on T channels. Acute ethanol exposure had a dose-dependent effect on T current in thalamic relay neurons of the dorsal lateral geniculate nucleus (Mu et al., 2003). Specifically, clinically low concentrations of ethanol (5mM and 10mM) enhanced T current. A depolarizing shift in the inactivation curve was observed which suggests that more channels were available for activation. This shift ultimately generated a larger window current which indicated that there was more T current available at resting membrane potential. At higher concentrations of ethanol (>10mM), T current was inhibited in a dose-dependent manner. The observed reduction was due to whole cell T current inhibition not requiring changes in channel kinetics.

In neurons from the rat thalamic reticular nucleus, native T current was almost completely blocked by acute ethanol application (200mM, IC50 ~100mM) (Joksovic et al., 2005b). Interestingly, in this study, much higher concentrations of ethanol were necessary to inhibit the CaV3.3 current expressed in HEK cells. Both CaV3.2 and CaV3.3 isoforms have been identified in the thalamic reticular nucleus (Joksovic et al., 2005a; Talley et al., 1999) suggesting that the inhibition observed in these neurons was an effect on CaV3.2 current. These results were supported recently by an investigation evaluating the acute effects of ethanol on the three different T channel isoforms, all expressed in an HEK cell line, which demonstrated that the CaV3.2 isoform was the most sensitive to ethanol (Shan et al., 2013). Specifically, ethanol inhibited the CaV3.2 T current in a dose-dependent manner, but had no effect on T current from CaV3.1 and CaV3.3 isoforms.
Also, in this study, acute ethanol exposure inhibited native T current isolated from dorsal root ganglion neurons (Shan et al., 2013), which highly express the Ca\textsubscript{v}3.2 isoform (Talley et al., 1999). Shan et al. (2013) further characterized the mechanisms of acute ethanol inhibition of T current and found the effects to be mediated by protein kinase C. Ethanol decreased T current amplitude and produced a hyperpolarizing shift in the T channel steady-state inactivation curve in the dorsal root ganglion neurons. This effect was blocked with a protein kinase C peptide inhibitor. Further investigation is underway to determine which protein kinase C isoform mediates these effects. An additional study has also demonstrated dose-dependent inhibition by ethanol on T current in rat hippocampal cultured cells (Mah et al., 2011). Multiple studies have now provided evidence demonstrating inhibition of T current by acute ethanol exposure. This inhibition occurs in a dose-dependent manner and appears to affect the Ca\textsubscript{v}3.2 isoform.

*Effects of chronic ethanol exposure on T channels*

In nonhuman primates, chronic ethanol self-administration reduced T channel-mediated burst firing in neurons of the lateral geniculate nucleus (Carden et al., 2006). A decrease in T current amplitude was also observed, however, there were no changes in T current kinetics. These recordings were conducted in brain slices of the dorsal lateral geniculate nucleus from macaque monkeys immediately following their last ethanol session. Interestingly, the Ca\textsubscript{v}3.2 and Ca\textsubscript{v}3.3 mRNA levels were increased in mouse thalamic neurons during chronic ethanol consumption (Nordskog et al., 2006) in which the C57Bl/6 mice were given a liquid ethanol diet chronically for a period of four weeks.
A more recent primate study demonstrated an upregulation in T current in the inferior olive neurons of primates following chronic ethanol consumption (Welsh et al., 2011). The authors suggested that this upregulation in T current after chronic ethanol exposure contributed to the hyperexcitability during withdrawal and symptoms such as tremor. It was noted that the ethanol was washed out of the slices prior to recording and that it could therefore be viewed as “acute withdrawal”. The monkeys, however, had access to ethanol prior to necropsy. These are different findings compared to what was observed in the Carden et al. (2006) study evaluating T current in the lateral geniculate nucleus. It should be noted that the monkeys in the Welsh et al. (2011) study consumed more ethanol than what was observed in the earlier study. Differences observed in effects on T current could be related to the differences in the amount of ethanol consumed.

Taken together, these studies suggest that functional inhibition of T current during ethanol exposure may lead to compensatory mechanisms that result in an upregulation of both T channels and current. The exact mechanism of the upregulation remains to be determined and further understanding of chronic ethanol exposure on T channel density and current is necessary. Overall, the evidence suggests that the compensatory changes may cause increased excitation during withdrawal. These changes, however, may be different depending on brain region and there is uncertainty in these different conditions as the ethanol-mediated effects will vary depending on the amount and length of ethanol exposure. These are questions that need to be addressed in future investigations.
**T channel dysregulation persists in ethanol withdrawal**

To date only one study has evaluated the direct effects of ethanol withdrawal on T channel expression and function. In this study, C57Bl/6 mice underwent an intermittent ethanol exposure paradigm. Tissue samples were collected and whole cell patch clamp recordings were made from midline thalamic nuclei (Graef et al., 2011). An upregulation in Ca\textsubscript{v}3.2 mRNA was observed in the third and fourth withdrawal periods. Ca\textsubscript{v}3.3 mRNA was increased only in the fourth withdrawal period, and Cav3.1 mRNA expression remained unchanged throughout the exposures and withdrawal periods. Thus, changes observed in exposure are indicative of what was observed during withdrawal as the Ca\textsubscript{v}3.2 isoform was affected the most by ethanol exposure and withdrawal. Importantly, Graef et al. (2011) observed enhanced T channel function that persisted through withdrawal. Specifically, there was a depolarizing shift in the T channel steady state inactivation curve, which suggests there were more channels available for activation at a more depolarized potential. There was a concomitant increase in the window current, which is suggestive of more T current available near resting membrane potential, and the midline thalamic neurons had increased burst firing. This enhanced T channel function was blocked by ascorbate, which has been shown to selectively block Ca\textsubscript{v}3.2 (Graef et al., 2011; Nelson et al., 2006) suggesting the increased excitability was mediated through the Ca\textsubscript{v}3.2 isoform. These changes were identified after only three exposures and withdrawal periods, and although the changes in mRNA were transient, the functional increase persisted for days following the fourth exposure. Within this investigation, a separate group of mice were chronically exposed to the intermittent paradigm for a total of four weeks. A depolarizing shift in the $V_{50}$ potential persisted and enhanced T channel
function was observed following the chronic exposure and withdrawal. These findings were consistent with those from the acute exposure and withdrawal experiments and support the findings of enhanced T current observed by Welsh et al. (2011) after chronic ethanol consumption in monkeys. Overall, these findings indicate that exposure and withdrawal cause early changes in T channel function that might lead to withdrawal symptoms of excitability. One way that this might produce complex consequences would be if ethanol disrupted other known functions of T channels, including its known role in sleep rhythms.

Additional evidence for T channel contribution in ethanol withdrawal

Disruptions in sleep caused by ethanol abuse and withdrawal have been described in both rodent and clinical literature (Allen et al., 1971; Bauer, 2001; Brower et al., 2001; Brower and Perron, 2009; Ehlers and Slawecki, 2000; Gillin et al., 1990; Landolt et al., 1996; Veatch, 2006; Wiggins et al., 2013). T channel activity within thalamic circuitry has been implicated in sleep-related oscillatory activity (Llinas and Steriade, 2006; McCormick and Bal, 1997; Steriade, 1993, 2005; Steriade et al., 1993), and it has been hypothesized that the alterations to T channel expression and function may in part be responsible for the sleep disruptions observed during ethanol exposure and withdrawal (Carden et al., 2006; Graef et al., 2011; Nordskog et al., 2006). This hypothesis was further supported by Wiggins et al. (2013). In this investigation, disruptions in diurnal EEG activity were observed in C57Bl/6 mice undergoing a chronic intermittent ethanol exposure paradigm (Wiggins et al., 2013). Specifically, EEG rhythms were disrupted with persistent changes in delta and theta activity. Treatment with ethosuximide (ETX),
a nonspecific T channel antagonist, decreased the disruptions compared to saline-treated animals during withdrawal. This study suggested that ETX may restore sleep activity, however, future studies are necessary to determine if ETX can translate into a treatment for alterations in sleep during exposure. This study does suggest that T channels may underlie such disruptions.

T channels were also implicated in the development of ethanol withdrawal-induced seizures (Riegle et al., 2014). In this study, mice undergoing ethanol withdrawal had increased spike and wave discharge activity (SWD), which is a type of nonconvulsive seizure activity that is mediated by T current within thalamic circuitry (Blumenfeld, 2003; Coenen and vanLuijtelaar, 2003; Crunelli et al., 1989; Huguenard, 1999; Huguenard and Prince, 1992; Huguenard and Prince, 1994; Jeanmonod et al., 1996; Marescaux et al., 1992; Porcello et al., 2003; Steriade, 2005; Suzuki and Rogawski, 1989; von Krosigk et al., 1993). Riegle et al. (2014) demonstrated a reduction in ethanol withdrawal induced SWD activity with acute ETX treatment in DBA/2J mice. Details of this study on seizure activity are described later in this review.

Alcohol withdrawal seizures

Evidence supporting the role for calcium channels during ethanol withdrawal now includes T channel activity as well. The contribution of T channel activity to hyperexcitability during withdrawal, with an emphasis on alcohol withdrawal seizure activity, is reviewed below. We highlight the importance of alcohol withdrawal seizure and the brain circuitry involved in the models of withdrawal-induced seizure activity. The potential for targeting T channels during ethanol withdrawal is also reviewed.
Alcohol withdrawal seizures have been described in many species including human, chimpanzee, monkey, dog, cat, rat, and mouse (Metten and Crabbe, 1996; N'Gouemo and Rogawski, 2006). In humans, alcohol withdrawal seizures develop in up to 33% of people undergoing alcohol withdrawal (Rogawski, 2005); however, this number can depend greatly on the amount of alcohol consumed, chronicity, and number of relapses experienced. Alcohol withdrawal seizures occur concurrently with ~25% of the other symptoms (Lechtenberg and Worner, 1990). Of those with seizures, 33% develop delirium tremens, and as many as 5-15% result in death (Erwin et al., 1998). Alcohol withdrawal seizures can precipitate more severe consequences such as status epilepticus and temporal lobe epilepsy (Hughes, 2009; Rogawski, 2005). In 9-25% of cases of status epilepticus, the cause was related to seizures induced by alcohol withdrawal (Hillbom et al., 2003; Hughes, 2009b). Thus, alcohol withdrawal seizures are a serious healthcare problem.

**Benzodiazepines**

Treatment strategies emphasize early intervention against neurotoxicity including seizures. Currently, benzodiazepines are the first line treatment for alcohol withdrawal symptoms (Ait-Daoud et al., 2006; Rogawski, 2005). Like ethanol, benzodiazepines, such as lorazepam and diazepam, modulate GABA_\text{A} receptor function (Rogawski, 2005) and quickly and effectively reduce seizure symptoms. Unfortunately, however, benzodiazepines are associated with several negative side effects that have raised questions about their use (Ait-Daoud et al., 2006). In particular, benzodiazepines have a high abuse potential. Other negative side effects include cognitive and motor
impairments, and the use of benzodiazepines may lead to rebound hyperexcitability that could increase the risk of seizure activity. It has been demonstrated that lorazepam exacerbated seizure activity in untreated withdrawal periods following an intermittent exposure and withdrawal paradigm (Becker and Veatch, 2002; Veatch and Becker, 2005). Both rodent and clinical studies have demonstrated rebound effects after benzodiazepine treatment was terminated (Chouinard, 2004; File and Wilks, 1990; Greenblatt et al., 1990; Loscher et al., 1996; Rundfeldt et al., 1995; Ward and Stephens, 1998; Woods et al., 1987). The development of tolerance to benzodiazepines has also been identified as a potential concern for their use (File and Wilks, 1990; Loscher et al., 1996; Rundfeldt et al., 1995; Veatch and Becker, 2005). Thus, identification of new treatment options is necessary and further understanding of the neurobiological mechanisms involved in alcohol abuse and withdrawal will facilitate such goals.

“Kindling”

Goddard et al. (1969) first characterized the phenomenon of “kindling” by demonstrating the development of seizure activity after repeated subthreshold electrical stimulations of the amygdala. A phenomenon with some parallels to kindling has been observed in the ethanol literature. In both clinical and animals studies that demonstrated that as the number of withdrawal periods increase, withdrawal symptoms worsen and the risk for seizure development increases (Ballenger and Post, 1978; Becker, 1994; Becker, 1998; Becker et al., 1997a; Becker et al., 1997c; Brown et al., 1988; Lechtenberg and Worner, 1990; Moak and Anton, 1996; Riegle et al., 2014; Veatch and Becker, 2002, 2005). The decrease in seizure threshold and increase in severity based on prior history
has been hypothesized as a “kindling-like” effect and underscores the importance of early intervention. In recognition that seizures may themselves be predisposing to increased seizure intensity and incidence (Ben-Ari, 2006; Bertram, 2007), it has been proposed that early and aggressive intervention may be necessary for preventing symptoms from progressively worsening (Becker, 1999; Malcolm et al., 2000; Myrick et al., 2001).

Models of alcohol withdrawal seizure

Electrographical correlates

Increases in abnormal oscillatory activity have been identified in multiple rodent and cat studies during ethanol withdrawal (Begleiter and Porjesz, 1977; Edmonds Jr et al., 1982; Guerrero-Figueroa et al., 1970; Hunter et al., 1973; Hunter et al., 1978; Hunter and Walker, 1978; Maxson and Sze, 1976; Perrin et al., 1975; Poldrugo and Snead, 1984; Riegle et al., 2014; Swartzwelder et al., 1979; Veatch and Becker, 2002; Veatch and Gonzalez, 1996; Walker and Zornetzer, 1974). This activity has been indicated by aberrant spiking, brief spindle episodes (BSEs) or spike and wave discharges (SWDs) as the electrophysiological signature of seizure events. BSEs or SWDs are nonconvulsive seizure events that occur at particular species-specific frequency ranges (Table 2).

Although the exact mechanism is not known for the development of BSEs or SWDs, thalamic involvement in spindle wave phenomena (Fuentealba and Steriade, 2005) suggests common underlying circuit mechanisms. These electrographical correlates differ from the behavioral correlates of withdrawal seizure described later in this chapter. BSEs or SWDs are spontaneous nonconvulsive events as the animals do not exhibit a motor component and do not need additional stimulation to induce the event. Behavioral
correlates include a motor component that has been described as myoclonic to tonic/clonic activity. In ethanol withdrawal, spontaneous tonic/clonic activity is not usually observed although there have been a few studies reporting such activity. An additional source is typically required to stimulate and assess such events during withdrawal. These types of seizure events are described in the behavioral correlate section.
Table 2. Models of Alcohol Withdrawal Seizure

<table>
<thead>
<tr>
<th>Model</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>BSEs or SWDs</td>
<td>Nonconvulsive epileptiform activity characterized by specific morphology (sharp spike and wave or spindle-like) with activity occurring in a particular frequency range (species dependent). Thalamic involvement has been suggested. These events are spontaneous and can be detected by EEG.</td>
</tr>
<tr>
<td>Representative Sources</td>
<td>(Riegle et al., 2014; Veatch and Becker, 2002; Walker and Zornetzer, 1974)</td>
</tr>
</tbody>
</table>

**Behavioral Correlates (Convulsive Seizure)**

<table>
<thead>
<tr>
<th>Model</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Handling-Induced</td>
<td>Mice are picked up by the tail and monitored for behavioral response. After immediate assessment on tail lift, the mouse is spun. The elicited seizure activity from the tail lift and spin is assigned a score based on severity. Mice in withdrawal have increased severity in handling-induced convulsions.</td>
</tr>
<tr>
<td>Representative Sources</td>
<td>(Becker, 1994; Becker and Hale, 1993; Goldstein and Pal, 1971; Metten and Crabbe, 2005)</td>
</tr>
<tr>
<td>Audiogenic</td>
<td>Mice or rats are exposed to an auditory stimulous during withdrawal. Behavioral response is monitored. Seizure activity is scored based on severity. Animals in withdrawal have increased sensitivity to audiogenic stimulation.</td>
</tr>
<tr>
<td>Representative Sources</td>
<td>(Celik et al., 2004; Clemmesen et al., 1988; Faingold and Riaz, 1995; N'Gouemo and Rogawski, 2006); non-ethanol related study (Jobe et al., 1973)</td>
</tr>
<tr>
<td>Chemoconvulsant</td>
<td>Animals are injected with a chemoconvulsant during withdrawal to evaluate threshold to seizure activity. Animals undergoing ethanol withdrawal have decreased seizure thresholds requiring lower doses of the drug to elicit a seizure. Behavioral monitoring and seizure scores are assigned to assess latency and severity.</td>
</tr>
</tbody>
</table>
### Electrical Stimulation

Animals can be electrically stimulated prior to ethanol exposure or following ethanol exposure. Different regions of the brain have been used for electrical kindling including the amygdala and hippocampus. Animals that are electrically stimulated prior to exposure kindle quicker during withdrawal compared to nonstimulated animals.

**Representative Sources**

- Grant et al., 1990; Kokka et al., 1993; Stephens et al., 2001; Szabo et al., 1984

### Spontaneous

Spontaneous seizures range from myoclonic to tonic/clonic activity that occurs without the use of any stimulation or drug to elicit the event. To detect during alcohol withdrawal, continuous video monitoring has to be utilized. These studies are limited due to the unpredictable nature of such events.

**Representative Sources**

- Geisler et al., 1978; McCown and Breese, 1990; Pinel and Van Oot, 1978; Veatch and Gonzalez, 1997, 1999

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BSE, brief spindle episode; SWD, spike and wave discharge; EEG, electroencephalography
In 1974, Walker and Zornetzer identified abnormal oscillatory activity in mice during ethanol withdrawal and characterized this activity as BSEs. It was also noted that the severity of the seizure events increased in the second withdrawal period compared to what was observed during the first withdrawal period. Several brain regions were involved in these withdrawal seizures including the thalamus, hippocampus, amygdala, and cortex. Ictal activity in both the thalamus and hippocampus preceded the appearances of tonic/clonic convulsions. In this early study, it was indicated that the thalamus could be an organizing region that could initiate and spread more sustained seizure activity throughout the brain.

It has also been demonstrated in cats that abnormal EEG activity preceded behavioral events (Perrin et al., 1975). Specifically, spiking activity was observed in the hippocampal and cortical regions. Other studies observed abnormal EEG development caused by ethanol withdrawal, however, the investigators indicated no relationship between electrographic and behavioral events (Guerrero-Figueroa et al., 1970; Hunter et al., 1973). Friedman (1980) suggested that the abnormal EEG observed in these different models is not causative as there was no direct link to behavioral activity. The aberrant EEG activity detected in these different studies did not coincide with the observed behavioral seizures. However, this argument does not address the possibility that the abnormal EEG activity could precipitate future, spontaneous behavioral manifestations of seizure events. If this were true, the events would not temporally coincide.

Walker and Zornetzer (1974) identified the possibility of this with the thalamus as being a key region for propagating the abnormal activity. Hunter et al. (1978) and Hunter and Walker (1978) further implicated the thalamus, including midline thalamic nuclei, as
a generator for aberrant seizure activity during ethanol withdrawal. These studies
demonstrated nonconvulsive epileptic activity in the thalamus prior to behavioral
manifestations of convulsive seizure activity during ethanol withdrawal. Although there
was activity in other brain regions as well, such as the amygdala and cortex, it was
hypothesized that the thalamus could organize and facilitate the spread of aberrant
oscillatory activity to several of the brain regions. These studies also demonstrated that
the epileptic activity developed in subcortical regions such as the thalamus and spread to
the cortex.

Poldrugo and Snead (1984) demonstrated epileptiform activity in Sprague-
Dawley rats undergoing withdrawal. The abnormal EEG events and behavioral correlates
of withdrawal seizure progressively worsened from the first to the second withdrawal
period with more frequent and generalized irregular activity in the second withdrawal
period. Epileptiform activity was observed in the thalamus, amygdala, hippocampus, and
cortex. Although the behavioral signs and electrographic events occurred concomitantly,
the electrographic events peaked earlier than the more severe behavioral stages classified
as hyperactivity and severe tremor. Also, the behavioral symptoms persisted longer in
the withdrawal period than the electrographical events.

A more recent study by Veatch and Becker (2002) identified BSEs in C3He mice
undergoing a multiple intermittent exposure paradigm. The BSEs increased with
successive withdrawal periods in a “kindling” like manner and was characterized as “high
voltage repetitive spikes occurring at 7-9 Hz”. The authors hypothesized that
thalamocortical circuitry was involved in the aberrant brief spindle activity. In this study,
the temporal profile of the electrographic and behavioral responses of seizure activity
were similar, both progressively increasing within each withdrawal (peak 6-8 hours) and with successive withdrawal periods. Interestingly, the authors noted that the BSEs preceded the behavioral activity during withdrawal.

We found similar results in DBA/2J mice undergoing an intermittent ethanol exposure paradigm (Riegle et al., 2014). The electrographical correlates of withdrawal-induced seizure activity were characterized as SWD events occurring in the 6-14 Hz frequency range. A progressive increase in SWD activity was demonstrated during each withdrawal, and the severity of the SWD activity increased with successive withdrawal periods in a “kindling-like” manner. Acute treatment with the T channel antagonist, ETX, blocked the increased SWD activity. We hypothesized that the increased SWD activity observed during ethanol withdrawal was mediated by enhanced T channel function and was facilitated by thalamocortical circuitry.

As previously mentioned a key area that supports this type of activity during ethanol withdrawal is the thalamus. The thalamus, with its extensive connectivity to other brain regions, has been identified as a key node in a network supporting seizure generation, facilitation, and propagation (Avanzini et al., 2000). In the thalamic circuit, there is a feedback loop system composed of GABAergic neurons of the thalamic reticular nucleus and glutamatergic neurons of the relay nuclei (Fuentealba and Steriade, 2005). Rhythmic activity within this loop is sustained by T current, which mediates synchronized burst activity during both normal and abnormal rhythms (Huguenard and Prince, 1992; Jeanmonod et al., 1996). Abnormal activity is then transmitted to hippocampus and cortex, with the thalamic node serving as a rhythmic pacemaker.
Studies investigating circuitry involved in temporal lobe seizure activity have identified midline thalamic neurons as key participants (Bertram, 2009; Bertram et al., 2008). Here, the midline thalamic neurons supported the generation and propagation of the seizure activity emerging from the hippocampus and amygdala. Furthermore, the disruptions in T channel activity during ethanol withdrawal were identified in the midline thalamic nuclei (Graef et al., 2011). Withdrawal seizures can precipitate long-term consequences such as status epilepticus and temporal lobe epilepsy. T channels are located within thalamic circuitry and the other connected regions (Perez-Reyes, 2003; Talley et al., 1999); thus it is reasonable to suggest that the disruptions observed in T channel activity during ethanol exposure and withdrawal may facilitate epileptiform activity within the thalamus (and potentially other brain regions) leading to a hyperexcitable network. This circuitry can generate the abnormal seizure activity observed in animals undergoing withdrawal. It is also plausible to propose that this aberrant thalamic oscillatory activity can be propagated to other brain regions such as the cortex, hippocampus, and amygdala and serve as a trigger for more severe consequences, like tonic/clonic seizure activity. More investigations, however, are necessary as few studies have included recordings from the thalamus.

There is strong evidence supporting the use of EEG and the detection of nonconvulsive (absence-like) seizures as a model for alcohol withdrawal seizure. Multiple studies have demonstrated that the aberrant electrographic seizure events increase with successive withdrawal periods as well as within each withdrawal period (Riegle et al., 2014; Veatch and Becker, 2002). These findings support data from the clinical literature that demonstrate that as patients underwent more relapses and
experienced more withdrawal periods, the threshold for seizure activity decreased (Brown et al., 1988; Lechtenberg and Worner, 1990). Also, many reports have demonstrated abnormalities in the EEG activity of alcoholics (Cohen et al., 1997; Coutin-Chruchman et al., 2006; Feige et al., 2007; Porjesz and Begleiter, 2003; Rangaswamy et al., 2003; Rodriguez Holguin et al., 1999; Salety-Zyhlarz et al., 2004). Although it is less common, nonconvulsive seizure activity initiated by alcohol withdrawal has been reported (Fernandez-Torre and Martinez-Martinez, 2007; LaRoche and Shivdat-Nanhoe, 2011). The authors hypothesized that although mental alterations are usually attributed to hallucinations and neuropsychiatric causes, the nonconvulsive seizure activity may contribute to the confusion and cognitive deficits that can occur during withdrawal.

Alcohol withdrawal seizures may be underestimated and misdiagnosed as it requires continuous EEG monitoring to determine if nonconvulsive, abnormal events are present. It is evident that individuals with multiple relapses have a lower seizure threshold; however, additional studies are necessary to determine if nonconvulsive activity may precipitate or influence the generation and progression of tonic/clonic convulsions. The physiological consequences of the abnormal EEG activity remain to be determined, and more work is needed to evaluate the differences in these types of withdrawal induced seizures. This is critical to our understanding of the potential impact the epileptiform activity can have on an individual.

**Behavioral correlates**

There are several types of animal models that can measure the behavioral correlates of alcohol withdrawal seizure. As previously mentioned, behavioral manifestations of
alcohol withdrawal seizure include myoclonic to tonic/clonic activity. Table 2 provides a general description of each model. We, however, do not review every study utilizing these models as there have been hundreds to date and goes beyond the scope of this review. Most studies utilizing these techniques are evaluating severity of alcohol withdrawal, potential treatment options, and neurochemical substrates involved.

I. Handling-induced convulsions

In 1971, the handling-induced convulsion model was introduced as a measure to characterize ethanol withdrawal-induced seizure activity (Goldstein and Pal, 1971), and it has been used in several studies evaluating withdrawal seizure activity and potential treatment options (Becker, 1994; Becker et al., 1997a; Becker et al., 1997c; Becker and Hale, 1993; Becker et al., 2006; Crabbe et al., 2012; Grant et al., 1990; Kosobud and Crabbe, 1986; Metten and Crabbe, 2005; Metten et al., 2010; Veatch and Becker, 2005; Watson and Little, 1994; Watson et al., 1994). In this model, behavioral assessment of seizure activity occurs upon tail lift and following a 360° spin in rodents undergoing ethanol withdrawal.

Currently little is known of the circuitry involved in handling-induced convulsions (N’Gouemo and Rogawski, 2006). It is thought that activation of the vestibular system contributes to these withdrawal-induced seizures. Brainstem areas including the periaqueductal gray and pontine reticular formation, which are also involved in audiogenic seizure induction, are thought to contribute to handling-induced convulsions as well. In a slightly different model of handling induced convulsions, using ethanol-naïve animals, the circuitry hypothesized included sensory (vestibular and somatosensory systems), motor, and hippocampal circuitry (Etholm et al., 2013).
Although this was not the same type of handling as it did not involve spinning, studies such as this one will help elucidate the mechanisms involved in handling-induced convulsions. Further investigation into the circuitry of these seizures induced by ethanol is necessary.

This model has been implemented in many intermittent exposure paradigms, and results have demonstrated that handling-induced convulsions progressively worsen within the withdrawal period as well as with each successive withdrawal period (Becker, 1994; Becker et al., 1997a; Becker et al., 1997c; Becker and Hale, 1993; Veatch and Becker, 2002). These mice were compared to mice that had undergone the same amount of time in the vapor chamber but did not receive the intermittent exposures which further emphasized the kindling-like effect. This suggests that these types of convulsions might involve mechanisms that may precipitate more severe consequences.

Importantly, T channels are distributed in the circuitry that has been implicated in handling-induced convulsions (Talley et al., 1999) and may play a role in these types of seizures. While direct measures on T channel activity during withdrawal in these particular regions remain to be determined, T channels have indirectly been implicated by testing the use of ETX in this model. Recently, ETX (250mg/kg) was found to reduce the severity of handling-induced convulsions in mice undergoing ethanol withdrawal (Riegle et al., 2014). DBA/2J mice underwent an intermittent ethanol exposure and withdrawal paradigm, which consisted of four ethanol exposures (16 hours each) and four withdrawal periods (8 hours each). Mice were tested for handling-induced convulsions at seven and eight hours in the fourth withdrawal period. Acute ETX treatment significantly reduced the severity of handling-induced convulsions compared to saline-treated mice. ETX-
treated mice also had significantly less tonic/clonic seizures compared to saline-treated mice.

These results are similar to a previous study evaluating the effects of ETX on withdrawal symptoms. Mice were exposed to three straight days of ethanol vapor and then tested using the handling-induced convulsion method with ETX treatment (Kaneto et al., 1986). ETX treatment reduced handling convulsions in the mice. While the focus of this study was on differences between barbiturate and ethanol withdrawal, it provides further evidence that T channels may play a role in handling-induced convulsions during ethanol withdrawal and that ETX may serve as a potential treatment option.

II. Audiogenic seizures

Another well characterized model of ethanol withdrawal seizure includes audiogenic stimulation to induce seizure activity during withdrawal (Celik et al., 2004; Clemmesen et al., 1988; Faingold et al., 2004; Faingold and Riaz, 1994; Freund and Walker, 1971; Hunter et al., 1978; Hunter et al., 1975; Kostowski et al., 1993; Little et al., 1986; Maxson and Sze, 1976; N'Gouemo and Rogawski, 2006; Riaz and Faingold, 1994; Rogawski, 2005; Watson et al., 1994). In this model, sound stimulation is used to elicit seizure activity during ethanol withdrawal; a detailed description of audiogenic seizures and the scale used to behaviorally score the animals can found in Jobe et al. (1973). Audiogenic seizures during ethanol withdrawal are primarily mediated in areas of the brain stem including the inferior colliculus, superior colliculus, periaqueductal gray, and the pontine reticular formation. Other evidence has demonstrated that the amygdala is involved in audiogenic seizures during ethanol withdrawal (Feng et al., 2007; Hunter et al., 1978). Finally, abnormal oscillatory activity was identified in the
hippocampus after audiogenic seizures were initiated (Celik et al., 2004; Hunter et al., 1973). These findings suggested that other networks beyond the initial development of the audiogenic seizures were recruited.

As with the handling-induced convulsions, T channels are distributed in the regions involved in audiogenic seizures and disruptions to these channels during withdrawal may contribute to the increased propensity for these types of seizures in withdrawal. Thus far, disruptions to T channels in this circuitry of withdrawal-induced audiogenic seizures have not been investigated and it remains to be determined if T channel antagonists, including ETX, would reduce the severity of audiogenic seizures initiated during ethanol withdrawal. It is important to note that ETX does reduce audiogenic seizures in ethanol-naïve mice (Bialer et al., 2004), which indirectly suggests that T channels may play a role in this type of seizure activity. We would predict that if T channels are disrupted in these regions during withdrawal that this disruption would further enhance the severity of audiogenic seizures during withdrawal. We also would predict that these types of withdrawal seizures could potentially be blocked by ETX. Other calcium channels have been implicated in the development of audiogenic seizures during ethanol withdrawal (N’Gouemo and Morad, 2003), and further evidence was provided in a study demonstrating that calcium channel antagonists reduced these seizures in rats (Little et al., 1986). These studies provide strong evidence that calcium channels play a role in the development of withdrawal related seizure activity, and we would expect that a disruption in T channels would contribute as well.
III. Electrical stimulation

Early studies demonstrated that electrical stimulation increased the sensitivity to seizure development during ethanol withdrawal (Geisler et al., 1978; McQuarrie and Fingle, 1958; Mucha and Pinel, 1979; Pinel, 1980; Pinel and Van Oot, 1977, 1978; Pinel et al., 1975). All of these studies led to the overall conclusion that electrically stimulated animals in withdrawal developed seizure activity more readily compared to controls. It is important to note that electrical stimulation preceded ethanol exposure and withdrawal in these investigations. The different techniques employed included electroconvulsive shocks (McQuarrie and Fingle, 1958; Pinel and Van Oot, 1977, 1978) and stimulation sites in the amygdala (Mucha and Pinel, 1979; Pinel et al., 1975) and in the hippocampus (Geisler et al., 1978). In support of the conclusions made by the early investigations, rats exposed to an intermittent intoxication and withdrawal paradigm that developed spontaneous seizures had enhanced kindling in the amygdala compared to animals that did not develop spontaneous seizures and control animals (Ulrichsen et al., 1998). In this study, however, electrical stimulation in the amygdala was not initiated until weeks after the last exposure to ethanol.

In contrast, other studies have demonstrated that chronic ethanol exposure and intermittent exposure paradigms delay the development of kindling in the hippocampus (Veatch and Gonzalez, 1997, 1999) and amygdala (McCown and Breese, 1990). Conflicting with their findings in the amygdala, McCown and Breese (1990) also reported a decrease in threshold in the inferior colliculus. One difference in these later studies compared to the earlier investigations was that the animals were exposed to chronic ethanol and withdrawal prior to electrical stimulations, whereas, in the majority
of the studies that demonstrated a decrease in kindling threshold, the electrical stimulation occurred prior to ethanol exposure and withdrawal. It is worthy to note that electrical stimulation in these studies was initiated at different time periods during withdrawal. The earliest stimulation occurred seven days following the final withdrawal period (McCown and Breese, 1990) and the latest at twenty days following the final withdrawal period (Veatch and Gonzalez, 1999).

Different amounts of ethanol exposure could also influence the observed results. Because seizure sensitivity typically occurs in the first forty-eight hours of withdrawal (Hillbom et al., 2003; Hughes, 2009b), studies that stimulate weeks later were not directly testing the effects of kindling on acute ethanol withdrawal induced seizure activity. Different animal models and strains were used as well as different electrical stimulation procedures, which could contribute to differences found among the studies. Based on the data, the amygdala, hippocampus, and inferior colliculus play critical roles; however, the exact circuitry and mechanism involved remain to be determined. What role, if any, of T channels play in this model and the potential use of ETX remains to be determined.

**IV. Chemoconvulsant-elicited seizure**

Another common strategy used to study ethanol withdrawal seizure has been to use a chemoconvulsant to elicit a seizure event during withdrawal. Severity of seizure and the dose of chemoconvulsant necessary to elicit a seizure can be compared in animals undergoing ethanol withdrawal to control animals. Most studies have demonstrated that ethanol withdrawal decreased the threshold to seizure induced by a chemoconvulsant. Reagents that have been utilized during withdrawal include pentylenetetrazole (PTZ)
(Chesher and Jackson, 1974; Hunt, 1973; Kokka et al., 1993; McQuarrie and Fingle, 1958; Peters and Steele, 1980; Ratcliffe, 1972; Stephens et al., 2001), picrotoxin (Szabo et al., 1984), glutamate agonists (Grant et al., 1990), strychnine (Ratcliffe, 1972), and flurothyl (Greer et al., 1976; Sanders, 1980). The development of these seizures and circuitry involved is dependent on the specific neuronal system that the chemoconvulsant is targeting.

Part of the usefulness of using chemoconvulsants to study ethanol withdrawal seizure stems from the ability to easily test potential treatments, like ETX and other calcium channel antagonists. For example, acute treatment with ETX reduced PTZ-induced seizures during ethanol withdrawal (Riegle et al., manuscript in prep). In this investigation, mice were exposed to an intermittent ethanol withdrawal paradigm and were tested during the fourth withdrawal period. Mice undergoing ethanol withdrawal had a lower threshold for seizure activity compared to mice that underwent identical handling but exposed to air only. Specifically, a subthreshold dose of PTZ (20mg/kg), which did not induce tonic/clonic seizure activity in air-exposed DBA/2J mice, elicited myoclonic and tonic/clonic seizure activity in mice undergoing ethanol withdrawal. Acute ETX treatment dose-dependently reduced this seizure activity during ethanol withdrawal. It remains to be determined if early changes in neuronal activity such as nonconvulsive epileptiform activity contribute to the increased sensitivity to the chemoconvulsant agents. Perhaps the neurochemical adaptations that lead to the abnormal oscillatory activity also lower the threshold for seizure induction by these chemoconvulsant agents. This study does provide further evidence that targeting T
channels and the use of ETX may be a potential strategy in the treatment of ethanol withdrawal.

V. **Spontaneous behavioral seizures**

There have been relatively few studies that have assessed spontaneous behavioral seizures during ethanol withdrawal (Clemmesen et al., 1988; Gonzalez et al., 1989; Hunter and Walker, 1978; Little et al., 1986; Poldrugo and Snead, 1984; Walker and Zornetzer, 1974). Spontaneous seizure activity that has been characterized ranges from myoclonic activity to tonic/clonic activity. Different models of exposure and withdrawal were utilized, thus it is difficult to make comparisons between studies. Also, in many of the studies seizure activity was only monitored for a limited time span or continuous monitoring was not utilized which could result in an underestimation of these events.

The circuitry underlying spontaneous seizures is still unknown (N'Gouemo and Rogawski, 2006). Based on these studies, it is likely that limbic regions are involved including the amygdala (Clemmesen et al., 1988; N'Gouemo and Rogawski, 2006). The investigation by Walker and Zornetzer (1974) also implicated other regions including the hippocampus and the thalamus. All of these regions highly express T channels (Talley et al., 1999). Thus, it is plausible that calcium channels contribute to the development of observed spontaneous tonic/clonic seizures. Although T channels are thought to mainly contribute to absence (nonconvulsive) seizures, reports have demonstrated that they do contribute to behavioral tonic/clonic seizures in the pilocarpine model of status epilepticus and temporal lobe epilepsy (Becker et al., 2008; Graef et al., 2009a; Su et al., 2002). As mentioned earlier, alcohol withdrawal seizures can lead to the development of
status epilepticus and temporal lobe epilepsy. Perhaps T channels are involved in this development of more serious, life-threatening seizures.

The difficulty in detecting spontaneous behavioral seizures during withdrawal, due to their unpredictable nature, has made it challenging to test therapeutics (N'Gouemo and Rogawski, 2006). The effects of therapeutics have been successfully tested in more robust, controlled models including the aberrant spontaneous electrographical correlates described earlier or the elicited handling-induced convulsion and audiogenic seizure models.

Alternatives to benzodiazepines: potential for anticonvulsants

Immediate attention is required during alcohol withdrawal seizure to reduce the risk of seizures and other adverse events. Currently, the first line treatment for alcohol withdrawal is with benzodiazepines, and as described previously, this treatment is not optimal. There is growing evidence that suggests a promising role for anticonvulsants in treatment for alcohol withdrawal symptoms as well as alcohol dependence (Ait-Daoud et al., 2006; De Sousa, 2010).

To expand on this idea, we tested the effects of ETX on withdrawal-related symptoms (Table 3). First, ETX alleviated disruptions in diurnal EEG-patterns in mice undergoing ethanol withdrawal (Wiggins et al., 2013), which suggests it might have some utility in treating alcohol withdrawal-related sleep disruptions. Acute ETX treatment also reduced both electrographical and behavioral correlates of ethanol withdrawal seizure severity (Riegle et al., 2014). In addition, ETX rescued mice from ethanol withdrawal-induced seizure events occurring prior to treatment. Follow-up studies with ETX have
demonstrated a reduction in withdrawal-induced seizure severity in a dose-dependent manner (submitted to *Neuropharmacology*) and acute ETX reduced PTZ-induced seizures during ethanol withdrawal (manuscript under preparation). Also, an important outcome of these studies was that ETX reduced the mortality rate due to ethanol withdrawal-induced seizure activity. These results are similar to the findings by Kaneto et al. (1986) demonstrating a reduction in withdrawal signs after ETX treatment. To further evaluate the role of ETX as a potential treatment option, an initial characterization demonstrated that it modestly reduced ethanol consumption in nondependent mice (submitted to *Neuropharmacology*) and evaluating its effect on ethanol withdrawal-induced anxiety is under investigation.
Table 3. T-type Calcium Channel Antagonists, Potential Treatments for Alcohol Dependence and Withdrawal

<table>
<thead>
<tr>
<th>AW Seizure Models</th>
<th>Ethosuximide</th>
<th>Zonisamide</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spike and Wave Discharge</td>
<td>↓ ¹</td>
<td>N/A</td>
</tr>
<tr>
<td>Handling-Induced Convulsion</td>
<td>↓ *</td>
<td>N/A</td>
</tr>
<tr>
<td>Chemoconvulsant</td>
<td>↓ *</td>
<td>N/A</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>AW Anxiety</th>
<th>TBD **</th>
<th>N/A</th>
</tr>
</thead>
<tbody>
<tr>
<td>AW Sleep Disruptions</td>
<td>↓ ²</td>
<td>N/A</td>
</tr>
<tr>
<td>Alcohol Consumption</td>
<td>↓ *</td>
<td>↓ ⁸</td>
</tr>
</tbody>
</table>

<table>
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<tr>
<th>Clinical Trials</th>
<th>TBD</th>
<th>↓ AW symptoms⁵</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>↓ alcohol craving³, ⁴, ⁷</td>
</tr>
<tr>
<td></td>
<td></td>
<td>↓ side effects³</td>
</tr>
<tr>
<td></td>
<td></td>
<td>↓ consumption⁴, ⁵, ⁶, ⁷</td>
</tr>
</tbody>
</table>

↓ = Decreased; AW, alcohol withdrawal; ¹ Riegle et al. (2014); ² Wiggins et al. (2013); ³ Rubio et al. (2010b); ⁴ Rubio et al. (2010a); ⁵ Arias et al. (2010); ⁶ Knapp et al. (2010); ⁷ Sarid-Segal et al. (2009); ⁸ Knapp et al. (2007); * Data collected by authors, manuscripts in preparation for publication; **Preliminary evidence (observed by authors) suggest ETX reduces ethanol withdrawal-induced anxiety in DBA/2J mice
Zonisamide, an anticonvulsant that targets T channels (Kito et al., 1996; Suzuki et al., 1992), has also demonstrated promise (Table 3). In a clinical study, zonisamide decreased withdrawal symptoms including anxiety and reduced craving compared to the patients treated with diazepam (Rubio et al., 2010b). Additional clinical trials have demonstrated that zonisamide reduced alcohol consumption (Arias et al., 2010; Knapp et al., 2010; Rubio et al., 2010a; Sarid-Segal et al., 2009). The patients treated with zonisamide also experienced fewer side effects (such as sedation) compared to the diazepam-treated group. Furthermore, zonisamide decreased ethanol consumption in both rats and mice (Knapp et al., 2007). To our knowledge there have been no studies testing the effects of zonisamide on alcohol withdrawal symptoms, including seizures, in preclinical studies. Combining this data with ETX and zonisamide together, it appears targeting T channels may be a promising strategy (Table 3).

Many other promising anticonvulsants directly or indirectly target calcium channels including topiramate (Zhang et al., 2000), pregabalin (Taylor et al., 2007), gabapentin (Dooley et al., 2002), and levetiracetam (Lee et al., 2009; Yan et al., 2013). Pregabalin reduced both the severity of handling-induced convulsions and electrographical correlates of withdrawal-induced seizure activity (Becker et al., 2006). Gabapentin decreased both the severity of handling-induced convulsions and audiogenic seizures during withdrawal, and reduced ethanol withdrawal-induced anxiety-like behavior (Watson et al., 1997). Treatment with topiramate reduced severity of handling-induced convulsions (Farook et al., 2007), increased PTZ seizure threshold (Cagetti et al., 2004), and decreased anxiety-like behavior (Cagetti et al., 2004) during withdrawal. For

Although all of these anticonvulsants act on multiple targets, it is important to highlight that a combination of mechanisms, which include calcium channels can significantly impact the circuitry involved in alcohol withdrawal symptoms. Also, while we cannot definitively conclude that the therapeutic efficacy is due to the sole action on calcium channels, there is an abundance of evidence that has demonstrated significant effects of ethanol on these channels. Thus, it is critical to continue investigating the role of calcium channels, including T channels, and the use of anticonvulsants. Important considerations about anticonvulsants include the low risk for abuse, fewer cognitive and motor side effects, and the other treatment potentials that they have on affective disorders including depression and anxiety.

Considerations

Several considerations must be made when reviewing the literature on ethanol withdrawal seizure. First, there are known sex differences. Men are at much greater risk of developing alcohol withdrawal related seizures (Hillbom et al., 2003; Hughes, 2009b). Most of the studies that have been conducted used only male animals; however, a recent study using male and female mice demonstrated sex differences in ethanol withdrawal seizure (Veatch et al., 2007). Male C3H/HeCr mice demonstrated increased severity of handling-induced convulsions with each successive withdrawal period. This “kindling-like” increase in seizure propensity was not observed in female mice. Further investigation demonstrated that these differences were not due to blood ethanol levels or due to the presence or absence of hormones, specifically estrogen. It will be important to
determine the differences in male and female susceptibility to alcohol withdrawal seizure and the mechanisms that could be contributing to such differences.

Also, strain differences must be considered when evaluating the literature. Investigations have been made to determine differences in seizure susceptibility in mice undergoing ethanol withdrawal (Crabbe et al., 1980; Crabbe et al., 1983; Metten and Crabbe, 1994, 2005; Metten et al., 2010). The most seizure susceptible strains during ethanol withdrawal include C3H/HeJ and DBA/2J mice, and the most resistant include C57Bl/6J mice. Thus, genetic factors must be considered as well. Some criticism has been made against the use of seizure-prone mice; however, for evaluating seizure activity during withdrawal, it appears to be a good model, as it supports the clinical literature with respect to what is observed after multiple detoxifications with an increased risk for seizure development.

**An alternative hypothesis**

It is clear that there are multiple types of seizures that can be elicited during alcohol withdrawal, ranging from nonconvulsive, aberrant oscillatory activity to tonic/clonic convulsions. Multiple studies have demonstrated that there is a temporal pattern in the development of these seizures (Gonzalez et al., 1989; Walker and Zornetzer, 1974; Watson and Little, 1995). However, it is important to note that the development of withdrawal symptoms, including seizures, is dependent on the model. Regardless, it is evident that different brain regions are involved with each model of withdrawal seizure.
Gonzalez et al. (1989) suggested that the temporal differences observed are indicative of different mechanisms and are therefore independent of each other. There is strong evidence that there is neuroanatomical specificity with these different types of seizures which is not surprising, given their different characterizing features. While we do not argue that different neurotransmitter systems are engaged and neuroanatomical specificity occurs, it does not rule out the possibility that early changes in withdrawal, such as T channel dysregulation, could precipitate future long-term consequences. In fact, Walker and Zornetzer (1974) demonstrated that the aberrant oscillatory activity preceded the tonic/clonic seizures in ethanol withdrawal, providing initial evidence that mechanisms underlying nonconvulsive seizures are altered early in withdrawal. It was suggested that this hyperactivity spread from the midline thalamus and reticular formation, and the circuitry in these particular brain regions could serve as an organizer for widespread activity. Development of BSEs also appeared prior to handling-induced convulsions in the investigation by Veatch and Becker (2002).

Because nonconvulsive seizure activity is observed prior to behavioral seizures and T channel antagonists inhibit this activity, we propose that the enhanced T channel function during withdrawal facilitates nonconvulsive seizure activity. We believe that this nonconvulsive activity and early changes in withdrawal can precipitate more severe consequences such as tonic/clonic convulsions. Early changes in T channel function and abnormal oscillatory activity have been observed (Graef et al., 2011; Riegle et al., 2014). It is also important to note that many studies evaluating behavioral correlates of withdrawal seizure activity do not include electrographical analyses as well.
Interestingly, Gonzalez et al. (1989) reported that they had observed tonic/clonic seizure and death due to these convulsions up to three days later. We have made similar observations in that 37.5% of our mice undergoing alcohol withdrawal died within 48 hours of the fourth withdrawal period (Riegle et al. 2014, submitted for publication). While we cannot confirm that the deaths were due to tonic/clonic convulsions in each case; severe convulsions resulting in death have been witnessed in our studies. Interestingly when we blocked T channels with ETX (250mg/kg) during each withdrawal period, all of the mice survived (Riegle et al. 2014, submitted for publication). It is unknown whether these early changes in withdrawal and aberrant oscillatory activity precipitate more severe consequences such as tonic/clonic seizures and/or higher mortality. It will also be interesting and of important clinical significance if these early changes are related to other withdrawal symptoms such as anxiety and sleep disruptions. Regardless if the seizures are independent of each other or if certain factors do trigger future events, these are important considerations that need to be addressed for novel treatments and management of alcohol withdrawal symptoms.

Conclusions

T channels are disrupted during ethanol exposure and withdrawal

Several studies provide direct evidence that ethanol and ethanol withdrawal affect T channel expression and function. Further research is necessary to determine the direct role of T channel activity during withdrawal; however, these findings suggest that disruptions in T channel activity may contribute to the development of withdrawal symptoms including sleep disturbances and seizure activity. It also remains to be
determined the mechanism by which these channels are being disrupted during ethanol exposure and withdrawal. Most of these studies were conducted in thalamic regions; however, as mentioned, T channels are expressed heterogeneously throughout the brain (Perez-Reyes, 2003; Talley et al., 1999). It will be important to investigate other brain regions especially in areas of interest such as the hippocampus, amygdala and other regions that are linked to the development of withdrawal symptoms.

**T channels are distributed in the circuitry involved in withdrawal seizures**

Several regions of the brain have been implicated in the development of ethanol withdrawal seizure activity. These regions include but are not limited to the thalamus, hippocampus, amygdala, cortical areas, and brain stem regions (Figure 1). T channels have been identified in each of these brain regions (Talley et al., 1999). Highly relevant to this review is the role of the thalamus in withdrawal seizure activity. Thalamic circuitry with the involvement of T channels is positioned to generate and propagate abnormal epileptiform activity. Its role in ethanol withdrawal was originally proposed by investigations that demonstrated abnormal EEG activity in the thalamus and further showed that the abnormal nonconvulsive activity preceded spontaneous behavioral convulsions (Hunter et al., 1978; Hunter and Walker, 1978; Walker and Zornetzer, 1974). Disruption to T channels in the thalamic circuitry may be the key precipitator of this epileptiform-like electrographic activity. Recent studies have demonstrated abnormal activity that most likely involved thalamic activity (Riegle et al., 2014; Veatch and Becker, 2002). Reduction in both electrographic and behavioral correlates of withdrawal
seizure by the T channel antagonist, ETX, further provides evidence for the involvement of enhanced T channel activity during ethanol withdrawal seizure (Riegle et al., 2014).
Figure 1. Circuitry involved in alcohol withdrawal seizure. Within the thalamic circuit, there is a feedback loop system composed of GABAergic neurons of the thalamic reticular nucleus and glutamatergic neurons of the relay nuclei. Rhythmic activity within this loop is sustained by T-type calcium current current, which mediates synchronized burst activity during both normal and abnormal rhythms. Abnormal activity is then transmitted to other brain regions, with the thalamic node serving as a rhythmic pacemaker. The amygdala, hippocampus, brain stem, and cortex have all been identified as important areas in withdrawal seizure activity, which also have reciprocal connections with the thalamus. T-type calcium channels are upregulated during alcohol withdrawal and abnormal epileptiform activity within the thalamus has been identified during alcohol
withdrawal. Thus, we propose that this enhanced T channel function contributes to the seizure activity within the thalamus and helps it serve as a rhythmic pacemaker for alcohol withdrawal seizure development. Green arrows, excitatory projections; Red arrows, inhibitory projections; T channel, T-type calcium channel; TRN, thalamic reticular nucleus; ↑, increase
Implications for ethoximide in the treatment of alcohol withdrawal

Here, we have reviewed the acute effects of ETX treatment during ethanol withdrawal. The details of these studies evaluating ETX in three different seizure models are in the constituent chapters that follow. These findings implicate T channel involvement in ethanol withdrawal seizure activity. They also further support the role of thalamic involvement as this brain region is most likely to contribute to the SWD activity observed in these studies.

Currently, to our knowledge, ETX has not been tested clinically for the use against alcohol withdrawal symptoms or alcohol dependence. As mentioned previously, zonisamide, which blocks T channels, has shown promising results in clinical studies. It is important to note that ETX is FDA approved for the treatment of absence epilepsy (Goren and Onat, 2007). Absence epilepsy is characterized by nonconvulsive seizures with SWDs as the electrographical signature, similar to what has been observed in studies evaluating spontaneous aberrant electrographic activity during ethanol withdrawal. ETX is tolerated by both adults and children, and the bioavailability is 95-100%. ETX is rapidly absorbed and has a half-life of 30-60 hours in adults and 20-40 hours in children. In a rating system comparing antiepileptics that are used for idiopathic generalized epilepsies, the pharmacokinetic properties of ETX rated very well with only two other anticonvulsants with higher scores (levetiracetam and topiramate) (Patsalos, 2005). Properties included absorption, kinetics, metabolism, drug interactions, and several other factors. Although ETX is well tolerated, side effects can occur including headache, drowsiness, fatigue, and gastrointestinal-related discomfort. Other more serious complications have been reported but are considered rare. For details of ETX and
pharmacokinetic properties, we refer you to the reviews by Goren and Onat (2007) and Patsalos (2005).

Anticonvulsants have demonstrated success and appear to be a promising strategy for the treatment of alcohol dependence and alcohol withdrawal. Further investigation is necessary to establish whether ETX will be an effective anticonvulsant in alcohol abuse and withdrawal, and future clinical studies testing the use of ETX should be considered.

A revised hypothesis

Based on recent findings demonstrating the role of T channels during ethanol exposure and withdrawal, additional support has been added to the original hypothesis that suggests calcium channels are important to the hyperexcitable state that develops during ethanol withdrawal. We also discussed the hypothesis that the thalamus can serve as a network organizer with its extensive circuitry with other brain regions and also facilitate seizure activity during withdrawal. Recent evidence with the potential contribution of T channels further supports the role of the thalamus and also contributes to our understanding of how the thalamus could serve such a role during ethanol withdrawal. In addition, we propose that the nonconvulsive electrographical correlates of seizure activity that occur during withdrawal are mediated by functional enhancement of T channel activity and may precipitate more serious consequences such as spontaneous behavioral convulsions, which further increases the risk for long-term consequences like status epilepticus or temporal lobe epilepsy. More work is necessary to evaluate these hypotheses. Also, it is critical to our understanding to determine how these early changes in T channels and early electrographical and behavioral seizure events relate to or affect
other withdrawal symptoms such as anxiety, learning and memory impairment, and other cognitive impairments.

In this dissertation, I include the studies demonstrating the effects of ETX in three of the ethanol withdrawal seizure models described in this introduction. The majority of my work has centered on characterizing the development of SWD events in DBA/2J mice and whether ETX would dose-dependently reduce this aberrant activity. I extended the characterization of ETX as a potential treatment and made initial assessments of its effects on ethanol consumption, anxiety-like behavior, and motor ability. Additional projects that go beyond the scope of this dissertation are included in the appendix.
References


CHAPTER 2

ETHOSUXIMIDE REDUCES ELECTROGRAPHICAL AND
BEHAVIORAL CORRELATES OF ALCOHOL WITHDRAWAL
SEIZURE IN DBA/2J MICE

Melissa A. Riegle, Melissa M. Masicampo, Erin H. Caulder, and Dwayne W. Godwin

This manuscript has been accepted for publication in *Alcohol* (2014) and is reprinted with permission. The experiments were performed by M.A. Riegle with the help of M. M. Masicampo, and E. H. Caulder. The manuscript was written and prepared by M. A. Riegle. Dr. Dwayne W. Godwin provided mentorship and editorial feedback.
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 CaV3.2 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 6-10Hz frequency range. We also evaluated the effects of ETX on handling-induced convulsions. Overall, we observed a decrease in handling-induced convolution 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
Introduction

Approximately 18 million Americans abuse or are dependent on alcohol, which results in significant economic and societal burdens (Grant et al., 2004; Thavorncharoensap et al., 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 et al., 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 et al., 2006). However, BZDs such as lorazepam produce rebound effects during untreated withdrawal periods (Veatch and 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. (2011) identified an upregulation of T channel expression and function, including a persistent upregulation of CaV3.2 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) (Goren and 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 (~1mm) and two anterior to lambda (~1mm) holes on each side of the midline (~1.25mm) 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 and 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 (Figure 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 8h (9:00 AM to 5:00 PM). Mice were subcutaneously treated with pyrazole (100mg/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 μL samples of blood were collected from the tail. Samples were immediately placed in trichloracetic acid (6.25%) and analyzed using a NAD-ADH enzyme assay (Sekisui, Kent, ME).
**Figure 1.** A. Schematic of intermittent ethanol exposure paradigm. Saline or ethosuximide (ETX, 250mg/kg) treatments were administered at 1:30pm 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 3:00pm. 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.
Mice were injected with ETX (250mg/kg, Sigma Aldrich; intra-peritoneal [i.p.]) or saline during each withdrawal period at 1:30 PM (Figure 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 et al., 1986) and its use in studies of absence seizures (Aizawa et al., 1997; Dezsi et al., 2013; Frankel et al., 2005; Marrosu et al., 2007; Nissinen and Pitkanen, 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 200Hz and band-pass filtered from 0.5 to 40Hz. 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 (Figure 1B) occurring between 6-14Hz. Briefly, a band pass filter from 6-14Hz 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 - 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 6-10Hz 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 et al., 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 et al., 2006; Veatch and Becker, 2002), withdrawal severity progressively increases over the time and occurs 6-8h into withdrawal.

To assess the acute effects of ETX, mice were treated with saline or 250mg/kg ETX at 6h into the 4th withdrawal period (3:00 PM). We evaluated handling-induced convulsions (HIC) as a measure of behavioral withdrawal severity 45 minutes 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 seconds. 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 and Kosobud (1990).
Table 1 Handling-induced convulsion (HIC) Scale

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

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 and Kosobud (1990). We did not make any modifications to the scale described by Becker.
**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 ± SEM with statistical analyses for significance provided within the results section.

Results

**SWDs in DBA/2J mice progressed 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 ([Figure 2A]; Linear regression, $R^2=0.6903$, $F=84.69$, $p<0.0001$, $n=8$). The mean±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.
Figure 2. A. Ethosuximide (ETX, 250mg/kg) decreased alcohol withdrawal-induced spike and wave discharges (SWDs). We analyzed the number of SWDs following saline or ETX treatment from 2:00-5:00pm. 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 (250mg/kg) rescued mice from SWDs prior to treatment. Within the group of mice exposed to ethanol treated with ETX, we compared the number of SWDs prior to each treatment from 10:30am-1:30pm to the number of events that occurred following treatment from 2:00-5:00pm. 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, 10:30am-1:30pm), immediately following treatment (posttreatment, 2:00-5:00pm), and at the point when ETX should be metabolized (washout, 5:00-8:00pm) during the fourth withdrawal of mice treated with ETX. During the washout period (5:00-8:00pm), 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 6-10Hz frequency range representing the range of SWD activity. Data for each withdrawal period was collapsed and normalized. ETX treatment (250mg/kg) decreased power in the 6-10Hz frequency range to a level indistinguishable from baseline. *, p<0.05; **, p<0.01; ***, p<0.0001
Table 2 Summary of SWD Events

<table>
<thead>
<tr>
<th>2:00-5:00pm</th>
<th>Air-Exposed Mice</th>
<th>Ethanol-Exposed Mice</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Saline (n=3)</td>
<td>250 mg/kg ETX (n=9)</td>
</tr>
<tr>
<td>1&lt;sup&gt;st&lt;/sup&gt; WD</td>
<td>55.67±13.5</td>
<td>38.00±6.8</td>
</tr>
<tr>
<td></td>
<td>56.00±2.5</td>
<td>31.22±5.2</td>
</tr>
<tr>
<td>2&lt;sup&gt;nd&lt;/sup&gt; WD</td>
<td>60.00±7.6</td>
<td>34.00±4.8</td>
</tr>
<tr>
<td>3&lt;sup&gt;rd&lt;/sup&gt; WD</td>
<td>103.30±33.9</td>
<td>39.56±5.7</td>
</tr>
</tbody>
</table>

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
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 (250mg/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 (Figure 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 (Figure 2A, p<0.0001). The mean±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 increased during ethanol withdrawal; ETX restored 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 (Figure 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 (Figure 2B, p<0.0001). In saline-treated mice undergoing withdrawal, SWD duration was significantly increased (Figure 2B; Linear regression, R²=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 (Figure 2A; Linear regression,
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 rescued 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 (250mg/kg) before (pretreatment, 10:30 AM - 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 suggests that ETX rescued mice from withdrawal-induced SWDs that occurred prior to treatment (Figure 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 - 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 returned as ETX was 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 et al., 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 - 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 treatments. During the washout period, SWDs were significantly higher compared to the number of events immediately following treatment (**Figure 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 reduced 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 6-10Hz. 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 6-10Hz compared to saline-treated mice (Figure 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 reduced 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 7h into withdrawal and the test was repeated 1h later (8h into withdrawal). Mice treated with ETX had a significantly lower HIC score compared to mice treated with saline during the first test (Figure 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 (Figure 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 (Figure 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 (Figure 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.
Figure 3  A. We measured the effect of ETX treatment on handling-induced convulsion. Mice treated with 250mg/kg ETX had a significantly lower HIC score compared to mice treated with saline. Mice treated with ETX also had less 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
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 et al., 2003b; Shan et al., 2013b; Wiggins et al., 2012). 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 (Figure 2A). We also demonstrated a modest increase in duration of events (Figure 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 et al., 1997a; Becker et al., 1997b). Consistent with our investigation, Veatch and Becker also demonstrated that brief spindle episodes increased in tandem with the number of withdrawal cycles in C3H/He mice (Veatch and Becker, 2002, 2005). Different strains show differences in sensitivity to withdrawal (Metten and Crabbe, 2005; Metten et al., 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 (Fuentealba and 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 and 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 et al., 1999). T channels are one of many factors that may contribute to hyperexcitability and regional specific effects of ethanol withdrawal (Chen et al., 2009; Reilly et al., 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 withdrawal 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 et al., 1989b; Huguenard and 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 (Figure 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 (Figure 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 3h 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 (Figure 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 6-10Hz frequency range of the SWD events. We found that ETX treatment reduced power in the same frequency range as the SWD
activity (Figure 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 (Figure 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\(^+\)-dependent K\(^+\) current (Crunelli and Leresche, 2002; Leresche et al., 1998). Evidence from a recent study also suggested that ETX may increase GABA transmission (Greenhill et al., 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 and Crabbe, 2005),
also possess a seizure prone phenotype (Beyer et al., 2008). Evidence from previous
studies supports our interpretations with the electrophorical experiments. Veatch and
Becker (2002) indirectly demonstrated that increased electrophorical 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 and Becker, 2005). We demonstrated a reduction in
HIC severity in DBA/2J mice treated with ETX, which further validates the use of
electrophorical 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 (Fernandez-Torre and Martinez-Martinez,
2007; LaRoche and Shivdat-Nanhoe, 2011) and although inconsistencies exist in these
findings, several abnormalities in EEG of alcoholics have been identified (Cohen et al.,
1997; Coutin-Chruchman et al., 2006; Feige et al., 2007; Porjesz and Begleiter, 2003;
Rangaswamy et al., 2003; Rodriguez Holguin et al., 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.
Acknowledgements:

This work was supported by NIH grants F31AA021322-01, T32AA07565, R01AA016852, and the Tab Williams Family Fund. The authors would like to express our gratitude to David Klorig, Hong Qu Shan, and Walter Wiggins for their helpful comments and suggestions throughout this study. The authors declare no competing financial or other conflicts of interests.
References


DOSE-DEPENDENT REDUCTION OF ETHANOL WITHDRAWAL SEIZURES BY ETHOSUXIMIDE

Melissa A. Riegle, Eugenia Carter, Jeffrey L. Weiner and Dwayne W. Godwin

This manuscript has been submitted to *Neuropharmacology* (2014) for publication. The experiments were performed by M.A. Riegle with the help of E. Carter. The manuscript was written and prepared by M. A. Riegle. Drs. Jeffrey L. Weiner and Dwayne W. Godwin provided mentorship and editorial feedback.
Abstract

Individuals abusing alcohol frequently cycle between drinking and withdrawal states which can result in increased anxiety, delirium tremens, insomnia, seizures, and other adverse symptoms. Withdrawal symptoms are a major component of relapse and represent a significant barrier to recovery. Increasingly, studies indicate a promising role for anticonvulsants as a treatment for alcohol withdrawal symptoms as well as alcohol dependence. Our lab has identified a disruption in the thalamic T-type calcium channel isoform, CaV3.2, during withdrawal that may underlie the generation and propagation of withdrawal-related hyperexcitability. We previously found that the anticonvulsant, ethosuximide (a T channel antagonist) could reduce ethanol withdrawal seizures in DBA/2J mice. In the current investigation, we tested whether ethosuximide could reduce withdrawal seizures in a dose-dependent manner. We further tested the effects of ethosuximide on ethanol consumption, locomotion, and measures of anxiety-like behavior in ethanol-naïve mice. We found that ethosuximide decreased ethanol withdrawal seizures in a dose-dependent manner and modestly reduced ethanol intake in nondependent mice. We observed no effect on anxiety-like behaviors in ethanol-naïve mice, and importantly, ethosuximide treatment did not impair motor activity. Overall these data suggest that ethosuximide can significantly attenuate ethanol withdrawal symptoms without effects on baseline anxiety measures and without significant motor impairment.

Keywords: alcohol withdrawal, seizure, ethosuximide, T-type calcium channel, anxiety, alcohol consumption
1. Introduction

Alcohol is the third highest risk factor for health related problems in the world and leads to over 2 million deaths per year (World Health Organization, 2011). Over 18 million Americans (Hasin et al., 2007) suffer with an alcohol-related problem, with significant societal and economic costs (Rehm et al., 2009). This impact persists despite the availability of pharmacological treatment options that include disulfiram, naltrexone, and acamprosate for alcohol abuse (Heilig and Egli, 2006) and benzodiazepines for alcohol withdrawal (Ait-Daoud et al., 2006).

Cessation of drinking after a period of chronic use can lead to alcohol withdrawal syndrome, comprising a range of negative symptoms, including hyperactivity, anxiety, delirium tremens, and seizures (Saitz, 1998). Seizures occur with ~25% of the other symptoms, and the frequency of withdrawals increases the odds of future episodes (Becker, 1998; Lechtenberg and Worner, 1990). Thirty-three percent of those with seizures develop delirium tremens, and of these, as many as 5-15% may die (Erwin et al., 1998). Early treatment is important, and may emphasize reducing seizures with benzodiazepines (including chlordiazepoxide and lorazepam), which like ethanol modulate GABA<sub>A</sub> receptor function. However, these drugs possess their own unique side effects (Ait-Daoud et al., 2006; Morris and Victor, 1987). Benzodiazepines have a high abuse potential, can cause cognitive and motor impairment, and can exacerbate seizure activity in untreated withdrawal periods (Ait-Daoud et al., 2006; Morris and Victor, 1987; Veatch and Becker, 2005). Thus, there is a critical need for novel targets and the development of new therapies for intervention.
Recent studies indicate a possible role for anticonvulsant drugs as a treatment for alcohol withdrawal symptoms as well as alcohol dependence (De Sousa, 2010). Several of these compounds have direct or indirect effects on calcium channels. A few examples include topiramate (Zhang et al., 2000), gabapentin (Dooley et al., 2002), levetiracetam (Lee et al., 2009; Yan et al., 2013), and zonisamide (Kito et al., 1996; Suzuki et al., 1992). It is also known that ethanol can acutely inhibit calcium channel function and that chronic use of ethanol can lead to a functional increase in these channels that may contribute to ethanol withdrawal symptoms (Walter and Messing, 1999). Thus, calcium channels as a potential therapeutic target and the use of anticonvulsants should be considered.

T-type calcium channels (T channels) may serve as a novel treatment target for alcohol abuse and withdrawal. We have demonstrated that ethanol exposure and withdrawal alter the physiological properties of T channels (Carden et al., 2006; Graef et al., 2011; Mu et al., 2003; Riegle et al., 2014; Shan et al., 2013; Wiggins et al., 2013). Acute ethanol exposure was found to inhibit T channel function via a PKC-dependent mechanism (Shan et al., 2013). During ethanol withdrawal T channel-mediated burst firing was enhanced in midline thalamic nuclei, due to a depolarizing shift in voltage dependency and an upregulation of the CaV3.2 isoform (Graef et al., 2011). In a preliminary study, ethosuximide (ETX), an anticonvulsant agent that blocks T channels, reduced ethanol withdrawal-induced seizure activity in DBA/2J mice (Riegle et al., 2014).

Here, we further assessed the effectiveness of ETX at reducing withdrawal-induced seizure activity, including the dose-dependency of this effect. As there is some
evidence that ETX may also interact with other neuronal proteins (Crunelli and Leresche, 2002; Greenhill et al., 2012; Ponnessamy and Pradhan, 2006), we also evaluated the effects of ETX on measures of anxiety-like behavior, locomotor activity, and ethanol intake in ethanol-naïve mice, as these behaviors might be sensitive to some of the off-target effects of ETX. Our results demonstrate that ETX decreased electrographical correlates of ethanol withdrawal seizures in a dose-dependent manner. In addition to its suppressive effects on ethanol withdrawal seizures, acute ETX treatment resulted in a modest reduction in ethanol intake in nondependent mice. Consistent with the T channel dependency of these effects, ETX did not impair motor function and had no effect on anxiety-like behavior in ethanol-naïve mice.

2. Methods

All experiments were approved by the Institutional Animal Care and Use Committee of Wake Forest School of Medicine. Experiments were conducted in agreement with the National Institutes of Health and United States Department of Agriculture guidelines, including measures to reduce the use of animals and to minimize pain and suffering.

2.1. Seizure Activity

2.1.1. Animals and Surgical Procedure

A tethered electroencephalography/electromyography (EEG/EMG) acquisition system was utilized (Pinnacle Technologies Inc, Lawrence, KS) to assess seizure activity in male DBA/2J mice (8-10 weeks old; Jackson Laboratory, Bar Harbor, ME) as
described in Riegle, et al (2014). To surgically implant the mice, ketamine and xylazine (100 mg/kg and 10 mg/kg respectively) were used to anesthetize the mice. Supplemental doses of ketamine were provided until areflexia was apparent. An incision (approximately 1 cm) was made at the midline to expose the skull surface. The skin was reflected back, and four holes were drilled through the skull with placement on each side of the midline anterior to bregma and lambda. Stainless steel screws were secured to the headmount and served as electrodes. Electrical continuity was maintained with silver epoxy, and dental acrylic was applied to secure the headmount to the skull. The incision was sutured, and the mouse was given at least one week of surgical recovery. Following recovery, baseline EEG activity was recorded for each mouse prior to ethanol or air exposure and any treatment.

2.1.2. Intermittent Ethanol Exposure Paradigm

A modified intermittent ethanol inhalation paradigm (Becker and Hale, 1993) was used as previously described (Riegle et al., 2014). In brief, mice were exposed to four ethanol exposure and withdrawal periods (Figure 1A). A Plexiglas vapor chamber located in the same room in which the mice were housed was used for each exposure. Ethanol (95%) was volatilized and delivered to the chamber by an air pump. The room was maintained on a 12 hour light/dark cycle with lights off at 6:00 pm and on at 6:00 am. Mice were exposed to ethanol for 16 hours (5:00 pm to 9:00 am). After each exposure, the mice underwent a withdrawal period lasting 8 hours (9:00 am to 5:00 pm). To maintain blood ethanol concentration (BEC) levels during exposure, mice were injected with pyrazole subcutaneously (100mg/kg; Sigma Aldrich, St. Louis, MO) at the
beginning of each ethanol exposure (5:00 pm). Pyrazole, an alcohol dehydrogenase inhibitor, was dissolved in saline and prepared daily. Samples (5µl) of blood were collected from the tail to measure BEC levels at the start of each withdrawal period. Samples were immediately placed in trichloracetic acid (6.25%) and analyzed using a NAD-ADH enzyme assay (Sekisui, Kent, ME). There were no differences between groups in BEC levels in each withdrawal period. For each withdrawal period the average BECs (mg/dl) were as follows: 1st withdrawal, 301.7±16.59; 2nd withdrawal, 316.4±18.64; 3rd withdrawal, 284.4±21.44; 4th withdrawal, 218.9±11.65. The fourth withdrawal period was significantly less compared to the other withdrawal periods (Kruskal-Wallis test; p=0.0006).
Figure 1. A. Schematic of one ethanol exposure and withdrawal period. Each mouse received a total of four exposures (16 hours each) and four withdrawal periods (8 hours each). EEG activity was recorded prior to the first exposure and during each withdrawal period. In a subgroup of animals, EEG activity was recorded two weeks following the 4th exposure. Air-exposed mice received the same handling and underwent the same paradigm represented in schematic, only with air instead of ethanol. The asterisk represents the time point treatments of saline or ETX (50, 150, or 250 mg/kg) were administered (1:30 pm). Treatments were administered at this time for maximal effects during the peak withdrawal time of seizure severity. Treatments were not administered during baseline or the 2 week follow-up EEG recordings. B. A representative raw EEG trace with spike and wave discharge (SWD) activity occurring in the 6-10Hz frequency range. NeuroScore software was used to detect SWD events; inclusion criteria are described in the methods section.
Mice were injected with ETX (50, 150, or 250mg/kg, Sigma Aldrich; i.p.) or saline during each withdrawal period at 1:30 pm, 4.5 hours into withdrawal (Figure 1A). Based on our previous investigation (Riegle et al., 2014) and other studies (Becker, 1994; Becker et al., 2006; Veatch and Becker, 2002), in this paradigm, symptoms progress in severity throughout the withdrawal period, peaking in the last three hours prior to ethanol exposure. ETX has a biological half-life of approximately one hour in mice (el Sayed et al., 1978). Control mice underwent identical handling, including pyrazole injections; however, they were only exposed to air, not ethanol. Control mice were treated with saline at the same time points as the experimental mice. Air-exposed mice were not treated with ETX in these experiments as it has been demonstrated that ETX decreased seizure-like electrographical events in naïve DBA/2J mice (Marrosu et al., 2007; Riegle et al., 2014). To reduce the number of animals in these investigations, we did not repeat this control. ETX was dissolved in saline and prepared daily.

2.1.3. EEG Analysis

To assess EEG activity, spike and wave discharge (SWD) events occurring between 6-14Hz were analyzed as previously described in Riegle et al. (2014). Briefly, EEG signals were recorded during each withdrawal period or the equivalent time frame for air-exposed mice. The EEG acquisition system was programmed to sample at a rate of 200 Hz, and the signal was filtered with a band pass from 0.5 to 40 Hz. NeuroScore software (NeuroScore 2.1, Data Sciences International, St. Paul, MN) was used offline to analyze the digitized EEG signals. A dynamic threshold protocol was applied to each recording to identify SWD events occurring between 6-14Hz (Figure 1B). A minimum
threshold value was determined by the root mean square derived from the signal. The amplitude threshold ratio was set at 2 with a maximum ratio set to 20. The software identified the number and duration of SWD events using the defined algorithm. To ensure the protocol was identifying seizure events accurately, the signals were manually checked for spurious events. This automated protocol was selected to remove bias, and removal of events identified by the manual checks did not alter the scoring. The number and duration of SWD events were evaluated for each withdrawal period following treatments from 2:00-5:00 pm, 5-8 hours into withdrawal (or equivalent time period for air-exposed mice). EEG recordings for baseline activity were analyzed for the same time period, 2:00-5:00 pm, prior to ethanol or air exposure. Mice were not treated with saline or ETX during baseline recordings. We also conducted a power spectral analysis on the recordings in the 6-10Hz frequency range, consistent with the expected dominant frequency of SWDs in DBA/2J mice (Marrosu et al., 2007; Reid et al., 2011) and what we identified as well. For the power analysis, values were normalized to control for amplitude differences across EEG recording rigs.

2.2. Anxiety-like Behaviors

Group-housed, ethanol-naive DBA/2J mice (Jackson Laboratory, Bar Harbor, ME) were used to test the effects of ETX on anxiety-like behavior. Four pilot mice were initially tested to establish appropriate light settings for the light/dark box and elevated plus maze, established models used to test anxiety-like behavior (Kliethermes, 2005). The first week of testing, 16 mice were randomly split into two groups (n=8 per group). One group received saline (control) and the other received 250mg/kg ETX treatment 30
minutes prior to behavioral testing in the light/dark box. The animals were given one week to recover from this testing and the treatment groups were switched and tested on the elevated plus maze. Thus, each subject only received one exposure to ETX on each assessment of anxiety-like behavior. Treatments were administered 30 minutes prior to testing on the elevated plus maze as in the light/dark box testing. The details are described below.

2.2.1. Light/Dark Box

Anxiety-like behavior was assessed using a standard light/dark box (Colbourn Inst., Whitehall, PA). The 26.7cm × 26.7cm Plexiglas arena was divided into equal dark and light sides with a black Plexiglas insert. Mice were placed in the light side (~11 lux), and the duration of the test was 5 minutes. Infrared sensors were used to detect movement of the mice in both sides. Entries and time spent in the light and dark sides were collected with TruScan2.03 software. Light box entries and time spent on the light side were used as measures of anxiety-like behavior and total distance traveled was used to assess treatment effects on general locomotor activity. The apparatus was cleaned with soap and warm water mixed with bleach between animals.

2.2.2. Elevated Plus Maze

Anxiety-like behavior was assessed using a standard elevated plus-maze (Med Associates, St. Albans, VT). The runways of the maze were 10.2 cm wide and 30 cm long elevated 72.4 cm above the floor. The closed arms had 40.6 cm high black polypropylene walls, and the open arms had 1.3 cm high clear lips. Mice were placed at
the junction of the open and closed arms facing an open arm. The lighting of the open arms was set to ~25 lux at the end of each arm. The duration of the test was 5 minutes. Infrared sensors were used to assess entry into the arms and amount of time spent in the arms, measures of anxiety-like behavior. Data was acquired using a lab computer interfaced with control units and programmed using MED-PC (Med Associates).

2.3. Ethanol Consumption

Group-housed DBA/2J mice (Jackson Laboratory, Bar Harbor, ME) that were used in the anxiety-like behavior study were separated into individual cages to test whether ETX treatment would reduce ethanol consumption. The mice were 12 weeks at the start of drinking assessment and remained single-housed for the duration of testing. Prior to the drinking procedure, all 20 mice were allowed at least one week recovery from behavioral testing and were naïve to ethanol. Mice underwent a fade-in procedure described in detail below. A latin-square design was used to randomly assign mice to treatment groups. At the end of two weeks, all mice (both control and experimental groups) received saline, 50, 150, and 250 mg/kg ETX. A within subject design was used to assess the effects of ETX on ethanol consumption (experimental group) and monosodium glutamate consumption (MSG; control group). MSG was the tastant added to the ethanol solution. All drinking occurred in the home cage of each mouse, and bottles were 25 mL graduated with 0.2ml accuracy.
2.3.1. Ethanol Fade-In Procedure

At the start of the experiment, all 20 DBA/2J mice were given access to a two-bottle choice between 100mM MSG solution and water for 24 hours Monday, Tuesday, and Wednesday. On Thursday, the choice was scaled to only 2 hours (8:00am-10:00am), and for the remaining time, mice had access to water only. This was continued on Friday and the following Monday. The MSG concentration was selected based on previous findings that evaluated ethanol consumption in DBA/2J mice (McCool and Chappell, 2012). In the prior study, 100 mM MSG was the preferred concentration of MSG, and DBA/2J mice had a higher level of ethanol intake with the MSG-fade compared to the sucrose-fade. On Tuesday, the mice were randomly split into two groups of 10. One group remained as the control group and was given the choice between 100mM MSG and water. The second group was introduced to a 2% ethanol+100mM MSG solution. A fade-in procedure was used to slowly increase the amount of ethanol in the MSG solution for the experimental cohort. The mice were given access to the 2-bottle choice for 2 hours (8:00am-10:00am) Monday through Friday. On weekends mice were only given access to water. The mice were given access to the mixture of 100mM MSG + increasing concentrations of ethanol (2%, 4%, and 5%) with each concentration spanning at least two consecutive days. The MSG concentration remained constant throughout the study. The control mice were always given access to MSG because we did not fade it out of the ethanol solution in the experimental cohort. DBA/2J mice do not readily drink ethanol (Belknap et al., 1993; Yoneyama et al., 2008), and at this point in the fade-in procedure, a stable drinking baseline had not been achieved and levels of ethanol intake were declining. To increase consumption, the mice were exposed to a three day forced
exposure to EtOH, which entailed 24-hour access to a 5% EtOH + 100mM MSG solution for three straight days. After this forced EtOH exposure, subjects were given one day to recover with access to only water. In the ethanol group, the average intake across the three day force was 6.05±0.21 g/kg ethanol. After the recovery, the two-hour access fade-in paradigm resumed; however, the mice were no longer given a choice between the ethanol+MSG solution and water. For 2 hours each day, Monday through Friday, the mice received only the ethanol+MSG solution or MSG solution (control mice) with access to water throughout the rest of the day. We continued the fade-in procedure until the ethanol concentration reached 10%. After we observed a stable drinking baseline for two weeks at 10% ethanol, we tested both the control mice (100mM MSG) and experimental mice (10% ethanol + 100mM MSG).

A 4X4 Latin-square design was used to randomly assign subjects to a treatment (Saline, 50, 150, 250 mg/kg ETX) on Tuesday and Thursday. Treatments were administered 30 minutes prior to the 2 hour access and were only given on Tuesday and Thursday allowing at least 24 hours in between treatments. On Monday, Wednesday, and Friday during the two weeks of testing, mice were given access to the solutions without any treatments. On the weekend, all mice were given access to water only. The percent difference between consumption with ETX treatment and saline treatment was calculated to determine the effects of ETX on consumption of MSG in the control group and the ethanol + MSG experimental group.
2.4. Statistical Analyses

Two-way repeated measures ANOVAs were used to determine differences between ethanol- and air-exposed mice over the course of four withdrawal periods for SWD count, duration, and power analyses. Bonferroni post-hoc tests were used to determine if ethanol-exposed mice were significantly different from air-exposed mice in SWD count, duration, and power. Additional post-hoc tests included linear regression analyses to determine if there was a significant increase in SWDs with progressive withdrawal periods. Nonparametric ANOVA tests were used to assess baseline activity and determine differences between doses of ETX and saline treatments for all analyses (SWD count, duration and power) for each withdrawal period. Dunn’s multiple comparison tests were used as post-hoc tests when appropriate. Nonparametric tests were used because of the different group sizes. A chi-square test for trend was used to determine if there was a trend for protection against mortality. For the measures of anxiety-like behavior and locomotor activity, t-tests were used. The ethanol consumption data violated assumptions for parametric tests with unequal variance, thus a nonparametric ANOVA (Kruskal-Wallis test) was used. All data are presented as mean ± SEM with statistical analyses for significance provided within the results section.

3. Results

3.1. Ethanol withdrawal increased SWDs, duration of events, and power in the 6-10Hz frequency range in DBA/2J mice

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, five to
eight hours into withdrawal (2:00-5:00pm). Using a repeated measures, two-way ANOVA, we compared mice treated with saline undergoing ethanol withdrawal to mice treated with saline exposed to air only. We observed a significant effect of time (Figure 2A; df: 4, F: 35.71, p<0.0001), treatment (df: 1, F: 36.76, p<0.0001), and significant interaction between these factors (df: 4, F: 15.09, p<0.0001). A Bonferroni multiple comparison analysis revealed a significant difference in the number of SWD events between ethanol and air-exposed animals in each withdrawal period (Figure 2A; WD 1, 2 p<0.001; WD 3, 4 p<0.0001; air, n=6, ethanol, n=8). The mean±SEM SWDs for each withdrawal period are presented in Table 1 for each group. We conducted a linear regression post-hoc test to determine if the SWDs increase with each successive withdrawal period in the ethanol-exposed mice. We observed a significant increase in the number of SWDs with each successive withdrawal period (Figure 2A; R²=0.6603, F=73.85, p<0.0001, n=8). There were no differences in baseline activity between the ethanol and air-exposed mice (p>0.05). We also included the number of SWD events two weeks following the fourth withdrawal period for the mice exposed to the intermittent ethanol exposure paradigm (represented as the POST bar in Figure 2A) to demonstrate that the withdrawal-induced seizures are not sustained and levels of activity return to what is observed prior to ethanol exposure. These measurements were only included for observation and were not included in the two-way ANOVA analysis as we did not evaluate the air-exposed mice at this time point.
Figure 2. Ethanol withdrawal increased SWDs, duration, and power in DBA/2J mice.  

A. We compared the number of SWD events in mice undergoing ethanol withdrawal to mice exposed to air only. Both groups of mice were treated with saline. Mice undergoing ethanol withdrawal had a significant increase in SWD events compared to air-exposed mice during each withdrawal period. Additional analysis revealed that within the ethanol-exposed mice, the number of SWD events increased significantly with each successive withdrawal period in a “kindling-like” manner. There were no differences in baseline activity between the two groups. The EEG activity was recorded for the mice undergoing ethanol exposure and withdrawal two weeks following the fourth exposure. We included this activity to demonstrate that their SWD activity had returned to baseline values; however, this value was not included in the two-way ANOVA as we did not record this activity in air-exposed mice at this time point.  

B. We analyzed the duration of the SWD events in mice undergoing ethanol withdrawal compared to mice exposed to air only. There was a significant difference in SWD duration between ethanol and air-exposed animals in the first three withdrawal periods. There were no differences in baseline duration and in the 4th withdrawal between the ethanol and air-exposed mice. Within the ethanol-exposed mice, the duration of SWDs lengthened with each successive withdrawal period.  

C. We performed a power spectral analysis to evaluate the effects of ethanol withdrawal on power in the SWD activity range 6-10Hz. We observed a significant difference in power between air and ethanol-exposed mice in the third and fourth withdrawal periods. There were no differences in baseline power and during the 1st and 2nd withdrawal periods. The power in the 6-10Hz frequency range did not
increase with successive withdrawals in the ethanol-exposed mice. *, p<0.05; 
***, p<0.0001
ETX dose-dependently reduced the number of SWD events in mice undergoing ethanol withdrawal (data ± SEM); * statistically significant compared to ethanol-exposed, saline-treated mice; # statistically significant compared to air-exposed mice

<table>
<thead>
<tr>
<th></th>
<th>Baseline (2:00-5:00pm)</th>
<th>Ethanol-Exposed Mice</th>
<th>Air-Exposed Mice</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>172.9±13.95 193.4±13.88 186.0±10.20 192.3±11.36</td>
<td>149.3±13.15</td>
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</tr>
<tr>
<td>2:00-5:00pm</td>
<td>Saline 50 mg/kg ETX 150 mg/kg ETX 250 mg/kg ETX</td>
<td>Saline</td>
<td></td>
</tr>
<tr>
<td>1\textsuperscript{st} WD</td>
<td>241.1±24.80# 174.9±14.48 61.67±7.57* 39.38±9.25*</td>
<td>106.7±12.48</td>
<td></td>
</tr>
<tr>
<td>2\textsuperscript{nd} WD</td>
<td>310.1±24.89# 221.1±9.54 62.89±9.21* 32.50±7.89*</td>
<td>156.3±12.17</td>
<td></td>
</tr>
<tr>
<td>3\textsuperscript{rd} WD</td>
<td>406.1±36.98# 306.7±32.82 93.33±9.57* 25.75±5.4*</td>
<td>183.0±16.97</td>
<td></td>
</tr>
<tr>
<td>4\textsuperscript{th} WD</td>
<td>441.8±27.19# 401.0±33.64 126.4±20.01* 39.50±10.42*</td>
<td>187.2±14.65</td>
<td></td>
</tr>
<tr>
<td>2 weeks post</td>
<td>201.7±7.67 189.0±12.91 176±9.38 154.6±22.52</td>
<td>N/A</td>
<td></td>
</tr>
</tbody>
</table>
Next we evaluated how ethanol withdrawal influences the duration of the SWD events. We observed a significant increase in the duration of SWD activity in mice undergoing ethanol withdrawal compared to air-exposed mice. A repeated measures, two-way ANOVA revealed a significant effect of time (Figure 2B; df: 4, F: 9.706, p<0.0001), treatment (df: 1, F: 7.510, p=0.0179), and interaction between these factors (df: 4, F: 2.739, p=0.0393). A Bonferroni multiple comparison analysis revealed a significant difference in SWD duration between ethanol and air-exposed mice in the first three withdrawal periods (Figure 2B; WD 1, 2, 3 p<0.05; WD 4, p>0.05; air, n=6, ethanol, n=8). There were no differences in baseline duration (p>0.05) and in the fourth withdrawal between the ethanol and air-exposed mice (p>0.05). We used a linear regression post-hoc test to determine if the duration of the SWD events increased progressively with each withdrawal. We observed a significant increase in the duration with each successive withdrawal period (Figure 2B; R²=0.1633, F=7.415, p=0.0097, n=8). The mean±SEM SWD duration for each withdrawal period are presented in Table 2 for each group.
<table>
<thead>
<tr>
<th></th>
<th>Baseline (2:00-5:00pm)</th>
<th>Ethanol-Exposed Mice</th>
<th>Air-Exposed Mice</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Saline 50 mg/kg ETX</td>
<td>150 mg/kg ETX</td>
</tr>
<tr>
<td>2:00-5:00pm</td>
<td>1.07±0.057 1.12±0.036 1.13±0.029 1.08±0.045</td>
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<td></td>
</tr>
<tr>
<td>1&lt;sup&gt;st&lt;/sup&gt; WD</td>
<td>1.36±0.12* 1.19±0.051 0.94±0.038* 0.84±0.050*</td>
<td>0.92±0.054</td>
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<tr>
<td>2&lt;sup&gt;nd&lt;/sup&gt; WD</td>
<td>1.54±0.11* 1.59±0.11 1.01±0.56 0.93±0.11*</td>
<td>1.02±0.060</td>
<td></td>
</tr>
<tr>
<td>3&lt;sup&gt;rd&lt;/sup&gt; WD</td>
<td>1.65±0.19* 1.88±0.15 1.44±0.099 1.0±0.080*</td>
<td>1.18±0.12</td>
<td></td>
</tr>
<tr>
<td>4&lt;sup&gt;th&lt;/sup&gt; WD</td>
<td>1.50±0.16 1.88±0.16 1.59±0.11 1.13±0.11</td>
<td>1.12±0.047</td>
<td></td>
</tr>
<tr>
<td>2 weeks post</td>
<td>1.07±0.033 1.03±0.042 1.19±0.12 1.16±0.14</td>
<td>N/A</td>
<td></td>
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</tbody>
</table>

ETX dose-dependently reduced the duration of SWD events in mice undergoing ethanol withdrawal. (data ± SEM); * statistically significant compared to ethanol-exposed, saline-treated mice; #statistically significant compared to air-exposed mice.
To further characterize the finding that ethanol withdrawal increased SWDs, we performed a power spectral analysis to evaluate the effects of ethanol withdrawal on power in the SWD activity range (6-10Hz). If withdrawal increased SWDs, we expected a power increase in this range. We used a repeated measures, two-way ANOVA to compare the power in the 6-10Hz frequency range between ethanol and air-exposed mice. We observed a significant effect of treatment (Figure 2C; df: 4, F: 10.34, p=0.0074); however there was no effect of time or a significant interaction between these factors. A Bonferroni multiple comparison analysis revealed differences in power between air and ethanol-exposed mice in the third and fourth withdrawal periods (Figure 2C; WD 3, 4 p<0.05; air, n=6, ethanol, n=8). There were no differences in baseline power and during the first and second withdrawal periods. A linear regression post-hoc analysis revealed that power in the 6-10Hz frequency range did not increase with successive withdrawals in the ethanol-exposed mice. Thus, the 6-10Hz power band of the EEG reflected the underlying changes in the SWD, but was less sensitive than individual counts of SWDs in characterizing the progression of seizures.

3.2. ETX decreased SWD events during withdrawal in a dose-dependent manner

First, we evaluated the number of SWD events during baseline activity prior to ETX or saline treatment and ethanol exposure. As expected, there were no differences in baseline seizure activity between groups (Kruskal-Wallis test, p=0.7702; n=8-9 per group). The mean±SEM SWDs for baseline activity are presented in Table 1 for each group.
ETX (150 and 250 mg/kg) decreased SWD events compared to saline-treated mice in each withdrawal period (Figure 3A-D; Kruskal-Wallis with Dunn’s Multiple Comparison test, p<0.0001; n=8-9 per group). The lowest dose of ETX (50mg/kg) did not significantly decrease the number of withdrawal-induced SWD events compared to the saline-treated mice during any withdrawal period. Mice treated with the higher doses of ETX (150 and 250mg/kg) also had significantly lower SWDs compared to the mice treated with the lowest dose of ETX (50mg/kg) (Figure 3A-D; Kruskal-Wallis with Dunn’s Multiple Comparison test, p<0.05). We recorded activity from a subgroup of animals in these treatment groups to determine the number of SWD events two weeks following the fourth withdrawal period. Two weeks later, the number of SWD events had returned to baseline levels and there were no differences between groups (Kruskal-Wallis test, p=0.1851; n=3-7 per group). The mice were not treated at this later time point. With data from our fourth withdrawal recordings, we calculated the ED50 for ETX to be ~96.65mg/kg. Overall, ETX decreased ethanol withdrawal-induced SWD events in a dose-dependent manner, with higher doses necessary to block hyperexcitability.
Figure 3. ETX decreased SWD events during withdrawal in a dose-dependent manner. A-D. In each withdrawal period, 150 and 250mg/kg decreased SWD events compared to what was observed in the saline-treated mice. The lowest dose of ETX (50mg/kg) was not effective. Compared to saline: **, p<0.01; ***,p<0.0001; Compared to 50mg/kg: #, p<0.05; ##, p<0.01; ###, p<0.0001; gray-hashed horizontal line is the combined average baseline level between the four groups.
3.3. ETX reduced the withdrawal-induced increase in duration of SWD events to baseline

We investigated the duration of SWD activity during baseline and each withdrawal period. As previously described, we determined that ethanol withdrawal significantly lengthens the duration of the SWD events with each successive withdrawal (Figure 2B). We observed dose-dependent effects of ETX on seizure duration. In the first withdrawal, ETX (150 and 250 mg/kg) reduced the duration of SWD events compared to the duration of SWDs observed in mice treated with saline (Figure 4A; Kruskal-Wallis with Dunn’s Multiple Comparison test, p<0.0001; n=8-9 per group). In the second and third withdrawal, only the highest dose of ETX (250mg/kg) reduced the duration of SWD events (Figure 4B-C; Kruskal-Wallis with Dunn’s Multiple Comparison test, p<0.005; n=8-9 per group). Finally, in the fourth withdrawal there were no differences in SWD duration between the saline and ETX-treated mice; however, the highest dose significantly differed from the lowest dose tested (Figure 4D; Kruskal-Wallis with Dunn’s Multiple Comparison test, p>0.05; n=8-9 per group). The highest dose also differed from the lowest dose in the first three withdrawal periods as well, and mice treated with 150mg/kg had significantly less duration of events compared to the mice treated with 50mg/kg in the second withdrawal. As with the number of SWD events, mice treated with the lowest dose of ETX (50mg/kg) did not have shorter SWD duration compared to mice treated with saline during any of the four withdrawal periods.
Figure 4. ETX reduced the withdrawal-induced increase in duration of SWD events in a dose-dependent manner. A-D. In the first three withdrawal periods, 250mg/kg decreased SWD duration compared to what was observed in the saline-treated mice. 150mg/kg decreased SWD duration compared to saline-treated mice during the first withdrawal period only. There were no differences in the fourth withdrawal with any of the doses compared to saline-treated mice. The lowest dose of ETX (50mg/kg) was not effective. Compared to saline: *, p<0.05; **, p<0.01; Compared to 50mg/kg: #, p<0.05;
##, p<0.01; gray-hashed horizontal line is the combined average baseline level between the four groups from panel A
There were no differences in baseline duration (Kruskal-Wallis, $p=0.6682$). The duration of the SWD events had returned to baseline levels two weeks following the fourth exposure, and there were no differences between groups (Kruskal-Wallis test, $p=0.6808$; $n=3-7$ per group). The mean±SEM duration of SWDs for baseline, each withdrawal period, and two weeks later are presented in Table 2 for each group.

3.4. ETX reduced power during ethanol withdrawal in a dose-dependent manner

We performed a power spectral analysis during each withdrawal period to evaluate the effects of ETX on power in the SWD activity range (6-10Hz). If ETX decreased the number of SWD events in a dose-dependent manner, we expected to observe a power reduction in this spectral band during the same time frame. In the first three withdrawal periods, ETX (150 and 250 mg/kg) decreased power compared to what was observed in saline-treated mice (Figure 5A-C; Kruskal-Wallis with Dunn’s Multiple Comparison test, $p<0.0003$; $n=8$ per group). In the fourth withdrawal, only the highest dose of ETX (250mg/kg) significantly decreased power in the 6-10Hz frequency range compared to that observed in saline-treated mice (Figure 5D; Kruskal-Wallis with Dunn’s Multiple Comparison test, $p=0.0025$; $n=8$ per group). The lowest dose of ETX (50mg/kg) had no effect on power.
Figure 5. ETX decreased power (6-10Hz) during ethanol withdrawal in a dose-dependent manner. A-D. In the first three withdrawal periods, 150 and 250mg/kg decreased SWD events compared to what was observed in the saline-treated mice. Only the highest dose, 250mg/kg, reduced power in the fourth withdrawal period compared to saline-treated mice. The lowest dose of ETX (50mg/kg) was not effective. Compared to saline: *, p<0.05; **, p<0.01; ***, p<0.0001; Compared to 50mg/kg: #, p<0.05; ##,
p<0.01; gray-hashed horizontal line is the combined average baseline level between the four groups from panel A
Mice treated with the highest dose of ETX (250mg/kg) had reduced power compared to the mice treated with the lowest dose of ETX (50mg/kg) during all four withdrawal periods (Figure 5A-D; Kruskal-Wallis with Dunns post-hoc test, p<0.05). There were no differences in power between the groups during the baseline recording (Kruskal-Wallis, p=0.1741). Two weeks later, the power in the 6-10Hz had returned to baseline levels at this time point and there were no differences between groups (Kruskal-Wallis test, p=0.9067; n=3-7 per group). The mean±SEM power (6-10Hz) observed for baseline and each withdrawal period are presented in Table 3 for each group.
Table 3. Summary of Normalized Spectral Power

<table>
<thead>
<tr>
<th></th>
<th>Baseline (2:00-5:00pm)</th>
<th>Ethanol-Exposed Mice</th>
<th>Air-Exposed Mice</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Saline</td>
<td>ETX 50 mg/kg</td>
</tr>
<tr>
<td>2:00-5:00pm</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1st WD</td>
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<td>53.5±8.36</td>
<td>45.47±4.07</td>
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<tr>
<td>2nd WD</td>
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<td>48.91±8.03</td>
<td>29.68±3.26*</td>
</tr>
<tr>
<td>3rd WD</td>
<td>67.5±5.45</td>
<td>49.38±6.82</td>
<td>28.10±3.21*</td>
</tr>
<tr>
<td>4th WD</td>
<td>72.21±8.14#</td>
<td>52.38±7.61</td>
<td>31.33±3.45*</td>
</tr>
<tr>
<td>2 weeks post</td>
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<td>61.23±9.24</td>
<td>39.38±7.03</td>
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<td>37.27±13.67</td>
<td>45.77±8.54</td>
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</tbody>
</table>

dose-dependently reduced EEG power (6-10Hz) in mice undergoing ethanol withdrawal. (data ± SEM); *statistically significant compared to ethanol-exposed, saline-treated mice; 
#statistically significant compared to air-exposed mice
3.5. ETX reduced the risk of mortality

We also assessed the mortality rate among the animals undergoing the intermittent ethanol vapor exposure paradigm. We monitored the mice during the four withdrawal periods and for two weeks following the fourth exposure. All the mice that did not survive died within 48 hours of the fourth exposure. Three out of eight mice treated with saline did not survive. Only two out of nine among the mice treated with the lowest dose of ETX (50 mg/kg) died. Among the mice treated with 150 mg/kg ETX, one died and eight survived. Finally, all the mice treated with the highest dose of ETX (250 mg/kg, n=8) survived. There was a linear trend in mortality reduction with escalating doses (Figure 6; chi-square test for trend, p=0.0393).
Figure 6. **ETX reduced the risk of mortality.** Mortality rate was assessed among all the mice undergoing the intermittent ethanol exposure paradigm. All mice that did not survive died within 48 hours of the fourth exposure. Importantly, all mice treated with 250mg/kg ETX survived and only 5 out of 8 mice treated with saline survived. For the number of mice that died: saline, 3; 50mg/kg, 2; 150mg/kg, 1; 250mg/kg, 0. For the number of mice that survived: saline, 5; 50mg/kg, 7; 150mg/kg, 8; 250mg/kg, 8. There was a linear trend in mortality reduction with escalating doses of ETX (p=0.0393).
3.6. ETX did not affect general anxiety-like behavior or motor activity

Two well-established measures of anxiety-like behavior were used to determine if acute ETX (250mg/kg) had any anxiolytic effects in ethanol-naïve mice. Our results showed that ETX had no effect on anxiety-like behavior in the light/dark box (Figure 7A-D). An ANOVA with a Tukey’s Multiple Comparison post-hoc analysis revealed the test was anxiogenic (p<0.0001) meaning that the mice spent significantly more time in the dark side compared to the light side. Acute treatment with ETX, however, did not reduce anxiety-like behavior in this assay as we observed no differences between the saline and ETX-treated mice. ETX did not affect number of entries into the light side, total movement time, or total distance (t-test; p=0.4270; p=0.3033; p=0.9778 respectively). These results demonstrate that ETX treatment had no anxiolytic or motor impairing effects in this assay. The mean±SEM values for each test are presented in Table 4.
Figure 7. ETX did not affect general anxiety-like behavior or impair motor ability.

A-D. In the light/dark box test, we evaluated effects of anxiety-like behavior in naïve DBA/2J mice. All mice spent significantly more time in the dark side compared to the light side (p<0.0001). ETX did not affect time spent in the dark side versus light side, number of entries into the light side, total movement time, or total distance (p>0.05; p=0.4270; p=0.3033; p=0.9778 respectively). Overall, the mice treated with ETX displayed the same anxiety-like behavior as the saline-treated mice in the light/dark box assay. ETX treatment did not impair motor ability. E-F. Further testing in the elevated plus maze confirmed that ETX did not affect anxiety-like behavior. In the elevated plus maze, all mice spent significantly more time in the closed arms compared to the time spent in the open arms, suggesting the test was anxiogenic (p=0.0001). There was,
however, no difference between saline and ETX treatment (p>0.05). All mice made significantly more entries into the closed arms (p=0.0001); however, mice treated with ETX were not different from saline-treated mice. Overall, both tests initiated anxiety-like behavior in the mice; however, ETX had no effect. Our results also demonstrated that ETX did not impair motor ability.
Table 4. Summary of Light/Dark Box

<table>
<thead>
<tr>
<th></th>
<th>Time Spent (Sec)</th>
<th>Number of Entries (Light)</th>
<th>Total Movement Time (Sec)</th>
<th>Total Distance Traveled (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Light</td>
<td>Dark</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saline</td>
<td>108.4±9.3</td>
<td>192.6±9.3</td>
<td>10.38±1.1</td>
<td>240.5±2.3</td>
</tr>
<tr>
<td>ETX (250mg/kg)</td>
<td>104.3±15.7</td>
<td>196.8±15.7</td>
<td>11.50±0.8</td>
<td>245.3±3.9</td>
</tr>
</tbody>
</table>

ETX had no effect on anxiety-like behavior in ethanol-naive DBA/2J mice. ETX did not impair locomotor activity. (data ±SEM)
In the elevated plus maze, we observed that all mice, regardless of treatment, spent significantly more time in the closed arms compared to the time spent in the open arms, suggesting that the test was anxiogenic (ANOVA with a Tukey’s Multiple Comparison post-hoc, p=0.0001). There was, however, no difference in these measures between saline and ETX treatment (Figure 7E; p>0.05). An additional evaluation was made on the number of entries into the closed and open arms between groups (Figure 7F). Again, all mice, regardless of treatment, entered the closed arms significantly more than the open arms (ANOVA with a Tukey’s Multiple Comparison post-hoc, p=0.0001). ETX treatment did not increase the number of entries into the open arms or reduce the number of entries into the closed arms (p>0.05). The mean±SEM values for each test are presented in Table 5.
ETX had no effect on anxiety-like behavior in ethanol-naïve DBA/2J mice. ETX did not impair locomotor activity. (data ± SEM)

<table>
<thead>
<tr>
<th></th>
<th>Time Spent (Sec)</th>
<th>Number of Entries</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Light</td>
<td>Dark</td>
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<tr>
<td>Saline</td>
<td>44.76±7.9</td>
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<tr>
<td>ETX (250mg/kg)</td>
<td>32.28±7.1</td>
<td>158.9±20.1</td>
</tr>
</tbody>
</table>
3.7. ETX modestly reduced ethanol consumption in DBA/2J mice

We used a modified two hour access, fade-in design to assess the effect of acute treatment with ETX on ethanol consumption in naïve DBA/2J mice. After a stable drinking baseline was achieved using 100mM MSG + 10% ethanol (Figure 8B), we conducted a Latin-square design to test the effects of multiple doses of ETX (50, 150, and 250 mg/kg). We observed a moderate reduction (~20%) in ethanol consumption after ETX treatment; however, there were no differences in consumption between the different doses (Figure 8C; Kruskal-Wallis test with a Dunn’s Multiple Comparison post-hoc, p=0.0208). Importantly, ETX treatment had no effect on MSG consumption alone in the control group.
Figure 8. ETX modestly reduced ethanol consumption in nondependent DBA/2J mice. A. Schematic of ethanol fade-in procedure (see methods for details). The dark box in the 5% period represents when a 3 day force was applied. 100mM MSG remained constant. B. A stable drinking baseline was established two weeks prior to testing. C. A 4x4 latin square design was used to test each dose of ETX (50, 150, and 250mg/kg) and saline treatment on ethanol + MSG and MSG alone consumption. ETX modestly reduced ethanol consumption by ~20% (overall effect, p=0.0208). There were no differences between doses. ETX, however, did not reduce MSG consumption in the control group.
4. Discussion

In this investigation, we further characterized the use of ETX as a potential treatment and highlighted the potential for T channels as a novel target for alcohol abuse and withdrawal treatment. Previous investigations (Graef et al., 2011; Riegle et al., 2014; Wiggins et al., 2013) and the current study provide evidence that increased T channel activity contributes to hyperexcitability during ethanol withdrawal and pharmacologically targeting T channels may alleviate withdrawal symptoms, specifically withdrawal seizures. We also conducted an initial characterization of the efficacy of ETX as a potential treatment for alcohol use disorders by investigating its effects on ethanol consumption and anxiety-like behaviors in ethanol-naïve mice. We demonstrated the following: ethanol withdrawal increased seizure severity; ETX dose-dependently suppressed ethanol withdrawal-induced seizure activity; ETX had no effect on anxiety-like behaviors in ethanol-naïve mice but did not impair motor function; ETX modestly reduced ethanol consumption; and ETX may improve mortality rates. We discuss each of the findings below.

4.1. Ethanol withdrawal progressively increased seizure activity

Previously, we demonstrated that SWD events increased in number and duration with successive withdrawal periods and observed an increase in power in the SWD frequency range of 6-10Hz during peak withdrawal in DBA/2J mice (Riegle et al., 2014). Here, we utilized the same intermittent ethanol exposure paradigm and demonstrated similar findings. Mice treated with saline exposed to the intermittent paradigm had a progressive increase in SWD events over the course of four withdrawal periods, whereas
the number of SWD events in air-exposed mice undergoing the same saline treatment and handling remained constant. We also demonstrated that mice undergoing withdrawal have SWDs with longer duration and their EEG activity have increased power (6-10Hz) compared to air-exposed mice. These findings suggest that this model is consistent and can be used to evaluate the electrographical correlates of ethanol withdrawal-induced seizure activity and test potential treatment options. Furthermore, these findings support both rodent and clinical literature suggesting that severity of withdrawal symptoms, including seizures, progressively worsen with more episodes of withdrawal (Becker, 1994; Becker, 1998; Becker et al., 2006; Brown et al., 1988; Lechtenberg and Worner, 1990; Veatch and Becker, 2002, 2005).

4.2. ETX dose-dependently reduced ethanol withdrawal-induced seizure severity

We observed that ETX treatment dose-dependently reduced the number, duration, and the representation of this activity in the spectral power of the EEG, with an estimated ED50 of ~96.65mg/kg. These findings support our previous data suggesting that ETX reduced ethanol withdrawal seizures (Riegle et al., 2014). In the previous study, we tested only one dose of ETX (250 mg/kg); we observed both a decrease in electrographic and behavioral seizure activity and that this dose could rescue the mice from increased seizure activity prior to treatment. The ED50 of ETX in other models of seizure activity has been highly dependent on the species of animal and the test. Specifically, the ED50 of ETX in different models was as follows: pentylentetrazole seizure in mice, 134mg/kg (Swiader et al., 2006); audiogenic susceptible mice, 328mg/kg (Bialer et al., 2004); Wistar Albino Glaxo/Rijksloijk rats, 21.5 mg/kg (Bialer et al., 2004); maximal
electroshock seizure test, >500mg/kg (Bialer et al., 2004). Interestingly, anticonvulsants were found to be more potent in rats compared to mice (Bialer et al., 2004). In our study, we used doses relevant to what has been demonstrated in other studies (Aizawa et al., 1997; Dezsi et al., 2013; Frankel et al., 2005; Kaneto et al., 1986; Marrosu et al., 2007; Nissinen and Pitkanen, 2007); however most of these studies were investigating seizure activity in epilepsy models. This is the first study to carefully evaluate the dose dependence of ETX in an ethanol withdrawal seizure model. It remains to be determined how the intermittent exposure paradigm affects ETX pharmacokinetics; however ETX has a short half-life in rodents of approximately one hour (el Sayed et al., 1978) and our previous study demonstrated that the seizure events return as the drug is metabolized (Riegle et al., 2014).

We recorded EEG activity in the mice to determine if the SWD activity remained elevated in the saline-treated mice two weeks following the fourth withdrawal. We found that the SWD events had returned to levels observed at baseline in all treatment groups, including the saline treated-mice, which had increased SWD events in the fourth withdrawal period. These findings are expected as the window for risk of seizure occurs within forty-eight hours of withdrawal (Hillbom et al., 2003; Hughes, 2009b), and a prior study has shown that four exposures of intermittent ethanol and withdrawal are not sufficient to induce enduring increases in SWDs (Veatch and Becker, 2002). We do not, however, know if the threshold in the saline treated mice permanently shifted, which might be the case with increased BEC exposure level or number of withdrawal periods. A previous finding in C3H/He mice demonstrated a shift to a more hyperexcitable state when more withdrawal periods occurred (Becker et al., 1997b). Further testing will be
necessary to determine if there are lasting consequences in these mice exposed to only four days of ethanol and withdrawal and if, at later time points, ETX treatment can be protective from any shifts in threshold excitability that might occur.

One concern with the seizure model employed in this study is that it does not typically induce robust behavioral changes, such as convulsive, tonic/clonic-like activity, changes that are often associated with alcohol withdrawal (Rathlev et al., 2006). We previously demonstrated that ETX reduced handling-induced convulsion severity, and mice treated with ETX had less tonic/clonic seizures compared to mice treated with saline in this model of ethanol withdrawal seizure (Riegle et al., 2014). This work is similar to a previous report demonstrating that ETX reduced ethanol withdrawal signs (Kaneto et al., 1986). Furthermore, several investigations in mice have demonstrated that the abnormal electrographical activity was increased at the same time period as handling-induced convulsions (Becker et al., 2006; Veatch and Becker, 2002, 2005). Clinically, many abnormalities have been observed in EEG recordings of alcoholics (Cohen et al., 1997; Coutin-Chruchman et al., 2006; Feige et al., 2007; Porjesz and Begleiter, 2003; Rangaswamy et al., 2003; Rodriguez Holguin et al., 1999; Salety-Zyhlarz et al., 2004), and two case reports have identified nonconvulsive activity in EEG recordings of patients during alcohol withdrawal (Fernandez-Torre and Martinez-Martinez, 2007; LaRoche and Shivdat-Nanhoe, 2011), thus providing strong support for the use of EEG recordings in rodent studies of ethanol withdrawal.

Functional changes to T channel activity can have a dramatic impact on normal brain function (Cheong and Shin, 2013). Observed increases in T channel activity (Graef et al., 2011) and SWD activity during ethanol withdrawal that can be treated with ETX...
have initiated a hypothesis: abnormal oscillatory activity facilitated by early increases in T channel activity in withdrawal may serve as a trigger that precipitates into more long-term, severe consequences such as tonic/clonic seizure activity. In support of this idea, T channel function has been reported to be disrupted in a model of limbic-acquired epileptogenesis (Becker et al., 2008; Graef et al., 2009b) suggesting T channels are important for not only nonconvulsive activity but also convulsive seizure activity, including the development of epilepsy. A recent investigation determined that ETX prevented epileptogenesis in the GAERS model (Dezsi et al., 2013). These are relevant findings because alcohol abuse and withdrawal can precipitate status epilepticus and lead to temporal lobe epilepsy (Hillbom et al., 2003; Hughes, 2009). It will be important to test this hypothesis as it will further our understanding of the role and consequences of early adaptations in alcohol abuse and withdrawal.

4.3. ETX did not reduce general anxiety-like behaviors or cause motor impairment

A recent investigation demonstrated that ETX increased GABA release via a presynaptic mechanism in the entorhinal cortex (Greenhill et al., 2012). Previous reports have also indicated an increase in GABA levels in frontal cortical areas and whole brain levels of rodents (Lin-Michell et al., 1986; Ponnusamy and Pradhan, 2006). Based on these data, we predicted that ETX might also have an anxiolytic effect and possibly motor impairing effects. Both studies, with the light/dark box and elevated plus maze, yielded similar results: acute administration of ETX had no effect on anxiety-like behaviors. We confirmed that each assay induced anxiety-like behavior as all mice spent more time in the dark box of the light/dark box test and more time in the closed arms of
the elevated plus maze. Yet, the mice treated with ETX displayed the same amount of anxiety-like behavior as the mice treated with saline. These results suggest that the acute effects observed in our study with ETX were most likely not due to its actions on GABAergic signaling and are more likely due to its effects on T channels.

The actions of ETX on GABAergic transmission remain to be fully determined in addition to the physiological relevance of its effect as there have been conflicting findings with the effect of ETX on anxiety-like behaviors (Dezsi et al., 2013; Shaw et al., 2009; Simiand et al., 1984). Another consideration is that all of the mice tested were ethanol naïve, and therefore most relevant to the assessment of ETX effects on basal levels of anxiety. It will be important, in future studies, to test the effects of ETX on ethanol withdrawal-induced anxiety as ethanol dependence is associated with marked increases in anxiety-like behavior and T channel activity (Breese et al., 2005; Graef et al., 2011; Kliethermes, 2005; Nordskog et al., 2006; Welsh et al., 2011). Thus, if increased T channel function contributes withdrawal-induced anxiety, ETX treatment may prove more efficacious in treating ethanol dependence-induced increases in anxiety-like behavior.

One major limitation related to the use of benzodiazepines for alcohol withdrawal treatment is that these drugs have been shown to cause motor impairment (Ait-Daoud et al., 2006). Importantly, ETX did not impair motor function in DBA/2J mice in any of the behavioral assays employed in this study.
4.4. ETX modestly reduced ethanol consumption in nondependent mice

Preclinical studies have demonstrated that a broad range of antiepileptic drugs, with widely varying mechanisms of action, can reduce ethanol intake (Breslin et al., 2010; Farook et al., 2009; Gabriel and Cunningham, 2005; Gardell et al., 1998; Knapp et al., 2007; Messiha et al., 1986; Nyguen et al., 2007; Roberto et al., 2008; Zalewska-Kaszubska et al., 2011; Zalewska-Kaszubska et al., 2013). Interestingly, one of these antiepileptics, zonisamide, acts on T-type calcium channels (Holder Jr and Wilfong, 2011; Kito et al., 1996; Suzuki et al., 1992). We, therefore, sought to conduct an initial assessment to determine if ETX had any effects on ethanol consumption to further evaluate its potential as a treatment option. Here, we demonstrated that all doses of ETX tested decreased ethanol consumption by approximately 20%. There were, however, no differences between doses. This suggests that lower doses of ETX may be effective at reducing consumption compared to doses necessary for reducing seizure activity, and the effects observed on consumption might be mediated through a different mechanism in a nonlinear manner. While it was a modest reduction, importantly, ETX did not affect consumption of the added tastant, MSG. Unfortunately, there was considerable variability in drinking among the mice and prior studies have demonstrated that DBA/2J mice do not readily drink as much ethanol as other murine strains, such as C57Bl/6J mice (Belknap et al., 1993; Yoneyama et al., 2008). However, DBA/2J mice have exhibited stronger conditioned place preference for ethanol compared to C57Bl/6J mice (Cunningham et al., 1992; Gremel et al., 2006). It would be interesting to test if ETX has an effect on this characteristic.
Again, it is important to note that this initial assessment was conducted in ethanol-naïve mice. Another study investigating gabapentin demonstrated that this drug selectively decreased ethanol intake in dependent, but not nondependent animals (Roberto et al., 2008). In fact, many studies have demonstrated that the pharmacology that regulates ethanol intake is different in ethanol dependent animals (Griffin lii et al., 2014; Griffin et al., 2009; Henderson and Czachowski, 2012; Roberto et al., 2008; Walker et al., 2008). Thus, it will be important in future studies to determine if ETX can reduce the escalation of ethanol intake associated with ethanol dependence.

We hypothesize that the effects we observed with ETX are mediated through T channels, but off target effects cannot be completely excluded. ETX’s primary mechanism of action is widely accepted to be inhibition of T channels (Gomora et al., 2001; Huguenard, 2002; Todorovic and Lingle, 1998). However, indirect effects on a persistent Na\(^+\) current and Ca\(^{2+}\)-dependent K\(^+\) current have been reported on administering higher concentrations of ETX (Crunelli and Leresche, 2002; Leresche et al., 1998). In addition, reports have demonstrated an effect of ETX at GABAergic synapses (Greenhill et al., 2012; Lin-Michell et al., 1986; Ponnuamay and Pradhan, 2006; Terzioglu et al., 2006). We also cannot exclude the role of the channel as a calcium portal as well as overt effects on excitability as a possible mechanism (Zhou et al., 1997).

Regardless of the target, our data demonstrate a dose-dependent reduction of withdrawal seizure activity that is consistent with the anti-seizure nature of the drug.
4.5. Conclusions

Overall, this is the first study to carefully evaluate the dose dependence of ETX in the treatment of ethanol withdrawal and evaluate its effects on ethanol consumption. We demonstrated that ETX dose-dependently reduced ethanol withdrawal-induced SWD events, duration, and power in the 6-10Hz frequency range. In our initial characterization on ethanol consumption, we observed a modest reduction with ETX treatment in nondependent DBA/2J mice. Lastly, ETX had no effect on anxiety-like behavior in ethanol-naïve mice, and importantly, ETX did not impair locomotor activity. These findings highlight the critical need to continue investigating the role of T channels during ethanol withdrawal, the neural substrates responsible for the therapeutic effects of ETX, and whether ETX can serve as a potential treatment option for patients suffering with alcohol abuse and withdrawal.
Acknowledgements

This work was supported by NIH grants F31AA021322-01, T32AA07565, R01AA016852, P01AA021099, and the Tab Williams Family Fund. The authors declare no competing financial or other conflicts of interests.
References


CHAPTER 4

ETHOSUXIMIDE REDUCES ETHANOL WITHDRAWAL SEIZURE MORTALITY

Melissa A. Riegle, Melissa L. Masicampo, Hong Qu Shan, Victoria Xu and Dwayne W. Godwin

This manuscript is in preparation to be submitted for publication. The experiments were performed by M.A. Riegle with the help of M.L. Masicampo, H.Q. Shan, and V. Xu. The manuscript was prepared by M.A. Riegle. Dr. Dwayne Godwin provided mentorship and editorial feedback.
Abstract

Alcohol withdrawal seizures are a serious risk when individuals undergo detoxification. Unfortunately, these seizures can lead to life-threatening, long-term consequences such as status epilepticus and temporal lobe epilepsy. Thus, alcohol withdrawal seizures are a major concern that requires immediate attention. Current treatment options include benzodiazepines; however, these treatments have a high potential for abuse and can cause rebound excitability. There is a strong need for safe, alternative treatment options. We recently identified a novel target, T-type calcium channels, affected by alcohol abuse and withdrawal. Treatment with ethosuximide, an antiepileptic drug that blocks T-type calcium channels, has demonstrated promising results against sleep disruption during ethanol withdrawal and ethanol withdrawal-induced seizure activity. Here, we expand on these findings to test whether ethosuximide can reduce the sensitivity to pentylenetetrazole-induced seizures during ethanol withdrawal. We used an intermittent ethanol exposure model to produce withdrawal-induced hyperexcitability in DBA/2J mice. Ethosuximide dose-dependently reduced seizure severity in mice undergoing ethanol withdrawal. Importantly, ethosuximide protected against ethanol withdrawal-induced mortality and did not cause rebound excitability. These results in addition to previous preclinical findings suggest that ethosuximide may be considered as a safe, effective alternative to benzodiazepines for alcohol withdrawal seizure treatment.

Keywords: alcohol withdrawal, ethosuximide, T-type calcium channel, seizure, pentylenetetrazole


Introduction

Alcohol abuse and dependence can cause compensatory mechanisms that generate a state of hyperexcitability when an individual undergoes detoxification (Ait-Daoud et al., 2006; Rogawski, 2005). These compensatory mechanisms create an imbalance in excitation and inhibition that can persist through withdrawal. Increased excitation can result in seizure activity. Alcoholics undergoing withdrawal commonly present with convulsive-like seizures (tonic/clonic seizures), and the risk for seizures progressively increases with each successive detoxification (Ballenger and Post, 1978; Becker, 1998; Brown et al., 1988; Lechtenberg and Worner, 1990; Rogawski, 2005). As many as 33% of individuals undergoing alcohol withdrawal develop seizures (Rogawski, 2005).

Unfortunately seizure activity is a serious, life-threatening problem that can lead to status epilepticus and temporal lobe epilepsy (Hughes, 2009; Rogawski, 2005). Alcohol withdrawal seizures are the trigger for status epilepticus in up to 25% of the cases reported (Hillbom et al., 2003; Hughes, 2009). Thus, alcohol withdrawal seizures are a serious concern and require immediate medical attention. Both rodent and clinical literatures have demonstrated that benzodiazepines, the first line treatment for alcohol withdrawal seizure are effective in the acute phase of seizures, but can exacerbate seizure activity and cause rebound effects when treatment is removed (Ait-Daoud et al., 2006; Becker and Veatch, 2002; Chouinard, 2004; File and Wilks, 1990; Greenblatt et al., 1990; Loscher et al., 1996; Rogawski, 2005; Rundfeldt et al., 1995; Veatch and Becker, 2005; Ward and Stephens, 1998; Woods et al., 1987). One goal of the present study is to find a safe, effective alternative as benzodiazepines have been shown to have negative side effects and high abuse potential (Ait-Daoud et al., 2006; Morris and Victor, 1987).
Compensatory mechanisms that ensue during ethanol withdrawal include a plethora of adaptations including increased calcium channel activity (Walter and Messing, 1999). T-type calcium channels (T channels) have been identified as one potential target that undergoes adaptation during ethanol exposure and withdrawal that could contribute to the hyperexcitable state observed during withdrawal. The T channel isoform, CaV3.2, is inhibited by acute administration of ethanol, through a PKC-dependent mechanism (Shan et al., 2013). During chronic ethanol consumption and withdrawal, T channel expression and function were enhanced (Graef et al., 2011; Nordskog et al., 2006; Welsh et al., 2011). Ethosuximide (ETX), a T channel antagonist, restored sleep activity that had been disrupted in mice undergoing ethanol withdrawal (Wiggins et al., 2013). We also found that ETX reduced the increased spike and wave discharge activity that was observed in mice undergoing ethanol withdrawal and decreased the severity of handling-induced convulsions and the number of tonic/clonic seizures (Riegle et al., 2014). These studies suggest that T channels are a novel target for intervention and ETX may be a potentially effective treatment for ethanol withdrawal symptoms. However, it is unclear whether benefits of this treatment extend to reductions in withdrawal-induced mortality.

A common strategy to evaluate alcohol withdrawal seizure and test the effectiveness of treatments has been to utilize chemoconvulsants to elicit convulsive-like (tonic/clonic) seizures in rodents undergoing withdrawal (Becker et al., 1998; Cagetti et al., 2004; Grant et al., 1990; Joshi et al., 2005; Kokka et al., 1993; Ripley et al., 2002; Stephens et al., 2001; Szabo et al., 1984). Here, we used pentylenetetrazole (PTZ), a GABA\textsubscript{A} antagonist (Psarropoulou et al., 1994; Squires et al., 1984), to elicit convulsive seizures in mice undergoing an intermittent ethanol exposure paradigm. We evaluate the
effects of ETX on the severity of PTZ-induced ethanol withdrawal seizures and mortality rate. We found a dose-dependent reduction in seizure severity, and importantly, ETX decreased ethanol withdrawal-induced seizure mortality. Lastly, we showed that ETX did not cause rebound excitability in an untreated withdrawal period.

Methods

For these experiments, the investigators followed the guidelines established by the National Institutes of Health and United States Department of Agriculture. Pain and suffering and the use of animals were minimized as much as possible. All experiments were approved by the Institutional Animal Care and Use Committee of Wake Forest School of Medicine. All mice used in these studies were group-housed and maintained on a 12 hour light/dark cycle (lights off: 6:00 pm, lights on: 6:00 am).

Experiment 1

Male, 6-8 weeks old DBA/2J mice (Jackson Laboratory, Bar Harbor, ME) underwent an intermittent ethanol exposure paradigm as previously described in Riegle et al. (2014). The model was adapted from a previous investigation of ethanol withdrawal seizure severity (Becker and Hale, 1993). Mice were placed in a Plexiglas vapor chamber for each exposure, located in the same room in which the mice were housed. The chamber was filled with volatilized ethanol (95%) utilizing an air pump. Exposure lasted 16 hours each day (5:00pm to 9:00am). After each withdrawal, mice underwent an 8 hour withdrawal period (9:00am to 5:00pm). Mice received ethanol exposure and
withdrawal for a total of four straight days. Control mice received air exposure instead of ethanol and were handled identically as the experimental mice.

All mice were injected subcutaneously with pyrazole (100mg/kg, Sigma Aldrich, St. Louis, MO), an alcohol dehydrogenase inhibitor, to maintain blood ethanol concentration (BEC) levels. Injections were administered immediately prior to each exposure. At the beginning of each withdrawal, blood (5 µl) was sampled from the tail for BEC measurements. The blood was immediately put in trichloracetic acid (6.25%) prior to analysis. A NAD-ADH enzyme assay (Carolina Liquid Chemistries Corp, Winston-Salem, NC) was utilized to measure the ethanol levels of each withdrawal period. There were no differences between treatment groups in BEC values. Thus, they were combined and averaged. For experiment 1, the BECs (mg/dL) were as follows: 1st withdrawal, 243.2±12.9; 2nd withdrawal, 205.0±12.0; 3rd withdrawal, 200.5±6.7; 4th withdrawal, 128.2±6.2. BEC values in the fourth withdrawal were significantly lower compared to the first, second, and third withdrawal periods (Kruskal-Wallis test with Dunn’s Multiple Comparison post-hoc test, p < 0.0001). Mice were excluded from this experiment if BEC levels were not between 80 and 200 mg/dL in the fourth withdrawal period.

All ethanol- and air-exposed mice were tested for seizure sensitivity 6-8 hours into the fourth withdrawal period. Mice were treated with intraperitoneal (IP) injections of saline or ETX (100 or 250mg/kg; Sigma Aldrich) 45 minutes prior to testing for seizure activity. To test for seizure activity, mice were injected with a subthreshold dose of PTZ (20mg/kg, IP; Sigma Aldrich). Immediately following PTZ injection, mice were video-taped and monitored for seizure activity for 30 minutes. A blind reviewer analyzed
the seizure activity and scored the seizures based on criteria listed in Table 1. Mice that
did not have a tonic/clonic seizure were given 1800 sec and 0 sec for latency to and
duration of tonic/clonic seizure.
<table>
<thead>
<tr>
<th>Score</th>
<th>Description of Behavior</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>No seizure activity</td>
</tr>
<tr>
<td>1</td>
<td>Tail twitching; head nodding</td>
</tr>
<tr>
<td>2</td>
<td>Myoclonic activity or tonus</td>
</tr>
<tr>
<td>3</td>
<td>Tonic/clonic convulsion</td>
</tr>
<tr>
<td>4</td>
<td>Severe tonic/clonic convulsion resulting in death</td>
</tr>
</tbody>
</table>
Experiment 2

To assess mortality due to ethanol withdrawal, male DBA/2J mice (6-8 weeks old) underwent the intermittent exposure paradigm as described in experiment 1. For experiment 2, the BECs (mg/dL) were as follows: 1<sup>st</sup> withdrawal, 259.9±43.4; 2<sup>nd</sup> withdrawal, 219.0±17.2; 3<sup>rd</sup> withdrawal, 227.2±16.7; 4<sup>th</sup> withdrawal, 171.4±15.3. There were no differences in BEC values between the treatment groups. Thus, they were combined and averaged. BEC values did not differ between withdrawal periods. No mice were excluded from this study.

Mice were administered saline or ETX (250mg/kg, IP) 45 minutes prior to testing. At 6-8 hours into the 4<sup>th</sup> withdrawal, mice were tested by injecting 40mg/kg PTZ (IP). A higher dose of PTZ was used in order to assess the effects of ethanol withdrawal on mortality and whether ETX can be protective. In control mice, this was a non-lethal dose that induced tonic/clonic seizures. Mice were monitored for one hour following PTZ administration. For this study, a blind reviewer was not used to assess mortality. Tonic/clonic seizure activity was evaluated on whether or not it was present, the mortality rate was assessed, and latencies to death and first tonic/clonic seizure were measured.

Experiment 3

Male DBA/2J mice (6-8 weeks old) underwent the intermittent ethanol exposure paradigm as described in experiment 1. There were no differences between treatment groups in BEC values. Thus, they were combined and averaged. For experiment 3, the BECs (mg/dL) were as follows: 1<sup>st</sup> withdrawal, 298.6±17.9; 2<sup>nd</sup> withdrawal, 300.7±20.3; 3<sup>rd</sup> withdrawal, 263.8±22.9; 4<sup>th</sup> withdrawal, 191.6±15.6. BEC values in the fourth
withdrawal were significantly lower compared to the first, second, and third withdrawal periods (Kruskal-Wallis test with Dunn’s Multiple Comparison post-hoc test, p < 0.0001). Two mice were excluded from this experiment, one each from the 100 and 250mg/kg ETX treatment groups because the BEC value for these mice was below 80 mg/dL in the 4th withdrawal.

Mice were administered saline or ETX (100 or 250mg/kg, IP) at 10:00am and 1:30pm during the first three withdrawal periods. Mice were not treated with saline or ETX during the fourth withdrawal period as this was the final, untreated withdrawal period. Seizure activity was assessed 8-10 hours into the fourth withdrawal period. To do this, mice were injected with PTZ (20mg/kg) as in experiment 1. Immediately following PTZ injection, mice were video-taped and monitored for 30 minutes. A blind reviewer viewed the activity and scored seizure severity based on the parameters and scale (Table 1) described in experiment 1.

**Statistical Analyses**

We used a nonparametric ANOVA to determine differences in seizure score, latency, and duration parameters. For experiments 1 and 3, the overall seizure 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 and for comparison in mortality rate in experiment 2. Nonparametric tests were used because sample sizes between groups differed.
Results

Experiment 1

In the first experiment, we assessed acute ETX treatment on ethanol withdrawal-induced seizure activity using the chemoconvulsant, PTZ, to elicit convulsive seizure activity. The effects of ETX (100 and 250mg/kg) are illustrated in Figure 1 (A-D). A Kruskal-Wallis test revealed that, in mice undergoing ethanol withdrawal, ETX (250mg/kg) significantly reduced the seizure score compared to mice treated with saline (Figure 1A; Dunn’s Multiple Comparison test, p = 0.0035; n= 5-10 per group). The lower dose of ETX (100mg/kg) tested was not significantly different from saline-treated mice. Air-exposed animals (not shown in Figure 1) were compared as well (Table 2). Air-exposed animals treated with saline or ETX (250mg/kg) did not have tonic/clonic seizures when administered PTZ.
Figure 1. Acute ETX treatment dose-dependently reduced PTZ-induced seizure activity during ethanol withdrawal. All panels represent mice 6-8 hours in ethanol withdrawal. A. Max seizure score assessed for mice undergoing ethanol withdrawal.
Acute treatment with ETX (250mg/kg) decreased seizure severity compared to what was observed in saline-treated mice. **B.** Number of mice with tonic/clonic seizures. None of the mice treated with 250 mg/kg ETX had a tonic/clonic seizure. **C.** Latency to tonic/clonic seizure. Mice without tonic/clonic seizures were assigned a latency of 1800 sec (the amount of time monitored). **D.** Duration of tonic/clonic seizure. Mice without tonic/clonic seizure were assigned a duration of 0 sec.; T/C, tonic/clonic; sec, second
Table 2. Summary of acute ETX effects on ethanol withdrawal seizure.

<table>
<thead>
<tr>
<th></th>
<th>Ethanol Withdrawal</th>
<th></th>
<th>Air Exposure</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Saline</td>
<td>ETX (100 mg/kg)</td>
<td>ETX (250 mg/kg)</td>
</tr>
<tr>
<td>Seizure Score</td>
<td>2.25 ± 0.35</td>
<td>0.88 ± 0.38</td>
<td>0.21 ± 0.15*</td>
</tr>
<tr>
<td>Mice with T/C Seizures**</td>
<td>5/10 (50%)</td>
<td>1/8 (12.5%)</td>
<td>0/7 (0%)</td>
</tr>
<tr>
<td>Latency (sec)</td>
<td>1336 ± 207.5</td>
<td>1749 ± 51.38</td>
<td>1800 ± 0</td>
</tr>
<tr>
<td>Duration (sec)</td>
<td>17.40 ± 7.9</td>
<td>2.25 ± 2.25</td>
<td>0.0 ± 0</td>
</tr>
</tbody>
</table>

Data are presented as mean ± SEM. Ethanol withdrawal saline, n=10; Ethanol withdrawal ETX (100 mg/kg), n=8; Ethanol withdrawal ETX (250 mg/kg), n=7; Air exposure saline, n=5; Air exposure ETX (250 mg/kg), n=5; *, significantly different from ethanol withdrawal mice treated with saline (Kruskal-Wallis test, Dunn’s Multiple Comparison test, p= 0.0035); **, significantly different (Chi-square test, p= 0.024) T/C, tonic/clonic; sec, second
There were significantly more mice with tonic/clonic seizures when treated with saline compared to when treated with ETX (Figure 1B; Chi-square, $p = 0.024; n= 5-10$). Mice treated with the higher dose of ETX (250 mg/kg) during ethanol withdrawal were similar to the air-exposed mice (Table 2), as none of these mice had tonic/clonic seizures.

Overall, there were no significant differences in latency to tonic/clonic seizure (Kruskal-Wallis test, $p = 0.0756; n=5-10$). Illustrated in Figure 1C includes the graph of only the mice that presented with tonic/clonic seizures. All of these mice were undergoing ethanol withdrawal and treated with saline except for one mouse that had been treated with the lower dose of ETX tested. Statistical comparisons cannot be made; however, the one mouse treated with ETX (100mg/kg) had a longer latency to the tonic/clonic seizure compared to the latency of the saline-treated mice.

There was an overall effect on duration of tonic/clonic seizure (Figure 1D; Kruskal-Wallis test, $p = 0.0240, n= 5-10$ per group); however, the Dunn’s Multiple Comparison post-hoc test revealed no differences between groups. The duration of tonic/clonic seizures for only the mice that presented with this type of activity is illustrated in Figure 1D as well. The mouse treated with ETX (100 mg/kg) that had a tonic/clonic seizure did have a shorter duration compared to the mice treated with saline; however, statistical comparisons cannot be made between these two groups.

Observations from these studies revealed that mice acutely treated with ETX (250mg/kg) during the fourth withdrawal behaviorally looked more like air-exposed control mice. These mice explored the area much more whereas saline-treated mice were more likely to stay in the corner of the cages with less exploration. Saline-treated mice undergoing withdrawal exhibited multiple seizure events ranging from myoclonic and
tonic activity to more severe seizures, like tonic/clonic seizure events. Acute treatment with ETX dose-dependently reduced ethanol withdrawal seizure severity. The mean±SEM values for these parameters are presented in Table 2.

Experiment 2

We sought to determine if acute treatment with ETX (250 mg/kg) could prevent mortality due to convulsive seizures during ethanol withdrawal. All mice treated with ETX during the fourth withdrawal period survived whereas only one mice treated with saline survived out of eight (Figure 2A). ETX treatment significantly reduced mortality (Figure 2A, Chi-square test, $p < 0.0001$, $n=8$ per group). We observed that each death was due to a severe tonic/clonic seizure. This dose of PTZ (40 mg/kg) induced tonic/clonic seizure activity in air-exposed control mice (Figure 2B); however none of these mice died. ETX (250mg/kg) reduced the number of tonic/clonic seizures compared to ethanol withdrawal and air-exposed mice treated with saline (Figure 2C, Chi-square test, $p<0.0001$, $n=8$ per group). In mice undergoing ethanol withdrawal, the latency to death and first tonic/clonic seizure were significantly greater in mice treated with ETX compared to mice treated with saline (Figure 2B, D, Kruskal-Wallis test with Dunn’s Multiple Comparison post-hoc test, $p=0.0006$ and 0.0003 respectively). The mean±SEM values for these parameters are presented in Table 3.
Figure 2. Acute treatment with ETX (250mg/kg) reduced ethanol withdrawal-induced mortality. A. The number of mice that survived and died in each treatment group. All of the mice undergoing withdrawal, treated with ETX survived. Only one mouse treated with saline undergoing withdrawal survived. B. Latency to death in each treatment group. Mice that did not die were assigned 3600 seconds (the amount of time monitored). C. The number of mice with tonic/clonic seizures. ETX significantly reduced the number of tonic/clonic seizures. Mice undergoing ethanol withdrawal were observed to have multiple tonic/clonic seizures whereas mice air-exposed or treated with
ETX would only have one tonic/clonic seizure if any. D. Latency to first tonic/clonic seizure. Mice without tonic/clonic seizures were assigned 3600 seconds. T/C = tonic/clonic; sec, second
Table 3. Summary of acute ETX effects on mortality by ethanol withdrawal seizure.

<table>
<thead>
<tr>
<th></th>
<th>Ethanol Withdrawal</th>
<th>Air Exposure</th>
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<tbody>
<tr>
<td></td>
<td>Saline</td>
<td>ETX (250 mg/kg)</td>
</tr>
<tr>
<td>Seizure Mortality</td>
<td>7/8 (87.5%)</td>
<td>0/8 (0%)</td>
</tr>
<tr>
<td>Latency to Death (sec)</td>
<td>1921 ± 416.5</td>
<td>3600 ± 0.0</td>
</tr>
<tr>
<td>Mice with T/C Seizures</td>
<td>8/8 (100%)</td>
<td>1/8 (12.5%)</td>
</tr>
<tr>
<td>Latency to first T/C Seizure (sec)</td>
<td>155.0 ± 38.7</td>
<td>3234 ± 366.0</td>
</tr>
</tbody>
</table>

Data are presented as mean ± SEM. Ethanol withdrawal saline, n=8; Ethanol withdrawal ETX (250mg/kg), n=8; Air exposure saline, n=8; T/C, tonic/clonic; sec, second
Observations of the mice revealed that the saline-treated mice undergoing ethanol withdrawal had multiple tonic/clonic seizures that were much more severe compared to the ETX-treated, ethanol withdrawal mice and air-exposed, control mice. These mice exhibited behavior such as running and bouncing around the cage as well as loss of postural control. Mice treated with ETX did not exhibit tonic/clonic seizure activity with the exception of one mouse. In this mouse, the seizure was mild and more like the tonic/clonic seizures of the air-exposed mice. Air-exposed, control mice did not have multiple tonic/clonic seizures as the saline-treated, ethanol withdrawal mice did, and the only tonic/clonic seizure observed in these mice was much milder with only loss of postural control. All of these mice were able to recover normally after the tonic/clonic seizure. Many of the mice treated with ETX explored and behaved normally.

**Experiment 3**

Lastly, we treated mice during each withdrawal period with saline and ETX (100 or 250 mg/kg) and examined seizure activity in a fourth, untreated withdrawal period. We observed no differences in the seizure score between the groups (**Figure 3A**; Kruskal-Wallis test, $p = 0.9497$; n= 6-12 per group). 75% of the mice that had been treated with saline, 66.7% that had been treated with 100 mg/kg ETX, and 55.6% that had been treated with 250 mg/kg ETX had tonic/clonic seizures in the untreated, fourth withdrawal period; these values were not significantly different (**Figure 3B**; Chi-square test, $p = 0.8750$; n= 6-12 per group). Latency to and duration of tonic/clonic seizures were not different between groups (**Figure 3C, D**; Kruskal-Wallis test, $p = 0.9347$ and 0.7598 respectively; n= 6-12 per group). Overall there were no differences between
groups, thus suggesting that ETX does not produce rebound excitability in a fourth untreated withdrawal period. The mean±SEM values for these parameters are presented in Table 4.
Figure 3. ETX treatment did not produce rebound excitability in a fourth, untreated withdrawal period. All mice presented in the panels are undergoing ethanol withdrawal. **A.** There were no differences in seizure score between saline or ETX (100 and 250mg/kg) treated mice in a fourth, untreated withdrawal period suggesting that ETX did not produce rebound excitability when treatment is stopped. **B.-D.** Number of mice with, latency to, and duration of tonic/clonic seizure were not different between treatment groups. Mice without a tonic/clonic seizure were assigned a latency to and duration of 1800 seconds and 0 seconds respectively. Mice were monitored for 30 minutes (1800 seconds). T/C, tonic/clonic seizure; sec, second
Table 4. Summary of ETX effects on rebound excitability.

<table>
<thead>
<tr>
<th></th>
<th>Saline</th>
<th>Ethanol Withdrawal</th>
<th>Ethanol Withdrawal</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>ETX (100 mg/kg)</td>
<td>ETX (250 mg/kg)</td>
</tr>
<tr>
<td>Seizure Score</td>
<td>2.79 ± 0.19</td>
<td>2.75 ± 0.36</td>
<td>2.55 ± 0.29</td>
</tr>
<tr>
<td>Mice with T/C Seizures</td>
<td>9/12 (75%)</td>
<td>4/6 (66.7%)</td>
<td>5/9 (55.6%)</td>
</tr>
<tr>
<td>Latency (sec)</td>
<td>799.3 ± 203.5</td>
<td>865.0 ± 318.8</td>
<td>874.2 ± 293.1</td>
</tr>
<tr>
<td>Duration (sec)</td>
<td>25.83 ± 10.26</td>
<td>29.50 ± 12.87</td>
<td>17.78 ± 7.47</td>
</tr>
</tbody>
</table>

Data are presented as mean ± SEM. Ethanol withdrawal saline, n=12; Ethanol withdrawal ETX (100mg/kg), n=6; Ethanol withdrawal ETX (250mg/kg), n=9; T/C, tonic/clonic; sec, second
Discussion

In these experiments, we utilized an intermittent exposure paradigm that included four ethanol exposures and four withdrawal periods. This is a well-established model that consistently generates increased excitability during withdrawal (Becker, 1994; Becker et al., 1997a; Becker et al., 1997b; Becker and Hale, 1993; Riegle et al., 2014; Veatch and Becker, 2002). Previous studies have established that mice undergoing withdrawal have increased sensitivity to chemoconvulsants and a reduction in seizure threshold (Becker et al., 1998; Cagetti et al., 2004; Kokka et al., 1993; Stephens et al., 2001). We demonstrate similar findings in that saline-treated mice undergoing withdrawal had increased seizure severity compared to air-exposed, saline-treated mice.

In the first experiment, mice undergoing withdrawal exhibited severe tonic/clonic seizure activity whereas none of the air-exposed, control mice had tonic/clonic seizure activity after PTZ injection. In the second experiment, increasing the dose of PTZ resulted in tonic/clonic seizure activity in air-exposed mice; however these mice only experienced one mild tonic/clonic seizure and all of these mice were able to recover back to ‘normal’-like behavior. Mice undergoing ethanol withdrawal, injected with the higher dose of PTZ, experienced multiple, very severe tonic/clonic seizures that resulted in death in 7 out of 8 the mice. Thus, the mortality observed in these mice can be contributed to the hyperexcitable state induced by ethanol withdrawal.

Acute treatment with ETX dose-dependently reduced ethanol withdrawal-induced seizures. In the fourth withdrawal period, mice treated with the higher dose of ETX had a significantly lower seizure score compared to saline-treated mice. ETX treatment also reduced the number of tonic/clonic seizures observed in the fourth withdrawal.
Specifically, only one mouse treated with ETX (100mg/kg) had a tonic/clonic seizure and none of the mice treated with the higher dose of ETX (250mg/kg) had tonic/clonic seizures. This is in comparison to the saline-treated mice undergoing withdrawal where 50% had tonic/clonic seizures. There were no differences in latency to tonic/clonic seizure and only a trend for a decrease in duration of tonic/clonic seizure. These results are most likely due to the increased variability observed among the mice treated with saline undergoing withdrawal. The effects of ETX treatment on seizure severity and number of tonic/clonic seizures is not due to differences in ethanol exposure as there were no differences in BEC levels between the different groups of mice undergoing withdrawal. These findings are consistent with our previous investigation demonstrating that ETX reduced spike and wave discharge activity and handling-induced convulsions, both induced by ethanol withdrawal (Riegle et al., 2014).

We further demonstrated that acute ETX treatment reduced mortality caused by severe convulsions during ethanol withdrawal. All mice treated with ETX during the fourth withdrawal period survived despite the increased dose of PTZ. These mice had significantly fewer tonic/clonic seizures. Mice undergoing withdrawal treated with saline had multiple, very severe tonic/clonic seizures. As mentioned earlier, ultimately, a severe tonic/clonic seizure resulted in death in 7 out of 8 of the mice undergoing withdrawal that had been treated with saline. The effects of ETX are not due to differences in ethanol exposure as there were no differences in BEC levels between the ETX and saline-treated mice undergoing withdrawal. These results support our findings from a different model that suggested a trend for a reduction in mortality after ETX treatment in mice undergoing withdrawal (Riegle et al., submitted for publication).
In previous studies utilizing a similar intermittent exposure paradigm, mice, treated with lorazepam or MK-801 during withdrawal, suffered from rebound excitability when treatment ceased (Becker and Veatch, 2002; Veatch and Becker, 2005). Under these conditions, withdrawal-induced seizures were exacerbated. We utilized a similar design and treated mice with ETX or saline during the first three withdrawal periods. We then stopped treatment and tested the mice in the fourth withdrawal period, which was considered an untreated withdrawal period. ETX was administered twice during each withdrawal period, once in the morning and once in the afternoon of the first three withdrawal periods, because ETX is metabolized very quickly in rodents with an approximate half-life of one hour (el Sayed et al., 1978). Seizure activity observed in the fourth withdrawal period was not different between the groups. Thus, prior ETX treatment did not cause rebound excitability in the fourth, untreated withdrawal period. This was an important finding, because benzodiazepines, the first line therapy for alcohol withdrawal, have been shown to cause rebound effects after treatment termination in both rodent and clinical studies (Becker and Veatch, 2002; Chouinard, 2004; File and Wilks, 1990; Greenblatt et al., 1990; Loscher et al., 1996; Rundfeldt et al., 1995; Veatch and Becker, 2005; Ward and Stephens, 1998; Woods et al., 1987).

It remains to be determined whether chronic treatment with ETX can prevent long-term consequences from chronic ethanol abuse and withdrawal. Our investigation indicated that short term treatment with ETX cannot prevent acute development of withdrawal seizure; however, as previously mentioned, ETX is metabolized quickly and we cannot confirm what the ETX levels were in the brain throughout the withdrawal
period. Also, we do not know if the intermittent exposure paradigm may have altered the metabolism of ETX.

One of the major concerns of alcohol withdrawal seizures is the risk of developing more severe, life-threatening consequences such as status epilepticus and temporal lobe epilepsy. Alcohol withdrawal seizures precipitate up to 25% of the reported cases of status epilepticus (Hillbom et al., 2003; Hughes, 2009b). Also, it is well-established that multiple detoxifications increase the risk for seizure significantly. These factors underscore the importance of early intervention. As mentioned, it is unknown if chronic treatment with ETX can alter kindling mechanisms and prevent more severe consequences. Interestingly, chronic treatment over several months with ETX has been shown to inhibit epileptogenesis (Dezsi et al., 2013). We have observed promising results with acute treatment with ETX; however, further investigation is necessary.

Our results indicate that when ETX is administered, it can be effective in reducing ethanol withdrawal-evoked seizures. Our previous investigation demonstrated that ETX reduced both electrographical correlates of withdrawal seizure and handling-induced convulsions in mice undergoing withdrawal (Riegle et al., 2014). We have expanded on these findings and demonstrate that ETX dose-dependently reduced more severe tonic/clonic seizures initiated by the chemoconvulsant, PTZ, during withdrawal. Most importantly it protected the mice against withdrawal-induced mortality. Lastly, ETX did not cause rebound excitability in a fourth, untreated withdrawal period. These results not only emphasize the potential use of ETX as an effective therapeutic option against alcohol withdrawal seizure, but also implicate T channels as a potential mechanism. A
combination of recent work has demonstrated the utility of employing this approved drug in preclinical studies to reduce potentially lethal alcohol withdrawal seizures.
Acknowledgements

This work was supported by NIH grants F31AA021322-01, T32AA07565, R01AA016852, and the Tab Williams Family Fund. The authors declare no competing financial or other conflicts of interests.
References


CHAPTER 5

DISCUSSION AND FUTURE DIRECTIONS
Summary of Results

This dissertation embodies several novel findings and contributions to the literature that will change our view of alcohol withdrawal. These are reviewed in the following paragraphs.

DBA/2J mice are a novel model of progressive alcohol withdrawal seizure. A significant goal of this dissertation work was to characterize ethanol withdrawal seizure severity in DBA/2J mice and evaluate the potential use of ethosuximide (ETX), a T-type calcium channel (T channel) antagonist. It was important to establish this model in DBA/2J mice prior to testing the effects of ETX, as this “kindling-like” progression in seizure severity has been demonstrated in previous preclinical and clinical studies (Ballenger and Post, 1978; Becker, 1994; Becker et al., 1997a; Becker et al., 1997b; Brown et al., 1988; Lechtenberg and Worner, 1990; Moak and Anton, 1996; Veatch and Becker, 2002). In this model, a significant increase was observed in the number and duration of spike and wave discharge (SWD) events in mice undergoing withdrawal. Not only was this abnormal oscillatory activity increased within each withdrawal period, it progressively increased across each successive withdrawal period.

ETX eliminates and suppresses withdrawal seizures. We observed that acute ETX treatment during ethanol withdrawal blocked the increase in number and duration of SWDs and reduced the observed increase in power in a dose-dependent manner (50-250 mg/kg; ED50 ~96 mg/kg). A within subject analysis also revealed that ETX rescued the mice from seizure events occurring prior to treatment. ETX is metabolized quickly in mice with a biological half-life of approximately one hour (el Sayed et al., 1978). In an
extended fourth withdrawal period, SWD events returned to levels intermediate of what was observed prior to and immediately following treatment.

*ETX reduces mortality due to ethanol withdrawal seizure.* In the follow-up study evaluating the dose-dependency of ETX, only 62.5% of the mice treated with saline undergoing withdrawal survived whereas all mice treated with the highest dose of ETX (250mg/kg) survived. This was the first attempt made to determine whether ETX reduced mortality in mice undergoing ethanol withdrawal. Further validation occurred in a separate investigation that demonstrated protection from ethanol withdrawal-induced mortality with ETX treatment. In this experiment, only one mouse treated with saline prior to pentylenetetrazole (PTZ) during withdrawal survived whereas all mice treated with ETX prior to PTZ survived.

*ETX blocks convulsive as well as nonconvulsive withdrawal seizures.* While it was clearly evident that ETX blocked electrographical correlates of ethanol withdrawal-induced seizure activity, it was important to evaluate its potential effectiveness in behavioral models of ethanol withdrawal seizure. In the clinical literature, presentation of convulsive, tonic/clonic seizure activity is often described in individuals undergoing alcohol withdrawal (Rathlev et al., 2006). Thus, two different but well-characterized behavioral models of ethanol withdrawal seizure were utilized: handling-induced convulsion (HIC) and PTZ. Acute administration of ETX reduced HIC severity and the number of tonic/clonic seizures observed in DBA/2J mice undergoing ethanol withdrawal. A subthreshold dose of PTZ caused increased seizure activity characterized as myoclonic to tonic/clonic activity in mice undergoing ethanol withdrawal compared to air-exposed mice. Acute treatment with ETX dose-dependently reduced the PTZ-induced
seizure activity in mice undergoing ethanol withdrawal. Observations made by the experimenters implied that the behavior of the ETX-treated mice in withdrawal appeared very similar to the air-exposed control mice.

**ETX did not produce rebound effects.** Previous investigations have demonstrated that benzodiazepines and NMDA antagonists can exacerbate seizure activity in an untreated withdrawal period (Becker and Veatch, 2002; Veatch and Becker, 2005). In our investigation ETX treatment did not produce excitatory rebound effects as we did not observe an increase in seizure severity in an untreated withdrawal period. A major concern with the use of benzodiazepines in treatment of alcohol withdrawal has been their propensity to cause rebound effects, which has been demonstrated in both preclinical and clinical studies (Chouinard, 2004; File and Wilks, 1990; Greenblatt et al., 1990; Loscher et al., 1996; Rundfeldt et al., 1995; Ward and Stephens, 1998; Woods et al., 1987).

**ETX produces a modest reduction in ethanol consumption in nondependent mice.** To determine the potential effectiveness of ETX in alcohol abuse, the first attempt was made to characterize its effects on ethanol consumption in nondependent mice. A modest reduction in ethanol consumption was observed in the nondependent mice and importantly, ETX had no effect on consumption of the tastant used in the study. This was the first study to evaluate the effects of ETX on ethanol consumption, and our findings, although limited, suggest that future studies are worthy of consideration.

**ETX effects do not appear to be mediated through GABAergic synapses.** Lastly, studies have demonstrated that ETX can increase GABA (Greenhill et al., 2012; Lin-Michell et al., 1986; Ponnusamy and Pradhan, 2006). We tested if the pharmacological
actions of ETX would produce an anxiolytic effect in ethanol-naïve DBA/2J mice. The light/dark box and elevated plus maze produced anxiety-like behavior in all of the mice; however, ETX-treated mice did not differ from the saline-treated mice. These results indirectly suggest that the effects of ETX on ethanol withdrawal-induced seizure activity are mediated through T channels or another mechanism other than an effect on GABA. Importantly, in these behavioral studies, the ETX-treated mice did not have impaired motor function, which has been demonstrated as another negative side effect of benzodiazepine treatment (Ait-Daoud et al., 2006). Based on previous work and what is known of the role of T channel activity in the development of SWD activity, we suggest that the most parsimonious explanation of ETX effects on seizure activity is that they are mediated through T channels.

**Potential Mechanisms of Ethosuximide**

A significant contribution of this body of work has centered on the effectiveness of and potential for ETX as a treatment option for alcohol withdrawal seizure. As previously mentioned in Chapters 2-4, it is widely accepted that ETX is a non-selective inhibitor of T channels; however, off-target effects have been proposed, thereby creating controversy over the pharmacological mechanism of ETX (Huguenard, 2002). We consider the evidence below.

Controversy was initiated when studies failed to replicate the inhibitory effect of ETX on T channels at physiologically relevant concentrations (Leresche et al., 1998; Pfrieger et al., 1992; Sayer et al., 1993; Thompson and Wong, 1991; Todorovic and Lingle, 1998). In Leresche et al. (1998), recordings were conducted in ventrobasal and
lateral geniculate thalamic slices prepared from rats and cats. These investigators observed a reduction in the persistent sodium current and Ca\textsuperscript{2+}-activated potassium current by ETX. These results were suggested to be the reason for the observed decrease in burst firing and increase in tonic firing patterns in the thalamic neurons. Broicher et al. (2007) also observed a reduction in persistent sodium current in both epileptic and nonepileptic thalamic rat slices. It should be noted, that in both of these studies, high concentrations of ETX were required to achieve such effects and were above the physiologically relevant range.

A more recent study found an inhibitory effect of ETX on G protein-activated inwardly rectifying K\textsuperscript{+} (GIRK) channels (Kobayashi et al., 2009). At clinically relevant concentrations, ETX blocked the cardiac isoform (GIRK 1/4) expressed in Xenopus oocytes. However, higher concentrations of ETX were required to inhibit the brain isoforms (GIRK1/2 and GIRK2) expressed in Xenopus oocytes with the EC\textsubscript{50} above therapeutic relevance. At similar concentration levels, ETX also inhibited GIRK channels in cerebellar granule cells. One consideration of this finding is that partial inhibition of GIRK channels by ETX may also reduce T current as GIRK channels facilitate the generation of T current by hyperpolarizing the neuron which in turn deinactivates T channels (Crunelli and Leresche, 1991).

Although the effectiveness of ETX on ethanol withdrawal seizure could be solely mediated through these other membrane currents, it is less plausible as high concentrations of ETX (above physiological relevance) were necessary to affect these indirect targets. Discrepancies in the effect of ETX on T channels are most likely the result of different models and preparation techniques used. Although T channels can be
found on the cell body (McKay et al., 2006), they preferentially localize to dendrites (Perez-Reyes, 2003); thus, the proper preparation for recording T channel activity is necessary to ensure that T channels are present. Many of the studies were conducted on isolated neurons which had significant dendritic loss suggesting that the recordings may have been conducted in the absence of T channels (Destexhe et al., 1998). Also, findings that demonstrate that the action of ETX is state dependent could also contribute to the controversial findings (Gomora et al., 2001). Only partial inhibition by ETX on T channels can have a profound effect in vivo since these channels pace the firing of sodium dependent action potentials, which occur in an all-or-nothing mode (Huguenard and Prince, 1994; Narahashi, 2000).

ETX has also been shown to mechanistically act on GABA levels and GABAergic transmission. Recently, ETX (250-500 µM) was shown to increase GABA release via a presynaptic mechanism in the entorhinal cortex (Greenhill et al., 2012). Additional reports have indicated an increase in GABA levels in frontal cortical areas and whole brain levels of rodents (Lin-Michell et al., 1986; Ponnusamy and Pradhan, 2006). However, doses that increased GABA levels in mice were considered toxic doses (500mg/kg), and lower doses of ETX (150mg/kg) that were tested did not alter GABA levels (Lin-Michell et al., 1986). The only dose of ETX that increased GABA in the frontal cortex of mice was 250mg/kg, lower doses (100 and 200 mg/kg) did not produce an effect (Ponnusamy and Pradhan, 2006).

Despite these findings, there has been strong evidence suggesting that the primary target of ETX is T channels. Early investigations demonstrated that T current, isolated from thalamic neurons of rats and guinea pigs, was partially blocked by clinically
relevant concentrations of ETX (Coulter et al., 1989a, b; 1990a, b). Specifically, there was a 40% reduction in amplitude of T current. The steady-state properties and kinetics of the channels were not altered by ETX. It was also found that a similar analog to ETX blocked T current (Coulter et al., 1990a). Further investigation provided additional support with a reduction in T current amplitude by ETX in cultured dorsal root ganglion neurons (Kostyuk et al., 1992) and thalamic slices (Huguenard and Prince, 1994) from rats.

One of the most compelling pieces of evidence in favor of a T channel mechanism of ETX came from a study that transfected mammalian cells with cloned cDNAs, constituting the three human isoforms of T channels (Gomora et al., 2001). All three isoforms were antagonized by ETX. Differential binding by ETX was observed, and this effect could be manipulated by differing experimental conditions. For example, the sensitivity of ETX increased significantly in more depolarized conditions, which suggested that ETX may prevent thalamic neurons from switching from tonic to burst firing mode. In more depolarized conditions, T channels exist in an inactivated state, allowing the neuron to maintain a tonic firing pattern (Steriade and Llinas, 1988).

A similar finding was reported in a study evaluating the effects of ETX on T current in multiple rat strains (Broicher et al., 2007). Broicher et al. (2007) demonstrated that the effectiveness of ETX application was enhanced as stimulation frequency increased. The authors observed partial inhibition of T current in thalamic slices from both epileptic and nonepileptic rats; however, physiologically relevant concentrations of ETX were more effective in the epileptic rats whereas higher concentrations were
stronger in the nonepileptic rats. The authors suggested that this effect could contribute to the ability of ETX to effectively inhibit SWDs.

Another important feature of T channels is the window current that persists at resting membrane potentials when the channels fail to inactivate completely (Crunelli et al., 2006; Gomora et al., 2001; Hughes et al., 1999; Lee et al., 1999; Perez-Reyes, 2003; Williams et al., 1997). This T-type window current can greatly influence both intracellular calcium levels and the excitation of neurons (Assandri et al., 1999; Chemin et al., 2000; Crunelli et al., 2006; Crunelli et al., 2005; Graef et al., 2011; Mariot et al., 2002; Perez-Reyes, 2003; Williams et al., 1997). Gomora et al. (2001) also demonstrated that ETX reduced window T current by ~30%. The results of this study suggested that ETX was physically blocking the pore. These findings are relevant to this dissertation as there was an increase by ~30% in T-type window current observed during ethanol withdrawal (Graef et al., 2011). These findings could relate to the potential mechanism of ETX on ethanol withdrawal hyperexcitability.

As described in Chapter 3, we predicted that if ETX increased GABAergic signaling, it would have an anxiolytic effect and possibly motor impairing effects. Drugs that enhance GABAergic transmission, such as benzodiazepines, are anxiolytic and can cause motor impairment. We therefore chose the highest dose of ETX (250 mg/kg) tested in our studies, which had also been previously shown to increase GABA (Ponnusamy and Pradhan, 2006). Contrary to our prediction, acute treatment of ETX (250mg/kg) did not reduce anxiety-like behavior or impair locomotor activity in ethanol-naïve mice (Riegle et al., 2014, submitted for publication).
It should be noted that other studies have reported inconsistent results with acute ETX treatment on anxiety-like behavior (Shaw et al., 2009; Simiand et al., 1984). These results are most likely due to the differences in animal models and methods for detecting anxiety-like behavior. In an open-field test, chronic treatment with ETX reduced anxiety-like behavior in the GAERS model (Dezsi et al., 2013). These conclusions were made based on an increase in number of entries made into the center and an increase in distances traveled. To date, whether or not ETX affects the GABA system, and the mechanism by which it might do so, remains elusive.

In our studies presented here, the doses of ETX were selected based on a previous investigation evaluating ETX in mice undergoing withdrawal (Kaneto et al., 1986) and its use in studies of absence seizures (Aizawa et al., 1997; Dezsi et al., 2013; Frankel et al., 2005; Marrosu et al., 2007; Nissinen and Pitkanen, 2007). We determined that ETX dose-dependently reduced ethanol withdrawal-induced SWDs with an estimated ED$_{50}$ of ~96.65mg/kg in DBA/2J mice. We also demonstrated a dose-response effect with ETX on ethanol withdrawal PTZ-induced seizures. We only tested two doses of ETX in this model (100 and 250mg/kg) and were therefore unable to calculate the ED$_{50}$. We can speculate that the ED$_{50}$ in this model would be comparable to other ED$_{50}$ values of ETX identified in epilepsy models (Bialer et al., 2004; Swiader et al., 2006).

In vitro concentrations of ETX between 250 µM – 750 µM are within the relevant therapeutic range (Crunelli and Leresche, 2002; Kobayashi et al., 2009; Leresche et al., 1998; Sherwin, 1989). These values have been compared to the plasma levels of ETX which occur between ~0.125-1.0 mmol/L (~20-140 µg/mL) in the clinically therapeutic window (Huguenard, 2002; Sherwin, 1989). We did not test the plasma and brain levels
of ETX in our mice undergoing ethanol withdrawal. However, based on other studies we would expect them to be in the therapeutic range (Bialer et al., 2004; el Sayed et al., 1978; Swiader et al., 2003; Swiader et al., 2006). We cannot exclude the possibility that ethanol exposure altered the metabolism of ETX. Further evaluation of this possibility is an important consideration if ETX is used clinically for the treatment of alcohol abuse and withdrawal.

Doses found to be toxic (>500 mg/kg) (Swiader et al., 2003) were not used in our studies. In our studies we did not observe any abnormal side effects as a result of ETX treatment and determined that the highest dose tested (250mg/kg) did not impair motor function. The effective dose of ETX can vary greatly in humans as it does in nonprimate models as well. As previously mentioned, the ED$_{50}$ of ETX in other models of seizure activity has been highly dependent on the species of animal and the test. Doses effective in our studies, however, are similar to effective doses in other models and presumably fall within a relevant pharmacological range. Because of this, we can speculate that the effects of ETX are mediated through T channels, as much higher doses were necessary to engage off target effects.

Perhaps most relevant to this dissertation is that ETX has been shown to reduce SWD events in numerous models and is the first line treatment for absence epilepsy (Goren and Onat, 2007). T channels, within thalamic circuitry, drive this abnormal, synchronized activity (Crunelli et al., 1989; Huguenard and Prince, 1992; Jeanmonod et al., 1996; Porcello et al., 2003; Suzuki and Rogawski, 1989). We have now also demonstrated that ETX can inhibit ethanol withdrawal-induced SWDs. Thus, we suggest that ETX disrupts the synchronized activity by inhibiting T channel function which
mediates such aberrant activity. It will be important to determine the therapeutic mechanism of ETX on ethanol withdrawal seizure as it could lead to more specific compounds and could facilitate our understanding of mechanisms involved in withdrawal hyperexcitability.

The role of T channels in alcohol withdrawal seizure

T channels initiate low threshold calcium spikes which facilitate the ability of neurons to switch from tonic to burst firing modes (Crunelli et al., 1989; Huguenard and Prince, 1992; Jeanmonod et al., 1996; Suzuki and Rogawski, 1989). Circuitry within the thalamus and its connectivity to other regions can sustain both normal and abnormal rhythmic activity mediated by T current (Huguenard, 1999; Huguenard and Prince, 1994; Jeanmonod et al., 1996; Llinas and Steriade, 2006; McCormick and Bal, 1997; Steriade, 2005; Steriade et al., 1993; von Krosigk et al., 1993). This type of abnormal activity has been characterized as absence-like epileptiform activity and has been identified in animal models of absence epilepsy and individuals with this disorder (Blumenfeld, 2003; Coenen and van Luijtelaar, 2003; Huguenard, 1999; Marescaux et al., 1992).

Both clinical and animal reports have clearly established the presence of abnormal oscillatory activity during alcohol withdrawal (Fernandez-Torre and Martinez-Martinez, 2007; LaRoche and Shivdat-Nanhoe, 2011; Riegle et al., 2014; Veatch and Becker, 2002; Walker and Zornetzer, 1974). This activity described during withdrawal is similar to the activity characterized in absence epilepsy. Also, it is known that T channel expression and function are disrupted by chronic ethanol exposure and withdrawal, and it was hypothesized that enhanced T channel expression and function could contribute to
hyperexcitability during withdrawal (Graef et al., 2011; Nordskog et al., 2006; Welsh et al., 2011). Enhanced T channel expression and function has also been identified in the hippocampus and thalamus of a tonic/clonic seizure model (Becker et al., 2008; Graef et al., 2009; Su et al., 2002) and our data suggest that ETX can reduce tonic/clonic seizure during ethanol withdrawal. The combination of these findings and the similarity in abnormal oscillatory activity that is mediated by T channel function during ethanol withdrawal provides a strong rationale to speculate that enhanced T channel activity contributes to ethanol withdrawal seizure activity.

The role of T channels in alcohol dependence

A disruption in T channel expression and function has been identified in chronic ethanol exposure models (Carden et al., 2006; Nordskog et al., 2006; Welsh et al., 2011). These studies were reviewed in Chapter 1. Briefly, chronic ethanol exposure can cause brain region specific differences in effects on T channel activity. Relevant to our studies, Nordskog et al. (2006) observed an upregulation in CaV3.2 and CaV3.3 expression after chronic ethanol exposure, and Welsh et al. (2011) found an upregulation in T current in primates after chronic self-administration. Similar alterations have been observed in other calcium channels (Walter and Messing, 1999). It was proposed that L-type calcium channels may enhance ethanol intake. Further support for this hypothesis was established in multiple animal studies that demonstrated a decrease in ethanol consumption after treatment with L-type calcium channel antagonists (De Beun et al., 1996; Fadda et al., 1992; Gardell et al., 1997; Pucilowski et al., 1992; Rezvani et al., 1991; Rezvani and Janowsky, 1990).
In chapter 3, we demonstrated that ETX modestly reduced ethanol consumption in nondependent DBA/2J mice. This was the first attempt to characterize the effects of ETX on ethanol intake. Additional support for these findings was demonstrated in an investigation that evaluated NP078585, a mixed N- and T-type calcium channel antagonist (Newton et al., 2008). In this report, NP078585 reduced ethanol seeking behavior, ethanol conditioned place preference, and ethanol self-administration. The authors of the report suggested that the effects were mediated through N-type calcium channels because there were no effects of the drug in mice lacking N-type calcium channels in the loss of righting-reflex paradigm. While T channels may not be involved in the loss of righting-reflex, these channels cannot be excluded from the other effects.

Zonisamide, which can block T channels, is an anticonvulsant that has also been shown to reduce alcohol consumption in clinical studies (Arias et al., 2010; Knapp et al., 2010; Rubio et al., 2010; Sarid-Segal et al., 2009). Considering these findings and the fact that previous reports demonstrated that chronic ethanol consumption alters T channel expression and function, it is possible that T channels may contribute to alcohol dependence. This hypothesis requires more extensive investigation as it should be noted that our study evaluated nondependent mice. Future studies utilizing models of ethanol dependence are necessary.

The exact role of T channels in alcohol addiction remains elusive. As mentioned earlier, the effects of ETX may be partially due to its effects on GIRK channels. ETX reduced GIRK-mediated currents induced by ethanol (Kobayashi et al., 2009), and GIRK knockout mice demonstrate less ethanol withdrawal-induced symptoms and have decreased conditioned place preference (Hill et al., 2003). Thus, Kobayashi et al. (2009)
suggested that these GIRK-mediated effects due to ethanol may be inhibited by ETX. This hypothesis still needs to be tested and a T channel dependent mechanism cannot be ruled out as GIRK channels facilitate T current (Crunelli and Leresche, 1991). More studies utilizing dependent animals will help verify the efficacy of ETX, and further investigation is necessary to determine its mechanism of action and the role of T channels in alcohol dependence.

Contrary to these findings, a recent report suggested that increases in calcium may be important for the treatment of alcoholism (Spanagel et al., 2014). Specifically, the investigators suggested that the biologically active ingredient of acamprosate, a clinically approved treatment for alcohol addiction (Heilig and Egli, 2006), was calcium (Spanagel et al., 2014). Spanagel et al. (2014) proffered that the effects of acamprosate and increased levels of calcium may activate calcium-activated potassium channels as these have been implicated in alcohol consumption and hyperactivity (Hopf et al., 2011; Mulholland et al., 2011). Despite these findings, acamprosate treatment has lacked efficacy (Kenna et al., 2004), and despite treatment, up to 70% of individuals relapse within one year and resume excessive drinking (Finney et al., 1996). Thus, the efficacy of a calcium treatment for alcohol addiction and withdrawal requires much needed investigation as there is strong evidence suggesting that calcium channel inhibitors can reduce both ethanol consumption and withdrawal symptoms.

The role of the thalamus in alcohol dependence and withdrawal

In the introduction, we reviewed the hypothesis that was first introduced by Walker and Zornetzer (1974) that suggested the thalamus could serve as an organizing
hub for ethanol withdrawal seizure activity. In our investigations, we clearly established that ethanol withdrawal increased the number and duration of SWD activity in a way that is consistent with thalamic involvement. Using a different mouse strain, similar findings were demonstrated by an increase in brief spindle episodes during ethanol withdrawal (Veatch and Becker, 2002). Although there might be independent mechanisms involved in these nonconvulsive seizures, they have similar electrophysiological signatures and are both hypothesized to involve thalamic circuitry. Such findings support the original hypothesis generated by Walker and Zornetzer (1974) and suggest thalamic involvement in ethanol withdrawal. Few studies have recorded from thalamic circuitry and investigated the role of the thalamus during ethanol withdrawal. The majority of studies have focused on other areas such as the amygdala and hippocampus. However, there is clear evidence for the potential role for neuroadaptions within the thalamus and thalamic circuitry in alcohol dependence and withdrawal suggesting more attention is warranted.

Taken together, enhanced T channel expression and function during chronic exposure and withdrawal in thalamic neurons (Graef et al., 2011; Nordskog et al., 2006), ETX mediated reduction of withdrawal-induced sleep disruptions (Wiggins et al., 2013), and the current data demonstrating that ETX reduced ethanol withdrawal seizure all provide further implication for the role of the thalamus in alcohol withdrawal hyperexcitability. In addition, in Chapter 1, we proposed that this abnormal oscillatory activity identified in the thalamus could precipitate more long-term severe consequences and may be a trigger for tonic/clonic seizure activity. This hypothesis still needs to be tested. However, in models of temporal lobe epilepsy, midline thalamic neurons are key participants in the generation and propagation of seizures (Bertram, 2009; Bertram et al.,
2008). Enhanced T channel expression and function within midline thalamic nuclei were also identified in a temporal lobe epilepsy model (Graef et al., 2009).

Additional neuroadaptations in GABA\textsubscript{A} (Choi et al., 2008; Jia et al., 2008), NMDA (Criswell et al., 2003; Gulya et al., 1991) and nicotinic (Booker and Collins, 1997; Yoshida et al., 1982) receptors have been identified in the thalamus as a result of ethanol exposure. Recently, an increase in glutamate release via a presynaptic mechanism was identified in the basolateral amygdala during ethanol withdrawal (Christian et al., 2013). This increase was found to be mediated by thalamic afferents into the internal capsule. The investigators suggested that this increased excitation may in part contribute to anxiety-like behavior observed during ethanol withdrawal. Thus, not only is there a role for the thalamus and its circuitry with other brain regions in the development of withdrawal-induced seizure activity and sleep disruptions, it could also contribute to other withdrawal symptoms such as anxiety as well. These additional findings support the need to continue evaluating changes within thalamic circuitry as well as its connections with other brain regions as it will help our overall understanding of the processes that persist during ethanol exposure and withdrawal.

**Additional Considerations**

**Do alcohol withdrawal seizures relate to other symptoms?**

We have discussed in great detail the consequences of alcohol withdrawal seizure activity as well as the possibility of early nonconvulsive-like activity precipitating future, more severe seizures and long-term consequences. We have yet to discuss how such
early abnormal oscillatory activity could relate to other symptoms of alcohol abuse and withdrawal.

In Chapters 2 and 3, we briefly reported on case studies that have described the presence of nonconvulsive seizure activity in patients undergoing alcohol withdrawal (Fernandez-Torre and Martinez-Martinez, 2007; LaRoche and Shivdat-Nanhoe, 2011). Additional evidence has suggested that alcohol abuse and withdrawal is an underlying source for the late onset of nonconvulsive seizure or absence-like epilepsy (Kaplan, 1996; Thomas et al., 1992). Not only do these studies provide support for the use of these models and the necessity for more EEG analyses in both clinical and animal investigations, these case studies also suggest that aberrant oscillatory activity may be a potential source for symptoms such as confusion and cognitive impairment.

For example, a patient undergoing alcohol withdrawal was reported to have both confusion and disorientation (Fernandez-Torre and Martinez-Martinez, 2007). Evaluation of EEG activity identified SWD activity occurring in tandem with the altered mental state. After treatment was administered to alleviate the epileptiform activity, the mental state improved. The patient was no longer confused or disoriented when the SWDs were absent from the EEG recording. While this is circumstantial evidence and these symptoms could be unrelated and independent of each other, the authors suggest a relationship that should be considered in future investigations. If there is such a relationship, this type of seizure activity during alcohol withdrawal may be under recognized as continuous EEG monitoring is required. Also, the underlying source of altered mental state and confusion may be commonly misdiagnosed.
As described in Chapter 1, alcohol withdrawal seizure involves numerous brain regions that are interconnected including the thalamus, amygdala, hippocampus, and brain stem. Learning and memory, key functional aspects of the hippocampus, are clearly disrupted by alcohol (White et al., 2000). Although it is still unknown, similar underlying circuitry, and perhaps a common source of disruption, suggests that there may be a direct link between cognitive deficits (such as confusion) and alcohol withdrawal seizure.

Animal and clinical studies related to different epilepsies also provide support for a link. Multiple studies have demonstrated that individuals with absence epilepsy (a nonconvulsive seizure type) have cognitive impairments and learning deficits (Blumenfeld, 2005; Loughman et al., 2014). Memory and cognitive deficits are common features in temporal lobe epilepsy as well (Bell et al., 2011; Stretton and Thompson, 2012).

T channels are disrupted during ethanol exposure and withdrawal (Carden et al., 2006; Graef et al., 2011; Nordskog et al., 2006; Welsh et al., 2011), in absence epilepsy (Chen et al., 2003; Cheong and Shin, 2013; Huguenard, 1999; Khosravani et al., 2004; Kim et al., 2001; Song et al., 2004; Tsakiridou et al., 1995; Zhang et al., 2013), and in models of temporal lobe epilepsy (Becker et al., 2008; Graef et al., 2009). The same adaptation underlying these seizures may also be a source for cognitive disruption as well. Welsh et.al (2011) suggested that the disruption in T current after chronic self-administration in the inferior olive of monkeys could contribute to cognitive disruption as the neurons and pacemaking features of the inferior olive are involved in learning, cognition, and integration of such features.
With this implication, it might be problematic to treat alcohol withdrawal with anticonvulsants as many antiepileptic agents can cause cognitive impairment (Ijff and Aldenkamp, 2013), which has also been a concern of the use of benzodiazepines in the treatment of alcohol withdrawal (Ait-Daoud et al., 2006). In a recent review, ETX was one of few anticonvulsants that did not significantly impair cognition (Ijff and Aldenkamp, 2013). If there is such a link, ETX treatment may alleviate some of the cognitive impairments by blocking alcohol withdrawal-induced seizures without major side effects.

**The Model**

The intermittent exposure paradigm and the vapor chamber used in these studies has been well characterized (Becker and Hale, 1993; Goldstein and Pal, 1971). Multiple clinical studies have demonstrated that as individuals undergo more detoxification, withdrawal symptoms including seizures progressively worsen (Ballenger and Post, 1978; Brown et al., 1988; Lechtenberg and Worner, 1990; Moak and Anton, 1996). This intermittent model (Figure 1) adapted from Becker and Hale (1993) has been used successfully to replicate the kindling-like phenomenon observed in clinical studies (Becker, 1994; Becker, 1998; Becker et al., 1997a; Becker et al., 1997b; Veatch and Becker, 2002). We established the same results utilizing this paradigm in DBA/2J mice (Riegle et al., 2014).
Figure 1. Schematic of intermittent ethanol exposure paradigm used in the studies of this dissertation. a, am; p, pm; WD, withdrawal
The paradigm used in all of the experiments evaluating ethanol withdrawal-induced seizure in this dissertation consisted of four exposures (16 hours) and four withdrawal periods (8 hours). In some of the studies, the fourth withdrawal period was extended.

While it was important to use a model that mimics what is observed clinically, alcoholism is a multifaceted, heterogeneous disease which requires multiple models. It can be challenging to develop models that account for the observed variability in duration of alcohol exposure, amount of alcohol consumed, and the number of detoxifications between individuals (Duka et al., 2004). Although we tested three different types of ethanol withdrawal-induced seizures, we only used one method of ethanol exposure. It will be necessary to evaluate how ETX impacts more chronic paradigms including self-administration, and whether it can affect kindling mechanisms in these alternative model systems.

All of the studies described in this body of work used DBA/2J mice. DBA/2J mice are considered seizure-prone animals (Ferraro et al., 2007) and are susceptible to audiogenic seizures early in development (~3-5 weeks old) (Hall, 1947; Jawahar et al., 2011). It can be argued that these mice do not represent a baseline population similar to non-drinking humans because they have a basal level of SWD activity (Marrosu et al., 2007; Reid et al., 2011; Riegle et al., 2014). It has been well established however that individuals abusing alcohol will experience multiple withdrawals and consequently be at greater risk for seizure activity and more “seizure-prone”. Perhaps the differences in seizure susceptibility in different strains of mice underlie the variation observed in the population as not all individuals undergoing detoxification will develop seizures. These
types of genetic comparisons are under investigation (Crabbe, 2013) and will facilitate better understanding of the models used. It will be necessary to test the effectiveness of ETX in other strains of mice and additional models with rats and primates; however, preliminary data from our lab suggests it can be effective in Thy1 mice that have originated from a C57Bl/6 line.

To date, the models of alcohol withdrawal seizure are limited. It is difficult to expose animals with enough ethanol to produce spontaneous seizures during withdrawal as would be seen in an individual undergoing alcohol withdrawal. Unfortunately, as we observed in our lab, inducing a withdrawal period that causes spontaneous seizures will often result in death. As discussed in Chapter 1, to date, there have been relatively few studies that have evaluated spontaneous ethanol withdrawal seizure. Spontaneous seizures are unpredictable and difficult to monitor, thus making it very challenging to test therapeutics (N'Gouemo and Rogawski, 2006). Despite these challenges, the continued evaluation of different animal models is necessary to improve our overall understanding of alcohol withdrawal seizure and is essential for better treatment development.

*Alcohol dependence and withdrawal beyond disruption to T-type calcium channels*

The main focus of this dissertation has been directed toward the role of T channels during exposure and withdrawal and their contribution to the development of withdrawal seizures. This was demonstrated in Chapters 2-4 by the inhibition of withdrawal-induced seizure activity with ETX treatment. While the body of this work significantly contributes to our understanding, it is important to note that the disruptions and rewiring of the brain resulting in alcohol dependence and withdrawal are complex. It
would be negligent to not believe that multiple systems beyond T channels contribute to the development of dependence and withdrawal and that these disruptions are brain region specific.

It is beyond the scope of this discussion to review all of the disruptions that result from alcohol exposure and withdrawal as there are many; however, we highlight a few significant contributors that have been identified. Importantly, as we discussed with the interaction of alcohol on T-type calcium channels, acute and chronic alcohol exposure can result in opposing effects on inhibitory and excitatory systems which can ultimately disrupt the homeostatic balance between such communication (Deitrich et al., 1989; Faingold et al., 1998). Thus, there appears to be an overall increase in inhibition observed during exposure and with ensuing compensatory mechanisms, an increase in excitation persists during withdrawal mediated by multiple mechanisms and interactions between ethanol and many neuronal proteins.

Complex interactions between ethanol and GABA neurotransmission has been well-studied and identified as an important target. Ethanol exposure enhances GABAergic tone, however, the mechanism of such effect varies and is brain region specific (Weiner and Valenzuela, 2006; Faingold et al., 1998; Deitrich et al., 1989). Such findings have linked GABA to the reinforcing effects of ethanol (Koob, 2003). During withdrawal, compensatory mechanisms occur decreasing GABAergic tone (Faingold et al., 1998; Koob, 2003; Weiner and Valenzuela, 2006). Such changes have been hypothesized to contribute to the increased hyperexcitable state during withdrawal including seizures (Veatch and Becker, 2005; Faingold et al., 1998).
The glutamatergic system has also been implicated in the development of alcohol addiction (Koob et al., 1998; Faingold et al., 1998; Krystal et al., 2003). As with other systems, glutamatergic neurotransmission is not immune to the effects of ethanol. Contributing to the overall increase in inhibition during exposure, ethanol blocks NMDA receptors (Krystal et al., 2003; Faingold et al., 1998; Lovinger et al, 1989). During withdrawal, however, enhanced NMDA function has been identified which can facilitate a hyperexcitable state (Floyd et al., 2003; Faingold et al., 1998). These compensatory mechanisms have been suggested to contribute to withdrawal-induced anxiety and seizure activity (Floyd et al., 2003; Veatch and Becker 2005; Faingold et al., 1998).

Ethanol exposure increases dopamine activity within the ventral tegmental area and nucleus accumbens, which are key nodes in the reward circuit (Brodie et al, 1990; Brodie and Appel, 1998; Brodie, 2002; Weiss et al., 1993). This is of particular interest to this dissertation because the ventral tegmental area sends projections to thalamic nuclei (McKenna and Vertes, 2004); however, it is unknown if these changes in the dopaminergic pathway contributes to the T channel upregulation observed after chronic exposure and withdrawal. In addition neuronal plasticity has been described within the amygdala that may have strong implications for vulnerability to addiction, specifically alcohol dependence (Koob, 1998; Francesconi et al., 2009). Impaired dopaminergic neurotransmission during withdrawal may contribute to the disruption within the amygdala and contribute to a negative affective state observed during withdrawal. As with other systems, alcohol has complex effects on dopamine. Alcohol increases dopamine levels, which contribute to the reinforcing effects of alcohol, and during withdrawal, the opposite occurs with hypodopaminergic activity that can precipitate a
negative state observed during withdrawal (Weiss et al., 1993; Weiss et al., 1996; Koob et al., 1998; Koob, 2003).

Ethanol-mediated changes to ion channels also contribute to the imbalance between inhibition and excitation. We reviewed ethanol-mediated alterations to both high and low threshold calcium channels in Chapter 1. Thus, we will not discuss these findings now, but highlight other ion channels that may contribute to the imbalance in inhibition and excitation. Both small and large conductance calcium-activated potassium channels are altered by ethanol exposure in a complex manner that fits the pattern observed with other systems (Dreixler et al., 2000; Hopf et al., 2007; Dopico et al., 1996; Martin et al., 2004; Knott et al., 2002). Such interactions contribute to the inhibitory effects of ethanol exposure and increased excitability during withdrawal. Interestingly, T-type calcium channels can activate small conductance calcium-activated potassium channels (Cueni et al., 2008), however, the effects of this interaction during ethanol exposure and withdrawal is unknown. Also, acute ethanol enhances the function of hyperpolarization-activated, cyclic-nucleotide gated (HCN) channels (Brodie and Apel 1998) which contributes to the increased inhibition mediated by ethanol. During ethanol withdrawal, HCN-mediated current is downregulated (Okamoto et al., 2006; Hopf et al., 2007). Recently, interactions between HCN and T channels were identified at the presynaptic terminals of pyramidal neurons in the entorhinal cortex which were found to influence synaptic plasticity (Huang et al., 2011). These interactions could be relevant to the mechanisms responsible for ethanol-mediated changes in neuronal plasticity. Such hypotheses still need to be tested.
Taken together, these studies clearly indicate that multiple mechanisms contribute to the imbalance in inhibition and excitation during ethanol exposure and withdrawal. It is apparent that alterations to multiple proteins play a role in rewiring the brain that eventually leads to a dependent state. Ultimately all of these systems are likely to contribute to withdrawal symptoms including seizures observed when an individual undergoes detoxification. With the complexity of such changes, it remains to be determined how T channels interact with these other neuronal proteins during dependence and withdrawal. We have just begun to scratch the surface of the role of T channels and the complexity of all these different neuronal proteins during alcohol exposure and withdrawal.

**Future Directions**

Our findings have initiated a plethora of avenues to explore. First, further investigation is necessary to solidify the treatment potential of ETX in alcohol dependence and withdrawal. Specifically, studies evaluating other withdrawal symptoms such as alcohol withdrawal-induced anxiety are necessary to determine the ability of ETX to treat a range of withdrawal-induced symptoms. These investigations are currently in progress. As previously mentioned, ETX did not reduce anxiety-like behavior in ethanol-naïve mice. If we do observe a reduction in withdrawal-induced anxiety-like behavior after ETX treatment, it would provide further implication for a T channel-mediated mechanism in alcohol withdrawal hyperexcitability.

Our investigation of ETX on ethanol consumption was conducted in nondependent mice. This was the first assessment of ETX in an ethanol drinking model.
It will be important to determine if ETX treatment can reduce the escalation of ethanol consumption associated with dependence. Also evaluating the effects of ETX in models of the rewarding and reinforcing properties of ethanol will be necessary. Specific models that evaluate such behavior include conditioned place preference and stress-induced reinstatement.

Lastly, evaluating chronic treatment with ETX and its long-term effects on susceptibility of alcohol withdrawal symptoms would provide valuable information on many levels. The relative contribution of early changes and symptoms including seizures to long-term consequences is unknown. Understanding the early neurobiological adaptations underlying alcohol withdrawal and whether pharmacological intervention with treatments such as ETX can prevent negative outcomes still needs further investigation.

While there is strong evidence suggesting that the effects of ETX are mediated through T channels, further investigation is necessary. Identifying the exact mechanism of therapeutic efficacy is necessary to the development of more specific compounds. It still remains to be determined if T channels are disrupted in other brain regions beyond the thalamus and inferior olive during alcohol exposure and withdrawal. We could speculate that an increase in T channel expression and function would be present during withdrawal in areas such as the amygdala, hippocampus, and inferior colliculus as these are all critical areas involved in alcohol withdrawal seizure and other alcohol withdrawal symptoms.

Finally, previous work and the results of these dissertation studies underscore the importance of investigating the mechanisms responsible for T-channel disruption during
withdrawal. As previously mentioned, the CaV3.2 T channel was the isoform affected by ethanol and ethanol withdrawal (Graef et al., 2011; Shan et al., 2013). Interestingly, CaV3.2 undergoes greater regulatory and modulatory control compared to the other isoforms. Several different intracellular pathways, reducing agents, oxidizing agents, and hormones can selectively modulate CaV3.2 activity (Huc et al., 2009; Iftinca and Zamponi, 2009). Protein kinases can affect CaV3.2, and of particular interest is protein kinase C (PKC) (Huc et al., 2009; Iftinca and Zamponi, 2009). Numerous studies report PKC-mediated effects on CaV3.2, however, the results are somewhat conflicting (Kim et al., 2007; Park et al., 2003; Park et al., 2006; Rangel et al., 2010) and the influence of PKC on T current during ethanol withdrawal is unknown.

A recent investigation in our lab has, however, determined that the inhibition of acutely administered ethanol on CaV3.2 was mediated by PKC (Shan et al., 2013). Also, PKC activity was found to be responsible for the observed disruptions of other voltage-gated calcium channels during chronic ethanol exposure and withdrawal (Gerstein et al., 1997; McMahon et al., 2000; Walter and Messing, 1999). It is known that PKC activity is altered during withdrawal and has been shown to mediate withdrawal symptoms (Dina et al., 2006; Jung et al., 2005; Newton and Ron, 2007; Olive et al., 2001). Taken together, this may suggest that PKC dysregulation during ethanol exposure may be responsible for the observed T channel effects and could be responsible for the rebound excitability caused by increased T channel function during withdrawal. The effects of ethanol exposure and withdrawal on PKC, however, appear to be brain region and isozyme specific and highly dependent on the model of exposure (Newton and Ron,
With this gap in our knowledge and previous work identifying a disruption in T-current during withdrawal, investigations are warranted.

Other mechanisms related to transcriptional regulation of T channels could serve as a possible source for T channel dysregulation during ethanol exposure and withdrawal. The transcription factor, neuronal restrictive silencing factor (NRSF), restricts the transcription of several genes, including the gene coding Caᵥ3.2, by binding upstream to a neuronal restrictive silencing element (NRSE) sequence (Kuwahara et al., 2005; van Loo et al., 2012). van Loo et al. (2012) further demonstrated a relationship between NRSF and the early growth response 1 (Egr1) transcription factor on regulation of the Caᵥ3.2 isoform and implicated that such regulatory mechanisms could contribute to pathological conditions such as epilepsy and cardiac arrhythmias. Specifically, these investigators found that Egr1 enhances Caᵥ3.2 activation by binding to the Caᵥ3.2 promoter, which could be counteracted by NRSF.

Under pathological conditions in other tissues such as those leading to cardiac hypertrophy, NRSF expression is inhibited resulting in increased expression of NRSE-containing genes, such as the gene coding Caᵥ3.2 (Yasui et al., 2005). Evidence from a kindling model of seizure activity suggests that inhibition of NRSF can accelerate kindling and enhance seizure (Hu et al., 2011). There have been mixed reports on the effects of ethanol on NRSF and its binding activity (Cai et al., 2011; Ishii et al., 2008; Qiang et al., 2005; Tateno et al., 2006). To date, its role in the development of alcohol withdrawal hyperexcitability remains to be determined. Preliminary data collected in our lab suggests that NRSF protein expression is decreased during ethanol withdrawal. Further, it has been demonstrated that ethanol withdrawal increases Egr1 expression and
binding activity during ethanol withdrawal (Beckmann et al., 1997; Matsumoto et al.,
1993). One possible mechanism could be a down regulation in NRSF and an
upregulation in Erg1 that results in increased expression of the CaV3.2 isoform, which
could then facilitate a hyperexcitable state during withdrawal.

These are unanswered questions that require further investigation including the
specific impact NRSF and Erg1 have on CaV3.2 expression in pathological brain tissue.
We also cannot exclude other factors that may contribute to the dysregulation of T
channels during ethanol exposure and withdrawal as there are numerous possibilities.
While T channels are presented in this dissertation as a fruitful and therapeutically useful
target for study, the precise mechanism of T channel dysregulation during chronic
exposure and withdrawal remains unknown.

Conclusions

Alcohol is the third highest risk factor for health related problems in the world
and leads to over two million deaths per year (World Health Organization, 2011).
Individuals abusing alcohol frequently cycle between drinking and withdrawal states,
which among other adverse side effects can induce withdrawal seizures. Alcohol
withdrawal seizures are a major component of relapse and represent a significant barrier
to recovery. We have demonstrated that ETX inhibits ethanol withdrawal seizures in
three different models. In addition, we were the first to test its ability to reduce ethanol
consumption which demonstrated promise. Lastly, ETX prevented ethanol withdrawal-
induced mortality. ETX is as advantageous as it is safe; it is already used clinically for
childhood epilepsy and does not have a strong potential for abuse, which has been a
major limitation of currently used treatments. We hope that these studies will lead to a clinical trial in the near future.
References


APPENDIX

ACTIVATION OF GROUP 2 METABOTROPIC GLUTAMATE RECEPTORS REDUCES BEHAVIORAL AND ELECTROGRAPHIC CORRELATES OF PILOCARPINE INDUCED STATUS EPILEPTICUS

Erin H. Caulder*, Melissa A. Riegle*, and Dwayne W. Godwin

* Co-first authors

This manuscript was published in Epilepsy Research (2014), volume 108, pages 171-181 and is reprinted with permission. The experiments were preformed by E. H. Caulder and M.A. Riegle. The manuscript was written and prepared by E. H. Caulder and M. A. Riegle. Dr. Dwayne W. Godwin provided mentorship and editorial feedback.
Summary

Novel treatments for epilepsy are necessary because many epilepsy patients are resistant to medication. Metabotropic glutamate receptors (mGluRs), specifically mGluR 2 and 3, may serve as antiepileptic drug targets because of their role in controlling synaptic release. In this study, we administered a Group 2 mGluR agonist, LY379268, one of two mGluR2-specific positive allosteric modulators, BINA or CBiPES, or a cocktail of both BINA and LY379268 in a series of experiments using the pilocarpine model of SE. In one study, groups received treatments 15 min prior to pilocarpine, while in a second study groups received treatments after SE had been initiated to determine whether the drugs could reduce development and progression of SE. We measured bouts of stage 5 seizures, latency to the first seizure, and the maximum Racine score to characterize the seizure severity. We analyzed mouse EEG with implanted electrodes using a power analysis. We found that pretreatment and posttreatment with LY379268 was effective at reducing both behavioral correlates and power in EEG bandwidths associated with seizure, while CBiPES was less effective and BINA was ineffective. These data generally support continued development of mGluR2 pharmacology for novel antiepileptic drugs, though further study with additional drugs and concentrations will be necessary.
Introduction

Temporal lobe epilepsy (TLE) is a chronic condition characterized by recurrent seizures that involve the medial or lateral temporal lobe. Antiepileptic drugs (AEDs) can be effective, but nearly 30% of patients are refractory to AEDs, and some medications possess negative side effects that reduce patient compliance. There is also a wide range of individual responsiveness to AEDs, therefore the development of novel pharmacological targets remains an important goal.

The pilocarpine model of TLE mimics the process of epileptogenesis, and possesses many characteristics of the human disorder. Pilocarpine administration results in an acute period of status epilepticus (SE) that is defined by continuous seizure activity lasting at least 30 min. After the initial period of SE, there is a “latent period” during which significant neural reorganization occurs followed by chronic lifelong susceptibility to spontaneous, recurrent seizures (Cavalheiro et al., 1996; Curia et al., 2008; Müller et al., 2009; Perez-Mendes et al., 2011; Turski et al., 1989, 1984, 1983). The maintenance and generalization of SE and the development of spontaneous recurrent seizures (SRS) is thought to occur through hyperglutamatergic activity via NMDA receptors in the hippocampus (Nagao et al., 1996; Priel and Albuquerque, 2002; Smolders et al., 1997). Therefore, pilocarpine administration in wild-type mice provides the opportunity to assess novel therapies that interfere with excessive glutamate signaling.

A potential target for such novel therapies are the Group 2 metabotropic glutamate receptors (mGluRs), comprising mGluR2 and mGluR3 (Alexander and Godwin, 2006a; Moldrich et al., 2003). Unlike ionotropic glutamate receptors, mGluRs do not transmit fast synaptic responses (Conn, 2003). mGluRs tend to produce longer
lasting effects than ionotropic glutamate receptors due to their G-protein involvement (Conn and Pin, 1997). The Group 2 mGluRs are coupled to the G_{i/o} protein and may inhibit glutamate release via inhibition of high threshold calcium channels, activation of potassium channels and/or by inhibition of neurotransmitter release (Anwyl, 1999; Cochilla and Alford, 1998; Scanziani et al., 1995; Takahashi et al., 1996). In particular, mGluR2 appears to be exclusively positioned outside of the active zone of synapses where it may only be activated during high frequency neuronal activity (Alexander and Godwin, 2005, 2006b; Cartmell and Schoepp, 2000; Knöpfel and Uusisaari, 2008; Shigemoto et al., 1997), similar to that which occurs during SE (Blumenfeld et al., 2009; Chen and Wasterlain, 2006; Morimoto et al., 2004; Racine, 1972). In most systems studied to date, mGluR2 is specifically expressed presynaptically (Petralia et al., 1996; Shigemoto et al., 1997), which may allow for interrupting hyperexcitable activity before it spreads across the synapse and brain. Thus, mGluR2 exhibits a distinctive localization that may lend itself to abolishing or reducing the activity at hyperexcitable synapses.

Several Group 2 mGluR agonists, such as LY354740, LY389795 and LY379268, have been found to be anticonvulsant in limbic and generalized motor seizure models (Attwell et al., 1998a,b; Kłodzin´ska et al., 2000; Miyamoto et al., 1997; Moldrich et al., 2001a,b; Monn et al., 1997). Also, the effects of Group 2 agonists can be abolished by pretreatment with Group 2 antagonists, revealing the specificity of the drugs and receptor system against seizures (Folbergrová et al., 2001). While it is difficult to specifically target mGluR2 because of a lack of specific agonists, the positive allosteric modulators (PAM) 3’-[(2-cyclopentyl-2,3-dihydro-6,7-dimethyl-1-oxo-1H-inden-5-yl)oxy]methyl]-[1,1´-biphenyl]-4-carboxylic acid (BINA) and N-[4’-cyano-biphenyl-3-
yl)-N-(3-pyridinylmethyl)-ethanesulfonamide hydrochloride) (CBiPES) have pharmacological specificity at mGluR2 (Ahnaou et al., 2009; Benneyworth et al., 2007; Bonnefous et al., 2005; Fell et al., 2010; Galici et al., 2006; Johnson et al., 2003, 2005; Sanger et al., 2012).

In the present study, we centered our investigation on the acute effects of the Group 2 mGluR agonist LY379268 and the PAMs BINA and CBiPES in treatment of pilocarpine-induced SE. To do this, we tested the hypothesis that administration of Group 2 mGluR active compounds prior to pilocarpine administration (pretreatment) would reduce the behavioral and electroencephalographic progression of acute SE. The second hypothesis tested was that administration of these compounds after SE initiation induced by pilocarpine (posttreatment) would decrease the progression to clonic/tonic (C/T) seizure in acute SE.

Briefly, we found that mGluR2-active compounds (including LY379268 alone and a cocktail of LY379268 + BINA) were capable of reducing the behavioral and electroencephalographic correlates of acute SE if given prior to pilocarpine. We also observed that these drugs could reduce the progression to C/T seizure activity of acute SE if given after pilocarpine administration, though higher doses were necessary. CBiPES alone was also capable of reducing some of the behavioral manifestations of seizure when given prior to pilocarpine.
Methods

Rotarod pilot study

All animal procedures were approved by the Wake Forest School of Medicine Institutional Animal Care and Use Committee. We performed rotarod trials in pilot animals (n = 36) in order to assess gross motor performance after administration of mGluR2 active drugs. There were six groups, with six mice in each group: saline (0.25 ml), diazepam (5 mg/kg), LY379268 (10 mg/kg), LY379268 (20 mg/kg), BINA (100 mg/kg), and cocktail (LY379268 (20 mg/kg) + BINA (100 mg/kg)). Mice were randomly assigned to a group and given four habituation trials on the rotarod apparatus (SDI Inc., San Diego, CA) on day 1. Trials lasted up to 180 s or until the mouse fell from the rod.

On test day (day 2), mice were given a preinjection trial and then tested again 10 min, 30 min, 1 h, and 5 h after injection. Time spent on the rotarod was measured and postinjection performance was compared to the preinjection trial within each group. The diazepam group was included as a positive control for a compound that is commonly known to interfere with motor performance and ability (Savi´c et al., 2009; Willerslev-Olsen et al., 2011).

Surgery

A tethered electroencephalography/electromyography (EEG/EMG) acquisition system (Pinnacle Technologies Inc., Lawrence, KS) was used for these studies. For surgical implantation of EEG electrodes, a subset of male C57Bl/6 mice (n = 48) were anesthetized using ketamine and xylazine (100 mg/kg and 10 mg/kg, respectively).
Supplemental oxygen was provided and atropine (0.04 mg/kg) was given preoperatively to suppress bronchial secretions. Once areflexia was apparent, a 1 cm incision was made and the skull exposed. Four pilot holes were drilled through the skull for placement of stainless steel screws. These screws terminated in a preamplifier headmount that was affixed to the skull using dental acrylic. Silver epoxy was used to cover each screw and maintain electrical continuity with the headmount. Two EMG leads were placed into the neck musculature. The incision was sutured around the headmount and topical antibiotic was applied. Mice were moved to a recovery cage and given ketoprofen (5 mg/kg) for pain management. Mice recovered at least one week before initiating any subsequent experiment. Systemic antibiotics were not given, as post-operative infection was considered exclusionary criteria for the study according to our protocol.

Behavior

During the pre- (treatment given before pilocarpine) and posttreatment (treatment given after pilocarpine) studies, we measured the onset and severity of their behavioral response to pilocarpine administration. The measures taken included: the latency to the first stage 5 C/T seizure, bouts of individual C/T seizures during SE, and the maximum Racine score. When an animal failed to reach a C/T seizure in response to pilocarpine, they were automatically assigned a latency score of 180 min, which was the maximum length of observation. These animals were assumed to have not developed a C/T response to pilocarpine, but still allowed for them to be included in their group for statistical comparisons. In the posttreatment studies, treatment was given when an animal reached a stage 3 Racine score, which is a readily apparent behavioral response to
pilocarpine. Seizure scoring was performed using an adapted Racine scale (Racine, 1972). The scale is as follows: 0 (lack of any apparent seizure activity), 1 (oral automatisms), 2 (head nodding), 3 (forelimb clonus), 4 (rearing), 5 (clonic/tonic seizure with rearing and falling), 6 (wild running/bouncing), and 7 (death as a consequence of pilocarpine and resulting seizures). The experimenters scoring the behavioral activity administered the treatments and were therefore not blinded to the groups. Group analyses were not conducted, however, until completion of all experiments. Electrographical recordings were consistent with the observed behavioral activity.

**Pilocarpine administration**

Pilocarpine (330 mg/kg) was administered to mice (n = 114) 30 min after an injection of methyl-scopolamine (1 mg/kg), which was used to inhibit peripheral effects of pilocarpine and reduce mortality. This dose of pilocarpine was chosen because it has been demonstrated in the literature to be the lowest concentration of pilocarpine used that most reliably elicits seizures (Curia et al., 2008; Turski et al., 1983). Pilocarpine was given outside of the home cage while mice were individually housed in monitoring cages. Animals were monitored for three hours for behavioral scoring. In a subset of animals, EEG monitoring was also performed during the first hour. EEG sampling occurred at a rate of 200 Hz with a preamplifier applied band pass filter from 0.5 to 40 Hz. For the pretreatment studies, drugs were given 15 min prior to pilocarpine. The groups in that study included saline (referred to as “Pilo Only”, n = 14), LY379268 (10 mg/kg, n = 14), BINA (100 mg/kg, n = 14), and cocktail, which received both LY379268 and BINA (10 and 100 mg/kg, respectively, n = 14). For the posttreatment studies, drugs were
given immediately after the animal had the first stage 3 Racine seizure, which is characterized by forelimb clonus. The groups in that study were saline (‘‘Pilo Only’’, n = 12), LY379268 (20 mg/kg, n = 12), BINA (100 mg/kg, n = 10), and cocktail, which received both LY379268 and BINA (20 and 100 mg/kg, respectively, n = 12). Finally, a second mGluR2-specific positive allosteric modulator (PAM), CBiPES (30 mg/kg), was tested for efficacy in both pre- and posttreatment behavioral experiments (n = 6 in both studies).

Drugs

LY379268 was a kind gift from Eli Lilly and Company and was dissolved in 0.9% saline. CBiPES was also kindly provided by Eli Lilly and Company and was dissolved in a vehicle containing 1% carboxymethylcellulose, 0.25% Tween 80 and 0.05% Dow Antifoam, with the pH adjusted to 7.4. BINA was a gift by Dr. Jeffrey Conn (Vanderbilt University, Nashville, TN) and was dissolved in a vehicle containing 10% Tween 80 and 10% NaOH with the pH adjusted to 7.4. Diazepam was manufactured by Hospira, Inc. (Lake Forest, IL). Methyl-scopolamine and pilocarpine were purchased from Sigma (St. Louis, MO) and both dissolved in 0.9% saline. All drugs were given intraperitoneally in a volume range of 0.25—0.5 ml.

Data analysis

Rotarod data were analyzed using a repeated-measures ANOVA with a Dunnett’s post hoc to test for significant differences within each group’s performance before and after injection. Digitized EEG signals were transformed into power spectral data using a
custom-written Matlab script. Analysis of that transformed data was performed using a Kruskal-Wallis test with Dunn’s post hoc analysis. The spectral bands analyzed were defined as follows: delta (0.5-3 Hz), theta (4-7 Hz), alpha (8-12 Hz), and beta (13-25 Hz). EEG spectral power data for each mouse was normalized to its baseline. Mice for each group were then averaged together. Histograms represent a fold change in spectral power for the defined bandwidths. If normality could be assumed for data that were continuous in nature, analysis was performed using a one-way ANOVA with Tukey’s post hoc test. If behavioral data were discrete in nature, a Kruskal-Wallis test with a Dunn’s post hoc was used. If the behavioral data were categorical in nature, then a Chi-square test of homogeneity was used to test for statistically significant differences.

**Results**

**Rotarod pilot study**

A repeated-measures ANOVA was performed within each experimental group with a Dunnett’s post hoc to determine if any group’s postinjection performance was significantly different from their preinjection performance on the rotarod. The diazepam group spent significantly less time on the rotarod 10 and 30 min after the injection ($p < 0.01$, $n = 6$, **Table 1**) compared to before the injection. No other group exhibited differences in gross motor ability at any time point after injection compared to before the injection. The means ± SEM for the time (in seconds) spent on the rotarod for each group at every time point are presented in **Table 1**. The mGluR-active drugs used in this study do not appear to significantly inhibit motor performance on the rotarod test.
Table 1. Drugs active at Group 2 mGluRs do not perturb gross motor ability. C57Bl/6 mice were used (n = 6 per group) to determine whether performance on a rotarod apparatus would be altered by injection with either saline, diazepam (5 mg/kg), LY379268 (10 mg/kg), LY379268 (20 mg/kg), BINA (100 mg/kg), or a cocktail of both BINA and LY379268 at the highest dose. Diazepam was used to demonstrate the effect of an antiepileptic drug that is also known to inhibit motor function. Time spent on the rotarod (mean ± SEM) was measured prior to injection, and then 10 min, 30 min, 1 h, and 5 h postinjection. A repeated measures ANOVA with Dunnett’s post hoc analysis was used to determine which time points postinjection were significantly different from the preinjection performance within each particular group. The only significant differences were found in the diazepam group, at 10 and 30 min postinjection. *, p < 0.01.

<table>
<thead>
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<th>Table 1</th>
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<td>Cocktail (LY379268 (20 mg/kg) + BINA)</td>
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<td>93.34 ± 25.74</td>
<td>98.68 ± 24.20</td>
<td>108.90 ± 21.36</td>
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Pretreatment study

The latency to the first C/T seizure was significantly increased for the LY379268 and cocktail groups compared to the Pilo Only group (one-way ANOVA with Tukey’s post hoc, \( p < 0.001, n = 14 \) for all groups, mean ± SEM for LY379268 = 148.2 ± 14.61 min, cocktail = 163.5 ± 11.63 min, Pilo Only = 61.29 ± 13.76 min, Fig. 1A). Also, the LY379268 and cocktail groups had a significantly increased latency to the first C/T seizure compared to the BINA group (one-way ANOVA with Tukey’s post hoc, \( p < 0.001 \) for the cocktail group, \( p < 0.01 \) for the LY379268 group, mean ± SEM for BINA = 76.07 ± 15.44 min, Fig. 1A). Mice pretreated with LY379268 had fewer bouts of C/T seizures during SE (Kruskal-Wallis test with Dunn’s post hoc analysis; \( p < 0.01 \), mean ± SEM = 0.71 ± 0.44 bouts) than Pilo Only mice (mean ± SEM = 5.36 ± 1.27 bouts, Fig. 1B). Mice pretreated with the cocktail also had significantly fewer bouts of C/T seizures compared to Pilo Only mice (\( p < 0.001 \), mean ± SEM for cocktail = 0.21 ± 0.15, Fig. 1B). Lastly, using a Chi-square test for homogeneity, it was found that there were significant differences between all of the groups Racine scores (\( p = 0.0008 \), mean ± SEM Racine score for Pilo Only = 5.07 ± 0.29, LY379268 = 2.70 ± 0.52, BINA = 4.79 ± 0.46, cocktail = 1.57 ± 0.54, Fig. 1C). Post hoc tests revealed that mice treated with LY379268 had a lower Racine score compared to Pilo Only mice; mice treated with the cocktail had a lower Racine score compared to Pilo Only mice and BINA mice.
Figure 1. Pretreatment with Group 2 mGluR active drugs reduces behavioral severity of pilocarpine induced SE. A. Latency to the first C/T seizure during SE was significantly increased for the LY379368 and the cocktail groups compared to the Pilo Only group (n = 14 in all groups, one-way ANOVA with Tukey’s post hoc, p < 0.001). The LY379268
and cocktail groups also had an increased latency to the first C/T seizure compared to the BINA group (one-way ANOVA with Tukey’s post hoc, p < 0.01 for the LY379268 group, n = 14; p < 0.001 for the cocktail group). B. Average bouts of C/T seizures during SE were significantly reduced with pretreatment with LY379268 compared to the Pilo Only group (Kruskal-Wallis test with Dunn’s post hoc, p < 0.01). Pretreatment with the cocktail also significantly reduced bouts of C/T seizures compared to the Pilo Only group (Kruskal-Wallis test with Dunn’s post hoc, p < 0.001). C. The Racine scores were also significantly different between all pretreatment groups (Chi square test for homogeneity, p = 0.0008). Post hoc tests revealed that mice treated with LY379268 had a lower Racine score compared to Pilo Only mice. Cocktail treated mice had a lower Racine score compared to Pilo Only and BINA mice. Seizures were considered C/T if they reached at least a stage 5 Racine score. The Pilo Only group received saline as a control pretreatment prior to pilocarpine. Compared to Pilo Only: *, p<0.05; **, p < 0.01; ***, p < 0.001. Compared to BINA: ##, p < 0.01; ###, p < 0.001.
**Fig. 2** represents sample EEG traces from one mouse in each treatment group measured during the pretreatment study demonstrating activity at baseline prior to pilocarpine administration and activity 60 min after pilocarpine administration. To account for amplitude differences at baseline across EEG recording rigs, EEG spectral data for each mouse was normalized to its baseline. Power spectral data 60 min after pilocarpine administration when Group 2 mGluR-active drugs were given as pretreatment is shown in **Fig. 3**. Fold change in power is represented on the $Y$ axis. Power in each frequency bandwidth (delta- 0 to 3 Hz, theta- 4 to 7 Hz, alpha- 8 to 12 Hz, and beta- 13 to 25 Hz) was averaged, normalized to baseline activity in that frequency bandwidth, and a Kruskal-Wallis test with Dunn’s post hoc was performed across groups within each frequency band.
**Figure 2.** *Pretreatment Study*, sample traces from one mouse representing each treatment group. *Baseline*, activity at baseline prior to pilocarpine administration. *One hour after Pilo*, activity 60 minutes after pilocarpine administration. To account for amplitude differences at baseline across EEG recording rigs, EEG spectral data for each mouse was normalized to its baseline. Column treatment groups, Saline, BINA (100mg/kg), LY379268 (10mg/kg), Cocktail (LY378268 (10mg/kg) + BINA (100mg/kg)).
Figure 3. Pretreatment with Group 2 mGluR drugs reduces power of pilocarpine induced SE in EEG recordings 60 minutes after pilocarpine administration. In the delta range, the LY379268 group shows decreased power compared to all other groups (p < 0.001, n = 6 in all groups). In the theta frequency range, the LY379268 group shows decreased power compared to the Pilo Only group (p < 0.001), the BINA group (p < 0.001) and the cocktail group (p < 0.01). The power is also decreased in the theta range for the cocktail group compared to the Pilo Only group (p < 0.05) and the BINA group (p < 0.01). In the alpha and beta range, the LY379268 and cocktail groups show decreased power compared to the Pilo Only and BINA groups (p < 0.001 for both comparisons). The Pilo Only group received saline as a control pretreatment prior to pilocarpine. The frequency ranges were defined as delta (0.5-3 Hz), theta (4-7 Hz), alpha (8-12 Hz) and beta (13-25 Hz). Power has been normalized to the baseline power for each mouse in the defined frequency range. Compared to Pilo Only: *, p < 0.05; ***, p < 0.001. Compared to
BINA: ##, p < 0.01; ###, p < 0.001. Compared to Cocktail: ^^, p<0.01; ^^^, p<0.001.

Column abbreviations: Pilo: Pilo Only, LY68: LY379268 (10 mg/kg), BINA: BINA (100 mg/kg), Cocktail: Cocktail (LY379268 (10 mg/kg) + BINA (100 mg/kg)).
Sixty minutes after pilocarpine administration (Fig. 3), the LY379268 group had significantly less power than the Pilo Only, BINA and cocktail groups (p < 0.001) in the delta bandwidth. In theta bandwidth, the LY379268 group continued to demonstrate less power than the Pilo Only and BINA groups (p < 0.001), as well as the cocktail group (p < 0.01). Also in the theta range, the cocktail group had significantly less power than the Pilo Only group (p < 0.05) and the BINA group (p < 0.01). Finally in the alpha and beta bandwidths, the LY379268 and cocktail groups had significantly less power than the Pilo Only group (p < 0.001). The EEG data suggests that LY379268 and the cocktail mitigated the abnormal power increases that were seen in control mice that received only pilocarpine with saline as pretreatment.

Taken together, the behavioral and spectral data suggest that LY379268 (10 mg/kg) alone can alter the course of SE when given prior to pilocarpine. LY379268 lessened pilocarpine-induced power changes and also increased the time until initial seizure onset, decreased the number of individual C/T seizures during SE and lowered the average maximum Racine score. BINA does not appear to provide protection against pilocarpine-induced power changes in EEG or in behavioral expression of SE. BINA alone did not increase the latency to the first C/T seizure, reduce the bouts of C/T seizures, or lower the average Racine score. The cocktail of both LY379268 and BINA does not seem to provide any more protection from SE than LY379268 does alone. In general it appears there is no added benefit of administering the cocktail as opposed to the mGluR2/3 agonist alone as pretreatment against pilocarpine-induced SE.
Posttreatment study

In the posttreatment study, treatment was administered when the mouse reached a stage 3 seizure after pilocarpine administration. There were no significant differences between groups in the latency to treatment administration (Fig. 4A). A stage 3 seizure occurred and treatment was given on average $23.74 \pm 2.69$ (mean $\pm$ SEM) min after pilocarpine.
Figure 4. Posttreatment with Group 2 mGluR active drugs reduces severity of pilocarpine induced SE behavioral measures.  **A.** The average latency to stage 3 Racine seizures did not vary between groups (one-way ANOVA with Tukey’s post hoc, p > 0.05, n ≥ 10 in all groups, mean latency for treatment administration was 24 min). **B.** Latency to the first C/T seizure during SE was significantly increased for the LY379368 and the cocktail groups compared to the Pilo Only group and the BINA group (one-way ANOVA with Tukey’s post hoc, p < 0.001 for both comparisons, n = 12 in these three groups). No mouse that received LY379268 posttreatment ever reached a stage 5 C/T seizure, therefore that entire group was scored as 180 min latency (see Methods), as that was the maximum length of observation time for this study.  **C.** Average bouts of C/T seizures during SE were significantly reduced with posttreatment with LY379268 and the cocktail compared to the Pilo Only group that received saline posttreatment (Kruskal—Wallis test
with Dunn’s post hoc, p < 0.01 for the cocktail group, p < 0.001 for the LY379268 group, n ≥ 10 in all groups). The LY379268 and cocktail groups also experienced fewer bouts of seizures than the BINA group (p < 0.05 for the cocktail group, p < 0.01 for the LY379268 group). 

D. The Racine scores were also significantly different between posttreatment groups (Chi square test of homogeneity, p = 0.0034, n ≥ 10 in all groups). Post hoc tests revealed a significant difference in Racine score between the Pilo Only group compared to the LY379268 and cocktail groups. These groups also had a significantly lower Racine score compared the BINA group. Seizures were considered C/T if they reached at least a stage 5 Racine score. The Pilo Only group received saline as a control posttreatment after animals reached a stage 3 Racine seizure. Compared to Pilo Only: **p < 0.01; ***p < 0.001. Compared to BINA: #p < 0.05; ##p < 0.01; ###p < 0.001.
A one-way ANOVA with Tukey’s post hoc analysis determined there was a significantly increased latency to the onset of the first C/T seizure for the LY379268 (n = 12) and the cocktail (n = 12) posttreatment groups as compared to the Pilo Only group (n = 12, p < 0.001, latency mean ± SEM for LY379268 = 180 ± 0 min, cocktail = 165.7 ± 9.68 min, Pilo Only = 61.78 ± 15.65 min, **Fig. 4B**). Also, the cocktail and LY379268 groups had significantly increased latencies to the first C/T seizure compared to the BINA group (n = 10, p < 0.001, latency mean ± SEM for BINA = 74.2 ± 17.93 min, **Fig. 4B**). LY379268 posttreated mice had fewer bouts of C/T seizures during SE than Pilo Only mice (p < 0.001, Kruskal-Wallis test with Dunn’s post hoc analysis, bouts mean ± SEM for LY379268 = 0 ± 0, bouts mean ± SEM for Pilo Only = 2.22 ± 0.66, **Fig. 4C**). The cocktail posttreated mice had significantly fewer bouts of seizures than the Pilo Only group (p < 0.01) and the BINA group (p < 0.05, bouts mean ± SEM for cocktail = 0.17 ± 0.11, bouts mean ± SEM for BINA = 2.2 ± 0.61). Lastly, using a Chi square test for homogeneity, the posttreatment groups had significantly different Racine scores (p = 0.0034, Racine score mean ± SEM for Pilo Only = 5.44 ± 0.29, LY379268 = 3.5 ± 0.15, BINA = 5.4 ± 0.3, cocktail = 3.67 ± 0.22, **Fig. 4D**). Post hoc tests revealed that mice treated with LY379268 had a lower Racine score compared to Pilo Only and BINA mice; mice treated with the cocktail also had a lower Racine score compared to Pilo Only and BINA mice.

**Fig. 5** represents sample EEG traces from one mouse in each posttreatment group demonstrating activity at baseline prior to pilocarpine administration and activity 60 min after pilocarpine administration. To account for amplitude differences at baseline across EEG recording rigs, EEG spectral data for each mouse was normalized to its baseline.
Sixty minutes after pilocarpine (Fig. 6), the LY379268, BINA and cocktail groups all exhibited significantly less power compared to the Pilo Only group (p < 0.001 for the LY379268 and cocktail groups, p < 0.05 for the BINA group) in the delta frequency range. Also in the delta range, the LY379268 group had significantly less power than both the BINA group (p < 0.001) and the cocktail group (p < 0.01). In the theta and alpha ranges, the LY379268 group showed less power compared to the Pilo Only and the BINA groups (p < 0.001), as well as the cocktail group (p < 0.05). Also in the theta and alpha ranges, the cocktail group showed significantly less power than the Pilo Only group (p < 0.001) and the BINA group (p < 0.01). Finally, in the beta range, all groups exhibited less power than the Pilo Only group (p < 0.001), and the LY379268 and cocktail groups showed less power than the BINA group (p < 0.001). Spectral power remained around baseline levels in the different frequency bandwidths in these groups whereas there was a significant increase in mice treated with saline (Pilo Only group).
Figure 5. *Posttreatment study*, sample traces from one mouse representing each treatment group. *Baseline*, activity at baseline prior to pilocarpine administration. *One hour after Pilo*, activity 60 min after pilocarpine administration. To account for amplitude differences at baseline across EEG recording rigs, EEG spectral data for each mouse was normalized to its baseline. Column treatment groups, saline, BINA (100 mg/kg), LY379268 (20 mg/kg), cocktail (LY378268 20 mg/kg) + BINA (100 mg/kg).
Figure 6. Posttreatment with Group 2 mGluR drugs reduces power of pilocarpine induced SE in EEG recordings 60 min after pilocarpine administration. In the delta frequency range, the LY379268 and cocktail groups show decreased power compared to the Pilo Only group (p < 0.001, n = 6 in all groups), as does the BINA group (p < 0.05). Also in the delta range, the LY379268 group shows decreased power compared to the BINA group (p < 0.001) and the cocktail group (p < 0.01). In the theta and alpha ranges, the LY379268 group demonstrated decreased power compared to the Pilo Only group (p < 0.001), the BINA group (p < 0.001) and the cocktail group (p < 0.05). Also in the theta and alpha bandwidths, the cocktail group had significantly decreased power compared to the Pilo Only group (p < 0.001) and the BINA group (p < 0.01). Finally in the beta range, all other groups had less power than the Pilo Only group (p < 0.001), and the LY379268 and cocktail groups also had less power than the BINA group (p < 0.001). The Pilo Only group received saline as a control posttreatment after animals reached a
stage 3 Racine seizure. The frequency ranges were defined as delta (0.5-3 Hz), theta (4-7 Hz), alpha (8-12 Hz) and beta (13-25 Hz). Power has been normalized to the average power at baseline in each frequency range. Compared to Pilo Only: *p < 0.01; ***p < 0.001. Compared to BINA: ##p < 0.01; ###p < 0.001. Compared to cocktail: ¤p < 0.05; ¤¤p < 0.01. Column abbreviations: Pilo, Pilo Only; LY68, LY379268 (20 mg/kg); BINA, BINA (100 mg/kg); Cocktail: cocktail (LY379268 (20 mg/kg) + BINA (100 mg/kg)).
The posttreatment data suggest that Group 2 mGluR activation with LY379268 reduces behavioral seizures, as evidenced by increased latency to a C/T seizure, reduced bouts of C/T seizures and decreased Racine scores compared to the Pilo Only group. The BINA group was never significantly different from the Pilo Only group, and the LY379268 group was never significantly different from the cocktail group on any behavioral measure. The EEG data generally demonstrated that after treatment was given, any activation of mGluR2/3 with an agonist or in combination with a PAM, some protection would be provided in the form of reduced power compared to Pilo Only animals. Since LY379268 was not found to be different from the cocktail group, there may be no added benefit of a PAM in treating C/T seizures with mGluR2 active drugs.

**CBiPES study**

We performed an additional round of behavioral studies using CBiPES (30 mg/kg, n = 6 in both the pre- and posttreatment groups), a recently created PAM that has been shown to potentiate the effect of LY379268 (Johnson et al., 2005) as well as mimic the antipsychotic effects of LY379268 (Fell et al., 2010). The same behavioral measures were recorded as in the previous experiments, including latency to the first C/T seizure, bouts of C/T seizures, and the maximum Racine score.

CBiPES (30 mg/kg, n = 6) given as pretreatment before pilocarpine resulted in significantly longer latency to the first C/T seizure compared to Pilo Only pretreatment (one-way ANOVA with Tukey’s post hoc, p < 0.05, latency mean ± SEM for CBiPES = 132.3 ± 23.84 min, Table 2). When given as posttreatment, CBiPES still significantly increased the latency to the first C/T seizure compared to the Pilo Only posttreatment.
group (one-way ANOVA with Tukey’s post hoc, $p < 0.05$, latency mean ± SEM for
CBiPES = 135.3 ± 28.33 min, Table 2). CBiPES also significantly reduced the bouts of
C/T seizures during SE compared to Pilo Only when given as pretreatment (Kruskal-
Wallis with Dunn’s post hoc, $p < 0.01$, Table 2). When given as posttreatment, CBiPES
did not significantly reduce the bouts of C/T seizures during SE compared to the Pilo
Only posttreatment group (Kruskal-Wallis with Dunn’s post hoc, $p > 0.05$, Table 2).
Finally, although differences were found in the latency to the first seizure and bouts of
seizures during SE, no significant difference in Racine scores were found between these
groups in either the pre- or posttreatment studies (Chi-square test for homogeneity, $p >$
0.05, Table 2).
TABLE 2

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<td>135.3 ± 28.33 min*</td>
<td>0.67 ± 0.49</td>
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Table 2. Pre- and posttreatment behavioral differences between the CBiPES and Pilo Only groups (Mean ± SEM). Latency to the first C/T seizure, bouts of C/T seizures and Racine score are presented for the experimental groups receiving pilocarpine only or CBiPES as pre- or posttreatment. Asterisks indicate significant differences between those groups.
Discussion

Our study indicates that activation of Group 2 mGluRs reduces the behavioral and electrographic course of acute SE induced by pilocarpine, as evidenced by reduced behavioral seizure and EEG spectral power in animals treated with Group 2 mGluR compounds either before or after pilocarpine administration.

Pilo Only animals in both the pre- and posttreatment experiments that received only saline as an intervention demonstrated a typical progression through acute SE induced by pilocarpine. The SE inhibiting effects of the compounds were not due to any gross motor disturbances as evidenced by consistent performance on the rotarod (Table 1). Our positive control diazepam, a commonly used AED, significantly inhibited motor performance. We did not observe similar effects with the mGluR active drugs, which suggests that inhibition of seizure activity by these compounds is not due to inhibition of motor behavior. Some studies have noted the motor inhibiting quality of higher doses of LY379268 (Cartmell et al., 1999; Feinberg et al., 2002), however, 20 mg/kg (highest dose used in our study) did not significantly reduce motor ability from observed preinjection performance.

In both pre- and posttreatment experiments, we observed a reduction in behavioral seizure in mice treated with LY379268 compared to the Pilo Only group (Figs. 1 and 4). LY379268 increased latency to C/T seizure, reduced C/T seizure bouts, and decreased Racine seizure score. Complimentary to these behavioral findings, we observed no increase in EEG spectral power during status in mice treated with LY379268 (Figs. 3 and 6). There was at least a 30-fold increase in spectral power in the different frequency bandwidths during status in control animals treated with saline prior to or after
pilocarpine administration. The spectral power in the defined frequency bandwidths of mice treated with LY379268 stayed relatively the same as to what was observed in their baseline activity. In the posttreatment study, the mice had reached a stage 3 Racine seizure prior to treatment, which required a higher dose of LY379268 compared to the pretreatment investigation. A pilot group of posttreatment mice given the same dose of LY379268 used in the pretreatment study was not significantly different from the Pilo Only mice on any behavioral measure (data not shown).

In both pre- and posttreatment studies, the cocktail of LY379268 and BINA did not provide greater protection against pilocarpine-induced SE than when the LY379268 was given alone. The mice in the cocktail group exhibited similar behavioral measures as those in the LY279268 group (Figs. 1 and 4). This suggests that the anticonvulsive effect of the cocktail is most likely due to LY379268 and not synergy between the BINA and the agonist. It has been previously hypothesized that simultaneous orthosteric and allosteric ligand binding at mGluR2 may increase receptor sensitivity to the endogenous and/or orthosteric ligand (Johnson et al., 2003). Mechanistically, allosteric modulation of mGluRs may increase receptor sensitivity, receptor availability, and/or efficacy of receptor homodimerization (Johnson et al., 2005; Kew and Kemp, 2005), however in our study, it does not appear that any of these allosteric modulations could overcome the changes produced by pilocarpine without the orthosteric ligand on board as well.

More studies are needed, but one plausible explanation of this effect may be that LY379268 activation of mGluR3 on glial cells tonically reduces pilocarpine-induced glutamate release, and the addition of BINA does not potentiate the effect as it is not pharmacologically active at mGluR3. Other explanations for BINA not adding any
benefit when used in combination with LY379268 are that the dose used was not in the effective range and that BINA may not have crossed the blood brain barrier. Previous studies examining BINA in models of psychosis and anxiety found significant effects on their measure of interest using lower doses and the same administration technique (Benneyworth et al., 2007; Galici et al., 2006). Therefore, we believe an appropriate dose of BINA was used in our seizure model, and although we did not confirm with cortical measures of BINA concentration, the significant effects in other studies would suggest that BINA is crossing the blood brain barrier at the administered concentrations.

In contrast, with CBiPES alone pre- and posttreatment, we did find a significant increase in the latency to the first C/T seizure compared to the Pilo Only group. CBiPES also reduced the bouts of C/T seizures in the pretreatment study. This supports previous work in anxiety models, such as stress-induced hyperthermia, in which CBiPES was found to mimic the effect of LY379268 when administered alone (Fell et al., 2010; Johnson et al., 2005; Sanger et al., 2012). However, in both pre- and posttreatment studies, CBiPES did not decrease the overall Racine score. Interestingly, CBiPES was more easily dissolved than BINA under our conditions, and while both drugs were entirely dissolved for injection, this cannot be excluded as a possible explanation of the difference in efficacy between these two PAMS. Both of these compounds have been characterized as selective mGluR2 PAMS (Ahnaou et al., 2009; Benneyworth et al., 2007; Bonnefous et al., 2005; Fell et al., 2010; Galici et al., 2006; Johnson et al., 2005, 2003; Sanger et al., 2012). However, off-target effects during seizure activity cannot be excluded. A ceiling effect may have occurred with the dose of LY379268 used in the cocktail groups. We did not test lower doses of LY379268 in combination with either

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modulator. Further evaluation of the two modulators is necessary to parse out differences in efficacy and to determine if a different combination of doses in the cocktail treatment could provide more of a synergistic effect.

Studies evaluating mGluR2/3 expression have demonstrated a reduction 24 h after pilocarpine-induced SE with reports of long term loss (Ermolinsky et al., 2008; Garrido-Sanabria et al., 2008), which consequently could lead to increased excitability in the brains of these animals. The results of the two studies suggest that delaying mGluR2/3 drug administration by 24 h or more after pilocarpine would yield little to no effect on hyperexcitability in epileptic rodents. Contrary to this, a recent study evaluating hippocampi of patients with TLE reported an increase in mGluR2/3 expression (Das et al., 2012). More specifically, studies have also demonstrated an increase in mGluR2/3 expression on reactive astrocytes in both animal models and patients with epilepsy (Aronica et al., 2003; Seifert et al., 2006; Steinhäuser and Seifert, 2002; Tang and Lee, 2001). The effect of our treatment schedule on the development (or lack thereof) of subsequent SRS in the chronic period of pilocarpine treated mice remains to be determined.

Overall we demonstrate that treatment with mGluR2 active compounds reduces acute SE behavior, and the observed decrease in EEG spectral power suggests that there is less synchronized activity typically associated with SE. Certainly, treating acute SE is most critical in a clinical setting, but preventing future seizures precipitated by an episode of SE should also be of consideration. Future studies are needed to determine if these drugs inhibit the development of SRS after pilocarpine, as well as the resulting neuropathological changes that occur with epileptogenesis. If it could be demonstrated
that these drugs also prevent or reduce the incidence of SRS, it would further support the
development of drugs that target Group 2 mGluRs for the treatment of epilepsy.
Acknowledgements

The authors wish to express our gratitude to P. Jeffrey Conn at Vanderbilt University for providing BINA. We would also like to thank Eli Lilly and Company for kindly providing the LY379268 and CBiPES. Also, we would like to express our gratitude to Hong Qu Shan, David Klorig and Walter Wiggins for their helpful comments and suggestions during the writing phase of this manuscript.

This work was supported by NIH Grants F31AA017048, T32AA7565, R21EY018159, P01AA021099, R01AA016852, and R01AA015568, Citizens United for Research on Epilepsy, and the Tab Williams Family Fund.
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MELISSA A. RIEGLE
mriegle@wakehealth.edu

EDUCATION

2009 – 2014
Wake Forest University School of Medicine
Winston-Salem, NC
Ph.D. in Neuroscience, April 30, 2014

2007 – 2009
Ball State University
Muncie, IN
M.S. in Biology, May 2009

2002 – 2006
Grand Valley State University
Allendale, MI
B.S. in Biomedical Health Science, Minor – Chemistry, May 2006

FELLOWSHIPS, HONORS and AWARDS

2012-2014: Individual Ruth L. Kirschstein National Research Service Award, (F31AA021322-01, NIAAA)
2013: 13th Annual WFU Graduate School Research Day Poster Award
2011-2012: Institutional NIAAA T-32 Alcohol Training Grant, WFU
2011: Fine Science Tools Travel Award, WFU
2010-2011: Institutional NIH Predoctoral Neuroscience Training Grant, WFU
2010: Mary A. Bell WNCSfN Research Poster Award, WFU
2008-2009: Graduate Assistantship, BSU
2008: Research Assistantship, BSU
2002-2006: Award of Excellence, GVSU
2002-2006: Storer Scholarship, GVSU
2002-2006: Academic All-American, GVSU

PUBLICATIONS


*Co-first authors

Submitted:

In Prep:


*Co-first authors

RESEARCH

2010 – present, Wake Forest University School of Medicine, Winston-Salem, NC
Advisor: Dr. Dwayne Godwin
Doctoral Candidate; Individual Ruth L. Kirschstein National Research Service Award (Predoctoral NRSA)

A Mechanism and Promising New Treatment for Alcohol Withdrawal Seizure
Our lab previously identified a disruption in T-type calcium channels during alcohol withdrawal. We believe the increased T channel activity contributes to hyperexcitability experienced during withdraw symptoms. This project required development of an alcohol withdrawal seizure model in our lab to test ethosuximide. Additional methods included: intermittent alcohol exposure paradigm, surgical procedures for EEG recordings, analysis of EEG activity, behavioral assays (elevated plus maze, light/dark box, activity boxes, and alcohol consumption), blood ethanol analysis, chemoconvulsant model of seizure, and behavioral seizure scoring.

Major findings:
- Alcohol withdrawal seizure severity increased progressively with successive withdrawals.
- Ethosuximide dose-dependently decreased alcohol withdrawal-induced seizures, modestly reduced alcohol consumption, and had no effect on general anxiety-like behavior or motor function. Ethosuximide reduced more severe tonic/clonic seizure activity during withdrawal, demonstrated by a reduction in handling-induced convulsions and PTZ-induced seizures. Importantly, ethosuximide decreased mortality in mice undergoing alcohol withdrawal. Preliminary data suggests ethosuximide can decrease withdrawal-induced anxiety.
- Overall, our data supports the hypothesis that T channels contribute to alcohol withdrawal hyperexcitability. T channels may serve as a potential target, and
further investigation will determine whether ETX or more selective compounds may hold promise as a novel treatment.

**Metabotropic Glutamate Receptors and Epilepsy**

We investigated if Group II metabotropic glutamate receptor (mGluR) agonists and mGluR2-specific allosteric modulators could interrupt or arrest seizures in pilocarpine-injected mice (model for status epileptic and temporal lobe epilepsy).  Methods included: surgical procedures for headmount implantation, behavioral and EEG analyses.

**Major finding:**
- Activation of Group 2 mGluRs reduces the behavioral and electrographic course of acute status epilepticus induced by pilocarpine, as evidenced by reduced behavioral seizure and EEG spectral power.

**2009, Wake Forest University School of Medicine, Winston-Salem, NC**

**Lab Rotation Advisor: Dr. Jeff Weiner**

The goal of this research was to gain insight into the effects of stress on the noradrenergic system.  The hippocampus is an essential regulator in the stress response and receives a high concentration of adrenergic terminals.  However, the role of excessive stress exposure on the noradrenergic system in the hippocampus remains unclear.  Isoproterenol, a selective β-adrenergic agonist, transiently increases potentiation at the Schaffer Collateral-CA1 synapse.  We hypothesized that isoproterenol will further increase potentiation in the Schaffer-Collateral pathway as a result of stress-induced impairment of the noradrenergic system.  To test our hypothesis, we stimulated the Schaffer-Collateral pathway and measured field excitatory post-synaptic potentials in the CA1 region of stress-induced and naïve Long Evans rats.  Methods included slice preparation; electrophysiology techniques including extracellular field recordings.

**Outcome:**
- There were no significant differences between the stress-induced and naïve rats; however, I did learn extracellular field recordings in this rotation.

**2007-2009, Ball State University, Muncie, IN**

**Advisor: Dr. Bartholomew Pederson**

*Thesis: Investigating the Role of Brain Glycogen during Seizure Activity in Mice*

We investigated the role and significance of brain glycogen during acute seizure activity in wild-type and genetically modified mice.  Methods employed included various biochemical assays such as glycogen and glucose determination, glycogen synthase and phosphorylase enzymatic activity, other metabolite assays deemed necessary, and microscopy.  The goal of this research was to gain insight and better understanding of seizure activity.

**Major finding:**
- Brain glycogen is utilized but not required for pentylenetetrazol-induced acute seizure in mice.
SELECTED ABSTRACTS

*Co-first authors

Riegle MA, Carter E, Weiner J, Godwin DW. 2013. Ethosuximide, a T-Type Calcium Channel Antagonist, as a Potential Treatment for Alcohol Dependence and Withdrawal. Society for Neuroscience Annual Meeting, San Diego, CA; WFU Graduate and Postdoc Research Day, Winston-Salem, NC
*presented poster; received poster award for translational category at WFU Research Day


Riegle MA, Caulder EH, Godwin DW. 2011. Alcohol withdrawal seizure severity is reduced by ethosuximide, a T-type calcium channel antagonist. Society for Neuroscience Annual Meeting, Washington DC *presented poster


Caulder EH, Riegle MA*, Godwin DW. 2010. Group 2 metabotropic glutamate receptor activation after pilocarpine administration inhibits status epilepticus. Society for Neuroscience Annual Meeting, San Diego, CA; Western North Carolina Chapter of Society for Neuroscience Poster Day, Winston-Salem, NC
*presented poster; received Mary A. Bell research poster award at WNCCSfN poster day


PROFESSIONAL MEMBERSHIPS

2010-present: Society for Neuroscience  
2009-present: Western North Carolina Chapter of the Society for Neuroscience  
2013-present: National Science Teachers Association  
2009-present: Brain Awareness Council, WFU

ACADEMIC CONFERENCES

2013: Society for Neuroscience Annual Meeting, San Diego, CA  
2013: 10th Annual Teaching and Learning Conference, Elon University, Elon, NC  
2013: Evidence-Based Learning and Teaching, Lilly Conference on College and University Teaching, Greensboro, NC  
2011: Society for Neuroscience Annual Meeting, Washington, DC  
2010: Society for Neuroscience Annual Meeting, San Diego, CA

PUBLIC SEMINARS AND GUEST LECTURES

*I have been invited to guest lecture at Salem College on April 25, 2014*

2014: Invited talk: “Ethosuximide Reduces Alcohol Withdrawal Seizure”, University of Michigan  
2013: Invited guest lecture: “Brainzzzz”, Salem College  
2013: Seminar: “Ethosuximide: A potential Treatment for Alcohol Withdrawal”, WFU  
2012: Invited talk: “Ethosuximide, a T-type Calcium Channel Antagonist Reduces Alcohol Withdrawal Seizure Severity in DBA/2J Mice”, ACUC Continuing Education Series, WFU  
2012: Graduate School Orientation: “Brain Awareness Council: The Benefits of Neuroscience Education Outreach”  
2011: Alcohol Training Grant Meeting: “Alcohol Withdrawal Seizure Severity is Reduced by Ethosuximide, a T-type Calcium Channel Antagonist”, WFU  
2011: Tutorial: “Alcohol Withdrawal Seizure Severity is Reduced by Ethosuximide, a T-type Calcium Channel Antagonist”, WFU  
2010: Tutorial: “Group II Metabotropic Glutamate Receptor Activation after Pilocarpine Administration Inhibits Status Epilepticus”, WFU
TEACHING EXPERIENCE

2012-2013: Adjunct Faculty: Anatomy and Physiology Lab, Salem College
2012-2013: Student Advisor, Outreach Course (GRAD 709, NEUR 791), WFU
2012-2013: Student Mentoring, Victoria Xu, undergraduate student, WFU
2011: Neuroanatomy Teaching Assistant, Neuroscience Program, WFU
2009-present: lesson plans and neuroscience lectures, Brain Awareness Council, WFU
2008-2009: Biology Lab Teaching Assistant, BSU

SERVICE

2011-2013: Student Representative, Neuroscience Program Recruitment Committee
2009-present: BAC, K-12 visits, Lending Library, Council Coordinator
2011-2012: MMARS Participant (Matching Matriculates with Returning Students), WFU

PROFESSIONAL REFERENCES

Dwayne Godwin, Ph.D.
Dean of Biomedical Graduate Programs
Wake Forest School of Medicine
Medical Center Boulevard
Winston-Salem, NC 27101
dgodwin@wakehealth.edu

Brian McCool, Ph.D.
Professor, Department of Physiology and Pharmacology
Wake Forest School of Medicine
Medical Center Boulevard
Winston-Salem, NC 27101
bmccool@wakehealth.edu

Carol Milligan, Ph.D.
Neuroscience Program Director
Wake Forest School of Medicine
Medical Center Boulevard
Winston-Salem, NC 27101
milligan@wakehealth.edu

Jeff Weiner, Ph.D.
Professor, Department of Physiology and Pharmacology
Wake Forest School of Medicine
Medical Center Boulevard
Winston-Salem, NC 27101
jweiner@wakehealth.edu