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Alcohol, Stress, and Sex in the Bed Nucleus of the Stria Terminalis

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Alcohol, Stress, and Sex in the Bed Nucleus of the Stria Terminalis

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by

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ABSTRACT

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Alcohol use disorder (AUD) costs the U.S. billions of dollars each year and is a leading cause of preventable death. AUD leads to many health complications, and those who suffer from AUD will often have stress and anxiety disorder comorbidities. To better understand this connection between AUD and stress and anxiety disorders, restraint stress (RS) and chronic-intermittent ethanol exposure (CIE) procedures were used on rats to analyze neuroinflammation via ELISA in the infralimbic and prelimbic cortices, and the bed nucleus of the stria terminalis (BNST). Levels of proinflammatory cytokines, TNF-α and IL-1β, were found to be elevated across different tests in both males and females. This study builds upon previous work in the BNST and offers new information for future studies of stress and alcohol in the region.

DEDICATION

I would like to dedicate this to my wonderful wife Abbigale, my parents Steve and Kathy Burski, and to Mark and Traci Gujer, my in-laws. They have been nothing but supportive and I could not have done this without them.

ACKNOWLEDGMENTS

I need to thank Dr. Russell Brown, Dr. Justin Gass, Dr. Liza Wills, and Loren Peeters who did so much to help me complete this project. You all were so welcoming and willing to help me whenever I needed, and I cannot thank you enough.

TABLE OF CONTENTS

LIST OF FIGURES

CHAPTER 1. INTRODUCTION

Alcohol Use Disorder

As of the most recent survey conducted in 2020 by the US Substance Abuse and Mental Health Services Administration, there are 138.5 million current alcohol users with 28.3 million of those people being diagnosed with alcohol use disorder (AUD), with males diagnosed double the rate as females. AUD can be defined as a pattern of alcohol use that leads to significant impairment or distress (Kranzler and Soyka 2018). In 2010, the Center for Disease Control (CDC) estimated that excessive alcohol use cost the United States $249 billion per year in health care (?), with ~$192 billion being attributed to binge drinking, which is commonly recognized as consuming alcohol in excess of five drinks for males and four for females over the span of 2 hours (Kranzler and Soyka 2018). Based on the CDC's cost estimation, this would mean that alcohol will cost each of the 329.5 million people in the United States approximately $755.69 per year, every year if the estimated cost remains constant. Aside from the fiscal consequences that alcohol use has, 9.8% of deaths in the United States and 5.9% worldwide can be attributed to alcohol use making it one of the leading causes of preventable death. (Kranzler and Soyka 2018, Smiley 2021). The physical toll that alcohol takes on the on the body leads to many problems which leads to an overall lower quality of health and life. Despite the large economic cost, and its mortality, a 2017 report found that only 7.7% of adults who reported needing treatment, received treatment specifically for AUD (Park-Lee et al. 2017).

The heavy drinking that characterizes AUD results in an increased chance of developing many different health problems such as diabetes, gastrointestinal diseases, numerous cancers, liver disease, and pancreatitis (Kranzler and Soyka 2018). Neurodegeneration caused by excessive and chronic drinking results in several changes to behavior and neurobiological

pathology related to impulsivity, decision-making, anxiety, planning, and a deficit in the response of the brain's limbic system (Crews and Vetreno 2014). Relapsing can be a chronic and frequent occurrence in those who suffer from AUD. The high occurrence of relapse result in a long-term impact of alcohol has on the rewarding systems of the brain which are altered in individuals with AUD and make it difficult to abstain from alcohol use due to symptoms of withdrawal that can be harmful and potentially fatal.

Stress

In its most basic sense, stress can be thought of as anything that, when observed or experienced, causes the body to move away from its homeostatic state into a state of stress response. When in this state, the body attempts to respond to the cause of a stressor with the goal of regaining homeostasis. These responses cause adaptations to cope with the stress placed on the body (Sinha 2008). To better respond to potential future stressors, areas of the brain critical to mediating the stress response may be modified. The sensory regions of the brain responsible for the observation of an individual's surroundings and emotional state, as well as limbic regions responsible for any physical or emotional response, are most important as to how stress is experienced (Sinha 2008). Exposure to these stress-inducing situations can lead to the development of an anticipatory state that is referred to commonly as anxiety (Daviu et al 2019).

Anxiety disorders are the most commonly occurring form of mental health disorder, afflicting over 18% of the U.S. adult population in a 2005 study (Kessler et al. 2005). A prominent finding across studies of mental health disorders is the considerably higher prevalence of anxiety disorders in females than males (30.5% in females vs. 19.2% in men) (Bruce et al. 2005). This skew is found not only in the diagnosis of anxiety disorders as a whole, but in most studied anxiety disorders such as social anxiety (15.5% vs. 11.1%), phobias (15.7% vs. 6.7%),

generalized anxiety (6.6% vs. 3.6%), panic disorders (5.0% vs. 2.0%), and post-traumatic stress disorder (10.4% vs. 5.0%) (Kessler et al. 1994, Kessler et al. 1995, McLean et al. 2011).

Posttraumatic stress disorder is a disorder caused by exposure to a traumatic experience or event that results in symptoms such as altered reactivity to certain situations, negative alteration to one's cognition or mood state, and intrusive memories (Wynn et al. 2017). PTSD occurs more than twice as often in females as it does in males.

Stress and Alcohol

Since alcohol is classified as a depressant, alcohol is known to have an anxiolytic, or an anxiety- reducing property. This anxiolytic ability of alcohol acts to temporarily reduce stressful or anxious feelings. However, alcohol itself can act as a stressor during periods of sobriety amidst chronic alcohol use, and long-term use can result in brain pathology. . In addition, alcohol can serve as a negative reinforcer, resulting in a cycle of substance abuse, abstinence, withdrawal, and then relapse to prevent succumbing to the negative state that the periods of withdrawal and abstinence cause (Becker 2017). The "self-medication hypothesis" put forth by Hallam (1978) postulates that when attempting to cope with anxiety disorders, men are likely to do so through substance abuse while women use agoraphobic, or fear avoidance, behaviors. In support of the self-medication hypothesis, individuals diagnosed with an anxiety disorder, 33.2% of men were diagnosed with alcohol abuse and 21.8% with drug abuse, far more often than in females who were diagnosed 15.0% and 10.0% across their lifetimes (McLean et al. 2011).

The stress-induced alterations to the brain, specifically the rewarding areas, can cause a sensitization of an individual to the rewarding aspects of a substance (McKee et al. 2011, Peltier et al. 2019). Stress is able to stimulate both the brain's stress response system and its reward

system which is believed to increase the impact of the substances of abuse that may be used to cope with the induced stress (McKee et al. 2011, Peltier et al 2019).

Bed Nucleus of the Stria Terminalis (BNST)

The BNST is a component of what is referred to as the "extended amygdala," a term which also includes the central amygdala, medial amygdala, and shell of the nucleus accumbens (Olmos and Heimer 1999). The BNST is a key structure that serves an integral role of "valence surveillance" (Lebow and Chen 2016). This term refers to the BNST's function as an integration and relay component between the limbic structures of the forebrain and nuclei of the hypothalamus and brainstem (Crestani et al. 2009). Information collected (i.e., Surveillance) from forebrain structures like the prefrontal cortex (PFC) is assessed and assigned a valence either positive or negative based on several factors including mood, motivation, and energy levels. This assignment of valence then allows the BNST to relay this information to the appropriate brain regions to result in the "correct" reaction to the given situation (Lebow and Chen 2016). The BNST has a high concentration of CRF and contains both GABAergic and glutamatergic projections. Projections from the BNST to the amygdala, hypothalamus, ventral tegmental area, and lateral septum allow this transfer of information to result in action. Signals to the BNST from the frontal cortex, locus coeruleus, ventral subiculum, ventral tegmental area, amygdala, and olfactory bulb relaying information from the surroundings are all used by the BNST to disseminate what valence should be assigned (Miles and Maren 2019).

BNST and Sex Differences

The BNST itself is a sexually dimorphic brain region. In humans, the BNST of males was found to be 2.47 times larger than that of a female (Allen and Gorski 1990). Additionally, the

BNST is involved in numerous sexually significant functions like aggression, sexual behavior, and gonadal hormone uptake further demonstrating the sex difference of the region (Hines, Allen, and Gorski 1992). Subpopulations of hormonal receptors for androgen, estrogen, and progesterone within the BNST have the potential to impact intake of environmental information which has the possibility to cause a different assignment of valence and therefore the reaction to situations when compared to male counterparts. (Lebow and Chen 2016).

BNST and Stress and Alcohol

The hypothalamic-pituitary-adrenal (HPA) axis is the predominant stress response system in the body. The BNST has been implicated as one of the most important HPA contributing regions of the brain in the presence of stress and therefore plays a key role in stress response (Forray and Gysling 2004, Snyder and Silberman 2021). By its neuronal projections to the paraventricular nucleus of the hypothalamus (PVN), the BNST can exert its influence on the HPA axis and therefore the stress response (Lebow and Chen 2016). Additionally, the BNST's GABAergic and glutamatergic neuronal projections to the ventral tegmental area (VTA) give it another area of influence on further regulatory sites of both anxiety and stress (Partridge et al. 2020).

The BNST's role in the assignment of valence is thought to have an impact on mood and the rewarding centers of the brain. This has been demonstrated through the administration of CRF antagonists to the region of alcohol-dependent animals, and the resulting reduction of alcohol self-administration (O'Dell et al. 2014). The specific sites at which the BNST may impact alcohol use is not known.. However, it is hypothesized that alcohol consumption as a coping mechanism for stress is well documented and is why the BNST is hypothesized to factor into AUD.

Neuroinflammation

Inflammation is a defense mechanism of the body that is triggered by the challenge of potentially damaging stimuli and the release of inflammatory mediators (Roberto et al. 2018). One type of inflammatory mediator is cytokines. Cytokines are glycoproteins that are a response to an immunological challenge and consist of a few protein classes; interleukins, chemokines, tumor necrosis factor, interferons, and growth factors (Roberto et al. 2017). Tumor necrosis factor (TNF) is a cytokine and an important role player in the inflammatory and homeostatic process (Probert et al. 2015, Roberto et al. 2017). In addition, the interleukin family of cytokines mediate the signaling between different cells of the immune system, regulate cell growth, cell differentiation, and are produced in a plethora of different cell types including both neurons and glial cells. IL-1β specifically is not always present in the brain. Instead, it is only synthesized upon the activation of the inflammasome pathway, which induces the production of pro-inflammatory cytokines (Vosshenrich and Di Santo 2002, Roberto et al. 2017). IL-1β is able to influence neuronal activity, excitability, and neurotransmission in a manner that can be region or cell-specific within the brain (Vezzani and Viviani 2015, Roberto et al. 2017). An important pathway activated by these proinflammatory cytokines is the mitogen-activated protein kinase (MAPK) pathway. This pathway plays an important role in cell proliferation, differentiation, development, and apoptosis.

Neuroinflammation, Stress, and Alcohol

Exposure to both stress and alcohol has been shown to induce changes to brain cytokine production and AUD is associated with consistently altered neuroimmune expression (Crews and Vetreno 2014). While the neuroinflammatory response functions to protect areas of the brain, too much neuroinflammatory activity can cause neurodegeneration in those areas of the brain.

Therefore, the initial challenge of either alcohol or a stress stimulus results in the protective properties of these cytokines, binge-like consumption of alcohol and stress events will lead to degeneration which will increase in severity as duration increases (Roberto et al. 2017).

IL-1β levels have been found to increase following exposure to alcohol in both neuronal and glial cell populations though exactly how IL-1β goes about impacting alcohol-related behaviors is not understood in its entirety (Lawrimore and Crews 2017, Roberto et al. 2017). A region of interest when addressing how IL-1β may impact alcohol-related behavior is the extended-amygdala. IL-1β has been shown to both increase as well as decrease the release of the extended-amygdala's primary neurotransmitter, GABA. However, in the majority of the extended-amygdala cells, IL-1β increases GABA release (Roberto et al. 2017). Either impact, whether an increase or decrease of GABA levels in the BNST would have vast implications for the projections of the area given that the BNST's projections have a large impact on areas important to stress/PTSD via the HPA axis as well as addiction pathways via the VTA, PFC, and insular cortex.

TNF-α levels in plasma of alcoholics has been associated with an increased craving of alcohol and a greater tendency for relapse (Kiefer et al. 2002). Anxiety-like behavior resulting from the experience of alcohol withdrawal has been associated with the elevation of TNF-α in the brain and is mediated by TNF-α activation of CRF1 (Breese et al. 2008, Knapp et al. 2011). CRF1 and its importance to the HPA axis as well as to the BNST which would mean elevated TNF-α levels would heavily impact this region of the brain and the stress response. This is because CRF1 activation via TNF-α leads to the release of corticotropin which is a potent mediator of the stress response thus an increase in TNF-α levels would lead to a heightened activation of CRF1 which in turn means a heightened response to stress.

Preclinical Models of Alcohol Use and Stress

Chronic Intermittent Ethanol

There are a few different ways to study alcohol use in an animal model, some of the more common methodologies include animal self-administration, administration by a researcher, and vapor inhalation. Each method has been shown to effectively create alcohol dependence, which is the overarching goal, but some are accompanied by disadvantages. For example, alcohol self-administration involves voluntary drinking of alcohol by the animal, which is a widely used method of inducing dependence. However, it is often difficult for animals to acquire alcohol self-administration and can take a longer time for dependency to be achieved. Administration by a researcher can be done via methods like a feeding tube to the stomach or by injections. This method is much more difficult to keep consistent blood alcohol levels (BALs), is often invasive, and the animal is more likely to overdose on alcohol given this way. Vapor inhalation is done by placing the animal in a sealed housing chamber and exposing them to vaporized alcohol. Vapor inhalation allows for the control of the exposure duration, frequency, dosage, and easier to maintain consistency of BALs. It is also a much quicker way of creating dependence than self-administration by eliminating the need for the animal's voluntary alcohol intake (Gilpin et al. 2008).

Vapor Inhalation gives flexibility in the experimental design and there are two types of methodologies: intermittent and continuous exposure. Chronic intermittent ethanol exposure lasts anywhere from 8-17 hours rather than a continuous 24 hours, giving animals the chance to experience intermittent cycles of withdrawal. A continuous model was used for many years, but intermittent has become the more commonly utilized pattern of exposure since its induction. The reason chronic intermittent ethanol exposure has gained popularity is due to the reduction in the

time it takes to produce alcohol dependence. Animals have shown behaviors of alcohol dependence after only 2 weeks of exposure whereas animals exposed continuously take 4 to 6 weeks to display the same behaviors (O'Dell et al. 2004, Gilpin et al. 2008). The cycle of chronic exposure followed by periods of withdrawal does well to mimic episodes of binge drinking. In the experimental space, binge drinking is defined as a pattern of alcohol consumption in which an individual consumes large quantities of alcohol in a short period of time, leading to drastic increases in blood ethanol concentration >80 mg/dL, equivalent to 0.08 BAC (Sanchez-Marin et al. 2022). The binge-like episodes and the greatly accelerated induction of alcohol dependence is what has made CIE gain this popularity as a model, because it appears to more closely mimic the human condition of binge-like alcoholism.

Restraint Stress

Restraint, or immobilization stress has been a popular method of inducing a stress response as well as exposing animals to high stress situations. The procedure is in part so commonly used due to its non-invasive nature. Being restrained causes both physical discomfort as well as mental discomfort in the animal all while the animal is not being harmed. The method results in emotional responses as well as physical ones like elevated heart rate, elevated blood pressure, and changes in body temperature that model stress and anxiety well (Reis et al. 2011). Restraint sessions as little as 90 minutes have been shown to achieve the desired response from the animal though session can last much longer. Restraint stress can be used both chronically and acutely depending on the desired outcome. Where chronic use is used to study repeated stress, acute use is used to model a single stressful event in an individual's life and is often used along with substance abuse studies.

This model does well to represent the onset of PTSD and has been shown to lead to behavior and symptoms consistent with the disorder in humans. The trauma that the animal experiences during its confinement is the trigger that leads to the desired behavior and outcomes. Similar to how PTSD can be spurned from a single traumatic event that occurs in a human's life such as a car accident, imprisonment, war, etc. which then leads to the behaviors and symptoms associated with PTSD.

Purpose of Current Study

The prevalence of AUD in the world costs billions of dollars in health care each year but even more important than the financial burden is the physical and mental health impacts caused by AUD costs people their lives, lives whose deaths are preventable. This project seeks to further the understanding of how neuroinflammation in the BNST may play a role in the pathologies of both stress and AUD as well as identifying potential sex differences in BNST cytokine activity. Based on the literature reviewed previously, the hypotheses of the current study are as follows:

Hypothesis 1: We hypothesized that rats undergoing restraint stress followed by CIE (RS/CIE) will show significantly increased levels of the cytokines TNF-α and IL-1β compared to the control group of rats that underwent neither protocol.

Hypothesis 2: We hypothesized that the any increase present in the TNF-α and IL-1β levels in the RS/CIE was not due to solely the restrain stress nor solely the CIE but rather summation of the RS/CIE as a whole.

Hypothesis 3: Therefore, we hypothesized that there would be an increase in the TNF-α and IL-1β levels in male RS/CIE rats compared to female RS/CIE rats

CHAPTER 2. METHODS

Animals and Housing Environment

A total of 12 male Long-Evans rats, 12 male Wistar rats, and 12 female Wistar rats were obtained from Charles River (Wilmington, MA) and were 46 days post-natal (PND) when received. Animals were assigned randomly to experimental groups which were exposed to stress and alcohol, stress only, or alcohol only. The control group was exposed to neither stress nor alcohol but tested simultaneously. The animals were housed as within-group pairs in a 12-h light/dark cycle with continual access to food and water when in their home cage. Animals were given two days after arriving to allow them habituation to their environment. All experimental procedures were conducted with the approval of the East Tennessee State University Animal Care and Use Committee.

Immobilization Stress

Animals assigned to a group that would receive stress were placed in adjustable immobilization restraint devices for a single 2-hour session after which the animals were returned to their home cages. Each animal was subjected to only one session of immobilization stress. The groups of animals assigned not to receive stress remained in their home cages during this session.

Chronic Intermittent Ethanol Vapor Exposure

Beginning on PND 51, animals assigned to a treatment group designated for alcohol exposure were subjected to a chronic intermittent ethanol (CIE) vapor protocol which is a well-established model of inducing alcohol dependence rodents (Gilpin et al. 2008, Gass et al. 2014 Griffin et al. 2014, Griffin et al. 2015). A total of six exposures over the course of 11 days with

the exposure days occurring on the Monday, Wednesday, and Friday of each week beginning at 0800 h and ending at 1700 h. Cage mates were placed in the same cage within the acrylic CIE chamber and were given continuous access to water, but not food. CIE chamber ethanol concentrations were monitored daily and kept in the correct range that has been shown to model binge-drinking levels of intoxication in rodents (Gilpin et al.2008).

Tissue Collection

Animals were euthanized three days following the conclusion of the CIE protocol on PND 65 via live decapitation. Animals who did not receive CIE remained in their home cages waited until those who did receive CIE were ready to be euthanized. Brains were frozen using a solution of isopentane on dry ice. Frozen brains were then placed into labeled vials and stored at -80°C until needed for analysis, and later brain areas of interested were dissected away into separate labeled vials.

Cytokine ELISA

Tissue was collected from the portions of the collected, frozen brains and used to conduct enzyme-linked immunosorbent assays (ELISAs). The brains were allowed to partially thaw before being placed on a brain block and sectioned at 1mm intervals to locate the desired brain areas. The areas of interest were then collected (~2mg) from each animal using a biopsy punch and deposited in microcentrifuge tubes. The mass of each tissue sample was obtained, and the tubes were stored at -80°C until ELISAs were performed.

TNF-α

TNF-α protein expression was analyzed in the infralimbic cortex (IFL), prelimbic cortex (PRL), and the BNST using TNF-α ELISA kit (Cat # EKF57956) from Biomatik (Wellington,

DE). Tissue was homogenized using 500µl of RIPA cell lysis buffer (150mM NaCl, 50mM Tris-HCl, 1.0% NP –40, 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate) along with phosphatase and protease inhibitors (P5726, P8340, P0044; Sigma-Aldrich, St. Louis, MO) added to the sample microcentrifuge vial and sonicated (Fisherbrand Model 505 Sonic Dismembrator, Waltham, MA) to break apart cell structure. Homogenized samples were then centrifuged at 5000G at 4°C for 5 minutes. All 96 wells of the ELISA plate were coated in anti-TNF-α antibody. TNF-α standard solutions from Biomatik (Wilmington, DE) were created via serial dilution from 250 pg/ml to 3.91 pg/ml and these solutions were used to create the standard curve. Tissue samples were diluted by a factor of 1:2 using provided dilution buffer. Each standard and tissue sample were pipetted 2 times onto the plate and when finished, the plate was covered and incubated at 37°C for 90 minutes. Following incubation, the plate was washed with the provided wash buffer twice. Biotin-conjugated anti-TNF-α detection antibody was added to each of the wells and the plate was covered and incubated at 37°C for 60 minutes. The plate was removed once again, washed 3 times, and the detection antibody signal amplifier HRP-streptavidin conjugate was added to each well. The plate was covered and incubated at 37°C for 30 minutes. After incubation, the plate was washed 5 times. TMB substrate was then added to each well to promote a greater signal from the HRP enzymatic reaction. The plate was covered and incubated at 37°C in darkness for 10-20 minutes. For a final time, the plate was removed and the HRP and TMB reaction was halted using the Stop solution. The finished plate was then placed in the Bio-Tek ELx 800 microplate reader (Winooski, VT) to measure optical density at a 450-nm wavelength.

IL-1β

IL-1β protein expression was analyzed in the infralimbic cortex (IFL), prelimbic cortex (PRL), and the BNST using IL-1β ELISA kit (Cat # EKF57939) from Biomatik (Wilmington, DE). Tissue was homogenized using 500µl of RIPA cell lysis buffer along with phosphatase and protease inhibitors added to the sample microcentrifuge vial and sonicated (Fisherbrand Model 505 Sonic Dismembrator, Waltham, MA) to break apart cell structure. Homogenized samples were then centrifuged at 5000G at 4°C for 5 minutes. All 96 wells of the ELISA plate were coated in anti- IL-1β antibody. IL-1β standard solutions from Biomatik were created via serial dilution from 2000 pg/ml to 31.25 pg/ml and these solutions were used to create the standard curve. Tissue samples were diluted by a factor of 1:2 using provided dilution buffer. Each standard and tissue sample were pipetted 2 times onto the plate and when finished, the plate was covered and incubated at 37°C for 90 minutes. Following incubation, the plate was washed with the provided wash buffer twice. Biotin-conjugated anti- IL-1β detection antibody was added to each of the wells and the plate was covered and incubated at 37°C for 60 minutes. The plate was removed once again, washed 3 times, and the detection antibody signal amplifier HRP-streptavidin conjugate was added to each well. The plate was covered and incubated at 37°C for 30 minutes. After incubation, the plate was washed 5 times. TMB substrate was then added to each well to promote a greater signal from the HRP enzymatic reaction. The plate was covered and incubated at 37°C in darkness for 10-20 minutes. For a final time, the plate was removed and the HRP and TMB reaction was halted using the Stop solution. The finished plate was then placed in the Bio-Tek ELx 800 microplate reader to measure optical density at a 450-nm wavelength.

ELISAs

Data collected from the Bio-Tek ELx 800 microplate reader was imported into Microsoft Excel. The wavelength data was converted to pg/ml using the previously determined standard curve. Data was then imported to GraphPad Prism for visualization and performance of a One-Way ANOVA to determine the effects of stress and alcohol exposure on TNF-α and IL-1β levels.

CHAPTER 3. RESULTS

TNF-α and IL-1β ELISA for RS/CIE Males

Independent t-tests were separately used to determine the effects of CIE/RS treatment on the levels of IL-1β and TNF-α in the infralimbic cortex, prelimbic cortex, and the BNST. A significant main effect of group was revealed in IL-1β protein levels($t_{(10)} = 2.81$, $p < 0.01$; **Fig. 1**) was found in the BNST in the CIE/RS group compared to the control group. There were no other significant changes in IL-1β. An independent t-test revealed a significant group main effect in TNF-α levels of the CIE/RS group in comparison to the control group in the PRL ($t_{(9)} = 2.23$, $p < 0.027$; **Fig. 2**) as well as the BNST ($t_{(10)} = 3.815$, $p < 0.002$; **Fig. 2**).

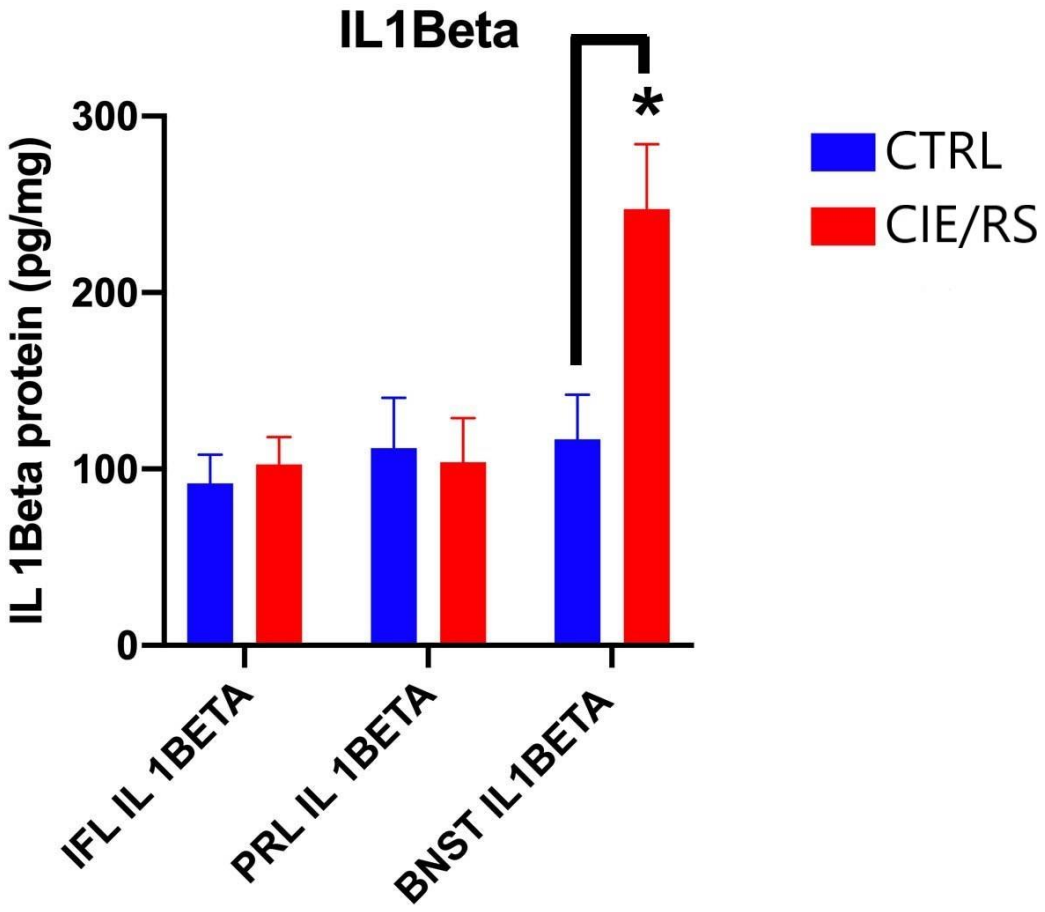

Figure 1. IL-1β Protein levels in the Infralimbic Cortex (IFL), Prelimbic Cortex (PRL), and the Bed Nucleus of the Stria Terminalis (BNST). Asterisks (*) indicate that IL-1β levels in the experimental, CIE/RS, group were significantly increased than those in the control group ($p < 0.05$). Error bars indicate the standard error of the mean.

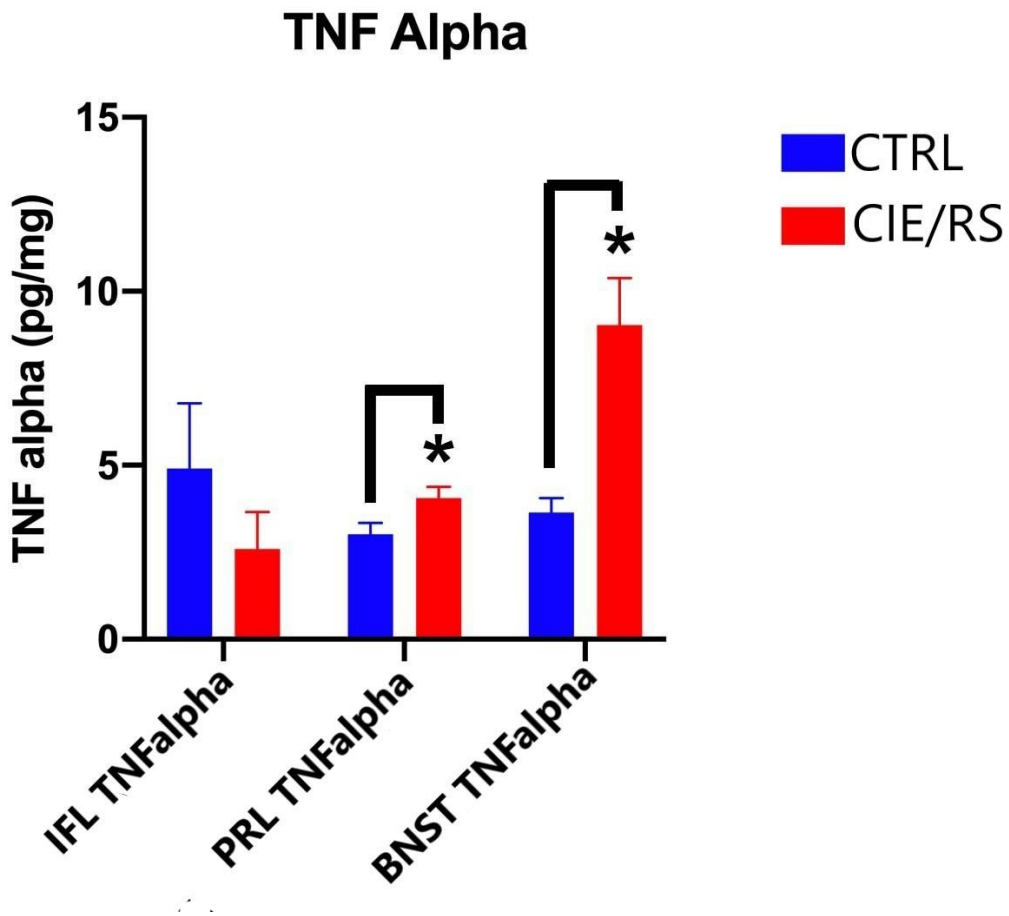

Figure 2. TNF-α Protein levels (pg/mL) in the Infralimbic Cortex (IFL), Prelimbic Cortex (PRL), and the Bed Nucleus of the Stria Terminalis (BNST). Asterisks (*) indicate that TNF-α levels in the experimental, CIE/RS, group were significantly increased than those in the control group ($p < 0.05$). Error bars indicate the standard error of the mean.

TNF-α and IL-1β ELISA for RS, CIE, and RS/CIE Males

One way ANOVAs were used to determine if there was any significant change between not only the control group of rats who received no treatment at all, but also control groups that only received Restraint Stress and those who only received Chronic Intermittent Ethanol

exposure. In the analysis of TNF-α in the PRL, a one-way ANOVA revealed a significant main effect of drug treatment $F(3,21)=31.04$, $p<.001$. Newman-Keuls post hoc analyses revealed that there was a significant increase in TNF-α levels between both the CIE and RS animals when compared to both the CIE/RS and control animal groups in the PRL as revealed by **(Figure 3)**. In the BNST, a one-way ANOVA revealed a significant main effect of drug treatment $F(3,23)=14.83$, $p<.001$. Newman-Keuls post hoc analyses showed that TNFα levels were also elevated in all three experimental groups when compared to control groups, but none of the experimental groups were significantly different from one another **(Figure 3)**. For IL-1β, the BNST was the only region where there were any significant differences observed. A one-way ANOVA revealed a significant main effect of drug treatment $F(3,22)=5.91$, $p<.005$. Newman-Keuls post hoc test revealed that the CIE and CIE/RS experimental groups were significantly higher than the control groups, but unlike the BNST TNF-α analysis, the RS group was not significantly different from the control group. **(Figure 4)**.

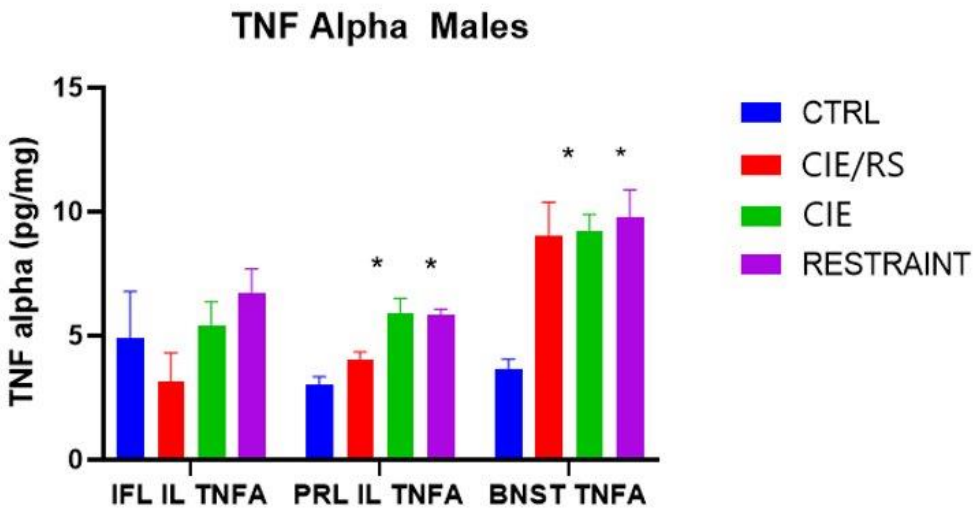

Figure 3. TNF-α Protein levels (pg/mL) in the Infralimbic Cortex (IFL), Prelimbic Cortex (PRL), and the Bed Nucleus of the Stria Terminalis (BNST) compared to controls for both CIE and RS. Asterisks (*) indicate that TNF-α levels in the experimental, CIE/RS, group were significantly increased than those in the control groups of no treatment (Blue), only CIE (Green), and only Restraint (Purple) ($p < 0.05$). Error bars indicate the standard error of the mean.

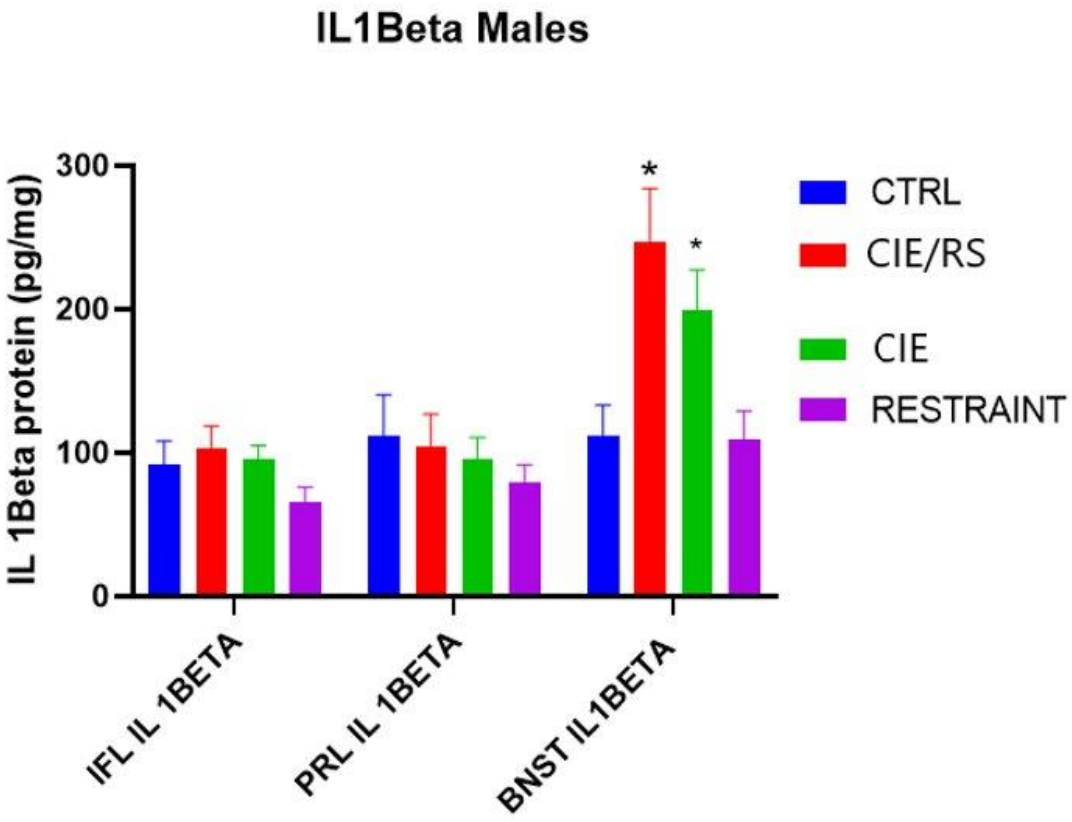

Figure 4. IL-1β Protein levels (pg/mL) in the Infralimbic Cortex (IFL), Prelimbic Cortex (PRL), and the Bed Nucleus of the Stria Terminalis (BNST) compared to controls for both CIE and RS. Asterisks (*) indicate that IL-1β levels in the experimental, CIE/RS, group were significantly increased than those in the control groups of no treatment (Blue), only CIE (Green), and only Restraint (Purple) ($p < 0.05$). Error bars indicate the standard error of the mean.

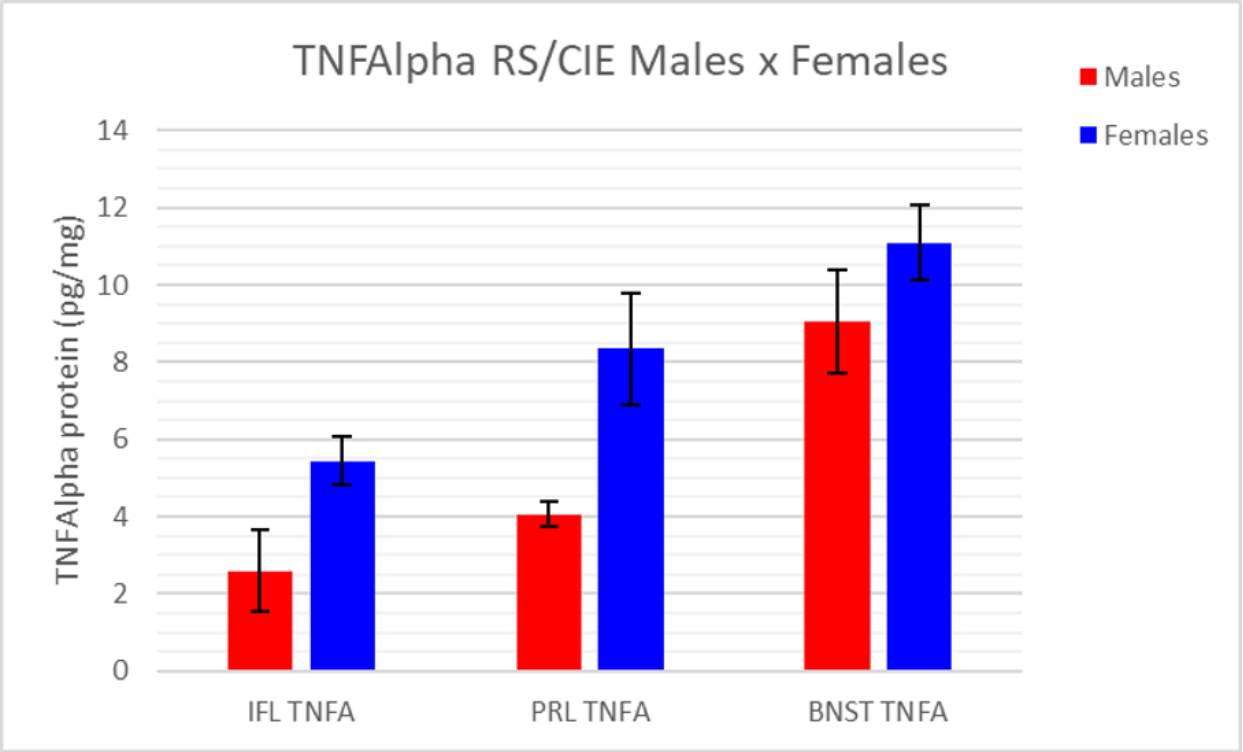

Figure 5. TNF-α Protein levels (pg/mL) in the Infralimbic Cortex (IFL), Prelimbic Cortex (PRL), and the Bed Nucleus of the Stria Terminalis (BNST) of Males and Females. Asterisks (*) indicate that TNF-α levels in the experimental, CIE/RS, group were significantly increased than those in the control group ($p < 0.05$). Error bars indicate the standard error of the mean.

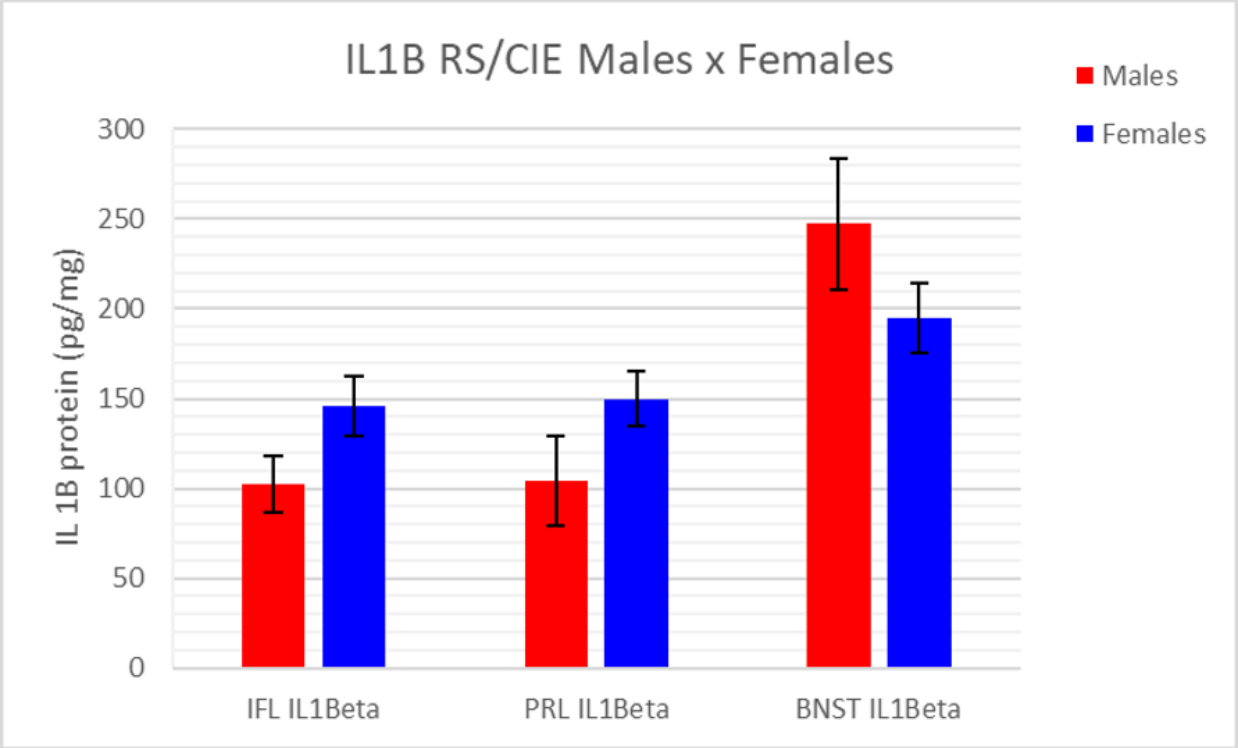

Figure 6. IL-1β Protein Expression (pg/mL) in the Infralimbic Cortex (IFL), Prelimbic Cortex (PRL), and the Bed Nucleus of the Stria Terminalis (BNST) of Males and Females. Asterisks (*) indicate that IL-1β levels in the experimental, CIE/RS, group were significantly increased than those in the control group ($p < 0.05$). Error bars indicate the standard error of the mean.

TNF-α and IL-1β ELISA for RS/CIE and Control Groups for Males and Females

There were some significant differences between the males and female RS/CIE groups found in the t-test that was conducted for some, but not all, brain regions for both TNF-α and IL-1β (**Figures 5 & 6)**. In both proteins, IFL and PRL brain regions showed significantly increased protein levels whereas the BSNT showed no significant difference in TNF-α and IL-1β.

When comparing the cytokine protein levels of the male and female control groups, we found no significant differences between males and females in IL-1β **(Figure 8**). In TNF-α, the

female control groups showed significantly elevated TNF-α levels when compared to males in both the PRL and BNST brain regions **(Figure 7).** A two-way ANOVA with the sex and group (Control and RS/CIE) as the two groups analyzed. The two-way ANOVA showed that there was a significant sex main effect ($F(3,22) = 11.96$, $p<0.003$) as well as a group main effect ($F(1,22) = 20.78$, $p<0.001$) in the levels of TNF-α found in the BNST. There was also a significant sex main effect in the TNF-α of the PRL ($F(1,22) = 19.84$, $p<0.001$). Levels of IL-1β in the BNST also showed a significant group main effect ($F(1,23) = 19.54$, $p<0.001$) but neither the PRL nor the IFL showed any significant effects.

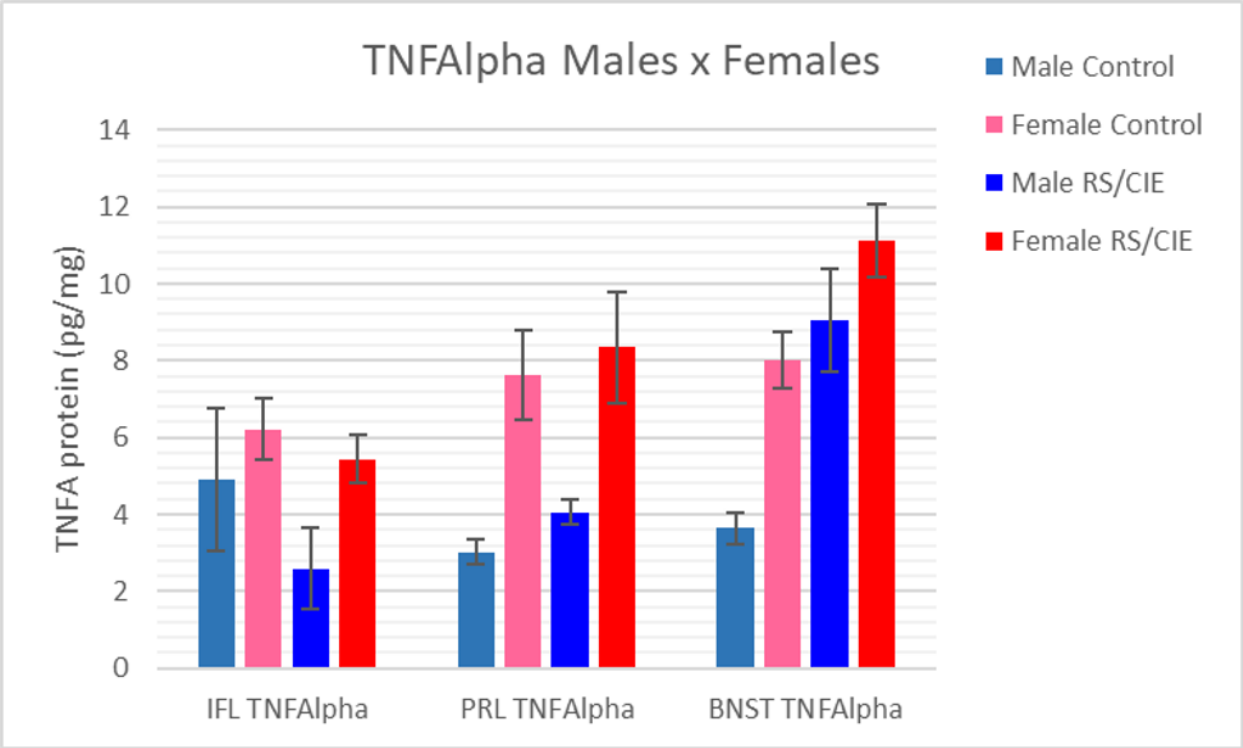

Figure 7. TNF-α Protein levels (pg/mL) in the Infralimbic Cortex (IFL), Prelimbic Cortex (PRL), and the Bed Nucleus of the Stria Terminalis (BNST) of Males and Females compared to the control group. Asterisks (*) indicate that TNF-α levels in the experimental, CIE/RS, group were significantly increased than those in the control group ($p < 0.05$). Error bars indicate the standard error of the mean.

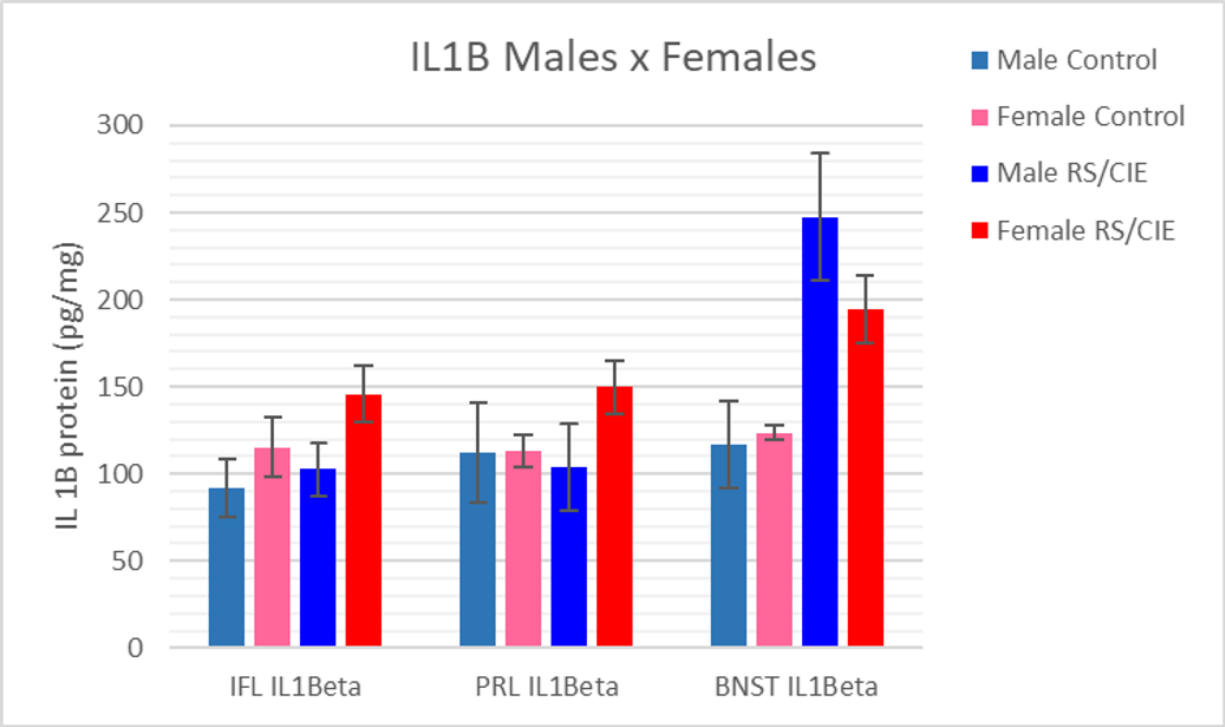

Figure 8. IL-1β Protein levels (pg/mL) in the Infralimbic Cortex (IFL), Prelimbic Cortex (PRL), and the Bed Nucleus of the Stria Terminalis (BNST) of Males and Females compared to the control group. Asterisks (*) indicate that IL-1β levels in the experimental, CIE/RS, group were significantly increased than those in the control group ($p < 0.05$). Error bars indicate the standard error of the mean.

CHAPTER 4. DISCUSSION

The aim of this study was to analyze the neuroinflammatory response occurring in the BNST during following stress and alcohol use. In the BNST specifically, TNF-α and IL-1β protein levels were found to be elevated during CIE/RS trial in males. In female CIE/RS trials, IL-1β protein levels in the BNST were elevated as well. TNF-α protein levels were elevated in CIE, RS, and CIE/RS trials in the BNST of males whereas IL-1β protein levels were elevated in CIE and CIE/RS trials in the BNST of females. When comparing the CIE/RS trials and the BNST of males and females, TNF-α protein levels between the sexes showed a difference in the experimental groups as well as the sex whereas IL-1β protein levels showed only a group effect in the BNST.

The existing information in this area of study shows that stress and anxiety disorders are more prevalent in females, 30.5%, than males, 19.2%. In those individuals that show a stress and anxiety disorder, men are much more likely to have a comorbid alcohol use disorder than females. The idea of men with comorbid stress and anxiety disorders and alcohol use disorders occurs more frequently is known as the "self-medication hypothesis" (Hallam 1978). The reason that these two are so often seen occurring together is in part to the anxiolytic effects of alcohol which can be used to quell the impact of stress and anxiety disorders.

These discrepancies that can be seen between the sexes in both stress/anxiety disorders as well as alcohol use are the reason that the BNST is a potential area of interest to this field of study. The BNST itself is 2.5 time larger in males as it is in females and this size difference is consistent in rodents. The BNST is also home to many receptors for sex hormones which could further implicate its potential contribution to this particular area of study.

The current study bolsters the findings of previous studies, while also introducing the angle of sexual dimorphism and how this may impact neuroinflammatory responses following stress and alcohol.

Hypothesis 1

The levels of TNF-α and IL-1β were consistent with our first hypothesis in that the RS/CIE group of males would have significantly elevated levels compared to the control group. For IL-1β, the only brain region that showed an elevation in protein levels was the BNST, showing over a twofold increase **(Figure 1).** Both the IFL and the PRL showed no significant alteration in the levels of IL-1β expression. Our analysis of TNF-α also showed some significant elevation in the levels of the protein similar to that in IL-1β. However, both the BNST and PRL showed these elevated protein levels in our TNF-α analysis compared to just the BNST being elevated in IL-1β . The BNST again showed around a twofold increase in protein levels, whereas the PRL showed a much smaller, but still significant, increase in TNF-α protein levels. The IFL region showed no elevated levels in TNF-α, just as it did for IL-1β protein levels. The BNST was the only brain region that showed significantly elevated levels in both the IL-1β and TNF-α assays, and both were elevated to similar degrees.

Hypothesis 2

The second hypothesis of ours was that any effect on the levels of cytokines found in the male rats during the first round of experiments would be as a result of the summation of both the RS and the CIE, not solely one or the other. We found that in male rats, levels of TNF-α in the BNST were consistently elevated between the three test groups of RS, CIE, and RS/CIE when

compared to the control group. This, however, was not the case for IL-1β. In the BNST, IL-1β was only elevated in CIE and RS/CIE groups when compared to the control group.

The mixed data obtained from this experiment does not allow us to definitively state that the increase seen in the CIE/RS males when compared to the control groups in both TNF-α and IL-1β is only caused by the summation of the effects of the CIE and RS rather than just one or the other. In TNF-α, the elevation of both the RS and CIE to similar levels as CIE/RS indicates that both factors contribute to elevated levels of this cytokine. However, IL-1β elevation may solely be due to the exposure of CIE as there was no significant elevation in the RS group when compared to the control group.

Hypothesis 3

We found that there was a significant sex difference in some of the brain regions in protein levels of IL-1β and TNF-α. For protein levels of TNF-α, CIE/RS males had elevated levels in both the IFL and PRL regions of the brain. In the BNST, there was not a significant difference in the levels of TNF-α. The levels of IL-1β protein showed the same pattern with both the IFL and PRL brain regions showing elevated levels in males compared to their female counterparts. Again, the BNST did not show any significant difference in the protein levels of IL-1β. When analyzing the control groups for both the males and the females, the levels of TNF-α were significantly elevated in both PRL and BNST regions of the brain in females compared to the males with no significant difference between the control groups for the IFL region. IL-1β protein levels in the control groups for males and females had no significant difference for any of the brain regions that were analyzed.

We then analyzed the data using a two-way ANOVA using sex and experimental group as the two conditions being analyzed. This test showed a significant effect in sex as well as experimental group in the protein levels of TNF-α in the BNST brain region. A significant effect in sex was also found for TNF-α in the PRL region. The IFL brain region showed no significant effects for neither TNF-α nor IL-1β. The BNST showed a significant experimental group effect for IL-1β protein levels. While the PRL region had a significant result for TNF-α , there were no significant effects for the protein levels of IL-1β in this region.

Sex Difference

Through the use of the two-way ANOVA, the only brain regions that showed any significant difference between males and females were the BNST and the PRL in TNF-α levels. Our data showed that females have an elevated levels of this cytokine compared to males in the control groups in both the PRL and the BNST, meaning that females may have already elevated levels of TNF-α at the normal resting state. The significant sex difference found in the same regions in the experimental CIE/RS groups, but this time with the higher levels in favor of the male groups, shows that males have a much more significant increase in TNF-α when exposed to the CIE/RS method compared to the increase of TNF-α in females. This finding is consistent with the idea behind the "self-medication" hypothesis which states that males are more likely to "cope" with stress/anxiety thorough the use of alcohol's anxiolytic properties.

CHAPTER 5. CONCLUSION

The literature continues to suggest that there is a difference between the sexes that causes the discrepancies that are seen in the prevalence of stress and anxiety disorders as well as the comorbidity of stress/anxiety disorders and the abuse of alcohol. This study served the purpose of investigating a potential area of the brain in the BNST that may contribute to this sex difference in stress/anxiety disorders and its comorbidity with alcohol abuse. This investigation focused on expanding upon the available information to this field of interest and helping contribute additional information about potential brain regions of potential impact to the area.

The test models consisting of RS, CIE, RS/CIE, and controls were tested for region activity of two inflammatory cytokines in TNF-α and IL-1β through the use of ELISA test kits. It was found that there was significant sex difference in the levels of males and females in the control groups, in favor of the females, indicating that females may have higher levels of certain cytokines at resting state. A significant sex difference was also found between males and females in the CIE/RS experimental groups, but this time in favor of the males, which indicated that males have a more heightened inflammatory response when met with CIE and RS challenges.

The increased inflammation that we found during this study could lead to detrimental effects on the subject if the patterns of CIE and stress continue through their life. Chronic neuroinflammation can lead to neuronal death as well as the furthering of degenerative disorders of the brain. The increased inflammation that was observed could lead to an individual having a much more challenging time breaking dependency with alcohol following a traumatic experience, like that which was modeled with the RS. Therefore, this elevated inflammation observed in males could be a reason as to why males are more likely than females to have a comorbid AUD and stress/anxiety disorder, such as PTSD.

In the future, this work could be used as a starting point for researchers to deeper analyze the BNST as a brain region implicated in the sexual discrepancies of stress/anxiety disorders and alcohol abuse. More in-depth analysis of the sub-nuclei of the BNST could lead to a better understanding of the root causes for the dimorphism of both disorders.

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