FUNCTIONAL CONSEQUENCES OF REPEATED ALCOHOL WITHDRAWALS ON THE ELECTROENCEPHALOGRAM OF MICE

BY

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TABLE OF CONTENTS

LIST OF ILLUSTRATIONS AND TABLES

LIST OF ABBREVIATIONS

PFC	prefrontal cortex
RE	nucleus reuniens
DG	dentate gyrus
CA1	cornu Ammonis 1 of the hippocampus
CA3	cornu Ammonis 3 of the hippocampus
Ent	entorhinal cortex
Sub	subiculum
CIE	chronic intermittent ethanol
LFP	local field potential
GABA	gamma-aminobutyric acid
EEG	electroencephalography
AUD	alcohol use disorder
SEM	standard error of the mean
BEC	blood ethanol concentration
Pyr	pyrazole
BL	baseline
WD	withdrawal
AP	anterior-posterior axis
L	lateral
DV	dorsal-ventral axis
AED	antiepileptic drug

ABSTRACT

Alcohol use disorder (AUD) is a chronic, relapsing brain disease characterized by the impaired ability to stop or control alcohol consumption to the point of adverse consequences in multiple facets of a person's life. Alcohol withdrawal (WD) is a clinical syndrome that affects those with a regular alcohol intake who suddenly decrease or stop consuming alcohol. Symptoms include neural hyperexcitability that can promote seizures and, potentially death. These seizures show similarities to kindling-like phenomena observed in epilepsy. Studying alcohol-withdrawal kindling is useful not only for the world of alcohol research but also for the development of new drugs and treatments. In this thesis, I performed continuous electrophysiological recordings from C57BL/6J mice undergoing chronic, intermittent ethanol exposure (CIE) and withdrawal in order to determine how multiple withdrawals increase epileptiform activity in tandem with increased numbers of withdrawal. Multiple withdrawals produced interictal spontaneous epileptiform spikes that intensified with increasing numbers of withdrawal, with statistically significant increase after three cycles of WD out of a total of 4 WDs. A second round of CIE replicated the effect in a subset of mice. Theta rhythms are important oscillations implicated in significant state-dependent hippocampal temporal processing, including coding and decoding of memories. During CIE, I found that while overall theta power did not change, theta frequency, measured in the dentate gyrus, shifted to lower values. Disruption of theta has been associated with compromised cognitive function in epilepsy. In summary, my work supports the idea that alcohol withdrawal produces a hyperexcitable state in a non-seizure prone mouse strain, with electrographic markers consistent with epileptiform activity and impaired cognition.

INTRODUCTION

Alcohol use disorder (AUD) is a chronic, relapsing brain disease characterized by the impaired ability to stop or control alcohol consumption to the point of adverse consequences in multiple facets of a person's life. Chronic alcoholism is a widespread global health problem, affecting 15 million adults in the United States in 2015. An estimated 88,000 people die annually from alcohol-related causes, making it the third leading preventable cause of death in the U.S. with 5.9 % of all global deaths attributable to alcohol consumption (NIAAA, 2018). Not only is alcohol abuse widespread, but AUD becomes particularly dangerous when users cease consumption abruptly, forcing them into withdrawal.

ACTIVITY OF ALCOHOL IN THE CENTRAL NERVOUS SYSTEM

To understand how alcohol withdrawal elicits its effects on the body, it is essential to understand the ways alcohol acts in the brain. Although alcohol affects many systems within the central nervous system, I have elected to review the three most relevant neurotransmitter systems: (1) gamma-aminobutyric acid (GABA); (2) glutamate; and (3) modulation by calcium. When alcohol is first introduced into the system, its acute effect is to potentiate GABAergic inhibition, primarily through interactions with $GABA_A$ receptors. The $GABA_A$ receptor is a conglomerate of five subunits, which determine how sensitive the receptor is to alcohol. For example, the α_1 subunit is the most susceptible to the acute effects of alcohol (Littleton, 1998; Matthews et al., 1998). In chronic alcohol exposure, neuroadaptation in the GABAergic system causes cells to downregulate the $GABA_A$ receptors containing the α_1 subunit (Papadeas et al., 2001), thereby making neurons less responsive to the effects of alcohol. Alcohol also decreases electrical activity

in the brain by inhibiting glutamatergic NMDA receptors. In a chronic alcohol state, there is an upregulation of NMDA receptors in neurons (Samson & Harris, 1992). Finally, calcium enters the neuron through channels that open when glutamate binds a nearby NMDA receptor. This influx of calcium stimulates the cell to release more neurotransmitter, potentiating the activity. When alcohol is administered, the flow of calcium through NMDA receptor-mediated channels is decreased, further reducing excitation (Hu & Ticku, 1995). Under chronic alcohol, an increase in calcium channels helps to counteract alcohol's depression of activity.

ALCOHOL WITHDRAWAL SYNDROME AND HYPEREXCITABILITY

Alcohol withdrawal is a clinical syndrome that affects those with a regular alcohol intake who suddenly decrease or stop consuming alcohol. Common symptoms include sweating, tachycardia, hypertension, fever, tremors, cravings, anorexia, nausea, vomiting, headache, anxiety, irritability, insomnia, and vivid dreams (Miller & Gold, 1998). More severe symptoms include hallucinations, seizures, delirium tremens, dehydration, and in some cases, death (Saitz, 1998). Seizures associated with alcohol withdrawal usually occur within 1-2 days after decreasing alcohol intake and are often characterized by a single generalized convulsion and loss of consciousness (Freedland & McMicken, 1993; Rogawski, 2005). Patients are at an increased risk for a second convulsion within six hours of the first seizure (Victor & Brausch, 1967). In fact, alcohol withdrawal is one of the most common causes of status epilepticus in the United States (Saitz, 1998), making alcohol withdrawal seizure a very serious health problem. Additionally, the risk for alcohol withdrawal-induced seizures increases with prior alcohol dependence and

withdrawal, longer duration of alcoholism, and prior seizures associated with withdrawal (Lechtenberg & Worner, 1991).

The neurochemical mechanisms of alcohol withdrawal are thought to follow Himmelsbach's neurochemical theory of drug-dependence, which states that physiological mechanisms in the brain that maintain homeostasis are responsible for both drug tolerance and the withdrawal syndrome (Himmelsbach, 1941; Littleton, 1998). Himmelsbach's model can be visualized as a see-saw. Prior to alcohol exposure, the brain is in homeostasis, or a stable state of equilibrium, and the inhibition vs. excitation ratio is balanced. Once alcohol is administered, the system enters the acute alcohol state, wherein GABAergic inhibition through $GABA_A$ receptors increases (Davies, 2003), glutamatergic excitation through NMDA receptors decreases (Narahashi et al., 2001), and calcium ion flow through voltage-gated Ca^{2+} channels (VGCCs) decreases (Oide et al., 2000). These changes serve to tip the scales toward inhibition and decreased calcium flow through VGCCs impairs the brain's ability to regulate its inhibition and excitation levels. To compensate for these effects of acute alcohol, the brain adapts by decreasing the sensitivity of $GABA_A$ receptors (Ticku & Burch, 1980), increasing the number of NMDA receptors (Hendricson et al., 2007), and increasing the number of VGCCs at the synapse (Itatsu et al., 1998). When alcohol is suddenly removed from the system the CNS immediately enters a withdrawal that is characterized by the opposite action of acute alcohol. Alcohol potentiates GABAergic inhibition and continued alcohol abuse leads to subunit changes (Cagetti et al., 2003) rather than decreased receptor density (Harris et al., 1998), which lowers overall sensitivity of $GABA_A$ receptors at the synapse. When

alcohol is suddenly removed from the system, GABA receptors remain less responsive, tipping the homeostatic balance toward excitation, resulting in neuronal hyperexcitability.

ALCOHOL WITHDRAWAL SEIZURE AND THE KINDLING HYPOTHESIS

The neurochemical theory of drug-dependence explains why and how withdrawal induces neuronal hyperexcitability, which is widely regarded as the pathology responsible for alcohol withdrawal-induced seizures. However, characterizing the seizures has posed a formidable challenge for investigators because alcoholics differ widely in their sensitivity to and severity of seizure associated with withdrawal (Figure 1). This phenomenon, known as “kindling,” occurs only in very particular models of electro- and chemo-convulsive stimulation and is not explained by Himmelsbach’s theory or the basic mechanisms underlying hyperexcitability. The characteristics of and mechanisms controlling alcohol withdrawal kindling are still being discovered, but it is clearly an integral part of the pathology of alcohol withdrawal-induced hyperexcitability and sets it apart from withdrawal syndromes from other drugs of abuse.

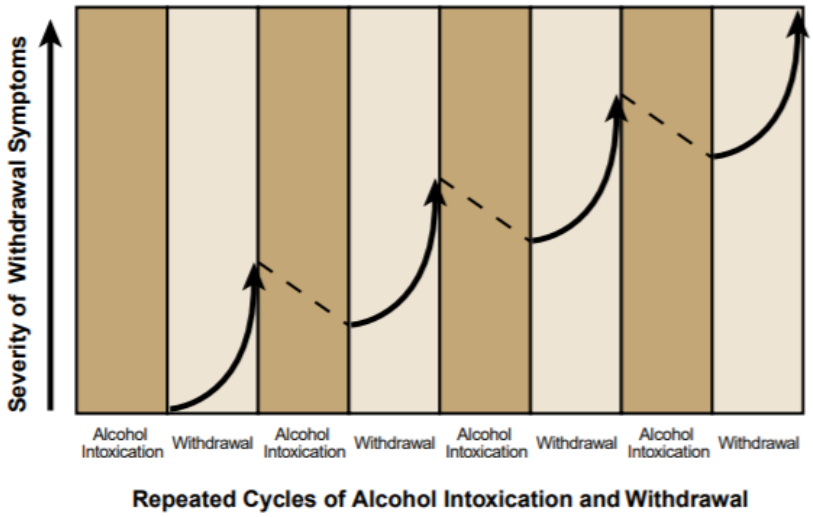

Figure 1. Diagram of the kindling phenomenon. Individuals undergoing repeated cycles of intoxication and withdrawal from alcohol experience increasingly severe withdrawal symptoms with each successive cycle. This phenomenon is known as "kindling." From "Kindling in Alcohol Withdrawal" by H. C. Becker, 1998, *Alcohol Health Res World*, *22*, 25-33. In the public domain.

First discovered by Delgado and Sevillano and further elucidated by Graham Goddard in 1967, kindling is defined as the phenomenon by which repeated administration of a subconvulsive electrical stimulus eventually results in the progressive intensification of seizure activity (Delgado & Sevillano, 1961; Goddard et al., 1969; McNamara et al., 1980). Ballenger and Post hypothesized in 1978 that the neural hyperexcitation that accompanies withdrawal from high levels of alcohol might serve as a stimulus to "kindle" the increased susceptibility to alcohol withdrawal seizures. They further theorized that during multiple intoxication and withdrawal cycles, withdrawal-induced changes in neuronal activity could serve as the kindling stimulus and that this

kindling might eventually culminate into full tonic-clonic seizures (Ballenger & Post, 1978; Becker, 1998). Therefore, alcohol withdrawal kindling can be defined as the propensity for subsequent withdrawal episodes to increase the risk for seizure (Lechtenberg & Worner, 1991).

PRIOR STUDIES CHARACTERIZING SEIZURE DUE TO ALCOHOL WITHDRAWAL-INDUCED HYPERACTIVITY

Efforts to better define alcohol withdrawal kindling using rodent models have shown that there exists an increased propensity for seizure during withdrawal in individuals with a history of seizure during previous withdrawal episodes and this phenomenon has been observed in both human alcoholics and mouse models of alcohol exposure (Becker et al., 1997; Veatch et al., 2007). Separate studies done in male Sprague-Dawley rats showed that animals exposed to a single 42-day continuous period of chronic ethanol exposure (Veatch & Gonzalez, 1997) and those that underwent repeated cycles of exposure and withdrawal (Veatch & Gonzalez, 1999) showed significant delays in the development of electrical kindling at CA3. However, local responses to electrical kindling remain unchanged by previous exposures and withdrawals, leading the authors to suggest that the changes seen in CA3 are due to alterations at the neural circuit level. Further experiments showed that alcohol withdrawal-associated changes in seizure-sensitive neural circuitry of the hippocampus persist long after cessation of ethanol exposure, these effects are greater after repeated withdrawal episodes, and L-type voltage-gated calcium channels are involved in this process (Veatch & Gonzalez, 2000).

Studying alcohol-withdrawal kindling is useful not only for the world of alcohol research but also for the development of new antiepileptic drugs (AEDs). An important distinction should be made between animal models of epilepsy and animal models of epileptic seizure. Epilepsy is characterized by spontaneous recurrent seizures and is well-modeled by transgenic and mutant animals, which are more closely related to human epilepsy. Conversely, an animal model of epileptic seizure is one in which an acute seizure is induced in an otherwise normal, non-epileptic animal (Löscher, 2011). Since my focus lies in alcohol withdrawal seizure rather than epileptogenesis, I chose the C57BL/6J mouse model. C57BL/6J was the first model of interictal epileptiform discharges associated with alcohol withdrawal by Walker and Zornetzer in 1974 (Walker & Zornetzer, 1974), it serves as the background strain for many transgenic animals and it is not seizure prone (and thus may be more generally reflective of human alcohol withdrawal).

Molecular and electrophysiological studies have shown that ethanol consumption and continued usage change the kinetics and population of ion channels present at the synapse. Animal model ethanol experiments have linked alcohol consumption to changes in serotonin, GABA, opioids. dopamine, protein kinases, G protein-coupled receptors, Ca^{2+} channels, and peptide systems (Crabbe et al., 2006). Recent studies have also shown that ethanol changes expression of L-type, N-type, and T-type voltage-gated Ca^{2+} channels in the brain (Walter & Messing, 1999). Specifically, our lab has taken an interest in studying the effects of alcohol withdrawal on T-type voltage-gated Ca^{2+} channels and their contribution to seizure-causing hyperexcitability. Using RT-PCR techniques, it was found that ethanol exposure and withdrawal causes an upregulation of

$Ca_V3.2$ in the nucleus reuniens immediately following two withdrawal periods and an increase in $Ca_V3.2$ and $Ca_V3.3$ following third and fourth withdrawals (Graef et al., 2011). Electrophysiological studies using whole cell patch clamp analysis showed that only T channel currents from the $Ca_V3.2$ isoform were significantly affected by ethanol; ethanol reduced current density and produced a hyperpolarizing shift in steady-state inactivation of only $Ca_V3.2$ channels (Graef et al., 2011; Shan et al., 2013). This increase in function of $Ca_V3.2$ channels during alcohol withdrawal leads to increased tendency for bursting, which further contributes to overall excitability of the system. Studies in seizure-prone DBA/2J animals showed that the T channel-mediated bursts were enhanced with multiple withdrawals and the T channel antagonist ethosuximide reduced withdrawal-mediated burst activity (Riegle et al., 2014). Blockade of T-type calcium channels with systematically-administered T channel antagonists improved mortality rates after withdrawal (Masicampo et al., 2018; Riegle et al., 2015).

ALTERED EEG IN RESPONSE TO ALCOHOL AND WITHDRAWAL

Some studies from human pre-surgical tests and recordings in chronic models of epilepsy suggest that interictal spikes and ictal discharges are generated by separate populations of neurons or different neuronal mechanisms (de Curtis & Avanzini, 2001; Gotman, 1991). Some have questioned whether kindling is a true effect, since results have shown that the interictal spike frequency does not increase before a seizure in both human (Katz et al., 1991; Lange et al., 1983; Lieb et al., 1978) and animal models (Gotman, 1984; Leung, 1990; Ralston, 1958) of epilepsy. In fact, spike rate has been shown to decrease before seizure (Karoly et al., 2016) and enhancement of interictal spiking has shown to suppress ictal events (Avoli et al., 2006). It is still possible, of

course, that the two may be causally linked. A period of high interictal spike activity could create a refractory state for ictal discharge, while a period that is free from interictal spikes might lead to a state where one spike is sufficient to trigger the ictal discharge (Morimoto et al., 2004). More work is required to explore the relationship between interictal and ictal discharges.

Studies of alcoholics undergoing detoxification have shown certain abnormal characteristics in EEG findings. One study comparing quantitative EEG analyses from 191 male alcoholic patients found that 42 percent of subjects showed decreased power in slow (delta and theta) bands and a concurrent increase in beta band, though a variety of shifts in band power were seen across individuals (Coutin-Churchman et al., 2006) that may be an indicator of brain atrophy associated with seizure (Hughes, 2009). In one study of aiming to characterize the clinical profile of patients with alcohol related seizures and identify their prevalence of idiopathic generalized seizure, researchers found that in scalp EEG recordings, 67% patients showed increased generalized fast low amplitude beta activity and 15% showed intermittent bursts of theta slowing. Specific epileptiform abnormalities of spike-wave discharges were observed in 2% of patients, with 1 patient showing dominant generalized spike-wave discharges, while another showed independent left frontal and bifrontal spike-wave discharges with focal fast frontal multi-modal spikes over a background of 7-7.5 Hz (Sandeep et al., 2013).

THE HIPPOCAMPAL NETWORK AND RHYTHMIC OSCILLATIONS

While depth electrodes are not feasible in humans, rodent models have the advantage of continuous *in vivo* electrophysiology, which allows sampling from systems that are similar in complexity to humans, but without the limitations of clinical necessity

and safety concerns of depth electrode recording in human patients. This method allows for spatial and temporal sampling of network activity in a controlled state where we can easily and safely test the effects of kindling and pharmacology (Baraban, 2009). One of the greatest strengths of continuous *in vivo* electrophysiology is the ability for multisite arrays, which allow for clean, site-specific local field potential recordings from multiple areas simultaneously (G. Buzsáki et al., 1988). Using this method in the withdrawal kindling-sensitive hippocampal network (Figure 2), we can study the network characteristics of alcohol withdrawal seizure.

Theta oscillations are defined as repeated patterns of brain activity measured by local field potential within the 6-10 Hz range. They most often occur during REM sleep (Jouvet, 1999; Montgomery et al., 2008) and voluntary motor activity (Vanderwolf, 1969). Theta oscillations are thought to be involved in learning and memory processes (Michael E. Hasselmo, 2005) such as memory encoding and retrieval (M. E. Hasselmo et al., 2002), context-dependent retrieval of sequences (M. E. Hasselmo & Eichenbaum, 2005), novel information buffering in entorhinal cortex for episodic encoding (Alonso & Garcia-Austt, 1987), and the phase relationship between PFC and hippocampus for memory-guided action selection (Hyman et al., 2005; Siapas et al., 2005).

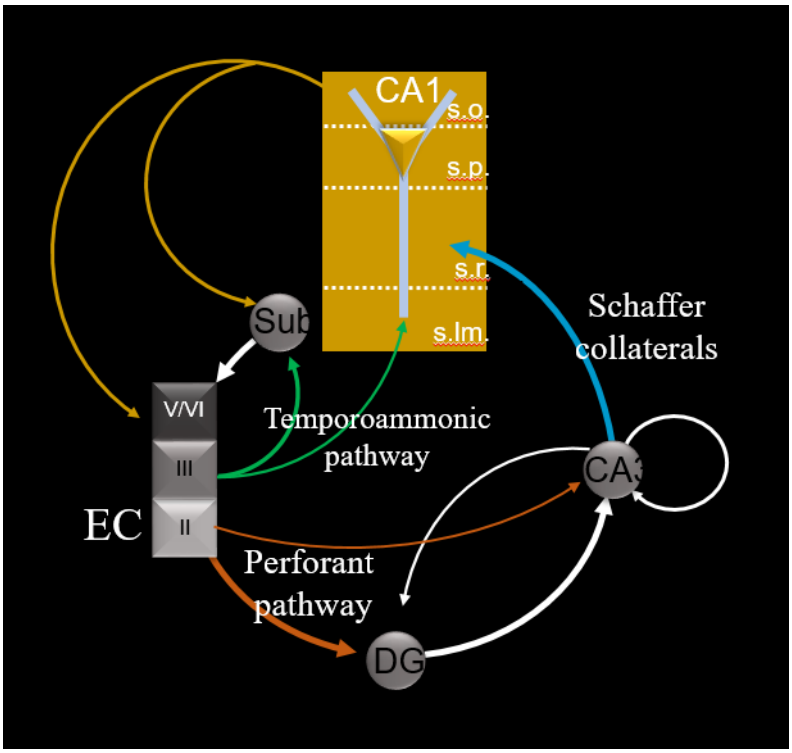

Figure 2. Hippocampal network loops propagate synchronous neural activity during temporal lobe seizure. The hippocampus network circuitry is a series of nested loops with multiple pathways by which activity can travel in the circuit. A synchronous population discharge in CA1 will re-enter the hippocampus through the entorhinal cortex and produce more high amplitude activity in dentate gyrus and CA3. Adapted by D. W. Godwin (from Buzsáki, 2011, p. 31).

SIGNIFICANCE AND HYPOTHESIS

With such a high risk for abstaining alcoholics to have seizure events, researchers have looked to characterize alcohol withdrawal seizures, their risk factors, and ability to be managed with medications. Much has been learned about the risk factors for withdrawal seizure and how it is affected by medication, gaps remain in our

understanding of and ability to detect measures of hyperexcitability in the brain. I hypothesized that with increasing rounds of ethanol administration and withdrawal will lead to increases in spontaneous interictal epileptiform spikes, consistent with development of network hyperexcitability. In addition to number of abnormal events, I expected that there would be significant shifts in frequency and power of the theta band associated with alcohol exposure and withdrawal.

METHODS

Subjects

The experimental subjects were nine C57BL/6J mice (Stock No: 000664, The Jackson Laboratory, Bar Harbor, ME, USA) weighing 23-28 g. All animals were 64-95 days old when implanted and housed individually in standard plastic mouse cages with ad libitum access to food and water. All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of Wake Forest University and in accordance with the National Institutes of Health's Guide for the Care and Use of Laboratory Animals.

Chronic implant surgery

Mice were weighed and received ketoprofen (5 mg/kg) and atropine (0.04 mg/kg) by intraperitoneal injection thirty minutes before beginning the procedure. They were anesthetized with isoflurane, shaved, and placed in a stereotaxic apparatus with skull landmarks bregma and lambda in the same horizontal plane (Buzsaki et al., 1983). Eight holes were drilled into the skull with a microdrill and a satellite array consisting of eight tungsten microwires (35 µm wide, 2 in long) was implanted into the brain, one wire at a time, and glued in to place. Placement of wires is shown in Figure 3A. Wires were placed in the right prefrontal cortex (PFC, AP: 5.58 mm, L: 0.37 mm, DV: -1.81 mm from the interaural plane), right nucleus reuniens (RE, AP: 2.98, L: 0.20, DV: -3.78), right dentate gyrus (DG, AP: 1.50, L: 1.39, DV: -1.61), right cornu Ammonis 3 of the hippocampus (CA3, AP: 1.34, L: 2.88, DV: -2.12), right entorhinal cortex (Ent, AP: 0.19, L: 3.90, DV: -2.73), right subiculum (Sub, AP: 0.88, L: 1.02, DV: -1.43), left CA1 (AP: 0.90, L: -2.36, DV: -1.20), and right CA1 (AP: 0.95, L: 2.36, DV: -1.20) (Klorig et al., 2019). Two

ground screws were placed into the skull above the cerebellum and one stabilizing screw was placed in the bone above the olfactory bulbs. The head cap was fixed to the skull with dental cement and black glue. Following surgery, mice were allowed to recover for between 10-29 days before their first recording.

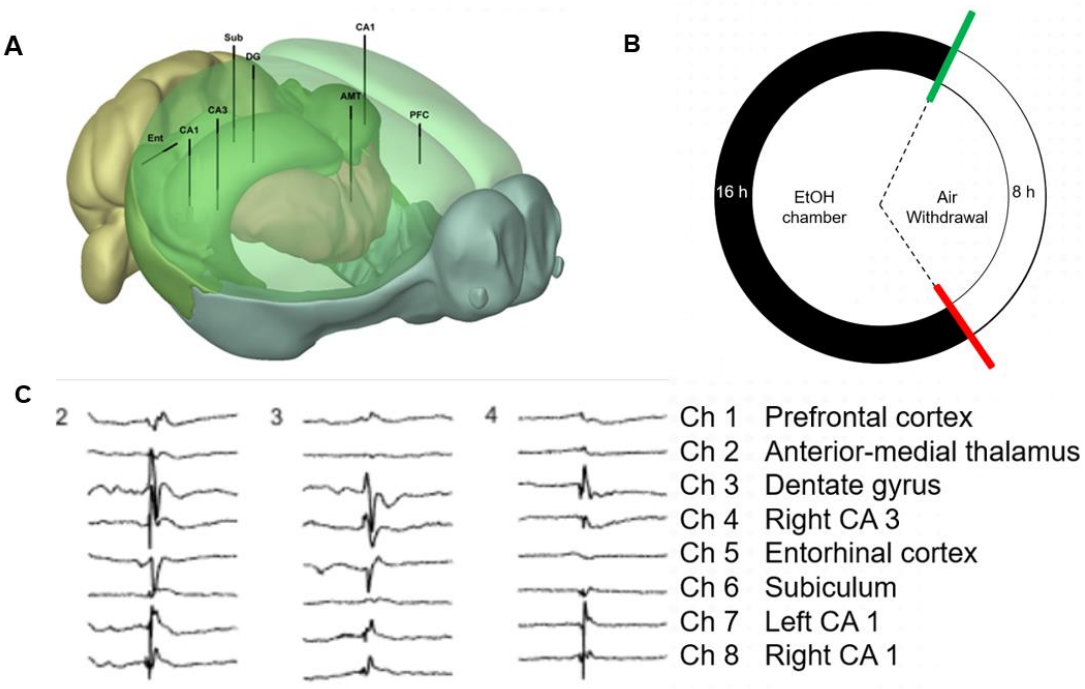

Figure 3. Overview of experimental design. A) Placement of 8 recording microelectrodes in satellite array for collection of local field potentials. PFC, prefrontal cortex; AM, anterior-medial thalamus; DG, dentate gyrus; CA3, ipsilateral (right) CA3; Ent, entorhinal cortex; Sub, subiculum; L CA1, contralateral (left) CA1; R CA1, ipsilateral (right) CA1. Adapted from (Klorig et al., 2019). B) Diagram of the chronic intermittent ethanol paradigm. The circle represents 24 hours, or one exposure and withdrawal episode. One round of CIE

consists of four consecutive days of exposure and withdrawal. C) Examples of interictal spontaneous spikes occurring across channels in real time.

Chronic intermittent ethanol exposure and experimental paradigm

Ethanol was chronically administered by a chronic intermittent exposure inhalation paradigm (Figure 3B) (Becker & Hale, 1993), which has shown to induce alcohol dependence and symptoms of withdrawal in rodents (Becker & Lopez, 2004; Becker et al., 2006; Graef et al., 2011). The experiment was designed to collect awake behaving recordings from animals at baseline and compare these within-subjects to the same animals' results after subsequent and consecutive withdrawals. Controls were within-subjects. For three days, subjects underwent baseline recordings, each 8 hours long. A baseline plus pyrazole recording was done to rule out pyrazole's effects on excitability. Since mice metabolize alcohol very quickly, subjects received an injection of pyrazole, an alcohol dehydrogenase inhibitor at 10 mg/kg at the end of the third day. fourth day was a pyrazole control baseline recording. After this recording, animals were injected with pyrazole and placed in the ethanol chamber, a specially designed plexiglass vapor chamber that delivers 95% ethanol volatilized in fresh air and delivered at 10 liters/min over the course of the 16-hour exposure period. After 16 hours, mice were removed from the ethanol chamber and 5 µl blood samples were collected via tail snip and stored in 45 µl of 6.25% trichloracetic acid prior to being analyzed for blood ethanol concentration (BEC) using an NAD-ADH enzyme assay (Randox Laboratories, U.K.). After four consecutive withdrawals, subjects went through a three-day post-withdrawal recording period to assess duration of changes in excitability.

Recordings

Unstimulated wideband depth recordings (0.3-30 kHz, sampled at 40 kHz) of local field potentials (LFPs) were measured using the SciWorks recording system (DataWave Technologies, Loveland, CO, USA), AM-3600 extracellular amplifiers (A-M Systems, Sequim, WA, USA), and T8G100 headstage amplifiers (TBSI, Durham, NC, USA) (Klorig et al., 2019). An individual animal was placed in a behavior box with bedding and wet chow and the headcap connector was attached to a headstage that was counterweighted to allow for full mobility. Recordings lasted eight hours and were done in a quiet, lit room with humidifier and heated to 75˚ F. Recording sessions were video recorded at 1080P using the Annke surveillance camera system 8 channel 3MP CCTV DVR recorder (Annke, Shenzhen, China). An infrared LED was placed in view of the video camera for precise temporal alignment between the video and recordings. Video was used for behavioral assessment.

Data analysis

Data analysis was performed using custom software written in MATLAB (MathWorks). For spike and ripple detection, raw data was extracted and decimated to a sampling frequency of 2000 Hz, the second derivative was calculated, and a sliding window of 10 ms was used to calculate the instantaneous root-mean-square (RMS) power via convolution. The max RMS within a time period was calculated, sorted by magnitude, and plotted for each channel over one hour sections of the 8 hour recordings. A threshold of RMS = 6.00 was chosen based on the channel with the largest amplitude deflection. For each RMS greater than 6.00, an event was detected and 20 ms snapshot of the raw trace was generated and saved. Each detected spike and ripple was verified visually. For

spectral power analyses, the raw data was filtered using a Butterworth filter of order 16 set to pass only the theta band (6-10 Hz). Next, a fast Fourier transform (FFT) with a window of 10 ms and overlap of zero was used to compute the power spectrum of frequencies within the theta band.

Statistics

Statistical testing was performed with MATLAB and Prism (GraphPad). Means are reported ± SEM. Comparisons were performed using repeated measures one-way ANOVA with Tukey's HSD test and paired t tests, where appropriate. All reported statistics are labeled with the test used and the sample size. Statistical significance is reported when the p-value was < 0.05.

RESULTS

In order to characterize alcohol withdrawal-induced hyperexcitability, *in vivo* electrophysiological recordings were done in the awake behaving mouse during consecutive withdrawals after a chronic intermittent ethanol administration paradigm. Statistical analysis of instances of interictal spontaneous spikes is shown in Figure 4. Baseline measures of spontaneous spikes were averaged across all three baseline recordings. Spike counts were summed across all eight hours of each recording. Figure 4A shows the total number of spikes present in each condition, sorted by subject. Spikes were summed across all 8 hours of the recording for each condition. Figure 4B summarizes the mean spontaneous spikes per condition, averaged across subjects for which all four days of withdrawal recordings exist ($n = 5$). Data were normalized to percent of baseline spikes within subjects prior to being averaged. There was no significant difference between mean spike counts for baseline and baseline with pyrazole recordings ($p > 0.05$, $n = 8$; paired t test), providing evidence that changes in spike prevalence was not significantly affected by pyrazole injection.

A repeated measures analysis of variance (ANOVA) test showed a significant difference between conditions ($F = 9.217$, $p = 0.0001$, repeated measures one-way ANOVA, $n = 5$). Further analysis using Tukey's HSD test for multiple comparisons showed no significant differences between spontaneous spike counts in baseline compared to WD1 (21.42 ± 11.75 % of baseline spikes, $p > 0.05$) or WD2 (28.72 ± 6.74 % of baseline, $p > 0.05$); however, significant differences were shown between baseline and WD3 (46.08 ± 14.34 % of baseline, $p = 0.0093$) and baseline and WD4 ($63.32 \pm$

13.50 % of baseline, $p = 0.0005$). There was also a significant increase in spontaneous spikes between WD1 and WD4 ($p = 0.0189$).

A total of eight of mice were implanted and used for these experiments; however, WD4 recordings were not useable in three test subjects. For one animal hour 5 of this recording became corrupted and was unreadable in MATLAB. Another animal expired during hour 6 of WD4. Both recordings were left out of analyses of WD4. The final animal only went through three withdrawals due to experimental error. Therefore, the analyses reported in Figure 4B were for a sample size of five.

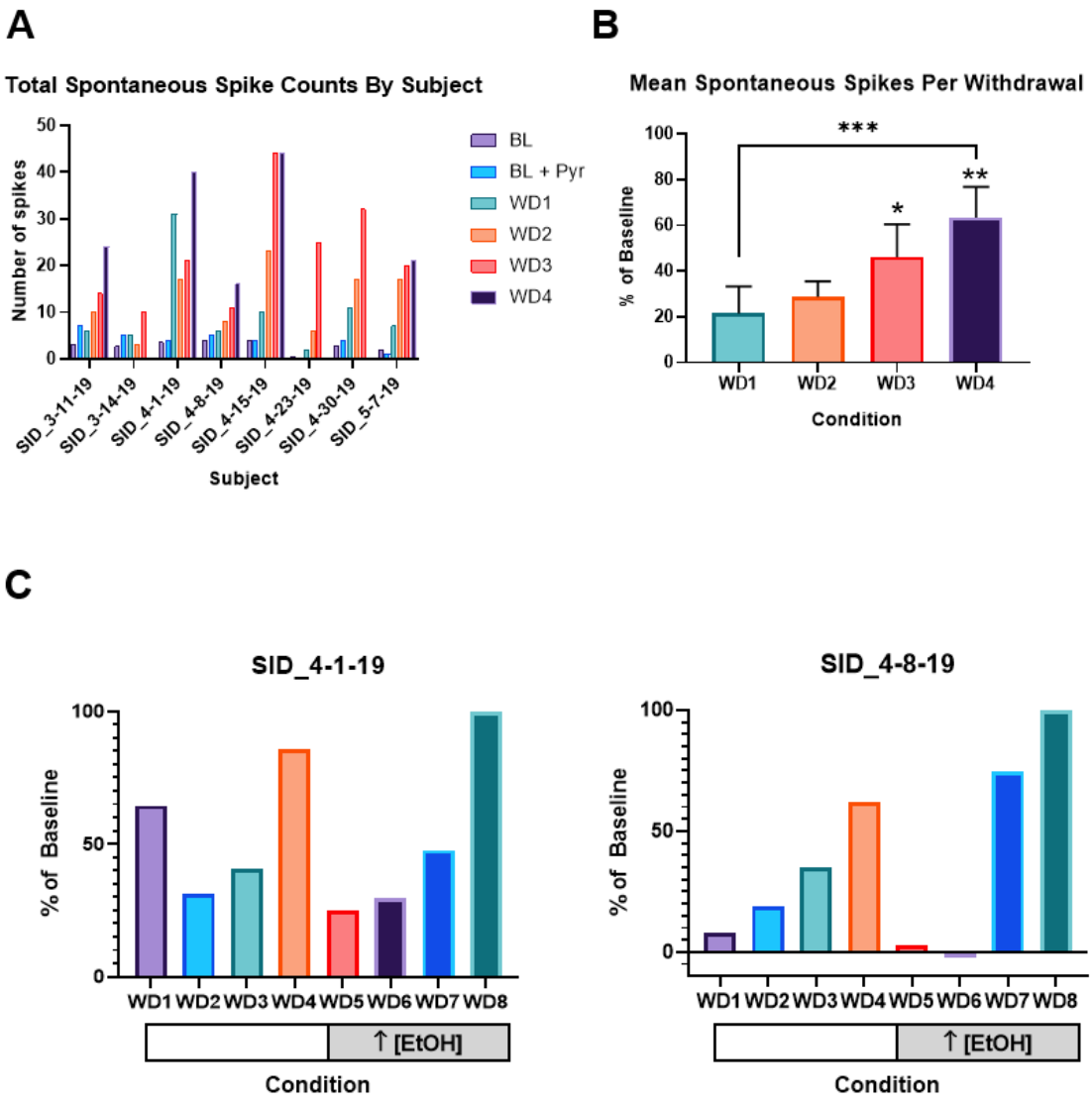

Figure 4. Spontaneous spike prevalence is increased with multiple consecutive withdrawals. A) Total spike counts per condition, organized by subject (n = 8). B) Summary of mean spikes per condition with SEM error bars. F = 9.217, p = 0.0001, repeated measures one-way ANOVA, n = 5. Baseline vs. WD3, *p = 0.0093, Tukey's HSD test. Baseline vs. WD4, **p = 0.0005. WD1 vs. WD4, ***p = 0.0189. C) Spontaneous spikes did not differ in preliminary test of two animals when ethanol concentration increased (n = 2).

After all subjects underwent one round of the experimental paradigm (3 days of baseline, one day of baseline plus pyrazole, 4 days of CIE, and 3 days of post withdrawal recordings), two animals went through the paradigm a second time with the alcohol chamber set to a higher concentration of ethanol. Figure 4C shows the percent of baseline spontaneous spikes per condition across all eight withdrawals for each subject. Due to animals being housed in cages with less air flow than usual, subjects had lower than expected BECs for CIE measurements during the first four withdrawals (118.9 ± 4.933 mg/dl, n = 8). To test whether an increase in alcohol concentration would affect the results of spike counts, a preliminary study was done using two subjects that had previously undergone one round (four exposures and withdrawals) of CIE. The average BEC for the increased ethanol concentration round of CIE was 171.4 ± 9.053 mg/dl (n = 2). For WD1-WD4, spike counts were normalized to the mean of spikes counts from BL (round 1), BL + Pyr (round 1). The same was done with BL (round 2), and BL + Pyr (round 2) recordings for WD5-WD8, then all normalized results were plotted as percent of baseline. A 2-way ANOVA of condition vs. regular CIE vs. increased concentration CIE on non-normalized spike counts did not show significant variation between condition and alcohol concentration ($p = 0.5598$). Post-hoc power analysis of the mean and standard deviations of spikes for basic CIE vs increased EtOH CIE from a 2-way ANOVA showed that an n = 4 would give sufficient power (> 80% power).

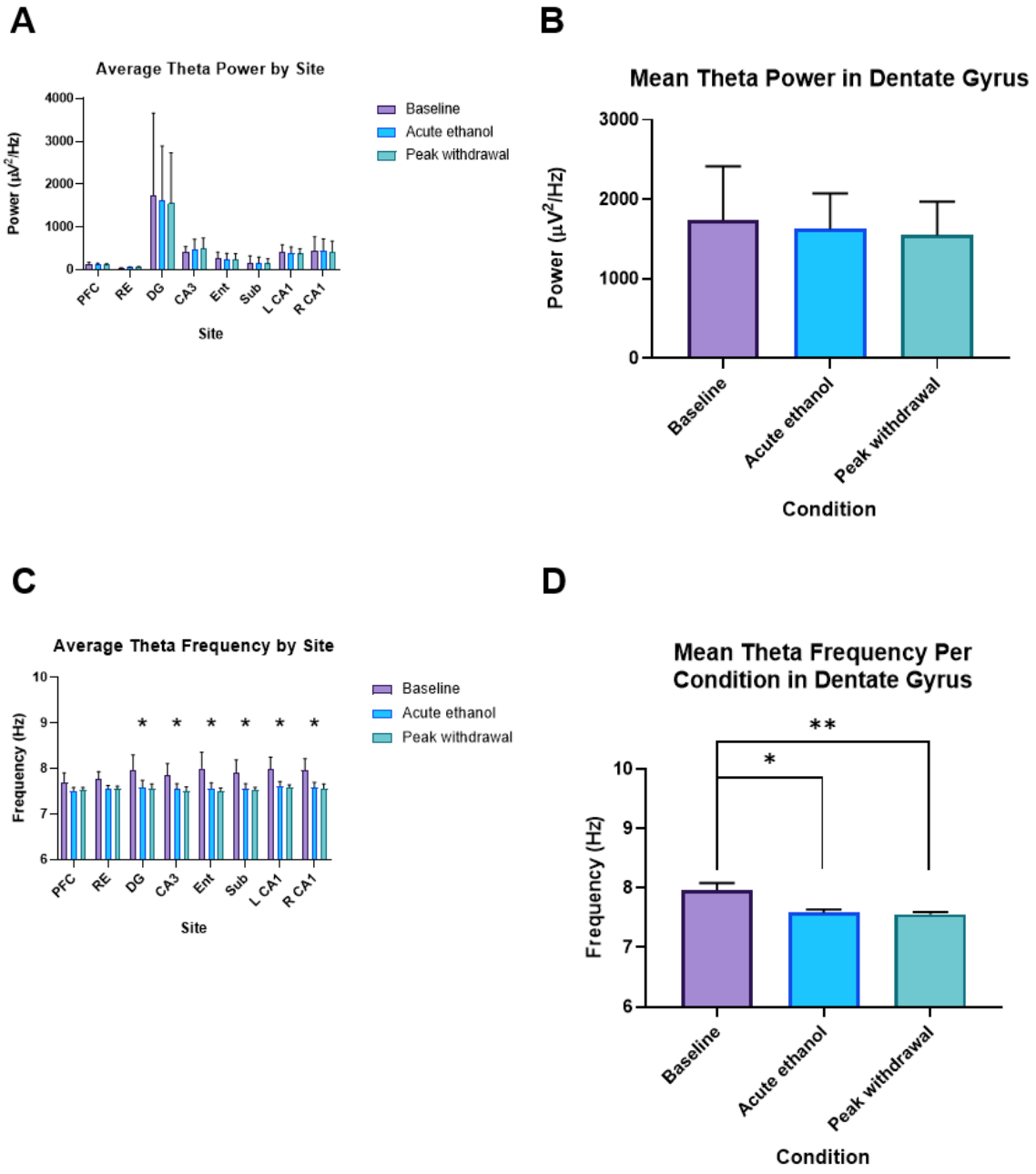

Figure 5. Spectral analysis of theta rhythm frequency is correlated with alcohol exposure. A) Hippocampal theta spectral power by channel site shows strongest theta power in dentate gyrus channel. Results are averaged results across baselines, acute ethanol (hour one of final withdrawal), and peak withdrawal (hour 6 of final withdrawal). B) Theta power did not significantly vary between

baseline, acute ethanol, and peak withdrawal conditions in the dentate gyrus. Repeated measures one-way ANOVA, n = 8. C) Average theta band frequency significantly decreased across all channels except PFC and RE. *$p < 0.0001$, $F = 101.9$, repeated measures one-way ANOVA and multiple t tests, n = 8. D) Theta band average frequency decreases with alcohol exposure and withdrawal in dentate gyrus. $F = 6.910$, $p = 0.0082$, repeated measures one-way ANOVA, Tukey's HSD test, n = 8. Baseline vs. acute ethanol, *$p = 0.0232$. Baseline vs. peak withdrawal, **$p = 0.0116$. Differences in mean between acute ethanol and peak withdrawal conditions were not significant ($p > 0.05$).

To test whether any large-scale frequency shifts would be affected by an increase in hyperexcitability due to intermittent withdrawals, the hippocampal theta band (6-10 Hz) was filtered and analyzed for differences in spectral density (Figure 5A) and average frequency (Figure 5C) between baseline, acute ethanol intoxication (CIE hour 1 of final withdrawal), and peak withdrawal (CIE hour 6 of final withdrawal). This data, shown in Figure 5 was averaged across subjects for the final withdrawal condition per subject (either WD3 or WD4). An overview of spectral power results by channel site (Figure 5A) shows that hippocampal theta power is strongest in the dentate gyrus channel., which is highlighted in Figure 5B for power and 5D for frequency. A repeated measures one-way ANOVA of average spectral power of the theta band did not appear to be correlated with condition (Figure 5A-B) ($p = 0.7123$). Figure 5C showed theta frequency averaged across all channels was significantly different between baseline, acute ethanol, and peak withdrawal conditions ($F = 101.9$, $p < 0.0001$, repeated measures one-way ANOVA, n =

8). Tukey's HSD test showed significantly decreased average frequency between baseline (7.747 ± 0.02446) vs. acute ethanol (7.562 ± 0.01022; $p < 0.0001$) and baseline vs. peak withdrawal (7.539 ± 0.01055; $p < 0.0001$), but not between acute ethanol vs. peak withdrawal ($p > 0.05$). Multiple t tests also showed that frequency decreased between baseline and acute ethanol (PFC, $p > 0.05$; RE, $p > 0.05$; DG, $p = 0.00637$; CA3, $p > 0.05$; Ent, $p = 0.01203$; Sub, $p = 0.00886$; L CA1, $p = 0.00492$; R CA1, $p = 0.00290$; multiple t tests with Holm-Sidak test, $n = 8$) and baseline and peak withdrawal (PFC, $p > 0.05$; RE, $p > 0.05$; DG, $p = 0.00106$; CA3, $p = 0.00460$; Ent, $p = 0.00035$; Sub, $p = 0.00214$; L CA1, $p = 0.00071$; R CA1, $p = 0.00079$) within all channels except for PFC and RE. A closer look at the dentate channel (Figure 5D) showed significant differences ($F = 6.910$, $p = 0.0082$, repeated measures one-way ANOVA, $n = 8$) between baseline (7.794 ± 0.08848), acute ethanol (7.583 ± 0.05644), and peak withdrawal (7.557 ± 0.03714) conditions. Further analysis showed significant decreases in frequency between baseline and acute ethanol ($p = 0.0232$, Tukey's HSD, $n = 8$) and baseline and peak withdrawal ($p = 0.0116$). No significant difference was observed between acute ethanol and peak withdrawal conditions ($p > 0.05$).

DISCUSSION

In this study, I used continuous *in vivo* electrophysiological local field potential recordings in C57BL/6J mice undergoing chronic intermittent ethanol exposure and multiple withdrawals to examine increases in epileptiform activity with multiple withdrawals. Total numbers of spontaneous epileptiform spikes significantly increased from baseline after three consecutive withdrawals and this effect intensified with increasing withdrawal cycle. This effect was also evident in a subset of mice that went through an additional round of CIE at a higher ethanol concentration; however, spikes did not significantly increase from the original CIE measures with increased ethanol concentration.

Analyses comparing spike counts between baseline and four subsequent withdrawal days showed that alcohol withdrawal causes a significant increase in spontaneous epileptiform spikes, beginning with the third withdrawal, and that this effect increased with continued withdrawals. These results are similar to those found by Walker and Zornetzer in C57BL/6J female mice, wherein the development of abnormal EEG activity (brief spike episode) was more severe during the second alcohol withdrawal period than after the first alcohol withdrawal period (Walker & Zornetzer, 1974). Previous work from our lab showed a progressive increase in spike-and-wave discharge (SWD) count and duration of events with each successive withdrawal period in DBA/2J mice (Riegle et al., 2014). Additionally, these results are consistent with those found by Veatch and Becker in male C3H/He mice, which showed increased brief spindle episodes with subsequent withdrawals (Veatch & Becker, 2002, 2005). Since different strains show highly variable differences in sensitivity to withdrawal (Crabbe et al., 1980; Metten

et al., 2010) this observed kindling-like effect is robust, appearing across multiple mouse strains. Though not previously reported, these results suggest that an increase in spontaneous spikes is a characteristic of alcohol withdrawal-induced hyperexcitability and in accordance with previous literature. this increase is exacerbated by consecutive withdrawals.

Spectral analysis of theta rhythms differentiated by hour was used to measure broad-scale differences in power and frequency between baseline, acute alcohol, and peak withdrawal conditions measured from the dentate gyrus. These analyses did not show an overall difference in theta band power in the presence or absence of alcohol. Analysis of mean frequency within the filtered theta band showed a significant decrease in mean frequency between baseline and acute alcohol and baseline and peak withdrawal conditions, however no significant difference was found between alcohol intoxication or peak withdrawal conditions. However, previous studies by Graef showed CIE disrupts baseline theta patterns (Graef et al., 2011). Previous studies in rats (Givens, 1995; Kaheinen et al., 1988) and rabbits (Whishaw, 1976) found that hippocampal theta activity shifted to a lower frequency following ethanol administration. Additionally, alcohol has long been linked to impairments in memory (Doss et al., 2018; Weafer et al., 2016; White, 2003). Since theta oscillations are necessary for spatial memory (Winson, 1978), changes in theta oscillations are associated with impairments in working memory (Goodman et al., 2019) and episodic memory (Hot et al., 2011) in Alzheimer's disease, and stimulus-locked theta is important for working memory (Tesche & Karhu, 2000), it is possible that any change in theta oscillations, such as the slowing reported here, would negatively affect memory. Recent schizophrenia research has posited that any change in

an oscillation (especially theta or gamma) will result in abnormal cross-frequency coupling (Moran & Hong, 2011), which has been linked to effective working memory in the human hippocampus (Axmacher et al., 2010).

A previous study by a student in our lab found that repeated bouts of CIE increased the relative power of the theta band, which regularly shifts with diurnal light-dark cycles throughout the day and that this change was long-lasting and disappeared in the presence of ethosuximide, a T-type calcium channel blocker (Wiggins et al., 2013). The fact that alcohol shifted the mean frequency within the theta band for my data but did not significantly affect power lead me to suspect that a problem with these could obscure a correlated result. Alcohol has been shown to cause sleep disturbances in both humans (Allen et al., 1977) and rodents (Mukherjee et al., 2008; Thakkar et al., 2015). Additionally, Wiggins et al (2013) reported that they did not see any significant differences in absolute power spectra during exposure and withdrawal conditions until they segregated the data by sleep score. Additionally, recent findings by Klorig (unpublished) reveal that shifts in seizure threshold associated with WD are most notable during sleep. Therefore, it is likely that overall theta power is behavioral-state dependent. Follow up studies will need to segregate data by behavioral state to better visualize shifts in theta power. In the future, video recordings of the recording sessions should be used to determine sleep vs. wake cycles and test for correlations with shifts in spectral power. Additional tests I plan to do in the immediate future include running spectral analyses for the other major frequency bands (ie: alpha, beta, gamma, delta) to see whether shifts differ across frequency bands.

In summary, my work supports the idea that alcohol withdrawal produces a hyperexcitable state in a non-seizure prone mouse strain, which elicits abnormal brain rhythms that are consistent with epileptiform activity. Spontaneous spikes significantly increased from baseline after three consecutive withdrawals and this effect intensified with increasing withdrawal cycle. A pilot study (n = 2) did not show significantly more spikes at higher alcohol concentrations. These results provide further evidence for the kindling hypothesis and suggests that the spontaneous spike is a hallmark of hyperexcitability in the abnormal mouse EEG. Analysis of frequency and power spectral density showed power did not significantly change in the presence or absence of alcohol for the theta band. However, average frequency within theta decreased significantly from baseline for acute and withdrawal conditions. This effect is likely due to alcohol exposure, rather than withdrawal. Since theta rhythms have been shown to be disrupted by CIE, I plan to look at whether behavioral state is confounding patterns of power.

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White, A. M. (2003). What happened? Alcohol, memory blackouts, and the brain. *Alcohol Res Health, 27*(2), 186-196.

Wiggins, W. F., Graef, J. D., Huitt, T. W., & Godwin, D. W. (2013). Ethosuximide reduces ethanol withdrawal-mediated disruptions in sleep-related EEG patterns. *Alcohol Clin Exp Res, 37*(3), 372-382. doi:10.1111/j.1530-0277.2012.01938.x

Winson, J. (1978). Loss of hippocampal theta rhythm results in spatial memory deficit in the rat. *Science, 201*(4351), 160. doi:10.1126/science.663646

CURRICULUM VITAE

Allison Taylor Goldstein

1802 Brantley St. Winston Salem, NC, 27103 allison.goldstn@gmail.com (602) 570-5478

EDUCATION

Wake Forest University, Graduate School of Arts and Sciences, Winston Salem, NC — August 2017 – Present

M.S. Biomedical Sciences—Neuroscience — GPA: 4.00

University of California in Los Angeles, Los Angeles, CA — September 2010 – August 2014

B.S. Neuroscience with minor in English — GPA: 3.40

RESEARCH EXPERIENCE

Research lab of Dr. Dwayne Godwin, Ph.D. — September 2017 – Present

Department of Neurobiology and Anatomy, Wake Forest Graduate School of Arts and Sciences, Winston Salem, NC

Student Researcher

- Utilized in-vitro and in-vivo techniques including electrophysiology, local field potential recordings, whole cell patch clamp, RT- and qPCR, and stereotactic surgery to study alcohol withdrawal-induced seizure in an optogenetic mouse model.
- Thesis project: Spike-and-wave discharges as a physiological biomarker for hyperexcitability in alcohol withdrawal-induced seizure in Thy1 transgenic mice

Banner University Sports Medicine and Concussion Center, Dr. Steven Erickson, M.D. — June 2016 – Present

Banner University Medical Center, Phoenix, AZ

Volunteer

- Proctored concussion baseline testing for youth athletes and assisted with data analysis for balance testing and symptoms scores for research publications

Biomedical Engineering Research with Dr. Rémy Wahnoun, Ph.D. January – June 2015

Barrow Neurological Institute at Phoenix Children's Hospital, Phoenix, AZ

Research Volunteer

- Analyzed seizure data using multiple pursuit and GAD signal processing to quantify μECoG, ECoG, and EEG signals from patients undergoing epilepsy treatment

Developmental Neurogenetic Disorders Lab, Dr. Vinodh Narayanan, M.D. December 2008 – July 2010

Barrow Neurological Institute, Phoenix, AZ

Research Assistant Intern

- Assisted in framing experiments regarding Rhetts Syndrome and Tuberous Sclerosis
- Techniques utilized to extract and identify DNA with specific gene sequences, and genotype and purify mice DNA include: mini-prep, PCR, restriction digest, agarose gel electrophoresis, mass spectrometry
- **Personal summer project**: inserted specific disorder-mirroring vectors into the genome of bacterial cells

Neuroproteomics Lab, Dr. Peter Pingerelli & Dr. Richard S. Burns December 2007 – December 2008

Barrow Neurological Institute, Phoenix, AZ

Research Assistant Intern

- Prepared laboratory experiments to identify specific proteins present in patients with Parkinson's disease and Parkinson's disease with dementia
- Used bioinformatics procedures to tag and identify proteins in a sample

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PROFESSIONAL EXPERIENCE

Herman | Goldstein Law Firm, Phoenix, AZ May 2016 – August 2017

Assistant paralegal

Phoenix Neurological Associates, Phoenix, AZ October 2015 – May 2016

Office of Dr. Barry Hendin, M.D.; Dr. Todd Levine, M.D.; Dr. David Saperstein, M.D.; Dr. Lori Travis, M.D.; Dr. Holly Hendin, M.D., Ph.D.; Ruth Jan Bland, NP-C; Charrid Simpson, NP-C

Medical Assistant

LEADERSHIP AND ACTIVITIES

Graduate Student Association, Wake Forest University December 2017 – Present

Community Service Committee Chair

- Planned community service and outreach opportunities available for all Wake Forest University graduate students to participate in

Casa Heiwa and Angelina Mentorship Program (CHAMPs), UCLA December 2010 – June 2014

Executive Director, Recruitment Director, and Transportation Director

- Provided academic and social support as a mentor for children of low-income families in the Angelina apartment building of downtown Los Angeles

Alpha Chi Chapter of Alpha Delta Pi Sorority, UCLA September 2010 – June 2014

Member

- Participated in the social association as a member of Alpha Delta Pi, which is dedicated to sisterhood, values and ethics, high academic standards and social responsibility