UNIVERSITY OF CALIFORNIA SAN DIEGO

Effect of Endostatin on Microglial Activation and Ethanol Seeking Behavior in Dependent Rats

A thesis submitted in partial satisfaction of the requirements
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Biology

by

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The thesis of Tejash Bharadwaj is approved, and it is acceptable in quality and form for publication on microfilm and electronically.

University of California San Diego

2021

DEDICATION

I dedicate this thesis to my family and my cousin Pragya for their continual support and love.

EPIGRAPH

"Your work is going to fill a large part of your life, and the only way to be truly satisfied is to do what you believe is great work. And the only way to do great work is to love what you do. If you haven't found it yet, keep looking. Don't Settle."

Steve Jobs

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I would also like to thank my committee members Professor Brenda Bloodgood and Professor Stacey Glasgow for providing me guidance through the project.

Material from this thesis is currently being prepared for submission for publication of the material. Tejash Bharadwaj was the sole author of this material.

ABSTRACT OF THE THESIS

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by

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Master of Science in Biology

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Professor Chitra Mandyam, Chair
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Alcohol use disorder, or AUD, is a brain disorder that affects millions of people with few FDA approved effective treatments at reducing relapse, with none preventing relapse episodes. Therefore, more research is necessary in order to find a targetable biochemical pathway for therapeutics to treat AUD and prevent relapse in alcoholics. Recent data in rodent models of moderate to severe AUD has demonstrated a correlation between higher levels of ethanol seeking behavior and increased levels of PECAM-1 in the prefrontal cortex of the brain, a region implicated in relapse to alcohol seeking. More notable is that increases in PECAM-1 is

associated with blood brain barrier (BBB) leakage, suggesting endothelial cell damage in relapsing alcoholics. Furthermore, published work from rodent studies and human postmortem tissue analysis have indicated severe neuroimmune responses in the brain that is correlated with increased relapse to drinking behavior. However, it remains unclear whether the activation state of microglia, a major player in the neuroimmune response that occurs due to alcohol metabolism, is associated with BBB leakage and endothelial cell damage in the prefrontal cortex. It was hypothesized that endostatin, an angiogenic inhibitor, would inhibit PECAM-1 and thus decrease BBB leakage. This was expected to reduce the peripheral neuroimmune response and prevent alteration in microglial activation. In order to test this, the brain tissue of male rats with moderate to severe AUD were analyzed. Specifically, quantitative immunohistochemistry and stereological cell analysis were used to evaluate microglial activation which was positively correlated to ethanol seeking behavior from the additional behavioral studies that were performed. It was found that endostatin reduced PECAM-1 expression in the prefrontal cortex, however, did not decrease ethanol seeking behavior of male rats. Additionally, microglial activation remained similar to the rats that were not treated with endostatin. Therefore, it is suggested that the peripheral immune response mediated by PECAM-1 is not directly linked to ethanol seeking behavior and microglial activation in the prefrontal cortex in male rats. From this, a new study investigating the internal neuroinflammation or immune response could help determine the mechanism to decrease microglial activation and thus lower relapse. This would be a significant advance in the effort to create an effective therapeutic for AUD.

INTRODUCTION

Alcohol use disorder (AUD) is a major societal and economic burden for the United States. AUD is a disorder characterized by the inability to refrain from alcohol consumption despite the consequences it has on one's health, occupation, or social life. (*Understanding Alcohol Use Disorder*, n.d.). There were approximately 15 million Americans that were diagnosed with AUD in 2018 (*Alcohol Use Disorder*, n.d.). The economic impact this disorder has is equally as bad with damages estimated to be 249 billion dollars (Office of the Surgeon General, 2019). Since AUD is considered a brain disorder due to the fact that it directly manipulates the reward pathway in the brain, there are very specific behavioral guidelines used to diagnose this condition that are outlined in the Diagnostic and Statistical Manual of Mental Disorders (*Alcohol Use Disorder: A Comparison Between DSM–IV and DSM–5*, n.d.). Anyone who meets two out of the eleven criteria within the span of a year is diagnosed with AUD (*Alcohol Use Disorder*, n.d.). AUD has the potential to destroy every aspect of one's life ranging from personal health and relationships to one's occupational prospects. Continuing to understand how AUD affects the brain and body and applying this knowledge to provide solutions for this complex disorder will improve the lives of millions in the United States and around the world.

AUD causes the dysregulation of various parts of the brain resulting in a cyclical phenotype that is characterized by three stages (Koob & Volkow, 2016); binge and intoxication, withdrawal and negative affect, and the preoccupation and anticipation stage. The preoccupation and anticipation stage involves severely compromised executive function like decision making which occurs in the prefrontal cortex (PFC) (Koob & Volkow, 2016). AUD is a complex disorder that not only involves the brain itself but also its vasculature. A key component of the brain that is affected by AUD is the blood brain barrier (BBB). The blood brain barrier serves a

dual function: to vascularize the brain and to tightly regulate the flow of molecules and ions between the blood and the brain (Daneman & Prat, 2015). Alcohol is able to readily diffuse through the lipid bilayer of the blood brain barrier and is metabolized to acetaldehyde and reactive oxygen species (ROS) (Kovacic & Cooksy, 2005). The disruption in blood brain barrier integrity due to alcohol related ROS results in altered glial plasticity of a variety of glial cells such as oligodendroglia and microglia in the PFC (Mandyam et al., 2017). Alterations in the plasticity of the PFC has been shown to impair executive function, which increases the probability of relapse (Trantham-Davidson & Chandler, 2015). Specifically, in the PFC, there is considerable evidence to show that the medial prefrontal cortex (mPFC) is involved in reinforcement behavior, or the pleasant feelings one gets after consumption of drugs or alcohol. Rodent studies have shown that the mPFC is also involved in the acquisition of drugs via lever pressing. (Van den Oever et al., 2010). Routtenberg and Sloan (1972) showed that using intracranial self-stimulation in the mPFC proved rewarding for the rats. The results showed that the rats experienced rewarding behavior as they would constantly press a lever to experience the electrical stimulation in the mPFC (Routtenberg & Sloan, 1972). Alterations in BBB structure in the context of AUD have hardly been studied, but are an important component in the physiology of the disorder. Therefore, studying the BBB in conjunction with the mPFC and the structural changes in the glia that accompany AUD, will allow us to see how the mPFC is altered, and if such alterations correlate with an increased propensity for relapse to alcohol.

In order to study the mPFC, rodent models are frequently used due to the fact that they are a functional homolog of the human medial prefrontal cortex (Vertes, 2006). Rats are also an excellent animal to use for alcohol studies because they compulsively drink and also express anxiety similarly to humans (Roberts et al., 2000). A variety of alcohol dependence models that

exist have been established with rats. An important model used to reliably induce alcohol dependence or AUD is alcohol vapor exposure in a vapor chamber (Gilpin et al., 2008). In order to develop alcohol addiction in rats, these animals must be exposed to chronically high levels of alcohol in one of two ways: chronic intermittent ethanol exposure (CIE) or chronic continuous ethanol exposure (Gilpin et al., 2008). Chronic continuous vapor refers to ethanol vapor being continuously on while the rat is in the vapor chamber, while CIE refers to ethanol vapor being activated for a certain number hours a day and then turned off for a certain number of hours a day (Gilpin et al., 2008). It has been shown that the chronic intermittent ethanol exposure is a superior model to develop dependence due to increased self-administration of alcohol after CIE exposure (O'Dell et al., 2004). After CIE exposure, the rats can be used to measure susceptibility of relapse during abstinence and withdrawal stages (Gilpin et al., 2008). Relapse can be simulated using the extinction-reinstatement model. The extinction phase extinguishes the rats' responses to ethanol-related environmental stimuli, and the reinstatement phase reintroduces these environmental stimuli in order to evoke a drug seeking response similar to relapse (Somkuwar et al., 2016). After this, the rats are euthanized and brain tissue can be extracted and analyzed to study in the context of relapse (Somkuwar et al., 2016).

It has been shown that rats that undergo chronic ethanol exposure demonstrate reduced blood brain barrier integrity (Somkuwar et al., 2017). Oxidative damage due to alcohol metabolism in the brain microvascular endothelial cells in the BBB causes BBB leakage resulting in a neuroinflammatory response due to various cytokines, chemokines, and leukocytes flooding in through the leaks of the BBB (Haorah et al., 2005; Mandyam et al., 2017). The metabolism of alcohol and the neuroinflammatory response it produces are mediated by a key molecule: platelet endothelial cell adhesion molecule (PECAM -1).

The interaction between the immune cells of the brain and the endothelium of the blood brain barrier is facilitated by adhesion proteins (Mandyam et al., 2017). PECAM-1 is a transmembrane adhesion protein that is expressed on the surface of the cell membrane and between the junctions of the cells of the blood brain barrier (Mandyam et al., 2017). PECAM-1 also mediates a pro-inflammatory response by allowing white blood cells to migrate through the blood brain barrier (Privratsky et al., 2010). It has been shown that rats under alcoholic oxidative stress, during protracted abstinence, had upregulated levels of PECAM-1 expression. This PECAM-1 expression in the mPFC has a positive correlation with BBB leakage indicating its role in compromising blood brain barrier integrity (Somkuwar et al., 2017).

As previously mentioned, rats during protracted abstinence experience an upregulation of PECAM-1 along with BBB leakage in the mPFC. The BBB leakage results in a peripheral immune response mediated by PECAM-1 and cytokines that flood into the BBB. This neuroinflammatory response is detected by receptors on microglia which causes these glial cells to activate (Kelley & Dantzer, 2011). Microglia are glial (non-neuronal) cells in the central nervous system which function as resident macrophages and are also involved in adaptive immunity. Activated microglia in the mPFC secrete various cytokines which further progresses the neuroinflammation in the brain due to chronic alcohol exposure (Walter et al., 2017). These glial cells perform a myriad of functions in the brain. During neuroinflammation they gain a reactive phenotype in the mPFC where they uncontrollably attack neurons and become neurotoxic (Kovacs, 2012). The neuroinflammation caused by PECAM-1 upregulation due to BBB leakage and microglia activation will ultimately alter the plasticity of the mPFC and could result in profound change in the reward pathway that contributes to relapse (Kovacs, 2012). Very few studies have considered the role of PECAM-1 and microglia in the context of alcohol use

disorder (Mandyam et al., 2017). Understanding the role of PECAM-1 in relation to neuroinflammation due to AUD could present us with new targetable mechanisms to prevent and hopefully reverse the glial plasticity changes of AUD in the brain of humans.

AUD conditions simulated in rodent models under a CIE paradigm have been shown to parallel the neuropathological conditions which occur in the brain of human alcoholics. There is strong clinical evidence which shows that the neuroimmune response facilitated by microglia plays an important role in the neuropathology we see in AUD in both rats and humans. In fact, previous research has shown that depletion of microglia in rats prevented escalation in voluntary alcohol intake without altering drinking in nondependent mice (Warden et al., 2020). Chronic ethanol exposure has shown increased immunoreactivity of Iba – 1, a protein expressed only in microglia, in the brains of human alcoholics, which is associated with heightened microglial activation. (He & Crews, 2008). However, this microglia activation is extremely dynamic in nature and thus various structural biomarkers such as soma size and branching complexity aid us in determining activation. Rodent studies using a CIE model done by Siemsen et al., 2020 have shown us that microglia activation characteristics are brain region dependent, and therefore a standard model cannot be used to identify activated microglia. For example, in the aforementioned study, it was found that the CIE rats did not show increased cell soma size, one of the characteristics of microglial activation, in the prelimbic (PL) cortex. However, there was a significant decrease in dendrite complexity, which is another characteristic of microglial activation in the PL cortex. This change was completely absent in the core of the nucleus accumbens, showing the brain region dependent effects of microglia. Due to the fact that activation characteristics of microglia cannot be pinpointed, it is likely that the CIE model in this

experiment will present varied characteristics of microglial activation which we hope to reverse using endostatin.

Unpublished observations in our lab have shown that endostatin, an angiogenic protein inhibitor, inhibits PECAM-1 and therefore reduces BBB leakage which results in reduced neuroinflammation. Endostatin is a 20kDa C-terminal fragment of type 18 collagen and is one of the most potent endogenously produced inhibitors of angiogenesis (Walia et al., 2015). Knockout studies of COL18A1 a gene that produces type 18 collagen has shown many deleterious effects including but not limited to softened glomeruli and delayed hyaloid vessel regression during development (Fukai et al., 2002; Kinnunen et al., 2011). This confirms the previous assertion that endostatin does indeed have significant antiangiogenic effects. However, the question still remains - how is microglia activation affected by the decreased neuroinflammation from the inhibitory effects of endostatin on PECAM-1? This question will help us determine whether the resident immune cells of the brain play a critical role in the complex biochemical pathway of relapse. As such, the aim of this study is to understand whether endostatin will be able to indirectly inhibit microglial activation and whether this biochemical effect will be able to significantly decrease the frequency of relapse. To answer this question, male rats were subjected to chronic ethanol exposure in a model similar to Somukwar et al., 2016. They were then given a subcutaneous injection of endostatin and forced into protracted abstinence using the extinction-reinstatement model which measures relapse. In order to study microglial activation, the rat's brain tissue was stained with Iba-1, a marker for microglia. A 3D modeling software was used to trace the microglia to see if they exhibited activation characteristics such as increased cell body size, reduced arborization, reduced number of intersection per unit distance from the soma, and reduced total dendritic distance (Wang et al., 2018).

MATERIALS AND METHODS

2.1: Animals

Fifty-four adult male 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-four 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=21) and ED (nondependent, n=23) 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 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

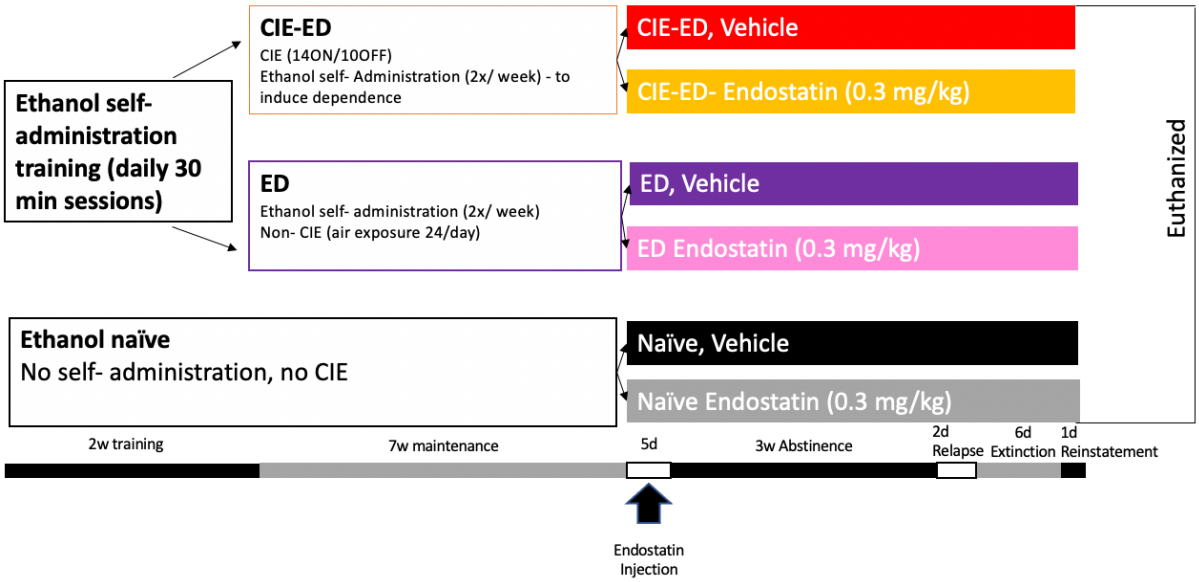

Figure 1. Schematic of experimental design. In the experiment male long evans rats were divided into three groups: one that was CIE (chronic ethanol exposure) (n = 21) to model moderate to severe AUD, an ED group (n = 23) which modeled casual drinking, and a control (n = 10) which wasn't given any alcohol. The CIE-ED and ED groups were given 2 weeks of training to self-administer alcohol via lever pressing. Then 7 weeks of maintenance, which means the CIE-ED rats were exposed to 14hr:10hr ratio of ethanol vapor to clean air while the ED rats were exposed to clean air 24hrs a day. Both alcohol groups are put in a box to self-administer ethanol twice a week. All three groups were subcutaneously given an endostatin or saline injection for 5 days. After the rats went through two days of relapse then 3 weeks of extinction followed a one-day period of reinstatement. After which, the rats were euthanized.

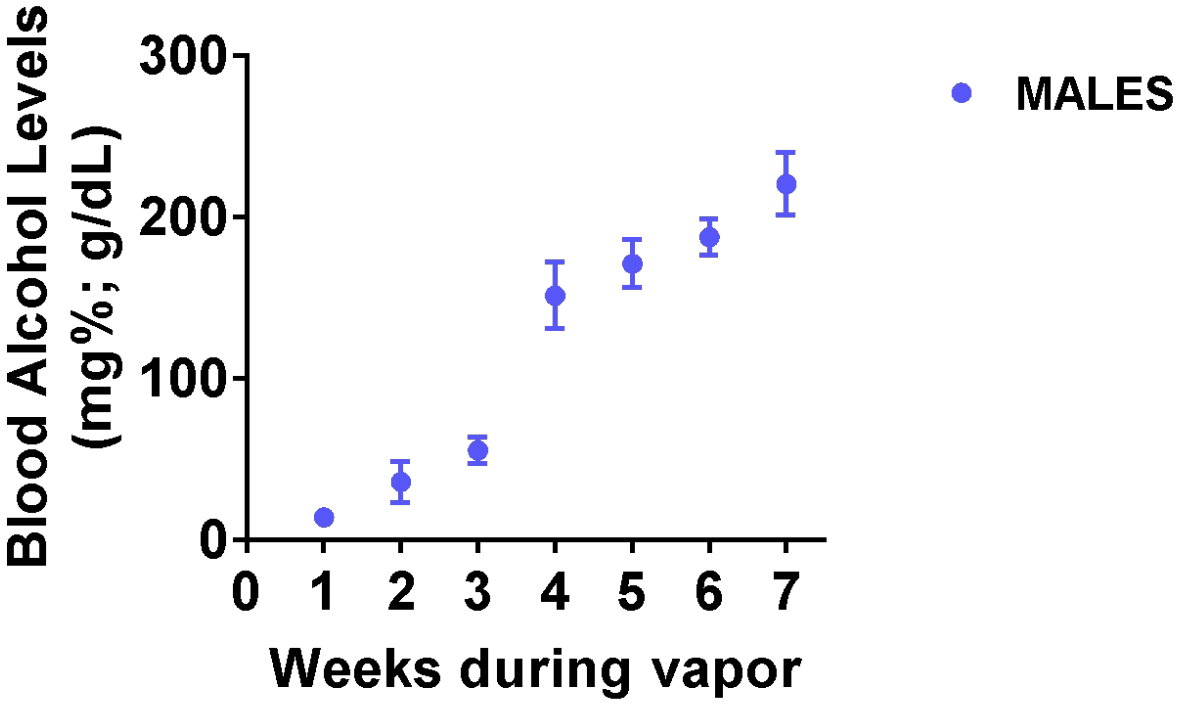

Figure 2. CIE-ED rats had a significant increase in blood alcohol levels (BAL) during week 4 through 7 to mimic moderate to severe alcohol use disorder. BAL was measure through tail bleeding once a week over the 7-week maintenance period. The error bars represent the standard error of the mean.

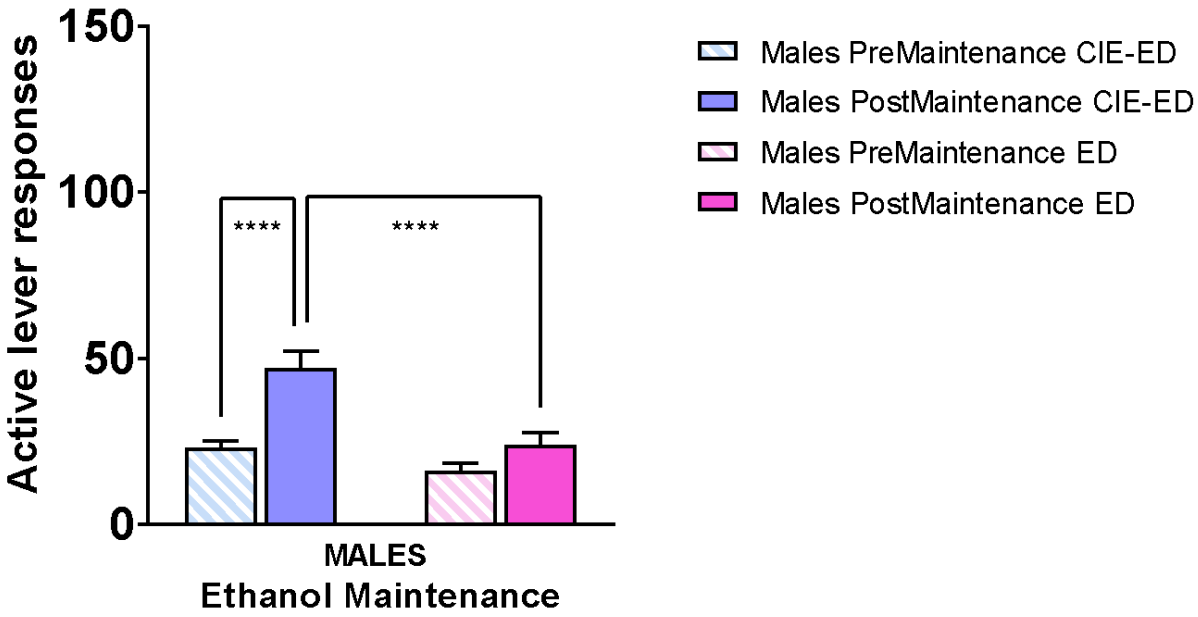

Figure 3. Chronic induced ethanol vapor exposure and ethanol drinking (CIE-ED) significantly increased drinking behavior. The figure shows active lever responses that were taken before vapor exposure and 7 weeks after vapor exposure in both alcohol dependent rats (CIE-ED) and alcohol nondependent rats (ED). $P < .0001$ between CIE-ED pre-vapor and post-vapor groups from post hoc analysis. There was no significant difference in active lever presses between pre-vapor and post-vapor ED.

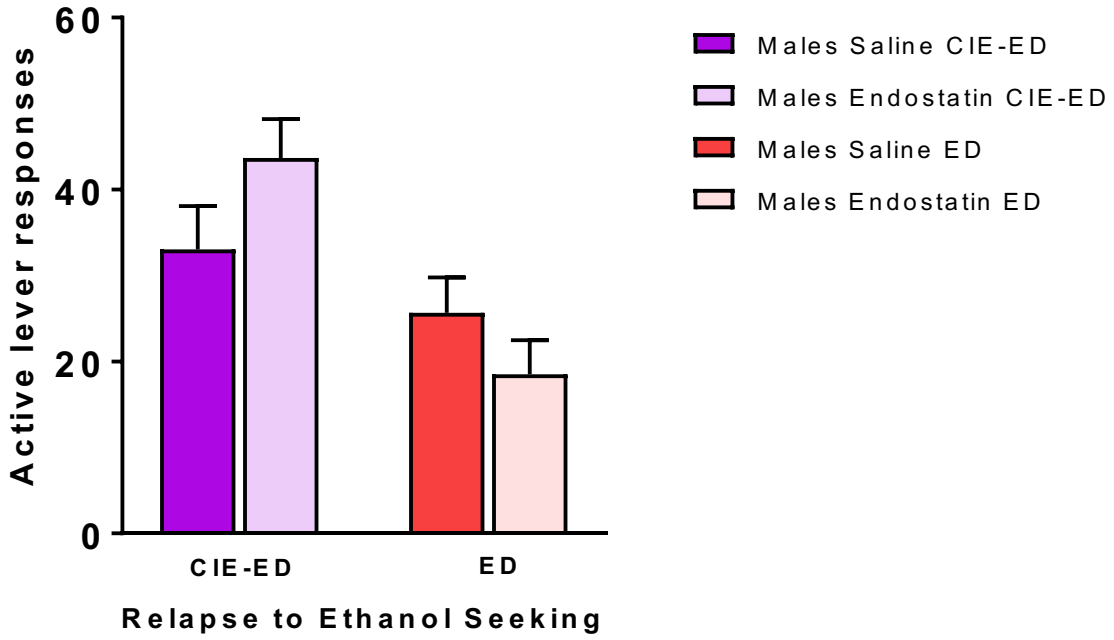

Figure 4. Endostatin did not significantly increase or decrease ethanol seeking behavior during relapse. The graphs above show the active lever responses (the lever that dispensed alcohol) during a single relapse session in both alcohol dependent CIE-ED rats and alcohol non-dependent ED rats that were both treated with saline or endostatin. The bar represents the mean of each group while error bars represent one standard deviation away from the mean.

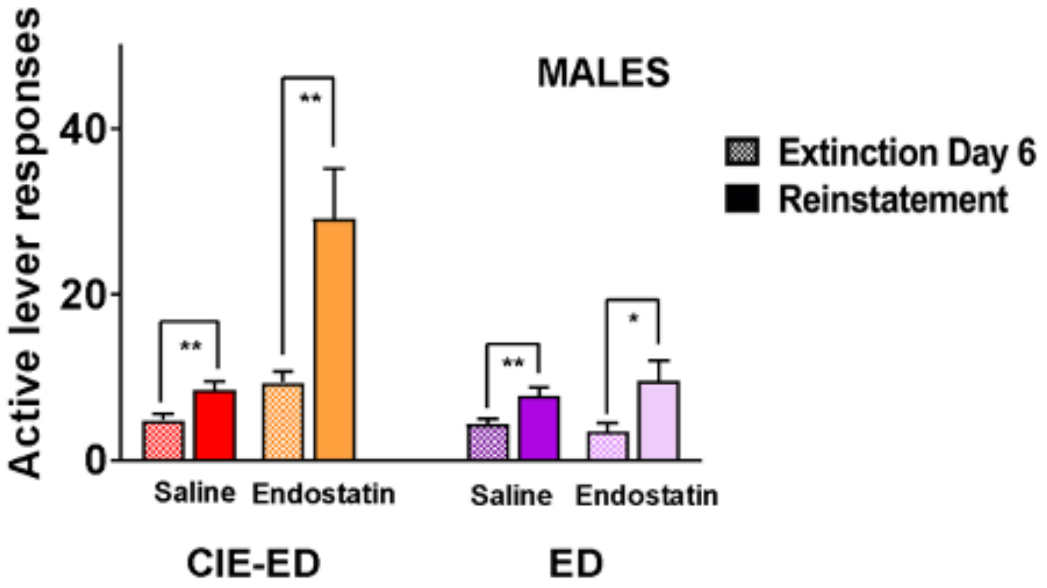

Figure 5. There was a significant increase in active lever responses during reinstatement in the CIE-ED and ED groups regardless of endostatin treatment. Active lever responses were measured during day 6 of extinction and reinstatement. The groups that were measured are alcohol dependent (CIE-ED) rats that were treated with saline and endostatin, and alcohol non-dependent ED rats that were treated with saline and endostatin. *$p < .05$ between the CIE-ED saline and CIE-ED endostatin. The error bars represent the standard error of the mean.

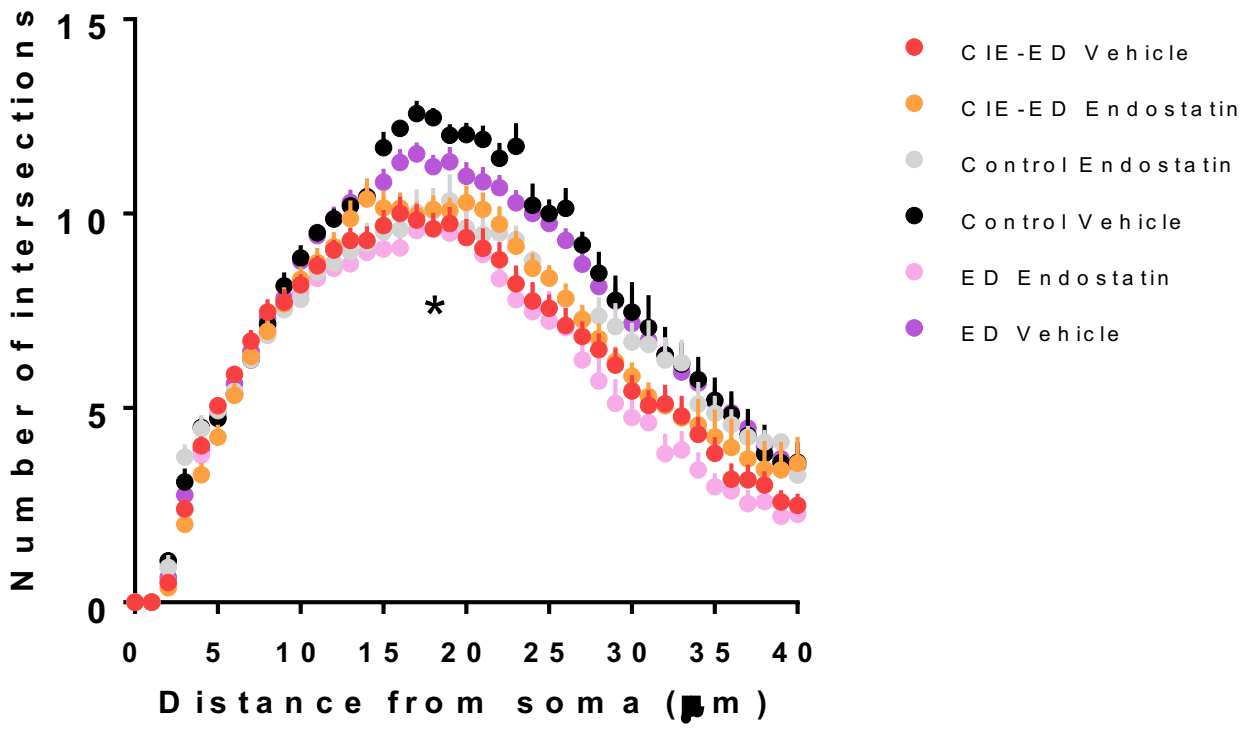

Figure 6. CIE-ED Vehicle rats show a significant decrease in the number of intersections relative to distance from the soma compared to Control Vehicle. The structural analysis of the microglia are shown in this graph as the number of intersection relative to the distance from the soma. The three groups of male Long Evans rats were the control which consumed no ethanol, chronic intermittent ethanol vapor exposure and ethanol drinking (CIE-ED), and ethanol drinking (ED). The three groups of rats were subsequently treated with saline (- control) and endostatin subcutaneously after the maintenance period. The mPFC was stained for IBA – 1, a marker for microglia. Stereochemical analysis was done using Neurolucida and data was extracted using Neurolucida Explorer. The error bars represent the standard error of the mean.

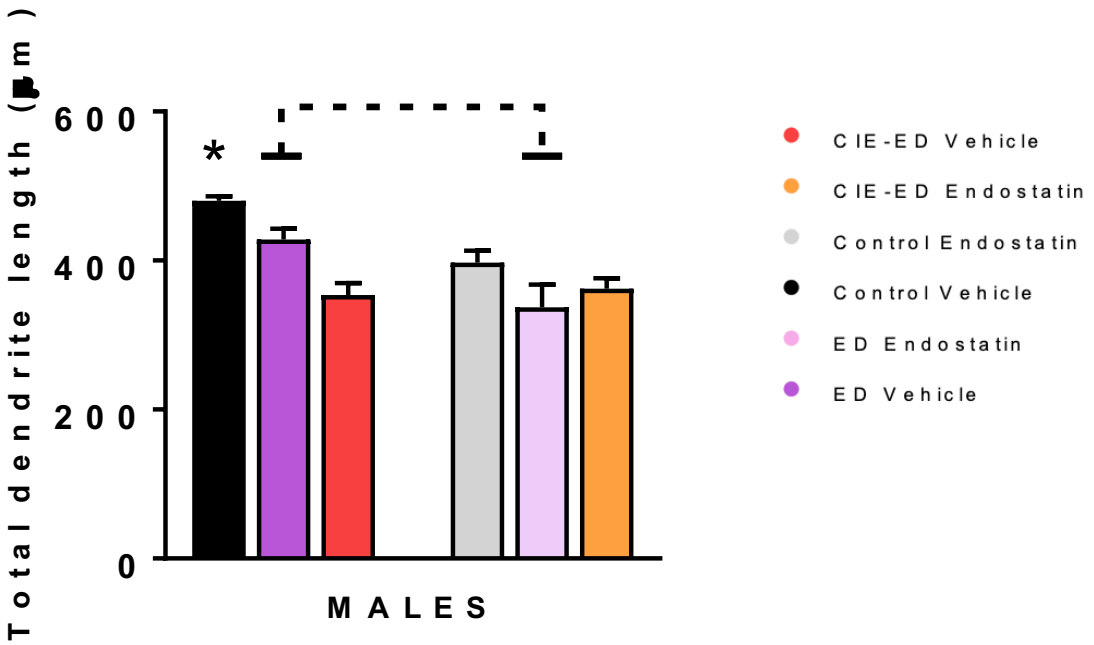

Figure 7. CIE-ED vehicle rats show a significant decrease in total dendrite length in microglia. The three groups of male Long Evans rats were the control which consumed no ethanol, chronic intermittent ethanol vapor exposure and ethanol drinking (CIE-ED), and ethanol drinking (ED). The three groups of rats were subsequently treated with saline (- control) and endostatin subcutaneously after the maintenance period. The mPFC was stained for IBA – 1, a marker for microglia. Stereochemical analysis was done using Neurolucida and data was extracted using Neurolucida Explorer. There was a significant decrease in dendritic length between the control vehicle and CIE-ED vehicle ($p < .0001$).

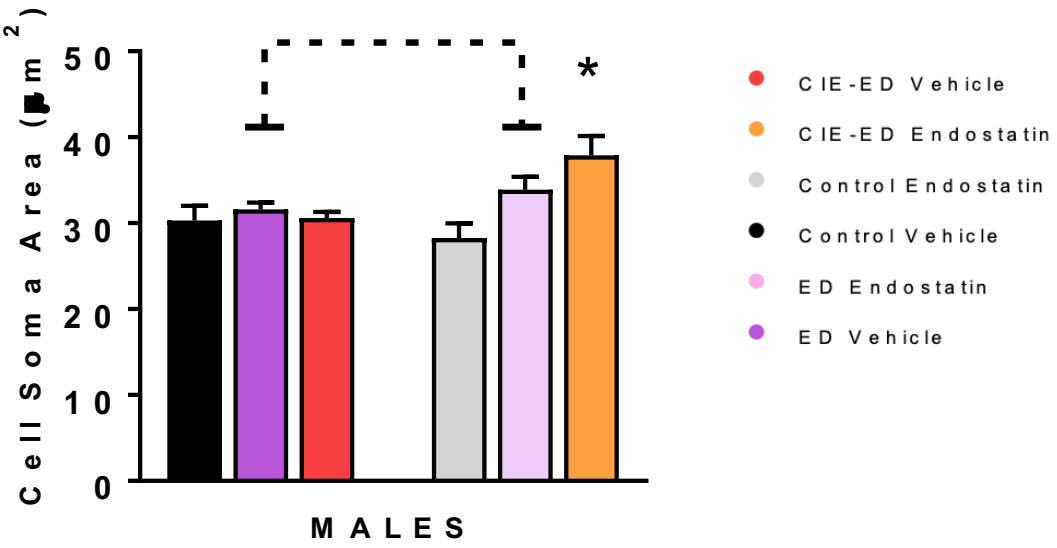

Figure 8. Endostatin significantly increases cell soma area in ED and CIE-ED rats. The three groups of male Long Evans rats were the control which consumed no ethanol, chronic intermittent ethanol vapor exposure and ethanol drinking (CIE-ED), and ethanol drinking (ED). The three groups of rats were subsequently treated with saline (- control) and endostatin subcutaneously after the maintenance period. The mPFC was stained for IBA – 1, a marker for microglia. Stereochemical analysis was done using Neurolucida and data was extracted using Neurolucida Explorer. There was a significant increase in cell soma area between the CIE-ED vehicle and CIE-ED endostatin groups ($p < .0001$).

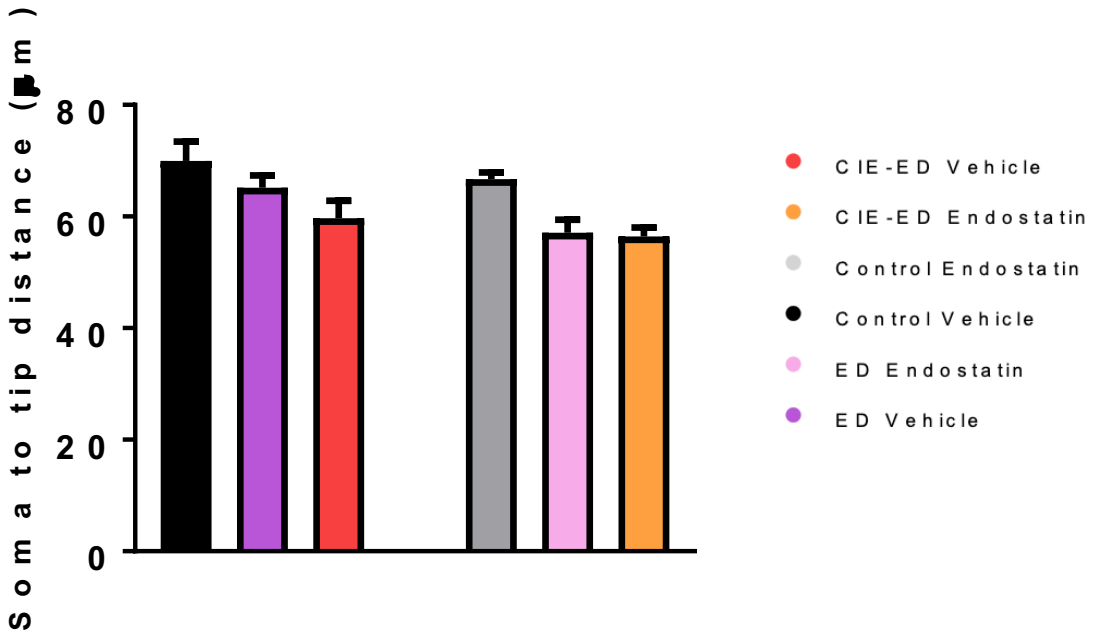

Figure 9. CIE-ED Vehicle rats show a significant decrease in soma to tip distance. The three groups of male Long Evans rats were the control which consumed no ethanol, chronic intermittent ethanol vapor exposure and ethanol drinking (CIE-ED), and ethanol drinking (ED). The three groups of rats were subsequently treated with saline (- control) and endostatin subcutaneously after the maintenance period. The mPFC was stained for IBA – 1, a marker for microglia. Stereochemical analysis was done using Neurolucida and data was extracted using Neurolucida Explorer. There was a significant decrease in soma to tip distance between the CIE-ED vehicle and control vehicle groups ($p = .0353$). Errors bars represent the standard error of the mean.

RESULTS

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

In male CIE-ED rats there was a significant increase in the BAL to about 1250-200% from weeks 3 to 4 which represents moderate to severe AUD. A two way ANOVA followed by post hoc analysis revealed a significant main effect between weeks and BAL's for the CIE-ED rats.

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 ($p < .0001$). There was not a significant difference in active lever presses in the ED pre-vapor or pos-vapor ($p > .05$).

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

To investigate the effects of endostatin on relapse in male rats, we treated two groups of rats: one with a dependent phenotype (CIE-ED) and one with a non-dependent phenotype (ED) subcutaneously with endostatin injections. Saline was used as a negative control as it was the vehicle for the endostatin injections. There was a significant increase in lever pressing for the CIE-ED compared to ED for both the saline and endostatin groups indicating that the CIE-ED rats were in fact dependent (two tailed t-test, $p < .05$). After a 3-week abstinence period the rats were put into operant-conditioning chambers with two levers: an inactive and active lever. The active lever dispensed a small amount of alcohol while the inactive lever didn't. The active lever count was measured to quantify ethanol seeking behavior. The results showed that for both ED and CIE-ED rats there was not a significant difference in active lever pressing to their respective

saline groups ($p > .05$, two tailed t-test). Endostatin ultimately did not decrease ethanol seeking behavior in male rats.

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

A paired t-test was used to compare the different experimental groups present in this data set. There was a significant difference in lever pressing between CIE-ED saline rats on day 6 of extinction and CIE-ED saline rats in reinstatement ($t = 3.302$; $p = .0045$). There was a significant difference in lever pressing between CIE-ED endostatin rats on day 6 of extinction and CIE-ED endostatin rats in reinstatement ($t = 3.882$; $p = .0015$). There was a significant difference in lever pressing between ED saline rats on day 6 of extinction and ED saline rats in reinstatement ($t = 4.109$; $p = .0009$). There was a significant difference in lever pressing between ED endostatin rats on day 6 of extinction and ED endostatin rats in reinstatement ($t = 2.15$; $p = .0843$).

Phenotypic analysis of the number of intersections relative to the distance from soma (Figure 6)

A two way ANOVA followed by a post-hoc analysis revealed that there was no significant interaction of alcohol treatment and endostatin on the number of intersections relative to distance from the soma ($F_{\text{alcohol X endostatin}}[200,2460]= 1.103$, $p = 0.165$)

Phenotypic analysis of total dendritic length (Figure 7)

A two way ANOVA followed by a post-hoc analysis revealed that there was significant interaction of alcohol treatment and endostatin on the total dendritic length of microglia ($F_{\text{alcohol x endostatin}}[2,57]= 4.85$, $p=.0114$). Endostatin alone had a significant effect on dendritic length of

microglia ($F_{endostatin}[1,57]= 10.32$, p= .0022). Alcohol alone had a significant effect on dendritic length of microglia ($F_{alcohol}[2,57]= 7.08$, p = .002).

Uncorrected Fischer's LSD showed a significant decrease in dendritic length between the control vehicle and CIE-ED vehicle (p < .0001). There was also a significant decrease in dendritic length between ED vehicle and CIE-ED vehicle (p = .0005). There was no significant decrease in dendritic length between CIE-ED vehicle and CIE-ED endostatin (p = 0.69)

Phenotypic analysis of microglial cell soma area (Figure 8)

A two way ANOVA followed by a post-hoc analysis revealed that there was a significant interaction of alcohol treatment and endostatin on microglial cell soma area ($F_{alcohol\ x\ endostatin}[2,57]= 4.08$, p=.0221). Endostatin alone did not have a significant effect on cell soma area ($F_{endostatin}[1,57]= 3.32$, p= .0738). Alcohol alone had a significant effect on cell soma area ($F_{alcohol}[2,57]= 4.11$, p = .0215).

Uncorrected Fischer's LSD showed that there was no significant difference in cell soma area between control vehicle and CIE-ED vehicle (p = .90). Interestingly, it was shown that there was a significant increase in cell soma area between CIE-ED vehicle and CIE-ED endostatin (p < .0001)

Phenotypic analysis of microglial soma to tip distance (Figure 9)

A two way ANOVA followed by a post-hoc analysis revealed that there was not a significant interaction of alcohol treatment and endostatin on microglial soma to tip distance ($F_{alcohol\ X\ endostatin}[2,57]= .40$, p=.6693). Endostatin alone did not have a significant effect on cell soma area ($F_{endostatin}[1,57]= 3.3205$, p= .0787). Alcohol alone did have a significant effect on cell soma area ($F_{alcohol}[2,57]= 4.501$, p = .0153).

Uncorrected Fischer's LSD showed that there was a significant difference in soma to tip area between the control vehicle and CIE-ED vehicle ($p = .0353$). However there was no significant difference in soma to tip area between the CIE-ED vehicle and CIE-ED endostatin ($p = .3390$).

DISCUSSION

This experiment consisted of a two-pronged goal: the first, to determine the qualitative changes that occur in microglia during protracted abstinence in male rats as a result of chronic ethanol exposure, and the second, to determine if endostatin would indirectly reduce microglial activation in CIE-ED rats during this protracted abstinence period. Our hypothesis was that endostatin would decrease microglial activation, which would reduce ethanol seeking behavior and in turn decrease the propensity of relapse.

Activated microglia have characteristics that include reduced arborization, reduced number of intersections per unit distance from the soma, and reduced total dendritic distance (Wang et al., 2018). We found that there were significant signs of microglial activation in our CIE-ED vehicle rats, indicating that microglial activation characteristics persist through long periods of abstinence. This is shown by the significant decrease in the number of intersections relative to distance from the soma, decrease in the total dendrite length, and decreases in soma to tip distance in the CIE-ED vehicle compared to the control vehicle (Figure 6; Figure 7; Figure 9). Interestingly, we did not see any significant increase in cell soma area in the CIE-ED vehicle rats which is a marker for microglial activation. This could be due to the fact that microglia show exceptionally dynamic morphological characteristics when activated (Erickson et al., 2019). Previous research done by Siemsen et al. found that rats under the CIE paradigm that were sacrificed 10 hours into withdrawal experienced no significant change in cell soma area. This could mean that this effect that we see extends through protracted abstinence.

The endostatin treated rats showed no decrease in microglial activation or ethanol seeking behavior. Using a 3-D modeling program, Neurolucida Explorer, we tested CIE-ED control rats with their endostatin counterparts and found no statistically significant difference in

total dendritic distance, number of intersections per unit distance from the soma, and soma to tip distance ($p > .05$, uncorrected Fischer's LSD). This indicated that the rats that were treated with endostatin had microglia that were activated to the same degree as the control rats. These results also translated into the ethanol seeking behavior in these rats (Figure 4). This behavior was determined by placing the different groups of rats into operant conditioning boxes one by one. As expected, we did see a significant increase in active lever pressing between CIE-ED and ED rats indicating a dependent phenotype for the CIE-ED rats. However, there was no difference in active lever pressing between the CIE-ED endostatin rats and their respective CIE-ED saline control. This indicated that the CIE-ED rats, mimicking moderate to severe alcohol use disorder, did not show signs of decreased ethanol seeking behavior in response to endostatin treatment. Overall, these results showed that despite the inhibition of PECAM – 1 by endostatin which rescued BBB integrity, microglial activation still occurred.

This unexpected result could be due to the extremely diverse set of conditions which can activate microglia. Unpublished observations in our lab show that in females, PECAM-1 inhibition via endostatin significantly reduced ethanol seeking behavior and significantly decreased microglia activation. Clearly, microglia in males are activated differently than in females. A plausible explanation for why male microglia activation occurred could be due to the fact that the microglia in these male rats simply were not activated by peripheral cytokines, as they are in females. The neuroinflammation that occurs in the brain as a result of chronic ethanol exposure is an attack that is waged on both sides of the BBB: in the periphery and within the BBB (Mandyam et al., 2017). The leaky BBB leads to an influx of a variety of peripheral immune factors like cytokines, chemokines, and leukocytes (Mandyam et al., 2017). These were the immune factors that we inhibited in this experiment by decreasing BBB leakage through

PECAM-1 inhibition via endostatin. There are a number of proinflammatory molecules and cytokines that are released within the BBB as a result of chronic ethanol exposure (Crews et al., 2015). An example is a signaling pathway that involves High Mobility Group Box Protein 1 (HMGB-1), which binds to TLR4 receptors. This binding results in the activation of a key transcription factor called Nf-kb that regulates the genes of a variety of cytokines that could potentially be involved in direct activation of these microglia (Crews et al., 2015). Since limiting the peripheral immune response with endostatin did not show promising results, inhibiting the inflammatory response that occurs within the BBB could result in significant inactivation of microglia.

A future direction for an experiment could be using an ELISA kit to determine which brain-signaling cytokines (instead of peripheral immune signaling cytokines) are involved in the activation of microglia. The inhibition of the pathways which release these cytokines could pose a possible mechanism to decrease microglial activation and in turn, to decrease ethanol seeking behavior in male rats during protracted abstinence.

Material from this thesis is currently being prepared for submission for publication of the material. Tejash Bharadwaj was the sole author of this material.

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