Neural Effects of Weekly Binge Alcohol: Sex Differences?

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ABSTRACT

In the U.S., 1/6 of adults report binge drinking about 4 times a month. There is also mounting evidence indicating that females may be more vulnerable to the neurotoxic effects of ethanol than males. Using a novel model of weekly binge ethanol exposure, we hypothesized that cellular damage would be greater and detectable earlier in female rats in comparison to male rats. Adult Long-Evans rats were administered 5g/kg ethanol (or an iso-caloric control dose) via intra-gastric gavage once-weekly. Neither BEC (177 mg/dl) nor behavioral intoxication measures differed over time, indicating that tolerance did not occur. Male rats, however, acted more behaviorally intoxicated than females. Brains were collected either 4 or 6 days following the final ethanol dose, and immunohistochemically processed for mature neurons (NeuN), microglia (Iba1), neurogenesis (DCX) and cellular activation (c-Fos). Stereology was used to quantify target cell populations in the hippocampus and medial prefrontal cortex (mPFC). We showed that binge ethanol administration for 11 weeks increases partial activation of microglia in the hippocampus and causes significant dentate gyrus (DG) cell loss despite an increase in neurogenesis in female rats. After 3 and 8 weeks, binge ethanol significantly decreased the number of NeuN+ cells in the DG of male and female rats in comparison to controls. 8 weeks of binge ethanol significantly increased the total number of microglia (Iba1) and the number of partially activated microglia in the hippocampus and mPFC in males and females. Despite no noted behavioral deficits during reversal learning, binged rats had increased cellular activation in the mPFC during testing, indicating decreased neural efficiency. Additionally, 8 weeks of ethanol influenced ultrasonic vocalizations in male rats, however had no effect on female rats. Overall, these results show hippocampal cell loss and an increased inflammatory response in ethanol-

vulnerable regions following repeated binge exposures in both male and female rats and showed changes in affect in male rats during behavioral testing.

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Alcohol Use

Alcohol abuse is the leading risk factor for premature death and disability among those aged 15-49 in the world (Lim et al., 2012), and roughly 13.5 percent of total deaths for people between 20 and 39 years old can be attributed to alcohol (WHO, 2018). Alcohol consumption is a common activity in America, with 70.1 percent of those over 18 reporting alcohol use within the past year, and 56 percent reporting alcohol use within the past month (SAMHSA, 2016). Although alcohol consumption in low to moderate quantities, defined as up to 1 drink per day for women and up to 2 drinks per day for men (USDA & USDHHS, 2015), has been shown to have some health benefits, such as decreased risk of heart disease, ischemic stroke and diabetes (USDA, 2015), heavy drinking can lead to a multitude of health problems. Excessive alcohol consumption has been linked to cancer, heart disease, liver cirrhosis, memory dysfunction, depression, anxiety, and brain damage to multiple regions, and has contributed to over 200 diseases and health conditions (NIAAA, 2000; WHO, 2018). According to the National Survey on Drug Use and Health, 15.1 million U.S. adults over the age of 18 had an alcohol use disorder (SAMHSA, 2016). Of those, merely 8.3 percent received treatment for their disorder (SAMHSA, 2016). An alcohol use disorder (AUD) is diagnosed as a medical condition outlined in the Diagnostic and Statistical Manual (DSM-V) (APA, 2013), as symptoms that cause distress or harm resulting from an individual's alcohol use. This condition is distressingly common and often underdiagnosed, with nearly 50 percent of American men and 25 percent of American women having symptoms that could be classified as having an AUD at some point during their lifetime (Goldstein, Dawson, Chou, & Grant, 2012). Although much research has been conducted on

American's drinking habits, other countries show similar AUD statistics (Bloomfield, Stockwell, Gmel, & Rehn, 2003; Grittner, Kuntsche, Gmel, & Bloomfield, 2013).

Consumption Pattern: Binge Drinking

When considering the physiological consequences of heavy drinking or alcohol abuse, it is important to consider the pattern of alcohol intake. Seventy-five percent of alcohol consumed by U.S. adults is in the form of binge drinking (CDC, 2018). Binge drinking is defined by the National Institute on Alcohol Abuse and Alcoholism (NIAAA, 2004) as drinking to the extent to bring an individual's blood alcohol concentration (BAC) to .08 g/dL or above. This typically is about four alcoholic drinks for women and five drinks for men in a two-hour span, and correlates with some motor impairment, diminished judgement and lessened self-control (USDA & USDHHS, 2015). In the U.S., one-sixth of American adults report binge drinking about four times a month, with an average of eight drinks per binge episode (CDC, 2012b) and 26.9 percent of adults above age 18 report binge drinking in the past month (SAMHSA, 2016). Of the 88,000-estimated annual alcohol related deaths, nearly half of those are as a result of binge drinking (Bouchery, Harwood, Sacks, Simon, & Brewer, 2011). Additionally, alcohol misuse cost the United States $249 billion in 2010, with three-quarters of that cost being related to binge drinking (Sacks, Gonzales, Bouchery, Tomedi, & Brewer, 2015).

A single alcoholic drink consists of 12-ounces of beer, 5-ounces of wine or 1.5-ounces of spirits (USDA & USDHHS, 2015). It is important to consider that depending on the recipe and drink maker, a single drink served likely contains a larger quantity of alcohol than a standard drink serving. For example, the classic margarita typically contains 2.5

ounces of spirits, 60-percent more than a “standard” alcoholic drink. Additionally, many individuals lack knowledge of what constitutes a standard drink and underestimate the pour of their drinks (White et al., 2005; Wilkinson, Allsop, & Chikritzhs, 2011), and are therefore inaccurate in self-reports of their own drinking behavior (Whitford, Widner, Mellick, & Elkins, 2009).

Although many studies of heavy alcohol use have examined college students, where getting drunk has become an almost ritualized part of a university education, 70-percent of binge drinking episodes involve those over the age of 26 (Naimi et al., 2003), and recent data shows that half the drinks are consumed by those over 35 years old (Kanny, Naimi, Liu, Lu, & Brewer, 2018). Additionally, binge drinkers over 65 report engaging in binge drinking more often than younger bingers, a reported 5 to 6 times per month (CDC, 2012b). While excessive alcohol use can be harmful at any age, the average age of first use of alcohol dropped to 14 years old in 2003 and has been decreasing over the years (Newes-Adeyi, Chen, Williams, & Faden, 2005). In fact, 22 percent of 10^{th} graders and 29 percent of 12^{th} graders report having engaged in binge drinking within the past 2 weeks (Johnston & Schulenberg, 2005). Research has indicated that young frequent binge drinkers are more likely to engage in risky behaviors (Grunbaum et al., 2004), and are also more likely to develop alcoholism or alcohol dependence in later years (Grant & Dawson, 1998).

The distinction between heavy alcohol consumption and binge drinking is an important one, yet it is often overlooked when categorizing drinkers. Heavy or at-risk alcohol consumption is defined as greater than 7 drinks per week for women, and greater than 14 drinks a week for men (USDA & USDHHS, 2015), however it is also important to consider how those drinks are spread across the week. A woman drinking a single drink a

night is unlikely to suffer any ill-consequences from her drinking, however drinking seven drinks during just one or two evenings is far more likely to be problematic due to a higher acute level of intoxication. The number of drinks consumed is by far the best predictor of BAC and level of intoxication, however many factors such as hydration, gender, and individual alcohol metabolism and tolerance can also influence the effects of alcohol. Aside from inducing high BACs, binge drinking differs from chronic heavy drinking in the pattern of intoxication. Binge drinking is typically characterized by repeated short episodes of heavy drinking, producing very high BACs, followed by longer periods of abstinence. Repeated episodes of excessive consumption followed by detoxification is likely responsible for the increased cognitive impairment and more physiological damage seen in binge drinking versus chronic drinking (Maurage et al., 2012).

Alcohol-Induced Brain Injury & Cognitive Impairments

Excessive alcohol intake is a contributing factor to at least 30 different diseases with systemic effects on the body (Rehm, 2011). Alcohol abuse is particularly damaging to the central nervous system (Gilpin & Koob, 2008), although the extent to which alcohol affects the brain is influenced by many factors. The quantity and frequency of alcohol intake, age at which drinking began, how long an individual has been drinking, current age, education, gender, general health, family history, and genetics can all influence the extent of damage resulting from heavy or problematic alcohol use (Parsons, 1996). Chronic alcohol consumption has been shown to cause gray and white matter loss, enlargement of the ventricles, and global brain shrinkage, particularly in cortical regions important for behavioral regulation and memory (F. T. Crews & Nixon, 2009; Kubota et al., 2001; K.

Nixon & Crews, 2002; Obernier, Bouldin, & Crews, 2002; Sullivan & Pfefferbaum, 2005). However, binge drinking does differ from the chronic drinking common among alcoholics, and the resulting type and severity of brain damage does depend on the on the specific pattern and quantity of alcohol intake (F. T. Crews & Nixon, 2009; Hunt, 1993; Lisdahl, Thayer, Squeglia, McQueeny, & Tapert, 2013; K. Nixon & Crews, 2002; Obernier, Bouldin, et al., 2002).

Binge drinking specifically has been shown to cause damage to several key areas of the brain, as well as inducing cognitive deficits in both the human and animal literature (Loeber et al., 2010; K. Nixon & Crews, 2002; Obernier, Bouldin, et al., 2002; Obernier, White, Swartzwelder, & Crews, 2002; Stephens & Duka, 2008; Stephens et al., 2005). Binge drinking is also more predictive of brain damage than examining the total lifetime alcohol consumption (Bobak et al., 2004; Hunt, 1993). Multiple periods of alcohol withdrawal have been associated with reduced neuroplasticity (Loeber et al., 2010), impaired cognitive abilities (Duka et al., 2004; Loeber et al., 2009; Stephens et al., 2005), microglial activation (McClain et al., 2011), and disruptions to cortical functioning (Duka et al., 2004; Duka, Townshend, Collier, & Stephens, 2003).

Two brain areas particularly susceptible to alcohol's damaging effects are the hippocampus and prefrontal cortex. These areas are of particular interest because of their critical role in cognition, connections with other brain areas, and implication in addiction. Both the frontal lobes and hippocampus are vulnerable to alcohol's damaging effects, and optimal functioning of both areas is critical for efficient brain function.

Effects of Alcohol on the Frontal Cortex.

The damage to the frontal lobe resulting from heavy alcohol consumption has been implicated in deficits in a wide arrange of cognitive tasks (Brown, Tapert, Granholm, & Delis, 2000; F. T. Crews, He, & Hodge, 2007; Konrad et al., 2012). Excessive alcohol consumption has been linked to deficits in tests of impulsivity (Stephens & Duka, 2008), executive control (Duka et al., 2004; Duka et al., 2003; Townshend & Duka, 2005; Weissenborn & Duka, 2003), cognitive evaluation of reward (Taren, Venkatraman, & Huettel, 2011), and risk-taking (Crone, Bullens, van der Plas, Kijkuit, & Zelazo, 2008). These higher cognitive functions governed by the frontal cortex, such as planning, executive functioning, inhibition, and working memory are thought to have a critical role in the cycle of alcohol abuse (Jernigan et al., 1991; Kubota et al., 2001; Sullivan & Pfefferbaum, 2005).

A decrease of prefrontal grey matter resulting from excessive alcohol consumption has been observed in the clinical population (Kubota et al., 2001; Nakamura-Palacios et al., 2014), however, in rodent studies, this loss of frontal lobe grey matter does not appear to be caused by neuronal death (Koss, Sadowski, Sherrill, Gulley, & Juraska, 2012). Rather, the grey matter loss observed may instead be a result of loss of cell body volume (S. H. Freeman et al., 2008; West, Maynard, & Leasure, 2018). Prior research examining the mPFC immediately following a 4-day model of binge alcohol administration, indicated that while there was no decrease in the number of neurons remaining, the neuronal cell bodies of both male and female animals that received alcohol had a nearly 20-percent decrease in volume (West et al., 2018). Although neuronal atrophy is not as severe or permanent as cell death, cell volume loss is still indicative of significant damage sustained from alcohol exposure, and it is unclear how long the volume loss persists.

Another consideration when evaluating alcohol-induced damage is cognitive efficiency. Brain damage can still occur even in the absence of observable cognitive or behavioral deficits, such as is evident in brain injuries such as asymptomatic or silent traumatic brain injury or stroke. Therefore, the lack of alcohol-induced behavioral deficits observed in some studies (Acheson, Ross, & Swartzwelder, 2001; Markwiese, Acheson, Levin, Wilson, & Swartzwelder, 1998), does not necessarily indicate a lack of alcohol-induced brain damage. Compensatory changes have been observed in human binge drinkers, as evidenced by a study conducted by Campanella and colleagues (2013), which found that despite comparable behavioral performance to controls, binge drinkers showed increased brain activity during a working memory task. There was also a positive association between brain activity and intensity or frequency of binge drinking (Campanella et al., 2013). This suggests that a history of binge drinking can cause neural damage which requires the brain to use more energy to perform at baseline levels.

Effects of Alcohol on the Hippocampus.

In addition to the frontal cortex, the hippocampus is also believed to have a critical role in addiction (Hyman, Malenka, & Nestler, 2006; S. A. Morris, Eaves, Smith, & Nixon, 2010), and is particularly sensitive to alcohol-induced damage (Agartz, Momenan, Rawlings, Kerich, & Hommer, 1999; Beresford et al., 2006; Sullivan, Marsh, Mathalon, Lim, & Pfefferbaum, 1995). Alcohol use disorder and problematic alcohol use, such as binge drinking is associated with reduced hippocampal volume (Wilson, Bair, Thomas, & Iacono, 2017). The hippocampus plays a vital role in learning and memory and alcohol-related damage to this area can cause deficits in tests of memory, learning, and spatial

navigation (Beatty, Hames, Blanco, Nixon, & Tivis, 1996; F. T. Crews et al., 2004; Pitel et al., 2009; Townshend & Duka, 2005).

In addition to cell death in the hippocampus, alcohol exposure can also inhibit cell proliferation, further contributing to hippocampal neurodegeneration (S. A. Morris et al., 2010; K. Nixon & Crews, 2002). Although the hippocampus is especially vulnerable to alcohol toxicity, it also is a neurogenic region that is capable of recovery from alcohol-induced damage. Neurogenesis, or the process by which new neurons are created, has been shown to occur throughout the human lifespan in the hippocampus (Boldrini et al., 2018; Eriksson et al., 1998), although there is some recent debate on the amount of neurogenesis that occurs in human adults (Sorrells et al., 2018). Reactive cell proliferation in the dentate gyrus of the hippocampus occurs within a week of alcohol abstinence, and produces cells that can grow and mature to repopulate the dentate gyrus granule neuron cell layer (K. Nixon & Crews, 2004).

Alcohol exposure has been shown to increase microglial activation and proliferation in the hippocampus (Barton, Baker, & Leasure, 2017; Barton, Lu, et al., 2017; McClain et al., 2011; West, Wooden, Barton, & Leasure, 2019). Microglia serve to support neurons and protect the central nervous system, and additionally may play a role in alcohol-induced neurodegeneration. A reduction in microglia may decrease trophic support, which may therefore increase the neuronal damage sustained from alcohol (F. T. Crews & Nixon, 2009; Miguel-Hidalgo et al., 2002). The process in which microglia alter their morphology and functionally differentiate in response to changes in their environment, often referred to as microglial activation or microglial priming, was traditionally described as proinflammatory and cytotoxic (Kreutzberg, 1996). However, microglial activation is a spectrum, and

identification of the particular activation state or phenotype can aid in understanding their role in injury response (Carson et al., 2007; C. Colton & Wilcock, 2010; Raivich et al., 1999; Schwartz, Butovsky, Bruck, & Hanisch, 2006; Town, Nikolic, & Tan, 2005). Partial cellular activation, or those that are in earlier activation states, appear to be therapeutic and play a role in repair and protection (Battista, Ferrari, Gage, & Pitossi, 2006; Shokouhi et al., 2010). Morphological changes to microglia, such as large somas and thick arbors, are indicative of partial microglial activation (Bollinger, Bergeon Burns, & Wellman, 2016; Norden, Muccigrosso, & Godbout, 2015; Perry & Holmes, 2014; Wolf, Boddeke, & Kettenmann, 2017). This is in contrast to higher activation states which result in a cytotoxic amoeboid phenotype which is associated with microglial phagocytosis and inflammation (Banati, Gehrmann, Schubert, & Kreutzberg, 1993; Kreutzberg, 1996; Raivich et al., 1999). It appears however, that heavy alcohol consumption results only in partial activation of microglia and does not elicit this harmful neuroinflammatory response. Binge ethanol exposure has been shown to prime microglia to enhance their response to a second binge (Marshall, Geil, & Nixon, 2016), which suggests that microglia may in fact, be working to regain homeostatic balance, rather than contributing to neurodegeneration (Marshall et al., 2013).

Assessing the Effects of Alcohol on Behavioral Outcomes

Heavy alcohol consumption has been linked to deficits in multiple cognitive tasks and can also impact emotional processing and mood. Areas such as the prefrontal cortex and hippocampus play a role in emotional processing and state regulation. A reduction in mature granule cells in the DG has been associated with worsened depression symptoms (Boldrini

et al., 2013). Therefore, a reduction in mature neurons in the granular cell layer resulting from alcohol exposure, like what has previously been observed (Maynard & Leasure, 2013; West et al., 2019), may worsen or induce depressive symptoms (Ji et al., 2012). Depressive-like symptoms following chronic ethanol exposure have been observed in rodents (Briones & Woods, 2013; Stevenson et al., 2009). In humans, those who are alcoholic dependent are two to three times more likely to present with comorbid depression or anxiety (Swendsen et al., 1998).

In rats, ultrasonic vocalizations (USV) allow for examination of affective state. Two frequencies of emissions are typically examined, 50-kHz and 22-kHz. The higher frequency, 50-kHz USV are typically emitted in pro-social interactions, such as during play or mating (Burgdorf et al., 2008; Burgdorf, Panksepp, & Moskal, 2011; Knutson, Burgdorf, & Panksepp, 1998; Panksepp & Burgdorf, 2000; Sales, 1972; Schwarting, Jegan, & Wohr, 2007), and can also be induced in a laboratory setting through interaction with a human hand (Schwarting et al., 2007). The emission of 22-kHz USV is associated with negative affect, such as from fear, pain, anxiety or stress (Calvino, Besson, Boehrer, & Depaulis, 1996; Cuomo et al., 1988; Knapp & Pohorecky, 1995; Wohr, Borta, & Schwarting, 2005). Investigation of adult vocalizations can thus allow researchers to examine associative mood states in the rat. Examination of cognitive deficits can be accomplished through use of a common spatial learning and memory task for use in rodents, the Morris water maze (MWM). Alcohol exposure has been shown to cause deficits in performance (Arendt et al., 1988; Lukoyanov, Madeira, & Paula-Barbosa, 1999; Maynard, Barton, Robinson, Wooden, & Leasure, 2018; Melia, Ryabinin, Corodimas, Wilson, & Ledoux, 1996; Obernier, White, et al., 2002; Schulteis, Archer, Tapert, & Frank, 2008; Shimizu, Matsubara, Uezono,

Kimura, & Shiono, 1998; White, Elek, Beltz, & Best, 1998). Therefore, using a combination of these tasks, changes in both cognition and affect resulting from alcohol exposure could be examined.

Sex Differences in Alcohol Vulnerability

Excessive or chronic alcohol use is associated with many negative consequences and health risks; however, females appear to have an increased risk of these harmful effects. Female alcoholics are at increased risk for cirrhosis, cardiomyopathy, nerve damage, heart disease and stroke (Ammendola et al., 2000; Fernandez-Sola et al., 1997; Loft, Olesen, & Dossing, 1987), despite shorter histories of alcoholism than male alcoholics. Women have lower rates of alcoholism than men (Kessler et al., 1994), begin drinking later in life, and consume less on average than men (Zilberman, Tavares, & el-Guebaly, 2003). Despite this, women progress to alcohol dependence more quickly than men (Hernandez-Avila, Rounsaville, & Kranzler, 2004; Randall et al., 1999; Schuckit, Anthenelli, Bucholz, Hesselbrock, & Tipp, 1995; Schuckit, Daeppen, Tipp, Hesselbrock, & Bucholz, 1998). This concept of increased physiological impacts of alcohol in females, despite that women tend to consume less and have shorter histories of excessive alcohol intake then men, is referred to as "telescoping" (Piazza, Vrbka, & Yeager, 1989). The existence of this telescoping effect, however, is still under debate and the theory is not universally accepted (Diehl et al., 2007; Goldstein et al., 2012; Keyes, Grant, & Hasin, 2008; Mann et al., 2005).

Both male and female alcoholics show a significant brain volume decrease in comparison to non-alcoholics, and heavy drinkers of both sexes show memory and learning deficits stemming from heavy alcohol intake (D. W. Hommer, 2003; Jacobson, 1986; Mann,

Batra, Gunthner, & Schroth, 1992; S. J. Nixon, Tivis, & Parsons, 1995). However, alcoholic men in these studies reported drinking excessively about twice as many years as the reported histories of the alcoholic women, indicating female vulnerability to alcohol-induced damage (D. W. Hommer, 2003; Mann et al., 2005; Mann et al., 1992). Compared to male alcoholics, female alcoholics show a greater overall brain volume decrease, smaller hippocampi (Agartz, Shoaf, Rawlings, Momenan, & Hommer, 2003), smaller corpus callosum (D. Hommer, Momenan, Kaiser, & Rawlings, 2001; D. Hommer et al., 1996), and frontal lobe grey matter reduction (Schweinsburg et al., 2003). In addition to increased cellular impacts resulting from excessive alcohol, females also exhibit cognitive deficits in hippocampal and frontal cortex mediated tasks. Female binge drinkers perform more poorly than male binge drinkers in such tasks as spatial working memory, attention and inhibition (Scaife & Duka, 2009; Squeglia, Schweinsburg, Pulido, & Tapert, 2011; Townshend & Duka, 2005).

Despite evidence pointing toward a vulnerability of the female brain to alcohol-induced damage, not all studies have shown this trend. While female alcoholics were found to have smaller cortical grey and white matter than alcoholic men, these differences could be accounted for by established sex-differences in intracranial volume (Pfefferbaum, Rosenbloom, Deshmukh, & Sullivan, 2001). Other studies failed entirely to show any indication of brain damage in alcoholic women (Kroft et al., 1991; Pfefferbaum, Rosenbloom, Serventi, & Sullivan, 2002).

Although a multitude of studies indicate that females are more vulnerable to alcohol neurotoxicity, studies investigating alcohol-induced damage primarily investigate only male samples (Stavro, Pelletier, & Potvin, 2013). Investigation into sex-differences is particularly important in the context of potential female susceptibility to alcohol-induced brain damage

(Agartz et al., 2003; D. Hommer et al., 2001; D. Hommer et al., 1996; Mann et al., 1992; Maynard & Leasure, 2013), especially when taken into consideration the increasing trend for binge drinking among American women (Breslow, Castle, Chen, & Graubard, 2017; Breslow & Smothers, 2004; Dwyer-Lindgren et al., 2015; Grant et al., 2017). According to the Centers for Disease Control and Prevention, 1 in 8 American women binges 3 times per month, 6 drinks per binge (CDC, 2012a). While the existence of a telescoping effect regarding alcohol-induced brain damage is still debated, much evidence suggests that heavy alcohol consumption is more harmful to females' health than it is to males' health.

Gender 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), with White and Hispanic women between the ages of 18 and 34, with household incomes over $75,000, being the mostly like to engage in binge drinking (CDC, 2013). While the prevalence of alcoholism and binge drinking is still higher in men than women, the gap is quickly closing. There has been 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). Additionally, while binge drinking among men above 60 has remained stable in the last two decades, prevalence in binge drinking in women of the same age increased by about 3.7% a year (Breslow et al., 2017). Multiple contributing factors likely account for this increase, including increased alcohol marketing toward women in recent years, more women who use alcohol to self-medicate for mood disorders (Dixit & Crum, 2000), as well as the normalization of women drinking in media and television. Increased social normalization of

women binge drinking and more alcohol marketing toward women is potentially problematic when considered in the context of women's apparent increased vulnerability to the toxic effects of alcohol (Agartz et al., 2003; Alfonso-Loeches, Pascual, & Guerri, 2013; Mann et al., 1992; Maynard et al., 2018; Sharrett-Field, Butler, Reynolds, Berry, & Prendergast, 2013; Wilhelm et al., 2016).

Project Overview

Despite growing evidence that the female brain is selectively vulnerable to the damaging effects of alcohol, few studies in either human or animal subjects have directly investigated sex-differences in alcohol-induced brain damage. Additionally, current rodent models of binge alcohol exposure do not typically relate to the most common human drinking patterns. Therefore, there is a need to investigate whether females are more susceptible to binge alcohol exposure than males, in addition to creating a model that will more closely mimic human drinking patterns and blood alcohol levels. The development of a clinically relevant rodent model of binge pattern AUD will aid future research efforts to investigate the mechanisms underlying female brain vulnerability to alcohol and the effects of binge alcohol on the brain.

Given the evidence that the female brain may be more susceptible to alcohol neurotoxicity (Agartz et al., 2003; D. Hommer et al., 2001; D. Hommer et al., 1996; Mann et al., 1992; Maynard & Leasure, 2013), it was predicted that using a rodent binge model, we would see evidence of brain damage in female rats with less ethanol exposure than what would be required for equivalent damage to be seen in male animals. Based on previous studies, we predicted that binge ethanol would cause neuronal damage to the frontal cortex

(Kubota et al., 2001; Nakamura-Palacios et al., 2014) and hippocampus (Maynard et al., 2018; Wilson et al., 2017), both of which have been shown to be vulnerable to ethanol damage. In the predicted event of hippocampal cell loss, we also would predict reactive cell proliferation, like what has been noted in response to alcohol abstinence following binge ethanol exposure (K. Nixon & Crews, 2004). We also predicted that there would be increased neuroimmune activation resulting from binge ethanol, as has been demonstrated from other models of ethanol exposure (Barton, Baker, et al., 2017; Barton, Lu, et al., 2017; McClain et al., 2011). Since ethanol exposure has been shown to affect both cognition (Maynard et al., 2018; Obernier, White, et al., 2002; Schulteis et al., 2008) and affect (Briones & Woods, 2013; Stevenson et al., 2009), we predicted that rodents with binge exposure would show deficits on behavioral tasks. Moreover, because it has been shown that the brains of human binge drinkers are less efficient during cognitive processing, we predicted that binged rats would show increased cellular activation during cognitive testing (Campanella et al., 2013; West et al., 2018).

This project aimed to identify damage that occurs from weekly binge alcohol exposure to the female brain and explore potential sex-differences in appearance and severity of brain damage and behavioral impairments. The overarching goal for this project was to examine sex differences in response to weekly binge exposure by investigating both cellular and behavioral outcomes in a rat model. More specifically, we attempted to determine whether females would manifest damage to vulnerable areas of the brain after weekly binge exposure and whether they would show greater behavioral impairments with fewer weeks of binge ethanol than males.

Aim 1: Examine the effects of repeated binge alcohol exposure on the female brain.

It was hypothesized that female rats that received weekly binge doses of alcohol would show significant brain injury when compared to same sex controls. Specifically, it was anticipated that binged rats will have significantly fewer granule neurons in the hippocampal dentate gyrus, decreased neuronal cell volume in the medial prefrontal cortex (mPFC), increased hippocampal neurogenesis, an increase in the number of activated microglia in the mPFC and hippocampus, and increased neuronal activation in response to behavior testing. These results combined would indicate that significant brain damage has occurred as a result of binge alcohol exposure, and further hint that the brain is attempting to compensate for sustained damage to key brain regions.

Aim 2: Determine whether a telescoping effect will result in earlier binge alcohol-induced brain damage in female rats.

It was hypothesized that female rats would manifest brain damage (as indicated by a decrease in remaining dentate gyrus granule neurons), with fewer dose of binge alcohol than male rats. It was also predicted that all significant cellular damage noted in female rats after 11 weeks (aim 1), would be detectable in female rats with fewer weeks than 11 weeks of ethanol exposure. It was also predicted that female rats would manifest more severe neural effects from ethanol damage, or show significant cellular changes with fewer weeks of exposure in comparison to male rats. Development of damage to these susceptible brain regions with fewer alcohol doses would indicate a sex-dependent telescoping effect and would provide evidence of female vulnerability to binge alcohol.

Aim 3: Determine whether a telescoping effect will result in earlier binge alcohol-induced behavioral deficits in female rats.

It was hypothesized that female binged rats would show behavioral deficits with fewer doses of alcohol than male binge rats receiving alcohol. Specifically, it was hypothesized that binged rats would emit fewer 50-kHz and more 22-kHz ultrasonic vocalizations compared with same-sex controls in response to play-sessions. On the Morris water maze task, it was hypothesized that binged rats would have longer escape latencies than controls, and that this deficit would appear more quickly in the female binge group than the male binge group.

Chapter 2: Examination of the effects of repeated binge alcohol exposure on the female brain

Many rodent models of AUD have been developed, employing techniques such as intraperitoneal injection, vapor inhalation, or oral administration. However, even though binge drinking has increasingly become a problem (Naimi et al., 2003), comparatively few rodent models of binge pattern AUD have been developed. Of the current models of binge alcohol exposure, many produce unrealistically high BACs that are not necessarily representative of the BACs of typical binge drinkers. While no animal model is without some flaws, the present model attempts to mirror the typical pattern of intake and level of intoxication of human binge drinkers.

Because few studies have attempted to estimate the average binge drinker's BAC through self-report or random sampling (Lange & Voas, 2000; Perkins, Linkenbach, & Dejong, 2001), there is no clear consensus on the average BAC of binge drinkers. One-sixth of the population in America drinks an average of eight drinks, four times per month (CDC, 2012b), which could yield a wide range of BACs, depending on factors such as sex, weight and time spent drinking. For reference, using the Widmark formula, the average 195 lb. American man and 168 lb. woman drinking eight standard drinks over the course of four hours would yield BACs of .10% and .17%, respectively. The current model involves intragastric administration of alcohol weekly and yields BACs ~ 174 mg/dl in female rats. These levels are consistent with likely intoxication levels in women binge drinkers.

Another important feature of this model is the lack of metabolic tolerance observed. Chronic alcohol administration can induce metabolic tolerance (Videla & Israel, 1970), meaning that alcohol is more quickly neutralized by the body. While habitual binge alcohol

consumption has been correlated with increased acute or behavioral tolerance (Fillmore & Weafer, 2012; Linsenbardt, Moore, Griffin, Gigante, & Boehm, 2011; Marczinski, Combs, & Fillmore, 2007), intermittent binge drinking does not produce the sustained high alcohol levels to necessary to induce metabolic tolerance (Esser et al., 2014; Tabakoff, Cornell, & Hoffman, 1986). The neuro-adaption that occurs with chronic alcohol consumption may delay the development of brain damage, whereas binge drinking does not induce tolerance and therefore leaves the brain vulnerable to toxic alcohol effects (Ward, Lallemand, & de Witte, 2009). An advantage of the current model is the lack of alcohol dependence or subsequent withdrawal symptoms observed. While dehydration and buildup of acetaldehyde resulting from alcohol intoxication can cause withdrawal-like "hangover" symptoms, such as nausea, fatigue and headache; alcohol withdrawal only occurs in those who are alcohol-dependent. Although binge drinkers are at higher risk than non-binge drinkers (i.e. light or social drinkers), for alcohol dependence (Dawson, 2000), only 10.5-percent of binge drinkers qualify as being alcohol- dependent (Esser et al., 2014).

This aim examined the effects of weekly repeated exposures of a 5 g/kg alcohol dose on the female rodent brain. Female rats were chosen for this aim because of the evidence of the susceptibility of the female brain to ethanol damage (Agartz et al., 2003; Alfonso-Loeches et al., 2013; Mann et al., 1992; Sharrett-Field et al., 2013; Wilhelm et al., 2016). Since this weekly binge model has never been used before, it was unknown what cellular damage would result, and therefore we hypothesized that if damage were to manifest, it would likely happen more quickly in female rats than in male rats. It was predicted that female rats receiving binge alcohol would show significant brain injury when compared with control females. Since the hippocampus and frontal cortex are two brain regions highly

susceptible to alcohol and critical for cognitive functioning, cellular analysis was focused on those two brain regions. It was expected that compared to same-sex controls, female binged rats would have an increase of activated microglia in the hippocampus and mPFC, reduced neuronal size in the mPFC, a decrease in remaining granule neurons in the DG of the hippocampus, increased neurogenesis in the DG and increased neuronal activation in response to a spatial navigation task in the in the mPFC and DG. Combined, these analyses allowed for examination of multiple cell types in two key areas of the brain that are essential for a wide range of cognitive functions.

Method

Statement of Ethics and Financial Support

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. Funding for collection of pilot data was provided by a University of Houston 2014-2015 Grant to Enhance and Advance Research awarded to J. Leigh Leasure.

Animals

The current aim used tissue collected from animals from a pilot experiment conducted in summer 2015. Eighteen female Long-Evans rats were randomly assigned to either the alcohol (n=9) or control group (n=9) and were housed in a reverse light-dark cycle housing room in groups of two or three per cage. Upon arrival at eight weeks of age, rats

spent a week acclimating to their new housing while also being handled daily to become accustomed to interaction with the experimenter.

Alcohol Administration

All food was removed from both the alcohol and control group roughly 8 hours prior to alcohol administration, however water was always accessible. The ethanol diet consisted of a 25% ethanol solution (190-proof alcohol with vanilla Ensure Plus™), and the control diet was an isocaloric mixture of Dextrose with vanilla Ensure Plus™, administered via intragastric gavage (adapted from: Nixon & Crews, 2002; Obernier et al., 2002; Majchrowicz, 1975). A single ethanol dose of 5 g/kg of animal body weight was given every 7 days at the end of the animals dark (active) cycle. Amount of fluid given to control groups varied by week and was dictated by the average volume of fluid that the same-sex ethanol group received for that dosing week. A total of 11 doses of alcohol or control diet were administered over the course of the study. Food was replaced in all home cages roughly 2 hours following alcohol or control diet administration.

Intoxication Measures

Blood was drawn via the lateral saphenous vein using heparinized capillary tubes, ninety minutes following each alcohol dose. This timepoint has been cited as the peak level of intoxication in rats following gavage alcohol dosing (F. T. Crews, Mdzinarishvili, Kim, He, & Nixon, 2006; Kelly, Bonthius, & West, 1987; K. Nixon & Crews, 2002). Blood was placed in 1 mL eppendorf tubes and immediately centrifuged following collection. Separated plasma from each sample was then transferred to a separate eppendorf tube, then

stored at -80 degrees Celsius until analysis. Samples were analyzed using a 5 µl aliquot in a GM7 Analyzer (Analox, MA, USA), in order to determine the blood ethanol concentration of each rat for each alcohol dose. Average blood concentration was 162.5 mg/dl ± 7.99 mg/dl and no metabolic tolerance to subsequent doses was noted.

Behavioral intoxication was also measured ninety minutes following each dose of alcohol. Behavioral intoxication scores were based on the 7-point scale designed by Majchrowicz (1975), with a score of 0 being no symptoms of intoxication and a score of 5 corresponding to loss of righting and eye blink reflex (see Table 1). The most severe behavioral intoxication symptoms noted were a delay in righting reflex and inability to elevate the abdomen (score=3) and the median observed behavioral intoxication for all doses was 1, corresponding with slight observable intoxication and mild loss of motor coordination. No progressive behavioral tolerance was noted after the second alcohol dose.

Table 2.1. Intoxication Scoring

Intoxication Score	Indications
0	Normal rat
1	Hypoactive, mild ataxia
2	Ataxic, abdomen elevated
3	Delayed righting reflex, ataxic with no abdominal elevation
4	Loss of righting reflex, retains eye blink reflex
5	Loss of righting reflex, loss of eye blink reflex

Table 2.1. Rat's behavioral intoxication was scored based on observed intoxication symptoms

Corticosterone Analysis

Fecal boli were collected from all rats 3 days following ethanol administration each week between 9:00-11:00 AM to determine if changes in corticosterone levels would result from repeated binge ethanol exposure. Whole fecal samples were stored at -80˚C until analysis. Samples were prepared by first thawing boli and weighing to .1 gram. To each

sample, 1 mL of 100% methanol was added and then samples were crushed, vortexed, then centrifuged for 10 minutes. Following centrifuging, 20 µl of the supernatant was removed and placed in a separate tube. Boli from three timepoints (following 2, 7, and 11 doses) was analyzed in triplicates using corticosterone ELISA kits according to the product instructions (Enzo Life Science; New York, USA) and compared between groups.

Tissue Collection & Processing

Four days after the 11th dosing of alcohol, approximately one hour after the completion of behavioral testing, rats were given an overdose of anesthetic (mixture of ketamine, xylazine and acepromazine) followed by intracardial perfusion with cold saline followed by 4% paraformaldehyde in 0.2M PBS (pH 7.2) until the limbs were stiff. The brain was extracted and post-fixed overnight, then refrigerated in 30% sucrose until sectioning. Frozen tissue was sectioned into 50 µm coronal sections on a microtome and sections were then stored in 96-well plates at -20 °C in cryoprotectant until further tissue processing.

Tissue Processing

To examine the effect of repeated binge alcohol exposure on the brain, tissue sections from the region of interest of each animal were processed with immunohistochemisty (IHC) in order to visualize specific cell types.

Standard IHC protocol

The IHC protocol consisted of first isolating the sections of interest by floating them in 0.1M PO_4. Sections in the region of interest were then placed in 0.1 M Tris Buffer (TBS) and rinsed for 10 minutes on a shaker at room temperature, three separate times. Sections were then placed for 30-minutes into a 0.6% Hydrogen Peroxide (H_2O_2) room temperature rinse. Following this, sections were again rinsed three times for ten minutes each in TBS. After all TBS rinses were complete, sections were transferred into blocking solution of 3% normal donkey serum (Sigma-Aldrich, St. Louis, MO, USA) for 60 minutes. Following the blocking step, sections were incubated in primary antibody in the cold room at 4 °C for 72 hours. After primary incubation, the sections were removed from the cold room and rinsed twice, for 15 minutes each in room temperature TBS. Following this rinse, sections were placed once again in blocking solution for 15 minutes and then placed in corresponding secondary antibody for 24 hours at room temperature (see Table 2). The following day, sections were rinsed in an additional three ten-minute TBS washes and then incubated in ABC/*Elite* Standard Kit at room temperature for 60 minutes. After three more ten-minute TBS rinses, the sections were reacted in diaminobenzidine (DAB), a non-florescent label. After a final three ten-minute TBS rinses, sections were mounted on gelatin coated slides and left to dry overnight. Once sections had fully adhered to the slide, they were counterstained with methyl green, cleared in alcohol followed by xylene and then cover slipped (Permount; Electron Microscopy Sciences, Hatfield, PA).

Table 2.2 Primary and Secondary Antibodies

Primary	Concentration	Manufacturer	Secondary	Concentration	Manufacturer
NeuN anti-guinea pig	1:1000	EMD Millapore	Donkey anti-guinea pig	1:250	Jackson ImmunoResearch
DCX anti-rabbit poly	1:100	Santa Cruz Biotechnology	Donkey anti-rabbit	1:250	Jackson ImmunoResearch
iba1 anti-rabbit	1:10,000	Wako USA	Donkey anti-rabbit	1:250	Jackson ImmunoResearch
c-Fos anti-mouse mono	1:100	Santa Cruz Biotechnology	Donkey anti-mouse	1:250	Jackson ImmunoResearch

Table 2.2. Primary and secondary antibodies, dilutions and suppliers used for immunohistochemistry

c-Fos IHC protocol

The IHC protocol used for the c-Fos antibody differed slightly from the standard IHC protocol. After selecting the tissue sections which fall in the region of interest, sections were floated three times in .05 M Phosphate Buffer (PBS) for ten minutes each. Following the PBS rinses, sections were placed for in a blocking solution of 5% normal donkey serum (Sigma-Aldrich, St. Louis, MO, USA), PBS and Triton-X for one hour. Following the blocking step, tissue was placed into primary antibody solution (c-Fos anti-mouse monoclonal, Santa Cruz Biotechnology, Dallas, TX, USA; 1:100), at room temperature on a shaker for 18 hours. The following day, sections were removed from the primary antibody solution and rinsed three times (10 minutes each) in PBS. After the PBS rinses, sections were placed for two hours at room temperature in secondary antibody (donkey anti-mouse biotinylated, Jackson ImmunoResearch, West Grove, PA, USA; 1:250). Following three more PBS washes, the sections were incubated in ABC for 45 minutes. Tissue was given two more rinses in PBS, followed by a single ten-minute rinse in TBS. Following the last rinse, all sections were reacted in DAB and then rinsed another three times in PBS before being mounted. Once tissue was mounted and dried overnight, slides were cleared in xylene for 5 minutes, then cover slipped.

Stereology

Stereology is a process that allows for unbiased estimation of cell population and associated parameters. The optical fractionator method within automated stereology software (StereoInvestigator, MicroBrightField, VT, USA), works by using the number of manually counted cells in small randomized frames to create an estimate for the total number of cells in the whole area or structure. In conjunction with an Olympus BX51WI upright microscope, the program first generates a grid of counting frames overlaying the area of interest so that within each counting frame, cells within a smaller sampling site can be manually counted by the experimenter. Cells within each counting frame are then averaged and this information can then be combined with the measured tissue thickness to estimate the total population of cells. Slides were coded to enable the experimenter to be blind to experimental condition and number of estimated cells was compared between groups.

Dentate gyrus neurons.

Both newly formed and mature granule cells in the dentate gyrus of the hippocampus were counted. The neuronal marker (NeuN) stains neuronal cell bodies, while excluding any dendritic projections, and another marker (DCX) is used to visually identify newly formed neurons. The DG was first traced using a 10x objective, then cells were counted using a 100x oil objective. Cells in every 12^{th} section of the brain were counted between Bregma 1.88 µm and -6.04 µm (Figure 2.1a), resulting in about 5 sections per brain. A counting frame of 20 x 20 µm and grid size of 200 x 200 µm was used to estimate the total number of mature and immature neurons in the dentate gyrus for each animal. A decrease in DCX+

cells would indicate a negative impact of binge alcohol on cell proliferation, consistent with what has been seen in other models of alcohol exposure (F. T. Crews & Nixon, 2009; S. A. Morris et al., 2010). An increase in DCX+ cells in binged rats could indicate compensatory mechanisms resulting from damage or neurogenesis occurring during alcohol abstinence. It was predicted that the rats that received weekly binge doses would have fewer remaining mature granule neurons and more new cells being generated than control animals.

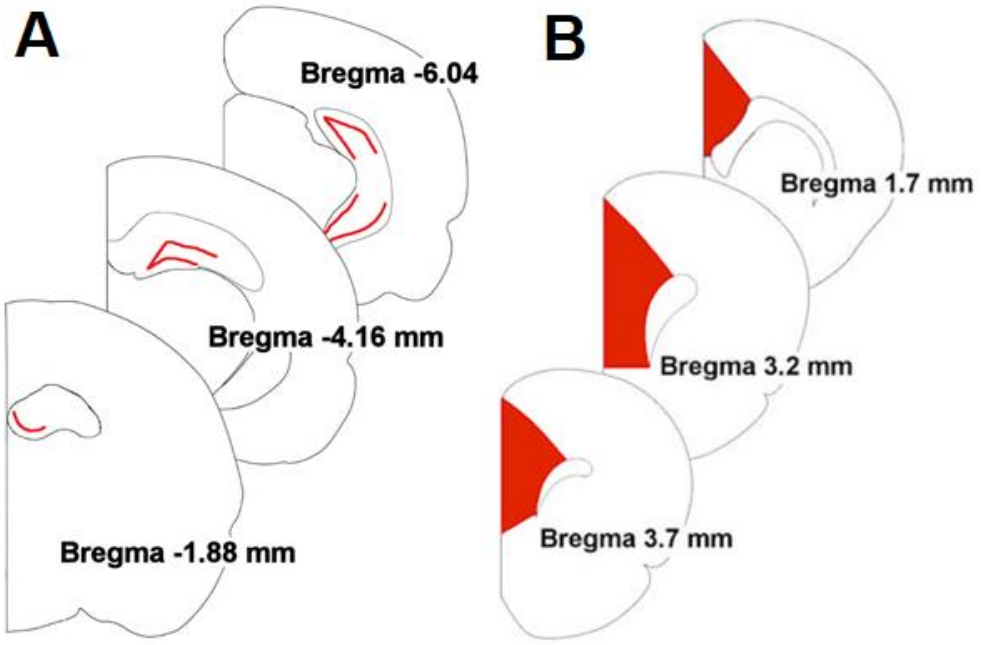

Figure 2.1. Illustrated area of hippocampus and dentate gyrus cell quantification outlined in red (A). Granule neurons (NeuN) and newly formed cells in the subgranular zone (DCX) were quantified using stereology. Illustrated area of medial prefrontal cortex cell quantification, an area that encompasses the anterior cingulate, prelimbic and infralimbic cortices (B).

Prefrontal cortex neurons.

Tissue from the medial prefrontal cortex (mPFC) was collected from 2 columns spaced 300 µm apart, yielding approximately 4-5 sections per brain. Neurons from these sections were counted and cell volume estimated using the Nucleator probe via StereoInvestigator. The traced area of interest included the infralimbic, prelimbic, and

anterior cingulate cortex between Bregma 3.9 µm and 2.2 µm (Figure 2.1b). The grid size that was used to count mPFC cells was 400x400 with a counting frame of 40x40. Area outline was traced at 4x and cells were counted at 100x magnification. The nucleator probe works within the optical fractionator workflow to measure the cell volume of each cell counted within the sampling frames. The nucleator probe generates four rays at randomized angles from a designated center point of the cell. The spot at which the rays intersect with the boundary of the cell is identified and marked (Figure 2.2), and the program then uses the mean measured radius in order to estimate the average volume of the cells counted. The estimated number of cells in the mPFC and the average neuronal cell body volume was quantified and compared between alcohol and control groups. It was hypothesized that rats in the binged group would have reduced neuronal cell volume but would not differ in number of remaining mPFC cells.

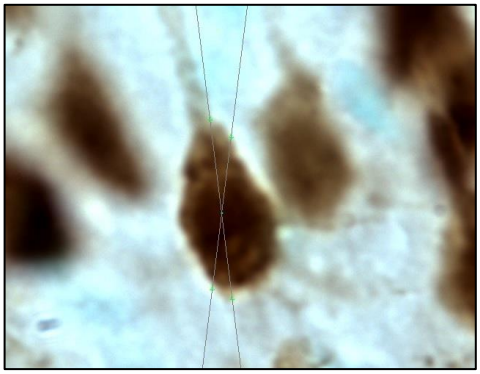

Figure 2.2. Screenshot of nucleator probe working within the optical fractionator workflow in StereoInvestigator software.

Hippocampal and mPFC microglia.

Tissue from the mPFC was collected from 2 columns spaced 300 µm apart between Bregma 3.9 µm and 2.2 µm (Paxinos & Watson, 2007), yielding approximately 4-5 sections per brain. Tissue from the hippocampus was collected from 1 column spaced 600 µm apart between Bregma 1.88 µm and -6.04 µm (Paxinos & Watson, 2007), yielding approximately

5 sections per brain. Staining used the standard IHC protocol listed above with Iba1 primary (iba1 anti-rabbit, Wako USA, 1:10,000). The estimated number of activated and total microglia from these sections was estimated using the optical fractionator workflow via Stereo-Investigator. In the mPFC, the area of interest was traced at 4x magnification, cells were counted at 40x and the grid size that was used to count mPFC cells was 400x400 µm with a counting frame of 100x100 µm. Area outline of the hippocampus was traced at 10x and cells were counted at 40x magnification. A 400x400 µm grid with counting frames of 100x100 µm was overlaid the area and used for quantification estimated. Partially activated microglia were categorized by a visibly larger and darker cell bodies, and thicker, bushy processes (Kreutzberg, 1996; Raivich et al., 1999)(Figure 2.3). The estimated number of microglia and the number of partially activated microglia in the mPFC and hippocampus was obtained and compared between the ethanol and control group. Based on previous data indicating that binge alcohol exposure results in morphological changes consistent with partial microglial activation (Barton, Baker, et al., 2017; Barton, Lu, et al., 2017; Marshall et al., 2016; McClain et al., 2011), it was hypothesized that binged animals would have more partially activated microglia in the mPFC and hippocampus than control animals, without any significant difference between groups in total number of microglia.

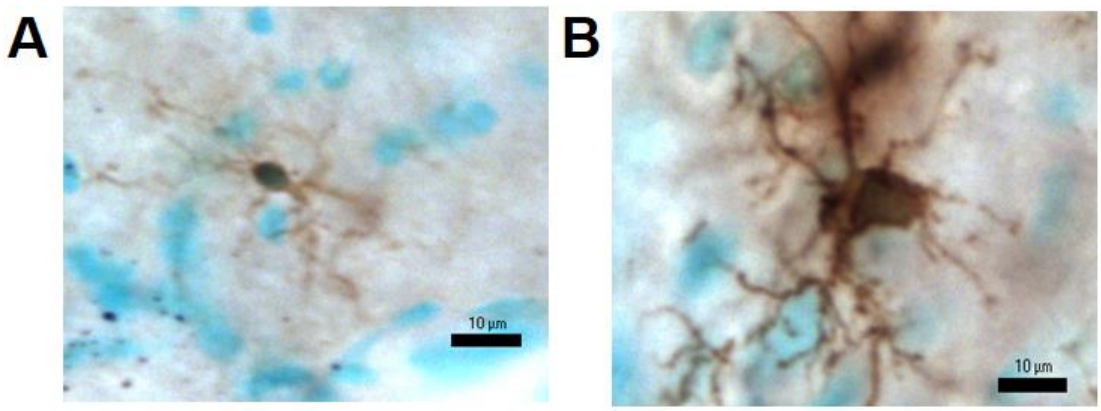

Figure 2.3. Image of visual differences between a microglial cell in a resting (A) and partially activated (B) activation state.

Hippocampal and prefrontal cell activation.

Neuronal activation in response to the Morris water maze task (outlined below) was examined using tissue from the mPFC and DG in the same Bregma cutoffs as previously stated. Staining used the standard c-Fos protocol outlined above and stained cells were counted using the optical fractionator workflow via Stereo-Investigator. In the mPFC, the area of interest was traced at 4x magnification, cells were counted at 40x and the grid size used to count mPFC cells was 400x400 µm with a counting frame of 80x80 µm. Area outline of the hippocampus was traced at 10x and cells were counted at 100x magnification. A 200x200 µm grid with counting frames of 40x40 µm overlaid the area and was used for quantification estimate. The estimated number of neurons in the mPFC and hippocampus activated by behavioral testing was obtained and compared between the alcohol and control group. It was hypothesized that binged animals would have more neuronal activation than control animals in the mPFC and hippocampus resulting from a memory and spatial navigation task. An increase in c-Fos-positive cells in binged animals indicates that the recruitment of more cells is required in order to obtain the same performance on the behavioral task as controls, thus implying less efficient brain processing.

Morris Water Maze

Morris water maze (MWM) testing was conducted every other week, beginning the first week on the day after gavage dosing. All testing occurred roughly midway through the rats' active cycle. Rats were brought into the room 10 minutes prior to testing to acclimate to the testing room. MWM testing was conducted with minimal white lighting, and water was kept at ambient air temperature. Water was colored with nontoxic white paint, and a large,

colorful, poster was hung on the wall to give additional spatial cues in the room. Rats were given 2 trials for 3 consecutive days with roughly 5 minutes between each trial; except for the first week, in which a single habituation trial was given on the first day of testing in addition to the standard 2 trials. After each week of testing, the location of the platform moved to a different quadrant. Rats were released pseudo-randomly and allowed to explore the arena freely. The trial ended when either the rat located and remained on the platform for 10 seconds or 60 seconds passed without the rat locating the platform. If animal did not locate the platform within the trial constraints, it was gently guided there by the experimenter, and allowed to remain for an additional 10 seconds to explore the surrounding visual cues. Water was agitated between trials to disperse any lingering olfactory cues, and the experimenter backed away from the tank and stood in the same location after release of each animal. An automated tracking system (Noldus; Amsterdam, Netherlands) recorded each trial.

Brain tissue was harvested roughly 1 hour following the final MWM trial on the 5th testing block (after 11 dosings). Therefore, cellular activation (c-Fos) analysis (outlines above) analyzed the performance of rats on the third day of MWM testing after 11 weeks of binge ethanol or control solution exposure.

Statistical Analysis

All comparisons between binge and control rats for all stereology outcomes, were analyzed with an independent group t-test using SAS 9.4 (SAS Institute Inc., Cary, NC, USA). Outcome measures were the quantitative results of tissue analysis and included, the number of remaining neurons in the DG and mPFC (NeuN+), the volume of neurons in the

mPFC, the number of total and activated microglia in the DG and mPFC (Iba1+), and the number of activated cells (c-Fos+) in the mPFC and DG. Examining the effect of dose number on blood ethanol concentration was analyzed using a repeated measures t-test. Because the Majchrowicz scale used to assess behavioral intoxication is based on a non-linear scale, behavioral intoxication scores for the factor of Week for was analyzed with a Friedman's test. Body weight, fecal corticosterone and MWM outcome data were analyzed with a repeated measures ANOVA for Diet as the measure of interest, and Week as a repeated measure. Simple correlations were run between related outcome measures using SAS. Results from statistical tests were deemed significant if the p-value was less than 0.05. Data was organized and graphed using Excel 2016 (Microsoft Office 360 Excel, WA, USA).

Results

Intoxication and Body Measures

There was no effect of Week on either blood ethanol concentrations [$t(47)=2.12$, $p=.08$] or behavioral intoxication on the Majchrowicz scale [$t(88)=1.32$, $p=.23$], indicating that additional binge alcohol exposure did not alter either metabolic or behavioral alcohol tolerance. Average BEC was 162.5 mg/dl ± 7.99 mg/dl, and median behavioral intoxication on the Majchrowicz scale was 1.

For body weight, there was no significant interaction between Week x Diet on body weight [$F(10,187)=.09$, $p=.99$]. There were significant main effects of both Week [$F(10,187)=22.62$, $p<.0001$] and Diet [$F(1,187)=9.82$, $p=.002$] on body weight, with binged females weighing less throughout the course of the experiment than control females, and all rats gaining weight over time.

There was no significant effect of Week [F(2,42)=.38, p=.69], Diet [F(1,42)=1.64, p=.21] or an interaction of Week x Diet [F(2,42)=.29, p=.75] on fecal corticosterone levels. This indicated that there were no persistent effects of ethanol exposure on corticosterone levels.

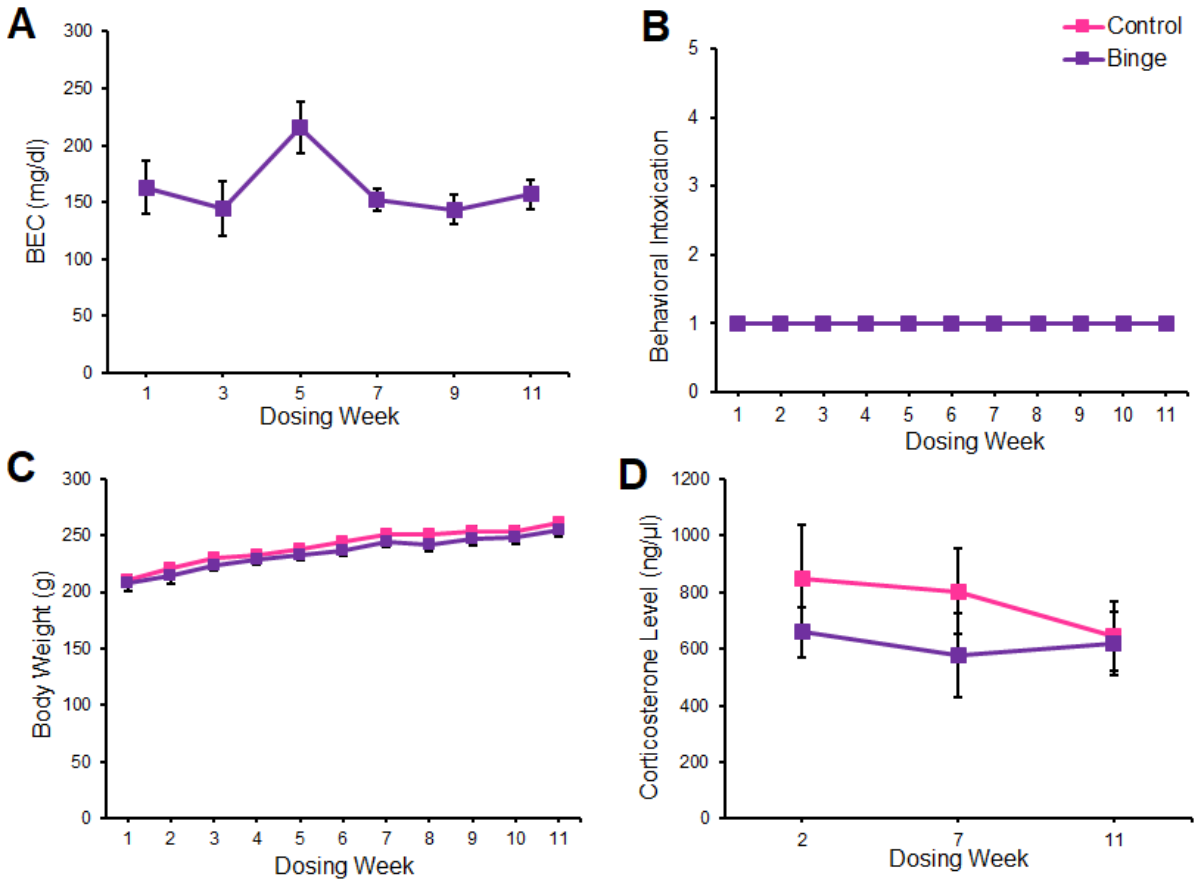

Figure 2.4. Neither blood ethanol concentrations (A) or behavioral intoxication (B) were significantly affected by additional binge alcohol exposure. Binged females weighed less than control females throughout the course of the experiment (C), however binged and control females did not significantly differ in fecal corticosterone levels (D).

Dentate Gyrus Neurons

There was an effect of Diet on NeuN+ cell in the DG [t(15)=3.57, p=.003] and DG DCX+ cells [t(15)=2.44, p=.03]. There was no significant correlation between the number of NeuN+ and DCX+ in cells in the DG [r=.27, p=.29]. Alcohol exposure decreased the

number of remaining granule neurons (NeuN) after 11 doses of binge alcohol but increased hippocampal neurogenesis (DCX).

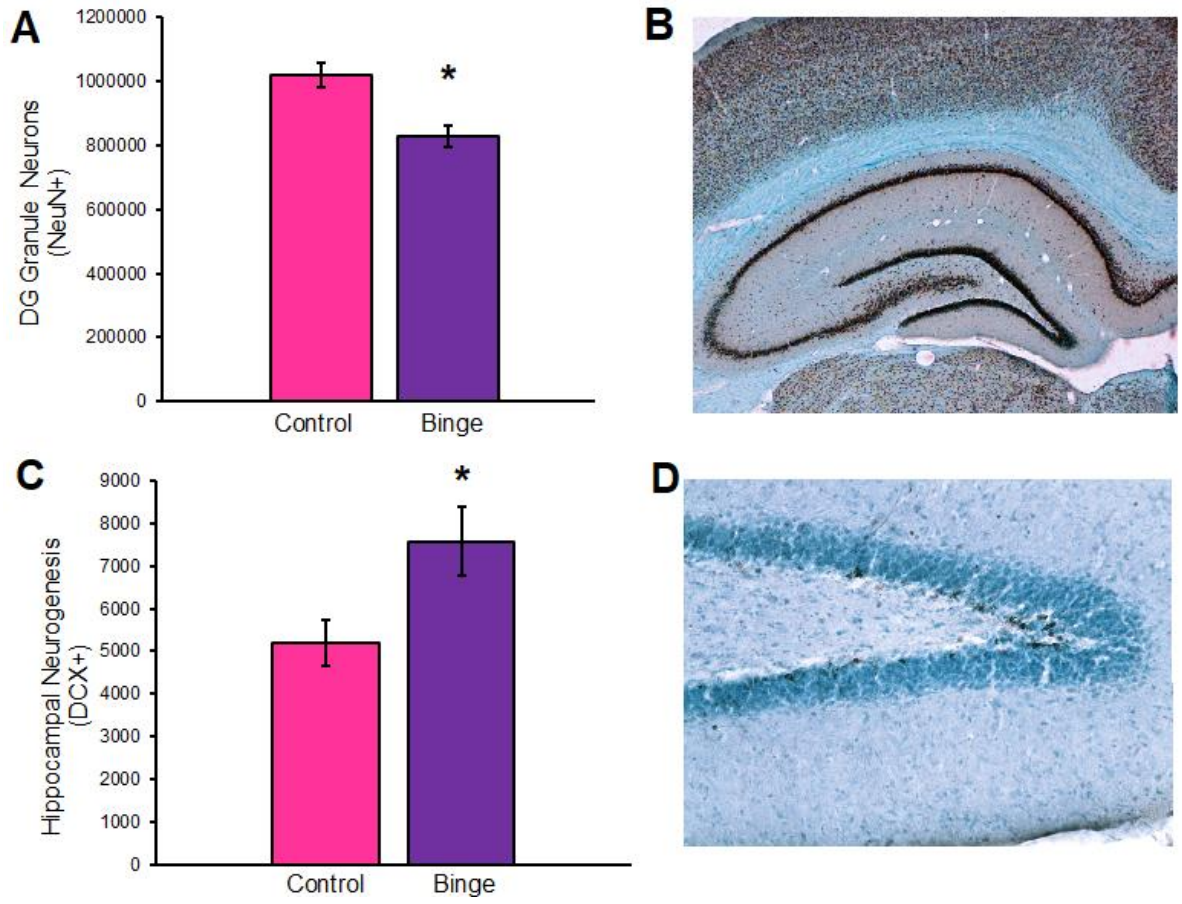

Figure 2.5. Binge alcohol exposure significantly decreased the number of DG granule cells remaining following 11 doses of binge alcohol (A). Panel B shows a representative image of NeuN+ cells stained in the DG of the hippocampus. Binge alcohol increased the number of newly formed cells in the DG (C). Representative image of DCX+ staining in the DG (D). * $p < .05$

Prefrontal Cortex Neurons

There was no significant effect of Diet on either NeuN+ cells in the mPFC [$t(15)=1.09$, $p=.29$] or nuclei volume of NeuN+ cells [$t(15)=.01$, $p=.99$]. Binge alcohol exposure did not result in neuron loss in the mPFC, nor in a decrease in nuclei volume.

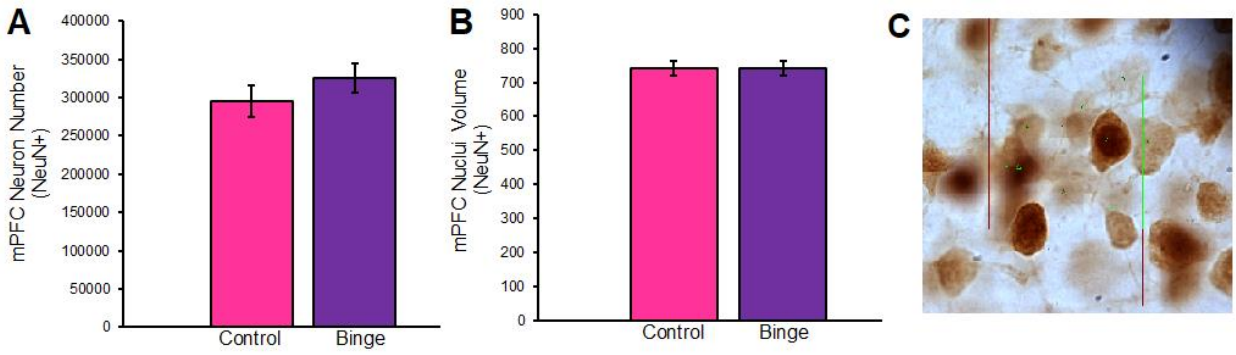

Figure 2.6. Neither the number of medial prefrontal cortex neurons remaining (A) nor mPFC nuclei volume (B) were significantly impacted by binge alcohol exposure. Panel C shows a representative image of stereological quantification of mPFC neurons.

Hippocampal and mPFC microglia

There was no significant effect of Diet on the total number of Iba1+ cells [$t(16)=1.36$, $p=.19$]. There was a significant effect of Diet on the number of partially activated Iba1+ cells in the hippocampus [$t(16)=2.18$, $p=.04$]. In the hippocampus therefore, binge ethanol exposure significantly increased the number of partially activated microglia but did not have an effect of the total microglial number.

In the mPFC, there was no main effect of Diet on either the total number of Iba1+ cells [$t(16)=1.99$, $p=.06$] or on the number of partially activated Iba1+ [$t(16)=1.62$, $p=.12$]. Therefore, ethanol exposure did not significantly increase microglial activation or total number of microglia in the mPFC.

There were also strong positive correlations between the overall number of microglia in the mPFC and hippocampus [$r=.81$, $p<.0001$] and between the number of partially activated microglia in the mPFC and hippocampus [$r=.71$, $p=.001$]. Rats with more total microglia in the hippocampus had higher microglial number in the mPFC, and rats with higher microglial activation in the hippocampus correspondingly had higher activation in the mPFC .

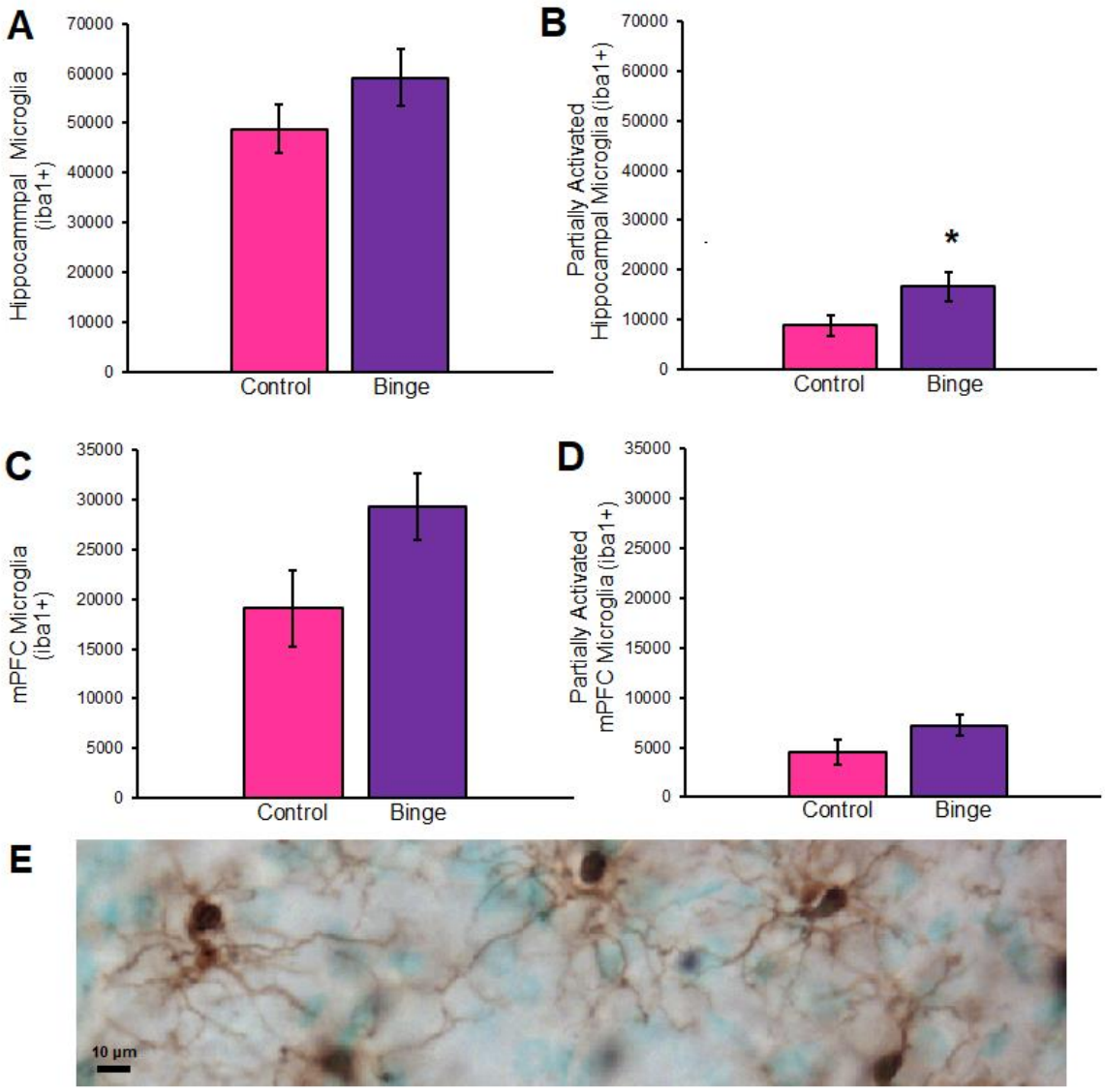

Figure 2.7. Eleven weeks of binge exposure did not significantly increase the overall number of microglia in the hippocampus (A), it did however, increase the number of partially activated hippocampal microglia (B). Binge alcohol did not significantly increase the overall number (C) of partially activated microglia (D) in the mPFC. Panel E shows representative image of stained Iba1+ cells.

Hippocampal and prefrontal cell activation

There was not a significant effect of Diet on the number of c-Fos+ cells in the DG during MWM behavioral testing [t(15)=1.72, p=.11]. In the mPFC however, there was a significant effect of Diet on c-Fos+ cells [t(16)=2.38, p=.03]. There was no significant correlation between c-Fos+ cells in the DG and mPFC during MWM testing [r=.04, p=.87].

Therefore, in the mPFC, but not the hippocampus, binge exposure significantly increased cellular activation in response to MWM testing.

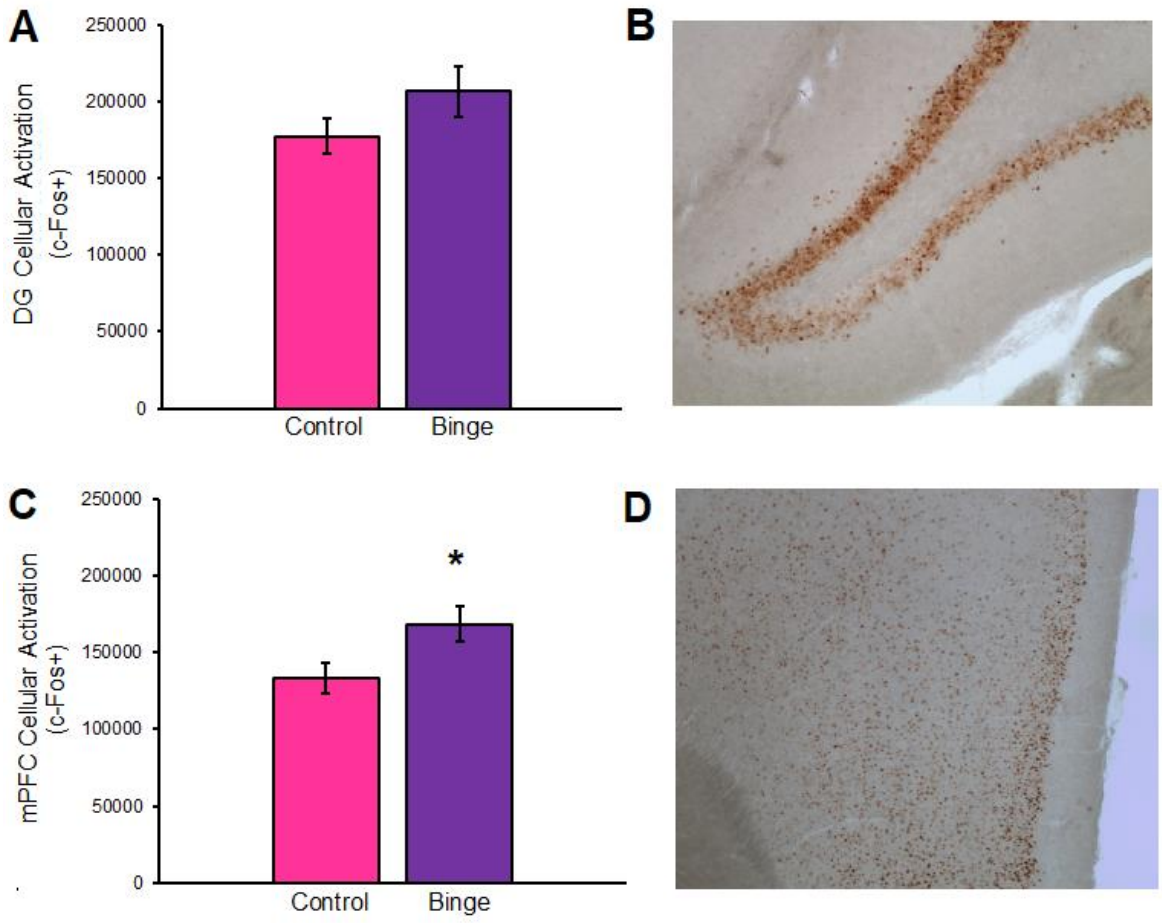

Figure 2.8. Binge dosing did not significantly increase cellular activation during MWM testing in the DG (A). Representative image of c-Fos+ cells in the DG of the hippocampus (B). 11 weeks of binge dosing significantly increased activation in the mPFC during MWM behavioral testing (C). Panel D shows a representative image of c-Fos+ cells in the mPFC. * p <.05

Morris Water Maze

For latency to the platform during MWM testing, there was no Week x Diet interaction [F(5,684)=.34, p=.89], nor any effect of Diet [F(1,684)=.09, p=.76]. There was a significant effect of Week [F(5,684)=8.24, p<.0001], with rats taking less time to find the platform during the later weeks of testing.

For the distance to the platform during MWM testing, there was no Week x Diet interaction [F(5,684)=.82, p=.54]. There was no significant effect of Diet [F(1,684)=.13, p=.72] on distance to the platform, however there was a significant effect of Week [F(5,684)=6.62, p<.0001]. Like with the latency to the platform, all rats had longer distances to the platform in the earlier weeks of testing and swam to the platform more directly during later weeks.

There was no significant Week x Diet interaction [F(5,684)=1.31, p=.26] on swim speed nor a significant effect of Diet [F(1,684)=.53, p=.47]. There was a significant effect of Week [F(5,684)=2.58, p=.025] on swim speed, with rats swimming more quickly during the earlier weeks of testing, and swimming more slowly in later weeks.

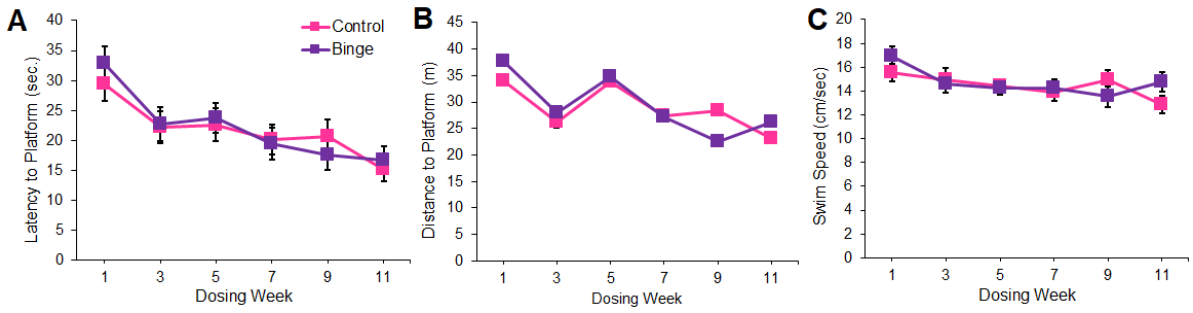

Figure 2.9. Binge dosing did not have a significant effect on Latency to the Platform (A), Distance to the Platform (B), or Swim Speed (C) during the Morris Water Maze.

Discussion

The current aim examined the effects of weekly binge ethanol on the female brain. Weekly ethanol administration produced an average BECs consistent with what could be reasonably achieved by a woman during a binge drinking episode. A weekly repeated 5 g/kg dose of ethanol resulted in an average BEC of 162 mg/dl and resulted in mild behavioral intoxication, without any observable withdrawal symptoms, and without resulting in detectable metabolic or behavioral tolerance over time. Binge drinking is thought to be a

particularly brain-damaging pattern of alcohol possibly because intoxication episodes are too infrequent for metabolic tolerance to develop (Esser et al., 2014; Tabakoff et al., 1986). Each binge episode therefore likely acts as a separate injury to the dentate gyrus, culminating in significant cellular loss.

After 11 weekly doses of ethanol, there was an 18.5% loss in DG granule neurons in binged female rats, an even greater magnitude of loss than was observed previously with a single 4-day binge ethanol model in female rats (Maynard et al., 2018). This amount of cellular loss is also what has been reported after months of chronic ethanol intake in rats (Walker, Barnes, Zornetzer, Hunter, & Kubanis, 1980). This indicates that there may be a limit to the magnitude of hippocampal cell loss resulting from ethanol exposure, whether it be from binge or chronic exposure.

Binge ethanol exposure has been shown to suppress neurogenesis in the DG (S. A. Morris et al., 2010; K. Nixon & Crews, 2002, 2004), and has been suggested as a potential mechanism of alcohol neurotoxicity. However, ethanol abstinence has been associated with increased neurogenesis (Galinato et al., 2018; K. Nixon & Crews, 2004; K. Nixon, Kim, Potts, He, & Crews, 2008), and in the current study rats were sacrificed 4 days after the last binge ethanol administration. Since we examined cellular outcomes after a period of ethanol abstinence, this likely explains why increased neurogenesis in binged animals was observed. This increase in newly generated cells (DCX), despite a significant loss in the number of mature neurons (NeuN) in the granule cell layer, indicates that changes in the cellular environment may be a likely mechanism for this loss. While cellular death is likely also a contributing factor, decreased trophic support would explain the inability for newly generated cells to mature to integrate into the granule cell layer. A decrease in neurotrophins

following binge exposure has been previously shown in female rats and was also associated with significant granule cell loss (Maynard et al., 2018). This increase in neurogenesis in binged animals was likely a compensatory mechanism aimed at repopulating the depleted cellular layer in the dentate gyrus. The loss of mature granule neurons observed after 11 weeks of binge ethanol, may have resulted in an upregulation of neurogenesis to repair the injured brain. An increase in neurogenesis and progenitor cells has been previously observed in other cases of neurological insults, such as TBI (Chirumamilla, Sun, Bullock, & Colello, 2002; Dash, Mach, & Moore, 2001; Villasana, Westbrook, & Schnell, 2014), hypoxia (Varela-Nallar et al., 2014; Zhu et al., 2005), and seizures (Parent et al., 1997; Scharfman, Goodman, & Sollas, 2000). Despite a noted compensatory increase in neurogenesis in binged rats, the attempt at cellular repopulation was unsuccessful, likely due to a decrease in trophic support in the cellular environment.

This hippocampal cell loss was also accompanied by an increase in microglia with morphological characteristics indicative of partial microglial activation. Ethanol has been previously implicated in the neuroimmune response (F. T. Crews et al., 2015; F. T. Crews & Vetreno, 2014; Qin & Crews, 2012; Vetreno & Crews, 2012; Vetreno, Patel, Patel, Walter, & Crews, 2017), which is consistent with the results from the current study. In the hippocampus, binged rats had an increase in microglia with morphology indicative of a partial activation, consistent with prior findings (Barton, Baker, et al., 2017; Barton, Lu, et al., 2017; McClain et al., 2011). This increase in microglial activation suggests that binge exposure primes microglia to respond to subsequent ethanol exposure. This is consistent with the idea that microglia can be partially activated in response to CNS injury to respond to future neural challenges (Banati et al., 1993; Block & Hong, 2005; C. A. Colton &

Gilbert, 1987; Kreutzberg, 1996; Marshall et al., 2016; Norden et al., 2015; Woodroofe et al., 1991).

Despite significant damage and neuroimmune activation observed in the hippocampus, cellular outcomes in the mPFC did not indicate observable damage to neuronal nuclei volume, neuron number, or an increase in microglial response, indicating that the hippocampus may be particularly vulnerable to binge ethanol. The frontal cortex is an area that has been previously shown to be vulnerable to alcohol neurotoxicity and we have previously observed decreased neuron nuclei volume (West et al., 2018), and an increased neuroimmune activation (Barton, Baker, et al., 2017) in response to the 4-day binge model of exposure. However, despite our predictions, there was no evidence of neuroimmune activation or damage to neuronal cell bodies observed in the mPFC after 11 weekly ethanol dosings. It should be noted however, that we did not examine changes at the dendritic level or examine other cellular markers which may have been vulnerable to ethanol toxicity.

Increased neuronal activation during the MWM task, indicates that despite comparable behavioral performance, rats exposed to 11 weeks of binge ethanol required more cellular resources than control rats. Binged rats had increased cellular activation in the mPFC than control rats during this spatial navigation task, indicating that despite no detectable behavioral impairments, brain damage may still have occurred. This increase in cellular activation is indicative of less efficient cognitive processing, meaning that binge ethanol may still have had a neurotoxic effect on the mPFC, despite no detectable grey matter loss. Although the increase in in hippocampal c-Fos expression was not statistically significant, this may be partially explained by the loss of mature neurons in the granular cell

layer. A similar amount cellular activation in binged rats in the dentate gyrus despite a decrease in the overall number of cells, means that a higher percentage of those hippocampal cells were being recruited to achieve the same performance as control rats.

There was no observed difference in stress levels between binged and control rats, and no increase in stress levels over time, according to fecal corticosterone analysis. Since the primary focus of this analysis was to determine if there were long-term changes in circulating stress hormones, fecal samples were collected 3 days after binge administration, and thus were not sensitive to any temporary increase in circulating corticosterone levels that would occur in the period directly following binge exposure. It is highly likely that there would be transient effects in corticosterone levels if samples were collected the day following ethanol exposure, however this analysis showed that any possible increase in stress levels as a result of binge exposure are not persistent.

This study focused on the effects of binge alcohol in female rats, due to increasing evidence that the female brain may be selectively vulnerable to alcohol exposure (Agartz et al., 2003; Alfonso-Loeches et al., 2013; Mann et al., 1992; Sharrett-Field et al., 2013; Wilhelm et al., 2016). Although only female rats were used in this aim, the rationale for not monitoring estrous cycle was both practical and scientific. We have previously shown that stage of estrous does not influence binge alcohol effects on remaining cells in the dentate gyrus (Maynard et al., 2018), and the pharmacokinetic parameters of ethanol absorption do not vary across the estrous cycle (Robinson, Brunner, & Gonzales, 2002). Additionally, since ethanol dosings occurred every 7 days and rats have a mean estrous cycle length of 4 days (M. E. Freeman, 1988; Long, 1922; Mandl, 1951), the stage of estrous on binge day was not the same each week, providing effective randomization within subjects.

Results from this study were used to inform the direction of the following aim, by evaluating vulnerable brain regions and cell types of interest. Aim 2 used significant cellular outcomes obtained from examination of females after 11 weeks, in order to determine whether the female brain is selectively vulnerable to recurrent binge ethanol exposure and examine after how many weeks of exposure it will take for cellular damage to occur in both male and female rats.

Chapter 3: Determine whether a telescoping effect will result in earlier binge alcohol-induced brain damage in female rats.

As previously stated, since the prevalence of binge drinking in women has been increasing in recent years (Dwyer-Lindgren et al., 2015), investigation into female vulnerability to binge alcohol exposure is of great importance. The current weekly model of binge exposure enabled investigation into the question of whether females would develop brain damage with fewer binge alcohol exposures. If brain damage was found with fewer doses of alcohol in females than in males, the theory of a telescoping effect of binge alcohol on the female brain would be supported. Based on previous research supporting female susceptibility to alcohol-induced damage (Agartz et al., 2003; Alfonso-Loeches et al., 2013; Mann et al., 1992; Maynard et al., 2018; Sharrett-Field et al., 2013; Wilhelm et al., 2016), and the results from Aim 1, it was hypothesized that female rats would show a decrease in dentate gyrus granule neurons, increased compensatory neurogenesis, increased neuroimmune activation in the mPFC and hippocampus, and increased cellular activation in the mPFC during behavioral testing with fewer doses of alcohol than male binged rats, when compared with same-sex controls. Damage to these vulnerable brain regions with fewer alcohol doses would indicate a sex-dependent telescoping effect and would provide evidence of female vulnerability to binge alcohol neurotoxicity.

Method

Binge Paradigm and Timeline

Behavioral intoxication measurement, blood ethanol concentration measurement, tissue collection, processing and stereology all followed identical methods as described in

the respective sections in the previous chapter. All dosing occurred at the end of the rats' active (dark) cycle. Weekly ethanol administration followed the same protocol as outlined in the previous chapter, with ethanol or control solution being given once weekly. However, to prevent an unnecessarily high volume of fluid being given to male rats in the final weeks of the experiment in the additional animals run for the second goal of this aim, a 50% ethanol solution (190-proof alcohol with vanilla Ensure Plus™), was used instead of the 25% solution used previously. The iso-caloric control solution was also altered accordingly to compensate for the higher concentration of alcohol in the ethanol solution. Water intake was monitored in the 14 hours following the first, second, third dose by removing lixits from cages, and placing two bottles filled with water into cages which were measured by weight to determine the amount drank. Food intake was measured in the 14 hours following dosing after all weeks of dosing by measuring the weight of food hoppers after food was replaced after blood collection, and again the following morning.

The first goal of this aim was to determine at what point male and female rats first begin to develop binge-induced brain damage, and to identify the number of doses at which granule cells first begin to significantly decrease for further analysis. Initially, tissue from 16 animals (4 per group; male binge, male control, female binge, female control) was collected after 5 alcohol or control doses, and the DG granule layer examined. Additional tissue was then evaluated with small groups of male and female binge rats after 3, 8 and 11 weeks to determine after how many weeks female and male rats first manifest cell loss. Damage was considered significant when binged rats had either a statistically significant decrease in granule cells when compared to controls, or when binged animals showed at least 10-percent

fewer DG granule cells compared to same-sex controls. All rats were intracardially perfused four days following their final assigned binge or control dose.

Information from this exploratory experiment was then used to inform the second goal for this aim. The dose at which detectable granule cell loss was first seen in females and males was identified and used to inform further cellular analysis. The number of doses chosen for this further analysis were 3 weeks and 8 weeks of control or ethanol gavage. Rats for the second part of the aim were also sacrificed four days following the last assigned ethanol or control dose, and the significant findings from Aim 1 were used to inform more in-depth cellular analysis after each of the two vulnerable timepoints.

An additional goal of interest was to determine whether gavage with control solution would induce cellular changes. Since the process of oral gavage is a stressor, even in the absence of ethanol, evaluation of the effects of weekly gavage alone was of interest. Therefore, an additional group of "cage control" rats that did not receive any gavage exposure was used in order to investigate the possible effects of gavage stress. These rats were group housed in the same room and all other conditions (apart from oral gavage) were identically matched to the experiences of the standard control rats. Cage control rats were sacrificed 2 days following the rats that received 8 weeks of oral gavage with control solution.

Animals

This aim used a total of 202 male and female Long-Evans rats across 5 cohorts. The first part of Aim 2 used a total of 40 male and female Long-Evans rats to determine at which point DG granule cell loss was first detectable. The second part of Aim 2 used 62 additional rats of the same age and strain to investigate sex-differences in the appearance and severity

of brain damage (n=10/group) for the measures of granule neurons (NeuN), neurogenesis (DCX) and microglia (iba1). The third goal, to investigate the effects of gavage exposure, used an additional 20 rats that did not receive any gavage dosing of either ethanol or control solution.

Eight-week-old animals were randomly assigned to either the alcohol, control, or cage control group, within their respective sex, upon arrival. All rats spent 10 days being handled and acclimated after transport to the animal care facility before receiving any experimental treatment. Food and water were always available throughout the experiment, apart from the fasting period prior to oral gavage when all food was temporarily removed from cages. Housing rooms were on a reverse light-dark cycle (9AM-9PM) and all gavage and behavioral testing was conducted under red light during the animals' active cycle. All animals remained group housed throughout the course of the experiment, except for a total of 6 males which had to be separated at varying points throughout the experiment due to fighting with cage-mates.

Immunohistochemistry and Stereology

Outcome measures deemed statistically significant from quantitative analysis of tissue from Aim 1, were used to further investigate sex-differences in appearance and severity of brain damage resulting from binge alcohol exposure. Remaining DG granule neurons (NeuN), newly generated DG neurons (DCX), and microglia in the mPFC and hippocampus (iba1) were all measured and compared after 3 and 8 weeks of binge ethanol exposure. All rats were sacrificed 4 days following the final alcohol dose, and cage control rats were sacrificed 2 days following 8-week binge and control animals. Tissue processing,

immunohistochemistry and counting parameters were identical to those outlined in Aim 1 (chapter 2). Tissue sections were analyzed using unbiased stereology (StereoInvestigator) and the estimated number of cells was examined as a function of Week and compared between groups.

Statistical Analysis

To pinpoint when a significant decrease in DG granule cells first emerges in both male and female rats, female and male rats were each compared to same-sex controls using independent sample t-tests for each dosing week. A group of same-sex control animals served as the baseline to which binged animals were compared against for each alcohol dose (3, 5, 8, 11).

The second part of aim 2, involved analysis of cell counts by using a three-way factorial design for the factors of Sex (2 levels: Male, Female), Diet (2 levels: Control, Binge) and Week (2 levels: 3, 8) as well as interactions of these variables. Outcome measures for cellular analysis were determined by the findings from the first aim: remaining DG granule neurons (NeuN), newly generated DG neurons (DCX), and microglia in the mPFC and hippocampus (iba1). Follow-up analysis of newly generated DG neurons (DCX) in animals that received behavioral testing was accomplished using one-way ANOVAs within sex, comparing control rats with no behavioral testing, binged rats with no behavioral testing, and binged rats that had behavioral testing after 8 weeks.

All rats used in Aim 2 were combined within their respective groups for intoxication analyses. Blood ethanol concentrations were compared using a mixed model ANOVA with Sex as a between-subjects factor and Week as a repeated measure. Body weights were also compared with a mixed model within Sex, with Diet as the main effects of interest and

Week as a repeated measure. For behavioral intoxication analysis, since the Majchrowicz scale used to assess behavioral intoxication is based on a non-linear scale (0-5), intoxication scores between were analyzed using a Mann-Whitney U Test, and analysis of the factor of Week for each Sex was analyzed with separate Friedman's tests.

To examine the effects of gavage stress, cellular analysis consisted of cage controls being compared to controls that received 3 and 8 weeks of gavage with control solution, within sex using a one-way ANOVA.

When appropriate, planned pairwise comparisons with Bonferoni adjustment were used to follow-up significant interaction effects. Results from statistical tests were deemed significant if the p-value was less than 0.05. All data was organized and graphed using Excel 2016 (Microsoft Office 360 Excel, WA, USA) and all statistical analysis was performed using SAS 9.4 (SAS Institute Inc., Cary, NC, USA). All group averages reported are represented by mean ± SEM.

Results

Body Weight

To investigate any changes in body weight between groups, mixed model analysis indicated no significant Diet x Week interaction [$F(7,654)=1.99$, $p=.055$] nor an effect of Diet [$F(1,654)=3.32$, $p=.07$]. There was a significant main effect of Week [$F(7, 654)=239.17$, $p<.0001$], with all male rats increasing their body weight throughout the course of the experiment. For body weight in female rats, there was no significant interaction of Diet x Week [$F(7,644)=.16$, $p=.99$] or a significant main effect of Diet [$F(1,644)=3.29$, $p=.07$]. Like in males, there was a significant main effect of Week in female

rats [F(1,644)=115.85, p<.0001], with all female rats increasing in body weight throughout the experiment.

To examine the effects of repeated gavage on body weight, in male cage control rats in comparison to standard controls, there was no significant Gavage x Week interaction [F(7,284)=.67, p=.70]. There was also no significant main effect of Gavage on body weight [F(1,284)=.81, p=.37]. There was a significant main effect of Week [F(7,284)=128.06, p<.0001], with both gavage and standard control males increasing body weight throughout the experiment. In female cage control rats, there was also no significant Gavage x Week interaction [F(7,284)=.16, p=.99], and no significant main effect of Gavage [F(1,284)=2.7, p=.10]. There was a significant main effect of Week [F(7,284)=39.25, p<.0001], with all female rats gaining body weight each week.

Intoxication Measures

To investigate whether male and female rats differed in behavioral intoxication in response to binge ethanol, Mann-Whitney U testing showed that there was a main effect of Sex [p<.0001] between female and male binged rats. Male rats acted significantly more intoxicated than female rats throughout the course of the experiment. Friedman's testing showed no main effect of Week in either male rats [p=.23] or female rats [p=.42].

To investigate sex differences in blood ethanol concentration, mixed-model analysis indicated that there was no significant interaction of the variables of Sex x Week [F(7,456)=.66, p=.71]. There were also no significant main effects of either Sex [F(1,456)=.26, p=.61], or Week [F(7,456)=1.02, p=42]. Males had an average BEC of 177 ±

2.6 mg/dl, and females had an average BEC of 176 ± 2.5 mg/dl.

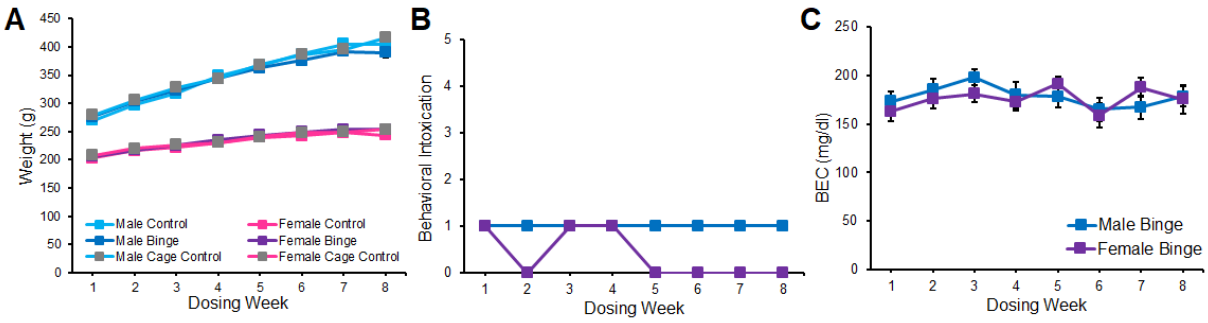

Figure 3.1. All rats gained weight throughout the course of the experiment. Binged rats did not differ from control rats and cage control rats did not differ from controls that received gavage exposure (A). Male rats acted more behaviorally intoxicated than female rats throughout the course of the experiment (B), however male and female rats did not differ in their blood ethanol concentrations (C). There was no noted tolerance that developed in either behavioral intoxication of blood ethanol concentration.

Food and Water Consumption Post-Binge

To determine whether ethanol influenced the amount of food intake in the hours following gavage, a mixed-model analysis with Day as a repeated measure and Diet and Sex as primary main effects. For food intake, there was no significant Sex x Diet x Week [$F(1,78)=1.59$, $p=.19$] interaction, nor any significant interactions of Sex x Diet [$F(1,78)=.27$, $p=.61$], Sex x Week [$F(7,78)=1.59$, $p=.17$], or Diet x Week [$F(7,78)=.69$, $p=.66$]. There was a main effect of Sex [$F(1,78)=68.67$, $p<.0001$] on food intake, however no main effects of either Diet [$F(1,78)=1.52$, $p=.23$], or Week [$F(7,78)=.78$, $p=.59$]. As expected, male rats ate more than female rats [Male: 13.9 g ± .7 g, Female: 10.1 g ± .3 g], however binged rats did not differ in food intake from control rats.

To determine whether ethanol influenced the amount of water intake in the hours following gavage, a mixed-model analysis with Day as a repeated measure and Diet and Sex as primary main effects. For water intake, there were no significant interactions of Sex x Diet x Week [$F(1,48)=.02$, $p=.98$], Sex x Diet [$F(1,48)=.15$, $p=.70$], Sex x Week

[F(2,48)=.71, p=.53], or Diet x Week [F(2,48)=.71, p=.59]. There were no significant main effects of Sex [F(1,48)=.31, p=.58], or Week [F(2,48)=1.18, p=.34], however there was a significant main effect of Diet [F(1,48)=12.51, p=.001] on water intake. As expected, given the dehydrating effects of ethanol, binged rats [18.1 mL ± .8 mL] drank more water in the hours following binge dosing than control rats [16.5 mL ± 1.2 mL].

Determination of Number of Binge Doses to Produce Cell Loss

To determine when both male and female rats first manifest damage from weekly binge ethanol, independent sample t-tests were used to compare the damage after each number of weeks versus same-sex controls. This information was then used for further analysis for the remainder of the aim. After 3 weeks of binge, male rats did not have a significant difference in NeuN+ cells in the DG in comparison to controls [-7.4%; t(12)=1.61, p=.21], female rats however, did show a significant reduction in NeuN+ cells in the DG [-11.4%; t(17)=5.1, p=.03]. After 5 weeks, male rats did not show a statistically significant decrease [-13.1%; t(10)=3.89, p=.06], female rats did show significant decrease in the number of NeuN+ cells [-12.2%; t(18)=4.9, p=.03]. After 8 weeks, male rats did not show statistically significant decrease in DG Neun+ cells [-12.3%; t(11)=3.93, p=.059], female rats did have significant decrease in NeuN+ cells [-15.1%; t(19)=8.96, p=.005]. After 11 weeks, male rats did not show a significant loss in NeuN+ cells [-11.1%; t(12)=3.56, p=.07], female rats on the other hand, as previously shown (Aim 1), did have significant loss in NeuN+ cells [-16.7%; t(22)=15.34, p=.0004]. Using the threshold of either statistically significant cell loss, or a 10% loss, female rats first showed cell loss after 3 weeks, and male rats after 5 weeks. However, it was decided that a short-term exposure (3 weeks) and long-

term exposure (8 weeks), would be more informative timepoints to use for further examination.

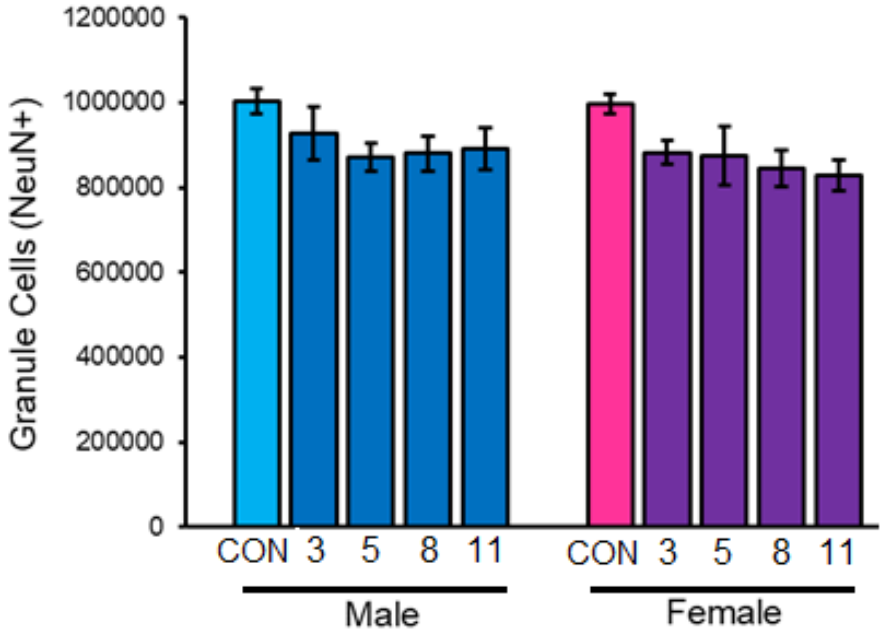

Figure 3.2. Pilot data examining remaining granule neurons in male and when female and male rats first develop damage to granule neurons. Based on these results, 3 and 8 weeks of binge exposure were chosen for further analysis.

Dentate Gyrus Granule Neurons

To analyze the effect of binge exposure on dentate gyrus granule neurons (NeuN), three-way factorial analysis showed no significant interaction of Sex x Diet x Week [$F(1,76)=.05$, $p=.0831$]. There were also no significant interactions of Sex x Diet [$F(1,76)=.34$, $p=.56$], Sex x Week [$F(1,76)=1.56$, $p=.22$], or Diet x Week [$F(1,76)=2.07$, $p=.15$]. There was no significant main effect of Week [$F(1,76)=.14$, $p=.71$], however there were significant main effects of both Sex [$F(1,76)=4.82$, $p=.031$] and Diet [$F(1,76)=17.61$, $p<.0001$]. Males overall had more NeuN+ cells in the DG than female rats and all binged rats had significantly fewer NeuN+ cells in when compared to control rats. When compared

to same sex controls, males had 6.8% fewer DG NeuN+ cells after 3 weeks and had 8.4% fewer DGGN NeuN+ cells after 8 weeks, females had 7.3% decrease after 3 weeks and 13.5% fewer NeuN+ cells after 8 weeks of binge exposure. There was a moderate effect size for binge in male rats [d=.77] and a large effect size for female rats [d=1.12]. Overall these results show that binged rats, regardless of the number of dosing weeks, had fewer remaining granule cells in the dentate gyrus.

To determine whether repeated gavage stress affected the number of NeuN+ cells in the DG, a one-way ANOVA indicated no significant effect of Group in males [F(2,28)=.49, p=.62], or females [F(2,28)=.12, p=.88]. This indicates that gavage exposure did not have any significant influence on the number of remaining granule cells in the DG.

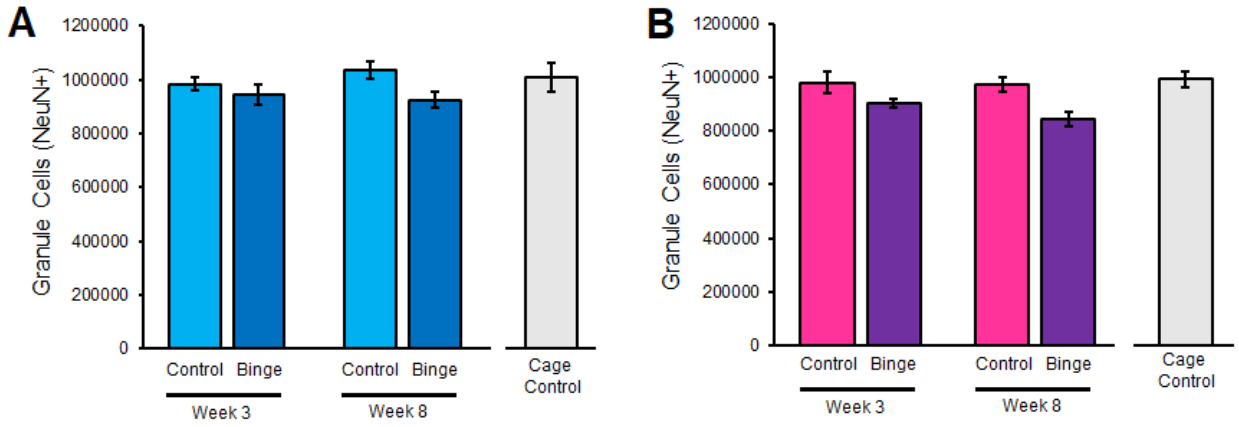

Figure 3.3. Examination of dentate gyrus neurons, binge exposure significantly reduced the number of remaining granule neurons in male (A) and female (B) rats in comparison to controls. There was no effect of gavage experience on the number of remaining granule neurons.

Newly Generated Dentate Gyrus Granule Neurons

A three-way factorial analysis indicated no significant Sex x Diet x Week interaction [F(1,76)=1.58, p=.21] on the number of DCX+ cells. There were also no significant interactions of Sex x Diet [F(1,76)=.02, p=.90], Sex x Week [F(1,76)=.17, p=.68], or Diet x Week [F(1,76)=.09, p=.76]. There were no significant main effects of Sex [F(1,76)=2.48,

p=.12], Diet [F(1,76)=.19, p=.66] or Week [F(1,76)=1.0, p=.32]. The results from the current aim indicate that binge ethanol exposure did not significantly influence neurogenesis after 3 or 8 weeks of exposure.

To determine whether repeated gavage stress affected neurogenesis in cage controls, there was no significant effect of Group on DG DCX+ cells in either male [F(2,28)=1.65, p=.21] or female [F(2,28)=1.55, p=.23] rats. These results indicate that neither gavage stress nor repeated binge exposure resulted in significant changes to neurogenesis.

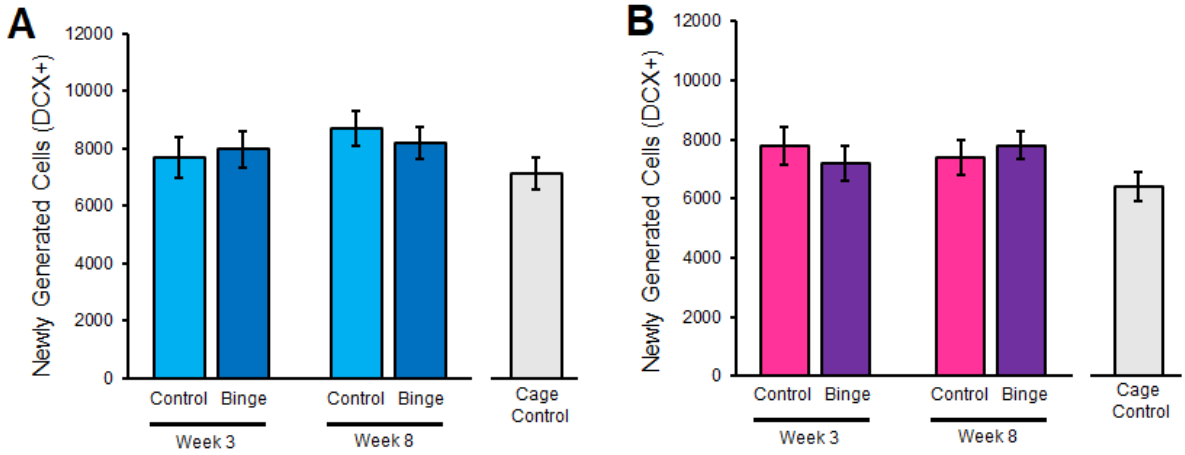

Figure 3.4. There was no significant effect of binge on neurogenesis in either male (A) or female (B) rats, nor any significant effect of gavage stress.

We had previously shown that 11 weekly binge alcohol dosings was associated with increased DCX+ cells in the DG of female rats. In the present study, we did not find an increased number of DCX+ cells in the DG in either males or females after 8 weekly dosings. The animals in the previous study had been behaviorally tested, whereas those in the present study were not. We reasoned that it was possible that the cognitive challenge of behavior testing, combined with binge exposure, had increased neurogenesis in the previous study. To examine this possibility, a small group of rats that received behavioral testing

after 8 weekly binge dosings were compared with rats from the present aim that received no behavioral testing. If rats that had behavioral testing had more DCX+ cells than rats that received no behavioral testing, then it may be true that a cognitive challenge is necessary for compensatory neurogenesis following weekly binge. Tissue processing and cell quantification of DCX+ cells in the dentate gyrus followed the same procedure as outlined in the previous chapters. One-way ANOVAs within sex were used to compare 8-week control and binged rats that received no behavioral testing, with 8-week binged rats from that received behavioral testing (chapter 4). While there was an increase in DCX+ cells in the DG after 8 weekly dosings plus cognitive testing (see Figure 5), one-way ANOVA testing indicated no significant difference between these groups in either male [$F(2,24)=0.6$, $p=.56$] or female rats [$F(2,24)=1.73$, $p=.20$]. These results indicate that the increase in hippocampal neurogenesis in binged animals that 5 days of cognitive testing was not enough to increase hippocampal neurogenesis in binged animals of either sex.

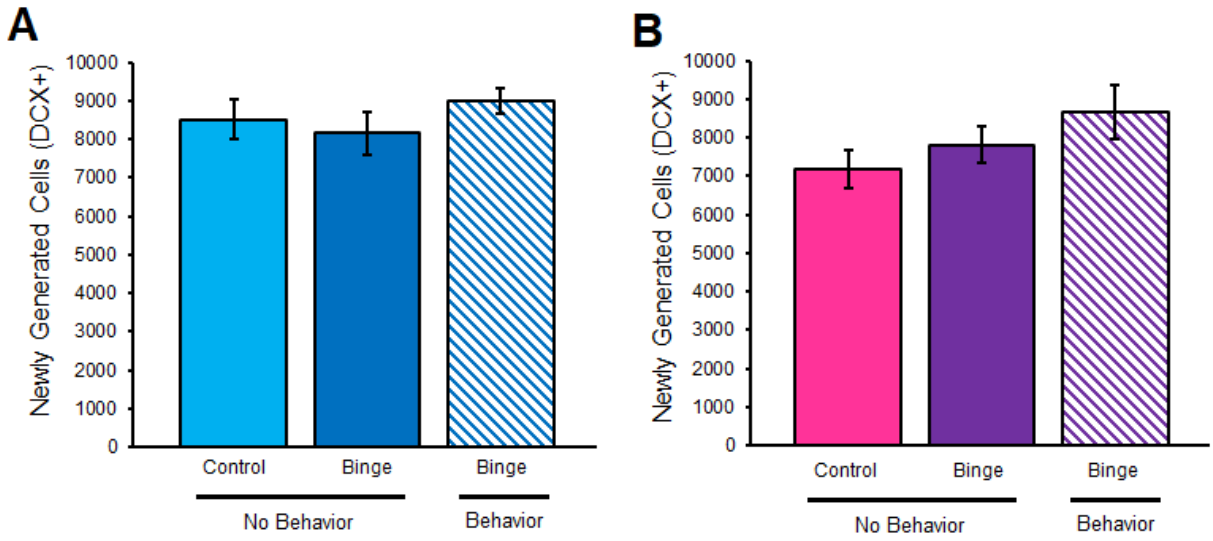

Figure 3.5. Behavioral testing in binged rats did not result in a significant increase in hippocampal neurogenesis in either male (A) or female (B) rats.

Hippocampal Microglia (Iba1)

To investigate the effect of binge ethanol on total hippocampal microglia, a three-way factorial design was used, and demonstrated no significant interaction of Sex x Diet x Week [F(1,76)=.46, p=.50] on the number of Iba1+ cells. There were also no significant interactions of Sex x Diet [F(1,76)=.001, p=.96], Sex x Week [F(1,76)=.01, p=.91]. There was a significant Diet x Week interaction [F(1,76)=9.94, p=.002]. There were also significant main effects of Diet [F(1,76)=13.13, p=.0005] and Dose [F(1,76)=57.88, p<.0001], but no significant main effect of Sex [F(1,76)=.4, p=.53]. Follow-up comparisons of the Diet x Week interaction showed that 8 weeks of ethanol exposure significantly increased number of Iba1+ cells in the hippocampus compared to 3 weeks of ethanol exposure [t(38)=60.38, p=<.0001], and compared to 8 weeks of control diet [t(38)=23.94, p<.0001]. Control rats that had 3 weeks of dosing had significantly Iba1+ cells than controls that received 8 weeks of gavage [t(38)=10.35, p=.002], but did not differ from rats that had 3 weeks of binge ethanol [t(38)=.12, p=.73]. After 8 weeks, there was a large effect size of binge in both male rats [d=1.28] and female rats [d=1.34] for total microglia in the hippocampus.

For the number of partially activated Iba1+ cells in the hippocampus, there was no significant interaction of Sex x Diet x Week [F(1,76)=.51, p=.48]. There were also no significant interactions of Sex x Diet [F(1,76)=.6, p=.44] or Sex x Week [F(1,76)=.04, p=.84]. There was a significant interaction of Diet x Week [F(1,76)=10.55, p=.002], and significant main effects of Diet [F(1,76)=17.75, p<.0001] and Week [F(1,76)=22.1, p<.0001]. There was no significant main effect of Sex [F(1,76)=.04, p=.85]. Pairwise comparisons of the Diet x Week interaction, showed that 8 weeks of binge exposure

significantly increased the number of partially activated Iba1+ cells in the hippocampus compared to 3 weeks of ethanol [t(38)=32.81, p<.0001] and compared to 8 weeks of control doses [t(38)=28.91, p<.0001]. Three weeks of receiving control solution did not significantly alter the number of activated Iba1+ cells in comparison to 3 weeks of ethanol solution [t(38)=.48, p=.49] or 8 weeks of control solution [t(38)=1.09, p=.30]. There was a large effect size of binge in male rats [d=1.33] and female rats [d=1.19] for the number of partially activated microglia in the hippocampus after 8 weeks of binge or control exposure. Overall, these results indicate that 8 weeks of binge ethanol increased the total number and number of partially activated microglia in the hippocampus in both male and female rats.

To determine whether repeated gavage stress affected hippocampal Iba1+ cells in control animals, a one-way ANOVA indicated a significant effect between standard control groups and cage controls in both male [F(2,28)=3.39, p=.048] and female [F(2,28)=4.01, p=.03] rats in the number of Iba1+ cells in the hippocampus. Follow-up examination of this effect in male rats indicated there was no significant difference between male cage control rats and those who had either 3 weeks [t(18)=2.07, p=.16] or 8 weeks [t(18)=1.25, p=.27] of gavage control solution. In females, pairwise comparisons indicated that cage controls significantly differed in total Iba1+ cells in comparison to 3 week gavaged controls [t(18)=5.66, p=.0247], but did not differ from 8 week gavaged controls [t(18)=.02, p=.89].

To determine the effects of repeated gavage stress on partial microglial activation in the hippocampus, a one-way ANOVA indicated no significant effect of Group on the number of partially activated Iba1+ cells in either male [F(2,28)=.82, p=.45] or female [F(2,28)=1.33, p=.28] rats. Analysis of the effects of gavage stress on hippocampal

microglia therefore indicate no effect of gavage stress on total microglia or the number of partially activated microglia.

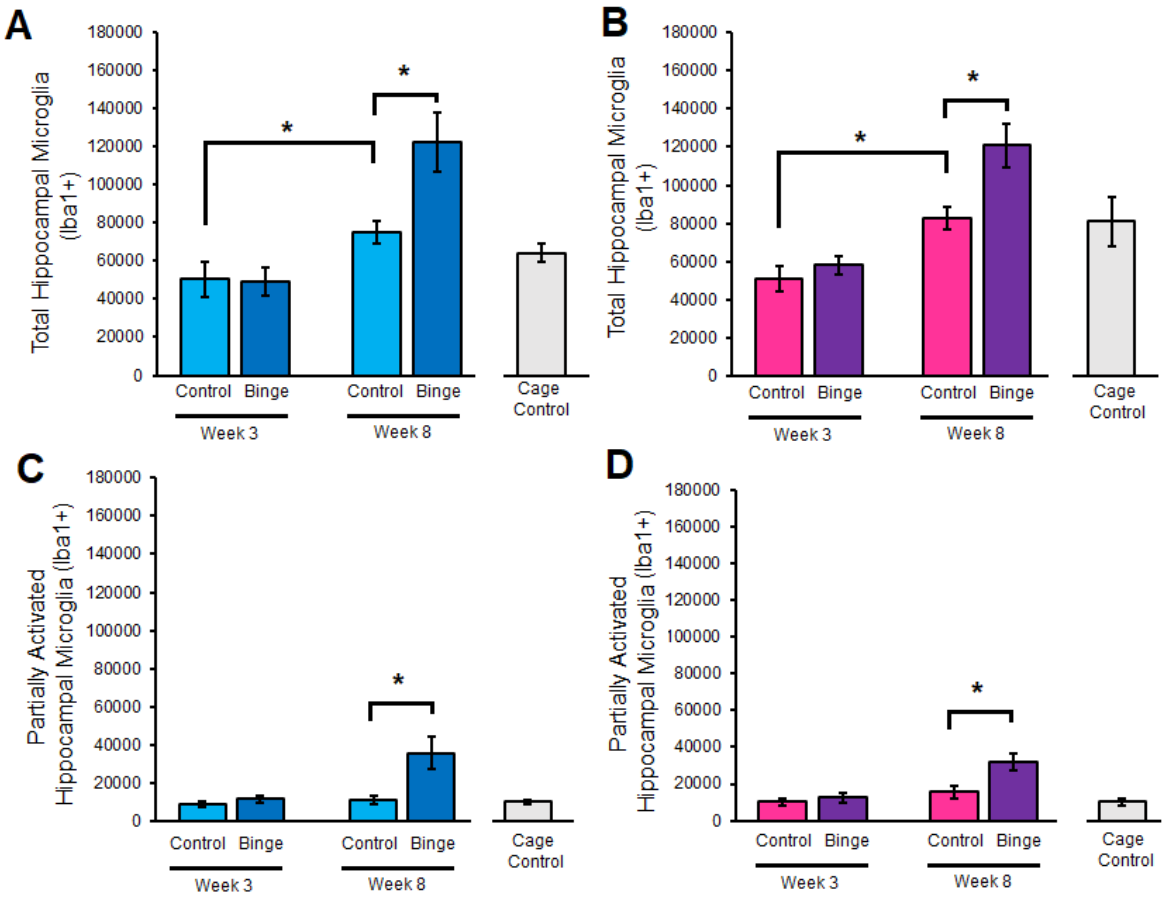

Figure 3.6. After 8 weeks of binge ethanol, both binged male (A) and female (B) rats had significantly more microglia in the hippocampus than control rats. There was also an effect of dose on control rats, with control rats after 8 weeks having more total microglia than control rats after 3 weeks. Similarly, 8 weeks of binge significantly increased the number of partially activated microglia in the hippocampus in both male (C) and female (D) rats. There was no effect of gavage on total microglia on either total number or number of partially activated microglia in the hippocampus.

Prefrontal Cortex Microglia (iba1)

To investigate the effects of binge ethanol on total microglia in the mPFC (iba1), three-way factorial analysis showed no significant interaction of Sex x Diet x Week [F(1,76)=.14, p=.70] on the number of Iba1+ cells in the mPFC. There were also no significant interactions of Sex x Diet [F(1,76)=.95, p=.33], Sex x Week [F(1,76)=.13,

p=.72]. There was a significant main interaction of Diet x Week [F(1,76)=26.11, p=<.0001]. There was no significant main effect of Sex [F(1,76)=1.97, p=.16], there were significant main effects of both Diet [F(1,76)=22.57, p<.0001] and Dose [F(1,76)=15.77, p=.0002]. Examination of the Diet x Week interaction indicated that 8 weeks of ethanol exposure increased the number of Iba1+ cells in the mPFC compared to both 3 weeks of binge [t(38)=41.67, p<.0001], and 8-week controls [t(38)=49.13, p<.0001]. After 3 weeks, control rats did not differ from binged rats [t(38)=.07, p=.80] or from control rats after 8 weeks [t(38)=.66, p=.42]. After 8 weeks, there was a large effect size of binge in both male rats [d=1.58] and female rats [d=2.54] for total microglia in the mPFC.

For partially activated microglia in the mPFC (iba1), there was no significant Sex x Diet x Week interaction [F(1,76)=.34, p=.56]. There were also no significant interactions of Sex x Diet [F(1,76)=.1, p=.76] or Sex x Week [F(1,76)=.28, p=.60] on the number of partially activated Iba1+ cells in the mPFC. There was a significant interaction of Diet x Week [F(1,76)=20.07, p<.0001], and significant main effects of Diet [F(1,76)=48.74,p<.0001] and Dose [F(1,76)=13.21, p=.0005]. There was no significant main effect of Sex [F(1,76)=.15, p=.70]. Further analysis of the Diet x Week interaction showed that 8 weeks of binge significantly increased partially activated mPFC Iba1+ cells compared to 3 weeks of exposure [t(38)=34.34, p<.0001] and in comparison to 8 weeks of control solution dosing [t(38)=68.51, p<.0001]. Control rats after 3 weeks did not differ from control rats after 8 weeks [t(38)=.37, p=.54], or from binged rats after 3 weeks [t(38)=3.26, p=.07]. There was a large effect size of binge in both male [d=2.32] and female rats [d=2.06] for mPFC partially activated microglia after 8 weeks. These results indicate that in both

male and female rats, 8 weeks of binge ethanol increased both microglia that were partially activated, as well as the total number of mPFC microglia.

To determine the effect of repeated gavage stress on total prefrontal cortex microglia in cage controls, a one-way ANOVA within sex indicated that in both male [$F(2,28)=3.59$, $p=.042$] and female [$F(2,28)=3.54$, $p=.043$] rats cage controls differed from gavaged controls. Follow-up pair-wise comparisons in male rats indicated a significant differences between cage controls and 3 week gavaged controls [$t(18)=5.94$, $p=.022$], but showed no difference between cage controls and 8 week gavaged controls [$t(18)=4.76$, $p=.038$] in the number of Iba1+ cell in the mPFC. In female rats, follow-up comparisons indicated no significant differences between cage controls and controls that received 8 weeks of gavage dosing [$t(18)=2.17$, $p=.15$]. There was a significant difference between female cage controls and 3-week standard controls [$t(18)=7.05$, $p=.013$], with cage controls having significantly less total mPFC microglia than controls after 3 weeks.

To determine the effect of repeated gavage stress on partially activated prefrontal cortex microglia, a one-way ANOVA within sex indicated that cage controls did not differ from standard control groups in either male [$F(2,28)=.58$, $p=.57$] or female [$F(2,28)=1.11$, $p=.34$] rats on activated Iba1+ cells in the mPFC. Overall, analysis indicated that cage controls did not differ from gavaged controls in the number of partially activated microglia, but did have less overall microglia than standard controls after 3 weeks.

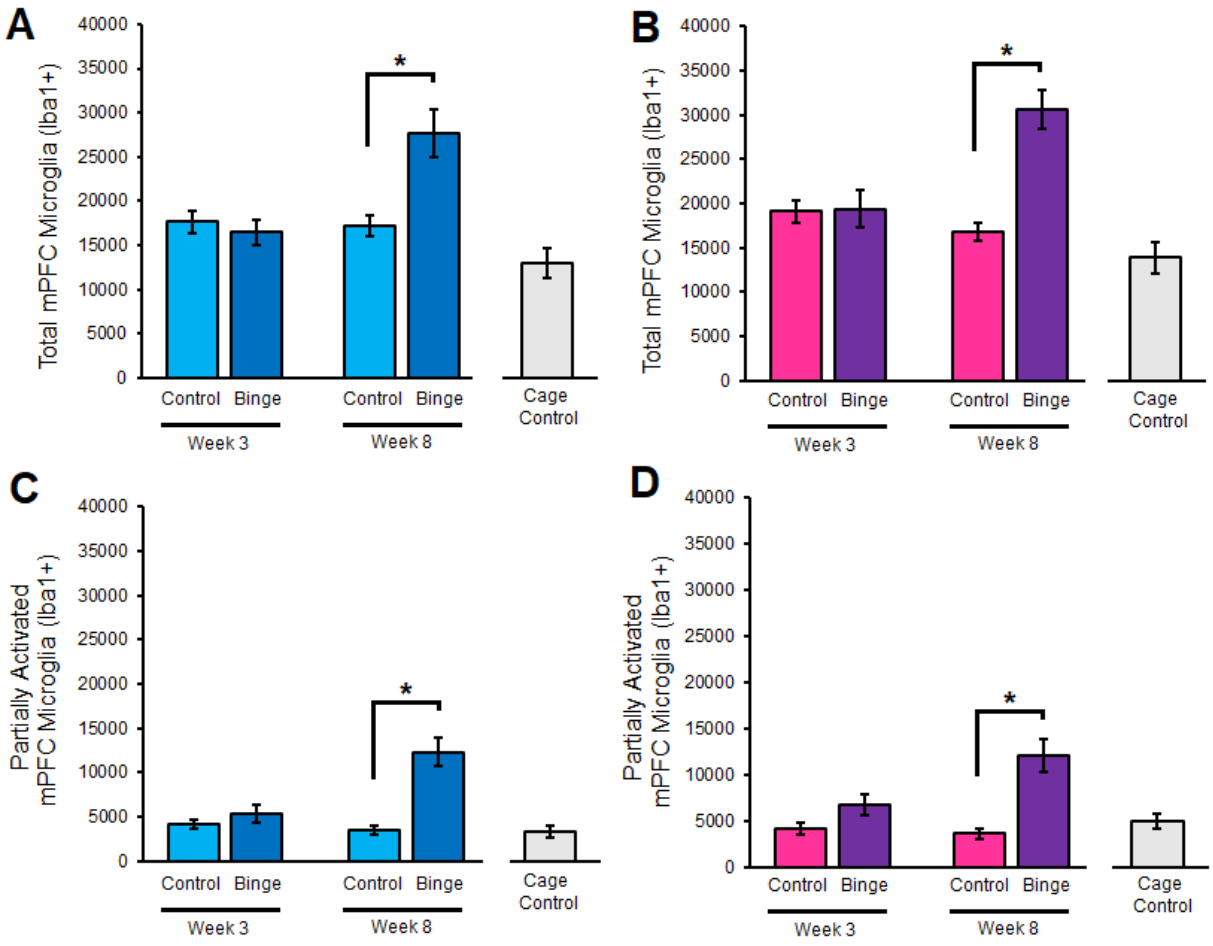

Figure 3.7. 8 weeks of binge ethanol exposure increased total microglia in both binged male (A) and female (B) rats compared to controls in the mPFC. After 8 weeks of binge, there was also a significant increase in the number of partially activated microglia in the mPFC of both male (C) and female (D) rats. There was no effect of gavage on total microglia on either total number or number of partially activated microglia in the mPFC.

Discussion

The first experiment of the current aim used a very limited group of animals (n=4-5/group) in order to investigate when DGGN damage first manifested in each sex. Sample sizes were kept small in an attempt to limit the number of animals needed for preliminary data. Because of the small sample size, this experiment was used exclusively to inform the second, main, experiment of this aim. Using a threshold of either 10% loss, or significant cell loss in comparison to controls, females first passed this threshold after 3 weeks, and

males after 5 weeks of ethanol. As there was expected to be unremarkable differences in cellular damage with only 2 weeks of difference, based off this exploratory study, it was decided that 3 weeks and 8 weeks of ethanol exposure would best provide information about both a relatively short versus longer term effect of weekly binge ethanol. Although in this exploratory experiment, female rats had an 11% cell loss after 3 weeks, and a t-test indicated this as a significant loss, when additional rats were run, this effect became somewhat less pronounced, and post-hoc comparisons did not show significant cell loss in female rats after 3 weeks.

Regarding sex comparisons in damage to the dentate gyrus in the main experiment, while both male and female rats overall had significant granule cell loss, there was a larger effect size in female rats than was present in female rats. Male rats overall had more DGGN neurons than did female rats, something that has been observed previously in our lab (Maynard et al., 2018). Female rats also had a larger percentage loss than male rats (Figure 3.3). After 3 weeks there was a 7.3% DGGN loss in female rats, and a 6.8% loss in male rats. After 8 weeks, this effect was more pronounced, with female binged rats having 13.5% fewer remaining granule cells than control rats, while male rats sustained only an 8.4% loss. Although there was no statistically significant difference in either males or females between 3 and 8 weeks of exposure, there was still a larger percent loss after 8 weeks in both sexes, though this loss was more pronounced in females. Overall, the DGGN results from this aim partly supported the hypothesis that both male and female rats would manifest significant cell loss from weekly ethanol exposure, but that this loss would be more severe in female rats. This partially supports previous literature indicating female susceptibility to chronic

ethanol damage (Agartz et al., 2003; Alfonso-Loeches et al., 2013; Mann et al., 1992; Maynard et al., 2018; Sharrett-Field et al., 2013; Wilhelm et al., 2016).

Contrary to the results found in Aim 1, the results from the current aim did not indicate a compensatory increase in neurogenesis. In the current aim, neither 3 or 8 weeks of ethanol exposure had a significant effect on neurogenesis in the dentate gyrus. There are a few explanations for this discrepancy. The first is that perhaps more cellular loss is needed to cause detectable compensation in hippocampal neurogenesis, as the results from aim 1 were measured after 11 weeks of exposure, versus 3 or 8 weeks. A second explanation lies in the experiences of the rats in from the first aim versus those in the current aim.

Female rats that received 11 weeks of ethanol had cognitive testing performed from the very start of ethanol exposure and therefore had much more interaction with the experimenter and exposure to multiple different testing rooms, both in the final week of the experiment, as well as throughout the entire experiment. The rats in this aim, had comparatively unenriched lives, as they received no behavioral testing, had far less contact with the experimenter, and had no enrichment items in their cages. Therefore, the more likely explanation of this discrepancy in results, is that a cognitive challenge is necessary to cause this compensatory upregulation in hippocampal neurogenesis. Although the experimental conditions still differ, this hypothesis can be in-part tested by examining the brains of rats that received ethanol and underwent behavioral testing. If it is true that a cognitive challenge is necessary to cause a compensatory increase in neurogenesis in binged rats, rats that received 8 weeks of ethanol and also underwent MWM and USV testing would show more neurogenesis than binged rats that had no behavioral testing.

After a lack of compensatory neurogenesis was found in binged rats that had no behavioral testing, follow-up testing was conducted in animals from aim 3 (chapter 4) to test the hypothesis that a cognitive challenge is necessary for a compensatory increase in hippocampal neurogenesis in binged rats. Due to limitations on the amount of available antibody (Santa Cruz Biotechnology: DCX anti-rabbit polyclonal), a small sample of male and female rats that had 8 dosing weeks (n=7/group) was stained for DCX to test this hypothesis. Binged rats that had exposure to behavioral testing did have more DCX+ cells than either binged or control rats that did not have behavioral testing; however, this difference increase was non-significant, perhaps due to the small sample size. Additionally, it cannot be discounted that this, albeit non-significant, increase could be the result of tissue collection after an additional 2 days of ethanol abstinence in comparison to rats that did not undergo behavioral testing.

There was no evidence in a decrease in neurogenesis resulting from weekly ethanol in rats that underwent behavior testing or those that did not. However, it should be noted that tissue for aim 2 was collected 4 days following the final does of ethanol, and tissue was collected after 6 days of ethanol abstinence for aim 3. Therefore, it cannot be stated that there was not a detectable decrease in hippocampal neurogenesis immediately following binge exposure. In inhibition of neurogenesis in the period immediately following ethanol exposure could contribute to the decrease in mature granule cells observed. An inhibition in neurogenesis has been noted immediately following other binge models neurodegeneration (S. A. Morris et al., 2010; K. Nixon & Crews, 2002), and a compensatory increase in cell proliferation was noted only after a period of alcohol abstinence (K. Nixon & Crews, 2004). Since we examined DCX+ cells after a short period of alcohol abstinence, it is possible that

an acute inhibition of neurogenesis occurred immediately following binge ethanol, followed by neurogenesis leveling out to control levels after a short period of abstinence from ethanol.

The neuroimmune activation in the mPFC and hippocampus after weekly binge exposure followed the same pattern, regardless of sex or region. After 8 weeks of ethanol, there was in increase in the total number of microglia in both male and female rats in both regions. Also, after 8 weeks, in both the mPFC and hippocampus in both sexes, weekly binge increased the number of partially activated microglia. These results indicate that regardless of sex, 8 weekly binge dosings increased neuroimmune activation. The lack of microglial response after 3 weeks is of interest when taken in the context of the start of, albeit non-statistically significant, hippocampal cell loss. After 3 weeks, there was not significant neuroimmune activation, indicated by microglial number and increased partial activation in the hippocampus, however there was after 8 weeks of binge exposure. The lack of response after 3 weeks of binge indicates that likely there is a tipping point that occurs between 3 and 8 weeks, where the brain is no longer able to prevent significant cell damage, and therefore begins to mount a neuroimmune response in attempt to prevent additional damage. This microglial activation response may contribute to the asymptotic curve that appears to prevent damage from occurring at a continual rate with additional weeks of exposure and may be helping to prevent further cellular damage.

If cell loss were to continue at the same rate as was initially seen in the first 3 weeks, male rats would have an 18% granule cell loss and female rats would have a 19% granule cell loss after 8 weeks. However, the rate of DG cellular depletion was not linear, and after 8 weeks male rats had lost only 8.4% and females had 13.5% fewer mature granule cell neurons. The observed increase in microglial response between 3 and 8 weeks likely

contributed to this decreased rate of change. Partial microglial activation in particular is thought to play a role in repair and protection (Battista et al., 2006; Shokouhi et al., 2010). An alternate model of binge ethanol exposure has also been shown to prime microglia, and that a second binge episode further increased microglial activation (Marshall et al., 2016; Marshall et al., 2013). This data supports the conclusion that increased partial microglial activation in response to repeated binge may be working to regain homeostatic balance and prevent further cellular damage.

Also of note is the increase in the number of total hippocampal microglia observed between control rats after 8 weeks in comparison to control rats after 3 weeks. Although this may at first appear to be as a result of additional dosings, cage controls that were sacrificed at the same time as 8-week control rats were indistinguishable from gavaged controls. This indicates that this increase in total hippocampal microglia over time is likely due to an age effect, rather than an effect of gavage stress. Although no published studies have quantified hippocampal microglia in rats comparing 13 weeks and 18 weeks old adults, there is evidence indicating an increase in microglia associated with aging (Mouton et al., 2002; Tremblay, Zettel, Ison, Allen, & Majewska, 2012). Additionally, this increase in total microglia in the hippocampus was not accompanied by an increase in the number of partially activated microglia, indicating that this increase is not due to neuroimmune activation.

To assess whether the stress of weekly gavage is itself enough to induce cellular damage, rats that were gavaged weekly with control solution were compared with rats that did not receive gavage. Aside from food removal prior to gavage, and tube insertion, the experience of these cage control rats did not differ from controls that were gavaged.

Therefore, if differences between these groups were observed, it would have to be as a result of either the stress of gavage or the process of digesting the control solution (i.e. high sugar, abdominal distention). None of the outcome measures that were explored in this study indicated that gavage exposure with control solution caused any significant negative changes to the brain, meaning the gavage procedure was not in itself stressful enough to result in cellular damage.

Overall these results indicate that weekly binge ethanol causes a significant depletion of mature neurons in the dentate gyrus, particularly in female rats. Eight weeks of ethanol also increased neuroimmune activation in the mPFC and hippocampus in both male and female rats. These results combined, indicate that while both male and female rats are susceptible to the neurotoxic effects of ethanol, there appears some evidence that the magnitude of cellular changes is more severe in female rats.

Chapter 4: Determine whether a telescoping effect will result in earlier binge-induced behavioral deficits in female rats.

In the previous chapter, the cellular effects of binge ethanol on the male and female brain were examined after both 3 and 8 weeks. Having established significant cellular damage in both sexes in response to repeated ethanol exposure, it is important to investigate whether they are accompanied by behavioral impairment. Excessive ethanol has been well documented to result in cognitive deficits and changes to mood state, therefore two tests were used in order to test for these changes. Using a combination of measuring ultrasonic vocalizations (USV) and the Morris water maze, changes to affect and cognition from weekly binge exposure were measured.

The Morris water maze (MWM), is a common spatial learning and memory task that has been shown to be sensitive to cortical and hippocampal damage (D'Hooge & De Deyn, 2001). Performance on the MWM is heavily dependent on hippocampal function, which as shown in the previous aims (chapter 2, 3) is a region vulnerable to weekly binge ethanol. Therefore, we hypothesized that if hippocampal cell loss and neuroimmune activation were to result in changes in cognition, this task would be likely to show these impairments. Performance on the MWM test is measured by the latency to find a hidden platform while swimming in opaque water, and/or by the distance traveled before the platform is located. Animals with cognitive impairments exhibit greater latencies or longer distance traveled before finding the platform. While simple in design, deficits in water maze performance have been shown to result from chronic alcohol (Arendt et al., 1988; Lukoyanov et al., 1999), and binge alcohol models (Maynard et al., 2018; Obernier, White, et al., 2002; Schulteis et al., 2008). While not all studies have demonstrated alcohol-induced performance deficits (Acheson et al., 2001; Markwiese et al., 1998), through lesion studies,

the MWM task has been shown to be influenced by both the hippocampus and frontal cortex (Galani, Weiss, Cassel, & Kelche, 1998; Kolb & Cioe, 1996; R. G. Morris, Garrud, Rawlins, & O'Keefe, 1982). Prior research examining sex-differences in alcohol-related behavioral impairment using the 4-day binge model demonstrated that female binge, but not male binge rats, had an impairment in Morris water maze acquisition (Maynard et al., 2018).

The hippocampus, particularly the ventral hippocampus, also has a significant role in emotional regulation and mood (Fanselow & Dong, 2010; Kheirbek & Hen, 2011). Therefore, cellular damage resulting from weekly binge would also likely impact rodents' affect. Detection and quantification of ultrasonic vocalizations (USV) allow for examination of affective state in rats. Two frequencies of emissions are typically examined. The higher frequency, 50-kHz USV are typically emitted in pro-social interactions, such as during play or mating (Burgdorf et al., 2008; Burgdorf et al., 2011; Knutson et al., 1998; Panksepp & Burgdorf, 2000; Sales, 1972; Schwarting et al., 2007). These 50-kHz vocalizations, which are commonly seen during juvenile play-wrestling, can also be induced in adults though "playful handling" or "stroking" by a human hand (Schwarting et al., 2007). In contrast to 50-kHz USV, the emission of 22-kHz USV is associated with negative affect. These vocalizations result from fear, pain, anxiety or stress (Calvino et al., 1996; Cuomo et al., 1988; Knapp & Pohorecky, 1995; Wohr et al., 2005). Examination of adult vocalizations can thus allow researchers to examine associative mood states in the rat. Generally, 50-kHz USV are associated with positive mood or affect states and 22-kHz USV are indicative of negative affect (Blumberg & Alberts, 1991; Burgdorf et al., 2008; Burgdorf et al., 2011; Mallo et al., 2007; Panksepp & Burgdorf, 2000; Sales, 1972). By evaluating USV,

particularly 50-kHz vocalizations emitted during play sessions or stroking, changes or differences in affect can be determined (D'Souza & Sadananda, 2017).

Additionally, USVs have been correlated with hippocampal neurogenesis, specifically that hippocampal cell proliferation was positively correlated with 50 kHz vocalizations and negatively correlated with 22 kHz vocalizations (Wohr et al., 2009). Therefore, on the face, the significant increase in DCX+ cells in female binged rats after 11 weeks previously observed (chapter 2), would indicate that binged rats would display more positive and less negative vocalizations, we predicted the opposite. It is likely that mood state or affect is tied more to overall hippocampal health than only neurogenesis and given that we observed significant hippocampal damage in both male and female rats, we predicted that rats with hippocampal neuroimmune activation and cell loss would result in fewer positive vocalizations (50 kHz) and more aversive vocalizations (22 kHz). Based on the results from both the previous aims and prior literature that the hippocampus is highly vulnerable to ethanol damage (Agartz et al., 1999; Beresford et al., 2006; Sullivan et al., 1995), these tasks were chosen because they are both hippocampal-dependent tasks and would therefore likely show any behavioral impairments that may exist due to hippocampal damage.

It was hypothesized that binged female rats would show behavioral changes in comparison to female controls earlier than male binge compared with male control rats. Specifically, it was predicted that binged rats would emit fewer 50-kHz and more 22-kHz ultrasonic vocalizations compared with same-sex controls, thus indicating a negative affect. On the Morris water maze task, it was hypothesized that binged rats would have longer escape latencies than same-sex controls. Consistent with the theory of alcohol-related

telescoping effects, it was anticipated that these changes in affect and cognition will appear more quickly in the female binge group than in the male binge group.

Method

Animals

A total of 80 Long-Evans rats (40 male, 40 female) were used to investigate sex-differences in behavioral impairment resulting from binge alcohol exposure. Eight-week-old animals spent 10 days becoming accustomed to the researcher and their new environment before gavage dosing. Rats were randomly assigned by sex, to either ethanol or control groups and were run in two equal cohorts. All rats were housed in groups of 2-3 rats per cage, and housing rooms were kept on a reverse light-dark cycle.

Binge Paradigm and Timeline

Weekly alcohol administration followed the same method as described in the previous chapters. Brain tissue for rats that underwent behavioral testing was also used in Aim 2 for analysis of cellular activation during MWM, therefore the results for behavioral intoxication, BEC and body weight for these animals was included in the analysis for these respective measures reported in the previous chapter (Aim 2). Measurement of USVs began 24 hours following the final ethanol dosing and was repeated for 5 consecutive days. The standard MWM testing began approximately 36 hours after the final ethanol dosing and was repeated for 4 consecutive days. Probe testing in the MWM was conducted an hour following the final trial on the fourth day of testing. Reversal MWM learning took place the day after the conclusion of the standard MWM testing and probe test, on the sixth day

following the final ethanol dosing. Animals were sacrificed and tissue was collected for c-Fos cellular analysis (Aim 2) 60-90 minutes following the final trial of MWM reversal testing for each rat.

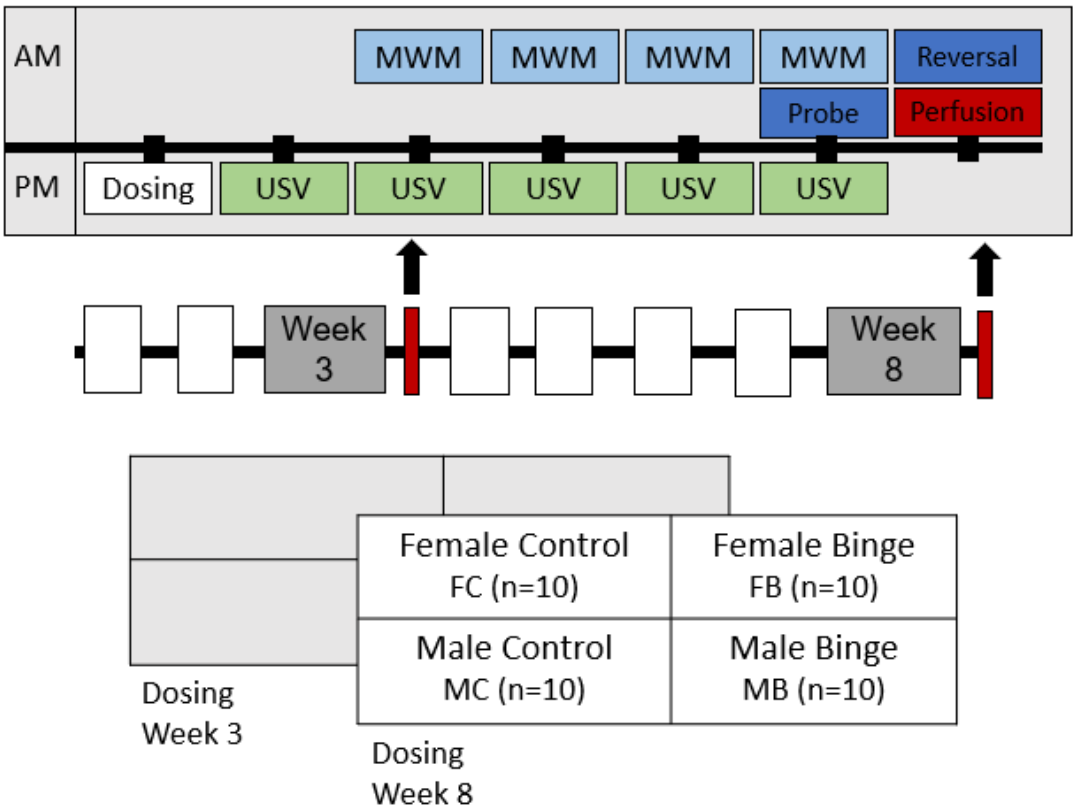

Figure 4.1. Timeline of behavioral testing. Ultrasonic vocalization testing was conducted for 5 consecutive days beginning 24 hours following ethanol or control dosing. Morris water maze acquisition testing began in the morning 2 days following the final dosing (4 trials/4 days). Probe testing was conducted roughly 1 hour following the final trial of MWM acquisition. Reversal learning was performed on the 6th day following dosing, and brain tissue was collected 60-90 minutes following reversal learning trials.

Morris Water Maze

Water maze testing took place the second day following the final dose of alcohol (roughly 36 hours). Water maze testing was conducted 1-2 hours into the animals' active (dark) cycle. Rats were brought into the room at least 10 minutes prior to testing to acclimate to the testing environment. The water maze apparatus was a large, circular plastic pool approximately 183 cm in diameter and 75 cm deep. A platform was hidden 2-3 cm

under the surface of the water, and the water was made opaque using non-toxic white paint. The water maze tub was located in the center of the testing room with 3 large black shapes measuring approximately 12 cm in diameter (circle, square and star) on the nearby walls to serve as visual cues. Rats had to learn the location of the hidden platform in reference to these visual cues to memorize the location of the hidden platform. Water was kept at slightly warmer than ambient air temperature (23 ± 0.5°C), by the addition of warm water before daily testing.

Four acquisition trials were given each day, for four consecutive days (adapted from (Verbois, Hopkins, Scheff, & Pauly, 2003)). The trial began when the rat was placed into the water, facing the experimenter. The rat was then released and allowed to swim freely for up to 60 seconds. The trial ended when either when the rat located the platform, or 60 seconds had elapsed. If after a minute, the rat had still not located the platform, it was gently led to the platform by the experimenter and allowed to remain there for 10 seconds to examine the visual cues from the platform location. Once the animal stayed on the platform for at least 5 seconds, the trial ended. Following the conclusion of each trial, the rat was removed from the water, dried off, and placed back into the home cage until the next trial. Each rat had approximately 5-minutes between each trial, and testing began at roughly the same time each day. Between trials the water was agitated to prevent any olfactory cues to the platform location, and the experimenter stood in the same location after release of each animal. The platform location remained constant throughout all 16 acquisition trials; however, the release quadrant changed each trial and was pseudo-randomly chosen.

After the conclusion of the fourth acquisition trial of day four, a single probe trial was given. For the probe trial, a single 60-second trial was given in which the platform was

removed from the tub. The rat's movements around the arena were tracked automatically. Following the probe trial, a 'reminder' trial was given to all rats. During this trial, the platform was returned to its original position to reinforce the location of the platform prior to reversal learning the following day. If necessary, rats repeated this reminder trial until they had located the platform within 30 seconds.

The following day, four reversal learning trials were given, in which the location of the platform was moved from its original location to the opposite side of the tank. Rats had four 60-second trials, following the same procedure as in the acquisition trials, in which to learn the new location of the platform. Rats were perfused and tissue was collected 60-90 minutes following the final reversal learning trial to examine cellular activation during this task.

An automated tracking system (Noldus; Amsterdam, Netherlands), tracked all animal movement within the trial time. Primary outcome measures evaluated were latency to platform, swim velocity and the distance to platform.

Tissue Processing & Analysis of Cellular Activation

Cellular activation in the mPFC (c-Fos) during MWM reversal learning was evaluated to assess whether binge would increase the amount of neural resources required during behavioral testing. Rats were perfused 60-90 minutes after the final reversal learning trial to examine cellular activation during this task Tissue processing, immunohistochemistry and counting parameters for mPFC c-Fos were identical to those outlined in Aim 1 (chapter 2).

Ultrasonic Vocalizations

All rats were acclimated to the testing room and USV apparatus for 4 days prior to testing. The first day of USV testing has been shown to have low predictive validity of future USV results (Mallo et al., 2007; Willey & Spear, 2012), therefore a session was conducted and recorded approximately 2 hours before the final gavage dosing, however this session served primarily to acclimate the rodents to the procedure and was excluded from statistical analysis. Beginning the day following the final alcohol dose (24 hours later), USVs were collected on 5 consecutive days. Testing was conducted in the evening during the animals active (dark) cycle under red light, 4-5 hours following the conclusion of MWM testing. All rats were brought into the testing room and allowed to acclimate for at least 15 minutes prior to testing. Each rat was individually transferred into the testing apparatus during vocalization testing and was then returned to the home cage following the testing session. The apparatus consisted of a sound box (measuring 60 H x 80 W x 60 D cm) with a large rectangular bin (measuring 13 H x 48 W x 35 D cm) lined with an absorbent pad and clean cage bedding, situated directly beneath the microphone. The box was spot cleaned between rats, and bedding was replaced between the testing of male and female rats, and at the end of each day.

Eliciting of USVs was designed to simulate the play of juvenile rats and has been shown to elicit 50-kHz USV signals in adults, often heard during play or during other positive social interactions (Burgdorf, Knutson, Panksepp, & Ikemoto, 2001; Mallo et al., 2007; Panksepp & Burgdorf, 2000). Sessions consisted of rapid finger movements across the back and neck, and flipping the animal on the back for quick “wrestling” sessions, combined with rapid finger movements across the animal’s ventral side (Cloutier, LaFollette, Gaskill,

Panksepp, & Newberry, 2018; Mallo et al., 2007; Panksepp & Burgdorf, 2000; Schwarting et al., 2007). Play-chasing also was a part of the sessions, in which animals could chase, or were gently chased by the experimenter's hand, behavior designed to mimic the play-chasing behavior seen in juvenile rats (Knutson et al., 1998). The testing sessions were comprised of alternating 15 seconds of experimenter interaction ("play"), with 15 seconds of rest (adapted from: (Burgdorf & Panksepp, 2001; Cloutier et al., 2018; Mallo et al., 2007)), repeated 6 times for a total of 3 minutes

Ultrasonic vocalizations were recorded with a Nutrick NC MX-HD microphone, placed 27 cm above the testing box and recorded with Avisoft SASLab Pro v5.1 software (Avisoft Bioacoustics, Berlin, Germany). Data from monitoring of each animal over the 3-minute testing period was visualized as a spectrogram, which was then examined and manually counted by the experimenter. The 22-kHz calls spanned a frequency range of 20-32 kHz, and vocalizations of 35-96 kHz were recorded as 50-kHz (Schwarting et al., 2007). The primary measure of interest was the number of 50-kHz and 22-kHz USV, which were compared between groups to examine whether there were changes in affect resulting from binge alcohol exposure. It was predicted that binged animals would emit fewer positive vocalizations (50-kHz) in response to play sessions and more negative USV (22-kHz) overall than same-sex control rats. While the number of 22 kHz and 50 kHz vocalizations were the primary outcome measure of interest, the frequency and duration of calls (22-kHz and 50-kHz) as well as the percentage of negative affect calls were also calculated and compared between groups.

Estrous Cycle Monitoring

Vaginal smears of a subset of female rats were taken at the same time each morning on behavioral testing days. Each sample was collected using a wet cotton swab and was then 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.

Statistical Analysis

Using SAS 9.4, comparisons between groups on the MWM was accomplished using a mixed-model analysis with Day (standard MWM) or Trial (reversal learning) as a repeated measure and Diet (binge or control) and Week (3 dosing weeks or 8 dosing weeks) as primary main effects within sex. Morris water maze testing focused on the average latency to the platform for each testing day during acquisition and reversal learning, however swim speed and distance to platform were also evaluated, as well as the amount of time spent in the platform quadrant during probe testing.

Analysis of c-Fos+ cells in the mPFC used a three-way factorial design for the factors of Sex (2 levels: Male, Female), Diet (2 levels: Control, Binge) and Week (2 levels: 3, 8) as well as interactions of these variables. To examine the effects of gavage stress, cellular analysis compared cage controls to controls that received 3 and 8 weeks of gavage with control solution, within sex using a one-way ANOVA.

Analysis of USV testing was similarly accomplished using a mixed-model analysis within sex to evaluate the main effects of Diet and Week with testing Day as a repeated measure. Outcome measures from USV testing included the average number of

vocalizations per session, the average frequency, and average duration of both 50-kHz and 22-kHz vocalizations.

When appropriate, comparisons with Bonferoni correction were used to further examine significant interactive effects. Results from statistical tests were deemed significant if the p-value was less than 0.05, data was organized and graphed using Excel 2016 (Microsoft Office 360 Excel, WA, USA), and statistical analysis was performed using SAS 9.4 (SAS Institute Inc., Cary, NC, USA).

Results

Morris Water Maze

Acquisition Trial Learning

For latency to platform location in male rats, there was no significant Day x Diet x Week interaction [$F(3,624)=.98$, $p=.40$]. There were no significant Day x Diet [$F(3,624)=.65$, $p=.59$], Day x Week [$F(3,624)=.66$, $p=.57$], or Diet x Week [$F(1,624)=1.94$, $p=.16$] interactions. There was no significant main effect of either Diet [$F(3,624)=.01$, $p=.95$] or Week [$F(1,624)=3.55$, $p=.06$]. There was a significant main effect of Day [$F(3,624)=84.4$, $p<.0001$], with all male rats finding the platform more quickly on the final day of MWM than at the start of testing. For latency to platform location in female rats, there was no significant Day x Diet x Week interaction [$F(3,624)=2.48$, $p=.06$]. There was no significant Day x Diet [$F(3,624)=.45$, $p=.72$] or Diet x Week [$F(1,624)=1.88$, $p=.17$] interaction. There was a significant Day x Week interaction [$F(3,624)=3.86$, $p=.009$], and a significant main effect of Day [$F(3,624)=69.01$, $p<.0001$]. There was no significant main

effect of either Diet [F(3,624)=.01, p=.92] or Week [F(1,624)=.14, p=.71] on latency to platform. Follow-up testing of the Day x Week interaction in female rats revealed that 3 and 8 week rats did not differ on testing day 1 [t(154)=.75, p=.39], or day 3 [t(154)=.11, p=.74], or day 4 [t(154)=2.55, p=.11]. On the second day of testing, female rats after 8 weeks had a longer latency to the platform than those after 3 weeks [t(154)=8.25, p=.004].

For swim velocity in male rats, there was no significant Day x Diet x Week interaction [F(3,622)=.09, p=.96]. There were no significant interactions between the factors of Day x Diet [F(3,622)=.64, p=.59] or Diet x Week [F(1,622)=.24, p=.62]. There was a significant Day x Week interaction [F(3,622)=4.04, p=.008], as well as a significant main effect of Day [F(3,622)=26.07, p<.0001]. There were no significant main effects of Diet [F(3,622)=.03, p=.85] or Week [F(3,622)=.78, p=.38]. Follow-up examination of the Day x Week interaction in male rats showed that 8 week rats swam faster than 3 week rats on the first testing day [t(154)=13.64, p=.0003], but that rats did not differ on day 2 [t(154)=.52, p=.47], day 3 [t(154)=.1, p=.76], or day 4 [t(154)=.14, p=.71]. For swim speed in female rats, there was no significant Day x Diet x Week interaction [F(3,622)=.82, p=.48]. There were no significant interactions between the factors of Day x Diet [F(3,622)=.14, p=.94] or Diet x Week [F(1,622)=.09, p=.77]. There was a significant Day x Week interaction [F(3,622)=5.34, p=.001], as well as significant main effects of Day [F(3,622)=31.74, p<.0001], Diet [F(3,622)=4.4, p=.037]. There was no significant effect of Dose [F(3,622)=1.29, p=.26]. Follow-up examination of the Day x Week interaction in female rats showed that 8 week rats swam faster than 3 week rats on the first testing day [t(154)=17.83, p<.0001], but that rats did not differ on day 2 [t(154)=.06, p=.80], day 3 [t(154)=.15, p=.70], or day 4 [t(154)=.68, p=.41].

For distance traveled in male rats during MWM testing, there was no significant Day x Diet x Week interaction [$F(3,622)=.56$, $p=.64$]. There were no significant interactions between the factors of Day x Diet [$F(3,622)=.24$, $p=.87$], Day x Week interaction [$F(3,622)=.77$, $p=.51$], or Diet x Week [$F(1,622)=2.19$, $p=.14$]. There was a significant main effect of Day [$F(3,622)=49.68$, $p<.0001$], but no significant main effects of Diet [$F(3,622)=.54$, $p=.46$] or Dose [$F(3,622)=1.82$, $p=.18$]. Male rats more directly to the platform on later days than on the first day of MWM. For distance swam in female rats, there was no significant Day x Diet x Week interaction [$F(3,622)=1.32$, $p=.27$]. There were no significant Day x Diet [$F(3,622)=.49$, $p=.69$], or Diet x Week [$F(1,622)=1.54$, $p=.21$] interactions. There was a significant Day x Week interaction [$F(3,622)=3.31$, $p=.02$] and a significant main effect of Day [$F(3,622)=35.24$, $p<.0001$]. There were no significant main effects of Diet [$F(3,622)=.02$, $p=.89$] or Dose [$F(3,622)=.81$, $p=.37$]. Further analysis of the Day x Week interaction in female rats revealed that 3 and 8 week rats did not differ on testing day 1 [$t(154)=.27$, $p=.60$], day 3 [$t(154)=.88$, $p=.35$], or day 4 [$t(154)=1.9$, $p=.17$]. On the second day of testing, female rats after 8 weeks had a longer distance to the platform than those after 3 weeks [$t(154)=7.67$, $p=.006$].

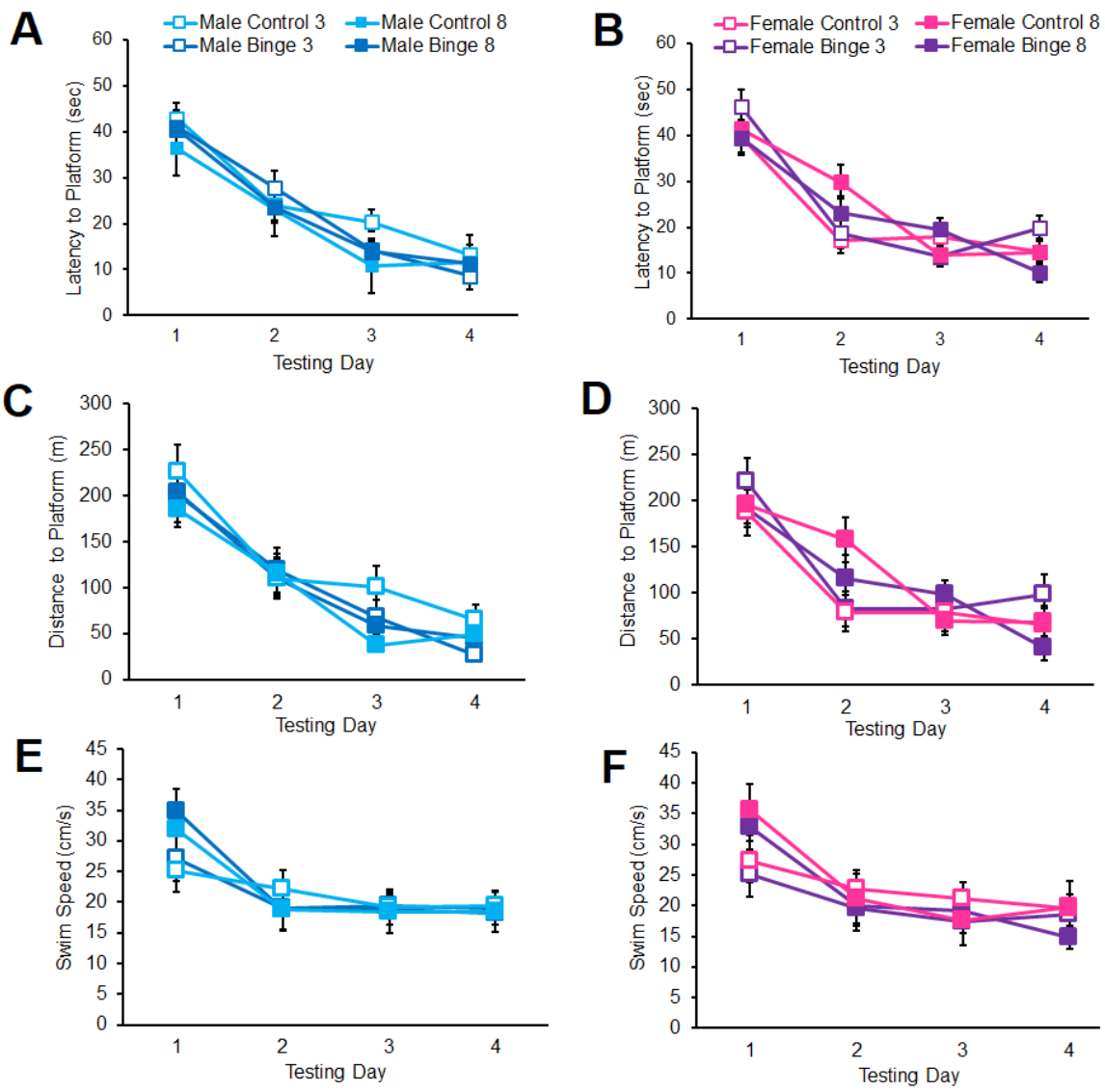

Figure 4.2. Binge ethanol exposure did not result in any detectable spatial learning impairments in latency to the platform in either male (A) or female (B) rats. Similarly, weekly binge did not impact the distance needed to locate the platform in either male (C) or female (D) rats. There were no binge effects in swim speed in male (E) or female (F) rats. All rats successfully learned the location of the platform over the course of MWM testing (4 days, 4 trials/day).

Probe Trial

During probe testing, for swim velocity in male rats, there was no significant Diet x Week interaction [$F(1,26)=.81$, $p=.38$]. There was also no significant main effect of either

Diet [F(1,26)=1.23, p=.29] or Dose [F(1,26)=1.88, p=.19]. For swim velocity in female rats, there was no significant Diet x Week interaction [F(1,26)=.66, p=.43]. There was also no significant main effect of either Diet [F(1,26)=.64, p=.44] or Dose [F(1,26)=.21, p=.66].

For time spent in the previous platform quadrant location in male rats, there was no significant Diet x Week interaction [F(1,26)=3.93, p=.058]. There was no significant main effect of Diet [F(1,26)=2.83, p=.10]. There was a significant main effect of Dose [F(1,26)=7.8, p=.010], with binged and control rats who had 8 weeks of gavage exposure spent more time in the previous platform location than those who had 3 weeks of ethanol or control doses. For time spent in the previous platform quadrant location in female rats, there was no significant Diet x Week interaction [F(1,26)=.95, p=.34]. There was also no significant main effect of either Diet [F(1,26)=.31, p=.58] or Dose [F(1,26)=.28, p=.60].

For the number of times entered into the previous platform quadrant location in male rats, there was no significant Diet x Week interaction [F(1,26)=.19, p=.67]. There was also no significant main effect of either Diet [F(1,26)=1.69, p=.21] or Dose [F(1,26)=.05, p=.83]. For number of times entered into the previous platform quadrant location in female rats, there was no significant Diet x Week interaction [F(1,26)=.18, p=.68]. There was also no significant main effect of either Diet [F(1,26)=.4, p=.53] or Dose [F(1,26)=.72, p=.40].

For the number of times entered into the previous location of the platform in male rats, there was no significant Diet x Week interaction [F(1,26)=3.24, p=.08]. There was also no significant main effect of either Diet [F(1,26)=.21, p=.65] or Dose [F(1,26)=2.99, p=.10]. For number of times entered into the platform location in female rats, there was no significant Diet x Week interaction [F(1,26)=1.58, p=.22]. There was also no significant main effect of either Diet [F(1,26)=.05, p=.83] or Dose [F(1,26)=.23, p=.64].

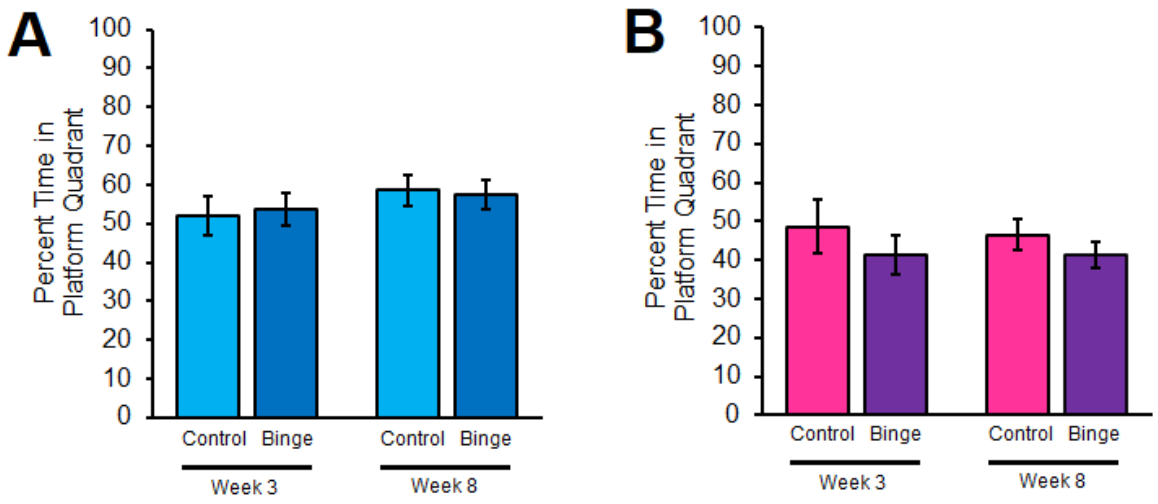

Figure 4.3. There were no effects of binge on percent time in the platform quadrant in either male (A) or female (B) rats during the 60 second probe trial. All rats, regardless of group, spent the most time in the quadrant where the platform was previously located.

Reversal Learning

For latency to platform location in male rats, during reversal learning, there was no significant Trial x Diet x Week interaction [$F(3,144)=.56$, $p=.64$]. There were no significant interactions of Trial x Diet [$F(3,144)=.37$, $p=.77$], Trial x Week [$F(3,144)=.34$, $p=.80$], or Diet x Week [$F(1,144)=.34$, $p=.56$]. There was no significant main effect of either Diet [$F(1,144)=2.19$, $p=.14$], or Dose [$F(3,144)=.04$, $p=.84$]. There was a significant main effect of Trial [$F(3,144)=37.45$, $p<.0001$], with male rats finding the platform more quickly with additional trials. For latency to platform location in female rats, there was no significant Trial x Diet x Week interaction [$F(3,144)=.5$, $p=.68$]. There were no significant interactions of Trial x Diet [$F(3,144)=2.32$, $p=.08$], Trial x Week [$F(3,144)=1.22$, $p=.31$], or Diet x Week [$F(1,144)=3.14$, $p=.08$]. There was no significant main effect of either Diet [$F(1,144)=.16$, $p=.69$], or Dose [$F(3,144)=.01$, $p=.91$]. There was a significant main effect of Trial

[F(3,144)=45.66, p<.0001], with female rats taking longer to find the platform on the first trials than with later trials.

For swim velocity in male rats, there was no significant Trial x Diet x Week interaction [F(3,144)=.59, p=.62]. There were no significant interactions of Trial x Diet [F(3,144)=.62, p=.61], Trial x Week [F(3,144)=.94, p=.43], or Diet x Week [F(1,144)=.06, p=.80]. There was no significant main effect of Dose [F(3,144)=1.76, p=.19]. There was a significant main effect of Trial [F(3,144)=5.15, p=.003] and Diet [F(1,144)=6.1, p=.016], with male rats swimming more quickly on the first trial than the last, and binged males swimming faster than control males. For swim speed in female rats, there was no significant Trial x Diet x Week interaction [F(3,144)=.22, p=.88]. There were no significant interactions of Trial x Diet [F(3,144)=.85, p=.47], Trial x Week [F(3,144)=1.33, p=.27], or Diet x Week [F(1,144)=1.13, p=.29]. There was no significant main effect of either Diet [F(1,144)=.33, p=.57], or Dose [F(3,144)=.01, p=.93]. There was a significant main effect of Trial [F(3,144)=7.96, p=.0001], with female rats swimming more slowly with additional trials.

For distance to the platform in male rats, there was no significant Trial x Diet x Week interaction [F(3,144)=.36, p=.78]. There were no significant interactions of Trial x Diet [F(3,144)=.02, p=.99], Trial x Week [F(3,144)=.57, p=.64], or Diet x Week [F(1,144)=.01, p=.93]. There was no significant main effect of either Diet [F(1,144)=2.53, p=.11], or Dose [F(3,144)=2.11, p=.15]. There was a significant main effect of Trial [F(3,144)=23.63, p<.0001], with male rats reaching the platform more directly with additional trials. For distance traveling in the MWM in female rats, there was no significant Trial x Diet x Week interaction [F(3,144)=.48, p=.70]. There were no significant interactions of Trial x Diet [F(3,144)=2.42, p=.07], Trial x Week [F(3,144)=.29, p=.83], or Diet x Week

[F(1,144)=1.81, p=.18]. There was no significant main effect of either Diet [F(1,144)=.4, p=.53], or Dose [F(3,144)=2.39, p=.12]. There was a significant main effect of Trial [F(3,144)=42.05, p<.0001], with female rats taking longer paths to reach the platform at the start of reversal learning.

For the time spent in the previous platform quadrant in male rats, there was no significant Trial x Diet x Week interaction [F(3,144)=.16, p=.92]. There were no significant interactions of Trial x Diet [F(3,144)=.21, p=.89], Trial x Week [F(3,144)=.37, p=.78], or Diet x Week [F(1,144)=.46, p=.50]. There was no significant main effect of either Diet [F(1,144)=1.04, p=.31], or Dose [F(3,144)=2.9, p=.90]. There was a significant main effect of Trial [F(3,144)=39.67, p<.0001], with male rats spending more time in the previous platform quadrant during the first trial than during later trials. For time spent in the previous platform quadrant in female rats, there was no significant Trial x Diet x Week interaction [F(3,144)=.32, p=.81]. There were no significant interactions of Trial x Diet [F(3,144)=2.62, p=.053], Trial x Week [F(3,144)=.51, p=.68], or Diet x Week [F(1,144)=1.74, p=.19]. There was no significant main effect of either Diet [F(1,144)=.32, p=.57], or Dose [F(3,144)=1.89, p=.17]. There was a significant main effect of Trial [F(3,144)=67.86, p<.0001], with female rats spending less time in the previous platform location with additional trials.

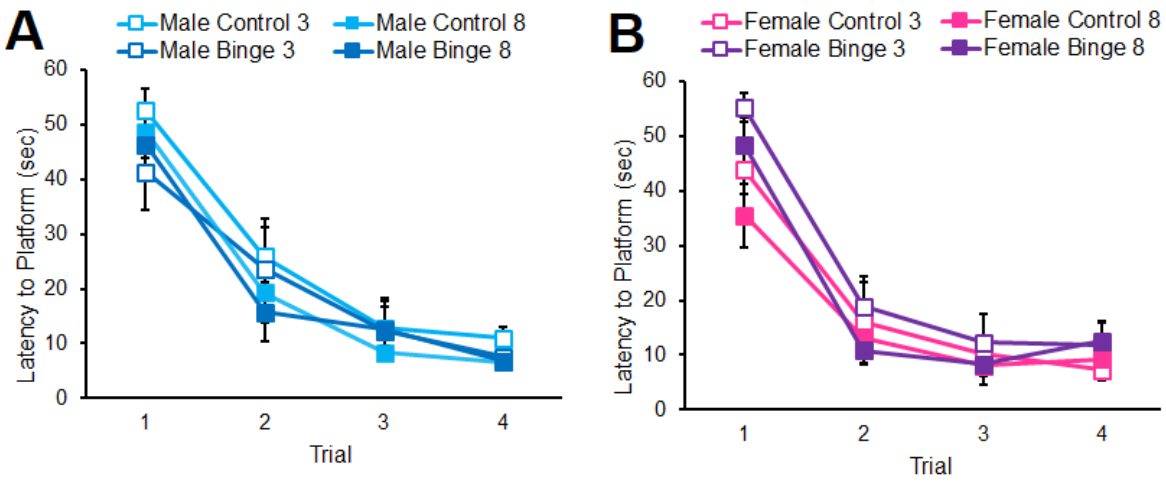

Figure 4.4. Binge ethanol did not have an impact on reversal learning in either male (A) or female (B) rats. All rats were able to successfully learn the new location of the platform during the 4 given trials.

Prefrontal Cortex Cellular Activation (c-Fos)

To determine if cellular activation in the mPFC during MWM reversal (c-Fos) was impacted by binge exposure, a three-way factorial design showed no significant Sex x Diet x Week interaction [F(1,76)=.28, p=.60]. There were also no significant interactions of Sex x Diet [F(1,76)=2.02, p=.16], Sex x Week [F(1,76)=.14, p=.71], or Diet x Week [F(1,76)=3.01, p=.09]. There was no significant main effect of Sex [F(1,76)=1.80, p=.18], however there were significant main effects of Diet [F(1,76)=5.54, p=.02] and Week [F(1,76)=9.05, p=.004]. There was a small effect size of binge in male rats [d=.19] and a large effect size of binge in female rats [d=.86] for cellular activation in the mPFC. These results from the current aim indicate that binge ethanol exposure significantly increased cellular activation in response to behavioral testing, and that 8 weeks of exposure increased cellular activation more than observed after 3 weeks.

To determine whether repeated gavage stress affected cellular activation in cage controls, a one-way ANOVA indicated no significant difference between cage controls and

control groups that received gavage doses in either male [F(2,28)=.92, p=.41] or female rats [F(2,28)=.76, p=.48]. Therefore, analysis of cellular activation during MWM reversal learning in cage controls indicated no significant effect of gavage stress. However, because cage controls also did not undergo behavioral testing, this also indicated that there was no significant increase in the amount of mPFC neural activation required during reversal learning in control rats.

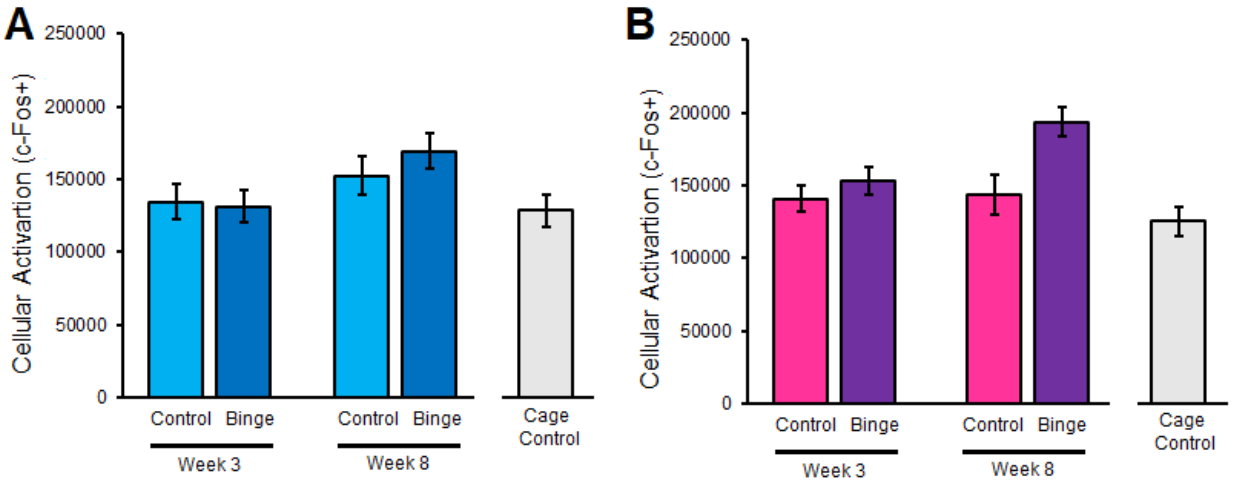

Figure 4.5. Cellular activation during MWM reversal learning indicated that binged male (A) and female (B) rats had significantly more cellular activation than control rats. Animals after 8 weeks of binge or control solution also had increased cellular activation during reversal learning than after 3 weeks. Behavioral testing did not significantly increase cellular activation in the mPFC in control animals.

Ultrasonic Vocalizations

50-kHz Vocalizations

For the number of 50-kHz vocalizations in male rats, there was no significant Day x Diet x Week interaction [F(4, 140)=.21, p=.93], nor any significant interactions of Day x Diet [F(4,140)=.25, p=.91] or Day x Week [F(4,120)=.26, p=.90]. There was a significant Diet x Week interaction [F(1,140)=5.53, p=.02], and a significant main effect of Diet

[F(1,140)=6.26, p=.013]. There were no significant main effects of Day [F(4,140)=.44, p=.78] or Week [F(1,140)=1.45, p=.23]. Follow-up examination of the Diet x Week interaction indicated that after 8 weeks, binged males made significantly more 50-kHz vocalizations than control males [t(18)=16.20, p<.0001]. Pairwise comparisons between control and binged males after 3 weeks [t(18)=.21, p=.65], binged males after 3 and 8 weeks [t(18)=1.13, p=.29] and control males after 3 and 8 weeks [t(18)=6.28, p=.013] were not significant. For the number of 50-kHz vocalizations in female rats, there was no significant Day x Diet x Week interaction [F(4,140)=.4, p=.81], nor any significant interactions of Day x Diet [F(4,140)=.93, p=.44], Day x Week [F(4,120)=1.03, p=.39], or Diet x Week [F(1,140)=1.06, p=.31]. There were no significant main effects of Day [F(4,140)=.79, p=.53], Diet [F(1,140)=1.49, p=.22], or Week [F(1,140)=.72, p=.40].

For the duration of 50-kHz calls in male rats, there was no significant Day x Diet x Week interaction [F(4,119)=.08, p=.99], nor any Day x Diet [F(4,119)=1.41, p=.24], Day x Week [F(4,119)=.31, .87], or Diet x Week [F(1,119)=.62, p=.43] interactions. There were also no main effects of Day [F(4,119)=.56, p=.69], Diet [F(1,119)=.14, p=.71], or Week [F(4,119)=.01, p=.93] on the duration of calls. For the duration of 50-kHz calls in female rats, there was no significant Day x Diet x Week interaction [F(4, 110)=.62, p=.65]. There were also no significant interactions of Day x Diet [F(4, 110)=.27, p=.90], Day x Week [F(4, 110)=.86, p=.49], or Diet x Week [F(1, 110)=3.07, p=.08]. There were no main effects of Day [F(4, 110)=1.39, p=.24], Diet [F(1, 110)=1.53, p=.22] or Week [F(4, 110)=.06, p=.80].

For the average frequency of 50-kHz calls in male rats, there was no significant interaction of Day x Diet x Week [F(4,110)=.37, p=.83], nor any significant Day x Diet [F(4,110)=1.33, p=.26], Day x Week [F(4,110)=.27, p=.90], or Diet x Week [F(1,110)=1.82,

p=.18] interactions. There were no main effects of Day [F(4,110)=.55, p=.70], Diet [F(1,110)=.13, p=.72] or Week [F(1,110)=1.48, p=.23] on the average frequency of 50-kHz vocalizations in male rats. For the average frequency of 50-kHz vocalizations in female rats, there was no significant interaction of Day x Diet x Week [F(4,109)=.14, p=.97]. There were no significant interactions of Day x Diet [F(4,109)=.14, p=.97], Day x Week [F(4,109)=2.33, p=.058], or Diet x Week [F(1,109)=.05, p=.83]. There were no main effects of Day [F(4,109)=.55, p=.70], Diet [F(1,109)=2.1, p=.15] or Week [F(1,109)=.01, p=.92] on the average frequency of 50-kHz calls in female rats.

22-kHz Vocalizations

For the number of 22-kHz vocalizations in male rats, there was no significant Day x Diet x Week interaction [F(4,140)=.21, p=.93]. There were also no significant interactions of Day x Diet [F(4,140)=.88, p=.48], Day x Week [F(4,140)=.97, p=.43], or Day x Week [F(1,140)=2.84, p=.09]. There were no significant main effects of Day [F(4,140)=.34, p=.85] or Diet [F(1,140)=.17, p=.69]. There was a significant effect of Week [F(1,140)=6.34, p=.013], in that male rats that had 8 weeks of control or ethanol gavage made more 22-kHz vocalizations than those receiving 3 dosings. For the number of 22-kHz vocalizations in female rats, there was no significant interaction of Day x Diet x Week [F(4, 139)=.65, p=.63]. There were also no significant interactions of Day x Diet [F(4,139)=1.11, p=.35], Day x Week [F(4,139)=.5, p=.74], or Day x Week [F(1,139)=.01, p=.995]. There were no significant main effects of Day [F(4,139)=.82, p=.50], Diet [F(1,139)=.05, p=.85] or Week [F(1,139)=2.17, p=.14].

For the duration of 22-kHz vocalizations in male rats, there was no significant Day x Diet x Week interaction [F(4,25)=.19, p=.83]. There were also no significant interactions of Day x Diet [F(4,25)=.18, p=.95], Day x Week [F(4,25)=.10, p=.98], or Day x Week [F(1,25)=.18, p=.68]. There were no significant main effects of Day [F(4,25)=.18, p=.95], Diet [F(1,25)=.05, p=.83] or Week [F(1,25)=.29, p=.59]. For the duration of 22-kHz calls in female rats, there was no significant Day x Diet x Week interaction [F(4,20)=.20, p=.84]. There were also no significant interactions of Day x Diet [F(4,20)=.28, p=.89], Day x Week [F(4,20)=.09, p=.96], or Day x Week [F(1,20)=.82, p=.38]. There were no significant main effects of Day [F(4,20)=1.7, p=.19], Diet [F(1,20)=.33, p=.57] or Week [F(1,20)=.08, p=.78].

For the average frequency of 22-kHz calls in male rats, there was no significant Day x Diet x Week interaction [F(4,22)=.29, p=.60]. There were also no significant interactions of Day x Diet [F(4,22)=1.28, p=.31], Day x Week [F(4,22)=.57, p=.69], or Day x Week [F(1,22)=4.04, p=.057]. There were no significant main effects of Day [F(4,22)=1.85, p=.15], Diet [F(1,22)=.69, p=.41] or Week [F(1,22)=2.29, p=.14]. For the average frequency of 22-kHz vocalizations in female rats, there was no significant Day x Diet x Week interaction [F(4,20)=.30, p=.58]. There were also no significant interactions of Day x Diet [F(4,20)=2.12, p=.12], Day x Week [F(4,20)=.93, p=.45], or Day x Week [F(1,20)=.22, p=.65]. There were no significant main effects of Day [F(4,20)=.29, p=.88], Diet [F(1,20)=.01, p=.94] or Week [F(1,20)=1.16, p=.29].

For the percent of 22-kHz vocalizations in male rats, there was no significant Day x Diet x Week interaction [F(4,119)=.42, p=.79]. There were also no significant interactions of Day x Diet [F(4,119)=.3, p=.88], Day x Week [F(4,119)=.69, p=.60], or Day x Week

[F(1,119)=.02, p=.89]. There was no significant main effect of Day [F(4,119)=.58, p=.67], however there were significant effects of both Diet [F(1,119)=5.27, p=.023] or Week [F(1,119)=10.21, p=.002]. Calls indicative of negative affect (22-kHz) accounted for a higher percentage of total vocalizations in binged males compared with control males. Also, male rats that had 8 control or binge doses had a higher percentage of 22-kHz calls than male rats that received 3 weeks of dosing. For the percentage of 22-kHz calls in female rats, there was no significant Day x Diet x Week interaction [F(4,113)=.44, p=.78]. There were also no significant interactions of Day x Diet [F(4,113)=.19, p=.94], Day x Week [F(4,113)=.71, p=.59], or Day x Week [F(1,113)=.64, p=.43]. There were no significant main effects of Day [F(4,113)=.27, p=.90], Diet [F(1,113)=2.97, p=.09] or Week [F(1,113)=1.75, p=.19].

To investigate the effects of estrus on vocalizations in female rats, a one-way ANOVA was used to compare the average number of daily vocalizations of rats that were in estrus, metestrus, diestrus and proestrus. As there were no significant main effects of Day, Diet or Week on USV in female rats, vocalization data was collapsed across the 5 days of testing and the number of vocalizations during each stage of estrus for each rat was compared between stages. Analysis comparing vocalizations during each estrus phase did not indicate any significant differences in the number of total vocalizations [F(3,94)=1.38, p=.23], 50 kHz vocalizations [F(3,94)=1.14, p=.33], or 22 kHz vocalizations [F(3,94)=.81, p=.54]. This indicates that the stage of estrus cycle did not appear to influence the vocalizations in female rats.

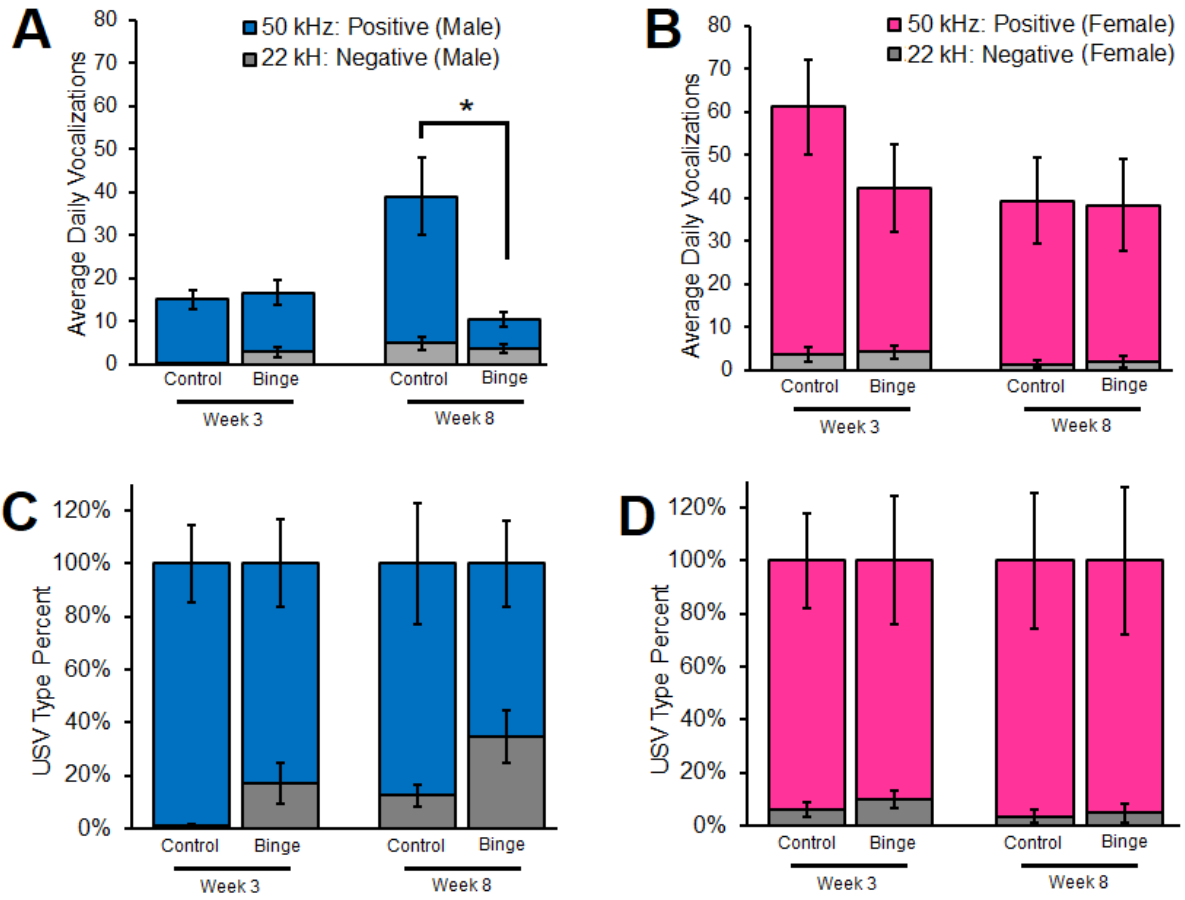

Figure 4.6. In male rats, there was an effect of time on negative affect vocalizations, with 8 weeks of either control or binge dosing resulting in more 22 kHz vocalizations than after 3 weeks. Also, after 8 weeks, binged male rats had less 50 kHz vocalizations than control rats (A). Neither binge exposure nor the number of weeks of dosing affected the number of either 22 kHz or 50 kHz vocalizations in female rats (B). Male rats after 8 weeks had a higher percentage of 22 kHz vocalizations than rats after 3 weeks, and binged rats had a higher percentage of negative affect calls than control rats (C). Female rats, regardless of group, did not significantly differ from one another in the percentage of 22 kHz vocalizations (D). Graphs are displayed as collapsed bars representing the means of 5 days of USV testing. *p<.0125

Discussion

There was no effect of binge alcohol on Morris water maze performance, including acquisition, probe and reversal trials, in either sex. This is consistent with what was found in females after 11 weeks of binge exposure in the first aim (see chapter 2). As the animals in that study were tested repeatedly across the 11 weeks, it is possible that practice effects had

masked any significant cognitive deficits resulting from binge exposure. Therefore, in the current aim, rats had testing only after either 3 or 8 weeks of dosing, rather than from the start of the experiment. Although deficits in MWM acquisition have been previously observed following a single 4-day binge ethanol exposure (Maynard et al., 2018; Obernier, White, et al., 2002; Schulteis et al., 2008), the current model of weekly binge did not result in detectable spatial navigation learning impairments.

All rats did successfully learn the MWM task and perform, and all rats found the platform more quickly over time and swam more directly to it with repeated practice at the task. During probe testing, all rats spent the most time in the platform quadrant, indicating that they knew where the platform location was. Finally, during reversal learning, rats all successfully learned the new location of the platform, and most found the platform within 10 seconds by the final trial. Collectively, these results indicate that rats understood the task and learned how to successfully navigate around the pool using extra-maze cues and remembered each day where the platform was located.

Although we predicted binged rats would manifest cognitive impairments in the MWM, no differences were noted in reversal learning, probe testing or MWM acquisition as a result of binge in either sex. Although MWM was conducted in Aim 1 (chapter 2), with no significant deficits manifested after 11 weeks, the lack of behavioral impairment was thought to be in part a result of practice effects. In the current aim, rats were tested only after 3 or 8 weeks of dosing, and therefore task acquisition could be analyzed without the confound of practice effects. However, in neither aim 1 (chapter 2) or the current aim were any changes in MWM performance uncovered.

A 4-day binge model of ethanol has previously been shown to result in MWM impairments (Maynard et al., 2018; Obernier, White, et al., 2002; Schulteis et al., 2008), indicating that the particular pattern of intoxication may be important. Similar cellular damage was noted with both models, however the accelerated changes that occur in a short span of time during the 4-day binge, may be far more damaging than neurodegeneration that compounded over the course of multiple weeks. Cellular damage in the current weekly binge model case was more gradual, with cellular depletion and neuroimmune activation becoming progressively more severe, which may have allowed for compensatory mechanisms, such as engagement of other brain regions or increased cellular recruitment, to make up for any cognitive deficits. Another explanation for the lack of behavioral deficits observed in the MWM, was simply that the task was not challenging enough to detect damage. Using fewer trials per day, employing a task that was more challenging, or one that got progressively more difficult over time may have been able to detect possible binge-induced deficits that the current MWM paradigm was not.

Although the hypothesis that binge would induce cognitive deficits in the MWM was not supported, this does not necessarily indicate that there are no cognitive changes that occur from binge, and changes on another task may indicate significant cognitive deficits. The results from cellular activation in response to behavioral testing indicated that rats that had binge ethanol exposure had significantly more cellular activation in response to MWM reversal learning. Also, all rats that had 8 weeks of exposure, of either ethanol or control solution, had more cellular activation than rats that only had 3 weeks of exposure. Cellular activation in the mPFC of cage controls, that received no behavioral testing, did not differ from standard control rats that underwent reversal learning. This indicates that in cognitively

intact rats, the task either was not challenging enough to require the recruitment of a larger number of mPFC neurons, or that this task relies primarily on other brain regions in rats.

The mPFC was examined in this aim based on the significant results observed in aim 1 (see chapter 2), however in the current aim, cellular activation was examined during MWM reversal learning, instead of a standard MWM spatial navigation paradigm. Multiple studies in rodents have shown that mPFC damage does not affect reversal learning (Birrell & Brown, 2000; Bissonette et al., 2008; Churchwell, Morris, Heurtelou, & Kesner, 2009; Cordova, Jackson, Langdon, Hewlett, & Corbett, 2014; Floresco, Block, & Tse, 2008), which may explain the lack of difference between cage controls and standard controls. Therefore, in future analyses, regions that have been shown to play a large role in reversal learning based on spatial navigation, such as the orbitofrontal cortex (Ghods-Sharifi, Haluk, & Floresco, 2008; Young & Shapiro, 2009) and hippocampus (Vila-Ballo et al., 2017), may be of interest to quantify.

Despite the lack of activation differences in control rats, rats that had significant DG cell loss (8-week binged rats) had increased cellular activation in the mPFC during reversal learning. Therefore, despite a lack of behavioral deficits observed, binged rats required the use of additional cognitive resourced to perform at control levels. A compensatory recruitment of additional brain regions during cognitive testing has been observed in chronic alcohol users (Parks et al., 2010), as well as in the aging population (Cabeza, Anderson, Locantore, & McIntosh, 2002; Grady, 2000; Hedden & Gabrieli, 2004; Mattay et al., 2002; Reuter-Lorenz, 2002). Therefore, although this task may not ordinarily have required the use of significantly more mPFC neurons, in rats with significant cellular damage, more mPFC neurons were recruited to achieve comparable behavioral performance. The results from c-

Fos analysis in the mPFC, indicated that although there were no deficits in performance during reversal learning in either male or female rats, binge exposure increased the amount of cellular activation required during testing. This supports our hypothesis and indicates that even with a lack of cognitive deficits, the brains of binged rats still had to employ more cognitive resources to perform at control levels.

Testing of ultrasonic vocalizations showed that weekly binge ethanol caused significant changes in affect in male, but not female rats. This result was surprising considering the evidence of female vulnerability to ethanol (Agartz et al., 2003; D. Hommer et al., 2001; D. Hommer et al., 1996; Mann et al., 1992; Maynard & Leasure, 2013) and the increased prevalence of mood disorders in women (Seedat et al., 2009; Seney & Sibille, 2014). Binge ethanol in males was found to increase the percentage of calls associated with negative affect (22 kHz). Male rats that had 8 weeks of either control or binge exposure overall had more 22 kHz vocalizations than those that had only 3 weeks of exposure. Cage control rats were not used for behavioral testing, therefore whether this effect was due to increased age, the stress of gavage, or some other environmental factor is unclear. Although the percent and number of 22 kHz calls was higher in 8-week rats, the number of 50 kHz calls was also significantly higher in control rats after 8 weeks compared with those after 3 weeks. Therefore, it appeared that additional weeks of exposure increased the number of calls associated with both positive and negative affect states in control males, perhaps attributable to additional weeks of handling and familiarity with the experimenter. In binged males however, additional weeks of exposure increased the number of calls associated with negative affect, without increasing the number of positive vocalizations. This indicates that

binge ethanol increases the percentage of vocalizations associated with negative affect, which is particularly apparent after many weeks of repeated exposure.

Although there was a robust effect of binge exposure influencing USVs in male rats, female rats did not show any such changes. Contrary to the hypothesis, binge ethanol did not have any effect on the vocalizations produced by female rats. Female rats overall produced far more vocalizations than males, consistent with existing evidence that ultrasonic vocalizations differ between males and females (Bowers, Perez-Pouchoulen, Edwards, & McCarthy, 2013; Donner & Lowry, 2013; Graham, Yoon, Lee, & Kim, 2009; Kosten, Miserendino, Bombace, Lee, & Kim, 2005; Wright & Brown, 2004). While individual male rats generally were consistent in the number of vocalizations, with rats being easily classified as either high or low vocalizers, female rats showed much more variability by day. However, like in other areas of animal research, studies examining USVs in adult female rats are limited and generally do not report on intra-subject variability. Also, of note, is that in a limited number of females, but not males, some darting and freezing behavior was observed. Darting is commonly perceived as an anxiety-like behavior, and is more common in females (Gruene, Flick, Stefano, Shea, & Shansky, 2015). In these animals, the number of vocalizations during these sessions was very high, although the percentage of calls in the 22 kHz range was not significantly higher than rats that did not display any darting or freezing behaviors. This behavior was not consistent across days and did not appear to be correlated with any specific estrous stage. Although sex hormones have only been found to play a small role in 22 kHz vocalizations (Inagaki & Mori, 2015), since these were intact females, fluctuations in sex hormones cannot be fully discounted as a contributing factor to the general variability observed in female rats.

There were no indications in any of our cellular outcomes that the female hippocampus was less vulnerable to weekly binge (chapter 3), therefore we do not have any explanation based on the cellular data for why only male rats showed changes in ultrasonic vocalizations. Other brain regions outside the hippocampus, such as the amygdala (Choi & Brown, 2003) and periaqueductal gray (Kroes, Burgdorf, Otto, Panksepp, & Moskal, 2007), are also associated with changes in 22 kHz vocalizations. Therefore, it is possible that in male, but not female rats, binged rats may have damage to other brain regions that may have impacted vocalizations. Although both male and female rats had a similar number of total 22 kHz vocalizations, female rats had far more 50 kHz calls, meaning the percentage of negative calls was very low for female rats (~4% of total vocalizations). When analyzing 22 kHz vocalizations, particularly in female rats, there appeared to be a floor effect with most rats not having any vocalizations in the 22 kHz range.

Although no changes in vocalizations were observed in female rats, this does not necessarily indicate that weekly binge ethanol does not impact affect in females. Ultrasonic vocalizations in response to a stressor (i.e. air puff, predator odor, or foot shock) or novel situation, or using other tests of affect (i.e. elevated plus maze, light/dark box, forced swim test, sucrose preference), may be used in the future to test for detectable changes in affect in female rats using this model of binge ethanol.

Although all efforts were made to account for potential confounding variables throughout the experiment, a single experimenter conducted gavaging of rats, blood draws, MWM testing and USV testing. Therefore, it is possible that rats did associate some levels of stress with the experimenter, which may have affected vocalizations. The likelihood of this does seem low however, as for both male and female rats, most vocalizations were in

the 50 kHz range, indicating that the interaction during testing with the experimenter was a generally positive experience for the rats.

Overall, the results from the current aim indicate that weekly binge ethanol negatively influenced affect in male, but not female rats. There were also no cognitive deficits in spatial learning, perseverative behavior or reversal learning observed as a result of binge exposure in either sex.

Chapter 5: Overall Conclusions

Excessive alcohol intake is a contributing factor to at least 30 different diseases, and has systemic effects on the body (Rehm, 2011). Binge ethanol is an extremely common form of alcohol consumption with one-sixth of American adults report binge drinking about four times a month (CDC, 2012b). Binge drinking in both humans and animals has been shown to cause damage to several key areas of the brain as well as inducing cognitive deficits (Loeber et al., 2010; K. Nixon & Crews, 2002; Obernier, Bouldin, et al., 2002; Obernier, White, et al., 2002; Stephens & Duka, 2008; Stephens et al., 2005). Two brain areas particularly susceptible to alcohol's damaging effects are the hippocampus (Agartz et al., 1999; Beresford et al., 2006; Sullivan et al., 1995) and prefrontal cortex (Kubota et al., 2001; Nakamura-Palacios et al., 2014; West et al., 2018). Models of binge have been shown to cause significant hippocampal cell loss (Maynard et al., 2018), increased neuroimmune activation (Barton, Baker, et al., 2017; Barton, Lu, et al., 2017; McClain et al., 2011), cognitive impairments (Maynard et al., 2018; Obernier, White, et al., 2002; Schulteis et al., 2008) and changes in affect (Briones & Woods, 2013; Stevenson et al., 2009).

Nearly 14 million US women binge drink about three times a month (CDC, 2013), and there has been a large increase in recent years in the number of women who are binge drinking (Dwyer-Lindgren et al., 2015). There is emerging evidence in both human and animals studies, that females appear to be uniquely vulnerable to the harmful neurological effects of alcohol in both human and animal studies (Agartz et al., 2003; Alfonso-Loeches et al., 2013; Mann et al., 1992; Maynard et al., 2018; Sharrett-Field et al., 2013; Wilhelm et al., 2016).

Based on the combination of these ideas, we predicted that in a clinically relevant model of weekly binge ethanol exposure, there would be resulting damage to the frontal cortex and hippocampus which would be more severe in the female brain. These experiments collectively examined the effects of weekly binge ethanol and whether female rats would be more susceptible than male rats to cellular damage and behavioral impairments.

Aim 1: Examine the effects of weekly binge ethanol on the female brain. (Chapter 2)

The hypothesis that female rats receiving binge alcohol would show significant brain damage in the hippocampus and frontal cortex when compared with control females was mostly supported. Specifically, it was expected that compared to same-sex controls, female binged rats would have a decrease in remaining granule neurons in the DG of the hippocampus (NeuN), increased neurogenesis in the DG (DCX), reduced neuronal size in the mPFC (NeuN), an increase of activated microglia in the hippocampus and mPFC (Iba1), and increased neuronal activation in response to a spatial navigation task in the in the mPFC and DG (c-Fos). As predicted, there was found to be a decrease in the number of remaining granule neurons, despite an increase in the number of DCX+ cells in the DG. Contrary to our hypothesis, there were no differences in neuronal size of neurons in the mPFC. The results from analysis of microglia in the hippocampus supported our predictions, with weekly binge resulting in an increase in microglial activation. However, in the mPFC, our hypothesis that there would be an increase in microglial activation was not supported, and the increase in total and partially activated microglia fell outside of significance. In partial support of our hypothesis, there was an increase in the number of activated cells in the

mPFC, but not in the DG, during MWM behavioral testing. Overall, these results showed that there were significant cellular changes in female rats resulting from 11 weeks of binge ethanol.

Aim 2: Examine sex differences in vulnerability to the neurotoxic effects of weekly binge ethanol. (Chapter 3)

The hypothesis that female rats would be more susceptible to cellular damage resulting from weekly binge ethanol was not supported. There were significant cellular changes resulting from weekly binge, as expected, however the changes seen generally did not differ between male and female rats. It was hypothesized that when compared to same-sex controls, female rats would show a decrease in remaining dentate gyrus granule neurons (NeuN) increased compensatory neurogenesis (DCX), increased neuroimmune activation in the mPFC and hippocampus (Iba1), and increased cellular activation in the mPFC (c-Fos) during MWM behavioral testing with fewer doses of alcohol than male binged rats. Damage to these vulnerable brain regions with fewer alcohol doses would indicate a sex-dependent telescoping effect and would provide evidence of female vulnerability to binge alcohol neurotoxicity. While the effect was greater in female rats, both binged male and female rats showed a decrease in the number of remaining DG granule neurons, which was not dependent on the number of doses recieved. Contrary to what was observed in aim 1, and thus contrary to our general hypothesis, binged rats of either sex did not differ from control rats in hippocampal neurogenesis. Moreover, both male and female rats had an increase in the number of partially activated microglia in both the mPFC and hippocampus after 8 weeks of binge. An additional tangent hypothesis was also examined to test whether the

stress of oral gavage was great enough to cause brain damage. It was predicted that oral gavage would not result in significant cellular changes, and this hypothesis was supported on all cellular outcomes measures. Overall the results from this aim supported the hypothesis that weekly binge would result in significant cellular changes, although we did not find evidence of selective female vulnerability to weekly binge ethanol.

Aim 3: Examine sex differences in behavioral impairments resulting from weekly binge ethanol. (Chapter 4)

Investigation of sex differences to changes in cognition and affect resulting from weekly ethanol did not show selective female vulnerability to behavioral changes. The hypothesis that binged rats would emit fewer 50-kHz and more 22-kHz ultrasonic vocalizations compared with same-sex controls, thus indicating negative affect, was supported only in male rats. This effect was directly opposite the expectation that female rats would show changes in affect with fewer weeks of binge. During the Morris water maze task, it was hypothesized that binged rats would have longer escape latencies than same-sex controls, and it was anticipated that these changes in affect and cognition would appear more quickly in the female binge group than in the male binge group. This hypothesis was also not supported, and there were found to be no effects of binge exposure on MWM acquisition, probe testing, or reversal learning. Finally, based on the results from Aim 1, it was predicted that weekly binge would increase the amount of cellular recruitment in the mPFC during MWM reversal learning, and that this effect would appear more quickly in binged females. This hypothesis was partly supported, as binged rats did have more cellular activation during reversal learning, however male and female rats did not significantly differ

in the amount of neural activity during MWM testing. Overall these results indicated that female rats were not more susceptible to behavioral impairments caused by weekly binge, however weekly binge resulted in affect changes in binged male rats. Additionally, more cellular activation was required in binged rats in both sexes to achieve control performance during MWM reversal learning, indicating a significant effect of weekly binge.

Conclusions

The significant decrease in the number of remaining mature granule neurons, which was observed in both the first and second aims, is compelling evidence of cellular damage from weekly binge ethanol. However, this decrease was not sex-dependent, and both male and female rats had significantly fewer DGGN after repeated weekly binge exposure. Statistical analysis did not indicate any significant interactive effects, and therefore there was no support of a difference in the magnitude of cellular depletion, aside from differences in effect size. As we did not examine underlying mechanistic changes, it cannot be determined exactly what factors contributed to this effect. However, the cellular damage observed from weekly binge is likely due to a combination of multiple factors, such as reduced trophic support, temporary decrease in neurogenesis, reduced cellular maturation, increased cell death, increased neuroimmune activation, and increased oxidative stress.

Previous analysis of the effects of binge ethanol have shown a suppression in hippocampal neurogenesis immediately following binge exposure (S. A. Morris et al., 2010; K. Nixon & Crews, 2002, 2004), which has been suggested as a potential mechanism of ethanol-induced neurodegeneration. On the other hand, a compensatory increase in cell proliferation has been seen after ethanol abstinence (Galinato et al., 2018; K. Nixon &

Crews, 2004; K. Nixon et al., 2008). Rats in this study were sacrificed 4 days after the last binge ethanol administration, therefore we predicted an increase in the number of DCX+ cells in the DG of binged rats. This hypothesis was supported in female rats after 11 weeks of ethanol (Aim 1: Chapter 2), however we did not observe an increase in DCX+ cells in male and female rats after 3 or 8 weeks of binge (Aim 2: Chapter 3). In both experiments, 4 days after the last ethanol dose, there was no evidence of a decrease in neurogenesis. However, based on the previously mentioned studies examining binge ethanol, it is probable that ethanol suppresses neurogenesis immediately following exposure, but that the number of newly generated cell had restored back to baseline levels after multiple days when the tissue was examined. An acute inhibition of neurogenesis combined with the inability of newly generated cells to mature therefore is a likely cause of the decreased number of mature granule neurons observed.

Unfortunately, when conducting follow-up analysis into neurogenesis following behavioral testing, there was not enough of the antibody to stain both 8-week control and binged rats (discontinued; DCX anti-rabbit polyclonal, 1:100, Santa Cruz Biotechnology). There was a slight, albeit statistically non-significant, increase in rats that underwent 5 days of behavioral testing after 8 weeks of ethanol which gave some limited support to the hypothesis that a cognitive challenge is necessary to spur a compensatory increase in neurogenesis. However, as there was not enough anti-body to stain more than a subset of rats, it cannot be stated that binged rats that received behavioral testing after 8 weeks would have differed from control rats that also underwent behavioral testing after 8 weeks. Tissue from rats that had behavioral testing was also collected 2 days later than tissue from rats that had no behavioral testing. If it is true that there is inhibition in neurogenesis immediately

following weekly dosing, which returns to baseline levels with abstinence, then an additional 2 days of recovery could account for the increase in DCX+ cells observed.

Spatial navigation tasks have been shown to increase neurogenesis in adult male rats, but not in female rats (Chow, Epp, Lieblich, Barha, & Galea, 2013; Yagi, Chow, Lieblich, & Galea, 2016), which is contrary to the hypothesis that bi-weekly MWM testing was partially responsible for increased neurogenesis in female rats. However, it does appear that there is a critical period for the influence of cognitive training on adult neurogenesis in which cognitive testing occurring within 6-10 days of cell birth is ideal for increasing cellular activation and survival (Epp, Haack, & Galea, 2011), which could explain why repeated cognitive testing would result in an increase in neurogenesis, whereas only a single bout of cognitive testing was not sufficient to result in a compensatory neurogenic increase. While the results did not support the hypothesis that a cognitive challenge is necessary to induce an increase in neurogenesis in binged rats, the non-significant increase, particularly in female rats that underwent behavioral testing, could lend some limited support to this idea.

Additionally, there is some disagreement about what precisely is stained by DCX antibodies. Although it has classically been considered to be a marker for neurogenesis, there is some evidence that doublecortin positive cells can be seen outside of neurogenic regions in the brain and can stain other cell types, such as non-neurogenic cells or oligodendrocyte precursor cells (Boulanger & Messier, 2017; Kremer et al., 2013). If it is true that the DCX antibody tagged more than just newly generated neurons, then the observed increase in female binged rats after 11 weeks in aim 1 (chapter 2), may have been reflective of an increase in other non-neurogenic cell types. In addition to other glial types, changes to oligodendrocytes and myelin have been shown to be affected by ethanol

(Lewohl, Wixey, Harper, & Dodd, 2005; Samantaray et al., 2015; Zahr & Pfefferbaum, 2017), therefore perhaps the increase observed in the first aim could have been in part a result a compensatory increase in oligodendrocytes, rather than only an increase in new neurons.

Despite evidence that microglial density has been shown to differ between males and female in multiple brain regions under standard conditions (Lenz, Nugent, Haliyur, & McCarthy, 2013; Mouton et al., 2002; Schwartz et al., 2006), in response to stress (Bollinger et al., 2016), and with other models of binge ethanol (Barton, Baker, et al., 2017), the results from our current experiment did not show any significant sex-differences in baseline microglia or in microglial response to binge. Rather, neuroimmune activation in both the mPFC and hippocampus was noted in both sexes after 8 weeks of binge ethanol, as indicated by an increase in total number and number of partially activated microglia. This is consistent with previous studies indicating an increased neuroimmune response in response to ethanol (F. T. Crews et al., 2015; F. T. Crews & Vetreno, 2014; Qin & Crews, 2012; Vetreno & Crews, 2012; Vetreno et al., 2017), and partial microglial activation in the hippocampus in response to binge (Barton, Baker, et al., 2017; Barton, Lu, et al., 2017; McClain et al., 2011). This partial increase in microglial activation suggests that binge exposure may be priming microglia to respond to subsequent ethanol exposure, which would be consistent with the idea that microglia can be partially activated in response to CNS injury to respond to future neural challenges (Banati et al., 1993; Block & Hong, 2005; C. A. Colton & Gilbert, 1987; Kreutzberg, 1996; Marshall et al., 2016; Norden et al., 2015; Woodroofe et al., 1991).

Analysis of changes in ultrasonic vocalizations resulting from weekly ethanol yielded unexpected results. Male binged rats after 8 weeks had far fewer positive (50 kHz) affect vocalizations, and a higher percentage of negative (22 kHz) vocalizations. In female rats, there were no observed differences in either 50 kHz or 22 kHz vocalizations, contrary to what was hypothesized. This is particularly surprising given the evidence of selective female vulnerability to ethanol (Agartz et al., 2003; D. Hommer et al., 2001; D. Hommer et al., 1996; Mann et al., 1992; Maynard & Leasure, 2013) and the higher rates of mood disorders in women (Seedat et al., 2009; Seney & Sibille, 2014). The cellular outcomes measured did not provide any explanation for this increased vulnerability to binge-induced affect changes in male rats, and significant sex differences in studies examining chronic alcohol typically show the opposite effect. However, whether this disparity is due to differences in the translational nature of rodent versus human brain differences to ethanol or is the result of sex-specific differences in other cellular outcomes that were not measured in this project is unclear.

Prior research has indicated that "stroking-induced" ultrasonic vocalizations were associated with increased hippocampal cell proliferation in a subgroup of rats that were highly responsive to the interaction (Wohr et al., 2009). Brains in aim 2 that were analyzed for neurogenesis (DCX) were not the same animals as underwent behavioral testing, and thus correlations between ultrasonic vocalizations and neurogenesis could not have been analyzed without additional staining. A subset of rats that underwent behavioral testing were stained for DCX to test the follow-up hypothesis of cognitive challenge increasing neurogenesis in binged animals, however the number of brains stained was very low and from only binged groups after 8 weeks. Additionally, while male rats were fairly consistent

with their vocalizations and had higher consistency in the number of vocalizations across days, and thus could theoretically be divided into high and low vocalizers, the number of vocalizations in female rats had much higher variability by day and could not easily be categorized, and therefore analysis examining cellular outcomes correlating to vocalizations was not feasible for this project.

The other behavioral measure examined in this dissertation, did not indicate any deficits in spatial working memory. There were no differences in MWM acquisition performance in either of the paradigms tested (aim 1: every other week, 2 trials x 3 days; aim 2: 4 trials x 4 days).There also were no observed differences in the amount of time spent in the platform quadrant between control and binged animals, nor any deficits found during MWM reversal learning. Previous studies using a 4-day binge model of ethanol, have shown to result in changes in MWM performance (Maynard et al., 2018; Obernier, White, et al., 2002; Schulteis et al., 2008), however differences using this weekly model of binge did not result in these same impairments. This further supports the idea that this model of weekly binge fundamentally differs in the way it impacts the brain and resulting behavior.

Cellular activation in the mPFC of control rats during reversal learning in the MWM did not significantly differ from cage control rats that had no behavioral testing, however binged rats had significantly more neural activation during testing than control groups. The mPFC was examined after reversal learning because of the significant results from aim 1 (chapter 2), that showed an increase in the number of c-Fos+ cells in the mPFC, but not the DG, after MWM. However, in rodents, mPFC damage does not always affect performance on reversal learning tasks (Birrell & Brown, 2000; Bissonette et al., 2008; Churchwell et al., 2009; Cordova et al., 2014; Floresco et al., 2008). This suggests that in rats without cellular

damage, this task was not challenging enough to require significantly more cellular activation in the mPFC, however binged rats had to employ a brain region not commonly used for this task to achieve the name performance as controls. Recruitment of additional brain regions during cognitive testing has also been observed in chronic alcohol users (Parks et al., 2010), as well as in the aging population (Cabeza et al., 2002; Grady, 2000; Hedden & Gabrieli, 2004; Mattay et al., 2002; Reuter-Lorenz, 2002). Additionally, EEG evaluation of the brains of human binge drinkers showed similar results, with binge drinkers showing increased brain activity despite similar working memory task performance (Campanella et al., 2013). Our findings support this compensatory hypothesis and indicate that even with a lack of cognitive deficits, the brains of binged rats still had to employ more cognitive resources to perform at control levels.

This project chose to focus on the hippocampus for measuring both cellular outcomes as well as behavioral changes, primarily due to the vast prior evidence indicating hippocampal vulnerability to ethanol damage. Stereological quantification of cellular outcomes analyzed the hippocampus as one structure, however the dorsal and ventral hippocampus functionally differ (Fanselow & Dong, 2010; Kheirbek & Hen, 2011; Lee, Kim, Cho, Kim, & Park, 2017). Although in this project the dorsal and ventral hippocampus were not separated for analysis, differences in location of hippocampal cell damage could explain the behavioral results. Spatial navigation and learning in the MWM is reliant primarily upon the function of the dorsal hippocampus, whereas affect changes measured using USVs are more indicative of the functioning of the ventral hippocampus (Fanselow & Dong, 2010). Both male and female rats overall had fewer remaining granule neurons in the DG and increased neuroimmune activation in the hippocampus, however it was not

evaluated whether the ventral and dorsal hippocampus were equally susceptible to these changes. Repeated binge ethanol had no effect on performance in any of the MWM paradigms evaluated in this study, which indicates that the cellular changes observed may have been driven by changes in the ventral, rather than the dorsal hippocampus. Given the results from USV testing, it could be hypothesized the ventral hippocampus is more susceptible to binge-induced damage in male rats, or that compensatory mechanisms in the ventral hippocampus were more effective in female rats.

Considering that other patterns of alcohol consumption have indicated that the female brain is particularly susceptible to both cellular damage and behavioral impairments, the general lack of sex-differences found in this project are of great interest. These differing results indicate that binge ethanol exposure only once a week, differs mechanistically from other patterns of intake that induce dependency, particularly in the male brain. Previous work showed that there was decreased trophic support in female rats (Maynard et al., 2018), which may explain why the brains of male rats did not show the same resulting damage seen in female rats from 4-days of binge . In the case of weekly binge, it is likely that the loss of trophic support would be observed in both male and female rats, which could be contributing the cellular changes observed in this model.

When comparing weekly binge ethanol to the previously used 4-day model of binge, there are differences in findings between the two models, primarily regarding sex differences. The 4-day binge in adult rats has been shown to result in a decrease in the number of granule neurons (Maynard et al., 2018), neuroimmune activation (Barton, Baker, et al., 2017), and delayed MWM acquisition (Maynard et al., 2018) only in female rats. The current experiments, however, did not replicate these findings, indicating a fundamental

difference in the way that the brain responds to different models of binge exposure. After 8 weeks of weekly binge, there was no evidence of selective female vulnerability, as there was after the 4-day binge model. A major difference between the two models which likely is responsible for these discrepant results is the ethanol dependence observed in rats who undergo the 4-day binge, as indicated by the withdrawal period that is observed during ethanol abstinence. Studies examining "telescoping" effects and which show selective female vulnerability to alcohol damage, are primarily comparing these results in alcoholics or chronic heavy alcohol users where neural adaptation and metabolic tolerance is likely to have developed (Agartz et al., 2003; Devaud, Smith, Grayson, & Morrow, 1995; Grobin, Matthews, Devaud, & Morrow, 1998; D. Hommer et al., 2001; D. Hommer et al., 1996; Mann et al., 1992). Therefore, it appears that the male brain is better protected only in cases where physiological tolerance develops. In the case of weekly binge, no metabolic tolerance or dependence was observed, which differs from the models used in other studies.

Based on the results from observable intoxication, dependency, cellular and behavioral outcomes, it seems that these models may be best modeling different patterns of human binge alcohol drinking. The weekly binge model likely better represents occasional binge drinkers, whereas the 4-day binge model is more valid for analysis of the brain changes occurring in habitual binge drinkers. In order to advance our understanding of how binge ethanol affects the brain in both males and females, it is important to have a variety of different animal models that can mimic the patterns and cellular damage observed in different populations of human drinkers.

Weekly binge administration in a rat model produced BEC similar to what could be expected in human binge drinkers and closely mimics the 4 episodes of binge per month

pattern of the typical binge drinker (CDC, 2012b). Therefore, the lack of sex differences found in this project likely has direct translational importance to human binge drinkers. This rodent model indicated that cellular damage from binge ethanol occurs gradually, and that changes in behavior were not observed until many weeks of repeated exposure, if at all. Due to the gradual compounding nature of the damage, binge drinkers likely would not notice any abrupt decreases in cognitive functioning which would alert them to be concerned about their drinking habits. However, the results from this project indicate that brain damage can still occur even without noted changes in behavioral task performance. Given the prevalence of binge drinking (CDC, 2012b; SAMHSA, 2016), it important that both men and women are aware of how binge drinking can affect the health of their brain, and this project proposes an additional rodent model to test the effects of binge in both sexes.

Limitations

Although all attempts were made to account for all extraneous variables, these experiments did have some limitations. Regarding face validity, while this rodent model closely mimicked the frequency, route of administration, and blood ethanol concentrations observed in human binge drinkers, this model did not imitate the age of drinking onset seen in the typical human binge drinker. In this experiment rats were first exposured to binge ethanol in adulthood; however, many humans start drinking in adolescence (Hingson, 2009; Hingson & Zha, 2009). Therefore, it may have been more representative of human binge drinking to first begin exposure during the rats' adolescence.

Some unavoidable technical and practical problems did arise throughout the course of this experiment. Due to a hurricane, half of the rats in aim 1 began receiving weekly

doses at 12 weeks of age, instead beginning doses at 9 weeks of age. Due to the threat of severe flooding, binge dosing of 24 rats began 2 hours early for the 6th dosing in binged and control animals (aim 2). Finally, for rats in aim 3 (chapter 4), an active fire alarm prevented data collection of USVs for the second day of testing for 15 8-week rats, and an overnight power-outage disrupted camera setting during the third day of MWM testing for 3-week rats resulting in unreliable measures of swim speed. Data for these animals on these behavioral outcome measures therefore had to be excluded from analysis.

Analysis of partially activated microglia was based on the judgement of the experimenter and was based on only visual characteristics of stained cells (larger and darker cell body, bushier processes). Classification of microglia based on more quantitative determination may have been more appropriate, such as setting standardized thresholds to categorize partially activated microglia based on cell body volume, diameter of processes, or measure of density. Additionally, other methods to better determine the characteristics and functioning of these microglia, such as the use of ELISA to investigate proinflammatory cytokines and growth factors to investigate microglial activation, could be used in the future.

Future Directions

Due to the time-consuming nature of stereological quantification, only a few cellular outcomes were measured in this dissertation. The choice to focus on these outcomes of interest in the hippocampus and prefrontal cortex was based on previous work done in our lab, as well as the body of evidence investigating the neurological impacts of ethanol damage. Although this project has advanced understanding of the effects of weekly binge ethanol, there are a multitude of other cellular outcomes and regions that could be explored.

Ethanol has also been shown to result in cellular death (Balaszczuk, Bender, Pereno, & Beltramino, 2011; Luo, West, Cook, & Pantazis, 1999; Obernier, Bouldin, et al., 2002; von Haefen, Sifringer, Menk, & Spies, 2011), dendritic changes (Yanni & Lindsley, 2000; Zhou et al., 2007), and loss of myelination (de la Monte, 1988; Pfefferbaum et al., 2002; Vargas, Bengston, Gilpin, Whitcomb, & Richardson, 2014; Zahr & Pfefferbaum, 2017), therefore investigation into these cellular changes may be of interest in response to this model of binge.

In lieu of investigation of additional cell types or brain regions, there are additional measures that could be explored using the measures that were already explored during this project. For instance, as mentioned previously, cellular analysis in the hippocampus did not differentiate between dorsal and ventral hippocampus. There is evidence that there are cellular and functional differences between the dorsal and ventral hippocampus (Fanselow & Dong, 2010; Lee et al., 2017), and therefore differentiating between these two halves of the structure may give more insight into precisely which regions have increased vulnerability to binge. Although it was not quantitatively analyzed, anecdotally, differences in homogeneity in microglia in the dorsal versus ventral hippocampus were striking, with visibly more total and partially activated microglia in the ventral hippocampus than were present in dorsal sections.

In female rats, there were not found to be any effects of 11 weeks of binge ethanol on a wide range of behavioral tests including, general locomotion indicated by running distance and speed, distance traveled in an open field apparatus, anxiety-like behavior indicated by time spent in the center of the open field, motor coordination indicated by rotarod performance, or spatial navigation or memory as indicated by performance during

MWM maze testing (West et al., 2019). There was also not found to be an effect of weekly binge ethanol on affect, as shown by the results of "stroking-induced" ultrasonic vocalizations in female rats; nor any effect in male or female rats in inhibitory control, as indicated by reversal learning. Although there were significant cellular changes resulting from weekly binge, these manifest into behavioral changes only in males after 8 weeks during USV testing. Despite this, there are additional behavioral tasks that may be of interest based on regions of ethanol vulnerability (frontal cortex, hippocampus, cerebellum) to measure, such as measurement of additional changes in affect (light/dark box, elevated plus maze, forced swim), more robust measures of frontal cortex function (radial arm maze, delayed discounting, 5-choice serial reaction task), or behavioral conditioning (operant chamber).

Neural activation the mPFC was examined in aim 3 (chapter 4) based on the significant results observed in aim 1 (chapter 2), however cellular activation was examined during MWM reversal learning instead of a standard MWM spatial navigation paradigm. The traditional MWM paradigm is a spatial navigation and memory task that relies primarily on hippocampal functioning (Bromley-Brits, Deng, & Song, 2011; D'Hooge & De Deyn, 2001), whereas reversal learning in the MWM still employs hippocampal function for the aspects of spatial navigation and memory, reversal learning primarily targets to functioning of the orbitofrontal cortex (Izquierdo, Brigman, Radke, Rudebeck, & Holmes, 2017; Xue et al., 2013). Therefore, in neither of these tasks is the prefrontal cortex the primary brain region activated. This is supported by the results that showed no significant difference in mPFC cellular activation (c-Fos) between controls during MWM reversal learning and cage controls that did not undergo any behavioral testing. Cellular activation in the hippocampus

in response to standard MWM testing was analyzed in aim 1 (chapter 2), and was shown not to result in significant changes, therefore analysis was not done in the larger group of rats in aim 3 (chapter 4). However, as the MWM paradigm did differ between these experiments (standard MWM acquisition versus reversal learning), changes in hippocampal activation may still have occurred. Studies in rodents have shown that mPFC lesions do not significantly affect reversal learning (Birrell & Brown, 2000; Bissonette et al., 2008; Churchwell et al., 2009; Cordova et al., 2014; Floresco et al., 2008). Therefore, in future analyses, regions that have been shown to play a large role in reversal learning based on spatial navigation, such as the orbitofrontal cortex (Ghods-Sharifi et al., 2008; Young & Shapiro, 2009) and hippocampus (Vila-Ballo et al., 2017) could be quantified.

As this was a new model of binge ethanol, the priority of this project was to examine cellular outcomes that were expected to be significant and to then investigate sex-differences in these outcomes. We did this through stereological analysis of cells stained using immunohistochemistry. While ideal for quantification and unbiased analysis of cellular measures, other less time-consuming methods of analysis, such as protein analysis of various other cellular markers using western blots or ELISA, could be very useful for enriching our understanding of both the cellular impacts of weekly binge. These additional analyses could also aim at investigating the potential underlying mechanisms of binge damage, particularly in comparison to other models of ethanol which induce dependency.

In aim 1, fecal corticosterone was analyzed to assess any persistent effects of binge ethanol over time and to investigate difference in stress levels between binged and control rats. However, there were no significant difference noted in samples that were collected 3 days following dosing. Ethanol is well-documented to cause increases in stress response and

circulating stress hormones (Blaine, Milivojevic, Fox, & Sinha, 2016; Patterson-Buckendahl, Kubovcakova, Krizanova, Pohorecky, & Kvetnansky, 2005; Rachdaoui & Sarkar, 2013), therefore these results were surprising. However, as these samples were collected multiple days after binge administration, any acute or short-term increases in corticosterone was unlikely to be detectable. In the future, since fecal corticosterone measures corticosterone levels in the last 6-12 hours (Harper & Austad, 2000), examination of any transient effects in corticosterone levels could be investigated by collecting fecal samples the day following dosing.

Overall Conclusion

In this dissertation, I aimed to use a novel model of rodent binge ethanol to investigate the effects of weekly binge ethanol and determine whether the female brain would be more vulnerable to these effects. Although there was no evidence of selective female vulnerability to weekly binge, there were significant cellular changes resulting from repeated weeks of exposure in both sexes. Specifically, there were fewer remaining DG granule neurons, increased neuroimmune activation in the mPFC and hippocampus, and increased compensatory brain activation in the mPFC during behavioral testing in binged rats. Behavioral testing indicated that in male rats, there was a decrease in the number of positive affect vocalizations in response to stoking-induced ultrasonic vocalizations. The lack of sex-difference in response to weekly binge ethanol, despite previous evidence of selective female vulnerability using other models of ethanol exposure, indicates that damage appears dependent on the specific pattern of ethanol intake. While not directly explored, the results of this dissertation allude to the importance of ethanol dependency when considering

the effects of ethanol on the brain and show that both the male and female brain are vulnerable to a pattern of ethanol consumption very commonly seen in human binge drinkers. This project further contributes to our understanding of the brain effects of binge ethanol and proposes an additional rodent model of social binge drinking.

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