BINGE ALCOHOL EFFECTS ON THE PREFRONTAL CORTEX

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A Thesis

Presented to

The Faculty of the Department

of Psychology

University of Houston

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In Partial Fulfillment

Of the Requirements for the Degree of

Master of Arts

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By

Rebecca K. West

December, 2016

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Abstract

Approximately 92% of U.S. adults who drink excessively report binge drinking in the past 30 days. Increased alcohol marketing in recent years has particularly targeted women, causing a 36% increase in the last 10 years in the number of women who are engaging in binge alcohol consumption. Since women appear to be more vulnerable to the harmful neurological effects of alcohol, this increase is of particular concern. One of the brain regions most affected by binge alcohol consumption is the frontal cortex, an area important for many functions and decision making of daily life. Loss of prefrontal grey matter resulting from heavy alcohol consumption has been documented, however this volume loss does not appear to be caused by a decrease in the number of prefrontal cortex neurons. This study aimed to determine whether the medial prefrontal cortex (mPFC) in female rats is more vulnerable to alcohol induced damage (compared to males), by examining neuronal volume and quantity as well as prefrontal- dependent behavioral tasks following binge alcohol exposure. To assess this, adult male and female Long-Evans rats were assigned to binge or control groups and exposed to ethanol using a well-established 4-day model of alcohol-induced neurodegeneration. Both male and female binged animals had significantly smaller average neuronal volumes than their respective control groups, as well as spatial working memory deficits detected during behavioral testing. No differences were found between binged male and binged female rats on neuronal volume, population or behavioral outcomes. These results support prior research indicating that frontal regions are vulnerable to binge alcohol damage.

Binge Alcohol Effects on the Prefrontal Cortex

Introduction

There were an estimated 88,000 annual American deaths from 2006-2010 due to alcohol-related causes (Center for Disease Control and Prevention, 2014), and in 2013, 5.9 percent of all deaths worldwide were attributable to alcohol consumption (World Health Organization, 2014). Alcohol marketing in recent years has particularly targeted women, causing an increase in the number of women who are engaging in binge drinking (Dwyer-Lindgren et al., 2015). Since females appear to be uniquely vulnerable to the harmful neurological effects of alcohol in both human and animal studies (Mann, Batra, Gunthner & Schroth, 1992, Leasure & Nixon, 2010; Maynard & Leasure, 2013; Agartz, et al., 2003; Hommer, et al., 1996, 2001), this increase is of particular concern. One of the brain regions most affected by binge alcohol consumption is the frontal cortex, an area responsible for decision making and behavioral control. This study determined whether the prefrontal cortex in female rats is more vulnerable to alcohol-induced damage than that of the male, by examining neuronal quantity and size, as well conducting behavioral tests designed to test frontal cortex functioning following a 4-day binge alcohol exposure model.

Alcohol Intake

Globally, among people ages 15-49, alcohol use is the leading risk factor for premature death and disability (Lim, et al., 2012). In addition to the well-known short-term health risks, excessive alcohol use can also lead to the development of serious problems such as heart disease, stroke, increase of various cancers, learning and memory problems, depression, anxiety,

unemployment, social problems, alcohol dependence and brain damage (National Institute on Alcohol Abuse and Alcoholism, 2000).

For women especially, there appears to be an increased risk of negative consequences from heavy alcohol consumption. Including deaths from alcohol-related incidents, heart disease, stoke, suicide, and liver cirrhosis, female alcoholics have death rates 50-100 percent higher than their male alcoholic counterparts (NIAAA, 2009). Additionally, women who consume as little as 3-6 drinks a week, have an increased risk of breast cancer compared to women who abstain (Chen et al., 2011). Moreover, women are likely to self-medicate mood or mental disorders with alcohol (NIAAA, 2008). For men, heavy drinking is defined as more than 15 drinks in a typical week, for women on the other hand, it is defined as nearly half that amount, eight drinks (CDC, 2014). For women, heavy drinking increases the risk of developing alcohol dependence or abuse as well as increases women's risk of violence and sexual assault. Despite that women tend to consume less and have a shorter duration of alcohol intake than men, long term heavy alcohol consumption is more likely to harm a women's health and cause possibly enduring damage to the brain (NIAAA, 2008).

Binge Alcohol Consumption

Among the different patterns of alcohol intake, binge drinking is the most common in the U.S. A reported one-sixth of U.S. adults binge drinks about four times a month with an average of eight drinks per binge (CDC, 2012). The National Institute on Alcohol Abuse and Alcoholism (NIAAA, 2004), defines binge drinking as consuming enough alcohol, usually four drinks for women and five for men over two hours, to bring a person's blood alcohol concentration (BAC) to 0.08 or above. One drink consists of 12-ounces of beer, 5-ounces of

wine or 1.5-ounces, also known as a “shot,” of spirits. Additionally many “mixed drinks” contain larger quantities of alcohol, which, (depending on the recipe) can contain 2-3 standard drinks of alcohol. Thus, for example, the average sized woman could reach the limit of binge drinking with roughly two “mixed drinks” at a bar. Of all the alcohol consumed in the U.S. by adults, 75 percent is in the form of binge drinking (CDC, 2014). Binge drinking is a risk factor for stroke (Sundell et al., 2008), cancer (Gupta et al., 2010), and dementia (Jarvenpaa et al., 2005; Gupta & Warner, 2008). In addition, binge drinking is associated with sexual assault, domestic violence, sexually transmitted diseases, unintentional pregnancies, alcohol poisoning, liver disease, heart disease, high blood pressure, poor diabetic control, sexual dysfunction and potential damage to multiple brain regions (CDC, 2014).

Heavy episodic (“binge”) drinking poses an especially serious risk for college students, where social gatherings often revolve around drinking games and getting drunk has become a ritualized part of a university education. Almost 40 percent of college students ages 18-22 report binge drinking in the past month (Substance Abuse and Mental Health Services Administration, 2013). While alcohol-induced blackouts are a fairly common result of a binge among heavy alcohol consumers, with 40 percent of college students having reported blacking out at least one time in the last year (White, Jamieson-Drake & Swartzwelder, 2002), they represent a potentially serious neurological blow. Although individuals often diminish the side effect of having gaps in their memory after a night of drinking, if other trauma resulted in such memory loss they would likely be very concerned. Drinking, however, is so imbued into social interactions, that the risks and potential damage caused by such patterns of consumption are dismissed. Additionally, a rise in popularity of drinking games actively encourages drinking large quantities of alcohol in a short span of time under the guise of friendly competition. This

combination not only increases drunk driving arrests, assaults, academic problems and unprotected sex, but also results in the death of an estimated 1,825 college students a year (Hingson, Zha & Weitzman, 2009). Additionally, 97,000 students ages 18-24 are victims of alcohol related assault or date rape, and more than 100,000 report having been too intoxicated to remember whether they gave sexual consent (Hingson et al., 2009).

Sex Differences in Alcohol Use

Nearly 14 million US women binge drink about three times a month with an average of 6 drinks during each episode (CDC, 2013). Even more alarming is that 1 in 5 high school girls report binge drinking, and by the time they reach 12-th grade, 62 percent of girls report alcohol use in the last 30 days (CDC, 2011). White and Hispanic women between the ages of 18 and 34, with household incomes over $75,000 are the most likely to engage in binge drinking (CDC, 2011).

For adults who drink, alcohol accounts for roughly 16 percent of the calories they consume (CDC, 2012). A 2013 poll (Jones & Saad), indicated that 57% of women surveyed expressed that they wished to lose weight, yet a woman who consumes 1 glass of wine a night, can gain about 10 pounds a year strictly from alcohol consumption. Capitalizing on this paradox, there is increased marketing towards women who want to lose weight yet still have their favorite cocktail. Concern over caloric intake can elicit disturbing patterns of binge drinking such as skipping meals in order to "save the calories" for later drinking or avoiding carb-laden beer or mixers in favor of drinking straight liquor (Johnston, 2013). Moreover, sweet wines, sweet and fruity flavored vodkas, and wine coolers or "chick beer" have increasingly emerged in recent years marketed directly at women consumers. Wine names including the words skinny, pink and

mommy are common, such as "Pink Zebra" and "Mommy's Time Out". These feminine wines have emerged adorned with pink foil tops and eye catching labels. All this has led to a median 18.9 percent increase in female binge alcohol consumption, compared with only a 7.3 percent increase in male binge consumption between 2005-2012 (Dwyer-Lindgren et al., 2015). While the prevalence of alcoholism and binge drinking is still higher in men than women, the gap is quickly closing.

Alcohol-Related Brain Impairment

The specific pattern of alcohol intake is a factor in the resulting type and amount of brain damage (Crews & Nixon, 2009; Nixon & Crews, 2002; Obernier, Bouldin & Crews, 2002). Binge drinking in adolescence and young adulthood is associated with damage to multiple brain areas, particularly the frontal cortex and hippocampus (Bengoechea & Gonzalo, 1990; Risher et al, 2015), as well as cognitive deficits in both human and animal studies (Duka et al., 2004; Loeber et al., 2010; Nixon & Crews, 2002; Obernier et al., 2002; Obernier, White, Swartzwelder, & Crews, 2002; Stephens & Duka, 2008; Stephens et al., 2005). A four-day binge model (Majchrowicz, 1975) has been used to simulate a multiple-day binge episode common among some alcoholics, and also provide a link between binge alcohol consumption and neural injury (Leasure & Nixon, 2010; Obernier et al., 2002; Tomsovic, 1974). Studies specifically utilizing this animal model of binge alcohol consumption have found neurodegeneration (Crews et al., 2004), cognitive deficits (Obernier et al., 2002; Crews & Nixon, 2009) and a reduction in neurogenesis (Nixon & Crews, 2004).

In contrast to the standard pattern of chronic alcohol consumption, alcoholics who binge drink will typically go through many periods of abstinence, which can result in multiple

withdrawal periods (Hunt, 1993). Multiple withdrawals from alcohol have been shown to cause a number of negative neurological effects such as reduced neuroplasticity, impaired cognitive functioning and cortical function disruption (Loeber et al., 2010; Duka et al., 2004; Loeber et al., 2009; Stephens et al., 2005; Duka, Townshend, Collier, & Stephens, 2003). Moreover, repeated episodes of binge drinking combined with multiple detoxification episodes are hypothesized to increase corticolimbic damage (Duka et al., 2004).

Frontal Cortex Susceptibility to Alcohol Damage

The frontal cortex is one of the brain areas most vulnerable to the damaging effects of alcohol. This area is responsible for a wide variety of critical functions (Kolb, 1984), including motor function, emotional processing, problem solving, spontaneity, memory, language, spatial orientation, judgement, impulse control and social behavior. Injury to the prefrontal cortex caused by alcohol consumption can impair health and influence addition trajectory by damaging the area responsible for reward evaluation (Taren, Venkatraman & Huettel, 2011) and cognitive evaluation of risk-taking behavior (Crone, et al., 2008).

Alcohol is known to have long-term damaging effects on the prefrontal cortex, which can affect these important cognitive functions mediated by the frontal lobe (Moselhy, Georgiou & Kahn, 2001; Tarter et al. 2004). Chronic alcohol consumption causes gray and white matter loss, enlargement of the ventricles, and substantial neuronal loss in the frontal lobes (Crews & Nixon, 2009; Kubota et al., 2001; Nixon & Crews, 2002; Obernier, Bouldin, & Crews, 2002; Sullivan & Pfefferbaum, 2005). Heavy episodic drinking is specifically related to lower white matter integrity in the corpus callosum (Pfefferbaum, Adalsteinsson & Sullivan, 2006) and prefrontal cortex (Vargas et al., 2014). Myelination is important for conductance speed in axons, thus

enhancing information processing and cognitive performance (Blakemore & Choudhury, 2006). Voluntary binge drinking reduces this important myelin density in the medial prefrontal cortex (mPFC), which is related to enduring white matter loss, working memory deficits and is correlated with higher relapse-like drinking in adulthood (Vargas et al., 2014).

Additionally, studies of alcoholic patients who have undergone multiple withdrawals suggest that previous experience of detoxification is also associated with prefrontal cortex dysfunction (Duka, Townshend, Collier & Stephens, 2003). Alcohol use during adolescence is related to increased impulsivity (Stephens & Duka, 2008), neuronal cell death (Crews et al., 2000), impairments in cognitive performance (Konrad et al., 2012; Brown, Tapert, Granholm & Delis, 2000; Crews, He & Hodge, 2007), and increased risk for alcoholism in adulthood (Grant & Dawson, 1998). The results from rodent studies are consistent with observations in human alcoholics and binge drinkers who show impaired cognitive function in executive control tasks sensitive to dysfunction of prefrontal cortex (Duka et al. 2003, 2004; Weissenborn & Duka 2003; Townshend & Duka 2005).

Females May Be More Vulnerable than Males to Alcohol-Induced Brain Damage

Alcoholic women manifest alcohol-induced brain damage after shorter histories of heavy drinking and less average daily consumption than their male alcoholic counterparts (Mann, Batra, Gunthner & Schroth, 1992), a phenomenon called telescoping. When compared to alcoholic men, women had greater global brain shrinkage and smaller hippocampi (Agartz, et al., 2003), as well as smaller corpus callosum size (Hommer, et al., 1996, 2001). Both men and women alcoholics had a reduction in frontal lobe white matter, however, only women had significant gray matter reduction (Schweinsburg et al., 2003). Additionally, female binge

drinkers perform more poorly than males on spatial working memory, attention, and inhibition-dependent tasks, which are mediated by the frontal cortex (Squeglia et al., 2011; Townshend & Duka, 2005; Scaife & Duka, 2009).

There has been an increase in alcohol marketing toward women in recent years, more women who use alcohol to self-medicate for mood disorders (Dixit & Crum, 2000), and an increasing number of women binge drinking. This in conjunction with the fact that women appear to sustain more damage to key brain areas required for memory and cognitive inhibition is cause for concern, and warrants further research on the vulnerability of the female brain to alcohol (Mann, Batra, Gunthner & Schroth, 1992).

Current Studies

Four days of binge alcohol exposure has been shown to cause a lasting loss of neurons in the hippocampus of female rats (Leasure & Nixon, 2010; Maynard & Leasure, 2013). Preliminary data also indicates binge-induced spatial navigation impairment in female rats, but not male. Additionally, a loss of prefrontal grey matter resulting from heavy alcohol consumption has also been well documented in human alcoholics (Kubota et al., 2001; Nakamura-Palacios et al., 2013; Rando et al., 2011). However, in rats, this volume loss does not appear to be caused by a decrease in the number of prefrontal cortex neurons (Koss et al., 2012). Frontal neocortical volume loss can occur not only from loss of neurons, but also from reduced neuronal cell size (Freeman et al., 2008). Reduced prefrontal cortical grey matter can cause cognitive deficits in functions such as inhibitory control and working memory. The present study aimed to determine whether females are more susceptible to binge alcohol effects on brain and

behavior. Using a rodent model of binge alcohol exposure, the volume of neurons in the prefrontal cortex was quantified and frontal function assessed in males and females.

Proposed Study

This experiment used two cohorts of rats to examine behavioral (Experiment 1) and neuronal effects (Experiment 2) on the prefrontal cortex using a rat model of binge-alcohol consumption. In both Experiment 1 and Experiment 2, male and female rats were divided into either alcohol or control groups and were administered either 4 days (12 doses) of alcohol or control diet (see Figure 1).

Experiment 1 assessed prefrontal cortical function following binge alcohol administration. The first behavioral task, food neophobia was conducted on day 4 following the last dose of alcohol. This task is intended to measure impulsivity by examining the latency to eat a novel food, a task shown to be dependent on frontal cortex functioning (Rudebeck et al., 2007; Mariano et al., 2009). The second behavioral test, rewarded alternation, began on the fourth day of alcohol abstinence. This task employs spatial working memory and has been previously shown to be sensitive to alcohol-induced damage in rats (Vargas et al., 2014). Rats successfully complete this task by recalling which of two areas they most recently visited and are rewarded for choosing the area they did not most recently visit.

> **Hypothesis 1a:** Binged female rats will display more impulsivity than control females, male binge or male controls during the food neophobia test, indicated by lower latency times on novel food consumption. There will be no difference between male binge and male control groups on novel food eating latency.

Hypothesis 1b: Binged female rats will have a lower percentage of correct alternations on the rewarded alternation, when compared with female control and both male binge and male control groups, however male binge rats will have the same percentage of correct alternations as male controls.

Experiment 2 used banked tissue from a separate experiment using the 4-day binge. Rats were sacrificed shortly following the last dose of alcohol. The neurons of this tissue were stained using immunohistochemistry and cell body volume and neuronal population was estimated using an unbiased method of cell quantification (stereology).

Hypothesis 2a: The cell bodies of neurons in the medial prefrontal cortex of binged female rats will have reduced neuronal cell volume, which would indicate atrophy of neuronal cell bodies, when compared with female control and both male binge and male control groups. However there will be no difference in cell body size between male control and alcohol groups.

Hypothesis 2b: There will be no difference in the number of remaining medial prefrontal cortex neurons between any of the groups (female binge, female control, male binge, male control).

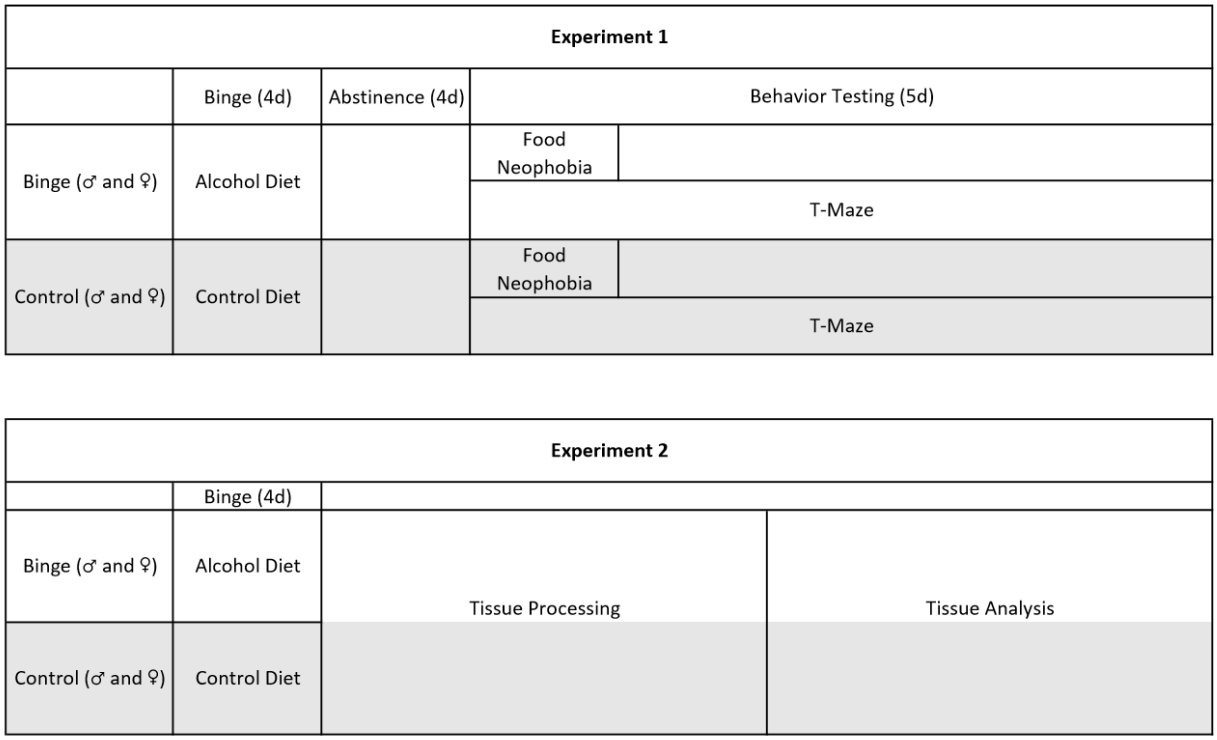

Figure 1. Experimental Timetable and Groups.

Methods

Ethics Statement

All experimental procedures were conducted in accordance with the Guide for the Care and Use of Laboratory Animals of the National Institute of health. The animal protocol was approved by the Institutional Animal Care and Use Committee at the University of Houston.

Animals

The behavioral aspect of the study (Experiment 1) included 39 male and female Long-Evans rats (10 male binge, 10 male control, 9 female binge, 10 female control). Rats were randomly assigned to their respective sex's alcohol or control group. Rats were housed in groups

of two or three per cage by gender. Upon arrival, rats spent about one week acclimating to their new environment and also being handled to become accustomed to the experimenter. Following this acclimation time, rats were randomly assigned to either the ethanol or control group and then underwent a four-day binge. Rats were allowed to gain weight up to 90% of their original body weight following the last dose of alcohol and then were tested on the food neophobia testing followed by a single five-day testing block on the rewarded alternation task. Each testing day was comprised of ten trials, for a total of 50 total trials per rat. All rats were sacrificed roughly one hour following their last trial of the rewarded alternation testing.

Neuronal cell volume and estimated neuronal population in the medial prefrontal cortex (mPFC) quantification were conducted using tissue collected from a previously run study (Leasure & Maynard). This experiment was conducted using 27 male and female Long-Evans rats (8 female binge, 6 female control, 7 male binge, 6 male control), sacrificed eight hours following the final alcohol dosing.

EXPERIMENT ONE

Estrous Cycle Monitoring

Some evidence suggests that female rats may have increased alcohol sensitivity (Roberts et al., 1998; Ford, Eldridge & Samson, 2002), during certain phases of the estrous cycle. Thus, in order control for this variability, all female rats received their first dose of control or alcohol diet when they were in the diestus stage of their cycle. Vaginal smears of both female binge and control groups were taken between 8:00 and 9:00 A.M. daily, beginning 5-7 days prior to the first alcohol or control dose and continuing until animals concluded behavioral testing. Each sample was placed on a glass slide, stained with cresyl violet, and then coverslipped. Stage of

estrous (proestrus, estrus, metestrus, diestrus) was determined by examining samples under a light microscope at 10x magnification.

Binge Paradigm

Food was removed from both control and experimental groups prior to the first dose, although water was always available in their home cages. Diets consisted of either an isocaloric control diet (Dextrose w/ vanilla Ensure Plus™) or ethanol diet (25% 190 proof alcohol w/ vanilla Ensure Plus™) given every 8 hours for 12 doses over 4 days via intragastric gavage (Nixon & Crews, 2002; Obernier et al., 2002; Majchrowicz, 1975). The initial loading alcohol dose for the animals receiving the ethanol diet was 5 g/kg of body weight. Following the initial dose, animals were dosed according to their behavioral intoxication based on the 7-point scale designed by Majchrowicz (1975). This was done to ensure that animals who acted more behaviorally intoxicated receive less alcohol at each subsequent dose and rats who acted less intoxicated receive more alcohol. Control animals were given the average volume of fluid that their respective gender alcohol group received for that particular dose. Following the last binge dose and subsequent withdrawal period, rats completed the food neophobia task, followed by a five-day T-maze testing block.

Blood Ethanol Concentration

Blood was drawn via the lateral saphenous vein ninety minutes following the seventh dose of ethanol. Samples were immediately centrifuged, then stored at -80 degrees Celsius until analysis. Extracted serum was analyzed using a 5 μl aliquot in a GM7 Analyzer (Analox, MA, USA), in order to determine blood ethanol concentration.

Withdrawal

Spontaneous withdrawal symptoms were monitored every 30 minutes for hours 10–26 after the last dose. This has been referenced to be the peak withdrawal period (Majchrowicz, 1975), with most severe symptoms peaking around 22-24 hours after the last dose (Maynard & Leasure, 2013). Withdrawal symptoms were scored by the scale created by Penland and colleagues (2001), at each 30-minute interval, the most severe symptom was recorded. Red lights on a reverse light-cycle were used throughout the monitoring period so as not to disrupt the animals' circadian rhythm.

Food Neophobia / Hyponeophagia

The food neophobia, or hyponeophagia, test relies upon the fact that rodents are naturally less inclined to eat unfamiliar foods in a potentially threatening, novel environment. Prior studies showed that rodents with orbitofrontal cortex lesions have lower eating latency to eating times than their control counterparts (Rudebeck et al., 2007; Mariano et al., 2009). This would indicate that binged animals who sustain frontal cortex damage from alcohol exposure would more readily consume a novel food while not differing from control animals in their latency to eat a familiar food.

Following the 4-day alcohol binge and recovery period, rats were permitted to gain up to 85-90 percent of their original free-fed weight before undergoing the food neophobia test. Rats had their food removed approximately 12 hours prior to hyponeophagia testing in order to provide motivation for sampling the novel food. Rats were presented with pieces of sweet corn

(Green Giant Original Niblets) in a clear custard dish located in the center of an open table. Each rat was placed in the testing location in a designated spot, facing away from the food dish.

The latency to make first contact with the food, latency to begin eating, and number of approaches was recorded. Each trial was a maximum of 5 minutes, with the time at which the rat began to eat the food marking the conclusion of the trial. If the rat failed to eat within 300 seconds, it was removed from the testing area and returned to its home cage for approximately 10 minutes. If the rat failed to eat the novel food on the first trial, it was retested on up to two more trials. If a rat failed to eat within three trials, the maximum latency of 900 seconds was recorded.

In order to rule out baseline hunger motivation, an additional set of trials using the same setup was employed in order to evaluate the amount of time it took each rat to eat their standard familiar food pellets. The latency to first approach, number of approaches, the time difference between the time the rat made first contact with food and the time it took to begin eating and the time difference between consumption of familiar vs. novel food, was compared between groups.

Rewarded Alternation

The rewarded alternation task relies on rodent's natural inclination to alternate areas of exploration in a new environment and rewards rodents for correctly alternating choice arms. Working memory, a prefrontal cortex dependent task, is employed by using a short inter-trial interval as rats must recall which arm they previously visited in order to obtain access to the reward. This task has been previously shown to be sensitive to alcohol-induced damage in rats (Vargas et al., 2014).

Following the four-day binge, rats were maintained at 85-90 percent of their original free feeding weight. Food restriction was necessary so that the rats were adequately motivated to seek out the food reward that was available if they chose the correct arm. Prior to the four-day binge, all rats were habituated in home cage groups to the T-Maze apparatus. During habituation all doors were raised and unlimited food reward pellets (chocolate 45mg Dustless Precision Pellets®) were present in both food dishes located at the end of each choice arm. Also during this time, food pellets were placed into each home cage so rats could become accustomed to searching out pellets in a familiar environment. Throughout T-maze testing, each rat was weighed and monitored daily and given food rations accordingly to maintain the range of 80-90 percent of their original free fed weight.

The T-maze is an apparatus made of clear Plexiglas with three guillotine doors secured in metal brackets which can be raised and lowered by the experimenter (see Figure 3 and Figure 4). The maze was spot cleaned between trials and wiped down with a 70 percent ethanol solution between animals. Pellet dust was present in both reward dishes so rats could not employ smell to determine which arm to visit. Rats received one forced trial followed by ten choice trials in succession each day, for five days.

Day one of behavioral testing began on the fourth day of alcohol abstinence and was conducted in the beginning of the animal's dark cycle. Each rat began testing by being placed in the starting area, and allowed to settle behind the starting guillotine door. On the first trial, one of the two reward arms was opened, forcing the rat toward one of the arms, chosen at random. The guillotine door was lowered behind the animal once both their back legs crossed into a chosen arm, thus confining it to that arm. Once the rat had spent either 10 seconds in the reward arm or eaten all the reward pellets, it was immediately picked up and placed back at the start. Trials 2-

11 were given with 15 seconds between each trial, with the rat able to freely choose which arm they wished to visit. Rats found a three pellet reward present only in the bowl in the arm opposite that of which they visited on the previous trial. Therefore, the animal had to employ the use of working memory to remember which arm they previously visited and go to the other arm to receive a reward. Latency times were recorded as the time between the opening of the starting door, to when the rat's hind legs crossed into their choice arm for each trial. The average latency times and the percentage of correct alternations were compared between groups.

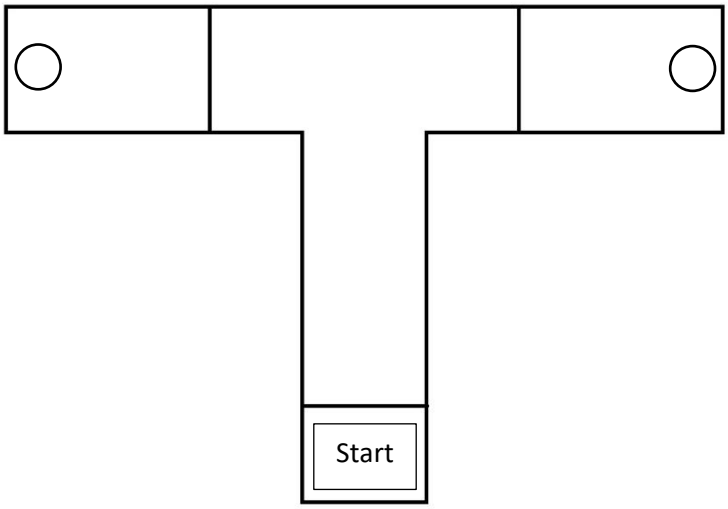

Figure 2. T-maze Apparatus, food reward locations are designated by circles

Figure 3. Photograph of a rat in the T-Maze.

EXPERIMENT TWO

Previously Collected Tissue

Tissue from a previously run study by Maynard and Leasure, was analyzed in order to determine whether there are sex-differences in damage to the medial prefrontal cortex sustained immediately following a binge drinking episode. Brain tissue was of 27 Long-Evans rats who were divided in a 2x2 design with the variables Sex and Diet (control or binge). These animals underwent the same procedure as explained above in the 'Binge Paradigm' section. Eight hours following the last dose of alcohol, animals were given an overdose of anesthetic and subsequently intracardially perfused with cold saline followed by 4% paraformaldehyde. The brain was extracted and post-fixed overnight, then refrigerated in 30% sucrose until sectioning. Tissue was sectioned into 50 µm coronal sections on a microtome. Sections were then stored in 96-well plates at -20 °C in cryoprotectant.

Tissue Processing and Analysis

Tissue sections from the prefrontal cortex of each animal were processed with immunohistochemisty (IHC) in order to label the neuronal cell bodies. The neuronal marker (NeuN) stains neuronal cell bodies, and excludes any dendritic projections. The IHC protocol began by floating previously sliced sections in 0.1 M PO_4 in order to isolate the sections from the PFC, then the sections of interest were placed in 0.1 M Tris Buffer (TBS) and rinsed three separate times in TBS at room temperature on a shaker. The sections were then transferred to a 30-minute 0.6% Hydrogen Peroxide (H_2O_2) room temperature rinse. After this sections were once again rinsed in TBS three times of ten minutes each. Following the third TBS rinse, they were blocked for 60 minutes in 3% normal donkey serum (Sigma-Aldrich, St. Louis, MO, USA), then incubated in the cold room at 4 °C for in the primary antibody (guinea pig anti-NeuN, EMD Millipore, Billerica, MA, 1:1,000). After 72 hours the sections were removed from the cold room and rinsed again twice (15 minutes) in TBS at room temperature. Following the rinse they were incubated in secondary antibody (donkey anti-guinea pig biotinylated, Jackson ImmunoResearch, West Grove, PA, USA, 1:250) for 24 hours on a shaker at room temperature. The following day, sections were rinsed in another three ten minute TBS washes and then incubated in ABC/*Elite* Standard Kit for 60 minutes. After an additional three ten minute TBS rinses, the sections were then reacted in diaminobenzidine (DAB), a non-florescent label. After a final four ten minute TBS rinses, the sections were then mounted on gelatin coated slides and left for 24 hours to allow sections to properly adhere to the slide. Once slides were dry, they were counterstained in methyl green and then coverslipped. Slides were coded to enable the experimenter to be blind to experimental condition.

Tissue from the mPFC was collected from 2 columns spaced 300 μm apart, yielding approximately 6 sections per brain. Neurons from these sections were counted and cell volume estimated using the Nucleator probe via Stereo-Investigator (MBF Bioscience). Stereology enables unbiased estimation of cell population and associated parameters, in this case, nuclear size. The area of interest encompassed the infralimbic, prelimbic, and anterior cingulate cortex (Figure 4). The optical fractionator workflow was followed in order to count the number of cells in the frame using a grid size of 400x400 and a counting frame of 40x40. Area outline was traced at 10x and cells were counted at 100x magnification. The optical fractionator generates a grid of counting frames overlaying the area of interest (in this case, the mPFC) so that each sampling site represents a small portion of the overall area. NeuN+ cells within each counting frame are counted and this information can then be combined with the measured tissue thickness to estimate the total population of cells. The nucleator probe works within the optical fractionator workflow in order to measure the cell area and volume of each cell counted within the sampling frames. The probe creates rays that emanate from the center point of the cell, which is arbitrarily designated by the investigator, and the points at which the rays intersect with the boundary of the cell is marked (see Figure 5). Thus the program generates an estimated population and measured volume of the cells counted. The estimated number of cells present and their average cell body volume were quantified and compared between groups.

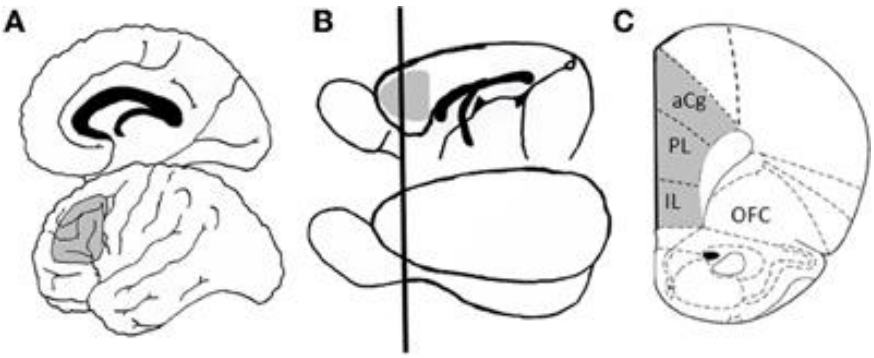

Figure 4. Area of interest for cell quantification in mPFC (adapted from Bizon, Foster, Alexander & Glisky, 2012)

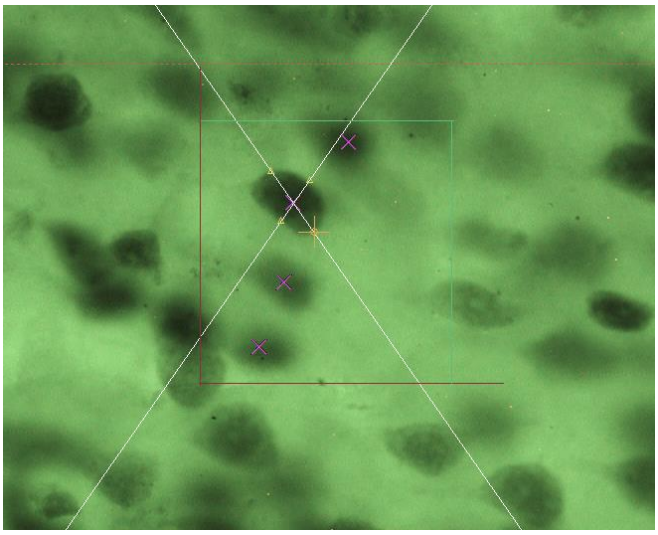

Figure 5. Screenshot of nucleator probe with 4 rays being used to measure cell volume (grid 400x400, 40x40 counting frame)

Statistical Analysis

Two-group comparison between male and female binge rats BEC (mg/dl), was analyzed with an independent group t-test. Ethanol doses between male and female binge groups were compared using a two-way mixed design for Sex, with Time as the repeated variable. Behavioral

intoxication and spontaneous withdrawal symptoms were measured using Wilcoxon testing since these values were measured using an ordinal scale. A three-way mixed factorial design with Time as the repeated measure was used to evaluate T-maze percent correct alternations and latency to choice arm for the variables Time, Sex and Diet as well as the interactions of these three variables. Data for investigation latency, latency to eat novel and familiar food and number of approaches for the food neophobia task was examined using two-way ANOVA with the variables of Sex and Diet as well as the interaction of these two variables. Prefrontal neuronal population and volumes also compared the variables Sex, Diet and Sex x Diet with two-way ANOVA testing. Pearson correlations were performed in order to examine relationships between behavioral intoxication and mean withdrawal score, novel and familiar eating latency, and correct T-maze alternations and latency time. Significance of these correlations was determined using the critical value table for Pearson's Correlation Coefficient. All values are presented as mean (± standard error of the mean) and error bars represent ± standard error of the mean. Planned Bonferroni-corrected post hoc comparisons were used when appropriate. Results from statistical tests were deemed significant if the p-value was less than 0.05 and all tests were run using SAS 9.4.

Results

EXPERIMENT ONE

Behavioral Intoxication and Withdrawal

Contrary to what is commonly observed in the human population (Mumenthaler, Taylor,

O'Hara & Yesavage, 1999), female rats acted less behaviorally intoxicated than male rats after the first 5g/kg dose [$t(17)=9.92$, $p=.006$] and less intoxicated overall, and thus received higher doses of alcohol [$F(1,17)=22.16$, $p=.0002$]. There was a main effect of Time on behavioral intoxication [$F(11, 226)=4.15$, $p<.0001$] which peaked during the second and third day of binge dosing (Figure 6). However there was found to be a Sex x Time interaction present for behavioral intoxication [$F(11,226)=36.09$, $p<.0001$]. Male rats displayed higher behavioral intoxication on doses 2-7 [$p<.05$], however had lower behavioral intoxication scores on the final alcohol dose than female rats.

Despite male rats receiving a lower alcohol dose and having higher behavioral scores, there was no effect of Sex on blood ethanol concentrations (mg/dl) [$t(17)=.18$, $p=.67$], measured 90 minutes following the seventh dose of alcohol (Figure 8). Mean blood ethanol concentrations were 378 (SD = 25.2) mg/dl for females and 405 (SD = 43.8) mg/dl for male rats, corresponding with high observed levels of behavioral intoxication.

There was no effect of Sex [$F(1,17)=.60$, $p=.47$] nor a Sex x Time interaction [$F(32, 623)=1.28$, $p=.18$] on withdrawal severity. However there was a significant effect of Time [$F(32, 623)=8.1$, $p<.0001$], on withdrawal severity, with spontaneous withdrawal symptom severity tapering off toward the end of the withdrawal monitoring time (Figure 8). There was no significant correlation between mean behavioral intoxication and mean withdrawal score [$r^2=.14$, $p=.58$].

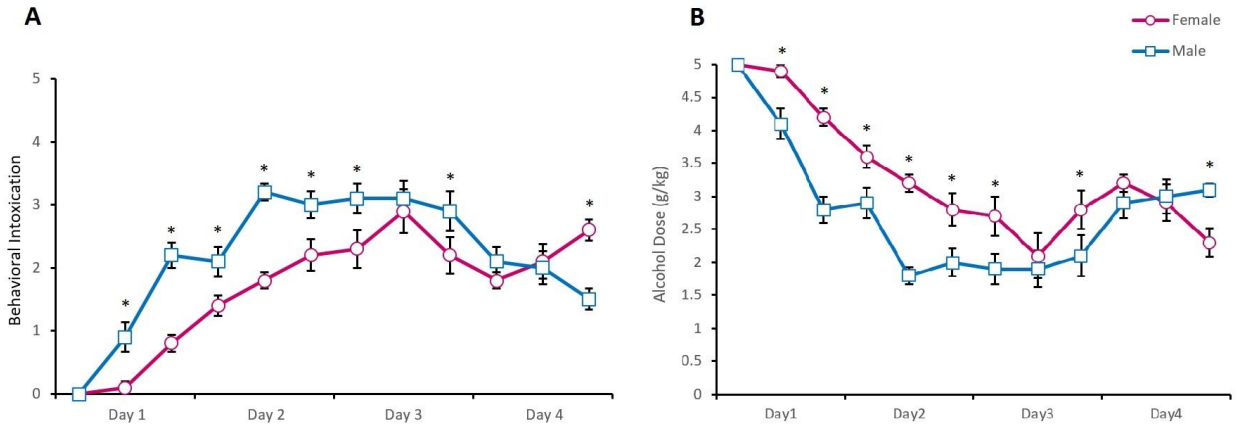

Figure 6. Males acted more intoxicated than female rats and thus received lower doses of alcohol over the course of four days of binge alcohol dosing.

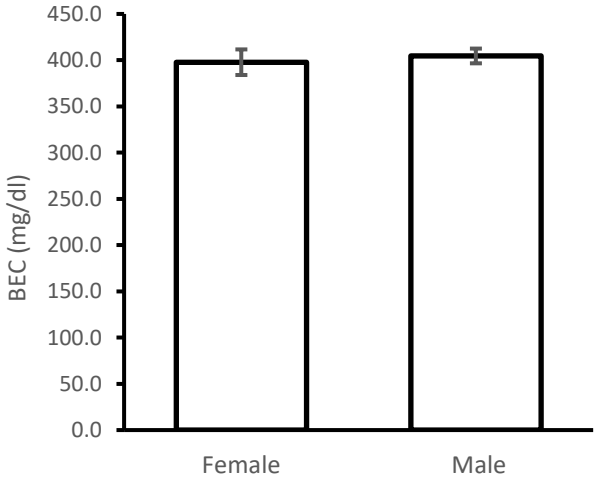

Figure 7. Male and female binge rats did not differ in blood ethanol concentration following the seventh dose of alcohol.

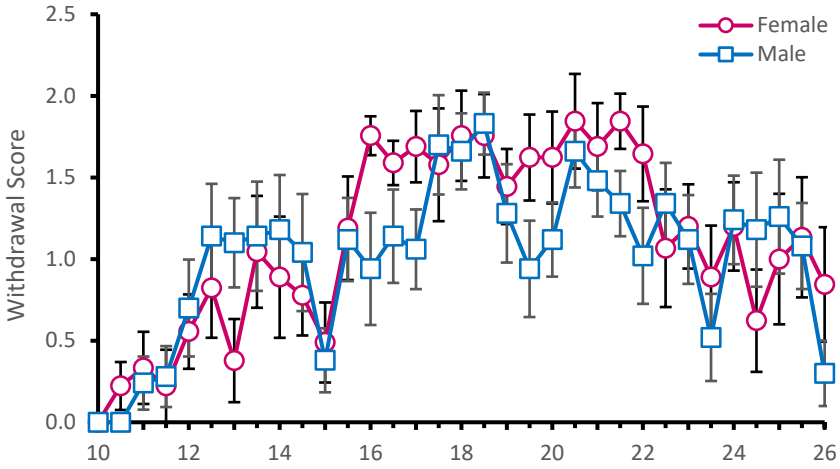

Figure 8. Male and female binge rats did not differ in peak withdrawal scores monitored 10-26 hours following the last dose of alcohol.

Food Neophobia Task

The results of food neophobia testing for impulsivity did not support Hypothesis 1a. A significant effect of Diet was found for both latency to consume novel [$F(1,35)=20.39$, $p<.0001$] and familiar foods [$F(1,35)=25.08$, $p<.0001$]. However the direction of this effect was opposite of the predicted hypothesis (figure 9), as binged animals took significantly longer to consume both novel and familiar foods. There was no main effect of Sex [$F(1, 35)=1.95$, $p=.17$] or a Sex x Diet interaction [$F(1,35)=.99$, $p=.33$] present on novel food consumption latency, nor was there a main effect of Sex [$F(1,35)=1.57$, $p=.22$] or a Sex x Diet interaction present for familiar food consumption latency. Additionally, there were no differences between groups for latency to initial approach [$F(1,35)=1.98$, $p=.13$] or number of approaches [$F(1,35)=1.49$, $p=.23$].No significant main effects or interactions existed when the difference between familiar and novel

eating times was compared [F(1,35)=.04, p=.99] and there was a strong correlation between novel and familiar eating times [r^2=.62, p<.0001].

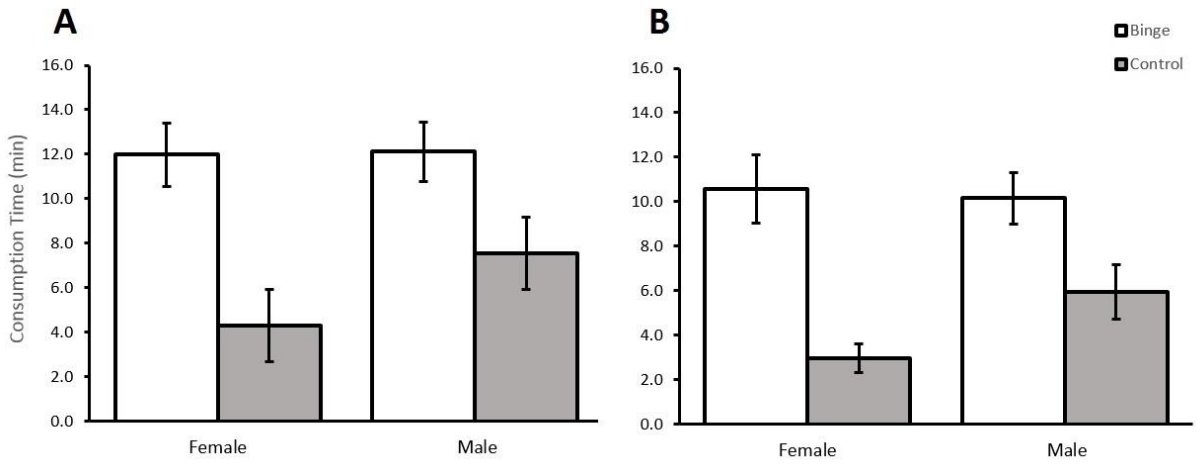

Figure 9. Female and male binged rats took significantly longer to consume both the novel (A) and familiar (B) foods in the food neophobia task.

Rewarded Alternation T-Maze

The results from the rewarded alternation T-Maze task partially supported Hypothesis 1b. While there were significant differences between female binge and control groups, male binge and control groups also differed in percentage of correct alternations (figure 10). There was a significant main effect of Diet [F(1,191)=31.24, p<.0001], and Day x Diet interaction [F(4,1946)=2.73, p=.03] on percentage of correct alternations. There was a significant effect of Diet on the first [F(1,191)=34.17, p<.0001], second [F(1,191)=17.28, p=.0002] and fourth [F(1,191)=5. p=.02], days of testing.

In addition to differences in percentage of correct alternations, there were also differences found in arm choice latency (figure 11). A 3-way mixed factorial showed a significant main effect of Day [F(4,1946)=9.71, p<.0001], Sex [F(1,191)=5.03, p=.03], and the Day x Diet

interaction [F(4,1946)=4.56, p=.002]. There was no main effect of Diet [F(1,191)=2.89, p=.10] and no other significant interactions. There was an effect of Diet on day 1 [F(1,35)=4.19, p=.05] and day 2 [F(1,35)=9.36, p=.004] and an fect of Sex on day 3 [F(1,35)=5.90, p=.02] and day 4 [F(1,35)=4.71, p=.04]. Additionally, there was no significant correlation between average latency times and average percent correct alternations [r^2=.19, p=.26].

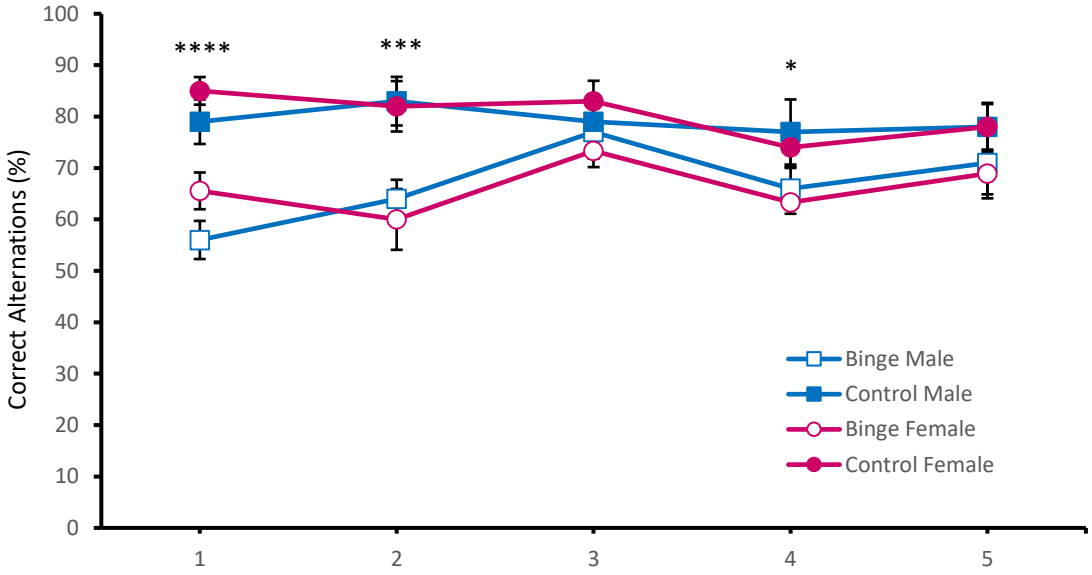

Figure 10. Both female and male binge rats had fewer correct alternations than control groups on the rewarded alternation T-maze task. *p<.05, ***p < .001, ****p<.0001 for diet

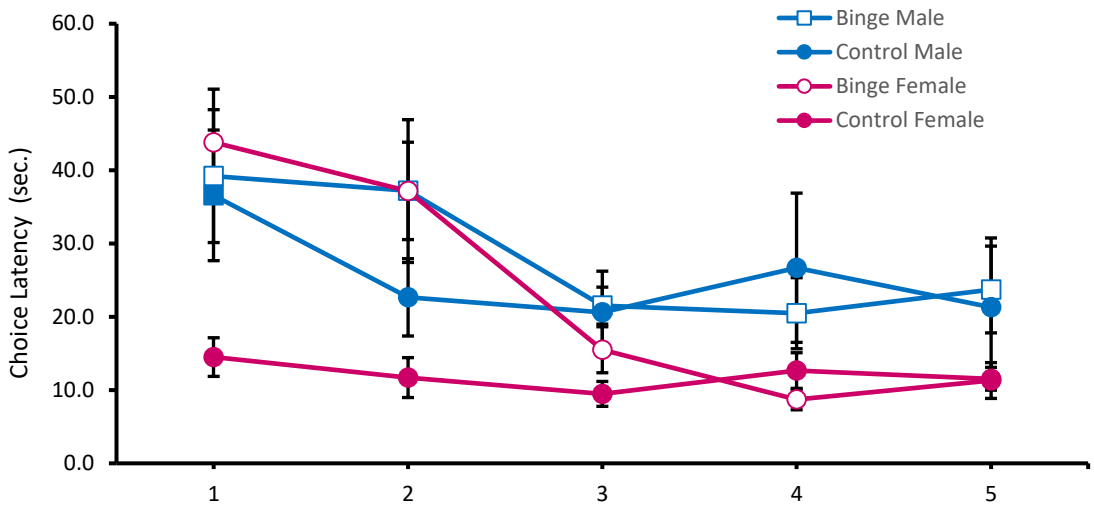

Figure 11. Male rats had longer overall latency times, however female binge rats had latency times comparable to those of the binge male rats on the first two days of rewarded alternation testing.

EXPERIMENT TWO

Medial Prefrontal Cortex Cell Volume and Population

Tissue from these rats were previously shown to exhibit a sex difference between male and female binge rats in number of remaining granule neurons in the dentate gyrus (Maynard & Leasure, unpublished). However results examining mPFC neurons only partially supported Hypothesis 2a. A two-way ANOVA revealed a significant main effect of Diet on neuronal volume [$F(1,23)=.44$, $p=.0004$], with no effect of Sex [$F(1,23)=.44$, $p=.52$] or an interaction of Diet x Sex present [$F(1,23)=.56$, $p=.46$]. The mean neuronal cell volume for all ethanol-dosed subjects was 19.9% smaller than those of the control groups. Hypothesis 2b was supported by these results. There was no main effect of Diet [$F(1,23)=2.73$, $p=.11$], Sex [$F(1,23)=.71$, $p=.41$]

or a Diet x Sex interaction [F(1,23)=.01, p=.92] present on estimated neuronal cell population in the mPFC.

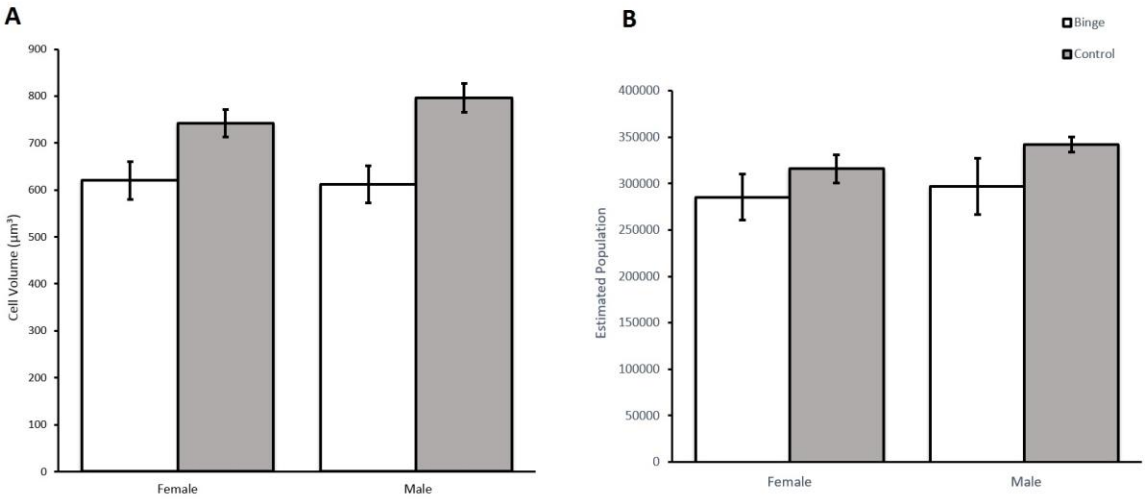

Figure 12. Both female and male binge groups had a significant reduction in neuronal volume (A), but not estimated neuronal number (B) in the mPFC in comparison to respective control groups.

Discussion

Prior research has supported the idea that the female brain is more vulnerable to the harmful effects of alcohol (Retson, Reyes & Van Bockstaele, 2015, Alfonso-Loeches, Pascual & Guerri, 2013; Wilhelm et al., 2015). However the results from this study do not support this assertion in a rodent model of binge alcohol consumption. Instead this experiment supports the conclusion that in the prefrontal cortex, male and female rats are both vulnerable to the toxic effects of excessive alcohol exposure.

The cell bodies of neurons in the medial prefrontal cortex of both the binged female and male rats had reduced neuronal cell volume, indicative of grey matter atrophy which has been previously observed in other brain areas during this model of alcohol intoxication (Obernier et al. 2002). This finding was contrary to the prediction that only female binged rats would have a reduction in cell body volume. Although this shrinking of cell body (soma) size observed in the mPFC is a marker of neurodegenerative cell death, there was not found to be a reduction in the number of remaining neurons in the medial prefrontal cortex. This indicates that although there was a damaging effect of alcohol on the prefrontal cortex, the injury was not a result of neuronal cell death, but rather of neuronal shrinking. Although cell volume loss usually is indicative of apoptotic volume decrease, which is not accompanied by compensatory mechanisms to counter the loss of cell volume (Bortner & Cidlowski, 2004, Gómez-Angelats & Cidlowski, 2002). In non-neurogenic regions, like the frontal cortex, the increase of grey matter seen in alcoholics during alcohol abstinence (van Eijk et al., 2012; O'Neill, Cardenas & Meyerhoff, 2006), most likely results from restoration of neuronal cell volume rather than formation of new neurons.

Additionally, there were no sex differences observed on percentage of correct alternations on the rewarded alternation task, also contrary to the hypothesis that only female rats who received alcohol would manifest cognitive deficits. However, during the first two days of testing, rats who received alcohol had 20% fewer correct alternations than control rats. A short inter-trial interval required rats to employ working memory, a hallmark function of the frontal lobe. Since both male and female binge animals had significantly fewer overall correct alternations, this indicates that 4-days of binge alcohol consumption did not differentially affect male and female spatial working memory on this task, and all animals that received alcohol had more difficulty employing working memory to recall which arm they previously visited. On the other hand, the

results of the food neophobia task were opposite of what was expected. Since binged rats were just as reluctant to eat the familiar food as the novel food, the results seem unlikely to be a result of impulsivity differences. The observed results may however be indicative of another underlying behavioral impact of binge alcohol or alcohol withdrawal such as anhedonia or other change in affect.

Previous research indicates that the female brain may be selectively vulnerability to alcohol (Retson, Reyes & Van Bockstaele, 2015; Wilhelm et al., 2015; Alfonso-Loeches, Pascual & Guerri, 2013), but the current study does not support this.. One possible explanation for the discrepancy is that female vulnerability to alcohol may be brain-region specific. Sex differences in reaction to alcohol are well cited (Valmas et al., 2014; Squeglia et al., 2011; Townshend & Duka, 2005; Scaife & Duka, 2009), as well as differences in impact of alcohol on various brain areas. For example, the hippocampus (Agartz et al., 2003) and corpus callosum (Hommer et al., 1996) in women appear to be selectively vulnerable to alcohol's damaging effects. Although the frontal cortex is an area commonly damaged by excessive alcohol consumption (Schweinsburg et al. 2003; Duka et al. 2003, 2004; Weissenborn & Duka 2003; Townshend & Duka 2005), it is possible that sex-specific vulnerability does not occur in the grey matter of this area of the cortex. Another proposed explanation may lie in the model of alcohol administration. Studies addressing sex differences of brain vulnerability to alcohol mainly rely on correlational studies which examine heavy drinkers, often failing to separate frequent binge drinkers from chronic alcoholics. Binge drinking is a pattern of alcohol intake which often manifests as alcohol abstinence followed by occasional bouts of heavy alcohol intake (CDC, 2012), and therefore differs from the daily drinking pattern of chronic alcoholics. While binge drinking consumption is a pattern of excessive alcohol intake, only a small percentage of binge

drinkers are alcohol dependent (Esser et al., 2014). Since binge alcohol consumption affects the body and brain differently than chronic alcohol consumption, it is not unreasonable that this four-day model of alcohol administration may affect the brain differently than other models of alcohol consumption.

Many studies indicate that females are more vulnerable to the harmful neurological effects of alcohol (Retson, Reyes & Van Bockstaele, 2015, Alfonso-Loeches, Pascual & Guerri, 2013; Wilhelm et al., 2015). However, prior work indicating that the frontal cortex of females specifically is more vulnerable to alcohol-induced damage has primarily focused on correlational studies in the adolescent or young adult human population (Schweinsburg et al., 2003, Squeglia et al., 2011, Townshend & Duka, 2005, Scaife & Duka, 2009), and has not yielded definitive results. The results from the present study contradict those found in these human studies, and instead find that in a rat model binge alcohol exposure, damage to the prefrontal cortex occurs regardless of sex. This study adds not only to the area of alcohol effects on the brain, but also aids in examining potential sex differences of alcohol susceptibility.

Study Limitations

Pilot testing of the food neophobia task on binged animals was not conducted prior to onset of the experiment and thus animals not consuming the food in the allotted fifteen total minutes of testing was not anticipated. Additionally not all rats consumed the offered reward upon choosing the correct arm in the rewarded alternation task. Likelihood to consume reward pellets was not correlated with weight or diet. The rewarded T-Maze task was chosen in lieu of an unrewarded, spontaneous alternation T-Maze task because during pilot testing, animals had lower latency times and were more motivated to complete multiple trials in succession even

when the reward pellets were not consumed. An additional minor limitation occurred during tissue processing, since sections were cut in vertical slices and were thus imaged in planar rather than isotropic sections, an unavoidable possibility for stereological bias was introduced. As seen in *figure 12*, although cell population differences were not significant, there was a decrease in both male and female binge rats when compared to their respective control groups, with a larger sample size this difference may have reached significance. In future work examining the brains of the animals who underwent behavior testing would be able to possibly reveal whether the cell body volume decrease persists 10 days after cessation of alcohol exposure, and whether there were differences in neuronal activation employed to complete the behavioral tasks

Conclusion

In this study, it was found that four days of binge alcohol exposure causes a decrease in neuronal cell body volume in the mPFC of male and female rats. Additionally, frontal cortex deficits in working memory were found in both male and female rats who had alcohol exposure. These results suggest that the repeated pattern of binge drinking seen in some alcoholics may cause detectable damage to the prefrontal cortex as well as accompanying cognitive impairments.

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