Title

Protracted Abstinence from Chronic Ethanol Experience alters Plasticity Related Proteins and Excitability of Pyramidal Neurons in the Rodent Medial Prefrontal Cortex

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Protracted Abstinence from Chronic Ethanol Experience Alters Plasticity Related Proteins and Excitability of Pyramidal Neurons in the Rodent Medial Prefrontal Cortex

A Thesis submitted in partial satisfaction of the requirements
for the degree Master of Science

in

Biology

By

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Professor Scott Rifkin

2019

The Thesis of Dvijen Purohit is approved, and is acceptable in quality and form for publication on microfilm and electronically:

Co-Chair

Chair

University of California San Diego

2019

EPIGRAPH

"The moment you give up, is the moment you let someone else win"
-Kobe Bryant

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ABSTRACT OF THE THESIS

Protracted Abstinence from Chronic Ethanol Experience Alters Plasticity Related Proteins and Excitability of Pyramidal Neurons in the Rodent Medial Prefrontal Cortex

by

Dvijen Purohit

Master of Science in Biology

University of California San Diego, 2019

Professor Chitra Mandyam, Chair

Professor Jose Pruneda-Paz, Co-Chair

The rodent medial prefrontal cortex (mPFC) is a brain region that is homologous to the human PFC, a brain region within the limbic system that has been implicated in executive functions and in alcohol relapse. Previous studies in rodents have demonstrated that inducing

ethanol dependence through chronic intermittent ethanol vapor exposure paradigms followed by forced protracted abstinence increases drinking during abstinence, and causes significant alterations in neuronal excitability and white matter/cerebrovascular composition in the mPFC. However, no studies have investigated the cellular and molecular changes associated with the neuroplastic adaptations that occur within this brain region during protracted abstinence. To investigate this, 69 adult male Wistar rats underwent seven weeks of chronic intermittent ethanol exposure and were then subject to either 1 day, 7 days, 21 days, or 42 days of protracted abstinence. Afterwards these animals were immediately euthanized and tissue from mPFC of their brains was collected for analysis via Western blotting or for analysis via *ex vivo* electrophysiology. We found that chronic intermittent ethanol experience followed by 21 days of protracted abstinence produced a significant decrease in levels of phosphorylated calcium calmodulin kinase II (CaMKII) along with a decrease in the ratio of glutamatergic subunits GluN2A/2B of the NMDA receptor. This reduction in CaMKII activity and the ratio of GluN2A/2B was associated with enhanced excitability of layer 2/3 pyramidal neurons in the mPFC. Together, the present results indicate adaptive biochemical changes in excitatory neurotransmitter systems in the mPFC and highlights the importance of exploring neuroplasticity changes that predict enhanced propensity to relapse alcohol-seeking behaviors.

Introduction

Alcohol Use Disorder (AUD) is a spectrum disorder varying from low to moderate to severe states (NIAAA 2016). Moderate to severe AUD is characterized by high relapse risk that affects approximately sixteen million people in the United States, including about 6.2% of the adult population as of 2015 (NIH 2018) (NIAAA 2016) (Seo and Sinha, 2015). The condition is defined by compulsive consumption of alcohol, loss of control of intake characterized by escalation of intake, and persisting negative emotional and physical states associated with cessation of usage (NIH) (NIAAA). Amongst individuals that suffer from moderate to severe AUD during their lifetime, fewer than 15% seek treatment for this disease, and approximately 27.3% of these individuals achieve at least partial remission (Dawson et al. 2005) (Cohen et al. 2007). Relapse rates amongst these individuals remain high as well with some estimates indicating that up to 80% recovering alcoholics fail to maintain abstinence on a long-term basis (Moos et al. 2006). To produce more effective therapies that both increase remission rates and decrease relapse amongst those suffering from AUD, a greater understanding of the neurobiological factors that contribute to compulsivity and escalation of usage along with factors that surround relapse needs further investigation.

To emulate moderate to severe AUD in animal models for the purposes of research, effective paradigms must replicate defining factors that characterize the disorder in humans to provide data that is both valid and translational. These defining factors include producing a dependence to alcohol in animals, one that produces physical and motivational withdrawal symptoms in humans during abstinence by enhancing anxiety-like behavior, and excessive drinking during relapse (Schulteis et al., 1996) (McBride et al., 1998) (Gilpin et al., 2008). One method to reliably produce alcohol dependence in animals is through alcohol vapor exposure

(Gilpin et al., 2008). Chronic intermittent ethanol exposure protocols (CIE) have been developed and utilized to study alcohol dependence in animals in a manner that allows the experimenter to control the dose of alcohol, the duration of exposure, and the pattern of exposure (Gilpin et al., 2008). This paradigm also allows the application of forced and protracted abstinence periods that induce withdrawal amongst animals following cessation vapor exposure. These withdrawal phenotypes are characterized by various motivational and somatic symptoms which include voluntary escalation in ethanol drinking, anxiety-like behavior, and reward deficits (Gilpin et al. 2008) (Schulteis et al., 1996) (McBride et al., 1998).

Within the rodent brain, the medial prefrontal cortex is one region that has been shown to pose significant neurobiological alterations as a result of alcohol dependence through CIE (Mandyam et. al 2012). This region is homologous to the human prefrontal cortex, a brain region within the limbic system that has been implicated in various executive functions, including regulation of goal-directed actions and memory-based decision making (Abernathy et al., 2010) (Weilbacher et al., 2016). Dysregulations in the mPFC have been implicated in various aspects surrounding alcohol addiction phenotypes, specifically in alcohol seeking behaviors, loss of self-control, and persisting relapse despite negative consequences (Abernathy et al., 2010) (Mandyam et al., 2012) (Seo and Sinha, 2014) (Seo and Sinha, 2015). Specifically, neuroplasticity in the PFC in response to alcohol, which refers to the functional and structural reorganization and changes in neural circuitry that occur during various stimuli responses, has been implicated in the maladaptive behaviors and suboptimal functioning that encapsulate addictive behavior and relapse propensity (Seo and Sinha, 2015) (Seo and Sinha, 2014) (Kalivas and O'Brien 2008). This highlights the importance in developing a further understanding of the exact molecular neuroplastic adaptations that occur at a synaptic level within the mPFC amongst alcohol

dependent individuals that persist during abstinence and determining how these changes contribute towards AUD.

Previous published studies by others and unpublished studies in this lab have investigated the effects of CIE and CIE followed by periods of either 7 or 21 days protracted abstinence (CIE-PA) on oligodendrocyte expression levels and subsequent white matter neuropathology in the mPFC of rats. These studies detected reduced expression of oligodendrocytes during CIE through measures of transcription factor Olig2 along with myelin basic protein (MBP) and a transient increase in MBP and myelin oligodendrocyte glycoprotein (MOG) concurrent with decreases in cerebrovascular integrity during protracted abstinence (Kim et al., 2014) (Mandyam et al., 2017) (Navarro and Mandyam, 2015) (Somkuwar et al., 2016) (Somkuwar et al., Unpublished) (Steiner et al. Unpublished). Recent *ex vivo* slice electrophysiology studies in the mPFC have demonstrated that acute ethanol treatment decreases sustained depolarization that occurs in pyramidal neurons during up-states, indicating that ethanol decreases N-methyl-D-aspartate-type glutamate receptor (NMDAR)-mediated excitatory postsynaptic currents (Tu et al., 2007)(Weitlauf & Woodward, 2008)(Woodward & Pava, 2009). Such mechanistic studies have been extended in animal models of chronic ethanol exposure to demonstrate that ethanol alters the functional and structural plasticity of pyramidal neurons in the mPFC. For example, chronic intermittent ethanol vapor exposure (CIE) produces significant, yet opposing effects on pyramidal neuron synaptic activity (persistent increase in NMDAR-mediated excitatory postsynaptic currents), and synaptic plasticity (aberrant increase in NMDAR-mediated spike-timing-dependent plasticity) compared with acute effects on slices, possibly through an NR1 and NR2B-mediated mechanism (Kroener et al., 2012). These adaptive changes in NMDARs during long-term ethanol exposure may be occurring to counterbalance the initial prolonged inhibitory

effects of ethanol on NMDAR activity, and may contribute to the aberrant neuronal excitability and neuronal toxicity observed during withdrawal and protracted abstinence (Grant et al., 1990) (Chandler, 2003) (Kroener et al., 2012). Notably, the altered functional plasticity of pyramidal neurons is associated with altered structural plasticity of pyramidal neurons (increased dendritic arborization and mature spine density), suggesting dysfunctional cortical networking in the mPFC (Holmes et al., 2012) (Kroener et al., 2012) (Kim et al., 2014). These electrophysiological findings suggest that CIE and CIE-PA produce distinct adaptations in the expression of proteins that regulate the excitability and plasticity of mPFC neurons.

This leads to the primary hypothesis of my thesis, which was to investigate whether CIE-PA will produce detectable differences in the expression levels of plasticity markers in the mPFC of rats and to determine if these findings will be associated with unpublished white matter neuropathological findings from our lab and published electrophysiological data by others. For the purposes of this experiment, the changes in neuroplasticity that occur in the adult rat medial prefrontal cortex during various time points of abstinence following CIE were investigated. Adult Wistar rats were subjected to seven weeks of CIE and were then subjected to 1 day, 7 days, 21 days, or 42 days of protracted abstinence (CIE-PA). Immediately after these rats completed their respective periods of protracted abstinence, they were euthanized and their brain tissues were processed to allow protein analysis and quantification via Western Blotting. This allowed a time-dependent analysis of the effects of abstinence on neuroplasticity following established alcohol dependence in these rats. Antibodies specific for NMDA and GABA receptor subunits along with ones specific for synaptic plasticity, including calcium calmodulin kinase II and post-synaptic density (PSD- 95) were used to evaluate relative levels of plasticity markers between the various time points of CIE-PA.

Materials and Methods

Animals

For this study, sixty-nine adult male Wistar rats (Charles River, Hollister, CA USA), 8-weeks old and weighing between 275-325g at the start of the experiment were used. Animals were housed in a temperature-controlled vivarium set at 22℃ with ad libitum access to food and water in cage units containing 2 to 3 animals. Animals were set on a reverse light cycle with alternating 12 hours light and 12 hours dark cycles, with the light cycle beginning at 8:00PM. All experimental protocols used in this study were approved by the VA San Diego Healthcare System Institutional Animal Care and Use Committee along with The Scripps Research Institute. These protocols were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85–23, revised 1996).

Chronic Intermittent Ethanol Vapor Exposure and Protracted Abstinence (CIE-PA)

During this experiment, animals were randomly assigned to groups that either experience CIE-PA or remain ethanol naïve (age-matched non-vapor controls). CIE-PA rats underwent cycles of ethanol vapor exposure daily on a 14 hours on/10 hours off schedule for the duration of seven weeks. During these 14 hours, 95% ethanol was vaporized in a heated flask and the resulting vapors were then immediately conveyed to the rat vapor chambers through controlled air flow. The vapor flow rate was calibrated to achieve a target blood alcohol level within the range of 125 to 250 mg/dL (27.2 to 54.4 mM) in these animals; this blood alcohol concentration has been shown to induce voluntary escalation in drinking behavior, in addition to both physical dependence and negative behavioral affect during ethanol withdrawal (Gilpin 2008, Griffin

2009a, Griffin 2009b, Griffin 2014, Lopez 2005). After seven weeks of vapor exposure, CIE-PA animals underwent varying durations of protracted abstinence from ethanol. CIE-PA animals were randomly assigned into four separate CIE-PA groups that were subjected to either 1 day (n=10), 7 days(n=9), 21 days (n=9), or 42 days (n=9) of protracted abstinence. After completing their designated periods of protracted abstinence, the animals were euthanized and their brain tissue was collected for post-mortem analysis. Age-matched non-vapor controls for 1 day (n=8), 7 day (n=6), 21 day (n=6), and 42 day (n=6) CIE-PA animals were sacrificed on the same day as their vapor exposed constituents.

Blood Alcohol Concentration (BACs) Measurements

To measure animal BACs and ensure that they were within the designated target range, blood samples (0.2mL) from CIE-PA animals were collected immediately after daily vapor exposure sessions via tail-bleeding. Blood samples were collected twice during the first week of vapor exposure, and once a week for the subsequent six weeks. After collection, the samples were centrifuged, and isolated plasma aliquots (5µL) were measured for ethanol content via an Analox AM1 analyzer (Analox Instruments USA Inc., MA, USA). Before each set of samples were measured, the Analox AM1 analyzer was calibrated using the reagents provided by Analox Instruments (25–400 mg/dL or 5.4–87.0 mM). When animal BACs fell outside of the target range of 125-250 mg/dL, ethanol vapor flow rate was adjusted accordingly. Mean BACs of CIE-PA animals throughout the course of seven weeks of vapor exposure were then determined.

Western blot analysis

Optimized western blot protocols were utilized in to measure levels of NMDA and GABA receptor expression along with levels of proteins related to synaptic plasticity at each time point during the abstinence period of the CIE-PA protocol. Immediately after rats completed their designated periods of protracted abstinence, they were subjected to isoflurane anesthesia and rapid decapitation along with their respective aged-matched non-vapor controls. After decapitation, brains were cut along the mid-sagittal axis, and the right hemisphere was fixated with 4% paraformaldehyde, while the left hemisphere was flash frozen in isopentane for tissue-punching. Afterwards, a Leica CM 1850 refrigerated microtome cryostat was used to cut 500µm slices from the mPFC of the left brain hemispheres, from which tissue punches were collected, homogenized via sonication with buffer (320 mM sucrose, 5 mM HEPES, 1 mM EGTA, 1 mM EDTA, 1% SDS, with Protease Inhibitor Cocktail and Phosphatase Inhibitor Cocktails II and III diluted 1:100; Sigma, St. Louis, MO) at 0℃, and then subsequently heated at 100℃ for five minutes. All samples were then subjected to a detergent-compatible Lowry method (Bio-Rad, Hercules, CA) in order to determine the protein concentrations in each sample. Samples were then mixed with 2x Laemmli Blue sample buffer containing beta-mercaptoethanol at a 1:1 ratio. Following this, aliquots containing 20µg of protein from each sample were ran on 10-12% SDS-PAGE gels, and were then transferred to PVDF membranes (0.2µm pore size). After transfer, 1.5% BSA (w/v) or 5% milk (w/v) in TBST solution (25 mM Tris-HCl (pH 7.4), 150 mM NaCl and 0.1% Tween 20 (v/v)) was used to block all blots at room temperature (20-25℃) for one hour. Blots were then incubated at 4℃ for 20-24h in solutions containing primary antibodies diluted in blocking buffer. These antibodies included an antibody to glutamate NMDA receptor subunit 2A (tNR2A) (1:500, Santa Cruz Biotechnology catalogue # SC-9056 (DC), predicted

molecular weight 175kDa, observed molecular weight ~200kDa), an antibody to phosphorylated glutamate NMDA receptor subunit 2A (pNR2A) (1:200, PhosphoSolutions catalogue # p1514-1325, predicted and observed molecular weight 180kDa), an antibody to glutamate NMDA receptor subunit 2B (tNR2B)(1:1000, Santa Cruz Biotechnology catalogue # SC-9057 (DC), predicted molecular weight 175kDa, observed molecular weight ~200kDa), an antibody to phosphorylated glutamate NMDA receptor subunit 2B (pNR2B)(1:200, Cell Signaling Technology catalogue # 4208S, predicted molecular weight 180kDa, molecular weight ~190kDa), an antibody to calmodulin kinase II (tCaMKII)(1:1000, ABCAM catalogue # ab52476, predicted molecular weight 70kDa, observed molecular weight ~54kDa), an antibody to phosphorylated calmodulin kinase II (pCaMKII)(1:1000, ABCAM catalogue # ab52683, predicted and observed molecular weight 50kDa), an antibody to the α6-subunit of rat $GABA_A$ receptor ($GABA_A$)(1:1000, PhosphoSolutions catalogue #850-GA6, predicted molecular weight 57kDa, molecular weight ~50kDa) and an antibody for post synaptic density (PSD-95)(1:200, ThermoFisher Scientific catalogue #MA-1045, predicted molecular weight 95kDa, molecular weight ~100kDa). After the primaries were incubated, the membranes were washed three times over a period of 15 minutes in TBST in order to was away excess primary antibody and were then subjected to a 1h, room temperature (24℃) incubation of secondary antibodies diluted in TBST. The secondary antibodies used included either a horseradish peroxide–conjugated goat antibody to mouse IgG1 (1:500, used for PSD-95, BioRad catalogue # 170-6516) or a horseradish peroxide–conjugated goat antibody to rabbit (1:500- 1:5000, used for all other proteins, BioRad catalogue # 170-6515). An additional three washes over a period of 15 minutes was then performed in order to wash away excess secondary antibody before immunoreactivity detection was conducted using SuperSignal West Dura chemiluminescence detection reagent

(Thermo Scientific) and visualized using an Azure Biosystems c600 imaging system. The software Image J (version 1.45S, NIH) was used to quantify the intensity of signals originating from each membrane. Coomassie Brilliant Blue staining was then utilized as a loading control for normalization and phosphorylated proteins were normalized to their respective total proteins.

Slice Preparation and Electrophysiology

Slices for electrophysiology were prepared from alcohol naïve (n=3 rats, n=13 cells) and 21day CIE-PA rats (n=3 rats, n=8 cells) according to our recently published protocol (Galitano et al. 2018). Rats from each group were subjected to brief Ketamine/Xylazine/Acepromazine anesthesia and perfused for 3 minutes with ice-cold, oxygenated modified sucrose artificial cerebrospinal fluid (ACSF). 330 µm-thick slices containing mPFC were cut using a Leica VT1200 vibratome and incubated at 34º C in an interface chamber containing the same modified sucrose ACSF solution for 30 min. Following incubation at 34º C slices were held at room temperature (23° C) in the same chamber for at least 45 min before initiating recordings. Recordings were made in a submersion-type recording chamber and superfused with oxygenated ACSF at 23° C at a rate of 2-3 ml / minute. Whole-cell patch clamp recordings were obtained using Multiclamp 700B amplifiers (Molecular Devices) and data was collected using pClamp 10 software (Molecular Devices). Data was low-pass filtered at 2kHz, and digitized at 10kHz (Digidata 1440A; Molecular Devices). In current-clamp experiments, currents were injected stepwise, in 20 pA increments. Step duration was 500 milliseconds (ms). Analysis of electrophysiological properties was conducted with Clampfit 10.4 software (Molecular Devices) as previously described (Galitano et al., 2018). In brief, resting potential is defined as the recorded membrane potential when cell membrane was initially broken into with a recording

pipette for the whole-cell patch clamp configuration. Action potential (AP) characteristics are based on a single AP evoked by intracellular current injection (ranging between 60 to 180 pA, 500 ms pulse). Peak amplitude is actual measured peak AP amplitude in mV. Spike threshold is defined as the voltage at which the second derivative of the membrane potential trajectory approaching a spike crossed a user-selected threshold of 20 mV/ms2. Half-width is the period measured in latency (ms) from the start of the AP to the time when the AP reached half of the peak amplitude. The amplitude of after-hyperpolarization (AHP) was measured from the threshold voltage to the most negative overshoot after repolarization. The slope of the rise to threshold was obtained by a linear fit from completion of the AHP to the threshold of the subsequent AP. Inter-spike intervals (ISIs) were measured as the time between first evoked AP peak to the second AP.

RESULTS

CIE consistently produces elevated BACs in animals within the target range (Figure 2)

The CIE-PA animals took two weeks of daily CIE sessions to reach the target range of blood alcohol concentration between 125-250 mg/dl. Once they reached this range by week 3, BACs remained consistently within target range for the remainder of the five weeks of CIE treatment.

CIE-PA causes a transient reduction in levels of CaMKII phosphorylation at Thr286 in the mPFC at the 7 day and 21 day timepoint (Figure 4)

Western blot analysis was conducted to investigate the effects of different durations of abstinence in CIE-PA on pCaMKII expression in the mPFC. Western blot analyses for each CIE-PA group were expressed as percentage differences from each group's respective age matched controls. Individual pCaMKII expression values were normalized as a percentage of total CaMKII (tCaMKII) expression in each rat, such that the pCaMKII value is calculated as a ratio of pCaMKII to tCaMKII. A two-way ANOVA test revealed a significant effect of CIE on pCaMKII levels in the mPFC [$F(1, 58) = 15.87$, $p<0.0002$]. Post-hoc analysis using a Holmes-Sidak's multiple comparison test revealed a significant decrease in pCaMKII levels with a mean difference of 69.02 ± 22.69 between the 7 day CIE-PA group and their age-matched controls ($p<0.0105$) along with a significant decrease in pCaMKII levels with a mean difference of 75.69 ± 22.69 between the 21 day CIE-PA group and their age matched controls ($p<.0059$). This continuous decrease was not observed in the 42 day CIE-PA group and therefore represents a transient decrease in pCaMKII levels in the rodent mPFC during abstinence. This marker was used to determine levels of calmodulin kinase II phosphorylation at the Thr286 position, which

has been demonstrated to increase the enzyme's calcium-dependent activity and has been implicated in roles surrounding synaptic plasticity, including LTP and LTD (Griffith 2004).

CIE-PA causes a transient reduction in t2A/2B ratios in the mPFC at the 21 day timepoint (Figure 4)

Western blot analysis was conducted to investigate the effects of different durations of abstinence in CIE-PA on the ratio of tNR2A/tNR2B expressed in the mPFC. Western blot analyses for each CIE-PA group were expressed as percentage differences from each group's respective age matched controls. A two-way ANOVA test revealed a significant effect of time course of abstinence on the ratio of tNR2A/tNR2B in the mPFC of CIE-PA animals [$F(3, 52) = 3.478$, $p<0.0223$]. Post-hoc analysis using Holmes-Sidak's multiple comparison test revealed that the ratio of tNR2A/tNR2B in the mPFC significantly decreased between the group that experienced 24 hours of protracted abstinence and the group that experienced 21 days of protracted abstinence [Mean difference=55.43, $p<.0479$]. Modifications in the ratios of 2A/2B subunits of the NMDA glutamate receptor has been hypothesized to be a homeostatic mechanism that facilitates the induction of plasticity through bidirectional potentiation of either LTD or LTP (Cui et al., 2013) (Shipton & Paulsen 2014). Specifically, a relative increase in the 2A/2B ratio has been observed to be a critical genetic factor in constraining and consolidation long-term memory and learned behaviors in the adult brain (Cui et al., 2013) (Jerusalinky et al., 2018) (Shipton & Paulsen 2014).

CIE-PA increases excitability of layer 2/3 pyramidal neurons in the mPFC at the 21 day time point (Figure 5)

We next studied the intrinsic excitability of layer 2/3 pyramidal neurons in rats that experienced 21 days of CIE-PA to associate the cellular changes with electrophysiology data (Figure 5). Depolarizing current injections generated fast action potentials with large amplitudes (Figure 5c & 5d). The number of spikes elicited by neurons from each group with increasing current injections in current-clamp recording were determined, and repeated measures two-way ANOVA demonstrated a significant number of spikes x treatment interaction [$F_{12, 216} = 3.09$, $p=0.0005$], and significant increases in the number of spikes over current injections [$F_{12, 216} = 88.8$, $p<0.001$) without a significant effect of treatment ($F_{1, 18} = 2.1$, $p=0.1$). Post hoc analysis demonstrates that CIE-PA rats have increased number of spikes with increasing current injections compared to controls at current injections 120pA to 140pA. At higher current injections (>240 pA), there were no significant differences in the number of spikes between controls and CIE-PA rats, indicating that neurons from both groups elicited property of regular spiking by depolarizing current injections (data not shown). The distinct intrinsic firing properties of CIE-PA neurons were associated with parallel alterations in interspike interval without alterations in AP threshold, rheobase current, interspike interval and AHP (Figure 5f). Unpaired t test demonstrated a significant reduction in inter-spike interval (ISI) amongst animals 21 day CIE-PA animals, measured as spike latency between first and second spike peak in neurons from CIE-PA rats compared to control rats ($p<0.05$, Figure 5f).

Discussion

The primary hypothesis of my thesis was to investigate whether CIE-PA will produce detectable differences in the expression levels of plasticity markers in the mPFC of adult male Wistar rats and to determine if these findings will be associated with white matter neuropathological and electrophysiological data discovered in the lab. What I found was that during CIE-PA, a significant and transient decrease in calmodulin kinase phosphorylated at Thr286 was detectable at the 7 day and 21 day timepoint of protracted abstinence and that this decrease was concurrent with a transient decline in the ratio of 2A/2B ratio of the NMDA receptor subunits. Autophosphorylation of calcium calmodulin kinase at Thr286 has been shown to alter interactions between the enzyme's regulatory domain and catalytic core and has been demonstrated to induce conformational changes that increase the activity of the enzyme and slow its off-rate (Griffith 2004). The Thr286 phosphorylated state has been implicated in its roles surrounding synaptic plasticity and reduced levels of Thr286 phosphorylation may be associated with resistance to plasticity related changes in neuronal circuitry (Griffith 2004) (Barcomb et al., 2014). Increases in the 2A/2B subunit ratio of the NMDA receptor have been observed to be a critical genetic factor in constraining and consolidation long-term memory and learned behaviors in the adult brain (Cui et al., 2013) (Jerusalinky et al., 2018) (Shipton & Paulsen 2014) and therefore the observed reductions in the 2A/2B in CIE-PA rodents at the 21 day timepoint may also signify a synaptic resistance to plasticity and learning. These findings offer a novel analysis towards the nueroplastic alterations that occur in the mPFC during CIE-PA. These results, when taken in concert with previous studies conducted in the lab that investigated white matter integrity, cerebrovascular integrity and neuronal excitability in the mPFC of rats that underwent

CIE-PA may elucidate a broader context of the dysregulations that occur in this brain region that contribute to alcohol addiction phenotypes.

Previous studies in this lab have detected reduced expression of oligodendrocytes during CIE through measures of transcription factor Olig2 along with myelin basic protein (MBP) and a transient increase in MBP and myelin oligodendrocyte glycoprotein (MOG) concurrent with decreases in cerebrovascular integrity after 7 days of protracted abstinence protracted abstinence (Supplemental Figure 1A)(Supplemental Figure 1B)(Kim et al., 2015) (Mandyam et al., 2017) (Navarro and Mandyam, 2015) (Somkuwar et al., 2016) (Somkuwar et al., Unpublished) (Steiner et al. Unpublished). Specifically, one study detected a transient increase in fractional anisotropy, or a reduction in vascular integrity, in the mPFC following 7 days of protracted abstinence following CIE (Somkuwar et al., Unpublished) (Supplemental Figure 1B & 1C). These findings in concert to the primary findings of this thesis may suggest that a combination of transient reduced vascular integrity and altered synaptic plasticity in the mPFC of rats at the 7 to 21 day timepoint may be a direct effect of withdrawal from CIE and may contribute persistence of alcohol addiction phenotypes in these animals.

This thesis has detected a transient change in plasticity markers in the mPFC of rodents undergoing CIE-PA at the 7 day and 21 day time point with regards to pCaMKII expression and the ratio of expressed 2A/2B subunits of the NMDA receptor and correlated these findings to previous findings in the lab regarding non-neuronal alterations including white matter pathology, cerebrovascular integrity, and intrinsic neuronal excitability in the mPFC in response to CIE-PA. In addition, these cellular alterations in the plasticity related proteins also correlate with previous published findings in the lab regarding neuronal alterations of layer 2/3 pyramidal neurons, including enhanced structural plasticity of these neurons, visualized as increased dendritic

arborization and complexity (Navarro and Mandyam 2015). To add to these neuronal findings, *ex vivo* electrophysiological studies have evaluated the intrinsic excitability of layer 2/3 pyramidal neurons. The reduced activity of CaMKII and reduced ratio of GluN2A/2B is associated with enhanced excitability of layer 2/3 pyramidal neurons. Increases and decreases in intrinsic excitability of pyramidal neurons may serve as a metaplastic mechanism for memory formation and retention (Milshtein-Parush et al., 2017) (Crestani et al., 2018), suggesting that functionally adaptive modulation of excitability in the PFC could promote network stability. Notably, CaMKII and GluNs are implicated in regulating intrinsic excitability of neurons in the cortex and elsewhere (Zhang et al., 2004) (Nelson et al., 2005) (Sametsky et al., 2009) (van Welie & du Lac, 2011) (Klug et al., 2012) (Milshtein-Parush et al., 2017) (Crestani et al., 2018), suggesting that altered expression of these proteins in the mPFC could correlate with changes in the neuronal excitability of layer 2/3 pyramidal neurons. These findings support the recent studies that have indicated causation between CaMKII and GluNs in oppositely regulating excitability of cortical neurons and their roles in learning and memory (Ohno et al., 2006) (Sametsky et al., 2009) (Milshtein-Parush et al., 2017) (Crestani et al., 2018).

Nevertheless, these results must be further investigated in order to establish causation and develop clinically translational approaches to treat AUD. The rodent mPFC is a brain region homologous to the human prefrontal cortex which has been implicated in various executive functions, including regulation of goal-directed actions and memory-based decision making (Abernathy et al., 2010) (Weilbacher et al., 2016); therefore, behavioral studies that test reward and memory in response to CIE-PA may prove to be useful in following up the findings of this thesis. Furthermore, this study solely employed the use of adult male rats to investigate the effects of CIE-PA on the brain. It has been demonstrated that males and females experience

addiction distinctly, specifically in the facets of acquisition, escalation, maintenance, withdrawal, and relapse (NIDA 2018)(Becker et al., 2017) therefore a continuation of this study investigating sex differences in the context of CIE-PA is worthwhile. Altogether, these findings suggest that future research should further characterize the behavioral implications of CIE-PA-induced neuroplastic, white matter, cerebrovascular, and electrophysiological changes in both males and females, in efforts to develop clinically translational treatments to treat AUD.

Figure 1: CIE-PA Experimental Design: Sixty-three adult male Wistar rats were used to complete this study. Thirty-seven rats were randomly selected to undergo seven weeks of ethanol exposure via CIE-PA protocol, before being subjected various periods of abstinence, either 24 hours, 7 days, 21 days, or 42 days. The remaining twenty-six did not undergo ethanol exposure and served as age-matched non-vapor controls. After finishing their respective periods of protracted abstinence, all animals were anesthetized with isoflurane, and immediately sacrificed along with age-matched controls.

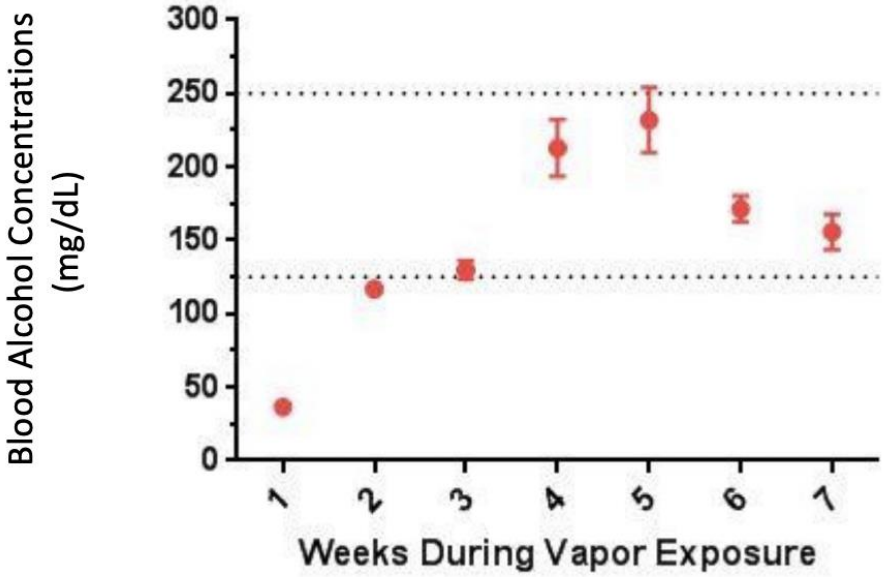


Figure 2: CIE produces stable elevated blood alcohol concentrations. This graph depicts weekly measures of blood alcohol concentrations (BACs) in mg/dl for CIE-PA animals over the course 7 weeks of CIE. The data points are expressed as mean concentrations from all animals and the area between the dotted lines indicate the target BAC range of 125 mg/dl and 250 mg/dl.

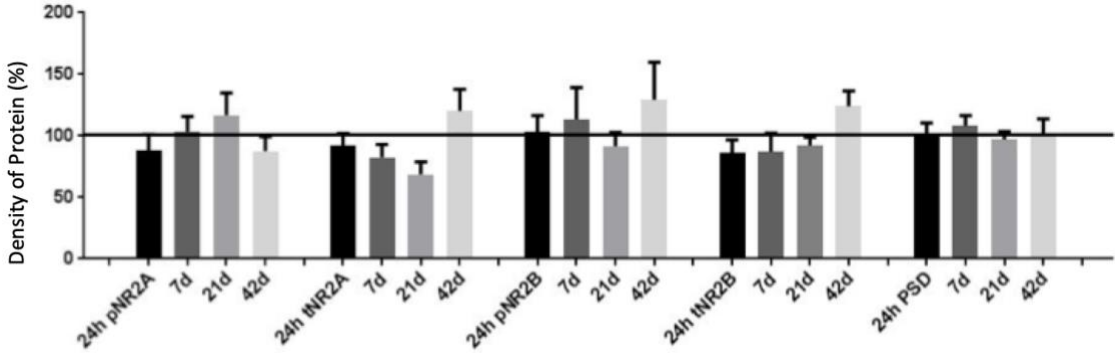


Figure 3: Quantified results from plasticity proteins analyzed in this experiment. Analysis of protein densities in CIE-PA animals are expressed as percentages of their respective controls. The solid line at 100 indicates control average. For CIE-PA animals, n = 10 for 24h, n = 9 for 7d, n = 9 for 21d, and n = for 42d. For age-matched non-vapor control animals, n = 8 for 24hr, n=6 for 7d, n = 6 for 21d, and n = 6 for 42d. No significant differences were found between CIE-PA animals and controls amongst these proteins.

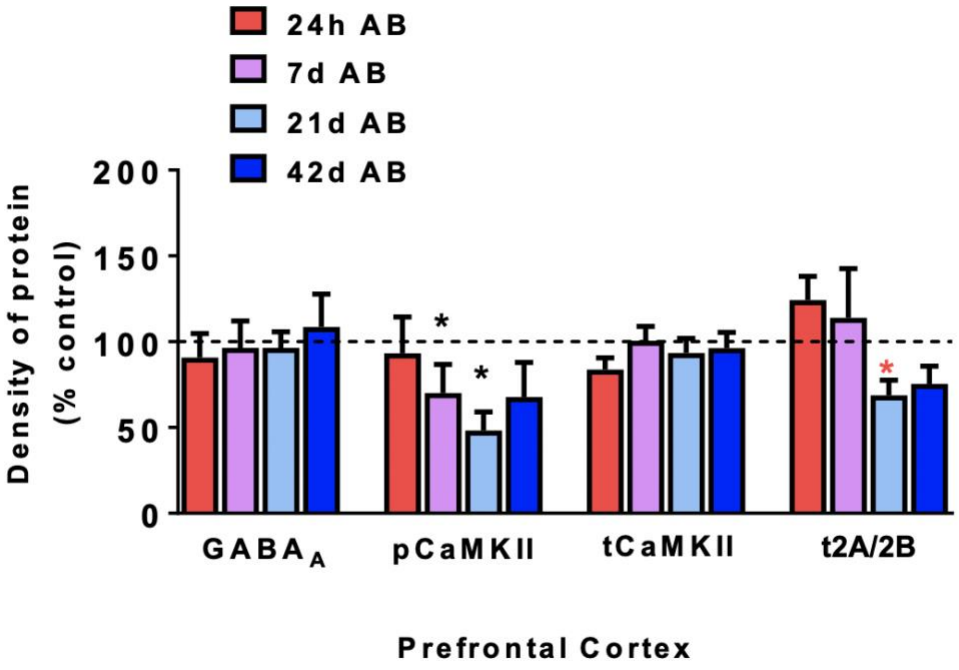


Figure 4: CIE-PA causes a transient decrease in pCaMKII levels after 7 and 21 days of abstinence along with a transient decrease in the ratio of tNR2A/tNR2B after 21 days of abstinence in the rat PFC. Analysis of protein densities in CIE-PA animals are expressed as percentages of their respective controls. The dotted line at 100 indicates control average. For CIE-PA animals, n = 10 for 24h, n = 9 for 7d, n = 9 for 21d, and n = for 42d. For age-matched non-vapor control animals, n = 8 for 24hr, n=6 for 7d, n = 6 for 21d, and n = 6 for 42d. Levels of CaMKII phosphorylated at Thr286 (pCaMKII) decreased following 7 days of abstinence and continued decreasing up to 21 days (*). This decreasing trend was not observed after 42 days of abstinence and therefore represents a transient decrease in pCaMKII. The ratio of tNR2A/tNR2B showed a decreasing trend following 7 days of abstinence although the difference was not significant and showed a significant decrease following 21 days of abstinence that did not persist following 42 days of abstinence (*). $GABA_A$ levels showed a trend towards increasing with duration of protracted abstinence although this difference was not significant.

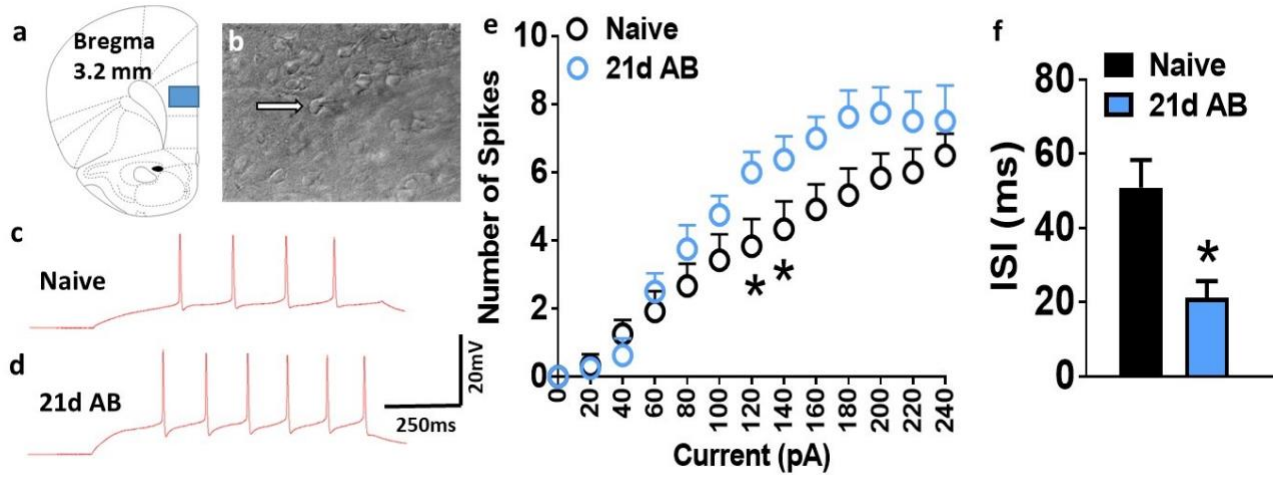
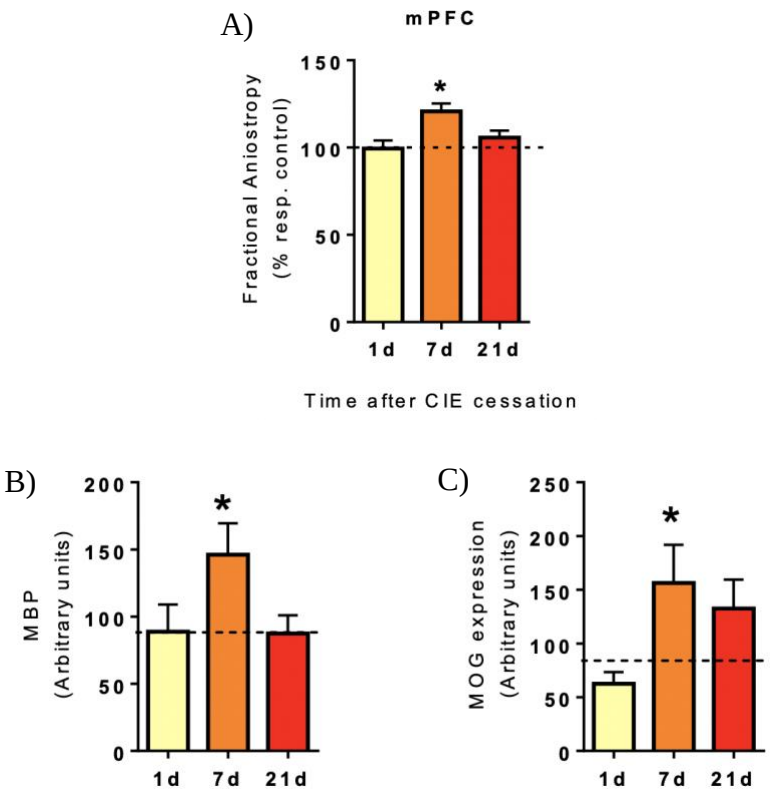


Figure 5: Intrinsic excitability of layer 2/3 pyramidal mPFC neurons in response to 21d Abstinence. Brain slices from ethanol naïve rats and rats that underwent CIE-PA were subjected to patch clamp whole cell electrophysiological recordings to determine intrinsic excitability in response to varying current injections. Slices at 3.2mm Bregma were used from each animal to isolate neurons in the mPFC for recording (a)(b). The excitability of PFC neurons was significantly higher in CIE-PA animals compared to ethanol naïve rats at 120pA and 140pA current injections (e). Each data point is represented as a mean value±standard error. The inter-spike interval was significantly lower in CIE-PA rats compared to ethanol naïve controls (f). There were no significant differences in several other electrophysiological properties examined (fast hyperpolarization, half width, action potential threshold, time of peak, rise slope to threshold, rise and decay tau and peak amplitude; data not shown).



Supplemental Figure 1: CIE-PA produces a transient increase in fractional anisotropy concurrent with transient increases in MBP and MOG expression in the mPFC after 7 days of PA. A) CIE-PA increases fractional anisotropy in the mPFC at the 7d protracted abstinence time point. Analysis of fractional anisotropy values in the mPFC of 1 day, 7 day, and 21 day CIE-PA animals are expressed as percent change compared with controls. B & C) Protein density analysis of myelin associated proteins MBP and MOG. Analysis of protein values in the mPFC of 1 day, 7 day, and 21 day CIE-PA animals are expressed as percent change compared with controls. n = 8 for 1 day age-matched non-vapor control animals, n=6 for 7d age-matched non-vapor control animals, n = 6 for 21d age-matched non-vapor control animals, and n = 6 for 42d age matched non-vapor control animals. *$p < 0.05$ compared with controls

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