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Neuroadaptations in the medial prefrontal cortex (mPFC) predict memory deficits dependent on the mPFC in alcohol dependent rats

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

Master of Science

in

Biology

by

Noah Steiner

Committee in charge:

Professor Chitra D. Manydam, Chair
Professor Douglass Forbes, Co-chair
Professor Randolph Hampton

2018

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The Thesis of Noah Steiner is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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Co-Chair

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Chair

University of California, San Diego

2018

DEDICATION

I dedicate this work to my parents, Robert and Jamie,

my siblings Tiki, Maya, Efi, and Shaya,

who have supported me and been my shoulder to lean on in my times of struggle.

I couldn’t have done this without them.

EPIGRAPH

"If the last to know he's an addict is the addict, then maybe the last to know when a man means what he says is the man himself."

Phillip K. Dick, A Scanner Darkly

TABLE OF CONTENTS

Signature Page iii
Dedication iv
Epigraph v
Table of Contents vi
Lists of Figures and Tables vii
Acknowledgements viii
Abstract of The Thesis ix

Introduction 1
Materials and Methods 7
Results 14
Discussion 17
Figures and Tables 22
References 29

LIST OF FIGURES AND TABLES

Table 1: Experimental conditions and groups 22

Figure 1: Blood alcohol levels, in mg/dL, measured via tail bleeding over 7 weeks of CIE 23

Figure 2: Trace fear conditioning (a) training at 72hrs after last CIE and (b) context retrieval 24hrs after training. 24

Figure 3: Dendritic arbor in layer 2/3 pyramidal neurons. Representative tracings from CIE/TFC, TFC, and CIE animals 25

Figure 4: TFC differentially affects expression of synaptic plasticity proteins. 26

Figure 5: Expression of oligodendroglial proteins in CIE rats is increased by TFC 27

Figure 6: TFC produces opposing effects on Ki-67 labelled proliferating cells in CIE 28

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ABSTRACT OF THE THESIS

Neuroadaptations in the medial prefrontal cortex (mPFC) predict memory deficits dependent on the mPFC in alcohol dependent rats

by

Noah Steiner

Master of Science in Biology

University of California, San Diego, 2018

Professor Chitra D. Manydam, Chair

Professor Douglass Forbes, Co-Chair

Chronic intermittent ethanol vapor exposure (CIE) produces dependence, alters the structure of pyramidal neurons, and decreases oligodendroglial progenitors in the medial prefrontal cortex (mPFC). The effect of early abstinence from CIE on these neuroadaptations are unclear and unknown and were investigated. Adult male Wistar rats were exposed to CIE for 7 weeks, after which they underwent 3 days of abstinence.

On the 4th day, CIE abstinent and age matched CIE naïve controls experienced trace fear conditioning (TFC) to determine the effect of early abstinence on neuroadaptations in the mPFC and retrieval of emotional memories, and the interactions between TFC and abstinence-induced neuroadaptations in the mPFC. To assess neuroadaptations, mPFC tissue was processed for Western blotting, immunohistochemistry and Golgi-Cox staining. CIE abstinent animals froze less during retrieval, indicating deficits in memory functions dependent on the mPFC. Abstinence enhanced dendritic complexity of layer 2/3 mPFC pyramidal neurons, as did trace fear conditioning. However, trace fear conditioning combined with CIE had no additive effect. Additionally, abstinence altered plasticity-related proteins in mPFC (increased total NR2A expression and decreased PSD-95) and TFC did not affect these adaptations. However, TFC produced profound alterations in oligodendroglial proteins (increases in transcription factor Olig2 and myelin basic protein (MBP)) and abstinence abolished these effects. Our findings indicate that neuroadaptations in the mPFC persist into early abstinence in CIE animals, and these deficits are associated with reduced memory functions dependent on the PFC.

INTRODUCTION

Abuse of alcohol in the United States is an issue that carries a heavy societal and economic burden. With more than 15 million American adults showing symptoms of Alcohol Use Disorder (AUD) and a negative economic externality of nearly 250 billion USD, the need for a solution is pressing (NIAAA 2017). AUD follows an established three-stage pattern: (1) binge/intoxication, (2) withdrawal, (3) anticipation/craving which follows a shift from impulsive behavior to an impulsive/compulsive disorder. (Koob, Le Moal M, 2008). Our interest lies in the anticipation stage where the prefrontal cortex (PFC) plays a pivotal role (Koob and Volkow, 2010). The PFC exhibits top down executive control on behavior planning; it synthesizes past experiences with a current situation thus informing decision making (Abernathy et al., 2010, Crews et al., 2009). In animal models, the PFC has been implicated in drug seeking and extinction (Moorman et al., 2015, Goldstein et al., 2011, Ishikawa et al., 2008). In human alcoholics, structural changes in the PFC have been documented including the loss of both white and grey matter (Jernigan et al., 1991, De la Monte et al., 1988). This change in brain structure may be associated with the loss of impulse control, leading to drug seeking and bingeing (Jentsch JD et al., 1999, Koob and Volkow, 2010).

AUD is a complex condition where multiple criteria must be satisfied before a person could be considered as having the disorder (DSM-V, American Psychiatric Association 2013). As a translational tool, animal models can help bridge the

molecular changes observed with behavior. To force a particular phenotype, genetic models are often used. For example, there are mouse strains bred to show either excessive or minimal ethanol consumption (Le et al., 1994). These high and low responders could then be used to measure a difference in anxiety or another behavioral trait. It should be noted that non-genetic models are also sufficient in displaying AUD-like phenotypes. By increasing ethanol in the diets of rats, researchers were able to negatively affect social interactions during withdrawal (Overstreet et al., 2002). Withdrawal from ethanol is considered to be a main driver of abuse and relapse (Roberts et al., 2000, Barkley-Levinson et al., 2012). One way to model the repeated cycles of withdrawal and binging seen in alcoholics is Chronic Intermittent Ethanol (CIE) vapor exposure. CIE forces ethanol dependence in exposed animals, where this dependence is seen as increased preference for ethanol (Roberts et al., 1996, Roberts et al., 2000). This dependence is achieved by, over the course of weeks, exposing the animals to extended periods of both ethanol vapor and regular air. Over the time course blood alcohol levels (BALs) in the animals increase, matching the BALs one would see in a human with AUD (Richardson et al., 2008). Once at target BALs, animals exhibit behaviors associated with alcohol dependence such as increased free choice ethanol drinking as compared to non-alcoholic animals (Becker at al. 2004).

Previous work from our lab has shown that acute withdrawal from CIE results in dendritic hypertrophy and increases in spine densities in layer 2/3 pyramidal neurons in the PFC, and these neuronal adaptations correlate with reduced proliferation of oligodendrocyte progenitor cells (OPCs) and differentiation

OPCs into pre-myelinating and myelinating oligodendrocytes, in rats (Kim et al., 2014). Additional work from our lab has shown that the structural adaptations in layer 2/3 pyramidal neurons persist into protracted abstinence (20d from last CIE exposure). However, contrary to the persistent effects of acute withdrawal and protracted abstinence on structural plasticity, rats that underwent protracted abstinence showed increased levels of OPCs and phosphorylated Olig2, a marker for myelinating oligodendrocytes (Navarro et al., 2016, Somkuwar et al., 2016). These findings indicate distinct alterations in neuronal and glial plasticity during acute withdrawal and protracted abstinence in CIE rats and suggest that evaluation of these factors during early abstinence from CIE (days of abstinence) may indicate a mechanism regulating these distinct differences during protracted abstinence.

In addition to the neuronal and glial adaptations during acute withdrawal and protracted abstinence, recent studies have examined the effect of abstinence on behaviors dependent on the PFC. For example, besides executive control and memory synthesis in impulsivity and decision making, the PFC is involved in encoding fear memories (Morgan et al., 1993). Long term storage of trace fear memory is seen in the PFC, where cue is what drives response rather than the context (Runyan et al., 2004). Rats with impaired PFCs are able to acquire cue and context as well as non-impaired animals during TFC training, but were unable to extinguish the fear memory during extinction (Morgan et al., 1993). Changes in the PFC have been seen in ethanol exposed brains (Holmes et al., 2012), and these changes may be implicated in cue driven drug seeking behavior (Koob and Volkow, 2010, Dayas et al., 2007). Mice that underwent CIE and delay fear conditioning (a

behavior that is shown to depend on proper functioning of the PFC (Quirk et al., 2000, Runyan et al., 2004, Heroux et al., 2017) showed no impairment in acquisition of fear memories, but did have reduced extinction of fear related cues compared to ethanol naïve controls. This deficiency is associated with increased dendritic length of neurons in the Prelimbic Cortex of the PFC. Electrophysiological measurements in these neurons during training showed firing times increased by 100-ms. Ethanol exposed mice had these firing times reduced during extinction, while control mice did not (Holmes et al., 2012). These behavioral deficits were observed during acute withdrawal. Changes in neuronal and glial plasticity have been seen during both acute withdrawal and protracted abstinence, but behavioral changes have only been investigated after acute withdrawal. By looking at behavioral changes during early abstinence, we hope to see if these early changes persist and if they can be correlated with any changes in plasticity.

In addition to studying the abstinence-induced effects on OPCs, structural plasticity of PFC neurons and behavior dependent on the PFC, biochemical studies have identified molecular adaptations that could regulate plasticity and behavior during abstinence. A receptor associated closely with synaptic plasticity, learning, and memory is the glutamatergic N-methyl-D-aspartate receptor (NMDAR) also known as the GluN system (Ron D. and Wang J., 2009, Bliss et al., 1993). Though primarily activated by glutamate, calcium flow through the channel can be mediated by Mg^{2+} or Zn^{2+} in a voltage dependent manner (Paoletti et al., 2007). With respect to alcohol abuse, GluN receptors have been implicated in withdrawal, seeking and relapse (Krystal et al., 2003). Acute ethanol exposure has been shown to inhibit

GlkuN excitatory postsynaptic potentials (EPSPs) in multiple brain regions including the PFC (Ron D. and Wang J., 2009). Single and whole cell recordings in the cortex show that ethanol inhibits NMDARs in a non-competitive manner at varying concentrations (Wright et al., 1996). In addition to suppressing receptor response time, alcohol has been shown to increase GluN2B, an NMDAR subtype, expression via phosphorylation of a tyrosine residue by Fyn (Miyakawa et al., 1997). Interestingly, in mice that were Fyn deficient, NMDARs did not develop tolerance to ethanol inhibition, suggesting that NMDAR ethanol tolerance (defined as reduced sensitivity to EtOH inhibition) is mediated by Fyn activity (Miyakawa et al., 1997). Chronic ethanol exposure, contrary to acute exposure, increases NMDAR spike timing in rat PFC as well as inducing structural changes to NMDAR expressing neurons (Kroener et al., 2012). Additionally, work in our lab has shown that in the PFC acute withdrawal increased total NMDAR expression, while protracted abstinence induced hypo-phosphorylation of the GluN2B subunit (Kim et al., 2014, Navarro and Mandyam, 2015). NMDAR activity is also crucial in the formation of fear memories in various brain regions (Davis, 2011). In particular, inhibition of NMDARs within the amygdala impaired extinction during training and retrieval (Baker et al., 1996, Dalton et al., 2008). In the mPFC, NMDAR activation seems necessary in order to consolidate extinction (Burgos-Robles et al., 2007). Blocking NMDAR activity in the mPFC during extinction training had no effect. Rather, deficits were seen in extinction retention (Burgos-Robles et al., 2007, Laurent et al., 2008). As previous work has shown that changes in NMDAR expression and activity have been seen in both acute withdrawal and protracted abstinence (Kim et al., 2014,

Navarro and Mandyam, 2015), it is not known how NMDAR expression behaves during early abstinence. Additionally, if there are any changes in expression, are these changes correlated with any changes in the extinction of fear memories.

The primary form of adult neurogenesis in the mPFC is gliogenesis (Arvidsson et al., 2002, Mandyam and Koob, 2012). Whether or not these newly formed glial cells replace damaged or dying cells is not clearly known. In the mPFC, most newly formed glial cells are of neuron-glia 2 positive (NG2+) type, known as oligodendrocyte progenitor cells (OPCs), as well as glial fibrillary acidic protein positive (GFAP+) astrocytes (Madsen et al., 2005, Mandyam and Koob, 2012, Somkuwar et al., 2014). NG2+ cells were seen to differentiate into myelinating oligodendrocytes (OLGs) but not astrocytes or neurons (Kang et al., 2010). As myelinating oligodendrocytes support healthy neurons, changes in myelin resulting from ethanol exposure and the resulting OLG response could be considered a form of plasticity. Previous research has shown that dependent drinking, but not non-dependent drinking reduced proliferation and survival of progenitor cells in the mPFC (Richardson et al., 2009). Our lab has also shown that during acute withdrawal from CIE, both OPCs and OLGs are reduced. This reduction in OLGs is indicated by increased phosphorylation of Olig2, an OLG associated transcription factor (Kim et al., 2014, Sun et al., 2011). During protracted abstinence OPC survival increased and Olig2 phosphorylation was reduced, indicating that more cells were differentiating into mature oligodendrocytes (Navarro and Mandyam, 2015). These data indicate that acute withdrawal and protracted abstinence have distinct effects on gliogenesis

and plasticity. Evaluating changes during early abstinence may elucidate a mechanism in how these differences in effect arise.

Taken together, findings from our lab show that acute withdrawal and protracted abstinence from CIE produce opposing changes in oligodendrocyte proliferation. It is unknown whether alterations in OPCs are evident during early abstinence, and what effect trace fear conditioning (TFC), in conjunction with abstinence, has on the fate of these cells. In addition, it is not known whether alterations in OPCs during early abstinence correlate with dendritic hypertrophy of pyramidal neurons and if TFC dysregulates their relationship. This project tested the hypothesis that early abstinence from CIE increases oligodendrocyte proliferation, and that exposure to TFC will reverse this process. Additionally, we examined the effect of dysregulated OPC proliferation as a result of abstinence and TFC on layer 2/3 pyramidal neurons. Immunohistochemistry was used to examine changes in OPC proliferation in the PFC. Golgi staining was used to determine structural changes in cortical neurons. Western blotting was used to analyze changes in proteins associated with neuronal and glial function

MATERIALS AND METHODS

ANIMALS

8-week-old male Wistar rats (n=38) (Charles River) were housed 2 per cage in a climate-controlled vivarium on a 12hr reverse light cycle (lights off at 8 am). Food and water was available *ad libitum*. All experimental procedures were carried out in strict adherence to the National Institutes of Health Guide for the Care and

Use of Laboratory Animals (NIH publication number 85–23, revised 1996) and approved by the Institutional Animal Care and Use Committee of The Scripps Research. (**Table 1**)

CHRONIC INTERMITTENT ETHANOL VAPOR EXPOSURE

During CIE, rat cages were housed in specialized chambers and exposed to a 14-h on 10-h off schedule for the alcohol vapors. Using a peristaltic pump (model QG-6, FMI Laboratory, Fluid Metering), 95% ethanol from a large reservoir was delivered to a heated flask at a regulated flow rate (95% ethanol vaporized at a drip rate of 2.5 - 4 milliliters per-minute for 14 hours a day followed by 10 hours of withdrawal). The vaporized ethanol was carried to the vapor chambers containing the rat cages by controlled air flow (regulated by a pressure gauge). The air and ethanol flow rates were optimized to result in blood alcohol levels (BALs) between 125 and 250 mg/dl of or 27.2 and 54.4 mM (Gilpin *et al.,*2008); these blood alcohol levels are roughly 2-3 times the BAL observed in binge drinking, but not high enough to abolish righting reflex (Ernst *et al.,*1976; Courtney & Polich, 2009). Additionally, intermittent exposure to ethanol vapor leads to higher self-administration than continuous exposure (O'Dell et al., 2004).

TAIL BLEEDING

Blood alcohol levels were sampled via weekly tail bleedings, in the 13th hour of the 14 hour cycle of ethanol vapor exposure. 5 µL blood plasma was measured for blood alcohol levels using an Analox AM 1 blood alcohol analyzer (Analox Instruments). The device measures the oxidation of alcohol by alcohol oxidase

($C_2H_5OH + O_2 \rightarrow C_2H_4O + H_2O_2$), with the consumption rate of oxygen directly proportional to the concentration of alcohol.

FEAR CONDITIONING

72 hours after last vapor exposure rats underwent trace fear conditioning training. Conditioning chambers (12.0"x9.0"x11.0", Med Associates) containing a house light and shock floor connected to a shock generator (Med Associates). A ventilation fan was used to isolate the chambers from external noise sources. During training animals were given 180 seconds to acclimate before being presented with a 30 second, 80dB tone, followed by a 45 second trace interval which terminated with a 1 second 0.5mA shock. The training was repeated for 5 cycles with a variable 20s-100s inter-trial interval (ITI) used to differentiate between trials. Context retrieval testing was conducted 24 hours after training. Animals were placed in the same context as conditioning and freezing was measured for three minutes for baseline behavior. The same 30 second 80dB tone used in conditioning was played followed by a 45s trace. Freezing in both training and testing was measured using video tracking software.

IMMUNOHISTOCHEMICAL ANALYSIS OF STEM-CELL PROLIFERATION

Medial prefrontal cortex sections 3.7 to 2.0 mm from bregma were mounted on Superfrost® Plus slides and dried overnight (Somkuwar *et al.,* 2016). Sections were incubated in antibody for Ki-67 (1:700, Rabbit polyclonal, Thermo Scientific) followed by biotin-tagged secondary antibodies and visualized with DAB. For Ki-67 analyses, all immunoreactive cells in the infralimbic, prelimbic and cingulate cortex

were counted. In addition to cell counting, area measures of the medial prefrontal cortex were also determined for each section for each animal using StereoInvestigator software (MBF), and the raw cell counts per section per animal were divided by the area of the prefrontal cortex and are indicated as cells per mm2 of the mPFC per animal.

WESTERN BLOTTING

Procedures optimized for measuring neuronal levels of both phosphoproteins and

total proteins were employed. All animals were sacrificed 96 hours after the last vapor exposure via rapid decapitation under light isofluorane anesthesia. Brains were quickly removed and flash-frozen in isopentane. Brain tissue was cut at the mid-sagital axis and one hemisphere was processed for Western blotting. Tissue punches from 500 µm thick PFC sections were homogenized for two minutes on ice by a bullet blender (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), heated at 100ºC for five minutes, and stored at -80°C until determination of protein concentration (via Bradford). Samples were mixed (1:1) with a Laemmli sample buffer containing β-mercaptoethanol. Each sample containing protein from one animal was run (30 µg per lane) on an 8-12% SDS-PAGE gel (Bio-Rad) and transferred to polyvinylidene fluoride membranes (PVDF pore size 0.2 µm). Blots were blocked with 5% milk (w/v) in TBST (25 mM Tris-HCl (pH 7.4), 150 mM NaCl and 0.1% Tween 20 (v/v)) for 16-20 h at 4°C and were incubated with the primary antibody for 24 hours at 4°C: antibody total GluN2A (1:500, Santa Cruz

Biotechnology cat. no. sc-9057, predicted molecular weight 178 kDa, observed band ~180 kDa), antibody to phosphorylated GluN2A (1:200, Cell Signaling cat. no. 4208S, predicted molecular weight 190 kDa, observed band ~190 kDa), antibody to oligodendrocyte lineage transcription factor 2 (Olig2; 1:100, generous gift from Drs. Charles Stiles and John Alberta, Harvard University, predicted molecular weight 37 kDa (Ligon et al., 2006), observed band ~37 kDa), antibody to myelin basic protein (MBP) (1:500, Abcam, cat. no. ab40390, predicted band size 18-23 kDa, observed band ~20kDa), antibody to myelin oligodendrocyte glycoprotein (MOG: 1:100 Abcam, cat no. ab32760, predicted band size 27 kDa, observed band size ~25 kDa).

Blots were then washed three times at 5min per wash in TBST, and then incubated for 1 h at room temperature (24 °C), appropriately with either horseradish peroxide–conjugated goat antibody to rabbit IgG1 (1:10,000, BioRad) or horseradish peroxide–conjugated goat antibody to mouse IgG1 (1:10,000, BioRad) in TBST. After another three washes for 5 minutes per wash with TBST, immunoreactivity was detected using SuperSignal West Dura chemiluminescence detection reagent (Thermo-Scientific) and visualized using HyBlot CL Autoradiography film (Denville Scientific) and developed. Net intensity values of the bands were determined using ImageJ (Research Service Branch, NIH) denisometric software. Following chemiluminescence detection, blots of protein outside of our molecular weights of interest were stained by Coomassie and analyzed in ImageJ for normalization purposes. 2-way ANOVA (analysis of variance) was used to compare values between groups.

GOLGI STAINING AND SHOLL ANALYSIS of DENDRITIC BRANCHING

To determine the effect of withdrawal and protracted abstinence from alcohol and trace fear conditioning on cortical pyramidal neuronal architecture, a group of vapor exposed fear-conditioned rats (n=4), vapor exposed non-fear conditioned rats (n=3), non-vapor exposed and fear-conditioned rats (n=4), and non-vapor exposed non-fear conditioned rats (controls, n=5) were killed by rapid decapitation under isofluorane anesthesia 96hrs after the last vapor exposure. The brains were quickly removed and cut mid-sagittal. One hemisphere for all experimental groups was then processed for Golgi-Cox staining. The other hemisphere was used for Western blotting analysis and immunohistochemistry. For Golgi-Cox staining, the brain was submerged in Golgi-Cox solution A+B (FD Neurotechnologies Inc.) for 8 days at room temperature, followed by solution C for 4 days at room temperature and stored at -80°C until processed for staining. The frozen brain tissue was then coronally cut on a cryostat to produce 100 µm-thick sections, which were then and stained with solution D+E and dehydrated according to manufacturer's instructions.

To evaluate pyramidal neuron morphology on the Golig-Cox stained sections, a Zeiss Axiophot microscope and a computer-based system (Neurolucida; MicroBrightField) was used to generate three-dimensional neuron tracings that were subsequently visualized and analyzed using NeuroExplorer (MicroBrightField). In order for a neuron to be selected for tracing and analysis the following four criteria were met: (1) the pyramidal neuron was in layer 2/3 of the medial prefrontal cortex, (2) the pyramidal neuron was distinct enough from other pyramidal and

interneurons to allow for identification of dendrites, (3) the pyramidal neuron was not broken, and (4) the pyramidal neuron exhibited dark, well filled staining throughout (Gould et al., 1990).

For each animal, 4 to 6 pyramidal neurons in layer 2/3 of the medial prefrontal cortex (3.7 to 2.0 mm from bregma; infralimbic and prelimbic cortices were combined) were traced with a 20x magnification lens (combined with a 10x eye piece). Both the apical and basal trees were traced, and morphological measurements were analyzed separately. For each reconstructed neuron, an estimate of dendritic complexity was obtained using the Sholl ring method (**Figure 3, a-c**). A 3D Sholl analysis was performed in which concentric spheres of increasing radius, in 20µm increments, were layered around the cell body until dendrites were completely encompassed. The number of dendritic intersections at each increment was counted, and results were expressed as total intersections and the number of intersections per radial distance from the soma.

STATISTICAL ANALYSIS

Changes in TFC acquisition was assessed as repeated measures two-way ANOVA (TFC session x treatment). Changes in fear retrieval was analyzed by one-way ANOVA. Cell counts for each marker (expressed as positive cells per mm^2) were analyzed by two-way ANOVA (EtOH vapor exposure x fear conditioning) or by Students-*t*-test. Significant interaction or ANOVA was followed by post-hoc analysis using Tukey HSD multiple comparisons test. All graphs and statistical analysis were

generated using Graph Pad version 6 for PC and $p<0.05$ was considered statistically significant.

RESULTS

BLOOD ALCOHOL LEVELS

BALs increased linearly with alcohol flow rates and remained within acceptable ranges (125-250 mg/dL) over the 7-week vapor exposure. Desired BALs were reached by week 3 of CIE experience. One-way AONVA shows a significant increase in BALs after week 2 ($F (6, 77) = 197.8$F, $P < 0.0001$). (**Figure 1**) CIE produces reliable and high BALs over 7 weeks of vapor exposure.

FEAR CONDITIONING

During trace fear conditioning acquisition, no significant difference in freezing was seen between CIE and control rats. During context retrieval (combined context and cue), ethanol exposed rats froze less than their non-vapor exposed counterparts. (**Figure 2**), indicating that ethanol vapor impairs the recall of fear memories in cue and context retrieval.

SHOLL ANALYSIS

Apical Dendrites: Under the condition of TFC 2-way ANOVA shows significant effect of distance ($F (30, 900) = 30.83$, $P < 0.0001$) and of of CIE ($F (1, 30) = 6.071$, $P = 0.0197$), as well as a significant interaction ($F (90, 2100) = 4.263$, $P < 0.0001$). Both CIE and FC increased dendritic branching compared to controls. (**Figure 3d**)

Basal dendrites: Under the condition of TFC 2-way ANOVA shows significant effect of distance ($F(13, 390) = 94.66$, $P < 0.0001$), significant effect of CIE ($F(1, 30) = 4.297$, $P = 0.0469$), and a significant interaction (CIE vs distance from soma) ($F(13, 390) = 3.893$, $P < 0.0001$), with ethanol exposed animals showing greater branching. Post-hoc analysis indicates a greater number of intersections between 30um and 90um from the soma ($p<0.01$, for 90um $p<0.05$), which indicates increased arborization closer to the soma. (**Figure 3e**) CIE induces hypertrophy in both basal and apical dendrites, while TFC only increases branching in apical dendrites. TFC does not increase synaptic branching in CIE animals.

SYNAPTIC PLASTICITY

Western blots probing from synaptic plasticity markers total and phosphorylated GluN2A, total and phosphorylated GluN2B and PSD-95 were ran to determine the effect of TFC and CIE on the mPFC. There was an effect of CIE ($F(1, 34) = 5.398$, $P = 0.0263$) and of shock ($F(1, 34) = 5.398$, $P = 0.0263$) on total NR2A levels. Compared to controls, CIE increased tNR2A expression. Foot shock reduced total NR2A levels in animals that experienced CIE and those that did not. Between experimental conditions, there was no significant difference in phosphorylated NR2A, or phosphorylated and total NR2B levels. (**Figure 4 a,b**) CIE increases expression of glutamate receptors, while TFC modulates this effect.

PSD-95, a scaffolding protein, was reduced in animals that received foot shock ($F(1, 34) = 5.293$, $P = 0.0277$). This was observed in rats that both did and did not experience CIE. (**Figure 4 c,d**)

GLIAL AND MYELIN PLASTICITY

Western blots probing for MOG, MBP, total and phosphorylated Olig2 were ran to determine the effect of CIE and TFC on the mPFC. 2-way ANOVA shows total levels of Olig2 are significantly increased by TFC ($F(1, 32) = 4.759$, $P = 0.0366$). (**Figure 5 a,b**)

MBP levels were significantly increased by foot shock ($F(1, 33) = 9.862$, $P = 0.0035$) compared to animals that did not experience shock. There is near interaction between CIE and foot shock ($F(1, 33) = 3.892$, $P = 0.0569$), but it does not reach significance. (**Figure 5 c,d**)

There was no significant effect of TFC or ethanol withdrawal on levels of phosphorylated Olig2 or MOG. CIE decreases the expression of glial and myelin associated markers, with TFC reversing this effect.

PROLIFERATION OF GLIAL PROGENITOR CELLS

2-way ANOVA showed no significant effect of CIE or shock but showed a significant interaction (CIE vs TFC) ($F(1, 23) = 7.407$, $P = 0.0122$) on the number of Ki-67 immunoractive cells. Student's t-test showed a significant decrease in Ki-67 counts in animals that underwent both CIE and TFC ($p=0.0188$) compared to those that only experienced CIE and compared to rats that only experienced TFC ($p=0.0206$). (**Figure 6a**) CIE increases expression of glial progenitors, TFC alone has no effect, but TFC with CIE normalizes the expression of these cells.

GLUCOCORTICOID RECEPTORS

CIE and TFC had no significant effect on total or phosphorylated levels of glucocorticoid receptors in the mPFC

DISCUSSION

The goal of this study was to investigate the effect of trace fear conditioning and protracted abstinence from alcohol exposure on neuron structure and oligodendrocyte proliferation in the mPFC. Disruption to the mPFC via inactivation and uncontrollable stress has been shown to degrade the ability of mice and rats to extinguish fear memories (Sierra-Mercado et al., 2011, Izquierdo et al., 2006). Specifically, protracted abstinence from ethanol after CIE has been shown to have this same effect (Holmes et al., 2012). The animals in our study showed deficits in retrieval of fear memories during early abstinence, showing that behavioral deficits can occur relatively early in withdrawal from chronic ethanol exposure.

Our study looked at withdrawal 24hrs after the last CIE exposure and shows that the changes observed during acute withdrawal, persist into early abstinence and continue into protracted abstinence. Changes in neuronal structure has been correlated with the behavioral deficits observed in rodents that were experiencing ethanol withdrawal (Holmes et al., 2012). In this study, increased dendritic branching was observed in both apical and basal dendrites in animals that experienced both CIE and TFC. Within basal dendrites, this increased arborization was due to ethanol vapor exposure; trace fear conditioning alone did not increase branching significantly compared to controls. In apical dendrites increased

branching due to both CIE and TFC is seen. CIE and TFC alone increase branching in apical dendrites, but there is no interaction between the conditions. Apical dendrites receive inputs from the basolateral amygdala (BLA), a region involved in the formation of fear memories (Gabbot et al., 2012, Hoover et al., 2007, Gale et al., 2004), thus the increased branching we observed in animals that experienced TFC may be the result of increased inputs from the BLA to the mPFC. In animals that experienced both CIE and TFC, the lack of increased branching may predict the behavioral deficits we saw in cue/context retrieval. The effect of ethanol exposure follows the trend previously seen in our lab showing that both acute withdrawal and protracted abstinence from chronic ethanol exposure increased dendritic length in the mPFC (Kim et al., 2014, Navarro and Mandyam, 2015). Modifications to dendritic structure are not necessarily required for differences in function (Lang et al., 2004). However, in the mPFC of rats experiencing ethanol withdrawal, pyramidal neurons with branching was showed changes in their electrophysiological properties, and these changes were correlated with decreased ability to extinguish fear memories (Holmes et al, 2012). Our study corroborates this structural and behavioral aspect of withdrawal from chronic ethanol exposure at an earlier time point.

Changes in NMDAR activity and expression is significantly affected by ethanol exposure and withdrawal (Carpenter-Hyland et al., 2004, Ron D. and Wang J., 2009, Kroener et al., 2012, Kim et al., 2014). For example, in the cortex, acute ethanol exposure inhibits NMDAR function (Wright et al., 1996, Wirkner et al., 2000, Yaka et al., 2003). NMDAR activity is also associated with neuronal plasticity and long-term potentiation (Bliss and Collingridge, 1993, Paoletti and Neyton 2006).

During chronic ethanol exposure and subsequent withdrawal, NMDARs are excessively active. This excessive activity leads to an influx of Ca2+ which is thought to be the cause of excitotoxic death (Lovinger 1993, Fada and Rossetti, 1998, Obernier et al., 2002). In fear conditioning NMARs are involved in extinction of fear memories (Santini et al., 2001, Suzuki et al. 2004). Specifically, NMDARs in the mPFC are required to consolidate extinction and abolishment of NMDAR activity in this region inhibits recall of extinction training (Burgos-Robles et al., 2007). Disruption of glutamate receptor activity in mice experiencing ethanol withdrawal was observed, where NMDARs containing the GluN1 subunit had increased spike times. This inhibition was correlated with deficits in fear memory extinction (Holmes et al., 2012). In this study total NR2A expression was increased due to CIE exposure, while expression of the post-synaptic scaffolding protein, PSD-95, was reduced because of TFC. Increased NR2A due to CIE parallels the hypertrophy observed in apical and basal dendrites in our study, providing a potential mechanism to explain what was observed. The increased dendritic branching observed in rodents during abstinence from CIE may be resulting from increased glutamatergic receptor expression (Holtmaat et al., 2009, Espinosa et al., 2009). In the animals undergoing ethanol withdrawal and, the increased expression of NMDARs may be driving this increased, potentially maladaptive, dendritic branching. It should be noted that while ethanol potentiates NMDAR activity in the hippocampus, this is not observed in the cortex (Yaka et al., 2003). The increased expression of NR2A follows what was seen with NR2B during acute withdrawal, further contributing to the idea that NMDAR dysregulation is associated with ethanol exposure and withdrawal.

Survival and proliferation of glial progenitors in the mPFC was shown to be reduced when exposed to ethanol (Richardson et al. 2009, Kim et al., 2014). As the mPFC has a role in the formation of drug seeking behavior (Koob and Volkow, 2010, Shaham et al., 2003), understanding how glia respond to ethanol in this region is critical. During acute withdrawal from ethanol, our lab has shown that proliferation and survival of oligodendrocyte progenitor cells (OPCs) is decreased in the mPFC. Olig2, a marker for pre-myelinating oligodendrocytes, was also shown to be reduced while its phosphorylated isoform was increased (Kim et al., 2014). This hyperphosphorylation is linked to reduced differentiation into mature myelinating oligodendrocytes (Sun et al., 2011). In protracted abstinence phosphorylated Olig2 was decreased, indicating that the OPCs in the mPFC were differentiating into mature oligodendrocytes. In parallel, MBP (associated with mature oligodendrocytes) increased as well (Navarro and Mandyam, 2015). In our current study OPCs were significantly decreased in animals that underwent CIE as compared to controls, indicating that the stem cell death we observe in acute withdrawal is occurring during early abstinence. Total Olig2 expression is reduced in early abstinent rats compared to ethanol naïve controls, however TFC normalizes Olig2 to levels comparable to control. There were no significant changes in phosphorylated Olig2, an indication that there is no change in OPC differentiation between experimental conditions. However, the early proliferative marker Ki-67 is increased in animals that experienced CIE, an effect opposite of what was seen during acute withdrawal. This increase in Ki-67 at the 4-day time point potentially predicts the rebound of myelin associated markers observed at the 21-day time point. Myelin

basic protein (MBP), a marker for mature oligodendrocytes, expression is reduced in early abstinent rats. TFC in conjunction with early withdrawal increases MBP expression significantly. When looking at these two markers together, during early abstinence, TFC seems to be upregulating the migration or survival of mature oligodendrocytes in the mPFC predicting deficits in fear memory formation.

Taken together, these data suggest that acute abstinence from alcohol coupled with TFC produces changes in the mPFC similar to what was observed during acute withdrawal. Morphological changes to apical dendrites in layer 2/3 pyramidal neurons observed in acute withdrawal persist into early abstinence (4 days), and these changes are also seen during protracted abstinence (21 days) with a normalization of basal dendrites. Accompanying this structural change was an increase in glial progenitors, but a decrease in glial and myelin associated markers. This indicates that the reductions to glial markers seen during acute withdrawal persist into early abstinence. The increase in proliferation of glial progenitor cells potentially predict the increase in glial and myelin associated markers seen during protracted abstinence. Animals that underwent CIE and TFC had deficits in fear memory retrieval, and these deficits are predicted by the reversals in protein expression seen in the CIE/TFC animals. This indicates that changes in the mPFC due to CIE occur early and persist long after the cessation of ethanol treatment, and these early changes in the mPFC due to CIE predict deficits in fear memory formation.

FIGURES AND TABLES

Table 1: Experimental groups and animal numbers.

Condition	Control	CIE
Control	No TFC/No CIE (n=10)	CIE (n=7)
TFC	TFC (n=10)	TFC/CIE (n=11)

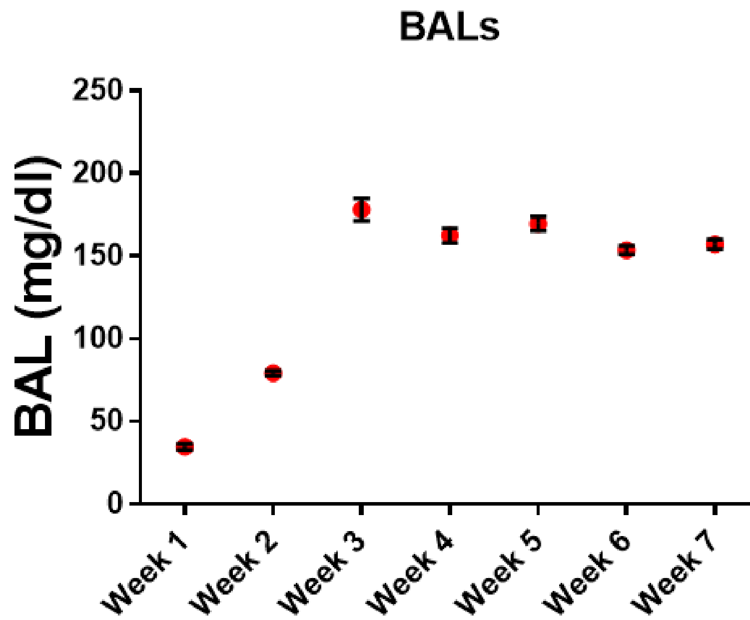


Figure 1: Blood alcohol levels (BALs),m in mg/dL, measured via tail bleeding over seven weeks of CIE

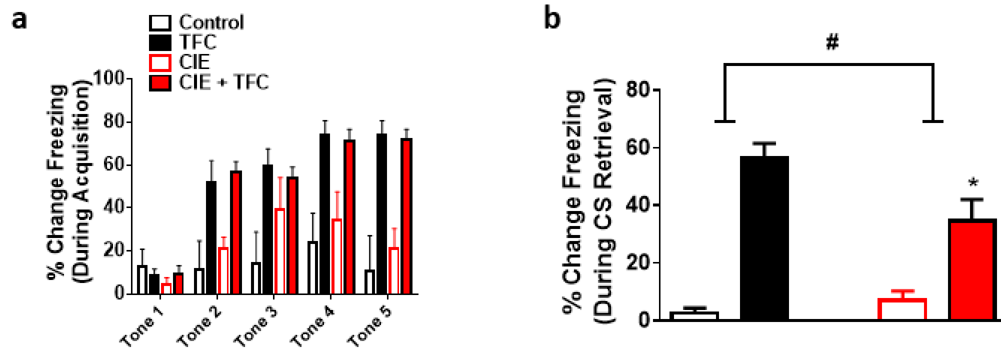


Figure 2: Trace fear conditioning (a) training at 72hrs after last CIE and (b) context retrieval 24hrs after training. #, main effect of TFC; *, different from non-CIE

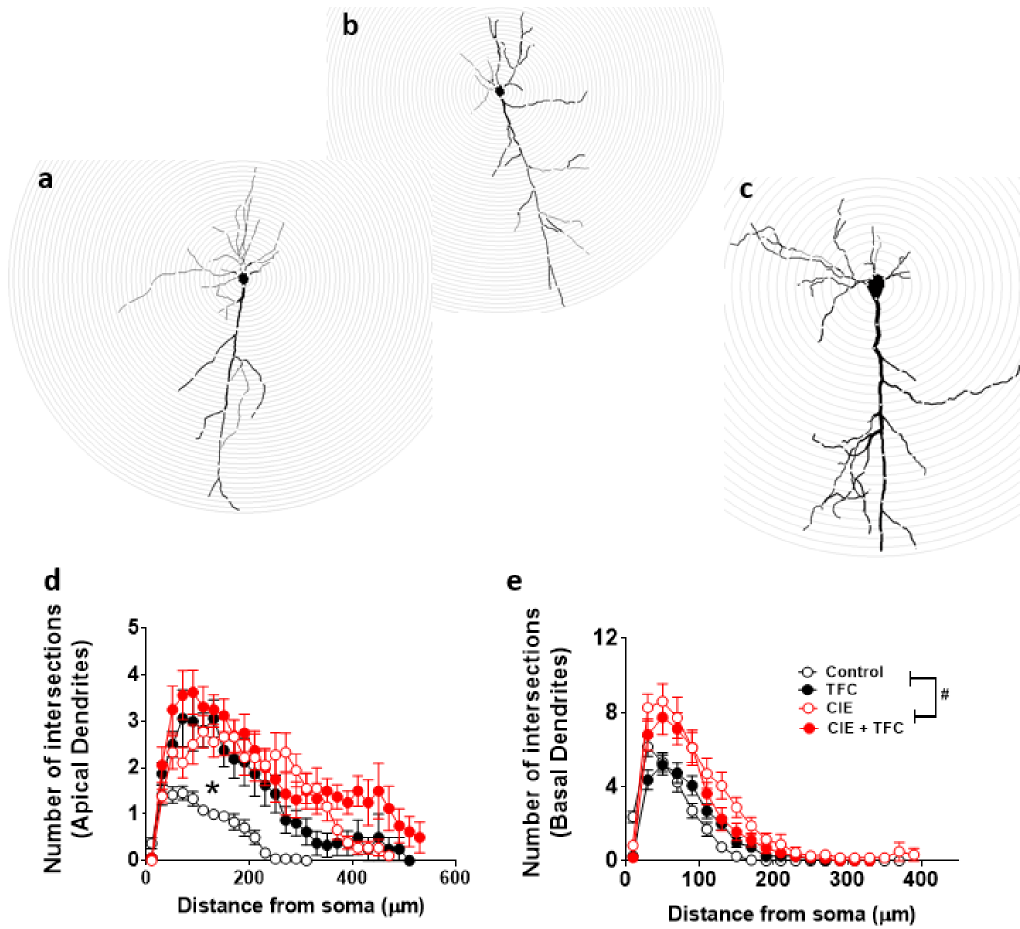


Figure 3: Dendritic arbor in layer 2/3 pyramidal neurons. (a-c) Representative tracings from (a) CIE/TFC, (b) TFC, and (c) CIE animals. Each concentric ring is 10μm. (d-e) Total dendritic intersections from control (n=), TFC (n=4), CIE (n=3), and CIE/TFC (n=4) in (d) apical and (e) basal dendrites. *, Different from all groups; #, main effect of CIE

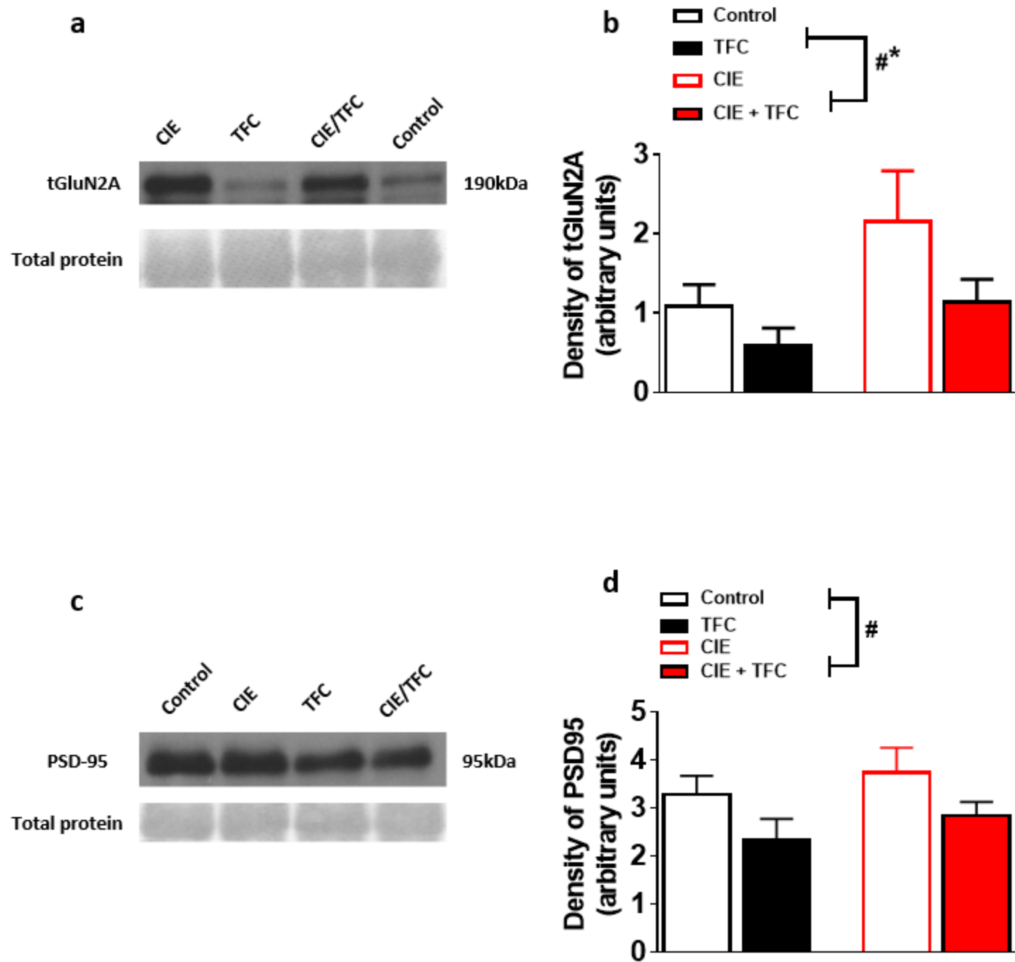


Figure 4: (a-d) TFC differentially affects expression of synaptic plasticity proteins. (a) Representative immunoblots of total GluN2A compared with Coomassie total protein staining. (b) CIE increases expression of total GluN2a, while TFC reverses this effect. (c) Representative immunoblots of PSD-95 compared with Coomassie total protein staining. (d) TFC decreases expression of the post synaptic scaffolding protein, PSD-95. #, main effect of TFC; *, main effect of CIE

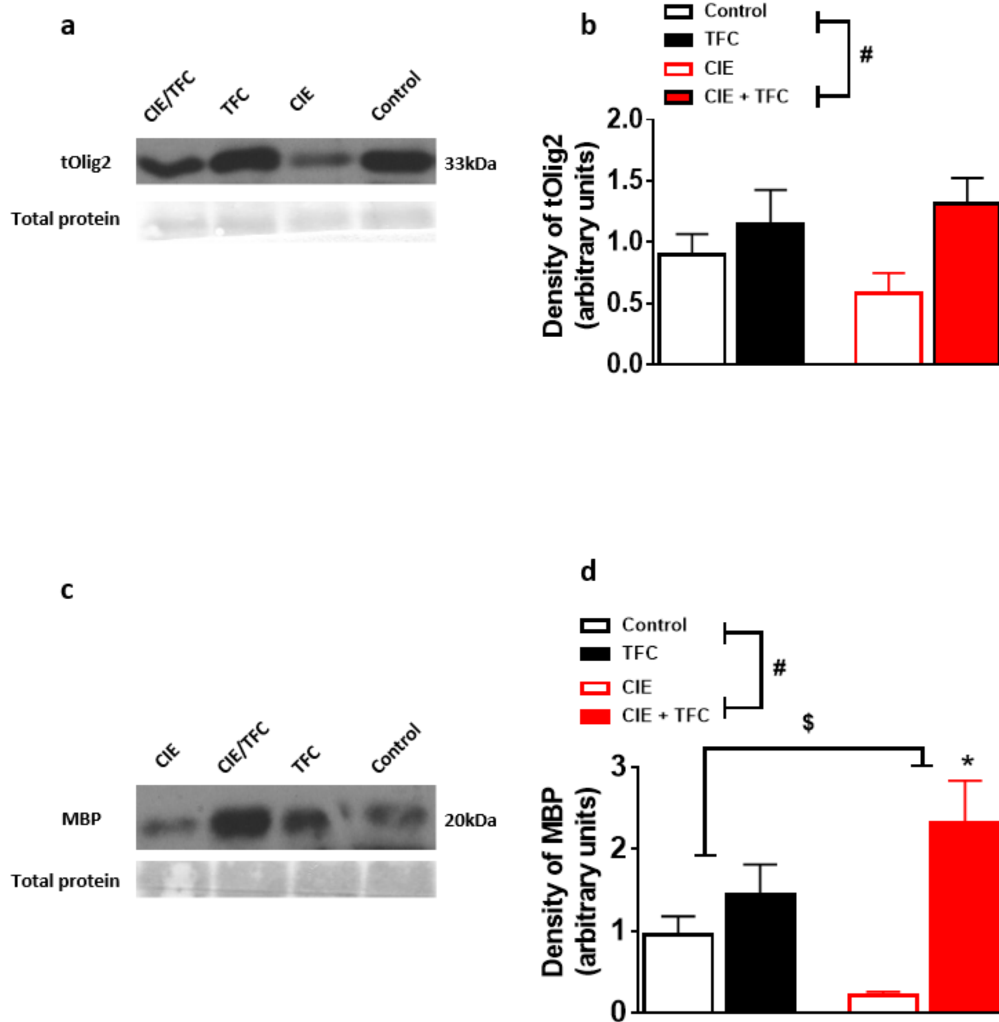
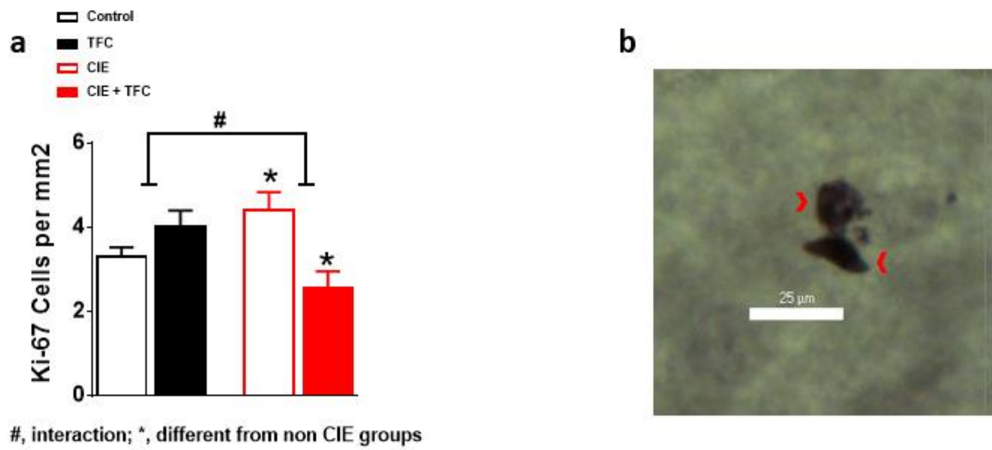


Figure 5: (a-d) Expression of oligodendroglial proteins in CIE rats is increased by TFC. (a) Representative immunoblots of total Olig2 compared with Coomassie total protein staining. (b) CIE reduces Olig2 expression while CIE reverses this change. (c) Representative immunoblots of MBP compared with Coomassie total protein staining. (d) CIE alone decreases levels of MBP, and when coupled with TFC abolishes this effect. #, main effect of shock; $, interaction; *, different from CIE



c

Interaural 12.20 mm

Bregma 3.20 mm

Figure 6: TFC produces opposing effects on Ki-67 labelled proliferating cells in CIE rats. (a) CIE increases expression of Ki-67 labelled oligodendroglial progenitors, TFC has no effect, CIE/TFC reduces progenitor survival. (b) Photomicrograph of immunostained Ki-67 cells in rat mPFC, indicated by red chevrons. (c) Adapted from Paxinos George and Watson *The rat brain in stereotaxic coordinate,* depicting in red the region where cell counting occurred.

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