# ETHOSUXIMIDE STABILIZES SLEEP-RELATED OSCILLATIONS AND REDUCES SLEEP FRAGMENTATION DURING ALCOHOL WITHDRAWAL

BY

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| | |
|---|---|
| **ADH** | ALCOHOL DEHYDROGENASE |
| **AED** | ANTIEPILEPTIC DRUG |
| **ANOVA** | ANALYSIS OF VARIANCE |
| **AWI** | ALCOHOL WITHDRAWAL-RELATED INSOMNIA |
| **AWS** | ALCOHOL WITHDRAWAL SYNDROME |
| **BEC** | BLOOD-ETHANOL CONCENTRATION |
| **BK** | BIG-CONDUCTANCE CALCIUM-DEPENDENT POTASSIUM CHANNEL |
| **BRF** | BRAINSTEM RETICULAR FORMATION |
| **BW** | BRIEF WAKENING |
| **BZD** | BENZODIAZEPINE |
| **$CA^{2+}$** | CALCIUM |
| **CIE** | CHRONIC INTERMITTENT ETHANOL |
| **CNS** | CENTRAL NERVOUS SYSTEM |
| **DFT** | DISCRETE FOURIER TRANSFORM |
| **EEG** | ELECTROENCEPHALOGRAPHY |
| **EMG** | ELECTROMYOGRAPHY |
| **ERP** | EVENT-RELATED POTENTIAL |
| **ETOH** | ETHANOL |
| **ETX** | ETHOSUXIMIDE |
| **EXP** | EXPOSURE |
| **FDA** | FOOD & DRUG ADMINISTRATION |
| **FMRI** | FUNCTIONAL MAGNETIC RESONANCE IMAGING |
| **GABA** | γ-AMINOBUTYRIC ACID |
| **GIRK** | G PROTEIN-MEDIATED INWARDLY RECTIFYING POTASSIUM CHANNEL |
| **$K^{+}$** | POTASSIUM |
| **KO** | KNOCKOUT |
| **LDW** | LONG-DURATION WAKE |
| **MESOR** | MIDLINE-ESTIMATING STATISTIC OF RHYTHM |
| **$NA^{+}$** | SODIUM |
| **NAD** | NICOTINAMIDE ADENINE DINUCLEOTIDE |
| **NE** | NOREPINEPHRINE |
| **NREM** | NON-RAPID EYE MOVEMENT |
| **PSG** | POLYSOMNOGRAPHY |
| **REM** | RAPID EYE MOVEMENT |
| **SCN** | SUPRACHIASMATIC NUCLEUS |
| **SD** | STANDARD DEVIATION |
| **SEM** | STANDARD ERROR OF THE MEAN |
| **SK** | SMALL-CONDUCTANCE CALCIUM-DEPENDENT POTASSIUM CHANNEL |
| **SRO** | SLEEP-RELATED OSCILLATION |
| **SWD** | SPIKE-AND-WAVE DISCHARGE |
| **SWA** | SLOW-WAVE ACTIVITY |
| **SWS** | SLOW-WAVE SLEEP |
| **TMN** | TUBEROMAMILLARY NUCLEUS |
| **TRN** | THALAMIC RETICULAR NUCLEUS |
| **VC** | VIGILANCE CYCLING |
| **VLPO** | VENTROLATERAL PREOPTIC NUCLEUS |
| **W** | WAKE |
| **WD** | WITHDRAWAL |
| **WK** | WEEK |
| **WT** | WILD TYPE |

**Abstract**

The central focus of my dissertation is the study of the effects of ethanol on brain rhythms, measured by electroencephalography (EEG), as they pertain to alcohol withdrawal-related insomnia (AWI). AWI is an important clinical problem for chronic alcoholics undergoing treatment for alcoholism. The severity of AWI is highly correlated with increased risk of relapse. Furthermore, the current first-line therapy for alcohol withdrawal, chlordiazepoxide, is inappropriate for long-term management of AWI due to potential for abuse and negative side effects. While much of the research into the mechanisms underlying AWI has focused on mechanisms of global inhibition and excitation, more specific mechanisms of coordinated activity implicated in the maintenance of sleep are adversely affected by chronic alcohol use. The work described herein employs the chronic intermittent ethanol (CIE) vapor administration method in mice to study the effects of ethanol exposure and withdrawal on sleep and oscillations in neuronal activity between the thalamus and cortex (or thalamocortical oscillations). Sleep-related oscillations (SROs) in neuronal activity are a subset of thalamocortical oscillations that occur during the rapid eye movement (REM) and, more importantly, non-REM (NREM) stages of sleep. T-type calcium channels are an important component of the cellular processes that generate and maintain SROs. In two complementary studies, we examined the effects of ethosuximide (ETX), a purported T-type calcium current inhibitor, and genetic suppression of a T channel subtype on SROs and sleep in mice during ethanol exposure and withdrawal. In the first study, we observed reversal of withdrawal-mediated disruptions in SROs with ETX treatment, which we predicted would help stabilize sleep during withdrawal. In the second study, we observed increased

stability of sleep during withdrawal in both ETX-treated and genetically modified mice. These results suggest, for the first time, an important role for T-type calcium channels in the treatment of AWI. Furthermore, these studies lay the groundwork for investigating the potential use of ETX, an FDA-approved drug with a minimal side effect profile, in the treatment of AWI.

# CHAPTER 1

# SLEEP-RELATED THALAMOCORTICAL OSCILLATIONS AND THE INSTABILITY OF SLEEP IN ALCOHOL WITHDRAWAL-RELATED INSOMNIA

## Introduction

Chronic alcohol abuse results in a wide array of deleterious effects on the central nervous system (CNS). Upon withdrawal from alcohol, these effects result in widespread CNS hyperexcitability leading to alcohol withdrawal syndrome (AWS). AWS comprises an array of symptoms including difficulty sleeping, anxiety, and – in severe cases – seizures. These effects relate back to both the toxic effects of ethanol as well as the accommodation of the CNS to the effects of chronic ethanol exposure. Difficulty sleeping is a common complaint related to clinicians by abstinent alcoholics and alcoholics undergoing withdrawal (Brower et al., 2001a). As a result, the impact of ethanol exposure and withdrawal on sleep has been examined in a plethora of studies conducted since the late 1960s.

More recently, the severity of alcohol withdrawal-related insomnia (AWI) has been correlated with the likelihood of relapse into alcohol use (Brower, Aldrich, & Hall, 1998; Brower et al., 2001b; Drummond et al., 1998; Gillin et al., 1994). While it is still unknown whether successful treatment of AWI can prevent relapse, AWI is nevertheless an important clinical problem in the treatment of AWS. Despite a considerable increase in knowledge with respect to the mechanisms of ethanol's effects on the CNS and sleep, successful treatment of AWI remains elusive.

The effects of ethanol on sleep in both alcoholics and nonalcoholic control subjects have been well described in the literature. Ethanol disrupts many, if not all, of the major neurotransmitter systems, complicating the study of specific mechanisms and, thus, the development of prospective treatments. Much of the existing literature focuses on the inhibitory effects of chronic ethanol exposure at the level of synaptic

communication. Hence, the primary focus in the study of ethanol exposure and withdrawal relates to effects on GABA and glutamate receptors, the primary inhibitory and excitatory neurotransmitters in the CNS, respectively. However, treatments based on these synaptic mechanisms have demonstrated mixed results. The current first-line therapy for AWS is the benzodiazepine (BZD) class of drugs. Long-acting BZDs, such as chlordiazepoxide, are preferred over short-acting drugs, such as lorazepam, due to decreased potential for abuse of the drugs. Given the abuse potential of these drugs, as well as possible pathology associated with withdrawal after extended treatment with BZDs, these drugs are only recommended for the acute treatment of AWS (Amato, Minozzi, & Davoli, 2011). Whereas, the problem of AWI often persists long after other symptoms of acute AWS have subsided.

Sleep is a complex neurobiological phenomenon characterized by widespread network oscillations and the involvement of various hormones, neuromodulators, and ion channels in the induction and maintenance of sleep. Therefore, a more complete understanding of ethanol-mediated sleep disruption will likely be necessary in the development of successful treatments for AWI. Furthermore, the most prominent features of AWI are those of sleep fragmentation and the instability of sleep states, implicating a potential role for the stability of sleep-related oscillations in the pathophysiology of AWI. Thus, in the ensuing review of the literature, we form a basis for the investigation of sleep-related oscillations as a mechanism and treatment for AWI, founded upon existing clinical evidence and recent work in our lab.

**Effects of Acute Alcohol Consumption on Sleep**

It is well known that ethanol is a CNS depressant that exerts a prominent sedating effect on nonalcoholics and sober alcoholics when administered at high doses or at stable blood ethanol concentrations (BECs). Interestingly, at low doses of ethanol with rising BEC, ethanol can exert excitatory effects. Furthermore, an excitatory rebound effect occurs once ethanol has been sufficiently metabolized after the cessation of drinking (Pohorecky, 1977). This biphasic relationship between BEC and the nature of its effects on the CNS may help explain the effects of acute ethanol consumption on sleep.

The most common subjective method for measuring sleep employs several physiologic measures, collectively termed polysomnography (PSG). Since the advent of this technique in the 1950s (Aserinsky & Kleitman, 1953), sleep is divided into two major stages – rapid eye movement (REM) sleep and non-REM (NREM) sleep. In humans, NREM is subdivided into four stages of increasing depth of sleep (as determined by the increasing level of stimulation required for arousal). Stages 3 and 4 are often referred to as slow-wave sleep (SWS) due to the predominance of low frequency oscillations in the electroencephalogram (EEG). In the absence of drugs or pathology, sleep occurs in approximately 90-minute cycles interrupted by periods of brief wakening (BW). The first half of the night is characterized by increasing depth of NREM sleep (i.e. increasing percent of sleep as SWS, SWS%) with REM% increasing in the second half of the night, predominantly occurring towards the end of a sleep cycle (for review, see Hobson, 2005).

Since the late 1960s, numerous clinical studies have examined the effect of acute ethanol consumption on sleep in both nonalcoholics and sober alcoholics. In several studies, consumption prior to bedtime was found to increase NREM% and/or SWS%

during the first half of the night, while decreasing NREM/SWS% during the second half of the night (Allen et al., 1971; Gross, Goodenough, Hastey, & Lewis, 1973; Johnson, Burdick, & Smith, 1970; MacLean & Cairns, 1982; Mello & Mendelson, 1970; Rundell et al., 1972; Wagman & Allen, 1975; Williams, MacLean, & Cairns, 1983; Yules, Freedman, & Chandler, 1966). This initial sedating effect of ethanol was reduced when ethanol was consumed 4 hours prior to bedtime (Yules, Lippman, & Freedman, 1967). Furthermore, tolerance to this sedating effect is often developed over 4-5 consecutive nights of ethanol consumption, but the effect has been observed to return after a period of abstinence (Williams & Rundell, 1981). Many of these studies also observed a suppression of REM sleep during the first half of the night (Gross et al., 1973; MacLean & Cairns, 1982; Rundell et al., 1972; Williams et al., 1983; Yules et al., 1966). In a subset of studies, increases in REM% over baseline values – termed REM "rebound" were observed during the second half of the night (Yules et al., 1966, 1967).

Inhibitory mechanisms are commonly thought to mediate the onset and maintenance of NREM sleep, whereas excitatory mechanisms are thought to mediate REM sleep (see España & Scammell, 2011, for a thorough review of the neurobiology of sleep). Thus, it is assumed that the potentiation of NREM and suppression on REM seen in the first half of the night are due to inhibitory effects ethanol, whereas REM rebound is thought to be due to metabolic rebound following sufficient metabolism of ethanol towards the middle of the night (Gross et al., 1973).

Given the sedating effect of acute ethanol consumption, many patients admit to using ethanol to facilitate sleep. At least 33% of insomnia patients admit to using ethanol to help them get to sleep (Brower, 2003). On the other hand, young adults who report

getting less sleep (< 7 hours/night) are more likely to be heavy alcohol users than those who report getting more sleep (Schuckit & Bernstein, 1981). These findings led the authors of this study to formulate the hypothesis that difficulty sleeping increases one's risk for developing alcohol dependence. Interestingly, in a study of chronic alcoholics, prior use of ethanol to facilitate sleep was not associated with an increased risk of relapse (Brower et al., 2001b).

In summary, acute ethanol consumption exerts a prominent sedating effect in both nonalcoholics and alcoholics, resulting in increased NREM sleep with suppression of REM during the first half of the night. This sedating effect provides the motivation for the use of ethanol as a sleep aid. However, this effect is short-lived, as the metabolism of ethanol during sleep results in a rebound effect during the second half of the night. Furthermore, tolerance to the sedating effect is developed in a matter of days.

**Sleep after Withdrawal from Alcohol**

The tolerance to the sedating effect of acute ethanol developed over a period of days is attributed to the adaptation of the brain to repeated ethanol exposure (Roehrs & Roth, 2001). Over periods of chronic ethanol abuse, it is well established that the brain continues to change as a result of both this adaptation to exposure and ethanol toxicity. Chronic alcoholics undergoing acute withdrawal from ethanol (on the order of days-to-weeks) demonstrate significantly fragmented sleep as demonstrated by increases in episodes of brief wakening (BW) during the night and increases in the number of transitions between sleep stages during a sleep cycle (Adamson & Burdick, 1973; Allen et al., 1971; Drummond et al., 1998; Johnson et al., 1970; Williams & Rundell, 1981).

Several studies have also noted a significant decrease in SWS and/or NREM sleep during the acute withdrawal period (Allen et al., 1971; Gross et al., 1973; Wagman & Allen, 1975; Williams & Rundell, 1981). The effect of acute withdrawal on REM sleep has been more variable, with some studies describing REM rebound during acute withdrawal (Johnson et al., 1970; Williams & Rundell, 1981) and others observing no change or decreases in REM sleep (Adamson & Burdick, 1973; Allen et al., 1971; Snyder & Karacan, 1985; Wagman & Allen, 1975). In a behavioral study of alcoholics undergoing acute withdrawal, subjective measures of sleep and reporting of sleep quality were found to be highly correlated with the more objective EEG-based findings (Mello & Mendelson, 1970).

While some alterations in sleep are reversed with continued abstinence (or subacute withdrawal, on the order of weeks-to-months), sleep fragmentation appears to persist after 1-2 years of abstinence (Drummond et al., 1998; Gillin et al., 1990). However, this finding was not consistent across all subjects studied, with some subjects' objective sleep measures normalizing after several months of abstinence (Drummond et al., 1998). Furthermore, age seems to be an important factor, with older alcoholics experiencing more severe and protracted sleep disruption than younger alcoholics (Brower & Hall, 2001).

The severity of sleep disruption during subacute withdrawal and sustained abstinence has been correlated with both decreased quality of life and increased risk of relapse (Brower et al., 1998; Brower et al., 2001b; Cohn, Foster, & Peters, 2003; Drummond et al., 1998). As mentioned in the previous section, it is interesting to note that despite this relationship, past history of using ethanol to facilitate sleep as an

independent variable was not found to be associated with increased risk of relapse (Brower et al., 2001b). Furthermore, it is yet unresolved as to whether treatment of alcohol withdrawal-related insomnia (AWI) itself is preventative of relapse (Roth, 2009). However, the combination of decreased quality of life, increased risk of relapse, and increases in all-cause mortality with long-term sleep deprivation (Cappuccio et al., 2010; Grandner et al., 2010) make the treatment of AWI a very important clinical problem.

## The Neurobiology of Proposed Treatments for Alcohol Withdrawal-Related Insomnia

In order to understand the motivation behind some of the proposed therapies and the pitfalls of others, it is important to understand what is known about the neurobiology of alcohol as it relates to sleep-promoting mechanisms in the brain. Ethanol is a small, lipid-soluble alcohol that affects a wide array of ion channels and receptors in the brain. It is best known for its direct effects on receptors of γ-amino-butyric acid (GABA), the primary inhibitory neurotransmitter in the brain. However, the initial focus of sleep research with respect to alcohol withdrawal was on the monoamine neurotransmitter serotonin.

While early theories of sleep implicated serotonin and norepinephrine as the principal neurotransmitters involved in the induction and maintenance of sleep (Jouvet, 1969), a more recent study demonstrated that serotonin receptor antagonists actually increased SWS in humans (Sharpley et al., 1994). These findings may explain why early attempts to treat AWI with serotonin analogs were met with questionable success (Asheychik et al., 1989; Zarcone, 1978). Despite the uncertain role of serotonin in sleep,

compounds with monoaminergic effects used to treat depression have been selected by clinicians for use in the treatment of AWI because of their sedative side effects (Friedmann et al., 2008). It is possible that the efficacy these compounds demonstrate in AWI is related to their actions on the norepinephrine (NE) system. Mice genetically modified to lack the enzyme responsible for synthesis of NE in the brain demonstrated enhanced sensitivity to the sedative effect of ethanol (Weinshenker et al., 2000). Over the course of chronic exposure, it is hypothesized that NE activity is upregulated to counteract this effect, as evidenced by increased NE activity during withdrawal (Hawley et al., 1985). Increased NE has an excitatory effect on the cortex and may, thus, contribute to the sleep fragmentation observed during sleep in abstaining chronic alcoholics.

Given the mixed results with the monoamine neurotransmitters, the focus in recent years has returned to effects on GABA. Initial reports suggested that ethanol directly enhances inhibitory ion conductance of postsynaptic $GABA_A$ receptors (Suzdak et al., 1986). More recent studies have suggested a primary effect on extrasynaptic $GABA_A$ receptors, producing a form of tonic inhibition on a wide variety of neuronal subtypes (Santhakumar, Wallner, & Otis, 2007). Interestingly, the balance between synaptic and extrasynaptic effects may reverse over the course of chronic ethanol exposure (Liang et al., 2006).

Compounds that potentiate the $GABA_A$ receptor have demonstrated efficacy in both animal and human studies of alcohol withdrawal (Aubin, Barrucand, & Auzépy, 1993; Rouhani et al., 1998). However, the majority of such compounds approved for clinical use (i.e. benzodiazepines and barbiturates) are burdened with a high potential for

abuse and significant withdrawal-mediated toxicity and, thus, remain controversial in the treatment of AWI (Roth, 2009). Recently, attention has turned to gabapentin, a medication developed as an antiepileptic drug that is also used for the treatment of neuropathic pain. Gabapentin was originally developed as a GABA analog, but has been subsequently shown to inhibit neuronal trafficking of voltage-gated calcium channels (Hendrich et al., 2008). This may result in effects on neuronal plasticity, as well as changes in probability of synaptic release and intrinsic (membrane) excitability of central neurons (Davies et al., 2007). The exact mechanism of gabapentin-mediated CNS inhibition is still under debate, but it has nevertheless demonstrated significant improvements in AWI in direct comparison with monoaminergic-based therapies without the controversy surrounding benzodiazepines (Karam-Hage & Brower, 2003; Myrick et al., 2009). Furthermore, gabapentin has also been shown to significantly decrease other symptoms of alcohol withdrawal and reduce the probability of drinking in the immediate post-withdrawal period (Myrick et al., 2009). However, the effects of gabapentin on AWI were not found to be different from placebo in a randomized, placebo-controlled trial (Brower et al., 2008).

The difficulties encountered in attempts to find an effective therapy for AWI have inspired many investigations of alternative uses for currently available drugs based on a combination of the known effects of ethanol on the neurobiology of the brain and known sedative side effects of drugs approved for other neuropsychiatric disorders. A clear efficacious therapy has not emerged from these attempts, nor has it been determined that successful treatment of AWI can, itself, prevent relapse to alcoholism. Much of the difficulty has arisen out of an uncoordinated approach between basic science and

clinicians. In the era of translational medicine, the solution to this problem may lie outside the classical approach of focusing on GABA and the other major neurotransmitter systems.

**Alcohol, Thalamocortical Oscillations, and Sleep**

Central neurons receive input within and between functional areas of the brain in the form of electrical impulses from other neurons. The electrical impulses are believed to carry information with respect to the sensory environment; active behavioral plans, as well as feedback from other systems in the body; and the internal state of the brain. These inputs are integrated in the dendritic tree of a neuron, generating a net potential that can be measured as electrical signal emanating from the region surrounding the neuron. Depending on the distance and electromagnetic properties of the recording device, these signals emitted from the region surrounding single neurons are summed to generate a population potential representing hundreds or thousands of neurons (Logothetis, 2008). Greater synchrony of inputs in time will generate a stronger, more regular potential. As the activity in a region changes, these potentials fluctuate. In practice, the potential is recorded with respect to another region of the brain (called a reference). Thus, changes in incoming activity generate fluctuations in the region that are often described as oscillations, a term borrowed from mathematics and physics due to the cyclical nature of these variations about the referenced signal. Hence, when the term "oscillations" is used in this manuscript, this refers to the synchronized integration of inputs across a region.

Not long after the invention of EEG revealed the first oscillations measured in the brain, the first investigation into the role of the thalamus as an oscillator demonstrated an

intricate relationship between thalamic and cortical oscillations (Dempsey & Morison, 1941). Later investigations revealed significant interactions between the thalamus and cortex during spindle oscillations observed in anesthetized cats (Andersen, Andersson, & Lomo, 1967). The spindles observed in these animals were quite similar to those observed in EEGs recorded during human sleep, thus, inspiring the theory that both types of spindles are generated by a common mechanism. Two independent groups identified intrinsic oscillatory activity capable of generating such rhythms in the isolated thalamus in a series of experiments in the 1980s (Llinás & Jahnsen, 1982; Steriade & Deschenes, 1984; Steriade & Llinás, 1988). It was subsequently shown that intrinsic thalamic oscillations during anesthesia and natural sleep were generated as a result of transitions between tonic (or regular) firing and burst firing modes of thalamic neurons, mediated by the voltage-dependent properties of T-type calcium current (Domich et al., 1986; Jahnsen & Llinás, 1984a, 1984b; Steriade et al., 1993). Such transitions are also implicated in other sleep-related oscillations, such as delta waves and the slow oscillation (Dossi, Nuñez, & Steriade, 1992).

The thalamocortical sleep-related oscillations (spindles, delta waves, theta rhythm, and the slow oscillation) are important components of the EEG recorded from the scalp or cortex during SWS (Llinás & Steriade, 2006). According to Steriade (2005), the slow oscillation describes a 0.5-4 Hz transition from periods of low cortical activity in a local region (or "down" state) to periods of desynchronized activity not unlike that found in the waking state ("up" state). When measured across larger regions of the brain, this activity appears as delta waves in the EEG – the hallmark of SWS. Sleep spindles are more commonly observed during up states and the frequency of their occurrence has been

correlated with increased memory consolidation (Diekelmann & Born, 2010). However, a recent study found that interrupting the continuity of sleep impaired memory independent of sleep time or depth (Rolls et al., 2011). Depth of sleep has also been implicated as important contributor to the restfulness of sleep (Benington & Heller, 1995). Taking the above results together, investigation into potential alterations in the mechanisms leading to the generation and maintenance of sleep-related thalamocortical oscillations could prove vital in the study of alcohol's effects on sleep.

As mentioned above, the T-type calcium current (or simply T current) plays a critical role in the generation of oscillatory activity in the thalamus. Three isoforms of the T-type calcium channel have been identified (see Perez-Reyes, 2003, for review). Ca(v)3.1 is the predominant isoform expressed in the brain and demonstrates fast inactivation kinetics. Whereas, Ca(v)3.2 is more widely distributed in the periphery and demonstrates slower inactivation kinetics and a slower recovery from inactivation. Ca(v)3.3 is expressed at similar levels to Ca(v)3.2 in the thalamic reticular nucleus and the hippocampus – where these two isoforms predominate over Ca(v)3.1 – and exhibits slower inactivation kinetics with a more rapid recovery from inactivation (Chemin et al., 2002; Klöckner et al., 1999; McRory et al., 2001; Talley et al., 1999). The differential kinetics of these isoforms gives rise to the unique firing profiles of neurons expressing high levels of T channels.

T current is typically activated after a period of hyperpolarization of thalamic neurons, such as occurs during the down state of SWS (Huguenard, 1996). Ca(v)3.1 is thought to be the primary source of T current in cortically projecting thalamic neurons, such as those found in the primary and higher-order "relay" nuclei; whereas, Ca(v)3.3

contributes significantly to the T current of reticular neurons, found in the thalamic reticular nucleus (Talley et al., 1999). The slower inactivation of Ca(v)3.3 and faster recovery from inactivation results in a firing profile consisting primarily of intense, rhythmic bursts in reticular neurons (Chemin et al., 2002). These reticular neurons project onto the relay neurons; thus, setting the pace of thalamocortical oscillations. Mice genetically modified to lack the Ca(v)3.1 and Ca(v)3.3 subtypes of T-type calcium channels demonstrate fragmented sleep and virtual absence of sleep spindles, respectively (Anderson et al., 2005; Astori et al., 2011). The role of Ca(v)3.2 in sleep is, as yet, undetermined. Certain alterations in T channels, resulting in a net increase in T current at more depolarized potentials may underlie pathological synchronization of thalamocortical oscillations (Becker et al., 2008; Graef et al., 2009; Jeanmonod, Magnin, & Morel, 1996; Steriade, 2005).

Several studies have examined the effects of ethanol on T-type calcium channels in thalamic neurons. Similar to the biphasic effects of ethanol on sleep, ethanol appears to exert biphasic effects on T current in thalamic neurons. At low concentrations, ethanol was found to enhance T current; whereas, at higher concentrations, ethanol inhibited T current (Mu et al., 2003). Recent work in our lab suggests that this effect of ethanol is selective for the Ca(v)3.2 isoform (Shan, Hammarback, & Godwin, unpublished data), as suggested by earlier results in another lab using octanol, a longer-chain alcohol (Joksovic et al., 2005). Furthermore, chronic intermittent ethanol (CIE) vapor administration in mice resulted in a progressive depolarizing shift in the inactivation of T current in midline thalamic neurons that persisted during withdrawal (Graef et al., 2011). Interestingly, this depolarizing shift appeared to demonstrate a dose-response relationship

with increasing numbers of exposure/withdrawal cycles reminiscent of the "kindling" hypothesis of alcohol withdrawal, for which the CIE paradigm was developed as a model (Becker & Hale, 1993). Under the "kindling" hypothesis, successive withdrawals from ethanol increase the severity of withdrawal symptoms (including sleep), similar to the progressive increase in severity of seizures observed in the kindling model of epilepsy – an effect supported by studies in chronic alcoholics (Brown et al., 1988).

Ethanol has also been shown to disrupt diurnal patterns of T channel expression in the midline thalamus (Nordskog, Hammarback, & Godwin, 2006). In the CIE model, alterations in T current voltage-dependence in the midline thalamus were associated with suppression of the diurnal variation in theta band (4-8 Hz) power in the cortical EEG (Graef et al., 2011). Theta power is particularly increased in the cortical EEG during NREM and REM sleep, though moderate theta power is normally observed during active wake (Lancel, 1993). Significant thalamocortical theta coherence is observed during NREM sleep in humans (Tsai et al., 2010). However, pathological increases in thalamocortical theta coherence during the waking state have been observed in a various neurological and psychiatric disorders, inspiring the term "thalamocortical dysrhythmia", used to describe a state of pathological thalamocortical oscillations (Llinás et al., 1999). Thus, the suppression in the diurnal pattern of theta power in the cortical EEG may represent a systems-level correlate of altered thalamocortical oscillations and, hence, T current dysregulation in the thalamus as a result of alcohol withdrawal. These findings carry significant implications for the proper generation and maintenance of thalamocortical oscillations following withdrawal from chronic ethanol. Furthermore,

they open up the possibility of T-type calcium channels as a target for pharmacologic therapy in AWI.

## Conclusion

Recent studies in animals and humans have begun to reveal the importance of thalamocortical oscillations in the proper maintenance of sleep and, conversely, the inappropriate appearance of these oscillations in the waking period as a potential contributor to neurological and psychiatric pathology. While there are significant associations between abnormal thalamocortical oscillations and known effects of alcohol withdrawal on sleep, the role of these oscillations in the pathology of AWI requires further investigation. Furthermore, it must be determined whether improvement in these abnormal rhythms is associated with improved outcomes in AWI. Future studies should investigate the therapeutic potential of drugs designed to inhibit the mechanisms leading to instability of sleep-related oscillations and subsequent fragmentation of sleep after withdrawal from chronic alcohol.

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# CHAPTER 2

# ETHOSUXIMIDE REDUCES ETHANOL WITHDRAWAL-MEDIATED DISRUPTIONS IN SLEEP-RELATED EEG PATTERNS

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**Abstract**

BACKGROUND: Chronic ethanol leads to disruptions in resting EEG activity and in sleep patterns that can persist into the withdrawal period. These disruptions have been suggested to be predictors of relapse. The thalamus is a key structure involved in both normal brain oscillations, such as sleep-related oscillations, and abnormal rhythms found in disorders such as epilepsy and Parkinson's disease. Previously, we have shown progressive changes in mouse thalamic T-type $Ca^{2+}$ channels during chronic, intermittent ethanol (CIE) exposures that occurred in parallel with alterations in theta (4-8Hz) EEG patterns. METHODS: Two groups of eight-week old male C57BL/6 mice were implanted with wireless EEG/EMG telemetry and subjected to 4 weeks of CIE vapor exposure and withdrawal. During the week after the final withdrawal, mice were administered ethosuximide (200 mg/kg) or saline. EEG data were analyzed via discrete Fourier transform and sleep scored for further analysis. RESULTS: CIE exposure produced changes in the diurnal rhythms of the delta (0.5-4Hz) and theta bands that persisted into a subsequent week of withdrawal. These disruptions were prevented with the T-channel blocker ethosuximide. Repeated ethanol exposures preferentially increased the relative proportion of lower frequency power (delta and theta), whereas higher frequencies (8-24Hz) were decreased. The ethanol-induced decreases in relative power for the higher frequencies continued into the subsequent week of withdrawal for both groups. Increases in absolute delta and theta power were observed in averaged NREM and REM sleep spectral data during withdrawal in ethosuximide-treated animals, suggesting increased sleep intensity. CONCLUSIONS: These results suggest that persistent alterations in delta and theta EEG rhythms during withdrawal from chronic intermittent ethanol exposure

can be ameliorated with ethosuximide and that this treatment might also increase sleep intensity during withdrawal.

## Introduction

Preventing relapse after withdrawal from alcohol represents a significant hurdle in overcoming alcohol dependence. Chronic alcohol use leads to disruptions in resting EEG activity and in the sleep patterns of alcoholics that can persist into the withdrawal period, where they have been suggested to be effective predictors of relapse (Bauer, 2001; Brower, 2001; Brower and Perron, 2009). In rodents, repeated ethanol exposures and withdrawals that model the binge/abstain consumption patterns of human alcoholics (Becker and Hale, 1993) have also resulted in similar EEG and sleep alterations (Ehlers and Slawecki, 2000; Veatch, 2006). It is still unresolved whether restoring normal sleep patterns alone can prevent relapse (Friedmann et al., 2008; Brower and Perron, 2009); however, understanding the underlying mechanisms and brain structures involved in progressive EEG and sleep pattern alterations that occur during chronic alcoholism and continue into protracted withdrawal will help provide the basis for additional avenues of adjunct therapies in the treatment of alcohol dependence.

The thalamus is a key brain structure involved in the generation and maintenance of normal brain rhythms during sleep, as well as different states of vigilance (Llinas and Steriade, 2006). Abnormal thalamic activity has also been implicated in several pathological conditions that are marked by abnormal increases in thalamocortical theta coherence (Sarnthein and Jeanmonod, 2007, 2008) and have been described as thalamocortical "dysrhythmias" (Jeanmonod et al., 1996; Llinas et al., 1999). These dysrhythmias are characterized by enhanced activity of T-type $Ca^{2+}$ channels (Steriade, 2005; Jeanmonod et al., 1996; Nelson et al., 2006), which generate characteristic bursts of action potentials that support intrinsic neuronal oscillations (Huguenard, 1996).

Results from our lab (Graef et al., 2011) have demonstrated that chronic ethanol exposure also produces alterations in thalamic T-type channel expression and function that occur in parallel with disruptions in EEG theta activity. These changes persisted into a subsequent week of withdrawal, but were ameliorated with the relatively selective T-type channel blocker ethosuximide (ETX). Such disruptions are consistent with observations of abnormal EEG activity in alcoholics (Porjesz and Begleiter, 2003; Rangaswamy et al., 2003), including increases in interhemispheric theta coherence that have also been suggested to arise from altered thalamocortical function (Porjesz and Rangaswamy, 2007).

In this study, we expanded upon our previous findings by investigating the effects of chronic intermittent ethanol exposures on the rhythmic patterns and relative power of different EEG power bands and the average spectrum during two types of sleep. We also assessed the efficacy of ETX as a pharmacologic intervention during a subsequent week of withdrawal. We found that chronic ethanol induced disruptions in the diurnal pattern of sleep-related EEG rhythms that continued into a subsequent week of withdrawal and could be restored to baseline rhythms with ETX. Furthermore, we observed significant increases in the absolute spectral power of these rhythms during REM and non-REM sleep in response to ETX treatment. These results provide new data on changes in important sleep-related rhythms that are elicited by withdrawal from ethanol, and further implicate T-type channel involvement in these CNS alterations.

## Materials and Methods

*Animals and Experimental Design*

All experiments were conducted with the advanced approval of the Institutional Animal Care and Use Committee at Wake Forest University School of Medicine. Ethanol was chronically administered by the inhalation route previously described by Becker and Hale (1993). Briefly, 18 individually housed 8-week old C57Bl/6 male mice (*Harlan, Inc. Indianapolis, IN, USA)* were placed in a sealed Plexiglas vapor chamber modified after Goldstein (1972) in a room with a 12hr light/dark schedule with lights on at 5am. Ethanol (95%) was volatilized and delivered to one of the chambers at a rate of 2.0 L/min by a vacuum pump. This, in combination with air being delivered to the chambers at a rate of 20 L/min, maintained the ethanol concentration in the chamber in the range of 14–16 mg per L of air (mean±SEM: 15.6±0.5mg/L). An 8hr period of abstinence allowed complete clearance of ethanol from circulation prior to the next cycle of intoxication (Becker and Hale, 1993; Becker, 1994). At the beginning of each exposure cycle (5pm), all mice (whether receiving room air or ethanol vapor) were treated with a subcutaneous injection of the alcohol dehydrogenase inhibitor pyrazole (100 mg/kg). Pyrazole is commonly employed in the CIE paradigm to stabilize blood-ethanol concentrations (BECs) over the course of repeated ethanol exposures. Pyrazole was prepared daily by dissolving in sufficient saline to achieve an injection volume of approximately 0.2 mL per animal. BECs achieved under these conditions remained relatively stable from one bout of intoxication to the next. Mean BECs were 221.9±12.7, 189.0±21.0, 149.3±11.2, and 155.5±10.5 mg/dl for weeks 1-4 of exposure, respectively. The mean BEC was 186.0-±9.4mg/dl for all four weeks of exposure. BECs for all mice were measured by taking 5µl

blood samples from the tail (stored in vials containing 45µl of 6.25% trichloroacetic acid) and analyzed using a NAD-ADH enzyme assay (Diagnostic Chemicals, Oxford, CT).

*Data Acquisition*

Mice were implanted subcutaneously with telemetric physiologic monitors (Model F20-EET; Data Sciences International (DSI), Arden Hills, MN) that simultaneously record electroencephalogram (EEG), electromyogram (EMG), temperature and activity. Briefly, animals were anesthetized with isoflurane followed by the implantation of electrodes for recording EEG signals and EMG signals. For placement of EEG wire leads, holes slightly larger than the coil diameter of the transmitter lead wire were drilled in the skull 2 mm on either side of midline suture half way between bregma and lambda. The exposed portions of the leads were placed between the skull and underlying dura. Wires were secured to the skull with dental acrylic. EMG leads were placed in the neck muscles and secured with sutures. The signal transmitter body was placed subcutaneously over the dorsal thorax. Mice were allowed to recover from surgery for one week prior to recording. The exposure paradigm during the 6-week recording period was as follows: one week of baseline and four weeks of intermittent ethanol exposure (5pm-9am on days 1-5, no exposure days 6 and 7), followed by 1 week of chronic withdrawal. One EEG and one EMG channel were continuously acquired over the entire six-week paradigm using the Dataquest A.R.T. acquisition system (DSI, Arden Hills, MN) at a sampling frequency of 500Hz. The EEG data was then band-pass filtered from 0.5-100Hz and the EMG data filtered from 10-100Hz for analysis. Average activity counts were obtained every 10s. During the withdrawal week, mice received isovolumic (0.2mL) injections of either

saline or 250mg/kg ethosuximide (ETX) daily at 5pm (corresponded to time of beginning of ethanol during exposure weeks 1-4) for the first 5 days. ETX was prepared by dissolution in sufficient saline to achieve an injection volume of approximately 0.2 mL per animal.

*Cosinor Analysis of EEG*

EEG Data were divided into 10-s epochs and analyzed with a conventional Discrete Fourier Transform (DFT) power spectra function using the software program NeuroScore (DSI, Arden Hills, MN). Using a customized MATLAB (The MathWorks, Natick, MA) program, epochs were then filtered into five power bands: delta (0.5-4Hz), theta (4-8Hz), alpha (8-12Hz), sigma (12-16Hz) and beta (16-24Hz). Normalized power was obtained by dividing the power in each 10-s epoch by the maximum power within its respective 24-hr period (5pm-5pm) for each frequency band. Normalized power was then further averaged into 1-h bins for Cosinor analysis (Nelson et al., 1979). Relative power for each band was determined by dividing the raw spectral power for that band by the total power for all frequencies analyzed (0.5-25Hz). Diurnal patterns were analyzed with a custom Cosinor analysis function written in MATLAB on all 24hr periods over the course of the six-week exposure paradigm for each animal. The Cosinor function, given by the following equation:

$$C(t) = \text{Mesor} + A*\cos[2\pi(t - \text{Acrophase}) / P]$$

determines through least squares approximation the Mesor (midline estimating statistic of rhythm), amplitude (A) and time of peak (Acrophase) values for the fitted cosine function from a predetermined set phase (P). For all animals, a custom MATLAB function was

written so that each 24-hr period (5pm-5pm) over the 6-week paradigm was double-plotted and fitted with Cosinor curves using a phase range of 18-32hrs in 10-m increments. A zero-amplitude test was then performed for each fit, using error estimates for the least-squares fit of the amplitude, to determine whether the amplitude of the fitted curve was significantly different from zero. The parameters from the best Cosinor fit, as determined by the lowest *p*-value, were returned. Data was then grouped and averaged for all 24hr baseline periods, exposure periods (weeks 1-4, days 1-5) and withdrawal periods (week 5, days 1-5), excluding any 24-hr periods that failed the zero-amplitude test (as indicated by the best Cosinor fit having a *p*-value greater than 0.05). Statistical analysis was performed between weeks both within each treatment group and between treatment groups.

*Sleep Scoring*

EEG, EMG, and activity data were sleep-scored in 10-s epochs using a customizable rodent sleep scoring algorithm available with NeuroScore. The primary measures employed by this algorithm are the delta and theta power bands, delta ratio, theta ratio, EMG threshold, activity threshold, and EMG and EEG artifact thresholds. These settings were adjusted for the analysis of each week for each animal according to manual verification of randomly selected sleep-scored epochs to account for individual variations in signal strength and noise over time. The algorithm proceeds as follows for each epoch analyzed: Any epoch containing a significant amount of artifact is discarded. If activity or EMG signal exceed their respective thresholds, the epoch is scored as wake. If the first condition is not met and the delta ratio exceeds its threshold, the epoch is scored as

NREM sleep. If the previous two conditions are not met and the theta ratio exceeds its threshold, the epoch is scored as REM sleep. If none of the preceding criteria are met, the default scoring is wake. The delta and theta bands remained constant for all animals and all time points at 0.5-4Hz and 6-9Hz, respectively, consistent with previous studies in this model (Veatch, 2006). As compared to the bands used in the analyses above, the theta band was narrowed for the sleep scoring algorithm. This was done to isolate the theta peak observed during REM sleep from any overlap with delta activity and, thus, minimize the number of false positive REM epoch identifications. The delta ratio is defined as the ratio of delta power to the total power in the spectrum from 0.5-25Hz and ranged from 0.35-0.45 in the present analysis. The theta ratio is defined as the ratio of theta power to delta power and ranged from 2-3. The EMG threshold ranged from 10-50μV, depending on the noise in the signal. The activity threshold remained constant across all animals at 0.1 counts per minute. Finally, the EEG and EMG artifact thresholds ranged from 0.5-1.0mV. Sleep epochs were scored as rapid eye movement (REM), non-REM (NREM), wake (W), or artifact, consistent with previous studies in this model (Veatch, 2006).

*Spectral Analysis of Vigilance States*

The DFT power spectrum of each sleep-scored epoch was exported in 1Hz bins from 1-25Hz in order to analyze the average spectral characteristics of individual vigilance states (NREM, REM, and W) across baseline, exposure, and withdrawal weeks. A custom MATLAB function was written to normalize each spectral bin to its maximum value in a 24-hr period. The function then binned the spectra by their associated vigilance state and

averaged the normalized power across all epochs for each animal in each week. The end result was an average spectrum for each vigilance state across the baseline, exposure, and withdrawal weeks, with the exception that the exposure week was further divided into averages for exposure and withdrawal days, respectively.

*Statistical Analysis*

Statistical analysis was performed between weeks both within each treatment group and between treatment groups using either MATLAB or Prism (Graph Pad Software, La Jolla, CA). One- or two-way ANOVA was used to assess effects of ethanol and/or withdrawal treatment for each group. Post-hoc tests were either Newman-Keuls Multiple Comparison tests or unpaired *t*-tests for planned comparisons. All statistical tests are specified in the Results section at first mention, and then clarified with any change in the choice of test.

## Results

*Multiple Intermittent Ethanol Exposures and Withdrawals Disrupt Diurnal EEG Patterns*

In order to determine the effects of chronic, intermittent ethanol exposures on frequency band patterns, surface EEG for two groups of mice (n = 5 in each group) was continually recorded for one week of baseline, four weeks of multiple intermittent ethanol exposures and one week of subsequent withdrawal.

Figure 1A shows that during the baseline week, a diurnal pattern can be seen for theta frequencies (4-8Hz), with lower normalized power exhibited during the animals' dark (active) period as compared to the light (inactive) period. In order to statistically compare variations in this diurnal rhythm, a Cosinor function was used to fit a cosine curve to each double-plotted 24-hr period for all power bands. The parameters derived from the fitted curves include the Mesor, which gives the estimated midline of the rhythm, the amplitude between the peak and trough of the curve, the time at which the peak of the curve occurs, and the length of the period. Figure 1 illustrates fitted Cosinor curves to the average normalized theta power for the baseline week (A), the fourth week of ethanol (B), and the subsequent withdrawal week for both saline (C) and ETX-treated mice.

Analysis between the baseline power band rhythms and the fourth week of intermittent ethanol exposures revealed that chronic ethanol significantly increased the Mesor for the theta power band ($F_{2,62} = 34.42$, $p < 0.001$, one-way ANOVA with Newman-Keuls *post-hoc*), as well as significantly decreasing the Mesor for the beta frequency ($F_{2,47} = 25.97$, $p < 0.05$; see Table 1). Additionally, the amplitude between the peak and trough of the fitted curve was significantly decreased for delta and theta

frequencies ($F_{2,47}$ = 7.762, $p < 0.05$, and $F_{2,47}$ = 16.64, $p < 0.001$, respectively), and the time of the peak was significantly shifted forward in time for the theta, alpha ($F_{2,47}$ = 3.829, $p < 0.05$), sigma ($F_{2,47}$ = 16.18, $p < 0.05$) and beta ($F_{2,47}$ = 3.728, $p < 0.05$) bands. Chronic ethanol exposure did not have a significant effect on the average length of the period for any frequency band (see Table 1).

These results indicate that multiple intermittent ethanol exposures disrupted diurnal EEG rhythms for the lower delta and theta bands, and shifted the peak of normalized power towards the end of each subsequent withdrawal period for all frequencies greater than 4Hz.

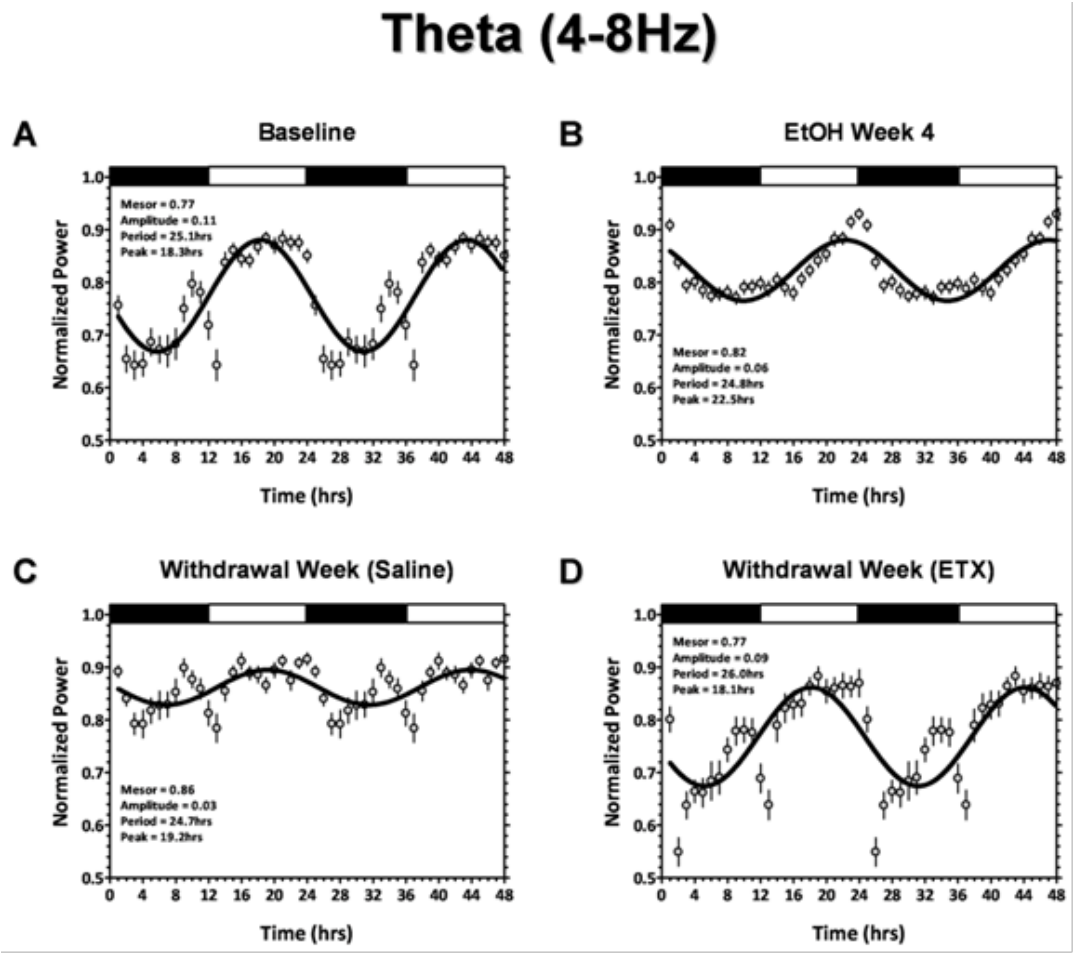


**Figure 1. Cosinor analysis of normalized theta (4-8Hz) EEG band during ethanol exposure and withdrawal.** An average 24-hr theta (4-8Hz) rhythm was compiled by double-plotting the mean of each respective time point during the first five days (M-F) of the baseline week **(A)**, the fourth week of ethanol exposure (EtOH Week 4), and the week of withdrawal with either saline or ethosuximide (ETX) treatment and fitted with a Cosinor function. The values for Mesor, amplitude, period and time of peak for each fitted curve are indicated on the graph. **(B)** The Mesor was significantly increased, the amplitude was significantly attenuated, and the peak was significantly shifted ($p < 0.05$ for each parameter) during EtOH Week 4. **(C, D)** Only the peak was restored in the saline group, whereas the Mesor and amplitude were also restored in the ETX group.

**Table 1.**

| | Mesor | | | | Amplitude | | | |
|---|---|---|---|---|---|---|---|---|
| ***Power Band*** | Base | EtOH (Wk4) | WD (saline) | WD (ETX) | Base | EtOH (Wk4) | WD (saline) | WD (ETX) |
| **Delta (0.5-4Hz)** | 0.80±0.01 | 0.83±0.03 | 0.84±0.02 | 0.81±0.03 | 0.09±0.01 | 0.07±0.01 * | 0.05±0.01 $ | 0.08±0.01 |
| **Theta (4-8Hz)** | 0.77±0.02 | 0.83±0.01 * | 0.85±0.02 | 0.74±0.02 %# | 0.12±0.02 | 0.08±0.01 * | 0.05±0.01 $ | 0.11±0.01 %# |
| **Alpha (8-12Hz)** | 0.75±0.02 | 0.74±0.01 | 0.84±0.04 | 0.71±0.01 # | 0.14±0.02 | 0.11±0.01 | 0.07±0.02 | 0.12±0.01 |
| **Sigma (12-16Hz)** | 0.75±0.02 | 0.75±0.01 | 0.81±0.03 | 0.74±0.02 # | 0.14±0.02 | 0.12±0.01 | 0.08±0.02 | 0.11±0.01 |
| **Beta (16-24Hz)** | 0.82±0.02 | 0.77±0.02 * | 0.82±0.05 | 0.82±0.01 | 0.09±0.01 | 0.11±0.01 | 0.05±0.01 % | 0.07±0.01 |

**Table 1 (cont'd).**

| | Period Length (hrs) | | | | Peak of Rhythm (hr) | | | |
|---|---|---|---|---|---|---|---|---|
| ***Power Band*** | Base | EtOH (Wk4) | WD (saline) | WD (ETX) | Base | EtOH (Wk4) | WD (saline) | WD (ETX) |
| **Delta (0.5-4Hz)** | 25.0±0.2 | 24.7±0.2 | 23.6±0.5 $ | 25.8±0.5 # | 17.6±0.6 | 19.7±1.3 | 17.5±1.7 | 16.4±1.1 |
| **Theta (4-8Hz)** | 25.1±0.2 | 24.8±0.2 | 24.7±0.3 | 26.0±0.3 # | 17.4±0.7 | 20.4±1.1* | 18.6±0.7 | 18.3±0.5 |
| **Alpha (8-12Hz)** | 25.1±0.3 | 25.3±0.2 | 25.8±0.2 | 26.2±0.4 | 18.4±0.5 | 22.0±0.7* | 19.5±0.5 % | 19.0±0.6 % |
| **Sigma (12-16Hz)** | 25.3±0.2 | 25.1±0.2 | 25.4±0.3 | 26.2±0.3 | 19.2±0.5 | 22.8±0.4* | 20.1±0.2 % | 19.3±0.8 % |
| **Beta (16-24Hz)** | 25.2±0.3 | 24.9±0.1 | 24.5±0.2 | 26.3±0.4 % # | 19.6±0.6 | 22.5±0.5* | 21.0±1.1 | 19.3±0.8 |

**Table 1. Values from Cosinor analysis for all EEG bands during baseline, fourth week of ethanol exposure and subsequent week of withdrawal.** (*$p<0.05$, repeated measures ANOVA, with Bonferroni's post test, compared to baseline (n=10; both treatment groups); $^{\$}p<0.05$, repeated measures ANOVA, with Bonferroni's post test, compared to baseline (n=5; within treatment group); $^{\%}p<0.05$, repeated measures ANOVA, with Bonferroni's post test, compared to ethanol week 4 (within treatment group); $^{\#}p<0.05$, unpaired *t*-test (between treatment groups)

*Ethosuximide Restores Altered EEG Patterns during the Withdrawal Period*

Since chronic, intermittent ethanol exposures and withdrawals disrupted diurnal EEG variations seen during the baseline week, we next sought to determine if these changes persisted during the week following the final withdrawal. In addition, we tested the effects of ETX treatment administered daily at the start of the dark period (5pm), corresponding to the beginning of each intermittent bout of ethanol exposure experienced during the previous four weeks.

Figure 1C shows the normalized theta power for saline-treated mice during the withdrawal period (hereafter, referred to as "withdrawal"), while Figure 1D illustrated the normalized power for ETX-treated mice during the same withdrawal week. Cosinor analysis of the theta bands revealed that both the significant increase in the Mesor ($F_{2,62}$ = 34.42, $p < 0.001$) and the significant decrease in amplitude ($F_{2,47}$ = 16.64, $p < 0.001$) observed during ethanol exposure persisted into withdrawal for saline-treated animals, however this returned to baseline levels for ETX-treated animals ($p > 0.05$ for both, when compared to baseline; see Table 1). In addition, the average period length of the fitted cosine curves for the delta band during the withdrawal week was significantly reduced compared to the baseline week for saline-treated mice ($F_{2,62}$ = 21.01, $p < 0.05$), whereas the average period for the fitted curves for ETX-treated animals was not statistically different from the baseline ($p > 0.05$).

While unaffected during ethanol exposure, derived values from the fitted cosine curves for the alpha and sigma bands revealed significantly greater Mesor values during the withdrawal week in saline-treated mice as compared to ETX-treated animals ($p < 0.05$ for both bands, unpaired *t*-test; see Table 1).

Derived values from the Cosinor analysis on the beta power band revealed a significant decrease in the Mesor during the fourth week of ethanol exposure ($F_{2,47}$ = 3.728, $p < 0.05$) that returned to baseline values for both treatment groups. The amplitude was unaffected during ethanol exposure but was significantly reduced during withdrawal regardless of treatment ($F_{2,47}$ = 11.31, $p < 0.05$ for both groups), whereas the period length was unaffected during ethanol exposure and withdrawal in saline-treated mice, but significantly increased during withdrawal in ethosuximide ($p < 0.01$, unpaired *t*-test).

*Ethosuximide Restores Relative Power in Low Frequency Bands during Withdrawal*

In addition to alterations in theta rhythms, we also observed changes in the relative power of all frequency bands over the course of ethanol exposure. During the fourth week of exposure, relative power in the lower frequency range (delta and theta; Figure 2A-B) was increased as compared to baseline measures ($F_{4,168}$ = 8.05, $p < 0.01$, for delta, two-way repeated measures ANOVA with Dunnett Multiple Comparison test; $F_{4,168}$ = 78.95, $p < 0.001$ for theta). Conversely, the higher power bands showed a decrease in relative power, with significant reductions in alpha ($F_{4,168}$ = 19.05, $p < 0.001$), sigma ($F_{4,168}$ = 22.72, $p < 0.001$) and beta ($F_{4,168}$ = 36.68, $p < 0.001$) power during the fourth week of intermittent ethanol exposure (Figure 2C-E).

During withdrawal, the significant increase in relative delta power seen during the fourth week of ethanol exposure persisted in saline-treated animals ($F_{1,18}$ = 12.14, $p < 0.01$); however, this increase was no longer evident for the ETX-treated mice (Figure 3A; $p > 0.05$). The observed ethanol-induced increases in relative theta power for both saline- and ETX-treated groups returned to values indistinguishable from their baseline week

(Figure 3B, $p > 0.05$ for both bands). Similar to the effect on delta, we observed a significant effect of withdrawal on the relative alpha power in the saline-treated, but not ETX-treated mice (Figure 3C; $F_{1,18} = 9.691$, $p < 0.01$). We also observed a significant main effect of ETX treatment for both delta ($F_{1,36} = 7.317$, $p < 0.05$, two-way ANOVA) and alpha ($F_{1,36} = 5.182$, $p < 0.05$) bands during withdrawal. We did not observe a significant main effect of ETX treatment for the sigma (Figure 3D, $p > 0.05$) and beta (Figure 3E, $p > 0.05$) bands, but with both treatment conditions pooled, a significant main effect of withdrawal was seen for both sigma ($F_{1,42} = 6.725$, $p < 0.05$, one-way ANOVA) and beta ($F_{1,42} = 8.275$, $p < 0.01$).

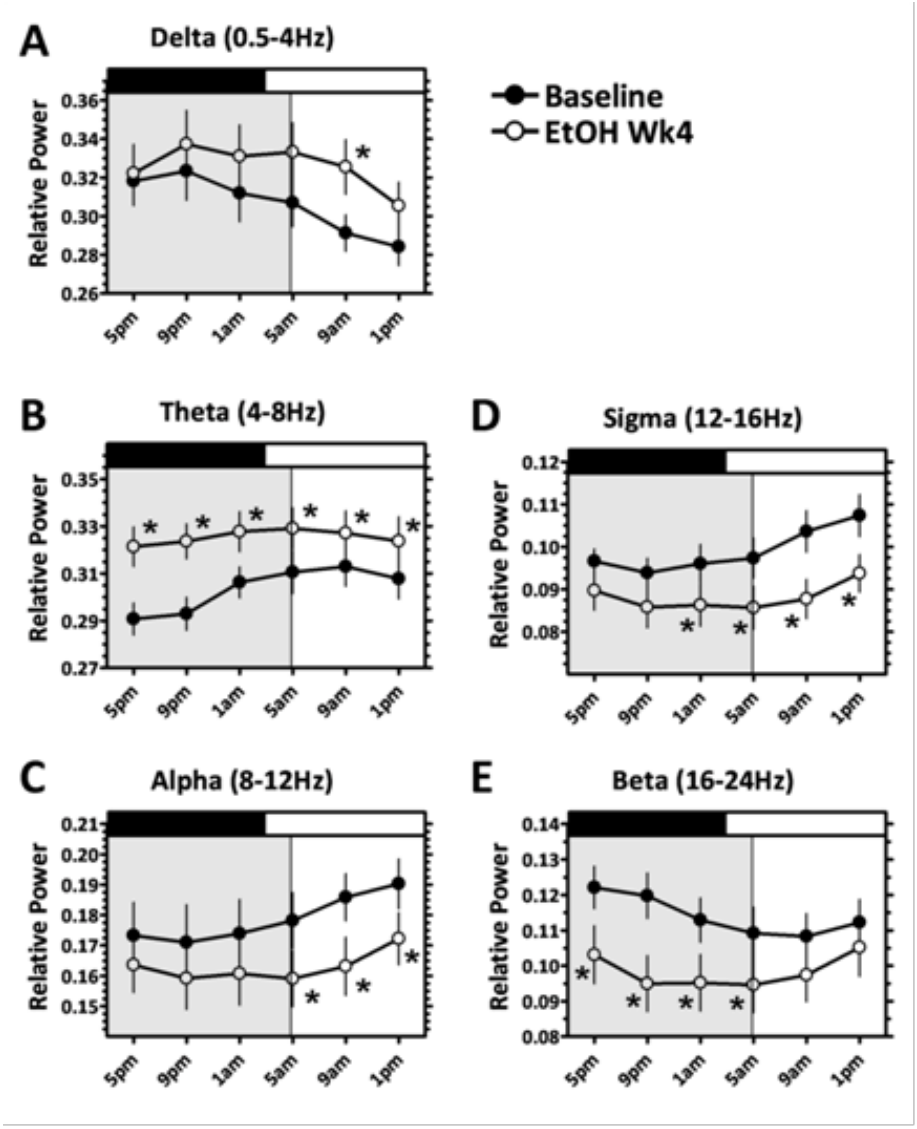


**Figure 2. CIE exposure increases relative power in low-frequency EEG bands.** Relative power for all power bands during the baseline week and fourth week or intermittent ethanol exposures over an average 24-hr period binned in 4-hr epochs. **(A)** Relative delta power was significantly increased during the light period after four weeks of intermittent ethanol exposure. **(B)** Relative theta power was significantly increased throughout both the dark and light periods. **(C-E)** Relative power in the alpha and sigma bands was decreased in the light period, while the beta and sigma bands showed

decreases in the dark period. Dark and light periods indicated by black and white bars at the top of the graph. Ethanol exposure is illustrated by the shaded region. *$p < 0.05$

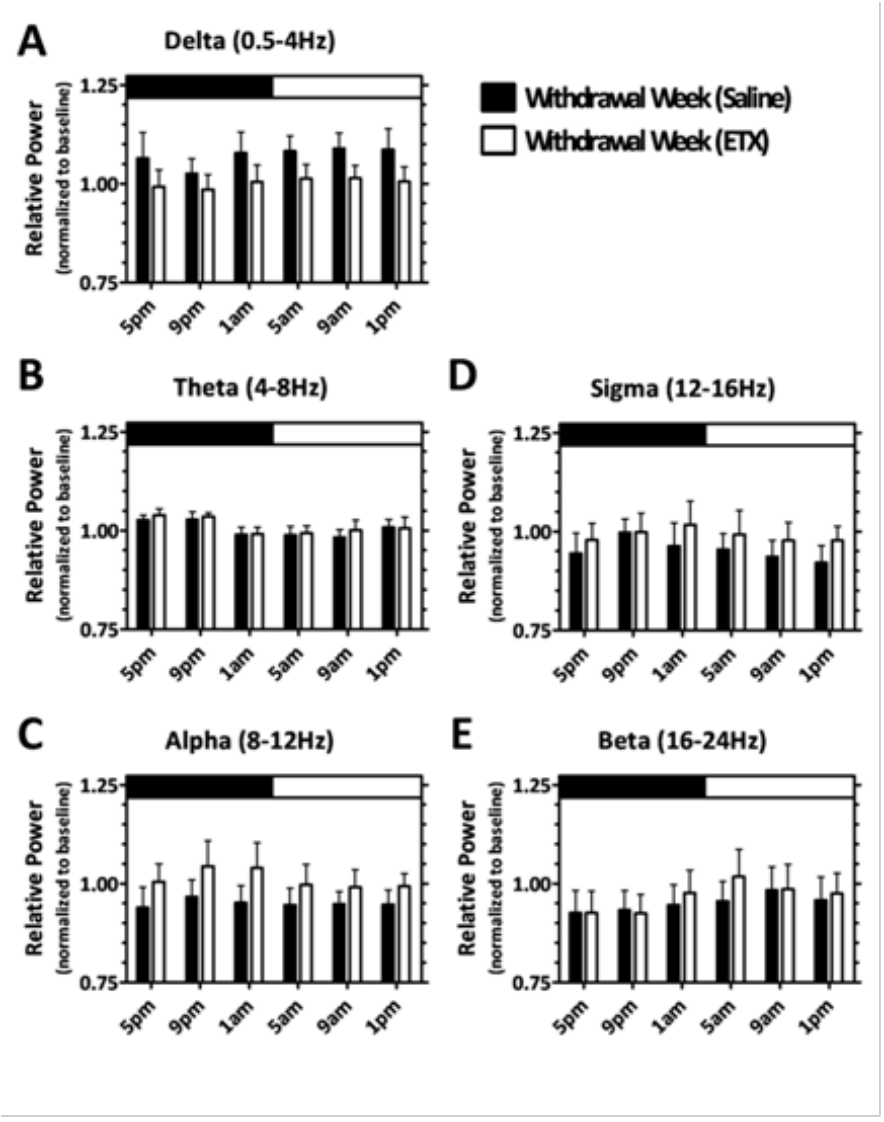


**Figure 3. Ethosuximide restores relative delta and alpha power during the withdrawal week.** Relative power for all power bands during the withdrawal week for both treatment groups over an average 24hr period normalized to the baseline and binned in 4hr epochs. Dark and light periods indicated by black and white bars at the top of the graph. There was a significant main effect of ETX treatment during withdrawal for both delta (**A**) and alpha (**C**) power bands ($p < 0.05$ two-way ANOVA, n=4 both groups), as well as a significant main effect of withdrawal in the saline-treated mice ($p < 0.01$, two-way repeated measures ANOVA, as compared to respective baselines). No significant

main effect of ETX treatment was observed for theta (**B**), sigma (**D**) or beta (**E**) bands. However, there was a significant main effect of withdrawal with both treatment groups pooled for the sigma (**D**) and beta (**E**) bands ($p < 0.05$, two-way repeated measures ANOVA, as compared to baseline).

*Ethosuximide treatment increases the spectral power of the sleep EEG*

Given the importance of the theta rhythm in the scoring of sleep EEG (particularly, in the differentiation between NREM and REM sleep), we assessed the EEG spectra of the individual vigilance states – NREM sleep, REM sleep, and wake. In other studies, increased power in the delta and theta power of sleep spectra have been attributed to increased sleep intensity (Borbély et al., 1981; Finelli et al., 2000) while decreased power has been correlated with decreased quality of sleep in chronic insomnia (Merica, Blois, & Gaillard, 1998).

We found that absolute spectral power was significantly increased in the high delta and low theta ranges of both the NREM and REM sleep spectra of ETX-treated animals, as compared to saline-treated controls. In the NREM spectra (Figure 4A), power was significantly increased in the 3-6Hz range ($p < 0.05$, unpaired *t*-test at each 1-Hz frequency band) with an increase in the 6-7Hz band ($p = 0.080$) that did not reach significance. In the REM spectra (Figure 4B), ETX-treated animals exhibited significantly higher power in the 2-7Hz range ($p < 0.05$) but increases did not reach significance in the 0.5-2Hz ($p = 0.079$ for 0.5-1Hz and $p = 0.059$ for 1-2Hz) or 7-8Hz bands ($p = 0.072$). No significant differences were observed between groups in the wake EEG spectra ($p > 0.05$; see Figure 4C). In a separate experiment, the effects of ETX administration were compared to the effects of saline in a group of ethanol-naïve animals (n = 4 in each group). No significant effects in the NREM or REM sleep spectra were observed in the measure of relative theta in the absence of ethanol ($p > 0.05$ for all 1-Hz frequency bands; see Figure 5).

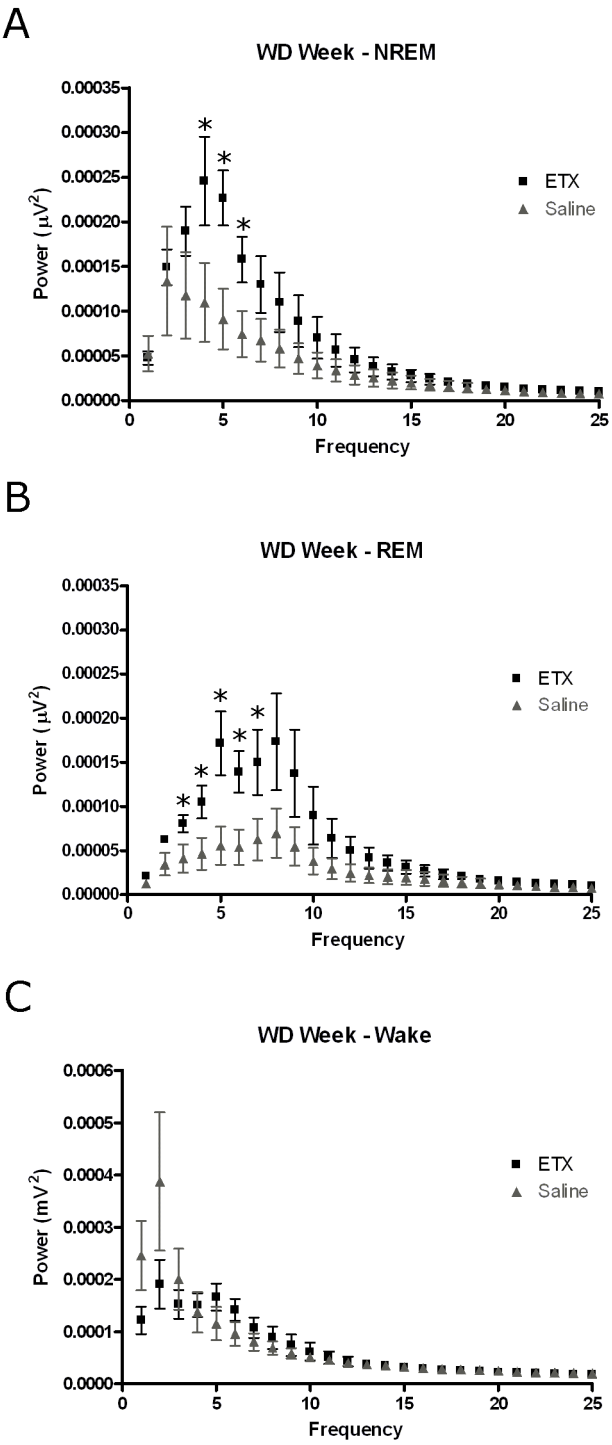


**Figure 4. Ethosuximide increases spectral power in the delta and theta bands of the sleep EEG after ethanol withdrawal.** Average power across all epochs scored as NREM sleep **(A)**, REM sleep **(B)**, or Wake **(C)** is plotted against frequency (1-Hz bands

from 0.5-25 Hz) for each treatment group during the WD Week. **(A)** Spectral power in the 4-7 Hz range (high-delta to low-theta band) of the NREM sleep EEG is significantly increased in the ETX group, as compared to saline-treated control. **(B)** Spectral power in the 2-7 Hz range (mid-delta to low-theta band) of the REM sleep EEG is significantly increased in the ETX group. **(C)** No significant differences were detected between the EEG spectra of the two groups during wake. One animal from the saline group was excluded from the analysis as an outlier as it demonstrated spectral power an order of magnitude greater than that of all other animals across all frequency bands analyzed in the spectrum. Data are presented as mean ± SD. * $p < 0.05$, unpaired *t*-test at the given frequency

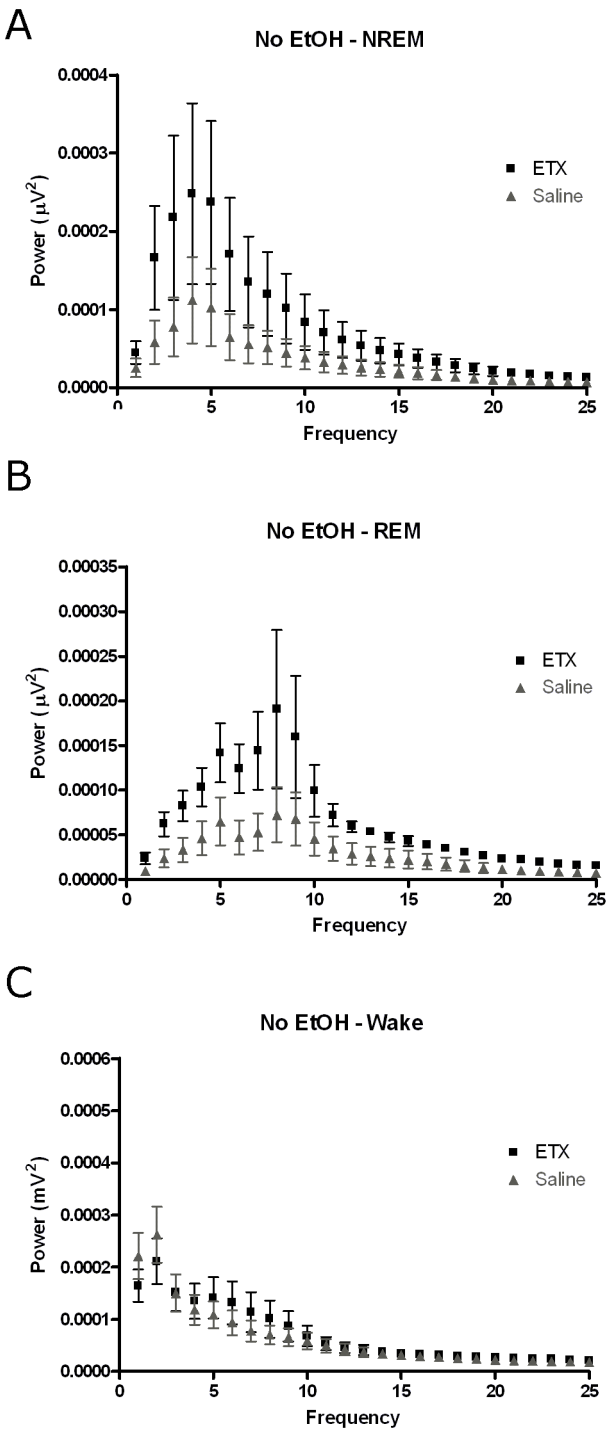


**Figure 5. Ethosuximide does not significantly affect the sleep EEG spectra in the absence of ethanol.** Average power across all epochs scored as NREM sleep **(A)**, REM sleep **(B)**, or Wake **(C)** is plotted against frequency (1-Hz bands from 0.5-25 Hz) in a

cohort of ethanol-naïve animals who received either ethosuximide or saline treatment. No significant differences were detected at any frequency for the three spectra ($p > 0.05$ for all comparisons, unpaired *t*-test). Data are presented as mean ± SD.

**Discussion**

In this study, we have demonstrated ethanol-mediated disruptions in both delta and theta diurnal rhythms that persist into withdrawal. These disrupted patterns could be ameliorated with ethosuximide (ETX), a purported T-type $Ca^{2+}$ channel blocker. In addition, we observed significant ethanol-induced increases in relative delta power and significant decreases in relative alpha power that continued into the subsequent withdrawal week that could be restored to baseline levels with ETX. Furthermore, when the absolute power spectra were aggregated according to sleep score, ETX increased power in the delta and theta bands of both the NREM and REM sleep spectra, without any effect on the wake spectrum. The significant ethanol-induced reductions in the higher frequency sigma and beta bands also persisted into withdrawal; however, ETX had no effect on the relative power of these frequencies ranges. Our results suggest that pharmacologically targeting alterations in rhythmic brain patterns that persist beyond the acute withdrawal window could be effective treatments in managing protracted withdrawal symptoms such as sleep disturbances. These types of interventions would be particularly beneficial as adjunctive therapies for treatment-seeking alcoholics since disruptions in sleep have been shown to be effective predictors of relapse (Bauer, 2001; Brower, 2001; Brower and Perron, 2009).

Abnormalities in the EEG of alcoholics have been well documented (for review, see Porjesz and Begleiter, 2003). The most consistent EEG deficiency found among alcoholics has been reduced sensory-evoked or event-related potentials (ERPs). Several studies have shown that the P300, a particular ERP that is believed to reflect the cognitive processing of a sensory stimulus rather than the stimuli's physical features, is

significantly attenuated in alcoholics (Cohen et al., 1997; Rodriguez Holguin et al., 1999). However, studies of resting spectral power have yielded conflicting results, with some showing increases in certain EEG power bands (Rangaswamy et al., 2003; 2005; Feige et al., 2007) and others reporting decreases (Saletu-Zyhlarz et al., 2004; Coutin-Churchman et al., 2006). In general, it appears that alcoholics exhibit both abnormal resting and evoked EEG signals as compared to control subjects.

In contrast to the abundance of investigations of EEG alterations in human alcoholics, there have been considerably fewer studies on chronic ethanol's effects on EEG bands in rodents. Ehlers and Slawecki (2000) reported that rats chronically exposed to ethanol in a vapor chamber demonstrated decreased spectral power in the delta, theta, alpha and beta bands during a four-hour recording period immediately following six weeks of multiple, intermittent exposures. In addition, they showed that the significant decreases in power remained five weeks later for both the delta and theta band, suggesting alterations in low-frequency EEG power can persist for a prolonged period during withdrawal. Kubota et al. (2002) also reported an overall decrease in spectral power for bands within the 2-25Hz frequency range in rats fed a three-week liquid ethanol diet, however this reduction was no longer apparent one week later. The authors of the latter study attributed this discrepancy to differences in administration methods (ethanol vapor versus liquid diet) and length of exposure (six weeks versus three weeks). In the present study, we did not find any significant differences in absolute power spectra values during our ethanol exposure and withdrawal paradigm until we segregated the data by sleep score. However, we did observe overall shifts in the relative proportion of power among bands that favored increases in lower frequencies and decreases in higher

frequency bands. In addition, our results showing that ethanol-induced alterations in delta and theta EEG rhythms continue into the subsequent week of withdrawal are consistent with the results of Ehlers and Slawecki (2000), which suggested that disruptions in the lower frequency bands are long lasting.

In this study, we treated ethanol exposed animals with a T-type channel blocker, ETX. ETX is a first-choice antiepileptic drug (AED) in the treatment of absence epilepsy, a non-convulsive seizure disorder characterized by generalized 3Hz spike-and-wave discharges (SWDs) recorded on the EEG (for review, see Hughes, 2009). ETX has been shown to inhibit low-threshold T-type $Ca^{2+}$ currents in both the thalamic ventrobasal nucleus (Coulter et al., 1989) and thalamic reticular nucleus of the rat (Huguenard and Prince, 1994). It is commonly held that ETX produces its antiepileptic effects by reducing T-type channel-mediated thalamic bursts, thereby disrupting the synchronized oscillations within this circuit that underlie SWDs. While some controversy has arisen over the specificity of ETX in blocking T-type currents (see Crunelli and Leresche, 2002), it has been suggested that ETX preferentially inhibits a T-type window current (Gomora et al., 2001) that arises from the interaction of the voltage-dependent activation and inactivation properties of whole-cell T-type current (Hughes et al., 1999; Crunelli et al., 2005).

Previously described experiments from our lab (Graef et al., 2011) demonstrated a progressive, functional upregulation of midline thalamic T-type channels, including alterations in steady-state properties that occurred in parallel with progressive alterations in the theta EEG band during chronic ethanol exposure and withdrawal. It was also noted in that study that disruptions in thalamocortical circuitry can give rise to disruptions in

normal thalamocortical rhythms, which have been shown to display a significant increase in medial thalamic T-type channel-mediated bursts, as well as augmented power and thalamocortical coherence in the theta (4-9Hz) band range (Jeanmonod et al. 1996; Llinas et al. 1999; Sarnthein and Jeanmonod 2007, 2008). In addition, increases in resting EEG theta coherence have been reported in alcoholics that were also suggested to arise from altered thalamocortical function (Porjesz and Rangaswamy, 2007). Considering that the persistent, ethanol-mediated alterations in theta rhythms we observed during the subsequent week of withdrawal could be ameliorated with ETX, it is possible that ETX is exerting its effects by inhibiting this functional increase in midline thalamic T-type channels, thereby restoring normal neural rhythms. Furthermore, it is possible that this effect of ETX is limited to sleep, when T-type channels are most activated, given the increase in sleep intensity observed in NREM and REM sleep spectra, but not the wake spectrum of ETX-treated animals. This beneficial effect of ETX would be in partial agreement with an earlier study where ETX was able to reduced withdrawal signs in ethanol-dependent mice (Kaneto et al., 1986). It is also interesting to note that in addition to its antiepileptic effects, ETX has been shown to be effective in other models of thalamocortical dysrhythmias, including neuropathic pain (Dogrul et al., 2003), Parkinson's disease (Gomez-Mancilla et al, 1992), and depression (Shaw et al., 2009).

Caution should be used in interpreting the role of thalamic T-type calcium channels from these studies. In our study, ETX was administered systemically and T-type channels are diffusely expressed throughout the nervous system (Talley et al., 1999; Perez-Reyes, 2003). Few studies have characterized the interaction between ethanol and T-type channel expression and function outside of the thalamus (Nordskog et al., 2006;

Newton et al., 2008). Additionally, despite known effects on T-type calcium channels (Gomora et al., 2001; Huguenard, 1996; Todorovic & Lingle, 1998), ETX has been suggested to also affect other currents, such as the persistent $Na^+$ current and $Ca^{2+}$-dependent $K^+$ currents (Leresche et al., 1998; Crunelli and Leresche, 2002). It is important to note that a decrease in T current-mediated burst firing was observed in the latter study. However, both of these studies were carried out at high concentrations of ETX, well above what is considered to be the physiologically relevant range. A separate study of the effects of low concentration ETX confirmed blockade of T-type calcium current at physiologically-relevant concentrations (Gomora et al., 2001). While no studies have investigated the effects of ethanol on persistent $Na^+$ currents, other studies have demonstrated significant modulation of both small (SK) and large (BK) $Ca^{2+}$-dependent $K^+$ channels during both acute and chronic ethanol exposure (Brodie et al., 2007). Additionally, G-protein mediated inwardly-rectifying K+ (GIRK) channels are also inhibited by ETX, where it has been shown to attenuate the ethanol-induced potentiation of GIRK currents; however, the concentration of ETX used in this study was well above the range of therapeutically relevant concentrations (Kobayashi et al., 2009). Future investigations involving localized application of more specific T-type channel inhibitors will clarify the exact role of T-type channels in the ethanol-mediated disruption of EEG rhythms.

In this study, we demonstrated chronic ethanol-induced disruption in both delta and theta EEG rhythms important for normal sleep, as well as relative delta and alpha power, which persisted into the subsequent week of withdrawal. These effects could be ameliorated with ETX, suggesting a role for T-type Ca2+ channels in underlying these

chronic ethanol effects. Since sleep disturbances during withdrawal have been implicated as potential cause for relapse, it will be important to determine if the increased sleep intensity we observed with ETX treatment translates into the restoration of disrupted sleep patterns and sleep-related symptoms associated with protracted withdrawal from chronic ethanol exposure. Moreover, the effects of alcohol withdrawal are complex and these same observations could have implications for other pathology resulting from hyperexcitability in this network, such as alcohol withdrawal-related seizure.

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CHAPTER 3

# DIFFERENTIAL EFFECTS OF ETHOSUXIMIDE TREATMENT AND GENETIC SUPPRESSION OF A T-TYPE $CA^{2+}$ CHANNEL SUBTYPE ON ALTERED SLEEP HOMEOSTASIS IN RESPONSE TO CHRONIC INTERMITTENT ETHANOL

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**Abstract**

BACKGROUND: Alcohol withdrawal-related insomnia is an important problem for chronic alcoholics and has been suggested to be a significant predictor of relapse. Previously, we have identified disruptions in sleep-related oscillations important for the maintenance of sleep that were partially reversed by administration of ethosuximide (ETX), a purported T-type calcium current blocker.

METHODS: Two separate experiments were conducted. In the first experiment, data from two groups of C57BL/6 mice (n = 5 in each group) were implanted with wireless telemetric monitoring devices and subjected to six weeks of continuous EEG, EMG, activity, and temperature recording. After one week of baseline, mice were subjected to 4 cycles of chronic intermittent ethanol (CIE) vapor administration, consisting of 5 days of CIE exposure and 2 days of subacute withdrawal (WD). During the final week of recording (sustained WD), mice were either administered saline or ETX (200 mg/kg). In the second experiment, a group of Ca(v)3.2-deficient mice (n = 6), bred on a C56BL/6 background, and a group of wild-type littermate controls (n = 6) were implanted with telemetry and subjected to 2 weeks of continuous recording, including 1 cycle of CIE vapor administration, as described above. Data from both experiments were sleep-scored and compared within and between groups using standard statistical methods.

RESULTS: Significant sedating effects of EtOH were observed in all groups from both experiments, as reflected by increases in non-REM sleep and vigilance cycling. In the first experiment, an increase in brief wakening observed during sustained WD was partially reversed by ETX treatment. In the second experiment, Ca(v)3.2-deficient mice demonstrated increased sleep during the WD period, as compared to baseline.

CONCLUSIONS: These results suggest that ETX may act to stabilize sleep during the WD period. Furthermore, the results of the second experiment suggest a role for Ca(v)3.2 in withdrawal-mediated sleep disruption.

## Introduction

Alcohol withdrawal-related sleep disruption plays a key role in the progression of alcoholism. Prolonged abuse of ethanol leads to long-term disruptions in sleep patterns, resting EEG activity, and profound disturbances in diurnal behaviors of alcoholics (Kubota et al., 2002). These disturbances last well beyond the cessation of ethanol consumption, persist into withdrawal, and have been suggested to be predictive of relapse during alcohol withdrawal (Brower, Aldrich, & Hall, 1998; Brower & Perron, 2010; Cohn, Foster, & Peters, 2003). Prevention of relapse represents a significant hurdle in overcoming alcohol dependence.

The thalamus is involved in the generation and maintenance of normal brain rhythms during both sleep and states of vigilance (Llinás & Steriade, 2006). Low-threshold thalamic bursts have been demonstrated to underlie certain aspects of both normal sleep oscillations and pathological states (Steriade, 2005). These bursts of action potentials are mediated by a class of T-type $Ca^{2+}$ channels that possess unique gating properties, forming the basis of generation of intrinsic neuronal oscillations that are a characteristic of the thalamus (Huguenard, 1996). Changes leading to altered T-type channel function have been associated with certain pathological conditions that are marked by abnormal increases in thalamocortical theta coherence (Sarnthein & Jeanmonod, 2007, 2008) and have been termed thalamocortical "dysrhythmias" (Llinás, Ribary, Jeanmonod, Kronberg, & Mitra, 1999).

Abnormal EEG activity in alcoholics has been well established (Porjesz & Begleiter, 2003; Rangaswamy et al., 2003), including increases in interhemispheric theta coherence that have also been suggested to arise from altered thalamocortical function (Porjesz &

Rangaswamy, 2007). In rodents, repeated ethanol exposures and withdrawals that model the binge/abstain consumption patterns of human alcoholics (Becker & Hale, 1993) increases the severity of subsequent withdrawal-related hyperexcitability, leading to altered sleep architecture (Ehlers & Slawecki, 2000; Kubota et al., 2002; Veatch, 2006), heightened anxiety (Overstreet, Knapp, & Breese, 2002), and abnormal EEG activity (Veatch & Gonzalez, 1996; Veatch & Becker, 2002). The neural substrates involved in the progressive disruption of EEG and sleep patterns form a network composed of medial thalamic, limbic, and prefrontal regions, all of which are especially vulnerable to the adverse effects of chronic ethanol (Fadda & Rossetti, 1998).

We have recently shown that chronic intermittent ethanol exposure in C57BL/6 mice alters normal daily variation of both thalamic T-type $Ca^{2+}$ channel mRNA expression levels and gating properties. These changes were associated with an increase in low-threshold bursts of action potentials during acute withdrawal periods and a progressive disruption in the mean peak frequency and normal diurnal variations in relative EEG theta power (Graef et al., 2011). Subsequently, we identified alterations in relative delta power and spectral power in the theta and delta bands of the sleep EEG, both of which were partially reversed by administration of ethosuximide (ETX), a purported T-type calcium channel antagonist (see Chapter 2).

In this study, we sought to determine the effects of pharmacologic and genetic inhibition of T-type calcium channels on sleep patterns after multiple withdrawals from chronic, intermittent exposure to ethanol vapor. While it is still unresolved if restoring normal sleep and EEG patterns alone can prevent relapse (Brower & Perron, 2010), furthering our understanding of the progressive EEG and sleep pattern alterations that

occur during chronic alcoholism and continue into protracted withdrawal will help provide the basis for additional avenues for therapy.

## Materials and Methods

*Animals and Experimental Design*

All experiments were conducted with prior approval of the Institutional Animal Care and Use Committee at Wake Forest School of Medicine. A total of 22 eight week-old male mice were used to carry out the experiments described below. Ethanol was administered via the chronic, intermittent ethanol (CIE) vapor inhalation procedure previously described by Becker and Hale (Becker & Hale, 1993). Briefly, animals were placed in a sealed Plexiglas vapor chamber modified from Goldstein (1972) in a room with a 12-hr light/dark schedule with lights on at 5am. Ethanol (95%) was volatilized and delivered to one of the chambers at a rate of 2.0 L/min by a vacuum pump. This, in combination with air being delivered to the chambers at a rate of 20 L/min, maintained the ethanol concentration in the chamber in the range of 14–16 mg/L of air (mean ± SD: 15.6 ± 0.5 mg/L). An 8-hr period of abstinence allowed clearance of ethanol from the blood stream prior to the next cycle of intoxication (Becker, 1994; Becker & Hale, 1993). At the beginning of each exposure cycle (5pm), all mice (both air and ethanol exposed) were treated with a subcutaneous injection of the alcohol dehydrogenase inhibitor pyrazole (100 mg/kg) to stabilize blood ethanol concentrations over the course of multiple exposures. Pyrazole was prepared daily by dissolving it in sufficient 0.9% saline to obtain a consistent injection volume of 0.2 ml per animal. Blood ethanol concentrations (BECs) achieved under these conditions remained relatively stable from one bout of intoxication to the next. BECs for all mice were measured by taking 5µl blood samples from the tail (stored in vials containing 45µl of 6.25% trichloroacetic acid) and analyzed using a

NAD-ADH enzyme assay according to the instructions of the manufacturer of the assay (Diagnostic Chemicals, Oxford, CT).

*Data acquisition*

Mice were implanted subcutaneously with telemetric physiologic monitors (Model F20-EET; Data Sciences International (DSI), Arden Hills, MN) that simultaneously record electroencephalogram (EEG), electromyogram (EMG), temperature and activity. We have previously described the implantation procedure in detail (see Chapter 2).

*Experiment 1: Effect of ethosuximide on sleep during sustained withdrawal from ethanol*

Ten eight week-old C57BL/6 mice (Harlan, Indianapolis, IN) were implanted with wireless telemetry and subjected to one week of baseline recording, four weeks of CIE vapor administration, and one week of sustained withdrawal. During each week of CIE vapor administration, mice underwent 5 days of exposure, followed by 2 days of subacute withdrawal, during which no ethanol was administered. During the final week of the experiment (sustained withdrawal), mice in the experimental group were administered ethosuximide (250 mg/kg, IP) dissolved in 0.2 ml of normal saline daily at 5pm. Mice in the control group received an isovolumetric injection of normal saline only. The mean BEC was 186.0 ± 9.4 mg/dl for all four weeks of exposure. An analysis of the EEG data from this experiment has been reported previously (see Chapter 2).

*Experiment 2: Effect of ethanol withdrawal on sleep in Ca(v)3.2-deficient mice*

The experimental group consisted of 6 eight week-old male B6.129-cacna1h<tm1Kcam>/J mice (Jackson, Bar Harbor, ME), a transgenic strain lacking the gene encoding for the Ca(v)3.2 isoform of T-type calcium channels (Chen et al., 2003). The control group consisted of 6 eight week-old male C57BL/6J mice, the background strain employed in the development of the transgenic strain. Both groups of mice were implanted with wireless telemetry and subjected to one week of baseline recording, five days of CIE vapor administration, and two days of sustained withdrawal. The mean BEC for the wild-type mice was 136 ± 20 mg/dL over the course of the experiment. The mean BEC for the Ca(v)3.2-deficient mice was 134 ± 15 mg/dL. No significant difference in BEC was detected between groups ($p > 0.05$, unpaired *t*-test). No significant effect of CIE treatment ($F_{1,20} = 1.99$, $p > 0.05$) on animal weight was detected. The mean weights of the wild-type and Ca(v)3.2-deficient mice were 30.3 ± 0.6 g and 28.7 ± 2.5 g, respectively, at the prior to surgery and 29.5 ± 2.1 g and 27.0 ± 2.8 g, respectively, at the end of the experiment. A significant main effect of strain ($F_{1,20} = 5.35$, $p < 0.05$) was detected; however, post hoc tests revealed no significant differences between groups before or after the experiment ($p > 0.05$ at both time points).

*Sleep Scoring*

EEG, EMG, and activity data were sleep-scored in 10-s epochs using a customizable rodent sleep-scoring algorithm available with the NeuroScore software package (DSI, Arden Hills, MN). We have previously described this algorithm in the context of rodent sleep analysis (see Chapter 2). Sleep epochs were scored as rapid eye movement (REM),

non-REM (NREM), wake (W), or artifact, consistent with previous reports in rodents (Simasko & Mukherjee, 2009; Veatch, 2006).

*Vigilance Cycling Analysis*

A custom MATLAB (The Math Works, Natick, MA) function was written to segregate vigilance cycles (VC) from episodes of long-duration wake (LDW). Briefly, this function removed artifact from the dataset, then combined adjacent bouts of the same type previously separated by artifact. Bouts of BW were then distinguished from bouts of LDW by the previously determined cutoff value of 300s duration, consistent with Simasko & Mukherjee (2009). A VC was identified as an episode of sleep (NREM or REM) followed by at least one other epoch of sleep or BW. To account for long periods of isolated NREM, we selected 300s as a cutoff for identifying an isolated bout of NREM (surrounded by two episodes of LDW) as a VC. All other isolated bouts of NREM were excluded from subsequent VC analysis.

*Cosinor Rhythmometry*

A custom MATLAB script was designed to bin the activity, temperature, and NREM sleep data into 1-hr bins in order to fit the parameters of the following cosinor equation to each dataset:

$$y = MESOR + Amplitude * \cos(2\pi * period + acrophase)$$

where MESOR is the midline estimating statistic of rhythm and acrophase describes the peak of the curve (Nelson et al., 1979). Fits were calculated using nonlinear least-squares regression for individual animals for each day across the baseline week, the fourth week

of CIE administration, and the sustained withdrawal week assuming a period of 24 hrs. The parameters were then averaged for each animal across the baseline week, the exposure days of EtOH Wk 4, the withdrawal days of EtOH Wk4, and the first 5 days of the sustained withdrawal week.

*Statistical Analysis*

Statistical analysis was performed between exposure periods both within each treatment group and between treatment groups using either MATLAB or SPSS (IBM Software, Armonk, NY). Two-way repeated-measures or mixed-model (between-/within-subjects) ANOVA was used to assess effects of ethanol, group/strain, and/or time of day for all datasets. *Post-hoc* comparisons were made between exposure/withdrawal periods and baseline and between treatment groups using Tukey's least significant difference procedure. All results are reported as mean ± SEM.

## Results

*Experiment 1: Effect of ethosuximide on sleep during sustained withdrawal from ethanol*

Electroencephalographic (EEG), electromyographic (EMG), activity, and body temperature data were continuously recorded from two groups of C57BL/6 mice (n = 5 in each group) during a baseline week, four weeks of chronic intermittent ethanol (CIE) administration, and one week of sustained withdrawal from ethanol. During the sustained withdrawal week, each group either received ethosuximide (ETX) or saline treatment. These data were sleep scored and analyzed for measures of sleep quantity, sleep architecture, and diurnal rhythmicity. Measures of sleep or wake time are reported as the total percentage of each period (dark or light) spent in the corresponding vigilance state. Measures of sleep architecture are reported within the "vigilance cycling" framework of sleep analysis (Mukherjee & Simasko, 2009; Simasko & Mukherjee, 2009). The diurnal rhythmicity of NREM sleep, activity, and body temperature was assessed via cosinor rhythmometry (Nelson et al., 1979).

*CIE vapor administration has a prominent sedating effect*

We first examined the effects of ethanol exposure on sleep time. Ethanol exposure in the CIE paradigm demonstrated a significant effect on the proportion of time spent in NREM sleep ($F_{5,108} = 17.15$, $p < 0.001$). During the ethanol exposure days of the first week of CIE administration (EtOH Exp 1), mice exhibited a significant increase in the percent time spent in NREM sleep (Fig. 6A) with a corresponding decrease in wake time (Fig. 8). The percent of the dark period (the active phase for mice under normal conditions) spent in NREM sleep increased from 25.0 ± 3.9% during the baseline week to 65.4 ± 3.9% by

the last exposure day of the first week of CIE ($p < 0.05$). The effect persisted through the fourth and final week of CIE administration (Exp 4; Fig. 6A, $p < 0.05$). For the light period, NREM sleep time increased from 53.2 ± 5.1% to 75.9 ± 5.1% during the EtOH Exp 1 ($p < 0.05$), but this effect was not observed in EtOH Exp 4 ($p > 0.05$). Interestingly, the animals did not appear to develop tolerance to the effects of ethanol exposure during the dark period, as the average NREM sleep time was not significantly different between EtOH Exp 1 and EtOH Exp 4 ($p > 0.05$ for both). No main effect of ethanol exposure was observed for the overall percent time spent in REM sleep (Fig. 7A, $F_{5,108} = 1.01$, $p > 0.05$).

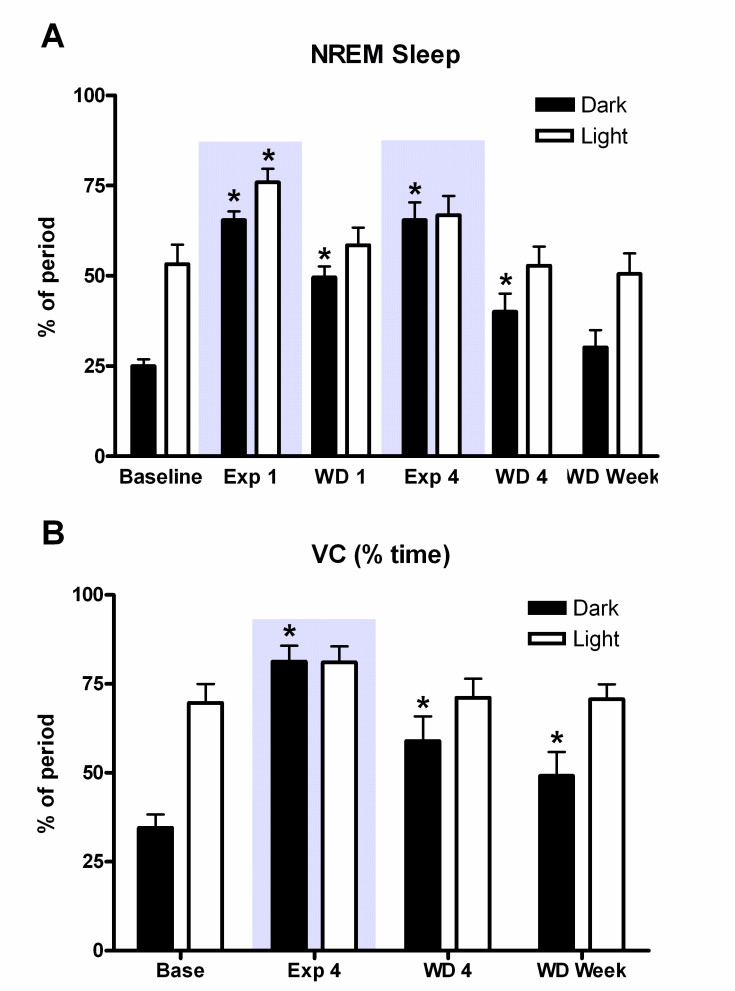


**Figure 6. CIE exposure has a prominent sedating effect in mice.** (A) Percentage of time spent in non-REM (NREM) sleep during the dark and light periods of baseline, the first and fourth weeks of CIE exposure (Exp 1 & Exp 4, respectively), the early withdrawal phase of these weeks (WD 1 & WD 4, respectively), and sustained withdrawal (WD Week). Grey shading indicates ethanol exposure. NREM was significantly increased in the dark period during Exp 1 & 4, returning to baseline during sustained withdrawal. (B) Percentage of time spent in vigilance cycling (VC). VC was elevated during the dark period of Exp 4 and remained elevated through sustained withdrawal. * $p < 0.05$

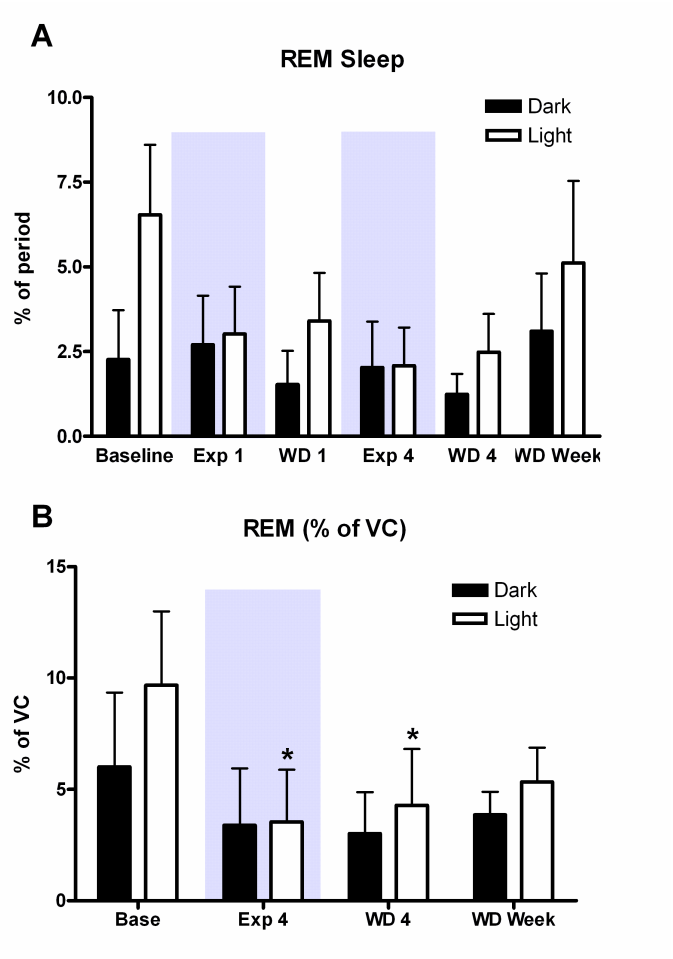


**Figure 7. CIE suppresses REM in the light period.** (A) Percentage of total time spent in REM sleep during the dark and light periods of baseline, the first and fourth weeks of CIE exposure (Exp 1 & 4, respectively), early withdrawal (WD 1 & 4, respectively), and sustained withdrawal (WD Week). No significant effect of CIE treatment on overall REM was detected. Grey shading indicates ethanol exposure. (B) Quantity of REM sleep expressed as a percentage of time spent in vigilance cycling (VC). REM was significantly suppressed as a % of VC during Exp 4 and WD 4. No increase in REM was observed during sustained withdrawal. * $p < 0.05$

To characterize effects on and within sleep architecture, we employed the vigilance cycling framework of sleep analysis (Simasko & Mukherjee, 2009). According to this framework, rodents alternate between episodes of long-duration wake (LDW) and vigilance cycling. A vigilance cycle (VC) is defined as a period during which alternation between NREM sleep, REM sleep, and brief wakening (BW) occurs without interruption by a period of long-duration wake. The results of this analysis are summarized in Table II.

During the dark period of EtOH Exp 4, the number and average duration of VC episodes were increased significantly ($p < 0.05$ for both) with a net effect of increasing the percent of total time spent in VC (Fig. 6B). There was a corresponding decrease in the number and duration of LDW episodes with a net effect of decreasing the percent of total time spent in LDW during the dark period (Fig. 8). Within vigilance cycles, there was an increase in NREM episode duration for both periods ($p < 0.05$ for both periods) and an increase in the average percent of the VC spent in NREM for the dark period ($p < 0.05$). Importantly, there was also a decrease in REM episode duration during the light period (Fig. 7B, $p < 0.05$).

## Table II. Vigilance cycling analysis parameters for Experiment 1

| Parameter | Baseline Dark Period | Baseline Light Period | EtOH Wk4 Dark Period | Exposure Light Period | EtOH Wk4 Dark Period | Withdrawal Light period | Sustained Dark Period | Withdrawal Light Period |
|---|---|---|---|---|---|---|---|---|
| **Long duration wake** | | | | | | | | |
| # episodes: | 24.2 ± 8.2 | 13.3 ± 8.0* | **10.6 ± 5.4†** | 10.0 ± 4.6 | 18.6 ± 7.1 | 13.7 ± 4.8* | 20.0 ± 8.6 | 13.2 ± 5.8* |
| Avg dur: | 1360 ± 730 | 1000 ± 420 | **670 ± 310†** | **710 ± 290†** | 950 ± 490 | 840 ± 410 | 1370 ± 1200 | 950 ± 420 |
| % time: | 64.3 ± 11.6 | 29.8 ± 16.1* | **18.5 ± 14.1†** | 18.7 ± 13.9 | **40.6 ± 21.6†** | 28.3 ± 16.8 | **50.1 ± 20.9†** | 28.9 ± 13.4* |
| **Vigilance cycling** | | | | | | | | |
| # episodes: | 14.7 ± 4.4 | 9.9 ± 4.1* | **9.3 ± 4.1†** | 8.5 ± 3.0 | 13.1 ± 3.3 | 10.4 ± 3.7 | 13.9 ± 8.6 | 9.9 ± 4.0* |
| Avg dur: | 1060 ± 320 | 4010 ± 3000 | **7260 ± 9750†** | 7220 ± 9710 | **2150 ± 1090†** | 5210 ± 7940 | 2890 ± 4920 | 6510 ± 10700 |
| % time: | 34.5 ± 12.0 | 69.7 ± 16.8* | **81.2 ± 14.2†** | 81.1 ± 14.1 | **58.9 ± 21.9†** | 71.1 ± 16.8 | **49.2 ± 21.1†** | 70.6 ± 13.5* |
| **NREM sleep** | | | | | | | | |
| Avg dur: | 145 ± 55 | 202 ± 103 | **269 ± 127†** | **293 ± 187†** | 142 ± 75 | 184 ± 112 | 113 ± 43 | 158 ± 83 |
| % VC: | 70.8 ± 10.6 | 75.1 ± 13.4 | **79.1 ± 10.5†** | 80.5 ± 12.0 | 66.7 ± 9.4 | 72.2 ± 13.4 | 62.8 ± 10.7 | 71.5 ± 13.6 |
| **REM sleep** | | | | | | | | |
| Avg dur: | 26.6 ± 14.3 | 37.9 ± 20.4 | 23.0 ± 13.8 | **21.9 ± 12.4†** | 18.2 ± 9.0 | **20.8 ± 13.7†** | 31.3 ± 16.7 | 30.1 ± 13.9 |
| % VC: | 6.0 ± 10.5 | 9.7 ± 10.5 | 3.4 ± 8.1 | 3.5 ± 7.4 | 3.0 ± 9.4 | 4.3 ± 8.0 | 7.4 ± 12.5 | 8.2 ± 12.7 |
| **Brief wake** | | | | | | | | |
| Avg dur: | 73.1 ± 25.8 | 62.0 ± 31.6 | 66.7 ± 14.9 | 61.4 ± 15.5 | 72.8 ± 12.3 | 65.5 ± 14.0 | 81.9 ± 21.5 | 64.1 ± 19.7 |
| % VC: | 23.2 ± 10.6 | 15.2 ± 10.0* | 17.5 ± 3.8 | 16.0 ± 5.2 | **30.3 ± 7.2†** | **23.5 ± 7.5*†** | **30.0 ± 5.5†** | 20.3 ± 5.6* |

*$p < 0.05$ (significantly different from dark period for same treatment)
†$p < 0.05$ (significantly different from baseline in same period)

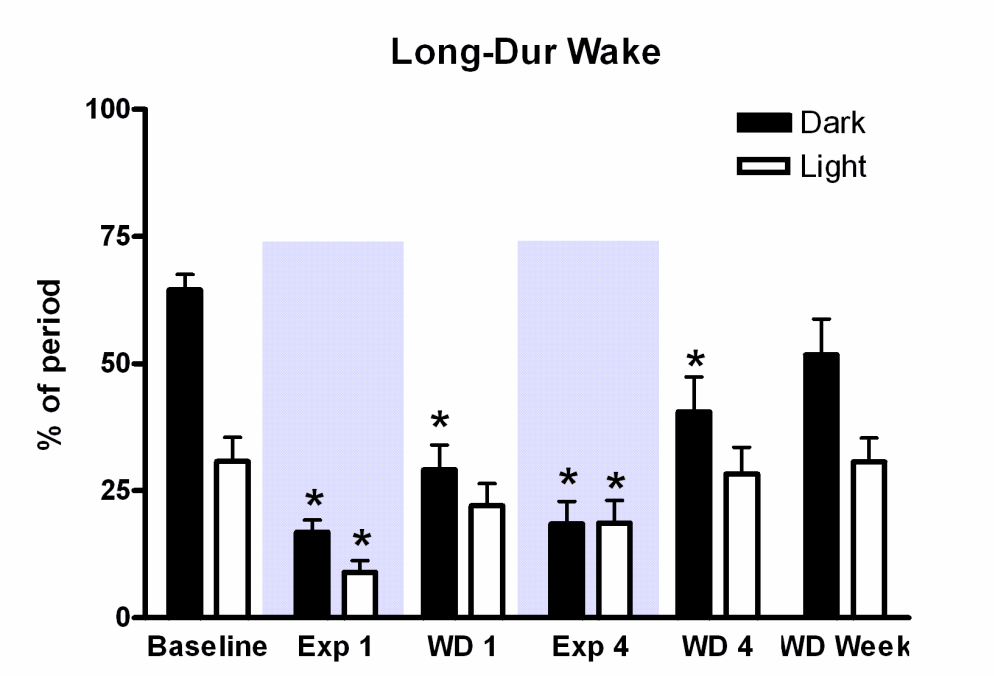


**Figure 8. CIE effects on LDW for Experiment 1.** Percent time spent in periods long-duration wakening during the dark and light periods of baseline, the first and fourth weeks of CIE exposure (Exp 1 & 4, respectively), the early withdrawal phase of both weeks (WD 1 & 4, respectively) and sustained withdrawal (WD Week). LDW was significantly decreased in both the dark and light periods during Exp 1 & 4. LDW was remained significantly decreased during the dark period of WD 1 & 4. * $p < 0.05$

*CIE exposure attenuates diurnal rhythms and decouples NREM from activity and temperature*

To examine the relationship between the increase in NREM sleep during ethanol exposure and possible alterations in activity and body temperature, we performed cosinor rhythmometry (Nelson et al., 1979) on data from baseline, Exp 4, WD 4, and sustained withdrawal for both the ETX and saline groups. As no significant main effect of ETX treatment was observed for any of the 9 parameters, the two groups were combined for statistical analysis. The results of this analysis are summarized in Table III.

Of note, we observed significant effects of CIE treatment on the midline-estimating statistic of rhythm (MESOR) and amplitude of the diurnal rhythm of NREM, activity, and temperature ($p < 0.05$ for all measures). Each of these measures was significantly attenuated during EtOH Exp 4 ($p < 0.05$, as compared to baseline for all measures). While the MESOR of NREM was partially reversed during WD 4 ($p > 0.05$, as compared to baseline), the MESOR of activity and the amplitude of both measures remained significantly attenuated ($p < 0.05$, as compared to baseline for each measure). Temperature MESOR and activity MESOR and amplitude remained attenuated during sustained withdrawal ($p < 0.05$ for each measure).

A significant effect of CIE treatment on acrophase was observed for activity ($F_{3,36} = 5.09$, $p < 0.01$) and temperature ($F_{3,36} = 12.40$, $p < 0.001$), but not NREM ($F_{3,36} = 1.71$, $p > 0.05$). The acrophase of both activity and temperature were similarly shifted in Exp 4 from 4.24 ± 2.06hrs and 3.78 ± 2.12hrs, respectively, to 7.37 ± 0.75hrs and 7.25 ± 0.59hrs, respectively ($p < 0.05$, as compared to baseline for both measures). Both values returned to baseline during WD 4 ($p > 0.05$ for both).

**Table III. Cosinor analysis of diurnal rhythms for Experiment 1**

| Parameter | Baseline | EtOH Exp 4 | EtOH WD 4 | Sustained Withdrawal |
|---|---|---|---|---|
| **Activity** (ct/min) | | | | |
| MESOR: | 391±125 | **153±55*** | **236±78*** | **286±61*** |
| Amplitude: | 313±134 | **101±51*** | **107±83*** | **160±66*** |
| Phase (hrs): | 4.24±2.06 | **7.37±0.75*** | 5.51±2.74 | 4.41±1.99 |
| **NREM** (% time) | | | | |
| MESOR: | 40.1±12.4 | **69.4±12.2*** | 50.1±13.9 | 41.5±15.6 |
| Amplitude: | 22.4±8.2 | **13.4±4.7*** | **11.3±7.5*** | 16.0±4.5 |
| Phase (hrs): | 17.3±0.5 | 16.7±1.0 | 17.1±1.4 | 17.5±0.4 |
| **Temperature** (°C) | | | | |
| MESOR: | 34.8±0.3 | **33.3±0.6*** | **33.6±0.6*** | **34.2±0.4*** |
| Amplitude: | 0.71±0.21 | 0.50±0.15 | **0.30±0.12*** | 0.51±0.14 |
| Phase (hrs): | 3.78±2.12 | **7.25±0.59*** | 3.97±1.88 | 3.38±1.37 |

*$p < 0.05$ (significantly different from baseline)

*Some effects of CIE exposure are partially reversed during the early withdrawal phase*

The prominent sedating effect observed during CIE exposure was partially reversed in the early withdrawal phase. During the two-day subacute withdrawal period following the first administration week, the percent time spent in NREM sleep exhibited a partial reversion to baseline (Fig. 6A). Specifically, the %NREM in the light period of both the first and fourth subacute withdrawal periods (WD 1 and WD 4, respectively) was not significantly different than that observed in the baseline week ($p > 0.05$ for both periods). With respect to sleep architecture, the number and duration of LDW episodes and the number of VC episodes during the dark period of WD 4 were also found not to be significantly different from baseline (Table II, $p > 0.05$). Furthermore, the duration (both periods) and the percent of VC spent in NREM (dark period) returned to their baseline levels, as well ($p > 0.05$ for all).

Some effects of CIE exposure remained during the early withdrawal phase. The duration of (Table II) and percent time spent in VC during WD 4 (Fig. 6B) remained significantly greater than baseline for the dark period ($p < 0.05$ for both) despite a trend towards a decrease from EtOH Exp 4 ($p = 0.067$). The reciprocal effect on percent time spent in LDW also remained during this time ($p < 0.05$). Furthermore, there was a continued effect of REM episode suppression during WD 4 ($p < 0.05$).

*Increase in BW during sustained withdrawal from CIE is partially reversed by ethosuximide*

There was a significant main effect of ethanol on BW during the first and fourth weeks of CIE administration ($F_{4,90} = 9.77$, $p < 0.001$). In the dark period, ethanol significantly

increased the percent time spent in BW during the Exp 1 and Exp 4 (Fig. 9A, $p < 0.05$ for both). This effect persisted into both WD 1 and WD 4 ($p < 0.05$ for both). While BW in the light period was not significantly increased by EtOH exposure (Fig. 9A; Exp 1: $p > 0.05$, Exp 4: $p > 0.05$), there were significant increases in BW during the light period of both WD 1 and WD 4 ($p < 0.05$ for both). The significant main effect of CIE treatment on BW was also observed during sustained withdrawal (Fig. 9B,C), during which 5 animals received saline treatment and 5 animals received ETX treatment ($F_{1,16} = 5.55$, $p < 0.05$ as compared to baseline for the dark period; $F_{1,16} = 11.92$, $p < 0.01$ as compared to baseline for light period). In the saline group, BW was increased from 5.9 ± 2.5% to 14.7 ± 2.5% in the dark period ($p < 0.05$) and from 8.9 ± 1.5% to 15.9 ± 1.5% in the light period ($p < 0.05$). However, in the ETX group, BW during both the dark and light periods was not significantly different from baseline ($p > 0.05$ for both).

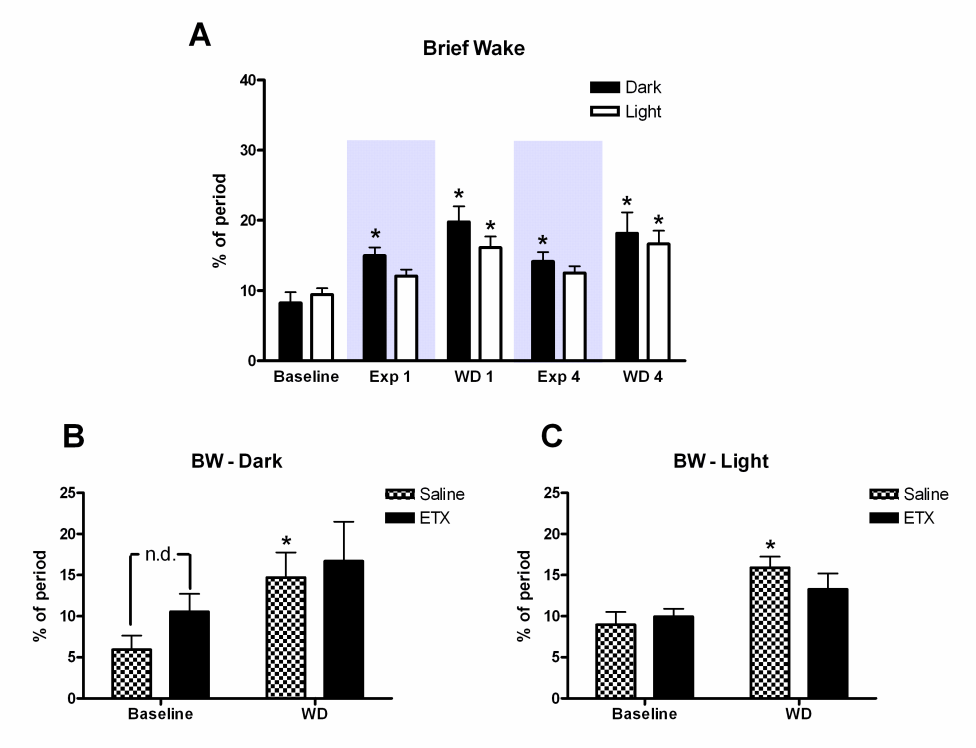


**Figure 9. ETX partially reverses withdrawal-mediated sleep fragmentation in the light period.** (A) Percentage of time spent in periods of brief wakening (BW) during the dark and light periods of baseline, the first and fourth weeks of CIE exposure (Exp 1 & 4, respectively) and early withdrawal (WD 1 & 4, respectively). Grey shading indicates ethanol exposure. BW was significantly increased during the dark period of Exp & WD during both weeks of CIE treatment (A). BW was significantly increased in the light period of WD only. Grey shading indicated ethanol exposure. (B) Comparison of percentage of time spent in BW during the dark period between mice receiving saline and ethosuximide (ETX) during baseline and sustained withdrawal. BW remains significantly elevated during sustained withdrawal in the saline group only. (C) Comparison of % time in BW during the light period between the saline and ETX groups during baseline and sustained withdrawal. BW remains significantly elevated during sustained withdrawal in the saline group only. * $p < 0.05$

*Experiment 2: Effect of ethanol withdrawal on sleep in Ca(v)3.2-deficient mice*

To examine the involvement of T-type calcium channels more directly, we performed a separate experiment based on prior results implicating the T-type calcium channel, Ca(v)3.2, in the development of hyperexcitability in the cortico-thalamo-hippocampal network implicated in the maintenance of sleep-related oscillations (Graef et al., 2011). In this experiment, a group of Ca(v)3.2-deficient mice (KOs, $n = 6$) and a group of wild-type controls (WTs, $n = 6$) were subjected to one week of baseline recording, five days of CIE exposure, and two days of sustained withdrawal. These data were sleep scored and analyzed for measures of sleep quantity and architecture. Measures of sleep or wake time are reported as the total percentage of each period spent in the corresponding vigilance state. Measures of sleep architecture are reported within the vigilance cycling framework, as described above. In order to determine the best approach to comparing the data from the WTs and the KOs, we initially compared the baseline data for NREM, REM, and BW. Unpaired *t*-tests revealed no significant differences among these measures for either the dark or light period at baseline ($p > 0.05$ for all). However, there was a trend toward increased REM at baseline in the KO group ($p = 0.096$). This was further highlighted by the analysis within the VC framework. There was a significant main effect of strain on the percentage of VC spent in REM ($F_{1,20} = 5.34$, $p < 0.05$); however, the post hoc tests comparing the two groups in the dark and light periods were not found to be significant ($p > 0.05$ for both). Thus, we determined that a direct comparison of the two groups would be appropriate for subsequent analyses.

*CIE exposure suppresses REM sleep of Ca(v)3.2-deficient, but not wild-type, mice*

There was a significant main effect of CIE treatment on percent time spent in NREM sleep in both the dark period ($F_{2,30} = 15.82$, $p < 0.001$) and the light period ($F_{2,30} = 6.09$, $p < 0.01$). WT mice exhibited increased NREM from 35.5 ± 16.5% to 61.6 ± 11.6% of the dark period ($p < 0.05$) during EtOH exposure days (Exp; Fig. 10A,B). However, the increase from 55.4 ± 10.3% to 67.7 ± 9.3% of the light period was not found to be significant ($p > 0.05$). KO mice exhibited increased NREM from 30.3 ± 2.8% to 57.3 ± 5.0% of the dark period ($p < 0.05$) and from 55.5 ± 5.1% to 66.7 ± 5.1% of the light period ($p < 0.05$) during Exp. Reciprocal changes were observed in percent time spent in LDW (Fig. 12). Furthermore, there were significant main effects of both CIE treatment ($F_{2,30} = 3.38$, $p < 0.05$) and strain ($F_{1,30} = 6.31$, $p < 0.05$) on percent time spent in REM sleep in the light period (Fig. 11A,B). Post hoc tests demonstrated decreased REM from 4.55 ± 0.8% to 1.78 ± 0.8% in the KO group during Exp ($p < 0.05$); whereas, all other differences were not found to be significant ($p > 0.05$ for all). No significant main effect of CIE treatment on BW was observed (Fig. 13).

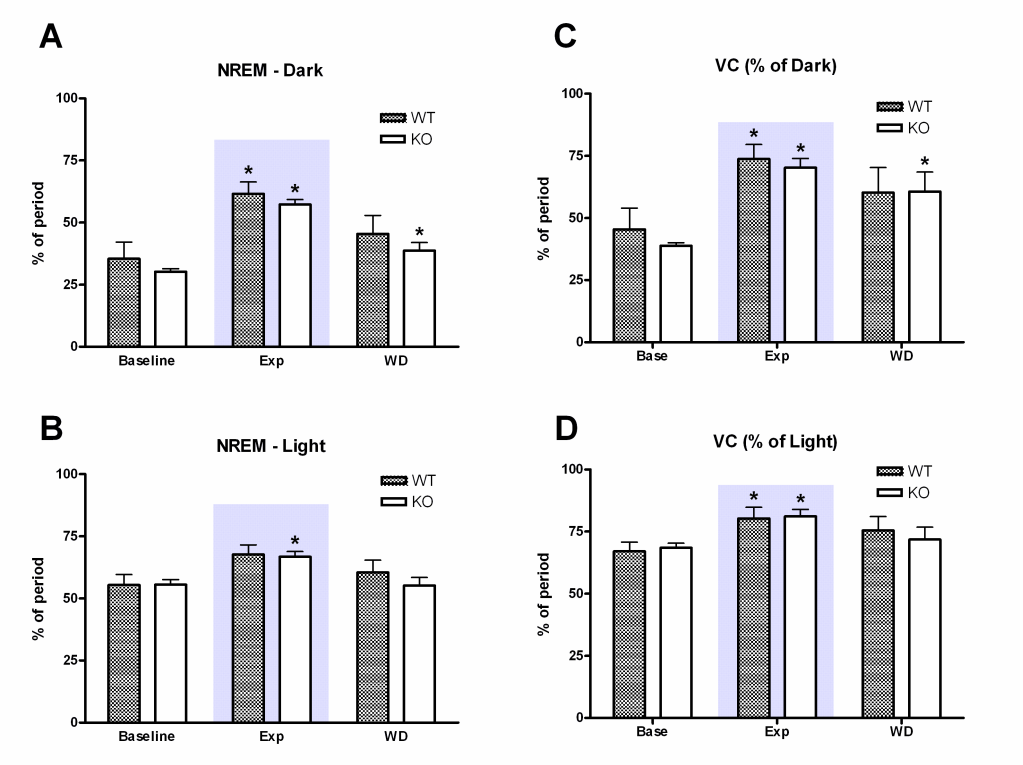


**Figure 10. Ca(v)3.2-deficient mice exhibit increased sleep during WD.** (A & B) Comparison of percent time spent in NREM sleep during baseline, one week of CIE exposure (Exp), and withdrawal (WD) between Ca(v)3.2-deficient mice (KO) and wild-type (WT) controls during the dark and light periods, respectively. NREM is elevated in both groups during the dark period of Exp (A). NREM remains elevated during WD in the KO group. (C & D) Comparison of percent time spent in VC during baseline, Exp, and WD between KO and WT groups. VC is elevated in both groups during both the dark (C) and light (D) periods of Exp. VC remains elevated in the KO group during the dark period of WD. * $p < 0.05$

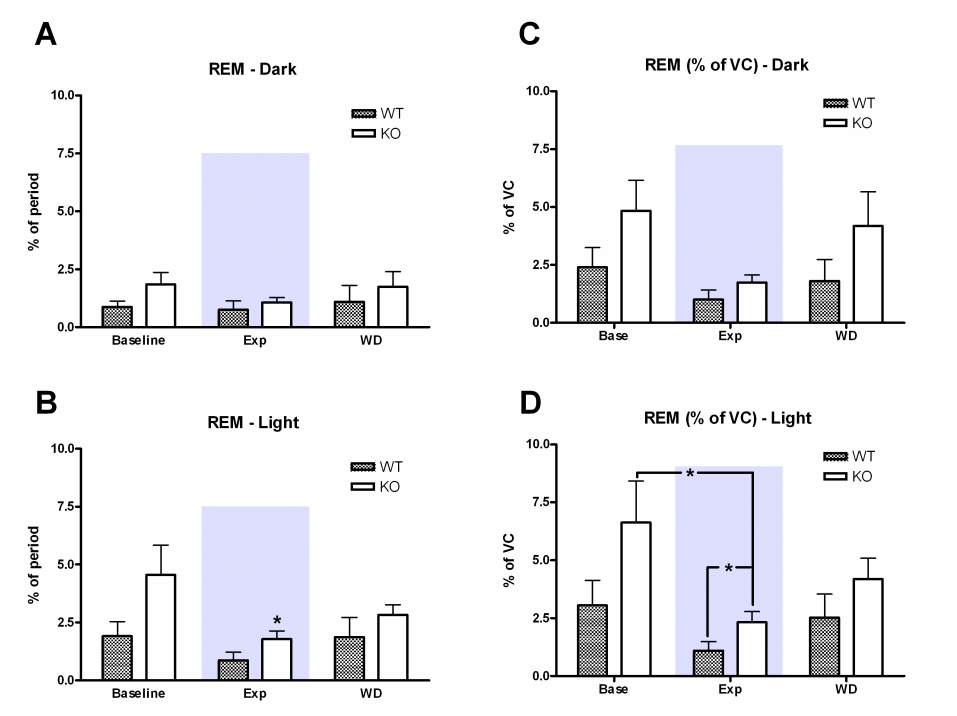


**Figure 11. CIE exposure suppresses REM in Ca(v)3.2-deficient mice during the light period.** (A & B) Comparison of percent time spent in REM during baseline, one week of CIE exposure (Exp), and withdrawal (WD) between Ca(v)3.2-deficient mice (KO) and wild-type (WT) controls. Grey shading indicates ethanol exposure. No significant effect of CIE treatment on REM was detected during the dark period (A). REM was significantly decreased in the KO group during the light period of Exp. No increase in REM was observed during WD. (C & D) REM expressed as a % of VC. No significant effect of CIE treatment was observed during the dark period (C). REM was significantly decreased during the light period of Exp in the KO group (D). However, the KO group exhibited a significantly greater amount of REM as a % of VC at this time point. * $p <$ 0.05

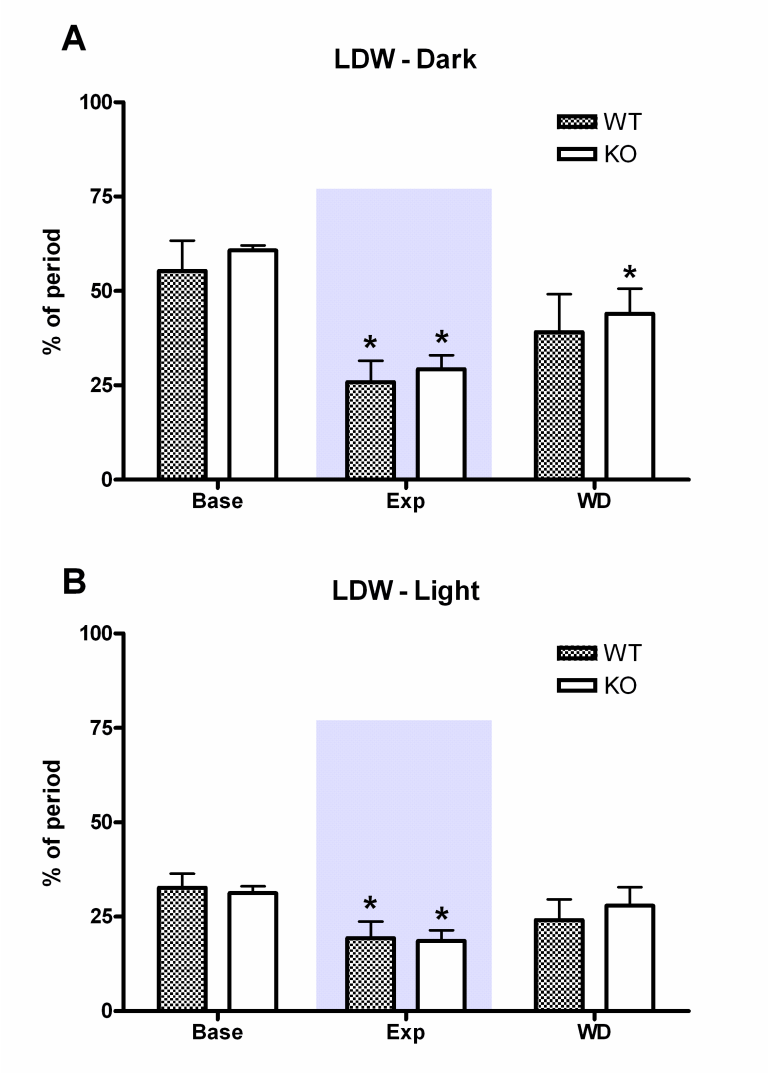


**Figure 12. CIE effects on LDW for Experiment 2.** Percent time spent in periods of long-duration wakening during the dark (A) and light (B) periods of baseline, one week of CIE exposure (Exp) and withdrawal (WD). LDW was significantly decreased in both groups during the dark and light periods of Exp. LDW remained significantly decreased during the dark period of WD in the KO group only. * $p < 0.05$

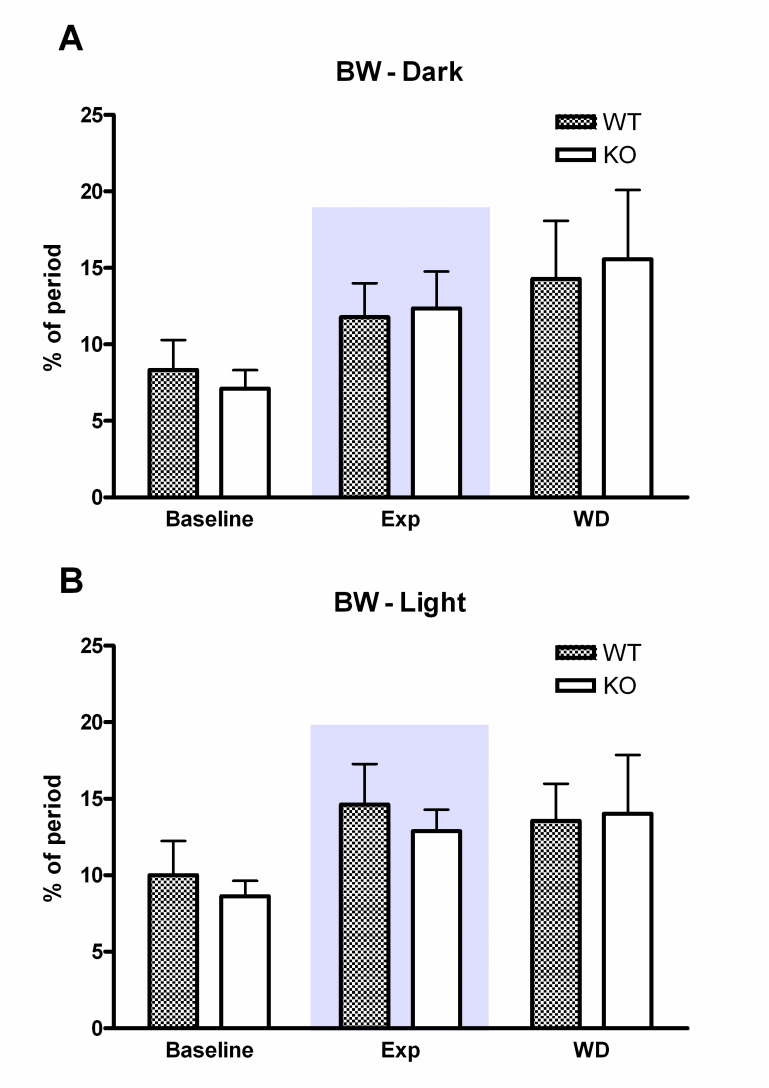


**Figure 13. No effect of CIE on BW in Ca(v)3.2-deficient mice after 1 week of exposure.** BW was not significantly increased in either the Ca(v)3.2-deficient mice (KO) or the wild-type (WT) controls during a single week of CIE exposure (Exp) and withdrawal (WD) during the dark (A) or light (B) periods. Grey shading indicates ethanol exposure.

Corresponding effects on sleep architecture were observed in the VC analysis, summarized in Table IV. There were significant main effects of CIE treatment on percent time in VC (Fig. 10C,D) during both the dark period ($F_{2,30} = 9.48$, $p < 0.001$) and the light period ($F_{2,30} = 5.00$, $p < 0.05$). Percent time in VC was increased in both groups during both the dark ($p < 0.05$ for both groups) and light periods ($p < 0.05$ for both). We found a significant main effect of CIE treatment on percent of VC spent in REM during the light period ($F_{2,30} = 4.51$, $p < 0.05$) with significant main effects of strain during both the dark ($F_{1,30} = 5.36$, $p < 0.05$) and light periods ($F_{1,30} = 6.44$, $p < 0.05$). Post hoc tests revealed decreased REM in the KO group ($p < 0.05$), but greater REM in the KO group ($p < 0.05$, as compared to WT), during the light period of EtOH Exp (Fig. 11C,D).

*NREM sleep remains increased in Ca(v)3.2-deficient mice during the withdrawal dark period*

Post hoc tests revealed the persistence of increased percent time spent in NREM during the dark period of subacute withdrawal (WD) in the KO group (Fig. 10A, $p < 0.05$), whereas the saline group returned to baseline ($p > 0.05$). This effect was also observed in the percent time spent in VC during the dark period of WD (Fig. 10C; $p < 0.05$ for the KO group, $p > 0.05$ for WT). No other significant effects were observed during WD.

## Table IVa. Vigilance cycling analysis parameters for Experiment 2, dark period

| Parameter | Baseline | | EtOH Wk 1 | Exposure | EtOH Wk 1 | Withdrawal |
|---|---|---|---|---|---|---|
| | Wild-type | Ca(v)3.2 KO | Wild-type | Ca(v)3.2 KO | Wild-type | Ca(v)3.2 KO |
| **Long duration wake** | | | | | | |
| # episodes: | 12.8 ± 2.6 | 16.0 ± 4.5 | 11.6 ± 5.0 | **12.4 ± 1.5*** | 18.3 ± 1.8 | 14.2 ± 4.2 |
| Avg dur: | 2220 ± 1400 | 1840 ± 680 | **900 ± 280*** | **1010 ± 380*** | 1050 ± 410 | 1340 ± 860 |
| % time: | 54.1 ± 20.9 | 60.7 ± 3.2 | **25.8 ± 14.0*** | **29.3 ± 9.1*** | 44.0 ± 18.7 | **38.8 ± 19.0*** |
| **Vigilance cycling** | | | | | | |
| # episodes: | 10.9 ± 2.7 | 12.4 ± 2.4 | 10.1 ± 4.1 | 10.7 ± 1.0 | 15.0 ± 1.3 | 11.8 ± 3.5 |
| Avg dur: | 1920 ± 900 | 1380 ± 200 | 6480 ± 7950 | **3620 ± 1600*** | 1650 ± 550 | **2410 ± 1040*** |
| % time: | 45.5 ± 20.9 | 38.8 ± 3.1 | **73.8 ± 14.2*** | **70.3 ± 9.2*** | 55.2 ± 18.9 | **60.7 ± 19.2*** |
| **NREM sleep** | | | | | | |
| Avg dur: | 175 ± 87 | 141 ± 36 | 273 ± 160 | 190 ± 50 | 261 ± 217 | 125 ± 41 |
| % VC: | 78.6 ± 6.0 | 77.7 ± 6.3 | 82.5 ± 7.1 | 81.1 ± 5.7 | 76.7 ± 14.5 | 72.8 ± 10.7 |
| **REM sleep** | | | | | | |
| Avg dur: | 22.6 ± 8.3 | 22.0 ± 9.3 | 16.4 ± 4.6 | 17.1 ± 5.1 | 20.0 ± 7.9 | 20.2 ± 6.9 |
| % VC: | 2.4 ± 2.1 | 4.8 ± 3.2 | 1.0 ± 1.0 | 1.7 ± 0.8 | 2.1 ± 2.4 | 4.2 ± 3.6 |
| **Brief wake** | | | | | | |
| Avg dur: | 49.7 ± 10.1 | 48.9 ± 13.1 | 56.6 ± 15.2 | 50.6 ± 11.0 | 66.2 ± 11.4 | 52.0 ± 11.3 |
| % VC: | 19.0 ± 5.6 | 17.5 ± 5.9 | 16.5 ± 6.5 | 17.2 ± 6.3 | 21.2 ± 12.4 | 23.0 ± 10.4 |

*p < 0.05 (significantly different from baseline for group)

## Table IVb. VC parameters for Experiment 2, light period

| Parameter | Baseline | | EtOH Wk 1 | Exposure | EtOH Wk 1 | Withdrawal |
|---|---|---|---|---|---|---|
| | Wild-type | Ca(v)3.2 KO | Wild-type | Ca(v)3.2 KO | Wild-type | Ca(v)3.2 KO |
| **Long duration wake** | | | | | | |
| # episodes: | 10.5 ± 1.8 | 10.3 ± 2.7 | 9.3 ± 4.9 | 9.9 ± 2.6 | 12.8 ± 3.2 | 10.3 ± 2.0 |
| Avg dur: | 1390 ± 480 | 1360 ± 360 | **890 ± 230*** | **780 ± 240*** | **990 ± 150*** | 1130 ± 420 |
| % time: | 32.7 ± 9.0 | 31.3 ± 4.4 | **19.3 ± 10.7*** | **18.6 ± 6.8*** | 28.4 ± 7.3 | 27.9 ± 12.1 |
| **Vigilance cycling** | | | | | | |
| # episodes: | 9.2 ± 1.8 | 9.1 ± 1.5 | 8.1 ± 3.8 | 8.6 ± 2.0 | 10.1 ± 1.7 | 9.2 ± 1.5 |
| Avg dur: | 3350 ± 870 | 3280 ± 470 | 8570 ± 9840 | 5060 ± 2980 | 3170 ± 740 | 3510 ± 1370 |
| % time: | 67.1 ± 9.1 | 68.6 ± 4.3 | **80.3 ± 11.0*** | **81.2 ± 6.9*** | 71.1 ± 7.4 | 71.9 ± 12.1 |
| **NREM sleep** | | | | | | |
| Avg dur: | 241 ± 187 | 150 ± 45 | 293 ± 183 | 192 ± 57 | 247 ± 177 | 128 ± 40 |
| % VC: | 82.2 ± 7.8 | 80.8 ± 5.2 | 84.0 ± 6.6 | 82.1 ± 2.9 | 81.0 ± 10.2 | 77.2 ± 9.4 |
| **REM sleep** | | | | | | |
| Avg dur: | 22.6 ± 10.4 | 23.3 ± 11.1 | 16.4 ± 4.1 | 16.8 ± 4.6 | 19.9 ± 7.1 | 20.2 ± 7.7 |
| % VC: | 3.1 ± 2.7 | 6.6 ± 4.4 | 1.1 ± 1.0 | **2.3 ± 1.1*†** | 2.9 ± 2.6 | 4.2 ± 2.2 |
| **Brief wake** | | | | | | |
| Avg dur: | 44.2 ± 12.0 | 40.1 ± 6.2 | 52.2 ± 17.6 | 47.9 ± 7.4 | 53.6 ± 17.0 | 42.1 ± 12.0 |
| % VC: | 14.8 ± 7.8 | 12.6 ± 3.8 | 14.9 ± 5.8 | 15.6 ± 3.3 | 16.1 ± 8.0 | 18.6 ± 9.3 |

*p < 0.05 (significantly different from baseline for group)

†p < 0.05 (significantly different from wild-type in same treatment period)

## Discussion

In the present study, we demonstrated a prominent sedating effect of chronic, intermittent ethanol (CIE) administration in mice, as reflected by changes in sleep time, sleep architecture, and behavioral/physiological diurnal rhythms. We observed that recovery from these alterations in sleep homeostasis was gradual and, in some cases, incomplete after withdrawal. Furthermore, we demonstrated differential effects of ethosuximide treatment and genetic inhibition of Ca(v)3.2 expression on recovery from CIE exposure. These results implicate a complex relationship between the effects of CIE treatment on T-type calcium channels and sleep homeostasis.

Our analysis fills in two important gaps with respect to previous studies of sleep in the CIE paradigm. The first is that we have been able to characterize sleep during the administration process, which provides important information for interpreting changes in sleep during withdrawal. A previous study found significant decreases in total sleep time after 4 days of CIE administration when compared to a group of air-exposed controls; however, the values in the treatment group were not significantly different from baseline and sleep was not recorded during exposure (Veatch, 2006). Second, our analysis is the first attempt to examine the effects of pharmacologic and genetic interventions in this model of alcohol-related sleep disruption.

As shown in Figure 6, we observed a progressive increase in vigilance cycling (VC) during the first week of CIE administration that was predominantly a result of increased NREM sleep time. We did not observe any significant fragmentation of VC by LDW as a result of chronic EtOH exposure, as was observed in a previous study in rats that employed a chronic liquid-diet method of EtOH administration (Mukherjee &

Simasko, 2009). This could be attributed to important differences in administration methods or species differences. In the CIE paradigm, consumption of EtOH is passive, as it is vaporized and pumped into the chamber where the animals are housed. This resulted in significant attenuation of daily activity revealed by cosinor rhythmometry. In the forced drinking paradigm, however, consumption of EtOH is an active process that requires the animal to be awake enough to drink from a bottle. Thus, the increase in activity required to drink EtOH may be sufficient to fragment VC with episodes of LDW and, thus, may mask the sedative effect.

In the present study, there was no evidence to suggest a development of tolerance to the sedative effect of EtOH, despite previous reports of tolerance to other behavioral effects of ethanol in the CIE paradigm (Becker & Baros, 2006). NREM sleep time during the fourth week of CIE administration in Experiment 1 was not significantly different from that observed in the first week. While this discrepancy is difficult to interpret, it may imply that by bypassing active consumption, passive models of EtOH administration lack the development of motivational processes in the brain to overcome the sedative effects and provide sufficient alertness to drink. As an addendum to this interpretation, it should be mentioned that the CIE model has been demonstrated to increase EtOH preference in mice, suggesting the development of dependence in this treatment paradigm (Becker & Lopez, 2004). Therefore, the likelihood is low that the absence of tolerance to the sedative effects of EtOH is attributable to a lack of chemical dependence. A final possibility is that absorption of inhaled EtOH in the lungs circumvents first-pass metabolism by the liver, increasing the opportunity for absorption into other tissues, such as the brain, prior to initial metabolism by alcohol dehydrogenase.

Another important effect of CIE exposure in Experiment 1 was the suppression of REM sleep identified through the vigilance cycling analysis. REM sleep was significantly suppressed during the fourth week of CIE exposure in the light period (a.k.a. the inactive phase in rodents), when considered as a percentage of the average vigilance cycle. This result is consistent with studies in other models of chronic alcohol exposure (Mendelson et al., 1978; Kubota et al., 2002); however, this is the first report of this phenomenon in the CIE paradigm. A sufficient amount of REM sleep is thought to play an important role in the restorative aspect of sleep (Endo et al., 1998). In this context, despite increase quantity of sleep during CIE exposure, the decreased quality of sleep could account for the increased sleep pressure reflected in continued elevation of VC during the dark period of sustained withdrawal in Experiment 1. Interestingly, mice did not demonstrate the phenomenon of increased REM known as "REM rebound", sometimes described in humans following alcohol withdrawal (Gillin et al., 1994). However, this phenomenon is inconsistent in human studies of alcohol withdrawal, raising the question of its importance in the pathology of alcohol withdrawal-related insomnia (Brower, 2001).

Many of the sedative effects of CIE administration demonstrated partial reversion towards baseline during the early withdrawal phase. By the WD Week, few effects persisted. As mentioned above, there was a residual increase in VC during the dark period of sustained withdrawal. However, the most prominent effect of successive withdrawals was an increase in brief wake (BW) that persisted through sustained withdrawal in the saline group. Interestingly, ETX administration appeared to partially reverse this increase (Fig. 9C). An increase in BW during the light period, in the absence of any significant increase in VC, implies that sleep is fragmented in the saline group

during sustained withdrawal as compared to baseline (Grandner, Hale, Moore, & Patel, 2010; Lo et al., 2004). A previous study in our lab identified increases in the delta and theta power of NREM and REM sleep spectra of this same cohort of mice treated with ETX (see Chapter 2). Taking these findings together, it appears that ETX may act to increase the intensity of sleep, thus stabilizing sleep states after withdrawal from ethanol.

As was discussed at length in Chapter 2, ethosuximide has been shown to inhibit T-type calcium current (Gomora et al., 2001; Huguenard, 1996; Todorovic & Lingle, 1998), though there is some debate as to the specificity of its action (Leresche et al., 1998; Crunelli & Leresche, 2002). To more specifically investigate the role of T-type calcium channels in alcohol-related sleep disruption, we compared the response of Ca(v)3.2-deficient (KO) mice to the CIE paradigm to that of wild-type (WT) controls. Our choice of strain was based upon previous findings in our lab implicating hyperexcitability of Ca(v)3.2 channels in the midline thalamus after multiple withdrawals from CIE (Graef et al., 2011). Initially, we observed significant suppression of REM sleep in KO mice during the first week of exposure that was not seen in the WT mice of either experiment at this time point. Furthermore, while KO mice experienced the sedative effects of CIE exposure to a similar degree as WTs, they appeared to be slower in recovering from these effects as demonstrated by a persistent increase in NREM sleep and VC during the dark period of the early withdrawal phase. Multiple studies have implicated increased sleep time and/or intensity during the dark period as an adaptive response to alterations in normal sleep homeostasis during the light period (Leemburg et al., 2010; Mukherjee & Simasko, 2009; Porkka-Heiskanen & Kalinchuk, 2011). Hence, while the KO mice may be more susceptible to REM suppression during CIE exposure,

the increase in sleep during the early withdrawal phase may actually indicate improved recovery of normal sleep homeostasis. Again, it should be noted that the KO mice did not exhibit increased REM during WD or REM rebound.

The present study identifies several important differences between ETX treatment and genetic inhibition of a specific T channel isoform, Ca(v)3.2: (1) Whereas, ETX did not appear to have a significant effect on NREM sleep or VC, Ca(v)3.2 KO may alter recovery during withdrawal; (2) Ca(v)3.2 KO may alter the REM response to EtOH exposure; and (3) while ETX may reduce sleep fragmentation during sustained WD, Ca(v)3.2 KO does not appear to have an effect on sleep fragmentation during exposure or subacute WD. However, caution must be taken when interpreting these differential effects for a number of reasons. First, the Ca(v)3.2-deficient mice were only subjected to one week of CIE exposure and withdrawal and their genetic deficiency was present throughout exposure; whereas, ETX-treated mice were subjected to four weeks of CIE exposure and withdrawal and only received ETX during sustained WD. Second, ETX is a blocker of all three T-type channel isoforms, and the contribution of these multiple isoforms to normal sleep is not completely characterized. Third, genetically modified mice are subject to developmental compensation, potentially affecting the other T channel isoforms or other physiologic processes in the brain. Future studies should examine the effects of temporally and/or regionally selective knock-down of Ca(v)3.2 specific to the forebrain in order to more precisely examine its role in WD. In our studies of wild-type mice, Ca(v)3.2 was implicated in withdrawal-related effects. In addition, acute administration of ethanol has recently been shown to selectively target the Ca(v)3.2 T channel isoform (Shan, Hammarback, & Godwin, unpublished results). Thus, acute

activation of Ca(v)3.2 may be a necessary condition for withdrawal-related effects, and its absence during ethanol exposure may affect the state of the system after withdrawal. Despite these concerns, both the group of mice receiving ETX during sustained withdrawal and the Ca(v)3.2-deficient mice demonstrated signs of increased sleep stability and/or intensity after withdrawal from CIE, potentially indicating increased quality of sleep. These effects further highlight the primacy of T-type calcium channels in sleep homeostasis. Future studies will be required to determine the potential of ETX as a therapeutic agent and/or T-type calcium channels as a therapeutic target in this and other models of chronic alcohol exposure.

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# CHAPTER 4

# MECHANISMS OF SLEEP, SLEEP-RELATED OSCILLATIONS, AND ALCOHOL WITHDRAWAL-RELATED INSOMNIA

## Introduction

The oscillatory properties of the thalamus have been the subject of intense research since the late 1930s, but the role of the thalamus in waking and sleeping processes has long been regarded as a simple relay of sensory information to the primary sensory cortices, active during waking and inactive during sleep. The active contribution of the thalamus to sensory processing has been recently reviewed in great detail (Sherman & Guillery, 2002). In this Chapter, we will focus on the mechanisms by which the thalamus generates and maintains sleep-related oscillations in the brain and the proposed functions of these oscillations in the maintenance of sleep. This discussion carries significant implications for the results presented in Chapters 2 & 3.

To facilitate a discussion of the thalamocortical processes that act to maintain the neurobehavioral coordination of sleep, we begin with a brief overview of the other mechanisms in the central nervous system (CNS) which contribute to the generation and maintenance of the sleep state in the brain, previously reviewed in great detail elsewhere (España & Scammell, 2011; Pace-Schott & Hobson, 2002). Subsequently, we discuss the identification of the various rhythms that characterize the electroencephalographic (EEG) profile of the sleeping brain and the role the thalamus is believed to play in the generation and maintenance of these rhythms. We then incorporate the effects of chronic alcohol exposure and withdrawal on sleep and sleep-related oscillations, presented in Chapters 2 & 3. Finally, we conclude with a discussion of the potential mechanisms by which ethosuximide appears to ameliorate these pathological alterations and the implications of these results for future research into the mechanisms and treatment of alcohol withdrawal-related insomnia (AWI).

**The Neurobiology of Sleep**

Generally speaking, there are several ongoing processes in the brain that regulate sleep. These processes govern sleep-wake transitions, sleep timing, and sleep homeostasis and have previously been reviewed in detail elsewhere (Pace-Schott & Hobson, 2002). A schematic of the brain regions involved in these processes is presented in Figure 1.

Throughout the day, exposure to natural light sets the phase of the central circadian clock via signals transmitted from the retina to the suprachiasmatic nucleus (SCN) of the hypothalamus. The period of this clock is set by the positive and negative feedback cycles generated by the "clock" genes to be approximately 24 hours, controlling the output of SCN neurons such that SCN firing peaks near the middle of the day (Gillette & Tischkau, 1999). These neurons exert an inhibitory effect on the ventrolateral preoptic (VLPO) nucleus of the anterior hypothalamus, which, in turn, exerts an inhibitory effect on the wake-promoting aminergic nuclei of the brainstem, the locus coeruleus and the dorsal raphe nucleus. Thus, when the output of the SCN decreases, the VLPO is disinhibited and increases its input onto these nuclei. Furthermore, a subset of neurons in the VLPO exerts an inhibitory effect on the histaminergic tuberomamillary nucleus (TMN) of the posterior hypothalamus, which projects inhibitory feedback onto the VLPO (Saper, Chou, & Scammell, 2001).

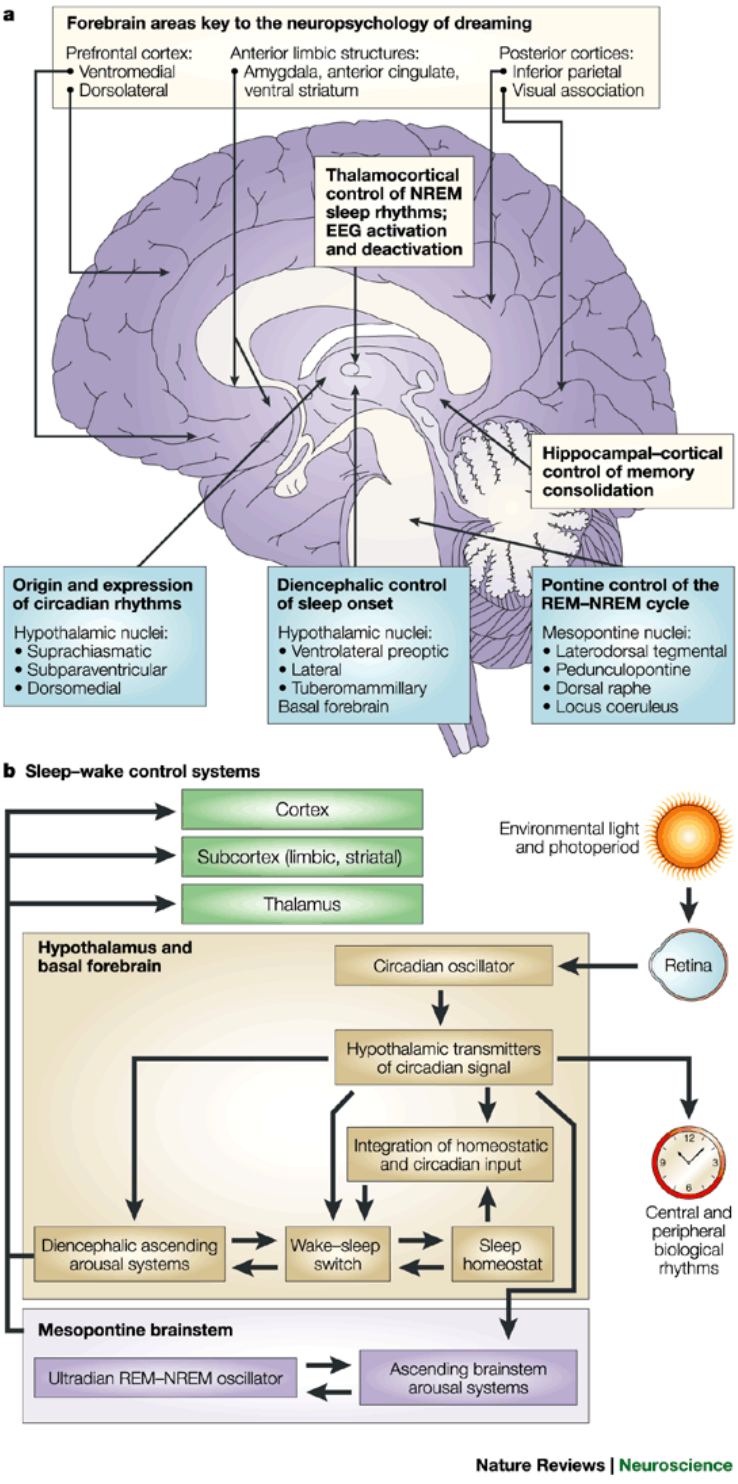


**Figure 14. A schematic of the brain regions involved in the regulation of sleep-wake cycles.** Reproduced from Pace-Schott & Hobson (2002).

The anterior VLPO and the posterior TMN form the sleep- and wake-promoting regions of the hypothalamus, respectively. Through mutual inhibition, this network manifests a bistability that can transition the CNS into the sleeping or waking state, depending on other homeostatic mechanisms. Adenosine is a proposed sleep-promoter (or somnogen) that may act to tip this balance towards sleep in concert with the circadian influences described above. Adenosine accumulated in the basal forebrain during wakefulness is believed to inhibit GABAergic neurons in the basal forebrain that project to the VLPO, thus, disinhibiting the VLPO (Strecker et al., 2000). This release of the VLPO results in inhibition of the wake-promoting regions of the TMN and brainstem, initiating NREM sleep.

The ultradian alternation of rapid eye movement (REM) and non-REM (NREM) states during sleep is primarily controlled by interactions between the pontine cholinergic and aminergic systems, as described by the reciprocal-interaction model (Hobson, McCarley, & Wyzinski, 1975). According to this model, the REM-off cells of the pontine aminergic system are tonically activated during waking, inhibiting the REM-on cells of the pontine cholinergic system. The activity of the REM-off cells wanes during NREM sleep, resulting in increased activity of the REM-on cells, eventually initiating a REM cycle. During REM sleep, activation of cells in the mesopontine junction results in activation of the brainstem reticular formation (BRF). As activity in the BRF builds, it reactivates the REM-off cells, completing the cycle.

To this point, we have given an overview of the complex interactions in the brainstem, hypothalamus, and basal forebrain that mediate sleep-wake transitions in the

CNS. These brain regions play an important role in the initiation of the NREM sleep, which is then maintained by the coordinated activity of thalamocortical oscillations.

**Thalamocortical Oscillations & the Maintenance of NREM Sleep**

NREM sleep is partitioned into four stages of increasing depth of sleep, based on EEG characteristics of cortical activity. The depth of sleep is defined by the transition from the low amplitude, high frequency activity of the waking brain to the high amplitude, low frequency (or slow wave) activity of the sleeping brain (see Pace-Schott & Hobson, 2002, for review). Slow wave activity (SWA) predominates in Stages III and IV of NREM, collectively referred to as slow wave sleep (SWS). Stage II NREM is characterized by the appearance of three coordinated thalamocortical oscillations: the slow (< 1 Hz) oscillation, the K-complex, and the sleep spindle. As the frequency of the slow oscillation increases during sleep, the appearance of spindles and K-complexes gradually transitions into delta waves (0.5-4 Hz).

The slow oscillation was initially described by Steriade and colleagues in a series of three papers published in 1993 (Steriade et al., 1993; Steriade, Nuñez, & Amzica, 1993a, 1993b). It was initially identified in cortical pyramidal neurons of anesthetized cats (Steriade et al., 1993; Steriade, Nuñez, & Amzica, 1993b) as a 0.2-0.9 Hz alternation between states of membrane hyperpolarization with low action potential firing (called the "down" state) and depolarization with high rates of action potential firing (called the "up" state). The third paper (Steriade, Nuñez, & Amzica, 1993a) described similar oscillations in thalamocortical relay neurons and neurons of the thalamic reticular nucleus (TRN) that were temporally correlated with the oscillations in cortex. The authors of this initial series

found that sleep spindles were clustered in the up states while delta waves were clustered in the down states. When the connections between the thalamus and cortex were severed, the slow oscillation persisted in the cortex, leading the authors to hypothesize that the slow oscillation was cortically generated (Steriade, Nuñez, & Amzica, 1993a). For over a decade, this view was widely accepted (Amzica & Steriade, 2002; Yuste et al., 2005); however, the role of the thalamus in the generation of the slow oscillation has recently been reexamined.

In their recent comprehensive review of the literature, Crunelli and Hughes (2010) present a compelling case for the central role of the thalamus in the generation of the slow oscillation. The slow oscillation has been extensively studied in anesthetized animals, but has also been identified and studied in the natural NREM sleep of both animals and humans. Studies have shown that the K-complex of Stage II sleep represents the transition from the down state to the up state and that spindles occur most frequently during the early phase of the up state, often preceded by a K-complex (Amzica & Steriade, 2002). The frequency of the slow oscillation increases as sleep deepens, resulting in a transition from the predominance of sleep spindles and K-complexes in Stage II to the delta waves of SWS (Crunelli & Hughes, 2010). This increase in frequency is consistent with findings in the thalamus, where increasing frequency of slow oscillations was observed with greater degrees of hyperpolarization (Blethyn et al., 2006; Hughes et al., 2002; Zhu et al., 2006); whereas, a similar study in cortex found decreased frequency of slow oscillations (Timofeev et al., 2000). Furthermore, T-type calcium channel-mediated burst firing often precedes the up state by 20-50 ms (Contreras & Steriade, 1995; Ushimaru, Ueta, & Kawaguchi, 2012) and such bursts from the thalamus

are the most effective means of generating up states in cortex (Beierlein et al., 2002; Cossart, Aronov, & Yuste, 2003; MacLean et al., 2005; Shu, Hasenstaub, & McCormick, 2003). The sum of the above results led Crunelli and Hughes to propose the three-oscillator hypothesis, whereby the oscillatory states of the TRN, thalamocortical relay neurons, and the cortex are all critical to the generation of slow oscillation dynamics (Crunelli & Hughes, 2010). Specifically, they implicate the role of "window" T-type calcium current, defined as the steady-state current occurring within a physiologically relevant range of membrane potentials (Hughes et al., 1999), in the generation of the membrane bistability responsible for these oscillatory properties of the thalamus in this model (Crunelli et al., 2005). Subsequent *in vivo* experiments and modeling studies have provided further support for this hypothesis (Crunelli et al., 2011; Ushimaru et al., 2012).

**Functional Implications for Normal Sleep and AWI**

With respect to their functional role in sleep, sleep-related thalamocortical oscillations are believed to be involved in sleep's physiologically restorative properties, neurobehavioral coordination of sleep, the consolidation of memory during sleep (see Hobson & Pace-Schott, 2002, for review). Here we will focus on the restorative properties of sleep-related oscillations (SROs) and the neurobehavioral coordination of sleep.

Borbély's two-process model of sleep homeostasis involves the interaction of circadian and homeostatic factors which drive sleep-wake transitions (Borbély & Achermann, 1999). In this model, SWA is the primary indicator of homeostatic sleep drive. Indeed, greater SWA during sleep is tightly correlated with degree of sleep

deprivation (Borbély, 2001). This effect is particularly pronounced in the frontal cortex, possibly indicating a greater need for restorative sleep in the areas controlling executive function and working memory (Finelli, Borbély, & Achermann, 2001). The waking correlate of SWA is theta (5-8 Hz) activity, which tends to rise with increasing time spent awake (Aeschbach et al., 2001; Finelli et al., 2000).

In chronic primary insomnia, individuals demonstrate lower theta power during waking hours (Wolynczyk-Gmaj & Szelenberger, 2011) and lower SWA during sleep (Merica, Blois, & Gaillard, 1998), suggesting dysfunction in sleep homeostatic mechanisms. In contrast, chronic alcoholics demonstrate increased theta power during waking hours (Rangaswamy et al., 2003) and decreased SWA during sleep (Brower et al., 2011), suggesting dysfunction in the generating mechanisms of SWA.

In Chapter 2, we presented results demonstrating alterations in the diurnal rhythm of theta power with in mice undergoing withdrawal from chronic intermittent ethanol (CIE) exposure. In the saline group, there was an elevation in the midline-estimating statistic of rhythm (MESOR) and a decrease in the amplitude of the theta rhythm. This decrease in the amount of variation in theta power, in combination with the increase in MESOR, suggests that mice undergoing withdrawal may experience less restorative sleep, thus, generating increased sleep pressure. However, in the group receiving ethosuximide (ETX), these rhythms were restored to baseline. Furthermore, mice receiving ETX demonstrated increased delta and theta power during NREM sleep than saline-treated controls. Taken together, these results suggest that ETX acts to reverse ethanol-mediated disruptions in sleep homeostatic mechanisms.

The other important mechanism of SROs is the neurobehavioral coordination of sleep required for the maintenance of NREM. The K-complex, representing the down-up state transition of the slow oscillation, has been implicated as a form of microarousal, whereby the down state of the slow oscillation buffers the cortex from complete arousal in response to sensory stimuli (Halász et al., 2004). Furthermore, sleep spindles may be involved in blunting brain responses to external stimuli. A recent study compared EEG and functional MRI (fMRI) responses to auditory stimuli between awake subjects and subjects in NREM sleep (Dang-Vu et al., 2011). FMRI responses to stimuli presented during sleep spindles were significantly blunted, consistent with previous studies of evoked potentials (Cote, Epps, & Campbell, 2000; Elton et al., 1997). The authors speculated that this was likely due to the nonlinear distortion of inputs (Sherman & Guillery, 2002) to bursting thalamocortical neurons during spindles. Stimuli presented outside of spindles often triggered a K-complex in the EEG with fMRI responses demonstrating activity resembling that observed during spontaneous and induced slow oscillations (Dang-Vu et al., 2008; Massimini et al., 2007). Given the role of thalamocortical oscillatory mechanisms in the generation of cortical up states and sleep spindles, it appears that both the down and up states of the slow oscillation are effectively buffered by the oscillatory properties of the thalamus during NREM sleep.

In Chapter 3, we presented results demonstrating fragmentation of sleep by brief wakening (BW) in mice undergoing withdrawal from CIE exposure. Given the lack of any observable increase in REM sleep during withdrawal and the mechanisms that maintain the ultradian alternation between NREM and REM once sleep has been initiated, it seems that the most likely cause of fragmentation is a failure to maintain

NREM. Administration of ETX during withdrawal reduced this fragmentation of sleep with BW to levels not significantly different from baseline. Taken together with the results of Chapter 2, we surmise that ETX acts to decrease fragmentation of sleep by stabilizing SROs during NREM.

**Potential Mechanisms of ETX Action in AWI**

In Chapter 2, we discussed the controversy surrounding the mechanism of action of ETX in detail. We revisit this discussion here to incorporate the results of Chapters 2 & 3. The pharmacologic mechanism of ETX action in the brain has been the subject of controversy over the last two decades (Huguenard, 2002). Early studies into its mechanism revealed partial inhibition of T-type calcium current in thalamocortical relay neurons (Coulter, Huguenard, & Prince, 1989a; Coulter, Huguenard, & Prince, 1989b). A subsequent study in rat sensory neurons found that ETX preferentially inhibited low-threshold T current over high-threshold L-type calcium current (Kostyuk et al., 1992). Whereas, a later study failed to observe an effect of ETX on T current in cat and rat thalamocortical neurons at similar concentrations; instead, implicating an effect on the persistent sodium current (Leresche et al., 1998). More recent studies have identified effects on calcium-dependent potassium currents (Crunelli & Leresche, 2002), G protein-mediated inwardly-rectifying potassium currents (Kobayashi et al., 2009), and GABA release probability at the synapse (Greenhill et al., 2012). However, it should be noted that the concentration of ETX administered in the last three studies was significantly higher than the therapeutically relevant range (Huguenard, 2002). Furthermore, recent

evidence confirmed partial blockade of the "window" T current (~ 30%) within this relevant range (Gomora et al., 2001).

A previous study in our lab demonstrated a progressive depolarizing shift in the inactivation of T current in midline thalamic neurons of mice undergoing multiple withdrawals from CIE exposure (Graef et al., 2011). This shift resulted in a net increase in the predicted "window" current of approximately 30% and was correlated with the alterations in delta and theta power presented in Chapter 2, which were reversed by ETX. We hypothesize that the increase in "window" current destabilizes SROs in the midline thalamus and that the partial blockade of "window" current afforded by ETX stabilizes these oscillations. To that effect, we carried out a computational study in a simple four-cell model of thalamocortical spindles and delta waves (Destexhe et al., 1996). The half-inactivation potential of the model T current was increased from -88 mV to -84 mV in model thalamocortical neurons to simulate the observed effects of withdrawal from CIE when the model was tuned to simulate sleep spindles (Figure 2) and delta waves (Figure 3).

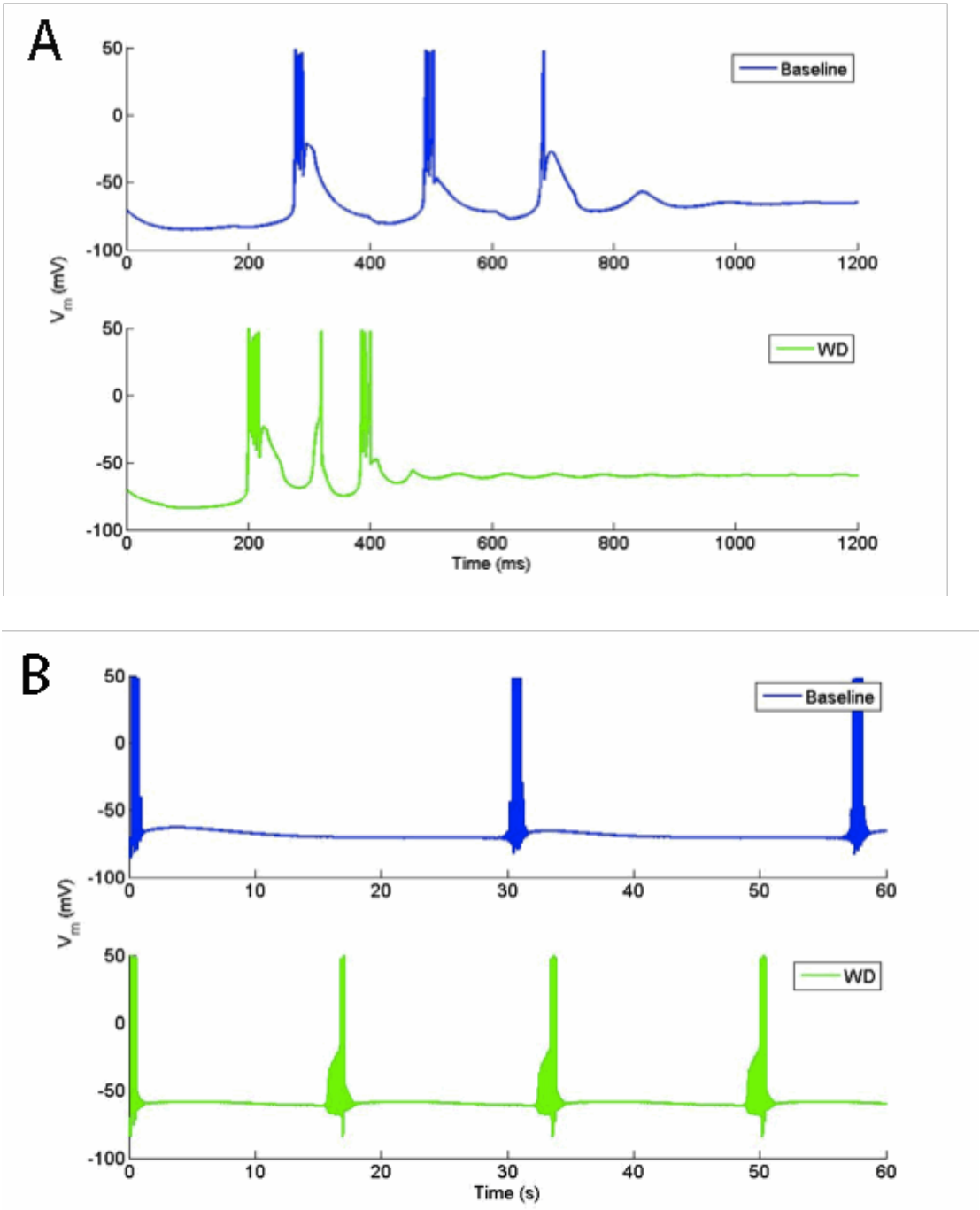


**Figure 15. Effects of depolarizing shift in half-inactivation of T-type calcium current on a model of thalamocortical spindles.** (A) 2-sec simulation demonstrating increased frequency of bursts. (B) 60-sec simulation demonstrating decreased inter-spindle period with increased "window" T current.

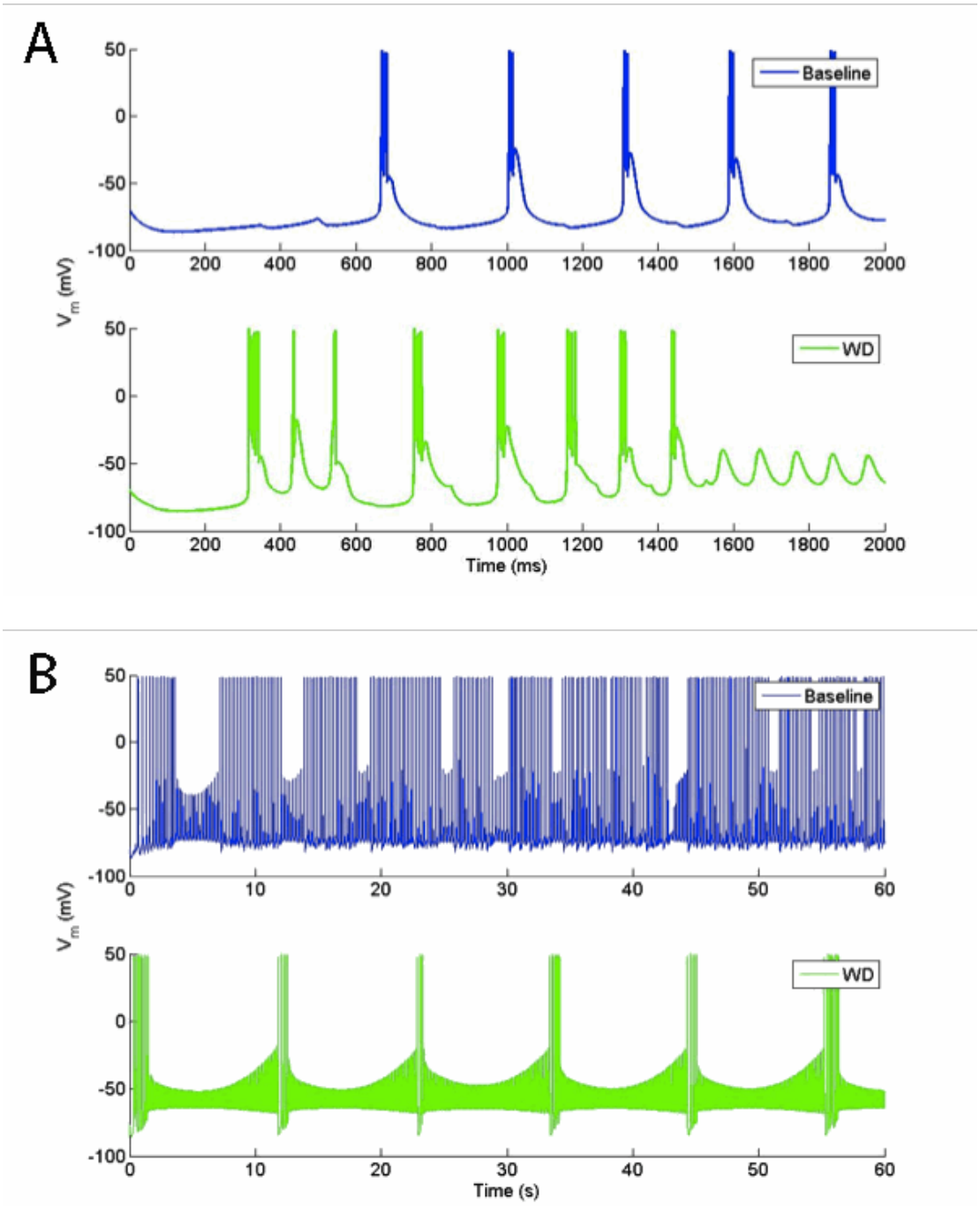


**Figure 16. Effects of depolarizing shift in half-inactivation of T-type calcium current on model of thalamocortical delta waves.** (A) 2-sec simulation demonstrating increased frequency of bursts. (B) 60-sec simulation demonstrating spindle-like oscillations emerging from increased "window" T current.

In the simulation of thalamocortical spindle oscillations, there was a significant increase in the frequency of bursts (Figure 2A), resulting in a decrease in the inter-spindle period (Figure 2B). A similar increase in burst frequency was observed in the simulation of thalamocortical delta waves (Figure 3A); however, this increase appeared to destabilize the delta waves, resulting in a spindle-like oscillation (Figure 3B). The three-oscillator hypothesis, described above, proposes that the transition in the slow oscillation from lower frequencies in lighter stages of NREM sleep (where K-complexes and spindles are the dominant features of the EEG) to deeper stages of NREM (where delta waves are the dominant features) is driven by the intrinsic oscillatory properties of the thalamus, particularly the "window" T current (Crunelli & Hughes, 2010). Taking this view together with the results of our simulation, we hypothesize that excessive "window" T current mediates the destabilization of delta waves observed in Chapter 2, which we believe underlies the sleep disruptions observed in Chapter 3. Conveniently, the estimated percentage increase in "window" current in this model of alcohol withdrawal is precisely the percentage that ETX is believed to inhibit.

**Conclusion: A Path to Translation**

In this Chapter, we have presented a case for excessive T-type calcium current as a novel therapeutic target in AWI. Furthermore, we demonstrated the efficacy of ETX, an FDA-approved antiepileptic drug with a minimal side-effect profile, in reversing withdrawal-mediated alterations in SROs and fragmentation of sleep. For these results to be translated to clinical studies, important steps need to be taken to replicate these results and ensure their validity.

First, the results demonstrating the ETX-mediated decrease in sleep fragmentation during withdrawal should be replicated in other models of alcohol withdrawal. The CIE vapor administration paradigm effectively models both alcohol dependence (Becker & Lopez, 2004) and the kindling-like effects of multiple withdrawals from ethanol (Becker, 1994). This paradigm also provides an excellent means of controlling for blood-ethanol concentration (BEC) in mice and rats, ensuring consistency and replicability of results. However, the passive nature of ethanol consumption fails to capture some of the behavioral aspects of chronic alcohol abuse. This, in turn, may have significant effects on the sleep behavior of animals during chronic ethanol consumption, as they are not required to awaken and actively seek out a source of ethanol to maintain a desired BEC. Thus, subsequent research should seek to replicate the study presented in Chapter 3 in active models of chronic alcohol consumption. As sleep fragmentation has also been demonstrated in rats to whom alcohol was chronically administered in their diet (Mukherjee & Simasko, 2009), this would seem a logical place to start.

Second, it will likely be necessary to replicate these results in higher organisms, such as nonhuman primates. While humans and rodents share common features within a sleep cycle (Lo et al., 2004), rodents are inherently polyphasic sleepers. As such, it is possible that polyphasic sleep may mask some features of AWI that are inconsistent between rodents and humans. Though nonhuman primates do sleep more than humans throughout the day, the increased degree of evolutionary homology may provide a more accurate assessment of the therapeutic potential of ETX in human AWI.

Finally, while our arguments presented in this Chapter are logically sound and well founded in the literature, there are gaps in experimental data that will need to be

filled in order to support our view that ETX stabilizes NREM sleep through inhibition of excess "window" T current. Subsequent research should focus on the use of intracellular and extracellular recordings *in vivo* to solidify this view in the following areas: (1) clarification of the effect of increased "window" T current on the oscillatory properties of thalamocortical neurons, (2) examination of effects of ethanol on T-type calcium channel function in the TRN, and (3) verification of ETX-mediated stabilization of thalamocortical oscillations after withdrawal from ethanol.

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Ushimaru, M., Ueta, Y., & Kawaguchi, Y. (2012). Differentiated participation of thalamocortical subnetworks in slow/spindle waves and desynchronization. *Journal of Neuroscience*, *32*(5), 1730-1746. doi:10.1523/JNEUROSCI.4883-11.2012

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# CURRICULUM VITAE

## Walter F. Wiggins

**Education**

- Wake Forest University; Winston-Salem, NC
  PhD in Neuroscience, May 2012
  Advisor: Dwayne W. Godwin, PhD
  Graduate GPA: 4.0 out of 4.0
  MD, expected May 2014
  Preclinical (MS1-2) Class Rank: 17 out of 119
  USMLE Step 1: 247/99
- Davidson College; Davidson, NC
  BS in Mathematics, *Cum Laude*, Minor in Chemistry, May 2007

**Honors/Awards/Fellowships**

- Ruth L. Kirschstein National Research Service Award for Predoctoral MD/PhD Students (F30)
  Funding Agency: NIAAA/NIH
  Project Title: The effect of alcohol withdrawal on molecular and behavioral circadian rhythms
  Dates: September 2010 – present
- NIH Intramural Fellowship, Medical Student Research Program
  Funding Source: WFUSM Dept. of Surgery
  Dates: June 2008 – August 2008
  Faculty Sponsor: Dwayne Godwin, PhD, Dept. of Neurobiology & Anatomy
  Amount: $4000

**Memberships**

- American College of Surgeons (Student Member)
- American Medical Association – Student Section
- North Carolina Medical Society – Student Section
- Society for Neuroscience
- Western North Carolina Chapter of the Society for Neuroscience

**Current Positions**

- Longitudinal Editor, USMLERx Step 1 Qmax
  MedIQ Learning, LLC; Elizabethtown, KY
  Dates: November 2011 – present
- Contributing Editor, USMLERx/First Aid Web Team
  MedIQ Learning, LLC; Elizabethtown, KY
  Dates: November 2011 – present

**Original Journal Articles**

1. **Wiggins WF**, Huitt TW, Graef JD, Godwin DW. Differential effects of ethosuximide treatment and genetic suppression of a T-type $Ca^{2+}$ channel subtype on altered sleep homeostasis in response to chronic intermittent ethanol. *In preparation*.
2. Plate JF, **Wiggins WF**, Haubruck P, Scott AT, Tuohy CJ, Saul KR, Stitzel JD, Mannava S. Aging influences the in vivo biomechanical properties of the rat gastrocnemius-achilles muscle-tendon unit. *Submitted*.
3. **Wiggins WF**, Graef JD, Huitt TW, Godwin DW. Ethosuximide reduces alcohol withdrawal-mediated disruptions in sleep-related EEG patterns. *Submitted*.
4. Haubruck P, Mannava S, Plate JF, Callahan MF, Saul KR, **Wiggins WF**, Schmidmaier G, Tuohy CJ, Smith TL. Botulinum neurotoxin A injections influence neural mechanisms during stretching of the gastrocnemius muscle-tendon unit. *Submitted*.
5. Mannava S, **Wiggins WF**, Saul KR, Stitzel JD, Smith BP, Koman LA, Smith TL, Tuohy CJ (2011). Contributions of neural tone to in vivo passive muscle-tendon unit biomechanical properties in a rat rotator cuff animal model. Ann Biomed Eng. 39(7):1914-24.
6. Mannava S, Callahan MF, Trach SM, **Wiggins WF**, Smith BP, Koman LA, Smith TL, Tuohy CJ (2011). Chemical denervation with Botulinum neurotoxin A improves the surgical manipulation of the muscle-tendon unit: an experimental study in an animal model. J Hand Surg Am. 36(2):222-31.
7. Graef JD, Nordskog BK, **Wiggins WF**, Godwin DW (2009). An acquired channelopathy involving thalamic T-type $Ca^{2+}$ channels following status epilepticus. J Neurosci. 29(14):4430-41.

**Book Chapters**

1. Godwin DW, **Wiggins WF**, Hayasaka S, Laurienti PJ, Stapleton JR (2011). Overcoming obstacles to creativity in geographically-fragmented environments: lessons from small world networks. In L. Book & D. Phillips (Eds.) *Creativity and Entrepreneurship: Changing Currents in Education and Public Life*. Edward Elgar Press. *Forthcoming*.
2. **Wiggins WF** & Paluzzi J (2011). Endocrine. In T. Le *et al*. (Eds.), *First Aid for the Basic Sciences: Organ Systems, 2nd Ed.,* New York: McGraw Hill Medical.

**Presentations**

1. **Wiggins WF**, Klorig DC, Godwin DW. A biophysically-plausible model of ChR2 implemented in the NEURON simulation environment. WNCSfN Annual Research Day, 2011. Poster.
2. Smith TL, Haubruck P, Saul KR, **Wiggins WF**, Stitzel JD, Smith BP, Tuohy CJ, Mannava S. In vivo biomechanics of neural mechanisms influencing stretching of the muscle-tendon unit. Biomedical Engineering Society Annual Conference, 2011. Symposium.
3. **Wiggins WF**, Huitt TW, Graef JD, Godwin DW. The effect of T-type calcium channel inhibition on ethanol withdrawal-related sleep disruption. WNCSfN Annual Research Day, 2010. Poster.

4. Mannava S, **Wiggins WF***, Callahan MF, Smith TL, Koman LA, Tuohy CJ. Innovations in tendon surgery: the neural contribution to surgical manipulation fo the muscle-tendon unit. North Carolina Orthopaedic Association Annual Meeting, 2010. Symposium.
   *Presenting author
5. "Neurodynamics: Chaos in the Brain"
   Date: October 15, 2009
   Location: Davidson College
   Brief Description: An hour-long lecture presented to the Mathematics Department about the use of chaos theory and tools from dynamical systems in the study of neural networks, highlighting examples from my graduate research.
6. Godwin DW, **Wiggins WF**, Hayasaka S, Laurienti P, Stapleton-Kotloski JR. Overcoming creative obstacles in geographically-fragmented environments: lessons from small world networks. WFU 1st Annual Creativity Symposium, 2009. Minisymposium.
7. **Wiggins WF**, O'Donovan CA, Wilson TW, Godwin DW. Imaging Childhood Absence Epilepsy with Magnetoencephalography. WFUSM Medical Student Research Day, 2008.
8. "Medical School Life"
   Date: February 12, 2008
   Location: Davidson College
   Brief Description: Panel discussion of medical school life with 3 other WFUSM medical students who are also Davidson alumni.

**Abstracts**

1. Plate JF, Haubruck P, **Wiggins WF**, Li Z, Stitzel JD, Smith TL, Tuohy CJ, Mannava S. Age-related changes influence the in vivo biomechanical properties of the rat gastrocnemius-achilles muscle-tendon unit. Orthopaedic Research Society Annual Meeting, 2012.
2. Mannava S, **Wiggins WF**, Callahan MF, Stitzel J, Koman LA, Tuohy CJ, Smith TL. Neurological influences and modulation of skeletal muscle tone. Society for Neuroscience Annual Meeting, 2010.
3. Mannava S, **Wiggins WF**, Stitzel J, Callahan MF, Smith TL, Koman LA, Tuohy CJ. Contributions of neural tone to muscle-tendon unit stress-relaxation properties. Biomedical Engineering Society Annual Conference, 2010.
4. Mannava S, **Wiggins WF**, Tuohy CJ, Koman LA, Poehling GG, Smith TL. Insights from a Simple Spring: Reconsidering Rotator Cuff Surgery. Eastern Orthopaedic Association Annual Meeting, 2010.
5. Huitt TW, Stapleton-Kotloski JR, O'Donovan CA, **Wiggins WF**, Wilson TW, Godwin DW. Imaging childhood absence epilespy with magnetoencephalography. Society for Neuroscience Annual Meeting, 2008.
6. Stapleton-Kotloski JR, Huitt TW, O'Donovan CA, **Wiggins WF**, Wilson TW, Godwin DW. Imaging childhood absence epilespy with magnetoencephalography. American Epilepsy Society Annual Meeting, 2008.

**Activities**

- Student Representative, Neuroscience Program Recruitment Committee
  Wake Forest University Graduate School of Arts & Sciences
  Dates: January 2011 – March 2012
- Web Communications Chair, Brain Awareness Council Executive Committee
  Wake Forest University Graduate School of Arts & Sciences
  Dates: January 2009 – present
- President, MD/PhD Student Association
  Wake Forest University School of Medicine
  Dates: June 2010 – June 2011
- Team Retention Chair, Winston-Salem Relay for Life
  American Cancer Society – Relay for Life
  Dates: October 2008 – January 2010
- WFUSM Ultrasounds
  Medical Student Male A Capella Group
  Dates: November 2007 – present
- Student Volunteer, DEAC Student-Run Free Clinic
  Wake Forest University School of Medicine
  Dates: September 2008 – present
- Student Member, Brain Awareness Council
  Wake Forest University School of Medicine
  Dates: September 2008 – present
- Medical Volunteer, Special Olympics of North Carolina Basketball & Cheerleading Competition 2009, 2010
  Dates: March 7, 2009; March 6, 2010
- Medical Volunteer, Ardmore/Knollwood Peru Mission 2011
  Chimbote, Peru
  Dates: May 2011
- Student Volunteer, "Share the Health" Student-run Health Fair
  Wake Forest University School of Medicine
  Dates: January 2008, January 2009, January 2010