UNIVERSITIY OF CALIFORNIA SAN DIEGO

Endostatin reduces relapse to ethanol seeking in dependent rats: Regulation by PECAM-1 and oligodendrocytes

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in

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by

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The Thesis of Nancy Yuanyuan Xing 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

2020

DEDICATIONS

I want to dedicate my thesis to my family members for their unconditional support, care, and encouragement.

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ABBREVIATIONS

AUD	Alcohol Use Disorder
PECAM-1	Platelet Endothelial Cell Adhesion Molecule - 1
OPCs	Oligodendrocyte Progenitor Cells
OLGs	Oligodendrocytes
ED	Ethanol Drinking
CIE	Chronic Intermittent Ethanol
CaMKII	Ca^{2+}/calmodulin-dependent Protein Kinase II
pCaMKII	Phosphorylated Ca^{2+}/calmodulin-dependent Protein Kinase II
tCaMKII	Total Ca^{2+}/calmodulin-dependent Protein Kinase II
BAL	Blood Alcohol Level

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ABSTRACT OF THE THESIS

Endostatin reduces relapse to ethanol seeking in dependent rats: Regulation by PECAM-1 and oligodendrocytes

by

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Master of Science in Biology

University of California San Diego, 2020

Professor Chitra D. Mandyam, Chair
Professor Cory Root, Co-Chair

Alcohol use disorder (AUD) produces a variety of mental damage; AUD is a serious public health issue. Current FDA approved medications to treat AUD are partially effective, warranting the need for better therapies. Animal models of AUD are currently used to discover new therapies due to their robust face and predictive validity. Previous studies suggested that rats with AUD had higher ethanol seeking behavior during abstinence that correlated with enhanced expression of platelet endothelial cell adhesion molecules (PECAM-1; angiogenesis marker), increased number of oligodendrocyte progenitor cells (OPCs) and altered neuronal activation in medial prefrontal cortex (mPFC). However, it is unclear whether PECAM-1 directly promotes ethanol seeking behavior by reducing neuronal activity and increasing proliferating OPCs. Thus, to investigate the role of PECAM-1 in alcohol-dependent rats, we

used endostatin, a broad-spectrum angiogenesis/PECAM-1 inhibitor. Results from behavior studies showed that in alcohol-dependent female rats, endostatin significantly reduced alcohol seeking during relapse. Postmortem tissue analysis using quantitative immunohistochemistry in the mPFC demonstrated that reduced seeking correlated with reduced PECAM-1, OPCs and neuronal activation. Western blotting analysis revealed that endostatin enhanced the activity of calcium calmodulin dependent kinase II (CaMKII), which could assist with normalizing the altered neuronal activity during abstinence. In conclusion, our analyses confirmed that the angiogenesis of PECAM-1 is responsible for enhancing ethanol seeking behavior by altering neuronal activation and oligodendrogenesis in female rat mPFC. Our research provided a possible mechanism to inhibit ethanol seeking and prevent the glial and neuronal damage caused by increased accumulation of PECAM-1 from alcohol drinking.

INTRODUCTION

1.1 Alcohol Use Disorder

Alcohol use disorder (AUD), or more commonly known as alcoholism, is defined as the inability to control both the physical and mental intention to drink, with obvious signs of negative emotional state when alcohol is inaccessible (Koob et al., 2016). About 20 million people in America have met the criteria for AUD (Traphagen et al., 2015); alcohol consumption is responsible for more than 85,000 deaths (3.5%) in the country, being the third leading cause of death (Mokdad et al., 2000). Alcohol abuse is responsible for heart disease, and dysfunctions in major organs such as liver, kidney, and lung (Traphagen et al., 2015, Munukutla et al., 2016). Kovacic and his colleagues examined the molecular basis of alcohol toxicity and revealed that alcohol induces oxidative stress (OS) and the production of reactive oxygen species (ROS) such as hydrogen peroxide and hydroperoxides. Notably, oxidative stress is resulted from the byproducts such as acetaldehyde, produced during alcohol metabolism and is directly linked to toxicity in the major organs of alcohol consumers (Kovacic et al., 2005).

Alcohol-induced oxidative stress is associated with altered function in the brain, particularly the hippocampus, corpus callosum and prefrontal cortex, with altering neuronal and glial plasticity (Mandyam et al., 2017). Alteration in the structure and function of prefrontal cortex impairs decision-making, which persists during protracted abstinence (Berre et al., 2014). This interference then possibly disrupts neurocircuitry such as the mesolimbic dopamine system, and dorsolateral frontal cortex/inferior frontal cortex/hippocampus circuits, contributing to chronic alcohol relapse (Koob and Volkow, 2010; Seo et al., 2013; Stephens and Duka 2008; Berre et al., 2014). More specifically, abstinence from alcohol reduces cognitive flexibility such as reversal learning in mice medial prefrontal cortex (mPFC), a functional area located in the frontal lobe of the brain (Badanich et al., 2011). The mPFC was found to control motivation instincts such as reward and alcohol seeking (Somkuwar et al.,2016). Studies used multi-electrode recording methods found that mPFC controls reward-related activities and consummatory behaviors (Horst, N. K., & Laubach, M. 2013); over-activity of mPFC may cause weakened control over

motivated behaviors, causing alcohol relapse (George et al., 2012). Accordingly, investigating mPFC allows us to study its role in facilitating reward seeking behaviors such as alcohol seeking and in intensifying propensity for alcohol relapse. A therapy to prevent or even reverse the impact of alcohol on people who suffer from AUD is revolutionary and it may be achieved by manipulating the specific pathways that were essential in causing alteration in the human brain.

1.2 The Chronic Intermittent Ethanol (CIE) Vapor Inhalation Model in Rats

Rodent models are excellent for studying neurological disorders because their behaviors are very close to that seen in humans (Vertes, 2006). Rats are well suited for AUD studies because alcohol dependent rats drink alcohol compulsively and express anxiety, comparable to human alcoholics (Roberts et al., 2000; Overstreet et al., 2002). Studies using rodent models for alcohol studies often conditioned rats with alcohol vapor to induce behavior of alcohol dependence, in a process called chronic intermittent ethanol (CIE) exposure (Gilpin et al., 2008). Rats are kept in alcohol chambers during chronic intermittent ethanol exposure, breathing ethanol vapor on a fixed schedule to establish voluntary drinking behavior, mimicking that of alcohol dependence. Once the rats adapt to alcohol dependence through CIE, they can also be used to measure relapse susceptibility during acute withdrawal and protracted abstinence. (Gilpin et al., 2008).

1.3 Sex Differences in Rodents Mimics the Gender Differences in Human in Drinking Behavior

Another important aspect of AUD is that there are significant sex and gender differences. Female Long Evans rats were used in this study. Female Long Evans rats tend to drink more than male rats because the rewarding effects of ethanol are enhanced in female rats due to higher number operant lever presses (Torres et al., 2014). Found previously, some ovarian hormones in females has also been correlated with enhanced ethanol seeking behavior, indicating that the increased ethanol seeking behavior in females might be associated with increased sensitivity to the enhancing effects of ethanol (Torres et al., 2014). Moreover, studies revealed that female rat's gastric alcohol dehydrogenase activity is increased,

allowing for faster alcohol metabolism and higher blood alcohol levels (Mezey et al., 1992). Enhanced activity of alcohol dehydrogenase activity allows female rats have faster absorption, distribution, and elimination of ethanol in the blood and brain, compared to the male rats (Priddy *et al*., 2017). Furthermore, female rats have less motivational withdrawal to alcohol during reinstatement and maintain ethanol drinking during relapse (Becker & Koob, 2016). Therefore, using female rats in AUD studies provide another scope to understanding differences in drinking behavior.

1.4 Blood Brain Barrier and Neuroinflammation

AUD in rats showed that chronic ethanol experience reduces blood-brain barrier (BBB) integrity (Somkuwar et al., 2017) and induces neuroinflammation in rats (Alikunju et al., 2011). Blood-brain barrier is a layer of endothelial cells that separates the brain from cells, proteins, and other agents in the blood (Baeten et al., 2011). Oxidative stress resulted from alcohol metabolism injures the microvessels on the BBB, causing persistent neuroinflammation and neurodegeneration; inflammatory responses occur immediately upon injury with recruitment of immune cells such as leukocytes to eliminate foreign antigens (Alikunju et al., 2011). Additionally, oxidative stress is often responsible for causing neuronal dysfunctions and neurological diseases such as Alzheimer and Parkinson (Alikunju et al., 2011).

1.5 Platelet Endothelial Cell Adhesion Molecule - 1 (PECAM-1)

Alcohol has been found to facilitate the production of cytokines in major organs such as livers, lungs, and brain (Alikunju et al., 2011); in addition, cytokine mediates the production of platelet endothelial cell adhesion molecules (PECAM-1). PECAM-1 is expressed on cerebral endothelial cells that construct the blood-brain barrier, mediating the interaction between endothelium and immune cells (Mandyam et al., 2017). PECAM-1 is pro-inflammatory and plays a role in recruiting white blood cells, such as leukocytes, to the injury site as response to brain injury and/or stress (Privratsky et al., 2010). Previous study elucidated that during abstinence from drinking, the expression of PECAM-1 in rat mPFC was positively correlated with reduced blood-brain barrier integrity, indicating the role of PECAM-1 in disrupting BBB under

alcohol-mediated oxidative stress (Somkuwar et al 2017). Disruption of blood-brain barrier leads to an influx of substances in the blood such as cytokines, leukocytes and toxins that can cause persisting damage and inflammation to the brain (Haorah et al., 2005; Alikunju et al., 2011). Remarkably, PECAM-1 is also known for its angiogenic capability - forming new blood vessels in the brain to support the growth of neural progenitor cells such as oligodendrocyte progenitors (Arai et al., 2009). For instance, newly born oligodendrocytes flourished closely to PECAM-1 in mPFC, confirming PECAM-1's role in supporting the growth of oligodendrocyte progenitor cells (OPCs) (Somkuwar et al., 2017). Since few studies examined PECAM-1 in relation to alcohol, more studies are needed to verify and confirm PECAM-1's role in mediating neuroinflammation in AUDs; studying PECAM-1 may contribute to alleviate and prevent inflammatory responses in alcohol abusers.

1.6 Oligodendrogenesis, Oligodendrocytes (OLGs), and Oligodendrocyte Progenitor Cells (OPCs)

Oligodendrogenesis is a process in which oligodendrocytes (OLGs) are produced throughout the lifetime of adult mammals. Oligodendrocytes are mature glial cells and are constantly replenished in adult mammals while they later mature into myelinating OLGs, providing protective myelination for axons to speed up neuronal signaling (McTigue et al., 2008). Oligodendrocytes were differentiated from oligodendrocyte progenitor cells (OPCs) that mediate neuron-glial interactions in the brain through generating and receiving action potentials. Notably, the proliferation and survival of oligodendrocyte progenitor cells and oligodendrocytes is supported by the cerebrovascular system (Mandyam et al., 2017). As previously mentioned, oligodendrocyte progenitor cells (OPCs) and oligodendrocytes located in proximity to PECAM-1 in rat mPFC during abstinence, suggesting supportive role of endothelial cells in enhancing the proliferation of OPCs and oligodendrocytes (Somkuwar et al., 2017).

1.7 PECAM-1, Oligodendrocytes and Neuronal Function during CIE and Abstinence

Kim et al., 2014 examined oligodendrogenesis and proliferation of oligodendrocytes during chronic intermittent ethanol exposure (CIE) and abstinence from alcohol dependence. During chronic

intermittent ethanol exposure, newly born oligodendrocyte progenitors and their capacity to proliferate into oligodendrocytes in the mPFC is decreased, along with reduction of related functional proteins such as myelin basic protein (MBP) (Kim et al., 2014). MBP is essential in the development of myelination in oligodendrocytes and is expressed on pre-myelinating oligodendrocytes and myelinating oligodendrocytes (Somkuwar et al., 2016). Consequently, reduced MBP usually associates with reduced number of oligodendrocytes. Yet, it is unspecified if the reduction of myelin basic protein (MBP) persists into prolonged abstinence and withdrawal. In other words, whether the impact of alcohol toxicity on the proliferation of oligodendrocytes lingers through abstinence or alleviates upon the absence of alcohol exposure was still undetermined. Previous findings from our lab illustrated that, opposite from what was observed during chronic intermittent ethanol exposure (CIE), the proliferation of oligodendroglial progenitors cells (OPCs) and myelin basic protein (MBP) levels in the mPFC of dependent rats were remarkably enhanced after withdrawal, compared to non-dependent ethanol drinking rats, who were regular drinkers that were not exposed to ethanol vapor (Navarro and Mandyam, 2015). In turn, the significant increase in proliferation of OPCs, myelination and functional proteins MBP in rat mPFC after withdrawing from alcohol suggested that alcohol-mediated oxidative stress altered neuroplasticity and mPFC function. Moreover, enhanced ethanol drinking was observed in dependent male rats during abstinence, correlated with increased oligodendrogenesis, significantly lowered neuronal activation and enhanced expression of PECAM-1 in the rat mPFC (Somkuwar et al., 2016). The above findings implicated that PECAM-1 may disrupt mPFC by reducing neuronal function, resulting in uncontrolled impulses for alcohol drinking. The increase in PECAM-1 and oligodendrogenesis also confirmed the role of PECAM-1 in supporting proliferation of oligodendrocytes and its participation in the situation of oxidative stress. Furthermore, oligodendrogenesis and neuroinflammation also escalated during abstinence in the male rat mPFC; the escalation was greater in alcohol dependent rats than non-dependent rats (Somkuwar et al., 2016), suggesting abstinence from alcohol may contribute to increased neuroinflammation and oligodendrogenesis in response to greater alcohol-mediated oxidative stress. Overall, PECAM-1 may be regulating neuronal function and the proliferation of oligodendrocyte

progenitor cells in mPFC in response to alcohol-induced oxidative stress. Nonetheless, it remains unknown if increased PECAM-1 expression contributes to the propensity for relapse by directly altering neuronal activity and the proliferation of premyelinating oligodendrocyte progenitors in the media prefrontal cortex; more studies are needed to find out the relationship between PECAM-1, ethanol seeking, neuronal function and oligodendrogenesis in the previously explored male subjects and unexplored female subjects.

1.8 Research Objective: Using Endostatin to Alter PECAM-1 Expression

As a continuation to previous research from our lab, this study used the similar paradigm of chronic intermittent ethanol exposure (CIE) model as reported in Somkuwar et al., 2016 with the additional use of FDA approved broad-spectrum angiogenesis inhibitor endostatin during early withdrawal and abstinence. Previous research using endostatin on tumors found that endostatin could successfully inhibit angiogenesis by interfering with angiogenic factors such as the vascular endothelial growth factor (VEGF) (Jia et al., 2004). We hypothesized that endostatin will reverse the impact of PECAM-1 by preventing angiogenesis. By hindering angiogenesis, we would be able to discover if reversing the effect of PECAM-1 could restore the change in neuronal function and oligodendrogenesis during withdrawal and abstinence, associating with alcohol relapse in AUD models. My lab investigated the role of PECAM-1 in enhancing the propensity to alcohol relapse after withdrawal and protracted abstinence from chronic intermittent ethanol (CIE) exposure, a preclinical model of moderate to severe AUD. Moreover, most of previous AUD studies used male rats as little to nothing was one on female rats. Thus, this AUD study focused primarily on female rats, intending to provide another scope to alterations in glial plasticity and drinking behaviors in addition to findings from male rats. Data shows that endostatin reduced ethanol seeking in female rats by reducing PECAM-1 expression in the mPFC. In addition to studying neuroadaptations in AUD, this research investigated glial plasticity, that is, how glial cells were altered in AUD. Therefore, the aim of my thesis was to determine whether endostatin also regulated/normalized other glial and neuronal alterations in the mPFC. I used immunohistochemistry

followed by quantitative stereological analysis to investigate whether endostatin altered the expression of glial progenitor cells (ki67), oligodendrocytes (Olig-2) and neuronal activation (cFos) in the mPFC (Dragunow & Faull., 1989; Yokoo et al., 2004). We regarded cFos immunoreactive cells as cells showing neuronal activation and Olig2 immunoreactive cells as premyelinating oligodendrocyte progenitors. I used Western blotting analysis to determine whether endostatin altered the levels of plasticity-related proteins in the mPFC. My experimental design consisted of 6 groups, with one ethanol naïve group, one ethanol nondependent group and one ethanol dependent (CIE) group. Each group had two subgroups with one treated with vehicle and the other treated with endostatin. Two-way ANOVA and Post-hoc tests were performed to identify significant group interactions and group differences. Pearson's product-moment correlation coefficient was used to determine the relationship between PECAM-1, neuronal function, and proliferation of oligodendrocyte progenitor cells and to conclude whether endostatin was able to alter the change resulted by PECAM-1.

RESULTS

2.1 Enhanced Ethanol Drinking and Blood Alcohol Level (BAL) During Maintenance

Active lever responding for ethanol significantly differed before and after 23 days of maintenance in both CIE-ED and ED groups (CIE-ED: $t_{(45)} = 6.499$, $p < 0.0001$; Fig. 2a; ED: $t_{(29)} = 3.169$, $p = 0.0037$; Fig. 2a). Unpaired t-test was also used to examine if CIE-ED had higher responding compared to ED. CIE-ED and ED did not differ in drinking behavior before vapor ($p = 0.9562$; Fig. 2a) while CIE-ED drank significantly more than ED rats did after vapor ($p = 0041$; Fig. 2a). Only BALs in CIE-ED rats were measured and recorded: a significant increase in BAL was observed starting week 4 compared with week 1 with a main effect between weeks, and BAL was maintained between 200-300 g/dL in CIE-ED rats between weeks 4 and 7 ($F_{weeks}[6, 216]= 68.95$, $p < 0.0001$; Fig. 2d).

2.2 Drinking During Abstinence: Endostatin Reduced Relapse to Drinking Behavior in Dependent Rats

Following 23 days of abstinence, CIE-ED-endostatin rats demonstrated lower drinking (lower active lever responses) compared to CIE-ED-vehicle rats when they were given access to ethanol, ($t_{(16)} = 2.547$, $p = 0.0223$; Fig. 2b). However, drinking behavior did not differ between ED rats with or without endostatin treatment ($t_{(12)} = 0.1643$, $p = 0.8724$; Fig. 2b). Unpaired t-test revealed that CIE-ED-endostatin rats and ED-endostatin rats did not differ in drinking behavior ($p = 0.3943$, Fig. 2b) while CIE-ED-vehicle drank more compared to ED-vehicle ($p = 0.006$, Fig. 2b).

2.3 Active Lever Pressing During Contextual Cued Reinstatement of Ethanol Seeking

Following 6 days of extinction under a different context (context B), the rats were subject to contextual cued reinstatement (context A, where lever pressing is paired with no light but with no alcohol delivery during reinstatement session). CIE-ED-vehicle and ED-vehicle and ED-endostatin all had higher lever pressing during reinstatement than that on 6th day of extinction session (CIE-ED-saline: $t_{(18)} = 2.198$, $p = 0.0421$; ED-saline: $t_{(12)} = 4.508$, $p = 0.0009$; ED-endostatin: $t_{(12)} = 4.883$, $p = 0.0005$; Fig. 2c). CIE-

ED-endostatin did not show difference in active lever pressing between reinstatement and 6th day of extinction ($t_{(18)}$ = 1.283, p = 0.2258; Fig. 2c)

2.4 Endostatin Reduced PECAM-1 Expression in mPFC

Immunohistochemistry was used to determine quantitative changes in PECAM-1 expression in the mPFC. Two-way ANOVA of PECAM-1 showed interaction between treatments and conditions and increased expression in ED and CIE-ED rats ($F_{treatment\text{-}condition}[2, 58] = 7.861$, $p = 0.0010$; $F_{treatment}[1, 58] = 26.82$, $p < 0.0001$; $F_{condition}[2, 58] = 7.244$, $p = 0.0016$; Fig. 3). Post hoc analysis demonstrated that PECAM-1 expression was greatly enhanced in CIE-ED-saline rats compared to control- and ED-saline rats (CIE-ED-saline x ED-saline: $p<0.0001$; CIE-ED-saline x control-saline: $p<0.0001$; Fig. 3). Endostatin greatly reduced PECAM-1 expression in ED- and CIE-ED-endostatin groups, compared to that in vehicle groups, respectively (ED-vehicle x ED-endostatin: $p<0.0231$; CIE-ED-vehicle x CIE-ED-endostatin: $p<0.0001$; Fig. 3)

2.5 Proliferation of Oligodendrocyte Progenitor Cells (OPCs) in the mPFC

Newly born progenitors were labeled with Ki67 using rabbit polyclonal anti-ki-67 (1:1000, catalog # RM-9106-S, Thermo Scientific) and were quantified. While two-way ANOVA did not show interaction between treatments and conditions ($F_{treatment\text{-}condition}[2, 62] = 1.836$, $p = 0.1680$; Fig. 4), the number of Ki67 expressing cells were significantly lower in ED groups than that in control and CIE-ED groups, both with and without endostatin, showing a main effect of alcohol ($F_{condition}[2, 62] = 13.46$, $p<0.0001$; Fig. 4). Endostatin did not have a large impact as there was no significant difference in all three conditions between treatments ($F_{treatment}[1, 62] = 0.2355$, $p = 0.6292$; Fig. 4). Post-hoc analysis showed lower expression of Ki67 in ED-saline compared to CIE-ED-saline rats ($p = 0.0008$); lower expression of Ki67 in ED-endostatin compared to CIE-ED-endostatin rats ($p = 0.0006$).

2.6 Endostatin Reduced Number of Oligodendrocyte Markers in the mPFC in CIE-ED Rats

Two-way ANOVA showed significant interaction between treatments and conditions ($F_{treatment-condition}[2, 60] = 4.477$, $p = 0.0154$; Fig. 5) and main effect of alcohol ($F_{condition}[2, 60] = 4.048$, $p=0.0224$; Fig. 5). However, Post-hoc analysis demonstrated that the number of Olig2 expressing cells significantly decreased in CIE-ED-endostatin group, compared to CIE-ED-saline group ($p = 0.0014$). Moreover, not only did CIE-ED-saline rats had greatly increased expression of Olig2 compared to ED-saline rats ($p = 0.0009$), but also did CIE-ED-endostatin showed no significant difference in Olig2 expression from that in control-endostatin group ($p=0.6186$) (Fig. 5).

2.7 Endostatin Reversed Neuronal Hyperactivation in the Medial Prefrontal Cortex in CIE-ED Rats

Two-way ANOVA showed significant group differences in cFos expression in mPFC ($F_{condition}[2,59] = 4.926$, $p = 0.005$; $F_{treatment}[1,59] = 5.273$, $p = 0.0252$; Fig. 6) while showing minimal interaction between treatments and conditions ($F_{treatment-condition}[2,59] = 3.035$, $p = 0.0556$; Fig. 6). Post hoc analysis demonstrated that neuronal activation increased in CIE-ED-saline rats compared to control-saline rats ($p = 0.0065$), and endostatin greatly reduced neuronal activation in CIE-ED-endostatin rats compared to CIE-ED-saline rats ($p = 0.0007$). In addition, cFos expression in CIE-ED-endostatin rats was not statistically different from that in control-endostatin rats ($p = 0.4105$) (Fig. 6).

2.8 Biochemical Analysis of Plasticity-related Proteins Revealed Enhanced Expression of pCaMKII in CIE-ED-Endostatin Rats

Western blot analyses of plasticity-related proteins (pCaMKII, tCaMKII) were conducted on mPFC tissue lysates (Fig. 7a). Two-way ANOVA indicated interaction between protein expression and conditions and main effect of conditions ($F_{CaMKII-condition}[2, 59] = 4.527$, $p = 0.0148$; $F_{condition}[2, 59] = 7.931$, $p = 0.0009$; Fig. 7b). Showed more specifically by Post-hoc analyses, significant increase of pCaMKII was observed in CIE-ED-endostatin rats, compared to control-endostatin and CIE-ED-saline

rats (CIE-ED-endostatin x control-endostatin: $p = 0.0004$; CIE-ED-endostatin x ED-saline: $p < 0.0001$; Fig. 7b).

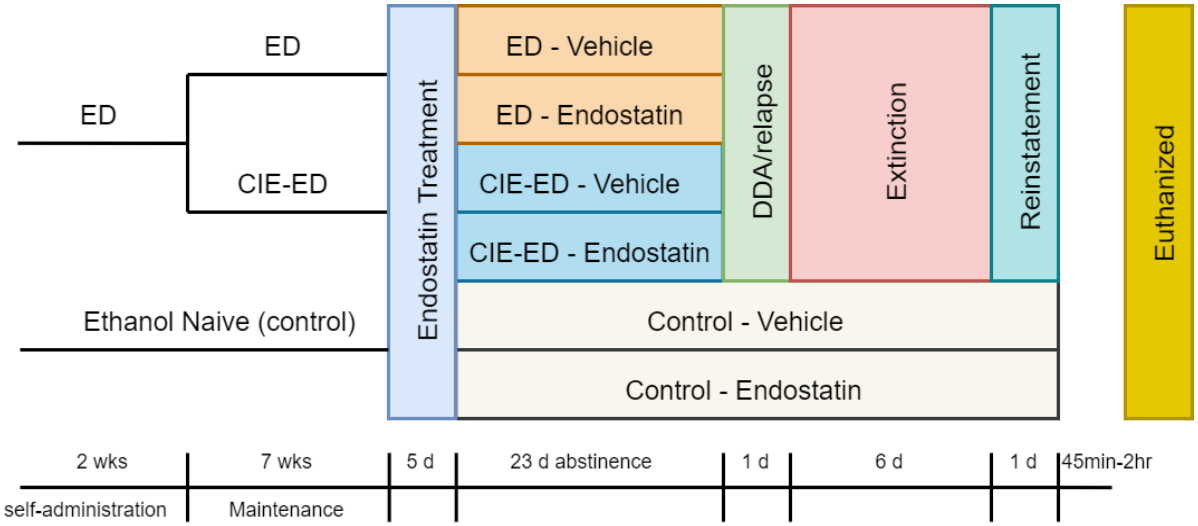

Figure 1. Schematic representation of entire experimental design
Animals were trained to self-administer ethanol for 2 weeks. They were then maintained to self-administer (in context A) ethanol for 7 weeks during which CIE-ED rats experienced ethanol vapors and ED rats experienced control air housing conditions. After 7 weeks, ED and CIE-ED rats were withdrawn from ethanol vapor or self-administration maintenance and were divided into two groups (vehicle or endostatin), to receive treatment. first injection was 2-3 hours after cessation of abstinence; the injection was given daily at the same time for both groups for 5 days. Vehicle rats received saline and endostatin rats received endostatin. After abstinence, rats were given one 30 min FR1 session to lever press for ethanol reinforcement, followed by 6 daily 30-min extinction sessions under a different cue-context combination (context B) and one session of cued-context reinstatement (context A) of ethanol seeking.

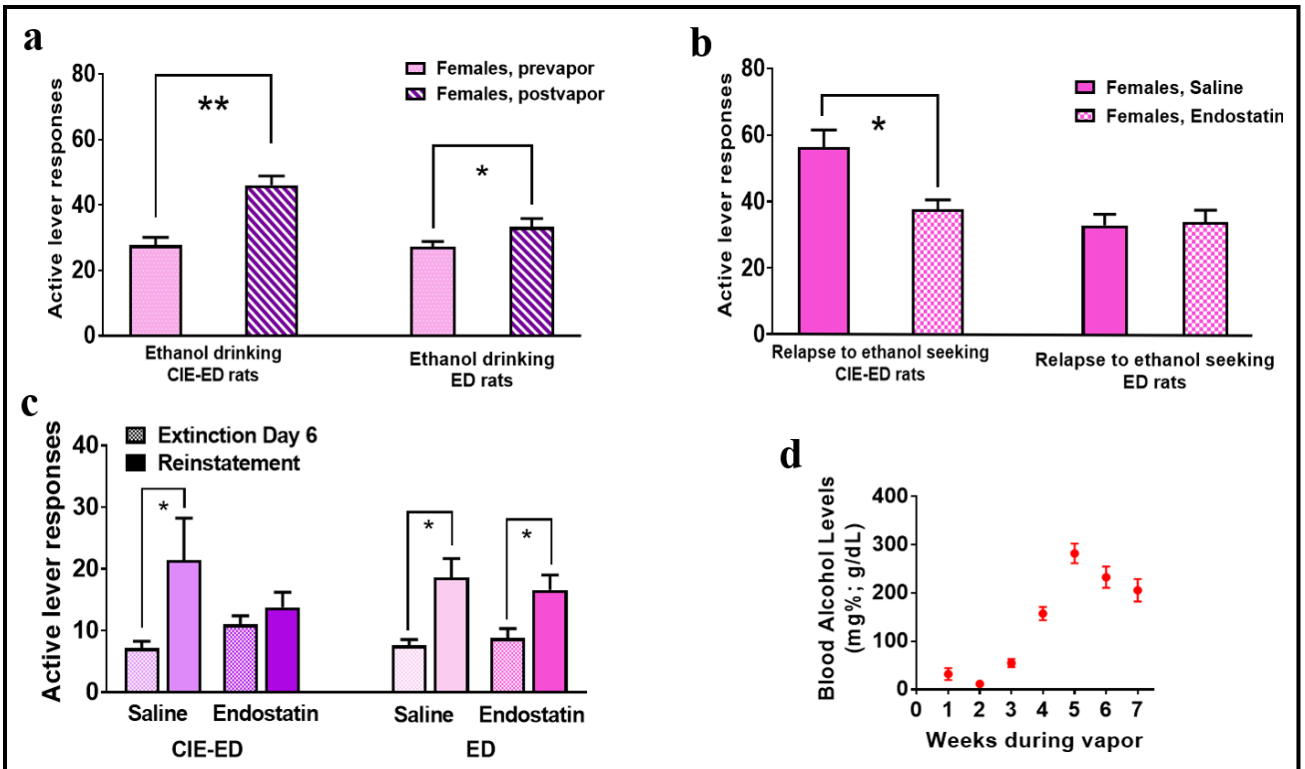

Figure 2. Active lever responses in CIE-ED and ED rats

(a) Active lever responses averaged from last three days of self-administration prior to separation into ED-vehicle and ED-endostatin and CIE-ED-vehicle and CIE-ED-endostatin groups. $^{**}p < 0.0001$ and $^{*}p = 0.0037$ in rats before and after vapor. **(b)** Active lever responses from ED-saline, ED-endostatin, CIE-ED-saline, and CIE-ED-endostatin rats during a single relapse session during protracted abstinence. $^{*}p = 0.0223$ between CIE-ED-saline and CIE-ED-endostatin rats. **(c)** Active lever responses during reinstatement, triggered by ethanol cues. $^{*}p = 0.0421$ in CIE-ED-saline rats; $^{*}p = 0.0009$ in ED-saline rats; $^{*}p = 0.0005$ in ED-endostatin rats. **(d)** Blood alcohol level (BAL) during self-administration. Group differences in (a)-(d) were calculated by Two-way ANOVA. Error bars represented standard deviation.

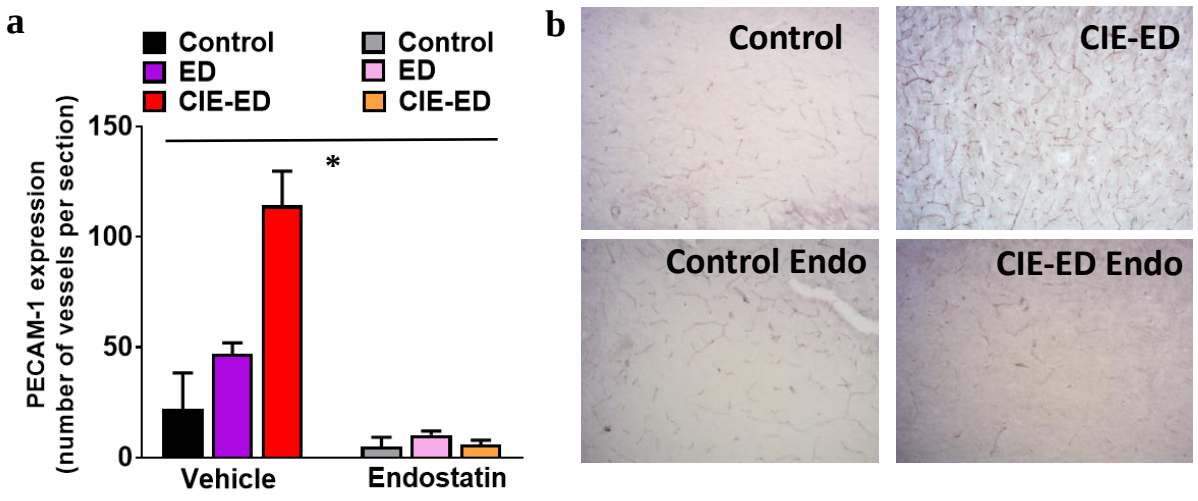

Figure 3. Evaluation of PECAM-1 expression in control, dependent and non-dependent female rats, received either saline or endostatin

(a) Quantity of vessels in female Long Evans rat mPFC. **(b)** Qualitative presentation of DAB stained PECAM-1 (as vessels) in the mPFC. Significant group differences were calculated using Two-way ANOVA and Post hoc analysis: *$p<0.0001$ between CIE-ED-vehicle, ED-vehicle and control-vehicle groups; *$p = 0.0231$ between ED-endostatin and vehicle groups; *$p < 0.0001$ between CIE-ED-endostatin and vehicle groups. Error bars represented standard deviation.

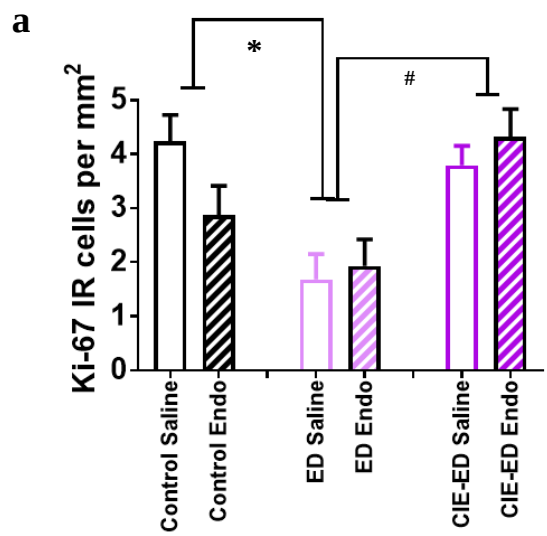

Figure 4. Evaluation of Ki67 expression using quantitative immunohistochemistry in control, dependent and non-dependent female rats, received either saline or endostatin

(a) Quantity of cells expressing Ki67 in female Long Evans rat mPFC, normalized to cells/mm^2. **(b)** DAB stained Ki67 cells in the mPFC with arrowhead pointing to immunolabeled cells. Significant group differences were calculated using Two-way ANOVA and Post hoc analysis: *p=0.0004 between Control-saline and ED-saline groups; $^{\#}$p = 0.0006 between CIE-ED- and ED-endostatin groups. Error bars represented standard deviation.

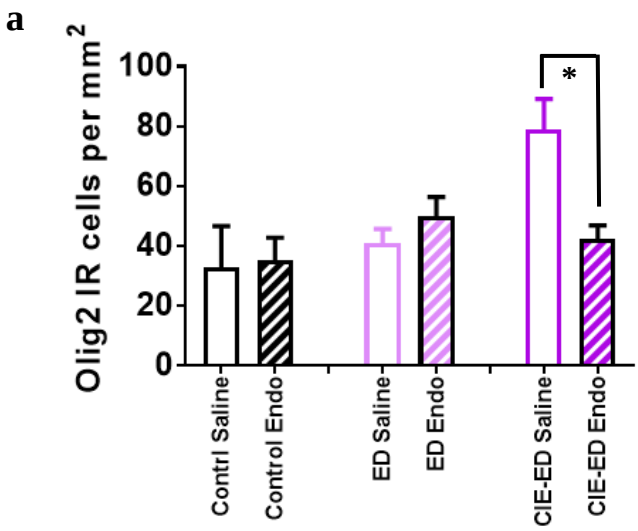

Figure 5. Evaluation of Olig2 expression using quantitative immunohistochemistry in control, dependent and non-dependent female rats, received either saline or endostatin

(a) Quantity of cells expressing Olig2 in female Long Evans rat mPFC, normalized to cells/mm^2. **(b)** DAB stained Olig2 cells in the mPFC with arrowhead pointing to immunolabeled cells. Significant group differences were calculated using Two-way ANOVA and Post hoc analysis: *p=0.0014 between CIE-ED-saline and CIE-ED-endostatin groups. Error bars represented standard deviation.

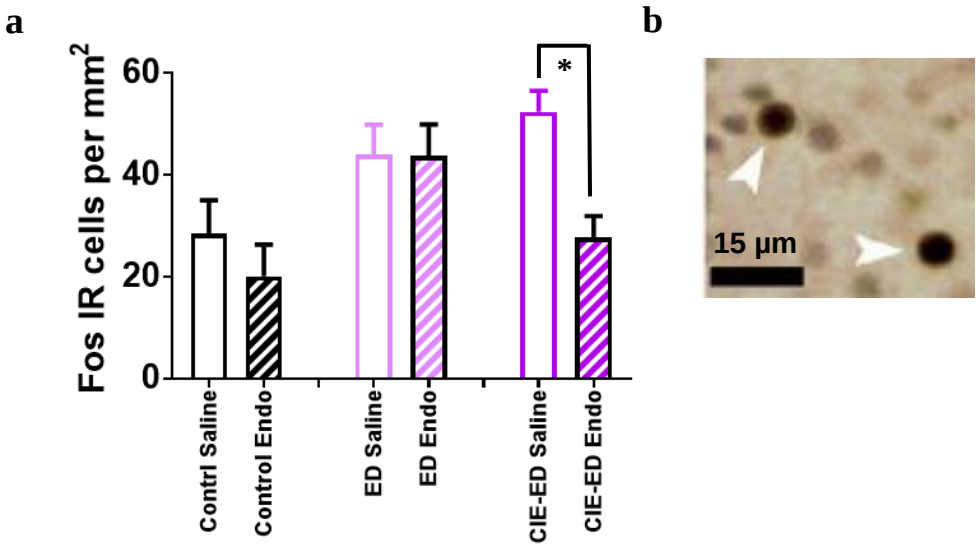

Figure 6. Evaluation of cFos expression using quantitative immunohistochemistry in control, dependent and non-dependent female rats, received either saline or endostatin

(a) Quantity of cells expressing cFos in female Long Evans rat mPFC, normalized to cells/mm^2. **(b)** Quantity of cells expressing cFos in female Long Evans rat mPFC, normalized to cells/mm^2. Significant group differences were calculated using Two-way ANOVA and Post hoc analysis: *p = 0.0007 between CIE-ED saline and CIE-ED-endostatin groups. Error bars represented standard deviation.

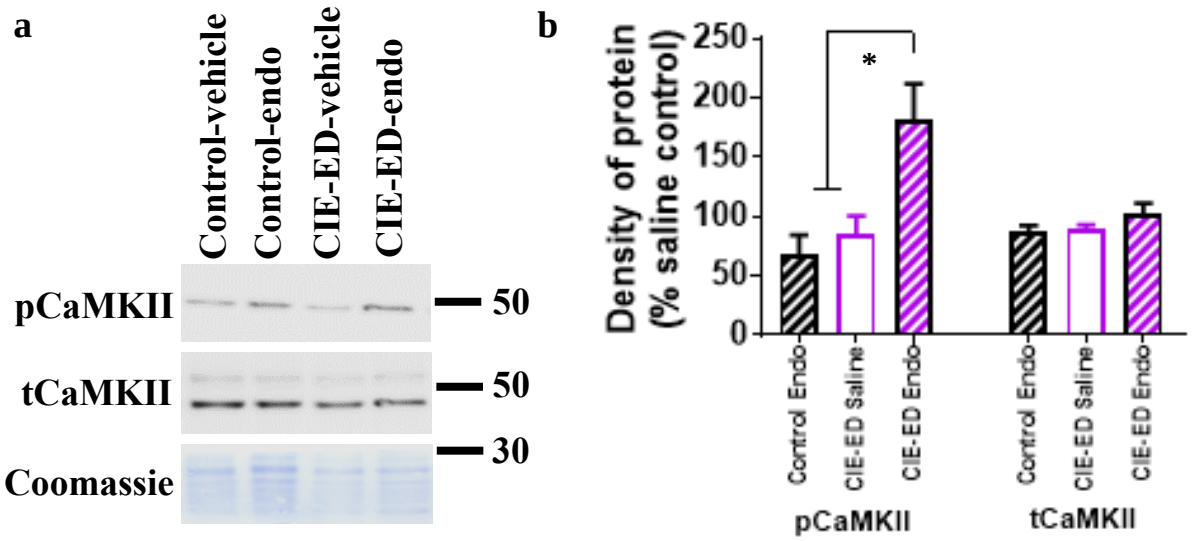

Figure 7. Western blot analyses of plasticity related proteins
(a) representative immunoblot of pCaMKII, tCaMKII and coomassie in all conditions with control-vehicle as control group **(b)** Densitometric analysis of pCaMKII and tCaMKII, indicated as percent change from control. Significant group differences were calculated using One-way ANOVA and Post hoc analysis: *$p<0.0001$ between CIE-ED-saline and CIE-ED-endostatin groups; *$p = 0.0004$ between control-endostatin and CIE-ED-endostatin groups. Error bars represented standard deviation.

DISCUSSION

Our first intention was to investigate the drinking behavior in female rats during chronic alcohol experience, relapse, and reinstatement. Our second intention was to study the alteration in glial plasticity in female rat mPFC during these sessions and its role in drinking behavior. Here, we report that both CIE-ED and ED rats had escalation in drinking during 7 weeks of maintenance with a larger increase in CIE-ED rats. The results indicated that female rats (both dependent and non-dependent) drank more and more when alcohol was made available. Supported by BAL results from CIE-ED rats, the procedure of chronic intermittent ethanol (CIE) vapor successfully introduced alcohol dependency in CIE-ED rats, who underwent forced ethanol vapor during maintenance; CIE increased and maintained alcohol drinking in CIE-ED rats that mimics moderate to severe AUD in human. Moreover, access to alcohol after 23 days of abstinence enhanced drinking in CIE-ED rats compared with ED rats, and the increase in drinking was restored in animals treated with endostatin. We also report that CIE-ED-saline, ED-saline, and ED-endostatin rats reinstated alcohol seeking to similar levels with CIE-ED-endostatin rats being an exception. CIE-ED-endostatin rats showed reduced ethanol seeking during reinstatement, compared to their last day of extinction session. Results suggest that endostatin was able to reduce ethanol seeking or the motivational intention to drink in CIE-ED female rats. To summarize the findings in behavior data, endostatin did not only reduce ethanol drinking, but also ethanol seeking in dependent CIE-ED female rats. Notably, a significant change in behavior between treatments was only observed in CIE-ED rats, who were supposedly alcohol dependent. Therefore, we could infer that endostatin was only capable of rescuing alteration in subjects with alcohol dependency, targeting something exclusively to alcohol addiction.

Previous findings revealed that PECAM-1 expression escalated with enhanced ethanol drinking in male rats (Somkuwar et al., 2016). Meanwhile, our data with female rats showed that enhanced PECAM-1 expression was correlated with increased ethanol drinking in female rats. In conclusion, accumulation in PECAM-1 was correlated with enhanced drinking in both sexes, indicating no sex difference. Discovered by another lab, endostatin could reverse the impact of PECAM-1 by preventing

angiogenesis (Jia et al., 2004), which is a key characteristic of PECAM-1. Confirmed by the results in this study, endostatin was sufficient at blocking PECAM-1's impact in the mPFC, as PECAM-1 expression was reduced to minimal levels in all ethanol groups treated with endostatin.

Previous studies reported that PECAM-1 supported the growth of neuroprogenitors such as OPCs by forming blood vessels to provide nourishment (Arai et al., 2009). Ki67 was used as a marker for proliferating oligodendrocyte progenitors. Immunohistochemistry showed no difference in Ki67 expression in all ethanol groups treated with saline or endostatin, suggesting no main effect of endostatin in altering the number of OPCs. However, a main effect of alcohol was observed as control and CIE-ED groups had relatively higher number of OPCs, compared to ED groups, suggesting that drinking behavior may be responsible for the alteration of OPCs.

Previous findings from our lab showed that rats experienced hyperoligodendrogenesis during protracted abstinence and the reduction in PECAM-1 was correlated with lowered oligodendrogenesis (Somkuwar et al., 2016; Somkuwar et al., 2017). We here report that there was a surge of oligodendrocytes in CIE-ED-saline rats, confirming previous findings from our lab. Further verifying previous results, endostatin was able to restore the number of OLGs in CIE-ED-endostatin rats back to similar level in that from the control-endostatin group. Hyperoligodendrogenesis during protracted abstinence may be a compensatory move for the brain during protracted abstinence when alcohol was not available (Somkuwar et al., 2017); endostatin could restore the alteration in OLGs in mPFC, reversing the impact from abstinence. As previously mentioned, OPCs are progenitors of OLGs, and the OLGs are replenished in the brain through oligodendrogenesis. Our results showed that although endostatin did not alter the level of OPCs, it reduced the level of OLGs in CIE-ED rats only, suggesting that hyperoligodendrogenesis only occurred in rats with alcohol dependency and endostatin needed a change to rescue a change. Thus, with the number of OLGs closely tied to chronic alcohol exposure and the alteration of OLGs in female rat mPFC may contribute to dysfunction in the brain and propensity of relapse (Somkuwar et al., 2016).

Lastly, investigation in neuronal activation in the female rat mPFC suggest that CIE-ED-saline rats had significantly higher Fos expression compared to ED-saline and Control-saline rats. Only seen in CIE-ED-endostatin rats, Fos expression was restored back to similar level to that in control-endostatin rats, indicating that endostatin could rescue neuronal hyperactivation in alcohol dependent rats only.

Taken together with findings from drinking behaviors, PECAM-1, OPCs, OLGs and neuronal activation, our data supported our hypothesis that restricting the impact of PECAM-1 using endostatin decreased ethanol drinking and seeking in female rats by directly preventing hyperoligodendrogenesis and neuronal hyperactivation in the mPFC of female rats. In female rat mPFC, restored neuronal activation was observed in CIE-ED-endostatin female rats, which correlated with reduced voluntary drinking during relapse and seeking during reinstatement. Decreased ethanol drinking and seeking behavior in CIE-ED-endostatin rats were also correlated with reduced hyperoligodendrogenesis. The results suggested that by using endostatin, we could reverse PECAM-1's alteration in neuronal activation and the proliferation of oligodendrocyte progenitor cells in female rats, reducing ethanol drinking and the motivation to drink.

Previous findings from our lab elucidated that decrease in PECAM-1 was correlated with enhanced ethanol drinking, increased neuronal activation, and decreased oligodendrogenesis in male rats (Somkuwar et al., 2016). In addition to findings from male rats, the current findings from my thesis demonstrates that in female rats, inhibiting PECAM-1 reduced oligodendrogenesis, indicating that OLGs played a role in relapse to drinking behavior and this was reduced by inhibition of PECAM-1. To explain the reduction in neuronal activation by endostatin, we hypothesize that the part of neuronal activity that was reduced may be responsible for controlling craving or incentive motivation (George et al., 2001; Karch et al., 2015; Kose et al., 2015); thus, lowered motivation for drinking in female rats may be responsible for the decrease in ethanol seeking behavior, which is supported by our behavior data as female rats had reduced ethanol seeking during reinstatement.

It is unclear if PECAM-1 produces different impacts in female and male rats, as our results in female rats suggested that PECAM-1 directly regulated neuronal activation and oligodendrogenesis in the mPFC. The results showed that the number of Fos and Olig2 expressing cells from CIE-ED-endostatin

female rats was reduced to similar level to that from the control female rats, implying that the reduction in neuronal activation and oligodendrogenesis was directly linked to the affect of endostatin. Alternatively, endostatin did not significantly affect control and non-dependent female rats may because endostatin was only targeting alcohol-dependent phenotypes. Increased oxidative stress resulted from alcohol metabolism led to elevated production of cytokines in dependent female rats, which caused upregulation of PECAM-1 and persisted neuroinflammatory responses (Alikunju et al., 2011). As the control and non-dependent rats did not have as much accumulation of PECAM-1 as dependent rats did, endostatin's impact in control and non-dependent rats was subtle. It was observed that endostatin treated control and non-dependent rats had slightly increased number of OLGs, which was opposite from the trend observed in dependent rats. An alternative hypothesis may be that the proliferation of premyelinating oligodendrocytes (OLGs) does not solely rely on PECAM-1. For instance, tumor necrosis factor-α (TNFα), a multipotent inflammatory cytokine, promotes proliferation of oligodendrocyte progenitors (Heather et al., 2001). TNFα may be produced upon alcohol metabolism and is reportedly related to inflammatory responses and neurological diseases such as Alzheimer's disease (Heather et al., 2001).

Since Fos was significantly altered in CIE-ED rats, western blotting was performed to investigate the mechanism. While several antibodies were probed as were mentioned in method section, including pNR2A, pNR2B, tNR2A, and tNR2B, there was no significant differences between control, ED, and CIE-ED groups with or without endostatin. However, a significant increase in pCaMKII (phoso-Ca^{2+}/calmodulin-dependent protein kinase II) was seen in CIE-ED-endostatin rats. pCaMKII is the active form of CaMKII and it is responsible for inducing LTP by translocating to synapses and positively regulating neurotransmitter release, enhancing synaptic transmission (Wang 2008). No difference in the level of tCaMKII was observed in all groups while Endostatin significantly increased autophosphorylation of CaMKII in CIE-ED rats only. This suggests that endostatin was only able to stimulate presynaptic release of neurotransmitters in alcohol-dependent phenotypes.

Conclusions and Future Directions

In conclusion, in female dependent rats, endostatin significantly reduced both alcohol drinking and seeking behavior, reversed hyperoligodendrogenesis and neuronal hyperactivation in mPFC, and increased pCaMKII, enhancing release of presynaptic release of neurotransmitter. In addition to summarizing the findings, future studies could be conducted to further investigate the cellular mechanism of endostatin at the synapse. As mentioned in the discussion earlier, endostatin was only able to impact female rats with alcohol dependency. Therefore, Fos could be co-labeled with GABA (inhibitory neurotransmitter) or glutamate (excitatory neurotransmitter) in the mPFC to find which neuronal phenotypes are altered by endostatin and which circuitry was normalized by endostatin's effect on pCaMKII. Since endostatin is an FDA approved drug, studying its affect in the brain may be contributing to new therapies to reduce the severity of alcohol use disorder and relapse in alcohol dependent individuals.

MATERIALS AND METHODS

4.1 Animals

Sixty-eight adult female Long Evans rats (Charles River) completed the study. All rats were 8 weeks old at the beginning of the study, and weighed approximately 160-180 g. The rats were maintained in reverse 12h light-12h dark cycle rooms and housed two/cage unless otherwise specified. Food and water were 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 were approved by the Institutional Animal Care and Use Committee at VA San Diego Healthcare System.

4.2 Ethanol Self-Administration

The behavioral experiments conducted herein are presented as a detailed schematic in Figure 1. Fifty-six experimentally-naïve rats were given one to two 14-hour lever-responding training sessions in the operant conditioning boxes (Med Associates Inc, VT), on an fixed-ratio 1 schedule (FR1; one response resulted in one reinforce delivery), where one press on the available lever resulted in the delivery 0.1ml of water to a sipper cup mounted on the wall in between the two levers. The operant conditioning boxes were housed inside sound attenuating chambers. During these sessions, the house-light and white noise were turned off. Then, rats were trained to respond for 0.1ml of alcohol (10% v/v) over four daily 2-h FR1 sessions; all other conditions remained the same as before. Subsequently, the rats were trained to discriminate between two available levers to obtain on 0.1 ml ethanol (10% v/v) during daily 30-min FR1 sessions. During these sessions, active (right) lever responding resulted in the delivery of ethanol, while responding on the inactive (left) lever was recorded but had no programmed consequence. Each ethanol delivery followed by a 4-sec time-out during which responding on the active lever did not result in the delivery of ethanol. During this time-out period, the cue-light above the active lever remained on; thus, the cue-light was paired with the delivery of ethanol. These 30-min

discrimination training sessions continued till stable responding was obtained, where stable responding was defined as less than 10% variation in active lever responding for 3 consecutive 30-min FR1 sessions.

Subsequently, the rats were divided into two groups; one group received chronic intermittent ethanol vapor exposure (CIE; see procedure below) while the other group was exposed to air in their normal housing condition (did not experience ethanol vapors) for a duration of 6-7 weeks. Henceforth, these rats will be called CIE-ED (alcohol dependent, n=30) and ED (nondependent, n=24) rats, respectively. All rats received two 30-min FR1 sessions per week (Tuesdays and Thursdays) during these 6-7 weeks. Responding was analyzed to determine escalation of self-administration compared to pre-vapor stable responding. After 7 weeks of CIE, CIE-ED rats were withdrawn from ethanol vapors and both CIE-ED and ED rats were withdrawn from ethanol self-administration. Both CIE-ED and ED rats were divided into two groups (vehicle or endostatin; see below) and maintained as described for the remainder of the study.

4.3 Chronic Intermittent Ethanol vapor exposure (CIE)

During CIE, rat cages were housed in specialized chambers and were exposed to alcohol vapors on a 14-h ON / 10-h OFF schedule. Alcohol (95% ethanol) from a large reservoir was delivered to a heated flask at a regulated flow rate using a peristaltic pump (model QG-6, FMI Laboratory, Fluid Metering). The drops of alcohol in the flask were immediately vaporized and carried to the vapor chambers containing the rat cages by controlled air flow (regulated by a pressure gauge). The air pressure and ethanol flow rates were optimized to obtain blood alcohol levels (BALs) between 125 and 250 mg/dl or 27.2 and 54.4 mM (Gilpin *et al.*, 2008a); these BALs are 2-3 times the BAL observed in binge drinking, but not high enough to abolish righting reflex (Ernst *et al.*, 1976; Courtney & Polich, 2009).

4.4 Tail Bleeding for Determination of BAL

For measuring BALs, tail bleeding was performed on the CIE-ED rats, once a week (every Tuesday), between hours 13-14 of vapor exposure (Gilpin *et al.*, 2008b). Rats were gently restrained

while the tip of the tail was pricked with a clean needle. Tail blood (0.2 ml) was collected and centrifuged at 2000 rpm for 10 min. Plasma (5 μL) was used for measurement of blood alcohol levels (BALs) using an Analox AM1 analyzer (Analox Instruments USA Inc., MA). Single-point calibrations were performed for each set of samples with reagents provided by Analox Instruments (100 mg/dl). When plasma samples were outside the target range (125–250 mg/dl), vapor levels were adjusted accordingly.

4.5 Endostatin Treatment

Endostatin (recombinant mouse endostatin) was purchased from a commercial source (BioLegend, Cat# 95453; 0.5 mg/ml). Endostatin was dissolved in sterile saline (vehicle). Endostatin or equal volume of vehicle was injected at a dose of 0.3 mg/kg s.c.. Control (ethanol naïve) and ethanol rats were injected with endostatin for 5 days. CIE-ED rats were given the first injection 2-3 hours after the cessation of 7 weeks of CIE and ED rats were injected on the same day at the same time.

4.6 Drinking during abstinence (DDA)

After 23 days of abstinence from CIE and ethanol self-administration, CIE-ED and ED rats, both with and without endostatin, were given one 30 min FR1 session to lever press for ethanol reinforcement (0.1 ml of 10% v/v ethanol) under cue-context conditions identical to that used for training and maintenance. Active and inactive lever responses were recorded.

4.7 Extinction

Following DDA, rats were subject to 6 daily 30-min extinction sessions under a different cue-context combination than that used for training and maintenance (Context B). Specifically, operant boxes different from those used for self-administration were used and the house-light and white noise were turned on, and no cue-lights were available following lever presses. Finally, the lever response did not result in the delivery of ethanol. Both lever responses were recorded.

4.8 Reinstatement

Following the 6th day of extinction, rats were subject to one session of cued-context reinstatement of ethanol seeking. Specifically, rats were introduced to operant chambers under conditions identical to training and maintenance (no house-light, no white noise; Context A). Active lever responses resulted in the presentation of the cue-light for 4 sec but did not result in the delivery of ethanol. Both active and inactive lever responses were recorded.

4.9 Brain tissue collection

Rats were killed by rapid decapitation and the brains were isolated and dissected along the midsagittal plane. The left hemisphere was snap frozen for Western blotting analysis and the right hemisphere was postfixed in 4% paraformaldehyde for immunohistochemistry. For tissue fixation, the hemispheres were incubated at room temperature for 36 hours and subsequently at 4°C for 48 hours with fresh paraformaldehyde replacing the old solution every 12 hours. Finally, the hemispheres were transferred to sucrose solution (30% sucrose with 0.1% sodium azide) for cryoprotection and storage till tissue sectioning was conducted (Cohen et al., 2015). Subsequently, the tissue was sliced in 40µm sections along the coronal plane on a freezing microtome. Every ninth section through the PFC (+3.7 to +2.5 mm from bregma; 4 sections per rat) was mounted on Superfrost® Plus slides and dried overnight and used for Ki-67 analysis. Two sections through the PFC (+3.2 and +2.7 mm from bregma) were mounted as described before and processed for PECAM-1, Olig-2 and cFos analysis. The sections were pretreated, blocked, and incubated with the primary antibody followed by biotin-tagged secondary antibody. Staining was visualized with 3,3′-diaminobenzidine chromogen (DAB; cat# SK-4100; Vector Laboratories, Burlingame, CA, USA).

4.10 Quantitative Immunohistochemistry Analysis for PECAM-1, Ki67, Olig-2 and cFos Labelled Cells

The following primary antibody was used for PECAM-1 immunohistochemistry (IHC): goat polyclonal, 1:500, catalog # AF3628, R&D Systems). PECAM-1 immunoreactive cells in the mPFC were examined and captured at 100× magnification (Figures X) with an AxioImager Microscope (Zeiss, Oberkochen, Germany). Cells in the mPFC were visually quantified using ImageJ software and used for analyses.

For Ki-67, Olig2 and Fos, quantitative immunohistochemical assay performed using a previously published optical fractionator method (Kim et al., 2015) using rabbit polyclonal anti-ki-67 (1:1000, catalog # RM-9106-S, Thermo Scientific). Ki-67 labelled cells were quantified in the mPFC with a Zeiss AxioImager Microscope equipped with Stereo Investigator 16 (MicroBrightField Bioscience, Williston, VT USA), a three-axis Mac 5000 motorized stage, a Zeiss digital MRc video camera, PCI color frame grabber, and computer work station. mPFC regions were contoured by referencing histological landmarks including corpus callosum, anterior commissure and rhinal fissure, using a 5x objective with a 10x eye piece and the above software (Paxinos and Watson, 1997). Cells were visually quantified within the contour using a 20x objective and a 10x eyepiece by an observer blind to the study using the following criteria - cells stained as dark brown to black, with the ability to focus the boundary of the cell within the mounted section thickness. A software generated 180 X 120 µm counting frame was systematically moved through the entire contoured area of the tissue to manually assess and count the Ki-67-positive (Ki-67+) cells. Mounted section thickness after immunohistochemistry was determined to be ~28 µm. The Ki-67+ cells always appeared in clusters of irregularly shaped dark stained cells. The overlapping-pair arrangement and the number of cells in each cluster were confirmed by focusing on different layers of cells along the Z-axis. Absolute cell counting (complete counting of all immunoreactive cells in the contoured area) was performed in the mPFC; the data are presented as total number of cells per unit area (cells/mm^2 based on mounted section thickness) per animal.

The following primary antibody was used for cFos: (1:1000, catalog # sc-52, Santa Cruz Biotechnology (Recinto et al., 2012)) and Olig-2 (1:10000; generous gift from Dr. Charles Stiles, Harvard). The sections were pretreated (Mandyam et al., 2004), blocked, and incubated with the primary antibody followed by biotin-tagged secondary antibody. Fos/Olig-2 immunoreactive cells were examined and quantified with a Zeiss AxioImager Microscope as described previously. Live video images were used to draw contours delineating the subregions of the PFC (anterior cingulate, prelimbic and infralimbic cortices). The fields of the brain regions for quantification were traced separately at 25x magnification. A 150 x 150 µm frame was placed over the regions of interest using the StereoInvestigator stereology platform followed by analysis using the optical fractionator method. The frame was systematically moved over the tissue to cover the entire contoured area and the labeled cells in each subregion falling entirely within the borders of the contour were marked and analyzed. Immunoreactive cells were quantified (absolute cell counting in the area contoured for analysis) and were summed up for each PFC subregion. Data were then summed up for the entire PFC and are presented in Figure 3-6.

4.11 Western Blotting

Procedures optimized for measuring both phosphoproteins and total proteins was performed as previously described (Kim *et al.*, 2014; Galinato *et al.*, 2015; Navarro & Mandyam, 2015; Staples *et al.*, 2015). Tissue was homogenized in a refrigerated bead mill homogenizer (Next Advance) in 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), heated at 95 degrees C for five minutes, and stored at -80 degrees C until determination of protein concentration by a detergent-compatible Lowry method (Bio-Rad, Hercules, CA). Samples were mixed (1:1) with a Laemmli sample buffer containing β-mercaptoethanol. Each sample containing protein from one animal was run (20 µg per lane) on 10% SDS-PAGE gels (Bio-Rad) and transferred to polyvinylidene fluoride membranes (PVDF pore size 0.2 µm). Blots were blocked with 2.5% (for phosphoproteins) or 5% milk (w/v) in TBST (25 mM Tris-HCl (pH 7.4), 150 mM NaCl and 0.1% Tween 20 (v/v)) for one hour at room temperature and were incubated with

the primary antibody for 16-20 h at 4 °C: antibody to total(t) GluN2A (1:200, Santa Cruz Biotechnology cat. no. sc-9056, predicted molecular weight 177 kDa, observed band ~170 kDa), antibody to pGluN2A Tyr-1325 (1:200, PhosphoSolutions cat. no. p1514-1325, predicted molecular weight 180 kDa, observed band ~180 kDa), antibody to tGluN2B (1:200, Santa Cruz cat. no. sc-9057, predicted molecular weight 178 kDa, observed band ~180 kDa), antibody to pGluN2B Tyr-1472 (1:200, Cell Signaling cat. no. 4208S, predicted molecular weight 190 kDa, observed band ~180 kDa); pCamKII Tyr-286 (rabbit polyclonal, 1:200, Abcam cat# ab5683, molecular weight 50 kDa); total CaMKII (rabbit polyclonal, 1:200, Abcam cat# ab52476, molecular weight 47 kDa); PSD-95 (rabbit polyclonal, 1:500, Millipore cat# 04-1066, molecular weight 95 kDa). Blots were then washed three times for 5 min in TBST, and then incubated for 1 h at room temperature with horseradish peroxide–conjugated goat antibody to mouse or rabbit in TBST. Following subsequent washes, immunoreactivity was detected using SuperSignalWest Dura chemiluminescence detection reagent (Thermo Scientific, Waltham, MA, USA) and images were collected using a digital imaging system (Azure Imager c600, VWR, Radnor, PA, USA). For normalization purposes, membranes were incubated with 0.125% Coomassie stain for 5 min and washed three times for 5–10 min in destain solution (Welinder & Ekblad, 2011; Thacker *et al.*, 2016). Densitometry was performed using ImageJ software (NIH). The signal value of the band of interest following subtraction of the background calculation was then expressed as a ratio of the corresponding coomassie signal (following background subtraction). This ratio of expression for each band was then expressed as a percent of the adult female sedentary rat included on the same blot.

4.12 Statistical Analysis

Ethanol behavior was analyzed with two-way ANOVA. Immunohistochemical data was analyzed by two-way ANOVA. Western blotting data was analyzed with one-way ANOVA. Specific group differences were determined using Post-hoc tests. GraphPad Prism was used for data analysis with significance set at $p<0.05$.

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