UNIVERSITY OF CALIFORNIA SAN DIEGO

Endostatin reduces relapse to ethanol seeking in dependent rats: Indirect regulation by microglia

A thesis submitted in partial satisfaction of the requirements
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by

Amin Andrew Shayan

Committee in charge:

Professor Chitra Mandyam, Chair
Professor Ralph Greenspan, Co- Chair
Professor Jon Armour
Professor Kenta Asahina

The thesis of Amin Shayan 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

DEDICATION

I dedicate this thesis to my family

Peiman, Sheida, and Matin for their empowerment

and unconditional love.

EPIGRAPH

“Everything negative - pressure, challenges - is all an opportunity for me to rise.”

Kobe Bryant

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AKNOWLEDGEMENTS

I would like to thank Dr. Chitra Mandyam for her guidance and support during my time as an undergraduate and graduate student working in her lab. The advice and knowledge that she has provided me as my principal investigator has been critical and invaluable throughout this process. I am truly grateful that she allowed me to work in her lab and learn about the neuroadaptations of the brain. She has devoted her time to teach me various techniques and aspects of professionalism that I will cherish forever.

I would also like to thank my committee members Professor Ralph Greenspan, Professor Jon Armour and Professor Kenta Asahina for joining me through this endeavor and providing me guidance.

ABSTRACT OF THE THESIS

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Alcohol use disorder (AUD) affects millions of people around the world and kills many, therefore, it is considered a serious public health issue. AUD is an economic burden, causes brain damage, and causes dysfunction of heart, liver, lungs, and kidney. Currently there are a few FDA approved medications and mutual help groups to combat

AUD, but none are successful in preventing relapse over long-term. Therefore, there is need for novel therapeutics to treat AUD. Previous studies suggested that in animal models of AUD, animals with moderate to severe AUD had higher ethanol seeking behavior during abstinence that correlated with enhanced expression of platelet endothelial cell adhesion molecule (PECAM-1, a marker for angiogenesis), increased blood brain barrier leakage, and altered neuronal activation in medial prefrontal cortex (mPFC), a brain region implicated in relapse to ethanol seeking behaviors. However, it was unclear how microglia, a cell type known to regulate neuroimmune responses was altered during abstinence. Observations from our lab show that endostatin reduces the expression of PECAM-1, prevents gliosis that occurs during abstinence, and decreased ethanol seeking behavior during relapse. Postmortem tissue analysis using quantitative immunohistochemistry in the mPFC demonstrated that ethanol seeking is correlated with microglial activation in the mPFC and endostatin reduced ethanol seeking and concurrently reduced microglial activation. Microglial deactivation was quantified using stereological methods and 3D structural analysis by investigating changes in soma to tip distance, cell soma area, total dendritic length, and number of intersections relative to distance from soma in microglial cells labeled with Iba-1. Phenotypic analysis revealed that the number of intersections relative to the distance from the soma recovered to normal levels after treatment with endostatin in alcohol dependent rats; which indicates a deactivation of microglia. Our research showed that endostatin can indirectly deactivate microglia and alter the neuronal response. Our research provides a possible mechanism of action to limit ethanol seeking behavior by indirectly deactivating microglia in the mPFC.

INTRODUCTION

1.1 Alcohol Use Disorder

Moderate to severe alcohol use disorder (AUD) affects 16 million people which translates to 6.2% of the population in the United States annually (NIAAA, 2015). 9.8 million men and 5.3 million women are affected by AUD, suggesting that addiction affects the sexes differently (NIAAA, 2015). In some cases, this disorder is fatal as a reported 88,000 Americans die from alcohol abuse annually. Almost 40-60% of alcoholics relapse within a year (Mokdad *et al*., 2004). In 2010, the U.S. spent 250 billion dollars on excessive alcohol use, which is mostly due to binge drinking (*Excessive Drinking Is Draining the U.S. Economy | Features | CDC*, n.d.).

There are treatment options to help people suffering from AUD, including mutual help groups and medication. Mutual help groups are the most common source of psychosocial treatment. Mutual help groups aim at using behavioral therapies to set individual goals and make changes for people suffering from addiction (*Alcohol Alert Number 81*, n.d.). The drugs used to treat alcohol addiction include disulfiram, naltrexone, and acamprosate. Disulfiram is a medication that creates an unpleasant reaction and acute sensitivity to the consumption of alcohol. Naltrexone is a medication that blocks dopamine receptors in the brain to decrease drinking urges (*Alcohol Alert Number 81*, n.d.). Acamprosate is an antagonist of the NMDA receptor and a positive allosteric modulator of $GABA_A$ receptor, which similarly reduces cravings for alcohol (Mason & Heyser, 2010). These drugs attempt to treat the problems; however, individuals still tend to relapse. There are also significant side effects associated with

these drugs. Therefore, there is a significant need to produce therapies to reduce relapse in alcoholics.

In humans, AUD affects many different organs such as the lung, liver, kidney, heart and brain (Traphagen *et al*., 2015). Alcohol produces reactive oxygen species (ROS) that contributes to oxidative stress among these organs. In the lung, ROS cause leakage of airways which leads to pneumonia (Traphagen et al., 2015). ROS can also accumulate in the liver and cause fatty liver disease. (Liangpunsakul *et al.*, 2016). In the kidney, ROS from alcohol consumption triggers tissue inflammation and fibrosis (Varga *et al*., 2017). Excessive alcohol use also affects the heart by causing hypertension and coronary heart disease (Piano *et al.*, 2017). Lastly, within the brain an excess consumption of alcohol induces cellular stress and triggers neuroinflammation (Mandyam et al., 2017). Additional research is required to study the relationship between AUD and its effects in the brain, and such studies will offer new therapeutic strategies to combat AUD.

Alcohol Use Disorder (AUD) can be conceptualized as occurring via 3 stages: binge/intoxication, withdrawal/ negative affect, and preoccupation/anticipation (Koob & Volkow, 2010). The binge/intoxication stage occurs during excessive alcohol consumption. During binge/intoxication, alcohol reinforcement is mediated by cellular changes in the brain region including the nucleus accumbens shell and core regions, which are part of the brain's basal ganglia. Neurobiological changes in the brain's basal ganglia, an area known for habit formation, can lead to compulsive alcohol seeking. The neurotransmitter that plays a role for positive reinforcement is dopamine (Koob & Volkow, 2010). The next stage is withdrawal/negative affect stage, which is driven by

the immediate absence of alcohol. The brain region called the central nucleus of the amygdala plays a role in the negative affect stage of AUD. The absence of alcohol activates stress neurotransmitters such as dynorphin, corticotrophin release factor and norepinephrine in this region to drive negative affect (Koob & Volkow, 2010). The last stage is preoccupation/ anticipation, which causes the long-term craving of alcohol. The brain regions including, prefrontal cortex, basolateral amygdala, and hippocampus play a role in the processing of contextual reinforcement during preoccupation/anticipation. The neurotransmitter in these regions that causes cravings is glutamate (Koob & Volkow, 2010).

1.2 Rodent Model of AUD

Rodent models are an excellent way to study AUD because their behaviors are very similar to humans. Rats are used to study AUD because they exhibit anxiety and alcohol compulsivity during withdrawal and abstinence (Giuliano et al., 2018). The paradigm – chronic intermittent ethanol vapor exposure (CIE) induces alcohol dependence in rats. Rats experience CIE in vapor chambers to mimic alcohol dependence behavior in humans (Nicholas W. Gilpin et al., 2008). Furthermore, the rodent model can be used to study alcohol dependence followed by relapse during a state of protracted abstinence. During protracted abstinence, a period of non-ethanol exposure, the brain region in the cerebral cortex that is most affected by alcohol use disorder is the medial prefrontal cortex (mPFC) (Somkuwar & Mandyam, 2019). The mPFC is a region in the brain that is known to control ethanol seeking behavior (Keistler *et al*., 2017).

1.3 Sex Differences in AUD

AUD affects males and females differently in rodents and these sex differences are similar to the gender differences observed in human alcoholics. This sex differences includes different neuronal changes in the mPFC in males and females due to different drinking habits, behaviors, and weight (Priddy *et al*., 2017).In my thesis, Female Long Evans rats were used to examine AUD and its effects in the mPFC. Female Long Evans rats tend to drink more than their male counterparts. The rewarding effects of ethanol are enhanced in female rats because females preform more operant lever presses to obtain ethanol, and therefore consume more ethanol than males (Torres et al., 2014). Therefore, the increased ethanol seeking behavior in females is associated with increased sensitivity to the rewarding effects of ethanol (Torres et al., 2014). It is also important to note that the estrous cycle has no effect on ethanol seeking behavior in female rats (Torres et al., 2014). The presence of ovarian hormones in females has been correlated with enhanced ethanol seeking behavior. In addition, female rat's gastric alcohol dehydrogenase activity is increased allowing for faster alcohol metabolism and higher blood alcohol levels (Mezey et al., 1992). Female rats have less motivational withdrawal to alcohol during reinstatement and maintain ethanol drinking during relapse (Becker & Koob, 2016). To explain ethanol seeking behaviors in female Long Evans rats, mPFC analysis is necessary.

1.4 Hyperoligodendrogenesis in CIE rats

Functional and mechanistic studies conducted in both rats and humans show that the mPFC plays a role in alcohol seeking behavior (Keistler *et al*., 2017). Previous studies have shown that alcohol induced stress during protracted abstinence is

correlated with hyperoligodendrogenesis, an increase in oligodendrocytes, in the mPFC. Oligodendrocytes mature into myelinating oligodendrocytes, which work to provide myelination to help axons propagate neuronal signaling (Pukos et al., 2019). Western blot analysis of oligodendrogenic progenitor cells (OPCs) [NG2, PLP, CNPase, MBP, MOG, Olig2] showed an increase in oligodendroglial markers in alcohol dependent rats (Somkuwar et al., 2016).

1.5 PECAM-1

The mPFC is composed of various cells, including endothelial cells expressing platelet endothelial cell adhesion molecule-1 (PECAM-1) (Albelda et al., 1991). PECAM-1 is a type-1 transmembrane adhesion protein, which contains a Ig superfamily that can undergo binding (Kirschbaum *et al*., 1994). PECAM-1 is expressed in intercellular junctions of endothelial cells in the blood brain barrier (BBB) (Somkuwar *et al*., 2017). PECAM-1 controls permeability of BBB by changing structures of junction protein expression in response to inflammation, stress, or injury (Mandyam *et al*., 2017). There are very few studies looking at the relationship between alcohol and PECAM-1; understanding PECAM-1's role in ethanol seeking behavior and the inflammatory response produced by ethanol may give us a better understanding to treat alcohol use disorder.

1.6 Hyperoligodendrogenesis, PECAM-1, and BBB Integrity during Protracted Abstinence in CIE Rats

Previous studies in our lab have established that hyperoligodendrogenesis is corelated with upregulation of PECAM-1 within endothelial cells of the mPFC in alcohol dependent rats during protracted abstinence (Somkuwar et al., 2016). The upregulation

of PECAM-1 in endothelial cells is positively correlated with dysfunctional blood brain barrier (BBB), evident as reduced barrier integrity through alteration of surface junction proteins (Somkuwar et al., 2017). The BBB is a layer of endothelial cells that regulates the movement of proteins, ions, and cells between the brain and the blood (Daneman & Prat, 2015). BBB leakage is an indicator of disrupted integrity, which has been observed during protracted abstinence in CIE-ED rats (Somkuwar et al., 2017). BBB integrity is characterized by an endothelial cell antigen, SMI-71. Reduced expression of SMI-71 is representative of BBB leakage during protracted abstinence (Somkuwar et al., 2017). Leakage of the BBB leads to a peripheral immune response, such as the release of cytokines into the brain (Haorah, 2005).

Cytokines are proteins released by immune cells in the central and peripheral nervous system; they are released by microglial cells to induce a pro-inflammatory response in the brains of rats and humans (Foster, 2001). The pro-inflammatory release of cytokines- IL-1, IL-6, and TNF-α have been seen to activate microglia (Smith et al., 2012). Microglia are a type of endothelial cell in the brain that function to enhance drug seeking behavior during protracted abstinence (Kovács, 2012). Excessive microglial activation causes neuroinflammation and neuronal damage in the mPFC in rats (Fu *et al.*, 2018). The characteristics of microglial activation include increased cell body into ameboid form, reduced total dendritic distance, reduced arborization and reduced number of intersections per distance from soma (Wang et al., 2018).

1.7 Using Endostatin to alter PECAM-1 and understand Microglial Activation

Existing studies have established that AUD is correlated with hyperoligodendrogenesis and PECAM-1 upregulation; which causes leakage of the

BBB and neuronal inflammation during protracted abstinence in the CIE model (Mandyam *et al*., 2017). Unpublished observations from our lab shows that inhibition of PECAM-1 systemically with endostatin administration, an angiogenic protein inhibitor, reduces excessive drinking during abstinence in alcohol dependent rats (unpublished observation). The subcutaneous injection of endostatin in rats effectively downregulates PECAM-1 in endothelial cells in the mPFC, which is associated with reduced blood brain barrier leakage (Yang *et al.*, 2019). Although the behavioral effects of endostatin are known to reduce ethanol seeking behavior during protracted abstinence, the downstream neurophysiological effects of endostatin on microglial activity is yet to be understood.

Ultimately, it is important to understand the changes in microglia activation in response to endostatin and chronic alcohol exposure in the mPFC. In turn, allowing us to define a targetable mechanism of action to limit ethanol seeking behavior in dependent rats. Previous studies have shown that endostatin downregulates PECAM-1, which increases BBB integrity and limits cytokine release. However, the question remains, how are microglia affected by endostatin in animal models of moderate to severe AUD during protracted abstinence? This overall research question can help determine if microglia deactivation may be responsible for limiting ethanol seeking behavior in dependent rats.

In order to address this question, female Long Evans rats were subjected to chronic induced ethanol vapor exposure and forced into a state of protracted abstinence. These rats were then treated with endostatin in an attempt to indirectly inhibit the activity of microglia in order to define the effects of endostatin, PECAM-1, and

CIE on microglia. The goal of this study was to identify the structural changes of microglia in response to endostatin and chronic alcohol exposure during protracted abstinence. 3- dimensional structural analysis was done on microglial cells labeled with Iba-1 to determine soma area, soma to tip distance, total dendritic length, and the total number of intersections of dendrites relative to the distance from the soma. We hypothesize that endostatin will downregulate PECAM-1; which will reduce the leakage of cytokines and limit structural changes to microglia to decrease ethanol seeking behavior during protracted abstinence. Understanding the neuroimmune changes in rat brains after moderate to severe AUD may give us a better understanding of the cellular mechanisms contributing to excessive drinking and alcohol relapse in humans suffering from AUD.

MATERIALS AND METHODS

2.1: 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.

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.

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 (N. W. Gilpin et al., 2008); these BALs are 2-3 times the BAL observed in binge drinking, but not high enough to abolish righting reflex (Courtney & Polich, 2009; Ernst et al., 1976).

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 (Nicholas W. Gilpin et al., 2008). 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.

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.

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.

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 lever response did not result in the delivery of ethanol. Both lever responses were recorded.

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.

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 (see below) 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 IBA-1 analysis. Two sections through the PFC (+3.2 and +2.7 mm from bregma) were mounted as described before and processed for PECAM-1, Iba-1 and SMI-71 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).

Immunohistochemistry

The following primary antibodies were used for PECAM-1 and SMI-71 immunohistochemistry (IHC): PECAM-1: goat polyclonal, 1:500, catalog # AF3628, R&D Systems; SMI-71: mouse monoclonal, 1:500, catalog # 836802, BioLegend. PECAM-1 and SMI-71 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.

Immunohistochemical assay of IBA-1 was performed on the mPFC using a previous published method. The mPFC was stained with rabbit anti IBA-1 (019-19741,1:1000; Wako) to view structural difference of microglial cells (Takashima et al., 2018).

Analysis of microglial cells in mPFC

Zeiss AxioImager A2 microscope was used to view the structures and Neuroleucida; Micro-BrightField (a computer-based program) was used to create three dimensional tracings that were analyzed by NeuroleucidaExplorer; Micro-BrightField (a computer-based program). The following criteria was used to access the efficacy of microglial cells: (1) the cell was in the mPFC (the region of interest), (2) the cell was

specifically defined from other cells, (3) the cell was fully intact and not broken, (4) the cell was stained dark enough to visualize the soma and dendrites. 6 cells were traced for each animal, 3 traces for each of the two sections. 40x magnification with an oil immersion lens (equipped with a 10x eye piece) was used to view and trace the cells. After tracing, soma area, soma to tip distance, total dendritic length, and 3D Sholl analysis was preformed to see the total number of dendritic intersections relative to the radial distance (starting from 0 μm and increasing in 1 μm in from the soma).

Statistical analysis

Ethanol behavior was analyzed with two-way ANOVA followed by post hoc analysis (Fisher's LSD). Immunohistochemical data was analyzed by two-way ANOVA followed by post hoc analysis (Fisher's LSD). GraphPad Prism was used for data analysis with significance set at $p<0.05$.

FIGURES

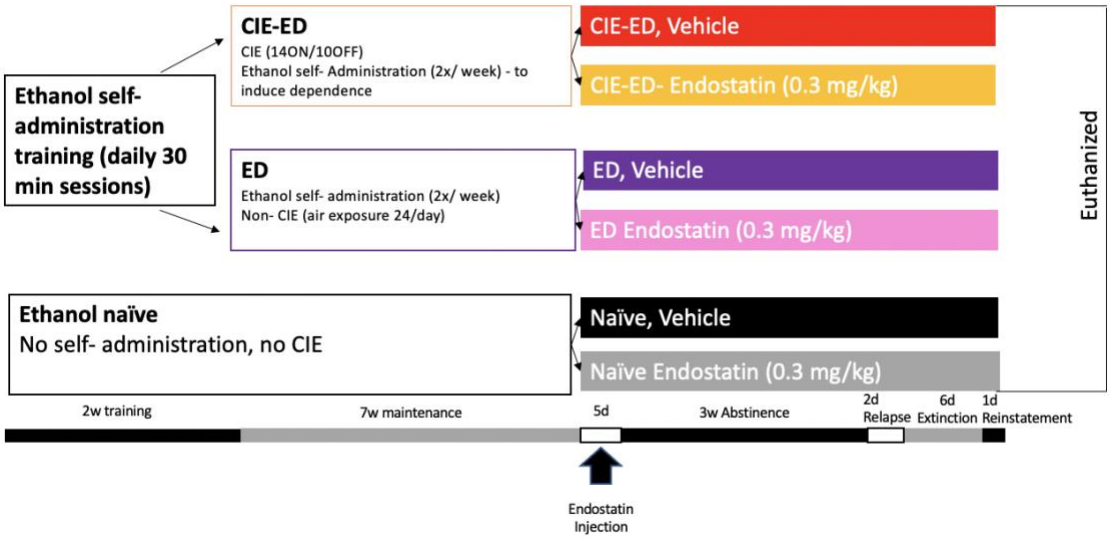

Figure 1. Schematic representation of experimental design. Female long evans rats went through 2 weeks of training, followed by 7 weeks of maintenance either by chronic intermitent ethanol vapor exposure and ethanol drinking (CIE-ED; n=30) or by ethanol drinking (ED; n=24). Control rats were not given any ethanol administration (Ethanol naïve; n=12). CIE-ED, ED, and ethanol naïve rats were then given either a saline injection subcutasneously for 5 days. This was followed by 3 weeks of abstinence. After abstinence, the rats went through 2 days relapse, 6 days extinction, and 1 day of reinstatement. They were then euthanized.

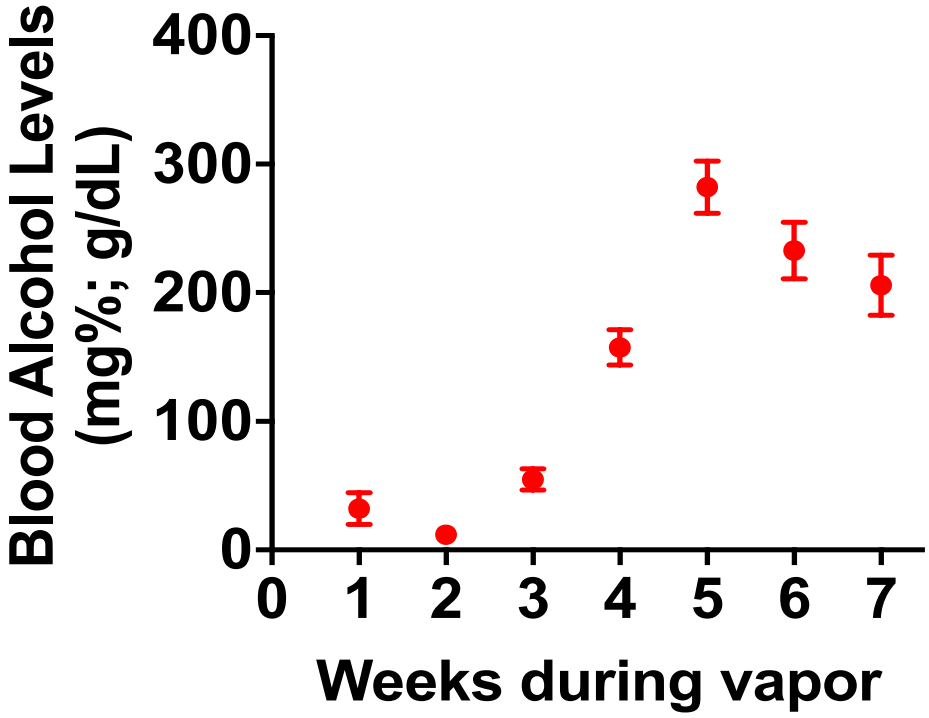

Figure 2. CIE-ED significantly increased blood alcohol levels to mimic moderate to severe AUD during week 5 to week 7. Blood alcohol levels taken during 7 weeks of maintenance for CIE-ED rats. For measuring BALs, tail bleeding was performed on the CIE-ED and ED rats, once a week. Error bars represent standard error of the mean.

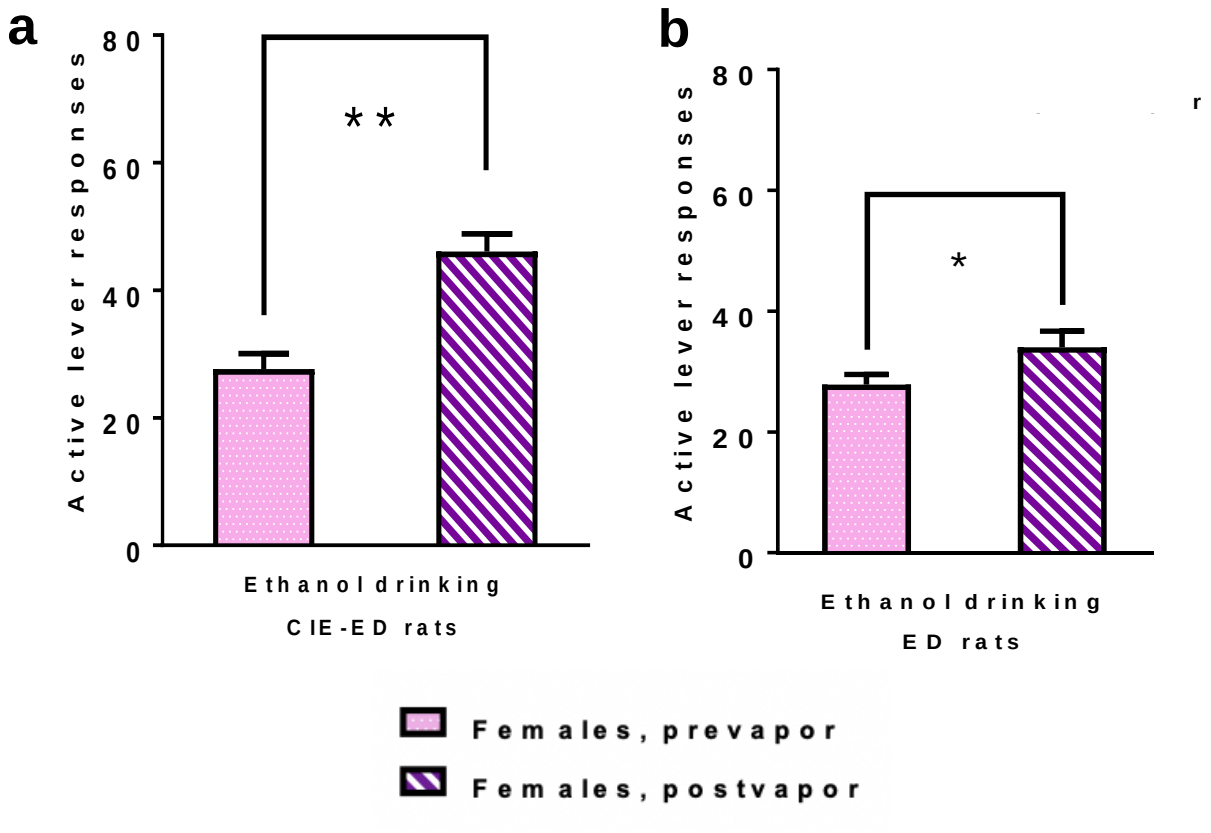

Figure 3.Chronic induced vapor exposure and ethanol drinking (CIE-ED) increased alcohol seeking behavior in CIE-ED rats. Active lever responses taken before vapor exposure and 7 weeks after vapor exposure in (a) alcohol dependent CIE-ED rats, (b) alcohol non-dependent ED rats. $^{*}p < .0001$ between CIE-ED pre- vapor and post- vapor groups by post hoc analysis. Error bars represent standard error of the mean.

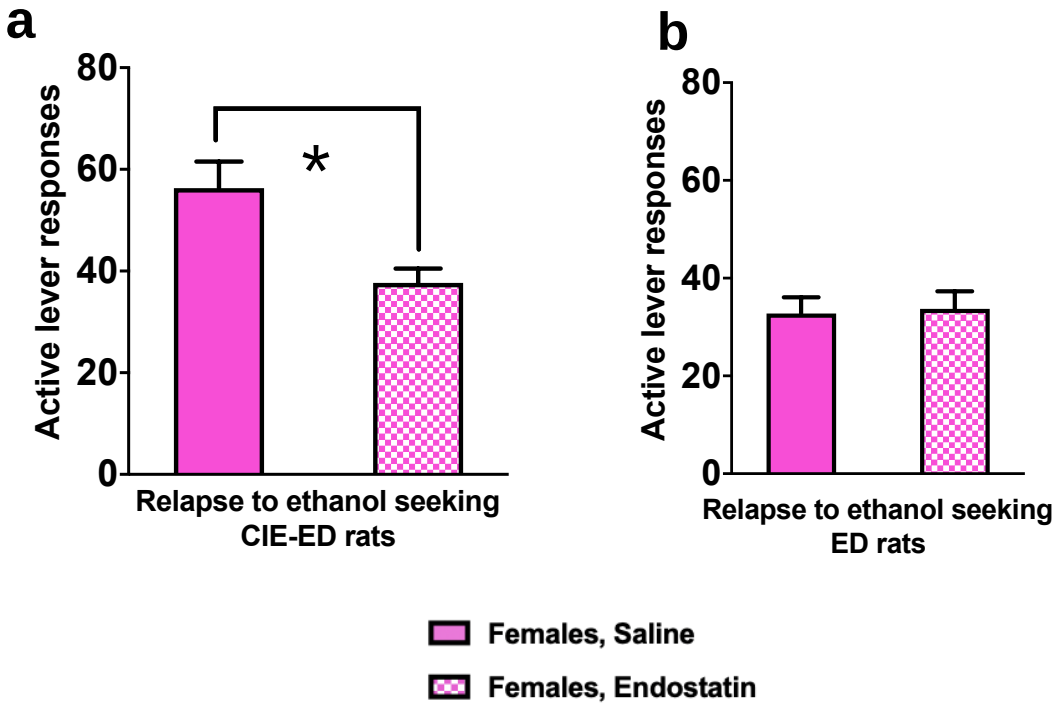

Figure 4. Endostatin significantly decreased alcohol seeking behavior in CIE-ED rats. Active lever responses during a single relapse session in (a) alcohol dependent CIE-ED rats, (b) alcohol non-dependent ED rats, treated with saline or Endostatin. $^{*}p < 0.05$ between CIE-ED saline and CIE-ED endostatin groups by post hoc analysis. Error bars represent standard error of the mean.

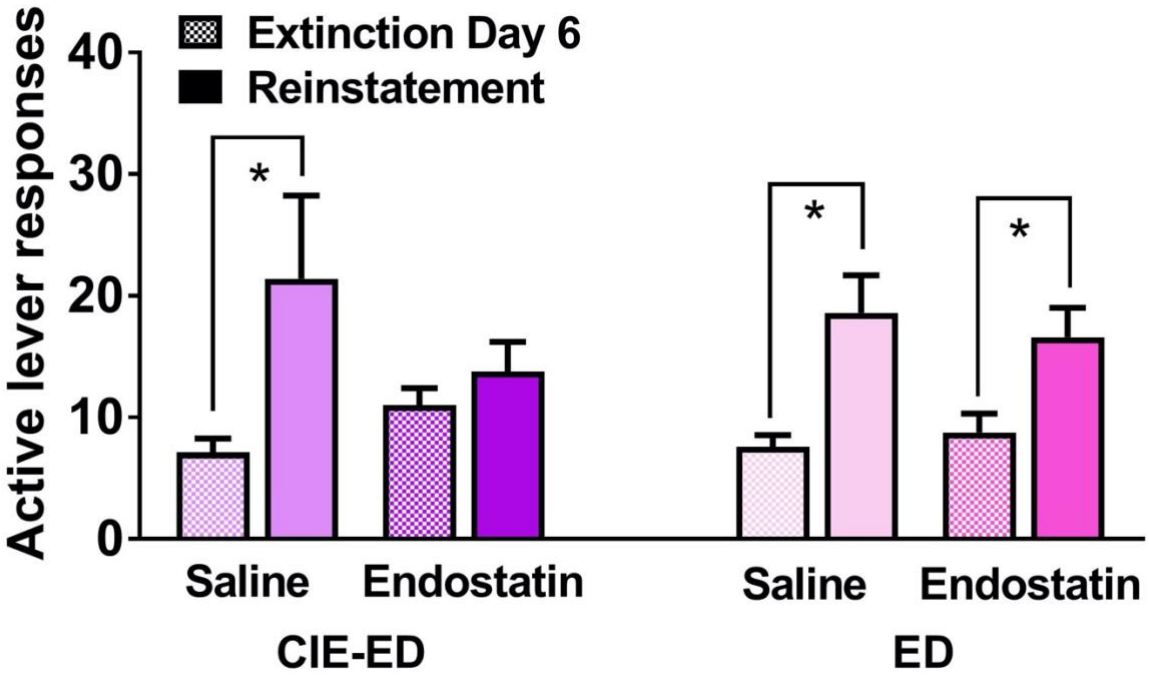

Figure 5. CIE-ED saline rats maintained active lever responses while CIE-ED endostatin rats decreased drinking during reinstatement. Active lever responses during day 6 of extinction followed by reinstatement in alcohol dependent CIE-ED rats, alcohol non-dependent ED rats, treated with saline or Endostatin. $^{*}p < 0.05$ between CIE-ED saline and CIE-ED endostatin groups by post hoc analysis. Error bars represent standard error of the mean.

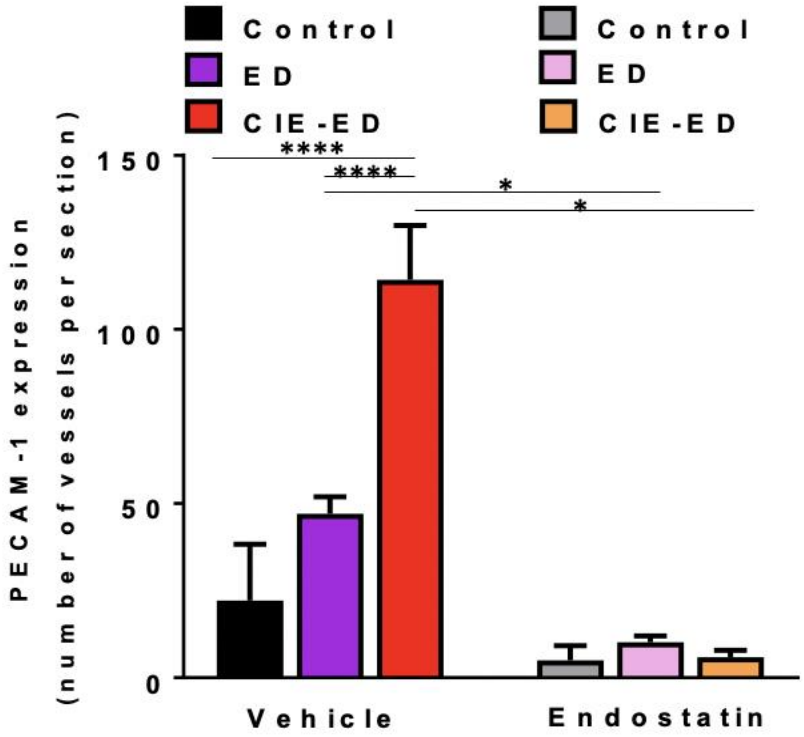

Figure 6. Endostatin significantly decreases PECAM-1 expression in CIE-ED endostatin rats. Immunohistochemical analysis of PECAM-1 expression in the mPFC. $^{*}p < 0.0001$ between CIE-ED vehicle and CIE-ED endostatin groups by post hoc analysis. Error bars represent standard error of the mean.

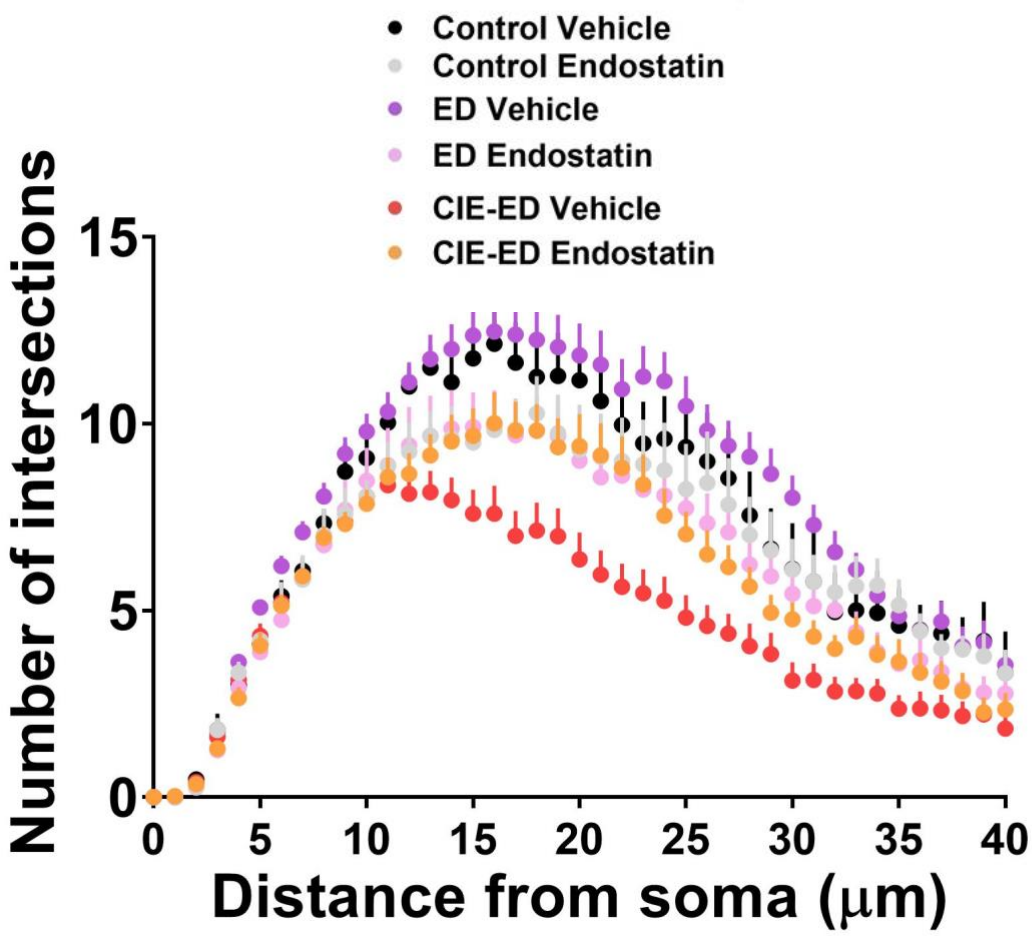

Figure 7. CIE-ED vehicle rats show significant decreases in number of intersections relative to distance from soma. Structural analysis of mPFC microglial cells dendrites are illustrated as number of intersections of dendrites relative to distance from soma. Female long evans rats were treated with no ethanol drinking, chronic intermittent ethanol vapor exposure and ethanol drinking (CIE-ED) or ethanol drinking (ED). They were subcutaneously treated with saline (- control) or endostatin after maintenance. The mPFC was stained for IBA-1, a marker for microglia. The stained microglial cell bodies were traced using Neuroleucida and quantified using Neuroleucida Explorer. Error bars represent standard error of the mean.

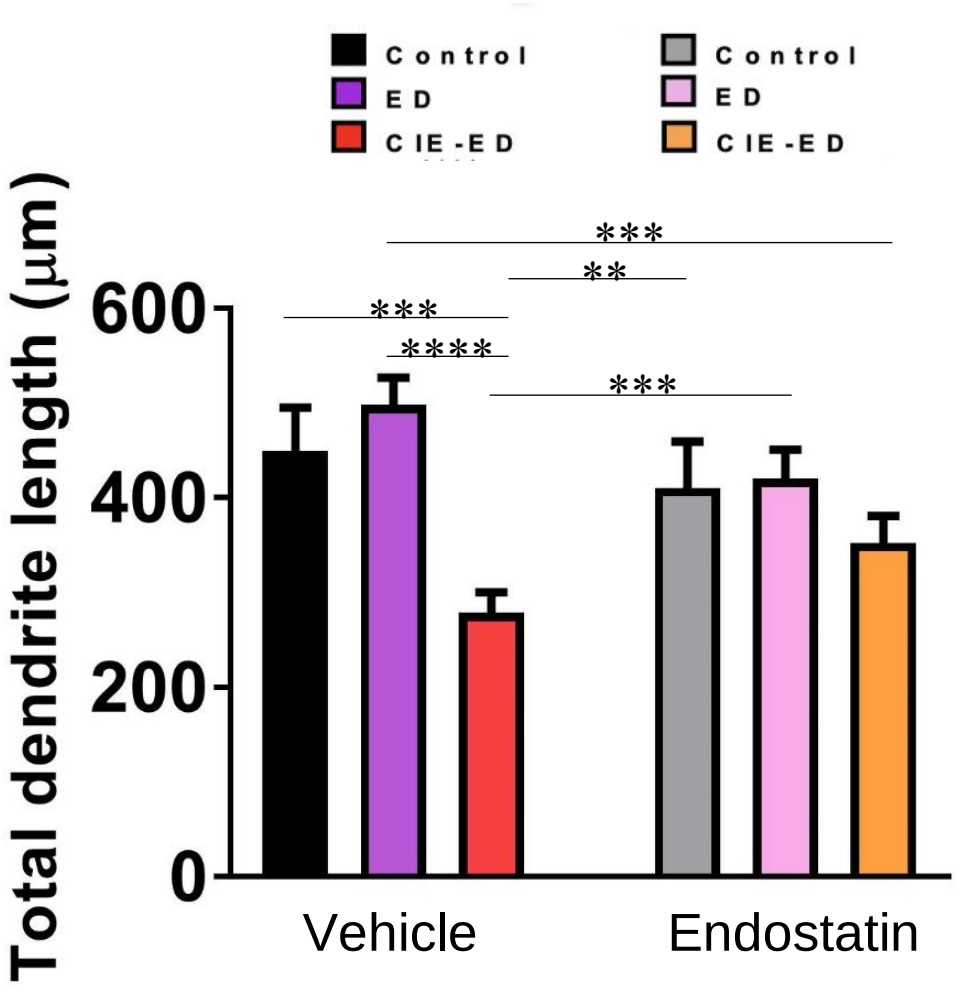

Figure 8. CIE-ED vehicle rats show a significant decrease in microglial total dendritic length. Structural analysis of mPFC microglial cells dendrites are illustrated as total dendritic length. Female long evans rats were treated with no ethanol drinking, chronic intermittent ethanol vapor exposure and ethanol drinking (CIE-ED) or ethanol drinking (ED). They were subcutaneously treated with saline (- control) or endostatin after maintenance. The mPFC was stained for IBA-1, a marker for microglia. The stained microglial cell bodies were traced using Neuroleucida and quantified using Neuroleucida Explorer. $^{*}p < 0.0001$ between ED saline and CIE-ED saline groups by post hoc analysis. Error bars represent standard error of the mean.

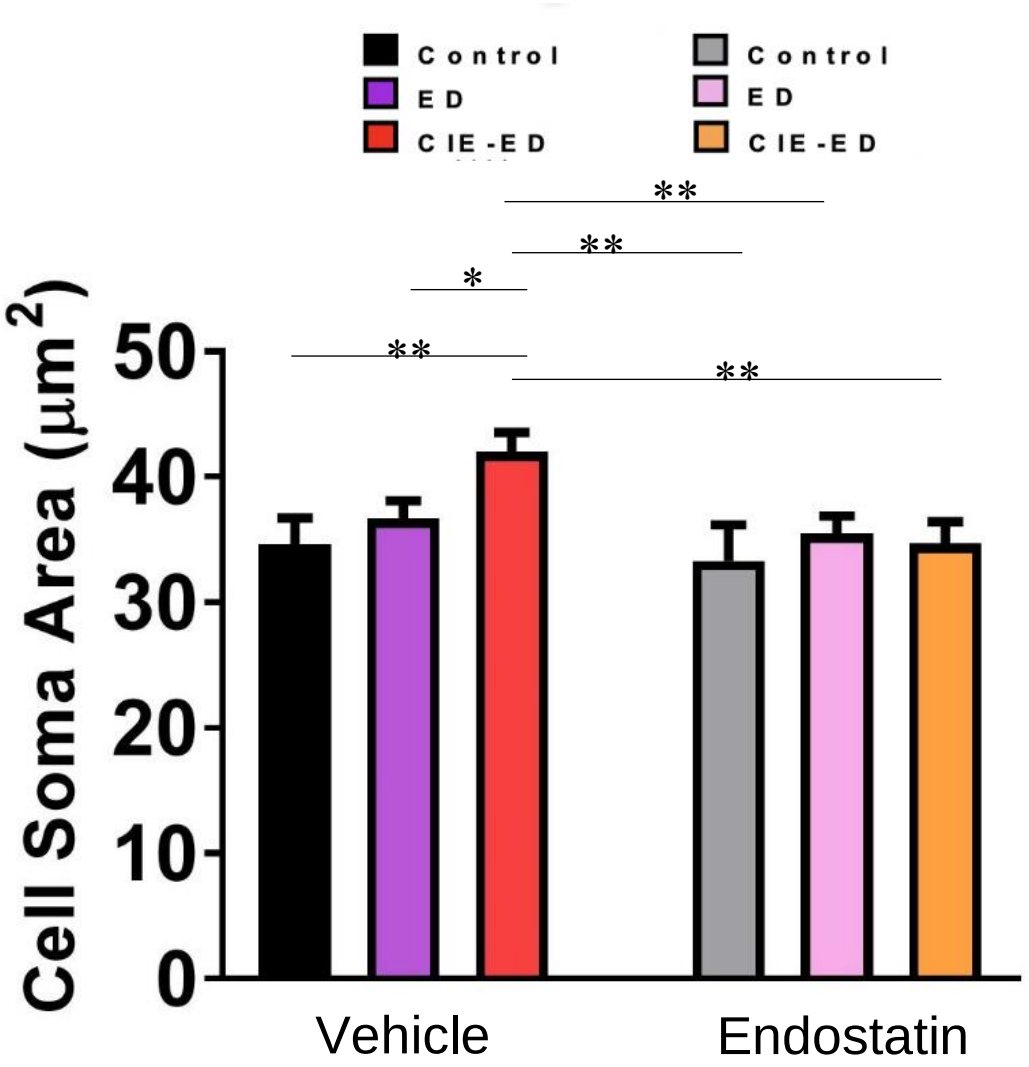

Figure 9. Endostatin rescues microglial cell soma area in CIE-ED rats. Structural analysis of mPFC microglial cells dendrites are illustrated as cell soma area. Female long evans rats were treated with no ethanol drinking, chronic intermittent ethanol vapor exposure and ethanol drinking (CIE-ED) or ethanol drinking (ED). They were subcutaneously treated with saline (-control) or endostatin after maintenance. The mPFC was stained for IBA-1, a marker for microglia. The stained microglial cell bodies were traced using Neuroleucida and quantified using Neuroleucida Explorer. $^{*}p < 0.0014$ between CIE-ED endostatin and CIE-ED saline groups by post hoc analysis. Error bars represent standard error of the mean.

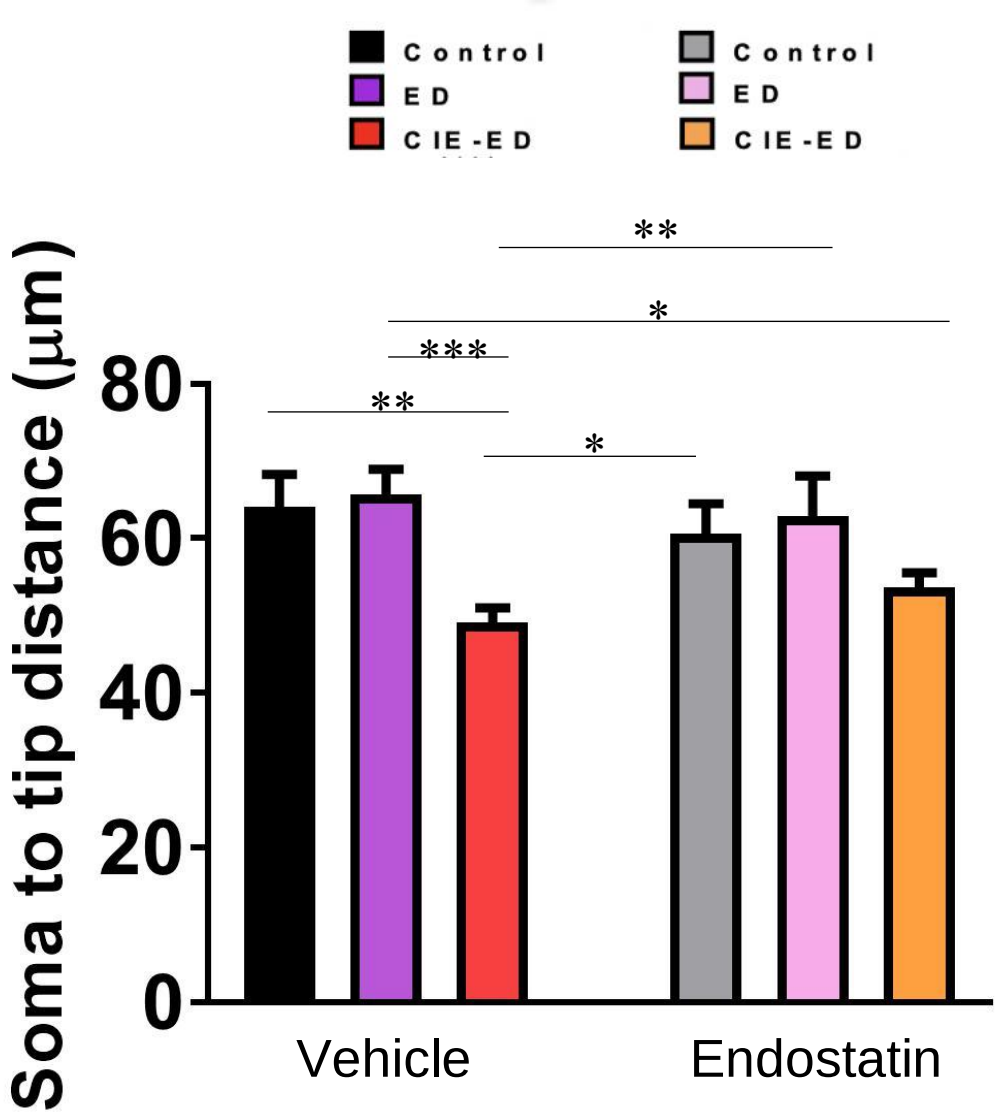

Figure 10. CIE-ED vehicle rats show a significant decrease in microglial soma to tip distance. Structural analysis of mPFC microglial cells dendrites are illustrated as soma to tip distance. Female long evans rats were treated with no ethanol drinking, chronic intermittent ethanol vapor exposure and ethanol drinking (CIE-ED) or ethanol drinking (ED). They were subcutaneously treated with saline (- control) or endostatin after maintenance. The mPFC was stained for IBA-1, a marker for microglia. The stained microglial cell bodies were traced using Neuroleucida and quantified using Neuroleucida Explorer. $^{*}p < 0.0069$ between CIE-ED vehicle and control vehicle groups by post hoc analysis. Error bars represent standard error of the mean.

RESULTS

Tail bleeding for blood Alcohol Levels (BAL) during 7 weeks of maintenance (Figure 2)

In female CIE- ED rats there is a significant increase in the blood alcohol levels to about 200-300 mg% from week 5 to 7 which represents moderate to severe alcohol use disorder. After running a two way anova with a post hoc analysis there was a significant main effect between weeks for CIE-ED rats BAL's (F_{weeks} [6,216]= 68.95, p<.0001).

Active lever responses of CIE- ED and ED rats (pre-vapor vs post-vapor) (Figure 3)

There was a significant difference in active lever responses in the CIE-ED separated groups: CIE-ED pre- vapor vs CIE- ED post- vapor ($t_{(45)}$= 6.499; p< .0001). There was less of a significant difference in active lever presses in the ED separated groups: ED pre- vapor vs ED post-vapor ($t_{(29)}$= 3.169; p= .0037).

Active lever responses of CIE-ED and ED rats (saline vs endostatin) during relapse (Figure 4)

There was a significant difference in active lever responses in the CIE- ED separated groups: CIE- ED saline vs CIE-ED endostatin ($t_{(16)}$= 2.547; p=.0223). There was not a significant difference in active lever responses in the ED separated groups: ED saline vs. ED endostatin ($t_{(12)}$= .1643; p=.8724).

Active lever responses of CIE-ED and ED rats (saline vs endostatin) during day 6 of extinction and reinstatement Figure (5)

A two-tailed t- test was used to compare the different experimental groups. There was a significant difference between CIE-ED saline rats in day 6 of extinction compared to CIE-ED saline rats in reinstatement ($t_{(18)}$= 2.198; p= .0421). There was not a significant difference in CIE-ED endostatin rats in day 6 of extinction compared to CIE-ED endostatin rats in reinstatement ($t_{12)}$= 1.283; p= .2258). There was a significant difference in ED saline rats in day 6 of extinction compared to CIE-ED saline rats in reinstatement ($t_{12)}$= 4.508; p= .0009). There was also a significant difference in ED endostatin rats in day 6 of extinction compared to ED endostatin in reinstatement ($t_{(12)}$= 4.883; p= .0005).

Immunohistochemistry of PECAM-1 (Figure 6)

Two way anova and post hoc analysis revealed that there was a significant interaction between alcohol treatment x endostatin on PECAM-1 expression ($F_{\text{alcohol X endostatin}}$ [2, 58)= 7.861, p=.0010). The analysis also showed a main effect of endostatin ($F_{\text{endostatin}}$ [1, 58]= 26.82, p<.0001) and alcohol (F_{alcohol}[2,58]= 7.244, p=.0016).

Uncorrected fisher's LSD showed a significant increase in PECAM expression in CIE-ED vehicle rats compared to control vehicle and ED vehicle rats. (p<.0001). There is also a significant decrease in PECAM-1 expression in ED endostatin rats compared to ED vehicle (p=.0231). The CIE-ED endostatin rats showed a significant decrease in PECAM-1 expression compared to CIE-ED vehicle rats (p<.0001).

Phenotypic analysis of the number of intersections relative to the distance from soma (Figure 7)

A two-way anova and post hoc analysis revealed that there was a significant interaction between alcohol treatment x endostatin on number of intersections relative to the distance from soma ($F_{\text{alcohol X endostatin}}$ [200,2280]= 3.115, p<.0001). The analysis shows a significant main effect on the number of intersections relative to distance from soma ($F_{\text{Number of intersections relative to the distance from soma}}$[40,2280]= 250.4, p<.0001), alcohol (F_{alcohol}[5,57]= 6.289, p=.0001), and subjects (F_{subjects}[57,2280]= 47.52, p<.0001).

Phenotypic analysis of total dendritic length (Figure 8)

A two-way anova and post hoc analysis revealed that there was a significant interaction between alcohol treatment x endostatin on total dendritic length ($F_{\text{alcohol x endostatin}}$[2,57]= 3.7, p=.0288). There was not a significant main effect of endostatin on total dendritic length ($F_{\text{endostatin}}$[1,57]= .2, p= .5944). There was a significant main effect of alcohol on total dendritic length (F_{alcohol}[2,57]= 13, p<.0001).

Uncorrected fisher's LSD showed a significant decrease in total dendritic length in CIE-ED vehicle compared to control vehicle (p=.0009). There was also a significant decrease in total dendritic length in CIE-ED vehicle compared to ED vehicle (p<.0001).

Phenotypic analysis of microglial cell soma area (Figure 9)

A two-way anova and post hoc analysis revealed that there was not a significant interaction between alcohol treatment x endostatin on cell soma area ($F_{\text{alcohol X endostatin}}$ [2,57]= 2.238, p= .1160). The analysis shows no significant main effect of alcohol on cell

soma area ($F_{alcohol}[2,57]=2.702$, p=.0757). The analysis also revealed a significant main effect of endostatin ($F_{endostatin}[1,57]= 4.719$, p=.0340).

Uncorrected fisher's LSD showed a significant increase of microglial cell soma area in CIE-ED vehicle compared to control vehicle (p=.0087). There was also a significant increase in cell soma area in CIE-ED vehicle compared to ED vehicle (p=.0174). Furthermore, Uncorrected fisher's LSD showed a significant decrease in cell soma area in CIE-ED endostatin compared to CIE-ED vehicle (p=.0014).

Phenotypic analysis of microglial soma to tip distance (Figure 10)

A two-way anova and post hoc analysis revealed that there was not a significant interaction between alcohol treatment x endostatin on soma to tip distance ($F_{alcohol\ x\ endostatin}[2,59]= .9215$, p=.4036). The analysis shows no significant main effect of endostatin on soma to tip distance ($F_{endostatin}[1,59]=.03173$, p=.8592). Furthermore, a two-way anova and post hoc analysis showed a significant main effect of alcohol treatment on soma to tip distance ($F_{alcohol}[2,59]= 9.451$, p=.0003).

Uncorrected fisher's LSD showed a significant decrease in soma to tip distance in CIE- ED vehicle compared to control vehicle (p=.0069). There was also a significant decrease in soma to tip distance in CIE-ED vehicle compared to ED vehicle (p=.0002).

DISCUSSION

The first goal of this study was to determine the effects of chronic ethanol exposure on microglia during protracted abstinence in female rats. The second goal of the study was to determine whether endostatin reduced microglial activation in CIE-ED rats during protracted abstinence. We hypothesized that alcohol indirectly activated microglia in CIE-ED rats and that endostatin limited ethanol seeking behavior in CIE-ED rats by indirectly deactivating microglia.

Here, we report that CIE-ED causes elevated blood alcohol levels to about 200-300 mg% from weeks 5-7 during maintenance (Figure 2). Additionally, active lever pressing was taken at two time points before vapor exposure and after vapor exposure. Active lever pressing significantly increased in CIE-ED rats after vapor exposure (Figure 3). Previous papers support that elevated blood alcohol levels and the increase in active lever pressing after vapor exposure mimic a dependent like behavior to alcohol, and therefore, confirm a model of moderate to severe alcohol use disorder (Nicholas W. Gilpin et al., 2008).

We can also report that endostatin significantly decreased active lever pressing in CIE-ED endostatin rats compared to CIE-ED vehicle (Figure 4). Endostatin's downstream effects are influencing ethanol seeking behavior in only alcohol dependent rats.

Furthermore, we looked at behavior in terms of active lever pressing during day 6 of reinstatement and extinction. This behavior was done to see if females maintained active lever pressing after extinction and during reinstatement. In both CIE-ED saline and ED saline rats they increased active lever responses during reinstatement (Figure

5). But with the endostatin injection CIE-ED endostatin rats were able to decrease reinstatement by decreasing active lever responses. Endostatin was not able to decrease reinstatement in ED endostatin rats seen. This shows that endostatin is specific to the dependent state.

Previous studies from our lab have demonstrated that during protracted abstinence CIE-ED rats are associated with hyperoligodendrogenesis (Somkuwar et al., 2016). Hyperoligodendrogenesis is associated with an upregulation of PECAM-1 (Somkuwar et al., 2016). Furthermore, PECAM-1 leads to BBB leakage and cytokine release (Somkuwar et al., 2017). This cytokine release can cause neuronal damage in the mPFC and influence behavior. We have made significant findings on microglial activation due to cytokine release and microglial deactivation by indirectly reversing the effects of chronic ethanol exposure. This is the first study that reveals the relationship between endostatin and reduced microglial cell body size during protracted abstinence in CIE-ED rats.

PECAM-1 expression in CIE-ED and endostatin rats mirror previous findings (Mandyam et al., 2017). CIE-ED vehicle increases PECAM-1 expression compared to the control vehicle. CIE-ED vehicle increased PECAM-1 at a higher magnitude than ED vehicle. Endostatin was able to decrease PECAM-1 expression in all experimental groups (Figure 6). A few mechanisms are speculated for increased PECAM-1 expression in dependent rats. For example, the consumption of alcohol produces acetaldehyde in the BBB. Acetaldehyde enhances xanthine oxidase and cytochrome-P450 expression which produces ROS. ROS induces oxidative stress causing cytokines

to transmit signals to the PECAM-1 promoter which increases the generation and upregulation of PECAM-1 (Mandyam et al., 2017).

We were also able to use the 3-dimensional sholl analysis to visualize number of intersections relative to the distance from the soma. We found a significant decrease in CIE-ED vehicle intersections compared to all other experimental groups (Figure 7). ED vehicle and ED endostatin rats did not affect the intersections relative to the distance from the soma. (Figure 7). This finding agrees with previous studies that there is a significant decrease at every intersection which indicates microglial activation in CIE-ED rats (Streit, n.d.). However, endostatin was able to reduce microglial activation; this was evident by the phenotypic change in Iba-1 cells in endostatin treated rats (increasing the number of intersections relative to the distance from the soma in CIE-ED rats).

Using 3 dimensional sholl analysis we were able to visualize the microglial cell soma area. An increased cell soma area that takes on an ameboid like form is a hallmark of microglial activation (Davis et al., 2017). This study was able to confirm this finding, where the CIE-ED vehicle rats showed an increase in cell soma area compared to the control vehicle rats concluding activated microglia (Figure 9). CIE-ED endostatin decreased cell soma area back to control vehicle levels. It is also important to note that cell soma area did not change in ED rats compared to the control. ED vehicle and ED endostatin did not have any effect on cell soma area (Figure 9). CIE-ED increased cell soma and activated microglia in CIE-ED rats. Endostatin was able to rescue cell soma area in only CIE-ED rats, and therefore, deactivate microglia back to resting levels.

Furthermore, the results show a significant decrease in total dendritic length and soma to tip length in CIE-ED vehicle rats compared to control vehicle (Figure 8,10).

Retracted microglial processes is a phenotypic characterization of microglial activation (Davis et al., 2017). ED vehicle and ED endostatin did not have any effect on total dendritic length and soma to tip distance (Figure 8,10). Therefore, CIE-ED vehicle activates microglia.

Endostatin did not have a significant impact on the control or non- dependent rats. This suggests that endostatin only works on rats with moderate to severe alcohol use disorder. Our findings suggest a targetable method of action to limit alcohol seeking behavior by targeting PECAM-1.

We believe that endostatin may be affecting the transcription of PECAM-1. Endostatin can reverse the impact of PECAM-1 by preventing angiogenesis (Jia et al., 2005). The results suggest that endostatin indirectly downregulates microglia and decreases ethanol seeking behavior. We speculate that the decrease in PECAM-1 is maintaining BBB integrity and reducing cytokine release. The decrease in cytokines limits a neuroinflammatory immune response, and therefore, microglia remains deactivated, as indicated by microglial cell soma area. The deactivation of microglia limits neuronal inflammation by limiting the production of interleukins and leukocytes (Streit et al., 2004). In sum, although correlational, these findings support the role of endostatin in the mPFC in modulating microglial activation and reinstatement of ethanol seeking in the context of chronic ethanol intake.

Future experiments will study which cytokines are causing the activation of microglia. An Elisa kit will be used to determine the cytokines involved. Cytokines could also be inhibited to see if endostatin was targeting cytokines directly or indirectly.

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