# SEX DIFFERENCES IN BINGE ALCOHOL-INDUCED BRAIN DAMAGE AND RECOVERY OF FUNCTION

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A Dissertation

Presented to

The Faculty of the Department

Of Psychology

University of Houston

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In Partial Fulfillment

Of the Requirements for the Degree of

Doctor of Philosophy

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By

Mark E. Maynard

May, 2016

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## ABSTRACT

Evidence suggests that women are more sensitive to the neurotoxic effects of alcohol and more vulnerable to the adverse medical consequences of heavy alcohol consumption than men. Despite this few studies have directly compared the consequences of binge alcohol between the male and female brain, and the mechanisms that underlie increased female vulnerability remain poorly understood. The present studies investigated sex differences in alcohol-induced neurodegeneration, and associated cognitive deficits and disruption of trophic support, using a rodent model of an alcohol use disorder (AUD). Binge exposure resulted in a significant loss of granule neurons and significantly more degenerating and dead cells in the hippocampal dentate gyrus of females compared to males. This was associated with a binge-induced spatial reference memory deficits in the Morris water maze for females but not males. Binge-induced neurodegeneration in the female hippocampus was associated with a decrease of BDNF, TrkB, CREB, and pCREB protein expression; however only BDNF was disrupted in the hippocampus of males. Further, we investigated if exercise-driven recovery from binge-induced neurodegeneration was associated with increased trophic support. One to two weeks of voluntary exercise reversed the binge-induced reduction of dentate gyrus granule neurons in females, likely via an increase in BDNF, pCREB, and Iba1 (microglia). We conclude that the female hippocampus is more sensitive to binge-induced neurodegeneration and associated cognitive consequences than males, like due to the disruption of protective tropic support. Voluntary exercise however, can enhance endogenous recovery processes by increasing trophic support that aids in recovery.

*Keywords*: alcohol, binge, sex differences, neurodegeneration, cognitive deficits, trophic support, exercise, neurorestoration

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# DEDICATION

This dissertation is dedicated to my parents, John and Tonie Maynard. Thank you both for all your love and support throughout this entire journey. Without you I would not be where I am or who I am today. To my mom especially, thank you for keeping me sane and always being there as the voice of comfort and reason.

# Sex Differences in Binge Alcohol-Induced Brain Damage and Recovery of Function

## Alcohol Use Disorders

Alcohol has a long recorded history of use and abuse. There is no consensus on when humans first began producing and consuming alcohol, however, the discovery of beer containers from the Stone Age suggests that humans have been making alcoholic beverages for at least 10,000 years (Patrick, 1952). It didn't take long for alcohol use and production to spread across the globe. Consuming alcohol was commonplace among many ancient civilizations, including the Chinese, Egyptian, Babylonian, Roman, and Mayan (Fei-Peng, 1982; Gately, 2008; Hyams, 1965; Lucia, 1963). Some cultures celebrated with alcohol, or used it ceremonially, socially, and even medicinally. Others, however, promoted moderation and condemned its abuse. Over the next several centuries, methods of alcohol production, such as brewing, winemaking, and distillation, improved and led to an increase in alcoholic beverage variety and availability. However, with increased access to alcohol came an increase in abuse and habitual drunkenness.

In the 18$^{th}$ and 19$^{th}$ centuries, doctors in hospitals became increasingly aware of the effects of heavy alcohol consumption on the human body and considered heavy drinking to be a disease. Doctors working in asylums saw an increased number of patients that suffered from mental illnesses as a result of their heavy drinking. The term "dipsomania" was coined in 1819 by German physician C. W. Hufeland to describe the medical condition characterized by habitual drunkenness and an uncontrollable craving for alcohol (Peters, 2007). Hufeland viewed the destruction of alcoholics' moral judgement as a consequence, not the cause, of the sickness. The concept of dipsomania was used as part of an effort by medical reformers to change attitudes about alcohol abuse from being criminally punishable

to being a medically treatable disease (Johnstone, 1996). However, views on alcoholism as an illness were generally restricted to the medical community and were divergent from the ideology of the growing temperance movement, where alcohol abuse was viewed as a moral failing. Instead, the increasingly pejorative views on alcohol of the time resulted in moral and social condemnation of alcoholics and helped fuel the temperance movement and prohibition of alcohol in the early 20th century.

After the failed prohibition of alcohol, alcoholism began to garner substantial public attention as a significant problem that needed to be addressed medically. However, alcoholism was categorized as a personality disorder in the United States until 1980, when it was officially defined as a diagnosable medical condition or disease in the third edition of the Diagnostics and Statistics Manual of Mental Disorders (DSM; Hasin et al., 1996). This distinction of alcoholism as a medical disease from a personality disorder was important because it implied that alcoholism was, and is, more than just a behavioral problem, and can be treated with appropriate interventions (White et al., 2002). Today, the way alcoholism is studied and treated is shaped by the understanding that it is a diagnosable medical disorder.

### *Understanding the Problem*

It wasn't until around 1850 that the most common term we use for alcohol abuse, "alcoholism", was devised by Magnus Huss, a Swedish professor of medicine (Marcet, 1862). Although alcoholism is now universally used as a broad term associated with problems resulting from drinking alcohol, it was originally used to describe alcohol poisoning (Lesch et al., 1990; Marcet, 1862). In the medical and scientific community the term alcoholism is viewed as poorly defined and outdated, therefore terminology and diagnostic criteria for alcoholism are more clearly defined in their diagnostic manuals.

In the DSM-V, the American Psychiatric Association (APA) categorizes problems associated with alcoholism under the umbrella term alcohol use disorder (AUD), with mild, moderate, and severe sub-classifications (APA, 2013). According to the DSM-V, anyone meeting any of two of the 11 criteria during the same 12-month period would receive a diagnosis of AUD (Table 1.1), the severity of which is based on the number of criteria met.

**Table 1.1 Criteria for Alcohol Use Disorder**

| Criteria for AUD | Severity |
|---|---|
| ▪ Had times when you ended up drinking more, or longer than you intended?<br>▪ More than once wanted/tried to cut down or stop drinking but could not?<br>▪ Spent a lot of time drinking? Or being sick or getting over aftereffects?<br>▪ Experienced craving – a strong need, or urge, to drink?<br>▪ Found that drinking, or being sick from drinking, interfered with taking care of your home or family? Or caused job troubles? Or school problems?<br>▪ Continued to drink despite causing trouble with your family or friends?<br>▪ Given up or cut back on activities that were important, interesting, or gave you pleasure, to drink?<br>▪ More than once gotten into situations while or after drinking that increased your chances of getting hurt (such as driving, swimming, using machinery, walking in a dangerous area, or having unsafe sex)?<br>▪ Continued to drink even though it made you feel depressed, anxious or added to another health problem? After having had a memory blackout?<br>▪ Had to drink much more than you once did to get the effect you want? Or found that your usual number of drinks had much less effect than before?<br>▪ Found that when the effects of alcohol were wearing off, you had withdrawal symptoms, such as trouble sleeping, shakiness, restlessness, nausea, sweating, a racing heart, seizure? Or sensed things were not there? | The presence of at least 2 of these symptoms indicates an **Alcohol Use Disorder (AUD)**<br><br>**Mild**: The presence of 2 to 3 symptoms<br><br>**Moderate**: The presence of 4 to 5 symptoms<br><br>**Severe**: The presence of 6 or more symptoms |

Table 1.1 Criteria for an AUD as defined by the DSM-V (APA 2013)

The criteria for an AUD are similar to the diagnostic criteria and definitions of addiction with an emphasis on the development of tolerance, showing signs of withdrawal, impaired control and impulsivity, preoccupation with the drug of choice, and continued use despite problems (APA, 2013). The International Classification of Diseases (ICD), a medical classification list by the World Health Organization (WHO), has an analogous definition for AUDs. The ICD differs from the severity spectrum of the DSM-V in that it takes a categorical approach, i.e. “alcohol harmful use” and the more severe “alcohol dependence syndrome”, that is similar to the “abuse” and “dependence” designations of the now replaced

DSM-IV (APA, 2013; WHO, 1992). Despite this difference, the criteria from both manuals used to define AUDs has been validated in studies predicting and identifying alcohol related problems within the general population (Grant et al., 2007; Hoffmann & Kopak, 2015). These guidelines for AUDs allow for diagnosis by clinicians, provide clear definitions for academic research, and offer the general population a way of understanding the differences between social and problematic drinking.

Using the DSM criteria to diagnose AUDs, studies have shown that alcohol abuse is a common problem in the United States. In 2013, approximately 17 million Americans (7.0%) of legal drinking age had an alcohol use disorder, including nearly 11 million men (9.4%) and 6 million women (4.7%; SAMHSA, 2013). When considering lifetime prevalence rates of AUDs, almost 50% of men and 25% of women could be diagnosed with an AUD at some point in their lives (Goldstein et al., 2012). Alcohol abuse is not unique to the United States either, as many other nations face similar prevalence rates (Bloomfield et al., 2003; Grittner et al., 2013). The WHO estimates that as of 2010 there were 208 million people worldwide who met criteria for an AUD (WHO, 2014). More importantly, nearly 88,000 people (approximately 62,000 men and 26,000 women) die from alcohol related causes annually, making it the third leading preventable cause of death in the United States (CDC, 2015).

### *Pattern of Intake: Binge Consumption*

When talking about alcohol abuse and AUDs, it is important to consider that there are several patterns of intake. The majority of problems from alcohol abuse stem from binge pattern drinking (Bouchery et al., 2011). Binge pattern drinking is defined by the National Institute of Alcohol Abuse and Alcoholism (NIAAA) as five or more drinks for a male, or four or more drinks for a female in a two hour period that results in a blood alcohol

concentration (BAC) of at least 0.08% (NIAAA, 2004). This pattern of intake is the most common amongst those with an AUD; 40-60% of alcohol abusers drink in this manner (Robin et al., 1998). Some reports even indicate that 92% of U.S. adults who drink excessively report binge drinking in the past 30 days (Town et al., 2006). Although college students commonly binge drink, 70% of binge drinking episodes involve adults age 26 years and older (Naimi et al., 2003). Additionally, while binge drinking is more common among young adults aged 18-34, binge drinkers aged 65 years and older report binge drinking more often – an average of five to six times a month (CDC, 2012) Nearly half of the 88,000 people that die from alcohol related causes and three-quarters of the $223.5 billion total cost of alcohol abuse are related to binge drinking (Bouchery et al., 2011).

The pattern of intake is crucial when considering consequences of alcohol abuse. In fact, binge pattern drinking is more predictive of neurodegeneration and other associated problematic outcomes than total lifetime alcohol intake (Bobak et al., 2004; Hunt, 1993). For example, an individual who abstains during the week and then chooses to have seven alcoholic drinks on a weekend night will consume the same lifetime quantity as an individual who drinks a single nightly drink. The individual that drinks in a binge pattern is much more likely to suffer the consequences from the acute effects of intoxication due to higher BACs. The consequences of high BACs can lead to dangerous behaviors, including poor-decision making (George et al., 2005), increased vehicular accidents (Villaveces et al., 2003), risky sexual behavior (Stappenbeck et al., 2013), and an increased tendency to be involved in violent crimes (Boles & Miotto, 2003). Many factors can influence BEC, such as sex, genetic differences in alcohol metabolism, and body mass index (Koob & Le Moal, 2005), however the number of drinks is the most common predictor of intoxication and has been shown to be

associated with greater risks and alcohol-related problems (Fillmore & Jude, 2011; Koob & Le Moal, 2005).

The key feature of binge pattern drinking is that the sustained period of elevated intoxication happens multiple times; one in six U.S. adults who binge drink report doing so at least four times a month, consuming about eight drinks per binge (CDC, 2012). It is thought that the repeated episodes of excessive drinking and high BACs, followed by periods of detoxification, are responsible for the greater degree of cognitive and structural deficits associated with binge pattern drinking, including permanent changes to organ systems. The biological effects caused by alcohol abuse are considered accountable for at least 30 different diseases and may leave many individuals more susceptible to countless others (Rehm, 2011). Alcohol and binge pattern consumption affect the central nervous system (CNS) in particular, causing significant impairments that may promote alcohol dependence (Gilpin & Koob, 2008).

### *Neurodegeneration & Cognitive Deficits*

The consumption of dangerous amounts of alcohol, the characteristic feature of an AUD, is associated with changes in physiology in addition to structural and functional abnormalities in the brain and other organs (Courville, 1955; Victor et al., 1959). The more global structural changes in the brain happen via cellular damage and altered neurotransmission due to excessive alcohol consumption (Crews & Nixon, 2009; Harper, 2009). The alcoholic brain damage discussed herein will refer only to damage that occurs from AUDs as opposed to neuronal loss that may be due to nutritional deficiency such as in Wernicke-Korsakoff's syndrome or enduring neuronal loss from prenatal exposure as seen in fetal alcohol spectrum disorders (Lewis et al., 2012; Thomson et al., 2012).

Although medical problems associated with alcoholism were understood in the 18th century, abnormalities in the CNS due to alcohol abuse weren't quantified until the late 20th century. Early research on alcohol-induced brain damage was based on deficits in cognition rather than quantitative measures of brain changes. The development of new sophisticated technologies such as magnetic resonance imaging (MRI) and diffusion tensor imaging (DTI) allowed for the detection of brain volume reductions in more specific brain regions within alcoholics compared to moderate and social drinkers (Harper & Kril, 1989; Pfefferbaum et al., 1992; Pfefferbaum et al., 1995; Sullivan et al., 1995; Zahr et al., 2011). This damage includes significant volume loss in cortical and subcortical brain structures due to shrinkage of both gray and white matter and enlargement of the lateral ventricles (Kubota et al., 2001; Mann et al., 2001; Pfefferbaum et al., 1992; Sullivan & Pfefferbaum, 2005).

Chronic alcohol-induced loss of brain mass is likely due to loss of neurons, glia, and their white matter fiber tracts, but may also include a reduction in the brain parenchyma, that is, the actual volume of surviving cells and their processes (Crews, 1999). Post-mortem examination of the brains of alcoholics show volume reductions and neuronal cell loss in a number of important regions, a finding that supports *in vivo* imaging of alcoholic brain morphology (Agartz et al., 1999; Harper & Kril, 1985; Phillips et al., 1987). Brain regions particularly affected are the hippocampus (Agartz et al., 1999; Beresford et al., 2006; Sullivan et al., 1995), cerebellum (Baker et al., 1999; Phillips et al., 1987; Sullivan et al., 2010), corpus callosum (Pfefferbaum et al., 1996; Pfefferbaum & Sullivan, 2002), and various cortical regions (Pfefferbaum et al., 1992; Pfefferbaum et al., 1997). White matter is another major target of alcohol toxicity in the CNS documented by MRI and postmortem studies (Buhler & Mann, 2011; Charness, 1993; de la Monte, 1988; Konrad et al., 2012). The

frontal lobes are especially vulnerable to excessive alcohol intake, resulting in deficits that may promote a cycle of further alcohol abuse (Jernigan et al., 1991; Kubota et al., 2001; Sullivan & Pfefferbaum, 2005). Neurodegeneration does not necessarily occur in all individuals that drink, pattern of intake is key because alcohol-induced brain damage correlates with chronic excessive alcohol consumption, like that seen in binge pattern drinking (Hunt, 1993; Lisdahl et al., 2013).

Behavioral changes associated with chronic excessive alcohol use are well documented but recent studies have begun to associate these cognitive deficits with neurodegeneration within specific brain regions and the integrity of their circuits (Alfonso-Loeches & Guerri, 2011; Zahr et al., 2011). For example, the frontal lobes, which regulate executive functioning and complex cognitive skills that underlie judgement, attention, risk taking, motivation, and mood, are the most affected region in the alcoholic brain (Kubota et al., 2001; Sullivan & Pfefferbaum, 2005). Alcohol-induced damage to this region is thought to be the cause of impaired judgements, blunted affect, poor insight, social withdrawal, reduced motivation, distractibility, and the attention and impulse-control deficits often seen in alcoholics (Crews & Boettiger, 2009; Oscar-Berman & Hutner, 1993; Parsons, 1993; Sullivan & Pfefferbaum, 2005; Sullivan et al., 2000a; Sullivan et al., 2000b) . It is believed that chronic alcohol consumption leads to alterations in the brain and frontal lobes more specifically that reduce behavioral control promoting a “spiral of addiction” by further alcohol abuse and neurodegeneration (Crews et al., 2004a). For example, an individual who begins with moderate volumes of alcohol may develop metabolic and/or behavioral tolerance resulting in the consumption of larger amounts in the future. The resulting increase in alcohol consumption over time can cause neurodegeneration in the frontal lobes and its connections,

compromising the ability of the individual to make good decisions and control future intake (Crews et al., 2005).

The hippocampus is another structure that is sensitive to alcohol-induced neurodegeneration and its dysfunction is associated with a range of cognitive deficits. Because of its integral roles in learning and memory, and connections with the frontal lobes and other areas involved with emotional processing, the hippocampus has been postulated to have a role in drug addiction (Hyman et al., 2006; Nixon et al., 2011). The compromised hippocampal integrity in alcoholics may underlie some of the behavioral deficits in executive functioning (Beresford et al., 2006; Chanraud et al., 2010) , as well as impairments in learning, memory, and visual spatial abilities (Beatty et al., 1996; Crews et al., 2004a; Pitel et al., 2009; Townshend & Duka, 2005). This dissertation focuses on alcohol-induced damage in the hippocampus and parahippocampal gyrus, and associated neuropharmacological changes and cognitive deficits.

Although some tissue volume recovery occurs within the first 5 to 6 weeks of abstinence from alcohol, studies covering longer periods of abstinence (6 to 8 months) have shown persistent significant shrinkage within the frontal lobe, anterior parietal lobe, temporal lobe, lingual lobe, cingulate gyrus, insula, thalamus, and cerebellum in chronic alcoholics compared to light/nondrinking controls (Cardenas et al., 2007; Chanraud et al., 2007). For example, cognitive and structural imaging data provide evidence that chronic alcohol abuse results in shrinkage of the parietal and occipital lobes, even in those that have abstained for an average of 6 years (Fein et al., 2009a). Shrinkage of the parietal cortex in chronic alcoholics is associated with deficits in spatial information processing (Fein et al., 2009a), such as visuospatial learning (Ryan & Butters, 1980; Sullivan et al., 1992; Wilson et al.,

1987) and mental rotation (Beatty et al., 1996; Brandt et al., 1983; Glenn & Parsons, 1991) . The olfactory bulbs are another area of neurodegeneration resulting in basic olfactory impairments that are only partially reversible with abstinence (Ditraglia et al., 1991). A lifetime of alcohol abuse may also result in prolonged alterations to white matter even after long periods of abstinence (Fein et al., 2009b).

Fein and colleagues present an interesting point that must be considered when drawing conclusions about studies investigating the adverse effects of alcohol dependence on the brain. The majority of publications on human alcohol dependence are based on a disproportionately large sampling of alcoholics that have or are being treated, which are then generalized to all alcoholics (Fein et al., 2010). In contrast to treated alcoholics, untreated alcoholics comprise the majority of alcohol-dependent individuals in the U.S; in 2013, only 8% of those meeting criteria for an AUD actually sought treatment at a specialized facility (SAMHSA, 2013) and around 25% reported ever having received any treatment in previous years (Dawson et al., 2005). There is some evidence that treatment naïve alcoholics may have less severe alcoholism (Fein & Landman, 2005), less severe comorbid psychiatric illness (Di Sclafani et al., 2008), and less impaired performance on decision making tasks associated and do not suffer from the full extent of the tissue loss seen in treated alcoholics (Gazdzinski et al., 2008). This is not to say that those that meet criteria for an AUD and never seek treatment are immune to the consequences of chronic alcohol consumption, but rather those meeting criteria for an AUD make up heterogeneous population that needs to be considered when drawing conclusions about samples.

### *Models of Alcohol-Induced Neurodegeneration*

Due to the complexity of AUDs, there are many models of alcohol exposure that investigate the nature of the various contributing factors to their progression, maintenance, and consequences. Alcohol consumption is a multifaceted behavior with different motivations; therefore no single model is enough to fully understand alcoholism. Therefore, the current review will focus on *in vivo* rodent models that produce alcohol-induced neurodegeneration and cognitive deficits that model human responses. Animal models provide the advantage of closely controlling factors that cannot be controlled in human studies, and allow the researcher to clearly test hypotheses to understand the effects of alcohol on health. In order to elicit alcohol-induced neurodegeneration, animal models have to use forced alcohol exposure because most animals do not voluntarily consume alcohol at the high concentrations associated with cell loss. Models using methods of forced alcohol exposure fall under four basic categories: injections, vapor exposure, chronic feeding, or intragastric gavage. These models have supported a direct link between excessive alcohol intake, neurotoxicity, and behavioral impairments, and mimic damage in brain regions and the cognitive deficits seen in alcoholic patients (Collins et al., 1996; Crews & Nixon, 2009; Obernier et al., 2002a; Walker et al., 1980). The current dissertation work presented uses the intragastric gavage method, but other methods will be discussed briefly.

Intermittent intraperitoneal (ip) injections of alcohol can produce evidence of neurodegeneration in the cerebellum, hippocampus, and neocortex (Lundqvist et al., 1995; Pascual et al., 2007). In these studies, animals received 3g/kg of alcohol via ip injection every two days for a month, with two days of abstinence between every two days of alcohol administration. Here neurodegeneration was dependent upon the repeated cycle of high

BACs and withdrawal like that seen in human binge pattern drinking (Lundqvist et al., 1995; Lundqvist et al., 1994). The neuronal damage to the hippocampus seen in this model was accompanied by impairments in object recognition (Barker & Warburton, 2011; Pascual et al., 2007). A limitation of this model is that ip injections do not mimic alcohol metabolism associated with the oral route of administration typical in humans (Adalsteinsson et al., 2006; Iwaniec & Turner, 2013).

Bypassing the typical oral route of administration is a limitation of alcohol vapor inhalation models of AUDs (Mattucci-Schiavone & Ferko, 1986; Ripley & Stephens, 2011), although there have been recent reports in popular media of a trend of alcohol inhalation (Sifferlin, 2013). Strengths of the vapor model include close control over dose, duration, and pattern of alcohol exposure, however this results in inconsistencies between studies and no accepted consensus on methodology for what dose, duration, and pattern to use exists (Gilpin et al., 2008). Chronic intermittent vapor exposure that mimics high BACs followed by withdrawal like that in human binge pattern drinking has shown evidence of neurodegeneration in the hippocampus (Ehlers et al., 2013) and other behavioral correlates associated with alcoholism, such as increased self-administration (Gilpin et al., 2009; O'Dell et al., 2004). However, bypassing the normal metabolic pathways of alcohol makes vapor inhalation problematic when translating to humans because behavioral outcomes are different when inhaling alcohol compared to oral administration (Mattucci-Schiavone & Ferko, 1986).

The final two model types (chronic feeding and intragastric gavage) have better face validity than the other models because they both use an oral route of alcohol administration (Bell et al., 2012). This is particularly important in light of recent evidence for the role of gut bacteria in regulating brain functioning, including neuroimmune and inflammatory processes

(see Gorky & Schwaber, 2016 for review). The chronic feeding models induce damage in the cerebellum and hippocampus (Cohen et al., 2007; Mellion et al., 2013; Walker et al., 1980) however they rely on self-administration over months. Alternatively, intragastric gavage models use force intubation over a relatively short timeline and can be done over the course of just a few days in rats, making them less time intensive and gives the researcher more control over the age of the animals (Collins et al., 1996; Crews, 2008; Hayes et al., 2013; Kelso et al., 2011; Leasure & Nixon, 2010; Maynard & Leasure, 2013; Qin & Crews, 2012a).

The experiments in the current series use a modified version of the Majchrowicz model, which exposes rats to alcohol over four days via intragastric gavage (Majchrowicz, 1975). This model mimics binge drinking seen in human alcoholics and produces BACs well above 300mg/dl necessary for neurodegeneration (Faingold, 2008; Tomsovic, 1974). Another benefit to this model is that it produces other traits characteristic of human AUDs including tolerance, and intermittent periods of intoxication and withdrawal (Crews & Nixon, 2009). This model was chosen for the current series of experiments because it produces characteristic traits of AUDs, is a model of binge drinking that has been widely investigated and replicated, and causes neurodegeneration in brain regions consistent with that of human chronic binge drinkers (Crews, 2008; Crews & Nixon, 2009; Faingold, 2008). Areas damaged in the binge drinking rodent model include the olfactory bulbs, frontal lobes and multiple corticolimbic structures including the agranular insular cortex, anterior piriform cortex, perirhinal cortex, and entorhinal cortex, as well as the hippocampal dentate gyrus (Collins et al., 1996; Corso et al., 1998; Crews et al., 2004a; Hamelink et al., 2005; Leasure & Nixon, 2010; Maynard & Leasure, 2013; Obernier et al., 2002b). These regions overlap with those found to be thinner in alcoholics (Fortier et al., 2011).

### *Mechanisms of Alcohol-Induced Neurodegeneration*

The extent of alcohol-induced neurodegeneration is dependent on many factors including pattern of intake, genetics, age, and varies by region of the brain. Animal models and studies of human alcoholics have increased the understanding of alcoholic brain damage and led to the following proposed mechanisms of neurodegeneration: oxidative stress, neuroinflammation, reduced cell genesis and altered glial functioning, and loss of trophic support. It is believed the involvement of these mechanisms results in the overall brain shrinkage seen in alcoholics characterized by the lack of ongoing cell generation (neurons and glia), cellular atrophy, and cell death (Crews & Nixon, 2009).

#### Oxidative Stress

Oxidative stress is associated with neuronal cell death in a variety of neurodegenerative diseases including Alzheimer's and Parkinson's disease (Reynolds et al., 2007). Oxidative stress reflects an imbalance between an increase in reactive oxygen species (ROS) and the depletion of antioxidant defenses needed to detoxify and repair resulting damage. This imbalance can cause toxic events that damage all components of cells, including proteins, lipids, DNA, mitochondria (Nixon et al., 2009; O'Rourke et al., 2005; Reddy et al., 2013), and disrupt normal mechanisms of cellular signaling. Postmortem studies of alcoholic brains indicate that there are increases in enzymes associated with ROS production (Qin & Crews, 2012b). Animal models of alcoholic brain damage concur with postmortem findings showing increases in cyclo-oxygenase-2 (COX-2) and other enzymes that are associated with tissue damage by producing free radicals (Knapp & Crews, 1999; Qin & Crews, 2012a, 2012b; Reynolds et al., 2007). Additionally, alcohol-induced oxidative stress can be due to ROS produced directly by the metabolism of high BACs within certain

brain regions (Haorah et al., 2005; Haorah et al., 2008). Although discussed individually as causative factors in degeneration, in reality oxidative stress and neuroinflammation contribute to each other's pathological processes and likely act in conjunction.

**Neuroinflammation**

Alcohol, like stress and other drugs, presents a challenge to homeostatic functioning in the brain. To compensate for the presence of these "challenges", the body initiates a process called neuroadaptation in an attempt to maintain normal functioning. Inflammation is part of this complex biological response of body tissue to noxious stimuli such as pathogens, foreign chemicals, or cellular damage. It is the result of a protective immune response that involves immune cells, blood vessels, and molecular mediators with the purpose of eliminating the initial cause of injury, clear our necrotic cells and tissue damage from the insult, and initiate tissue repair. Too little inflammation can lead to progressive tissue destruction and compromise survival; in contrast, chronic inflammation can lead to a host of diseases brought on by the simultaneous destruction and healing by the inflammatory process. It was originally thought that the blood brain barrier (BBB) made the CNS an immune-impervious system (Murphy & Sturm, 1923), however the discovery of the innate immune cell, microglia, and the many associated proinflammatory genes and signals have changed this view (Neuwelt & Clark, 1978; Penfield, 1925). Microglia are a type of glial cell that play a dynamic role in neuronal homeostasis by taking on a proinflammatory or anti-inflammatory phenotype depending on their environment. Neuroinflammation is strongly influenced by the hypothalamic-pituitary adrenal (HPA) axis and its regulation of glucocorticoids, such as cortisol, therefore their responses may play a role in alcohol-induced neurodegeneration. In the brain, long term elevations in glucocorticoid concentrations are

associated with neurotoxicity (Erickson et al., 2003) and enhanced vulnerability to insult (Mulholland et al., 2005). High levels of glucocorticoids are associated with increased oxidative stress and ROS (Assaf et al., 2012; You et al., 2009), and can lead to neurodegeneration and cell death in the hippocampus, among other areas (Sato et al., 2010).

Notably, alcohol abuse is associated with HPA axis dysregulation which is a risk factor for developing an AUD (Stephens & Wand, 2012). In both males and females, glucocorticoid levels are increased with alcohol exposure and withdrawal (Gianoulakis et al., 2003). Associations between alcohol abuse and neuroinflammation have been characterized in many regions of the brain (Lippai et al., 2013; Liu et al., 2007; McBride et al., 2012), including the hippocampus (He & Crews, 2008). Alcohol increases expression of proinflammatory genes, those containing the DNA for components that promote inflammation, by two mechanisms that are dependent on high BACs (Crews & Nixon, 2009). First, high levels of alcohol can cause the leakage of endotoxin from the gut and liver into the blood and result in increases in proinflammatory cytokines that can be transported to the brain and activate proinflammatory genes (de la Monte et al., 2009; Qin et al., 2008; Qin et al., 2007). Cytokines are signaling molecules that can change immune and nerve cell function. Second, the presence of high alcohol concentrations directly increases the transcription of brain proinflammatory genes and decreases the transcription of brain pro-survival genes. Transcription factors are proteins that regulate gene expression by binding to sequences of DNA and control the transcription of DNA to RNA, which is then translated into proteins needed for many components in the brain. Postmortem human alcoholic brains studies find neuroimmune gene expression correlates with lifetime of alcohol consumption

suggesting that persistent neuroimmune signaling that is repeatedly increased by alcohol consumption contributes to the chronic relapsing nature of alcoholism (Crews et al., 2013).

An important transcription factor that is altered by alcohol and implicated in neurodegeneration is nuclear factor κB (NF-κB; Zou & Crews, 2006) which is well known for its roles in inflammatory and immune response signaling, including control of cell division and programmed cell death (O'Neill & Kaltschmidt, 1997). The presence of alcohol increases the DNA binding of NF-κB, resulting in increases in transcription of proinflammatory genes (Zou & Crews, 2006). Activation of NF-κB transcription increases proinflammatory enzymes and cytokines, including TNF-α, that promote a proinflammatory cascade that can further activate NF-κB transcription and the neuroimmune response (Kaltschmidt et al., 2005; Mattson & Camandola, 2001; Vallabhapurapu & Karin, 2009). Increased levels of NF-κB have been found in dying neurons following trauma or ischemia and in patients with Alzheimer's disease and Parkinson's disease (Bethea et al., 1998; Schneider et al., 1999; Terai et al., 1996; Zou & Crews, 2006). It is not surprising then that NF-κB is activated by alcohol and oxidative stress to induce a proinflammatory cascade in the brain that contributes to neurotoxicity (Madrigal et al., 2006) and mediates alcohol-induced inhibition of neurogenesis (Zou & Crews, 2012). Post mortem analysis of alcoholic brains show an increase in NF-κB gene transcription (Okvist et al., 2007) and large differences in other genes related to NF-κB transcription, proinflammatory genes, and neurodegeneration (Liu et al., 2004; Liu et al., 2006; Okvist et al., 2007). Other studies have shown that human genetic variations in NF-κB genes are associated with increased risk for alcoholism and particularly early-onset alcoholism (Edenberg et al., 2008). Animal models corroborate human findings of an increase in alcohol-induced proinflammatory gene

expression with neurodegeneration and alteration of genes involved with alcoholism (Crews et al., 2006a; Qin et al., 2008). Evidence from human and animal models support the involvement of a proinflammatory cascade, particularly increased NF-κB expression, as a potential key factor in alcohol induced brain damage.

Evidence suggests that alcohol shifts the balance of proinflammatory versus pro-survival signaling toward inflammation during alcohol intoxication, creating an environment that is vulnerable and less resilient to neurodegeneration (see Crews & Vetrino, 2014 for review). Substantial support for this mechanism of alcohol induced neurodegeneration comes from studies that block neurodegeneration. Antioxidants, such as butylated hydroxytoluene (BHT), given to rats prior to and after alcohol administration prevented the increased binding of NF-κB-DNA, proinflammatory gene induction, the loss of neurogenesis, and neurodegeneration (Crews et al., 2006a; Hamelink et al., 2005; Zou & Crews, 2006; Zou & Crews, 2012). Other studies have reported similar findings that administration of antioxidants was protective against binge-induced brain damage by decreasing oxidative stress (Hamelink et al., 2005). Therefore administration of antioxidants and drugs that block NF-κB can inhibit alcohol-induced neurodegeneration.

**Inhibition of Neurogenesis**

The mechanisms covered up to this point have focused on cell death resulting from oxidative stress and neuroinflammation as the mechanism for alcohol-induced neurodegeneration. However, alcohol also inhibits ongoing cell genesis, neurogenesis in particular, providing another potential mechanism by which alcohol results in neurodegeneration (He et al., 2005; Herrera et al., 2003; Jang et al., 2002a; Jang et al., 2002b; Nixon & Crews, 2002). The process of neurogenesis is comprised by four components in the

formation of a new neuron from a neural stem cell (NSC): proliferation, differentiation, migration, and survival (Gage, 2000; Kempermann et al., 2004). In the adult mammalian brain, neurogenesis occurs in two specific regions: the hippocampal subgranular zone of the dentate gyrus (DG) and the subventricular zone of the lateral ventricles (Altman & Das, 1965; Doetsch et al., 1999; Eriksson et al., 1998). The disruption of ongoing neurogenesis occurs in many neurodegenerative diseases and conditions, including Alzheimer's disease, stress, and depression (Marxreiter et al., 2013; Mu & Gage, 2011; Ransome et al., 2012). Although NSCs and the functional purpose of adult neurogenesis are still being understood, evidence from a variety of neurodegenerative disorders show a direct correlation between inhibition of neurogenesis, DG volume loss, and impairment of hippocampal-dependent behaviors and functions (Marxreiter et al., 2013; Mu & Gage, 2011; Ransome et al., 2012). Increased neurogenesis on the other hand, is associated with improvements in hippocampal-related behaviors (Gould et al., 1999; Kempermann et al., 2004; Nilsson et al., 1999; Santarelli et al., 2003; Snyder et al., 2001; van Praag et al., 1999a).

Evidence from *in vitro* models of alcohol exposure have repeatedly shown that alcohol alters NSCs' ability to form colonies, proliferate, differentiate, and survive (Crews & Braun, 2003; Hao et al., 2003a, 2003b; Tateno et al., 2005), while most *in vivo* models primarily report alcohol intoxication inhibits NSC proliferation (Jang et al., 2002a; Jang et al., 2002b; Nixon & Crews, 2002). Alcohol's inhibition of proliferation is quite profound with many studies reporting a 40-60% decrease in the number of proliferating cells in adults (Nixon, 2006). Reductions in proliferation and long-term survival of newborn cells have consistently been observed using multiple markers of the stages of neurogenesis in the Majchrowicz model used in the current series of experiments (Crews et al., 2006a; He et al.,

2005; Nixon & Crews, 2002, 2004). Additionally, alcohol treatment during neurogenesis blunts the growth of progenitor's dendritic arbor (He et al., 2005). The inflammatory processes associated with alcohol may also inhibit neurogenesis (Monje et al., 2003), another possible mechanism via which alcohol-induced activation of proinflammatory cascades may result in neurodegeneration.

**Inhibition of Gliogenesis and Altered Glial Functioning**

The health and functioning of mature and newly generated neurons is heavily reliant on glia, the resident support network of the brain. Networks of glial cells (including astrocytes, oligodendrocytes, and microglia) and cerebral vasculature are responsible for enhanced neuronal proliferation and survival, synaptic transmission, and immune protection to name a few (Araque & Navarrete, 2010; Auld & Robitaille, 2003; Barres, 2008; Parpura et al., 2012). This is accomplished by providing metabolic and trophic support to neurons (Barres, 2008; Barzilai, 2011; Benarroch, 2005; Peters et al., 1991; Quaegebeur et al., 2011; Sierra et al., 2010; Streit, 2002; Zhao et al., 2013; Ziv et al., 2006), monitoring and cleaning the extracellular environment (Nimmerjahn et al., 2005), releasing signals for neural development (Butler et al., 2010), maintaining homeostasis (Heneka et al., 2010; Zlokovic, 2008), and by having a direct influence on synaptic firing and plasticity (Heneka et al., 2010; Ullian et al., 2001; Wake et al., 2009). Therefore, disruption of the generation and functioning of glia may also contribute to alcohol-induced neurodegeneration. In fact, glia may be more sensitive to the effects of alcohol than neurons (Miguel-Hidalgo et al., 2002). Postmortem analysis of the hippocampi of human alcoholics revealed a significant 37% loss of glial cells, including astrocytes and oligodendrocytes (Korbo, 1999). Other studies using various methods have found reductions in the number of astrocytes following chronic alcohol

exposure (Matsuda-Matsumoto et al., 2007; Miguel-Hidalgo, 2005; Rintala et al., 2001). Alcohol causes astrocytes to degenerate, leaving a void in trophic and metabolic support (Kimelberg & Aschner, 1994) and a reduced ability to eliminate ROS (Dringen, 2000) which may contribute to neurons degenerating. Chronic alcohol consumption can also cause reactive astrogliosis and alter astrocytic functioning causing them to produce ROS and contribute to neurodegeneration through proinflammatory signaling (Baydas & Tuzcu, 2005; Gonthier et al., 1997; Jin et al., 2013). The loss of oligodendrocytes due to chronic alcohol can also contribute to neurodegeneration by reducing maintenance and generation of myelin, and disrupting another source of trophic support (Du & Dreyfus, 2002; Wilkins et al., 2001; Wilkins et al., 2003). The loss of pro-neurogenic astrocytes and oligodendrocytes contributes to irreversible alcohol-induced brain damage (Skuja et al., 2012).

The role of microglia in alcohol-induced neurodegeneration is not as well understood. Microglia take on different phenotypes or activation states depending on their environment. In non-pathological conditions, microglia exist in a "resting" state where they are constantly surveying their environment, responding to minute changes or invaders (Braat et al., 2006; Michelucci et al., 2009; Nimmerjahn et al., 2005). When microglia encounter a noxious stimuli (i.e. cellular damage, ROS, etc.) they alter their morphology and enter a varying continuum of activation states based on the intensity, type, and duration of the insult (Harting et al., 2008; Lai & Todd, 2008). Activated microglia can take on an anti-inflammatory or proinflammatory states, each accompanied by alterations in protein expression. Proinflammatory microglia, considered fully activated, can influence other mechanisms of alcohol neurodegeneration. They can increase and maintain chronic neuroinflammation, as well as decrease neurogenesis via prolonged secretion of proinflammatory cytokines (Ekdahl

et al., 2003; Gaur & Aggarwal, 2003; Monje et al., 2003). Activated microglia can contribute to oxidative stress because they are activated by ROS, which can lead them to produce and be a source of further ROS (Block & Hong, 2005; Block et al., 2007; Reynolds et al., 2007). Anti-inflammatory microglia, or partially activated, provide many benefits such as secreting anti-inflammatory cytokines and growth factors, such as BDNF and IGF-1, that can increase neurogenesis and survival of newly populated cells (Battista et al., 2006; Correa et al., 2010; Ekdahl et al., 2009; Kiyota et al., 2012; Ziv et al., 2006). Many studies indicate that, like astrocytes, during alcohol exposure there is a reduction of microglia proliferation (Koss et al., 2012; Teixeira et al., 2014) followed by an increase following chronic alcohol exposure (Crews et al., 2006a; He & Crews, 2008; Marshall et al., 2013; McClain et al., 2011; Nixon et al., 2008; Ward et al., 2009b).

Various models of alcohol abuse agree that microglia are activated to some extent following alcohol exposure (Kelso et al., 2011; McClain et al., 2011; Ward et al., 2009a; Zhao et al., 2013), however there is disagreement on the phenotype (activation state) and consequences of alcohol-activated microglia. Some groups have discussed microglia activation during intoxication as initiating a neuroinflammatory response that results in neurodegeneration (Crews et al., 2011; He et al., 2005; Qin & Crews, 2012a, 2012b; Qin et al., 2008). However, it is possible that timing of the microglia activation and immune response is a consequence, not a source, of alcohol-induced damage, and is a necessary function to restore homeostasis. Therefore the observation of "activated" microglia alone is not equivalent or informative about the inflammation state (Graeber et al., 2011). For example, increases in TNF-α concentrations following alcohol exposure may contribute to neurodegeneration (Crews et al., 2006b; Qin et al., 2008), but acute increases in

proinflammatory cytokines may also promote neuroprotection (Song et al., 2013; Turrin & Rivest, 2006). Furthermore, other studies of alcohol induced neurodegeneration have not observed proinflammatory cytokines during intoxication and suggest microglial activation may be involved with recovery (Kohman & Rhodes, 2013; Marshall et al., 2013; McClain et al., 2011; Nixon et al., 2008; Varnum & Ikezu, 2012; Zahr et al., 2010). Other studies describe more classical signs of activation of microglia and neuroinflammation such as increases in MHC-II (Ward et al., 2009a) and phagocytic activity (Zhao et al., 2013).

A key factor to consider with microglia activation and their contribution to neurodegeneration is the timing of activation. Acute activation of even proinflammatory microglia may be necessary for recovery as they migrate to damaged areas to take on a "housekeeping" role by removing debris from an injured site (Nimmerjahn et al., 2005) and a "nursing" role by secreting neurotrophic factors (Streit, 2002). This migration is triggered by signals released by damaged neurons and other microglia that can also promote the proliferation of more microglia (Davalos et al., 2005; Gao & Ji, 2010; Kloss et al., 1997; Raivich et al., 1991). The neuroimmune response increases microglia in the area that are supporting damaged cells and removing those beyond repair (Carson et al., 2007).

However, studies have shown that persistent activation of microglia following an initial damaging event can impact future microglia responses to other immune challenges (Dilger & Johnson, 2008; Norden & Godbout, 2013). These 'primed' microglia exhibit a more robust proinflammatory response upon secondary activation and subsequent chronic activation. Chronic activation of microglia is associated with neuronal loss and has been proposed as a mechanism within various neurodegenerative diseases (Amor et al., 2010). For example, in traumatic brain injury (TBI), microglia activation persists well after the initial

focal brain injury and causes secondary damage outside of the original injury (Lenzlinger et al., 2001; Ramlackhansingh et al., 2011). It is possible microglia that are acutely activated in response to high BACs become primed after several subsequent episodes of high BACs (characteristic of AUDs). These 'primed' microglia can become chronically activated and contribute to neuroinflammation and further neurodegeneration (Bilbo & Schwarz, 2009; Norden & Godbout, 2013). The 4-day binge model used in the current series of experiments has been shown to increase microglia proliferation and activation but there was no evidence the microglia had become phagocytic (McClain et al., 2011) and instead increased expression of anti-inflammatory cytokines (Marshall et al., 2013). A controversial study found that repeating the 4-day binge model four times, with three days of withdrawal between binge-episodes, produced phagocytic microglia and proinflammatory cytokines present during and immediately after intoxication (Zhao et al., 2013). Supportive microglia acutely activated as a consequence of alcohol-induced damage can potentially be shifted by chronic alcohol consumption into chronically activated microglia and a source of further neurodegeneration.

**Loss of Trophic Support**

The loss of trophic support may be both a cause and a consequence of the other mechanisms associated with alcohol-induced neurodegeneration, highlighting the critical role of trophic and growth factors in the health and functioning of the brain. Three factors of particular interest that will be discussed, and are implicated in alcohol-induced neurodegeneration, are the transcription factor cAMP-responsive element-binding protein (CREB), the neurotrophic factor brain-derived neurotrophic factor (BDNF), and the growth factor insulin-like growth factor 1 (IGF-1). Contrary to proinflammatory factors like NF-κB, these factors promote overall health of the brain including cell proliferation, growth, and

survival, therefore their disruption by alcohol can shift the balance of pro-survival signaling toward pro-inflammation, creating an environment that is vulnerable and less resilient to neurodegeneration (Crews & Nixon, 2009).

**cAMP-responsive element-binding protein (CREB)**

CREB family transcription factors have a complex and multifaceted role in diverse processes such as neurodevelopment, synaptic plasticity and neuroprotection (Sakamoto et al., 2011). CREB promotes neuroprotection by regulating the transcription of other pro-survival factors to protect neurons from excitotoxicity and apoptosis (Lonze & Ginty, 2002; Mantamadiotis et al., 2002) and is critical for proliferation, differentiation, survival, and maturation of all types of cells, including new neurons in the rodent hippocampus (Fujioka et al., 2004; Nakagawa et al., 2002). Activation of CREB increases the downstream expression of many neurotrophic and growth factors, such as BDNF and IGF-1, which contribute to neuroprotection, and structural and synaptic plasticity (Conti et al., 2002; Gao et al., 2010; Kitagawa, 2007; Lambert et al., 2001; Tabuchi et al., 2002). CREB helps protect against oxidative stress driven neurodegeneration by regulating ROS detoxification and increasing expression of antioxidants (Bedogni et al., 2003; Kronke et al., 2003), while disruption of CREB-mediated transcription results in increased vulnerability to ROS-induced cell toxicity (Lee et al., 2009). Due to its pivotal role in brain plasticity, CREB is also important for learning and long-term memory (Barco et al., 2002; Dash et al., 1990; Kaang et al., 1993).

CREB dysregulation has been implicated in several disorders of the CNS, including Alzheimer's disease, Parkinson's disease, Huntington's disease, ischemia, schizophrenia, addiction, and depression (Carlezon et al., 2005; Chalovich et al., 2006; Lonze & Ginty, 2002; Ma et al., 2007; Nestler, 2002; Nucifora et al., 2001; Sawamura et al., 2008; Walton &

Dragunow, 2000). In the context of the current study, reduced CREB transcription contributes to alcohol neurotoxicity and alcoholism (Zou & Crews, Wand, 2005; 2006). The activation of oxidative stress by alcohol coincides with the loss of CREB transcription (Zou & Crews, 2006), while binge alcohol treatment results in a decrease in pCREB (activated CREB) immunoreactivity that is most pronounced in regions of the brain showing degeneration (Bison & Crews, 2003). Studies of hippocampal slice cultures have found reductions in CREB regulated BDNF that coincided with sensitivity to neurodegeneration (Zou & Crews, 2006). Thus a reduction in CREB transcription also results in reduction of available neurotrophic support leaving neurons sensitive to insults, including oxidative stress, and vulnerable to degeneration. On the other hand, increasing the transcription of CREB through the use of drugs or antioxidants blocks neuroinflammation and alcohol-induced neuronal death (Pandey et al., 2005; Pluzarev et al., 2008; Zou & Crews, 2004)

**Brain-derived neurotrophic factor (BDNF)**

Another important factor abundantly expressed in the brain is brain-derived neurotrophic factor (BDNF). BDNF is a member of the neurotrophin family that has a vital role in neurogenesis, including the proliferation, differentiation, and growth of progenitor cells (Huang & Reichardt, 2001; Lee & Son, 2009). Many studies have shown that BDNF is critical for plasticity and the normal cellular maturation process, including migration, dendritic arborization, synaptogenesis, axonal path-finding, and the induction and maintenance of LTP (Alonso et al., 2002; Chan et al., 2008; Hall et al., 2000; Heldt et al., 2007; Lu & Chow, 1999; McAllister et al., 1997; Reichardt, 2006; Tolwani et al., 2002). As part of the neurotrophin family, BDNF protects against neuronal cell death in the hippocampus (Alonso et al., 2004). BDNF controls neuronal survival and plasticity through

binding to the high-affinity receptor tyrosine kinase B (TrkB; Givalois et al., 2001), which activates the intracellular mitrogen-activated protein kinases (MAPK; originally called ERK) pathway (Moonat et al., 2010). Connections between BDNF and CREB contribute to their respective roles in brain plasticity. Signaling via activation of the BDNF-MAPK pathway increases CREB transcription and phosphorylation and expression of CREB-mediated anti-apoptotic genes (Arthur et al., 2004; Watson et al., 2001; Xing et al., 1998). CREB phosphorylation can also increase transcription of downstream BDNF in a positive feed-back loop (Conti et al., 2002; Katoh-Semba et al., 2008; Tabuchi et al., 2002). There are many sources of BDNF in the brain, as it is produced/secreted by neurons (Brady et al., 1999; Mannion et al., 1999), particularly dentate gyrus neurons (Ziv et al., 2006), microglia (Nakajima et al., 2002; Ziv & Schwartz, 2008), oligodendrocytes (Du & Dreyfus, 2002; Wilkins et al., 2003), and astrocytes (Dougherty et al., 2000; Jean et al., 2008; Zafra et al., 1992). Both BDNF and TrkB have a widespread distribution in the CNS (Conner et al., 1997; Drake et al., 1999; Yan et al., 1997a; Yan et al., 1997b), with a high concentration in the hippocampus and dentate gyrus granule cells specifically (Conner et al., 1997). Due to this abundant distribution and role in plasticity, BDNF is important for cognition, learning, and memory (Alonso et al., 2002; Heldt et al., 2007; Koponen et al., 2004; Pietropaolo et al., 2007; Yamada & Nabeshima, 2003).

It should not be surprising then that insufficient supply of BDNF has been implicated in many degenerative diseases of the central nervous system. Many reports have documented evidence of decreased expression of BDNF in neurological disease such as Parkinson's (Howells et al., 2000; Murer et al., 2001), Alzheimer's (Ferrer et al., 1999; Phillips et al., 1991), and Huntington's (Zuccato et al., 2001; Zuccato et al., 2003). Dysregulated BDNF

signaling may also be involved in negative affective behaviors, such as depression (Altar, 1999; Nair & Vaidya, 2006), where effective antidepressant treatments have been shown to increase BDNF mRNA and protein (Altar et al., 2003; Chen et al., 2001; Dias et al., 2003; Nair & Vaidya, 2006). Inflammation negatively affects the expression of BDNF; proinflammatory cytokines like those released with chronic alcohol consumption causes a significant reduction in BDNF in multiple brain regions (Calabrese et al., 2014; Guan & Fang, 2006; Lapchak et al., 1993).

Alcohol has profound effects on BDNF expression that appear to be dependent on the alcohol dose and brain region, with a pattern towards reduced BDNF with higher chronic doses (Moonat et al., 2010; Stevenson et al., 2009) that corresponds with alcohol-induced neurodegeneration (Davis, 2008) and reduced survival of new DG granule neurons (Herrera et al., 2003; Nixon & Crews, 2002). Small doses of alcohol, like those in acute alcohol consumption, result in a significant increase in BDNF mRNA in areas like the dorsal striatum of mice and rats (Jeanblanc et al., 2009; McGough et al., 2004). However, chronic alcohol exposure results in a reduction of BDNF in several cortical areas, and reverses the increased striatal BDNF levels (Darcq et al., 2015; Logrip et al., 2015; Logrip et al., 2009; Pandey et al., 2004). Short term increases in BDNF might be a reactive response to alcohol for neuroprotection and recovery however chronic alcohol consumption disrupts the brains ability to respond with BDNF. Significantly reduced BDNF secretion by chronic alcohol treatment suggests BDNF might be related to alcohol-induced cell damage (McGough et al., 2004; Sakai et al., 2005). In a study looking at alcohol's effect on the BDNF-ERK signaling pathway, Kim and colleagues found decreased expression of BDNF, TrkB, and pERK in the hippocampus with increasing alcohol administration (Kim et al., 2012). Alcohol-induced

disruption of the BDNF-ERK pathway is another mechanism that disrupts the neuroprotective and pro-neurogenic signaling of BDNF (Sanna et al., 2002). Alcohol also reduces signaling through the BDNF-CREB feedback loop, resulting in a loss of trophic support (Zou & Crews, 2006).

Accumulating evidence implicates a role for BDNF in negatively regulating alcohol intake (Hensler et al., 2003; McGough et al., 2004; Pandey et al., 2006) and reduced function of BDNF in the development of alcoholism (Matsushita et al., 2004; Uhl et al., 2001; Xu et al., 2007). The possible link between reduced BDNF and risk of alcoholism and neurodegeneration is supported by the finding of reduced BDNF in patients with alcohol dependence, with even lowers levels in those with a positive family history of alcohol dependence (Joe et al., 2007; Zanardini et al., 2011). An alcohol-induced reduction in BDNF may contribute to neurodegeneration by reducing the trophic support needed for neuroprotection, and altering signaling that leaves a vulnerability to future problem drinking and alcohol dependence.

**Insulin-like growth factor 1 (IGF-1)**

Insulin-like growth factor 1 (IGF-1) is an important trophic factor for a wide spectrum of development and growth actions on both the central and peripheral nervous systems. Several lines of experimental approaches have shown that IGF-1 promotes the survival and proliferation of neuroprogenitor cells, and stimulates differentiation of neurons and glia, leading to increased neurogenesis and synaptogenesis (Aberg et al., 2000; Aizenman & de Vellis, 1987; Anderson et al., 2002; Masters et al., 1991; Poulsen et al., 2005). Evidence indicates that IGF-1 also participates in normal functioning of the brain such as regulating release of hormones and neurotransmitters (Ceda et al., 1987; Kar et al., 1997)

and resistance against oxidative stress (Holzenberger et al., 2003). High concentrations of IGF-1 are primarily produced and released by the liver (Clemmons et al., 1995) and are able to cross the blood brain barrier via IGF receptors on the choroid plexus (Carro et al., 2001). Other sources of IGF-1 production are muscles and the brain cells, including microglia (Carro et al., 2001; Ziv & Schwartz, 2008), astrocytes (Madathil et al., 2013; Wine et al., 2009), and oligodendrocytes (Du & Dreyfus, 2002; Wilkins et al., 2001). The signaling of IGF-1 is influential on and influenced by BDNF and CREB as well. For example, increases in IGF-1, via exercise or peripheral administration, increase BDNF mRNA (Carro et al., 2001; Ding et al., 2006). Much like the reciprocity between BDNF and CREB, the activation of CREB regulates activity of IGF-1 (Lambert et al., 2001; Thomas et al., 1996). On the other hand, IGF-1 binding to the IGF-1 receptor (IGF-1R) signals the MAPK cascade and activates CREB transcription and phosphorylation (Aberg et al., 2000; Aberg et al., 2003; Choi et al., 2008). In association with its role in learning, memory and neurogenesis, IGF-1R is located through the brain, with highest densities in the hippocampus and parahippocampal gyrus (Adem et al., 1989).

Circulating IGF-1 has been shown to be protective against behavioral deficits and cognitive dysfunction following brain injury (Madathil et al., 2013; Ozdemir et al., 2012), while disruption of IGF-1 signaling in the brain is associated with a reduction in neurogenesis and survival. For example, IGF-1 null mice show a significant reduction in the number of dentate gyrus granule neurons and CA regions of the hippocampus (Beck & Hefti, 1995). Most IGF-1 null mice, and all mice lacking IGF-1 receptors, die at birth, indicating how important IGF-1 is for growth and survival. A number of studies indicate that alcohol-induced toxicity is mediated in part by interference with the production and functioning of

IGF-1 (Cohen et al., 2007; de la Monte et al., 2001; de la Monte et al., 2008; Hallak et al., 2001; Sonntag & Boyd, 1988). Alcohol mediates its effects on IGF-1 signaling by inhibiting gene expression and impairing receptor binding (de la Monte et al., 2005; Soscia et al., 2006), which results in increased apoptosis (de la Monte et al., 2001; Ikonomidou et al., 2000; Zhang et al., 1998), mitochondrial dysfunction (de la Monte et al., 2001), and increased oxidative stress (Cohen et al., 2007; de la Monte et al., 2008). Chronic alcohol may also contribute to cell death by increasing proinflammatory cytokines that inhibit IGF-1 and its pro-survival signals (Venters et al., 1999). It appears that the impaired binding of IGF-1 in alcohol dependence corresponds to an inability to promote survival and recovery from neurodegeneration. .

**Sex Differences in Alcohol-Induced Neurodegeneration**

Alcohol affects women differently than men, yet compared with alcoholic men, alcoholic women have received little research attention. Epidemiological studies show that compared to men, women start to drink later in life (Zilberman et al., 2003), consume less per occasion, are more likely to be abstinent (York & Welte, 1994), and have lower rates of alcoholism (Kessler et al., 1994). Yet evidence suggests women progress faster to alcohol dependence (Hernandez-Avila et al., 2004; Randall et al., 1999; Schuckit et al., 1995; Schuckit et al., 1998), and are more vulnerable than men to the adverse medical consequences of heavy alcohol consumption (Ashley et al., 1977; Hommer et al., 2001; Prendergast, 2004). The course of alcohol use disorders is thus considered by some to be accelerated or compressed in women compared to men, and has been labeled the "telescoping" effect by investigators (Piazza et al., 1989) although with mixed support (Diehl et al., 2007; Goldstein et al., 2012; Keyes et al., 2008; Mann et al., 2005).

For example, negative health outcomes such as cancers, gastrointestinal diseases, cardiovascular diseases, and liver damage are more pronounced in women than men despite the same level of consumption (Ma et al., 1999; Ma et al., 1993; Pequignot et al., 1974; Rehm, 2011). Evidence also indicates that women are more sensitive to the neurotoxic effects of alcohol than men (Hommer et al., 2001; Hommer, 2003), and cognitive and structural impairments in the brain emerge faster in the course of the disease for women than for men (Mann et al., 1992; Nixon et al., 1995). Although alcoholism may be less likely to affect women, women with AUDs have higher rates of medical problems and a 50-100% significantly higher mortality rate than men with AUDs (Smith & Weisner, 2000; Walter et al., 2005). Women with AUDs are also more likely to have co-occurring psychiatric diagnoses such as major depression, anxiety, post-traumatic stress disorder (PTSD), or panic disorder, and are more likely to have a history of physical or sexual abuse, which can lead to greater dependence on alcohol (Karoll, 2002). It is alarming then that the prevalence rates of binge drinking and AUDs are increasing among young women (Keyes et al., 2008; Zilberman et al., 2003) and not just in the United States (SAMHSA, 2013). Despite this, few experimental studies have directly compared alcohol-induced brain damage between males and females, and those that have are inconclusive.

### *Clinical Evidence for Sex Differences in Vulnerability to Alcohol*

The most common method for investigating alcohol-induced neurodegeneration in the brains of living alcoholic men and women is neuroimaging. With methods like computerized tomography (CT), MRI, and DTI, researchers can compare alcohol-induced brain shrinkage by measuring reductions of gray and white matter, and increases in cerebrospinal fluid (CSF), which is an indication of the size of the lateral ventricles inside the

brain that increases in size as the brain shrinks. It is important to note that the following studies controlled for age and used separate sex matched nonalcoholic control groups, two factors independent of alcoholism that can affect sex differences in brain structure (Hommer, 2003). Two early studies reported that male and female alcoholics had significantly larger amounts of intracranial CSF than control subjects, indicating greater brain shrinkage among alcoholics of both sexes (Jacobson, 1986; Mann et al., 1992). Women in both studies however, reported about half as many years of excessive drinking as alcoholic men. Other studies that have compared total cerebral volume among alcoholic men and women compared to controls. Using high resolution, full volumetric MRI scanning, strong evidence for greater brain shrinkage among alcoholic women than alcoholic men was found, even though alcoholic women started heavy drinking later in life and had consumed less alcohol in their lifetimes (Agartz et al., 2003; Hommer et al., 2001). On the other hand, Mann and colleagues found the same relative brain volume reductions in female alcoholics compared to female controls as male alcoholics compared to male controls using a global atrophy index (Mann et al., 2005). However, in this study and their previous work, this similar reduction in brain volume occurred despite the fact that women had a lower total lifetime dose of ethanol consumption than men, indicating an increase vulnerability to alcohol induced neurodegeneration (Mann et al., 2005; Mann et al., 1992). More recent work from the Hommer group revealed that alcoholic women had a larger decrease in cortical thickness compared to control women than did alcoholic men compared with control men (Momenan et al., 2012). However, other research has failed to find significant differences indicating increased vulnerability in women; some have pointed to relative vulnerabilities in men

(Pfefferbaum et al., 2001), and others found no evidence of brain abnormalities among alcoholic females (Kroft et al., 1991; Pfefferbaum et al., 2002).

Still fewer studies have compared the volumes of specific brain structures of alcoholic and nonalcoholic men and women. Evidence indicates that alcoholic women have significantly larger reductions in hippocampi volume (Agartz et al., 2003) and smaller corpus callosums (Hommer et al., 1996) than alcoholic men. Another study comparing male and female alcoholics found a significantly greater deficit in concentrations of N-acetylaspartate (a marker of neuronal integrity) in the frontal lobe gray matter of alcoholic women than men (Schweinsburg et al., 2003). A more recent study investigating prefrontal cortex gray and white matter volume in adolescents with an AUD found that females and not males had a significant reduction in the volume of both (Medina et al., 2008). Abnormalities in the prefrontal cortex may contribute to further heavy drinking and neurodegeneration. Likewise, binge drinking during adolescence is associated with sex-specific differences in frontal, temporal, and cerebellar brain activation which relates to cognitive performance and increased vulnerability to the neurotoxic effects of heavy alcohol use in females compared to males (Squeglia et al., 2012).

### *Potential Mechanisms that Underlie Sex Differences*

It has been shown that women are more sensitive to the many harmful effects of alcohol abuse than men, yet the underlying mechanisms of increased female vulnerability remain poorly understood. First, equal dosages of alcohol consumed by men and women generally result in women having higher blood alcohol concentrations (BAC). Women tend to have more body fat and thus lower total body water, therefore alcohol is diluted less resulting in a BAC that may be around 30% higher in women than in men (Cederbaum,

2012; Ely et al., 1999). First pass metabolism of alcohol by the stomach may also contribute to higher BACs in women due to lower concentrations of alcohol metabolizing enzymes (e.g. alcohol-dehydrogenase, ADH) than men (Baraona et al., 2001; Frezza et al., 1990; Pozzato et al., 1995). Unmetabolised alcohol continues to circulate in the blood to organs and other tissue until it is completely broken down by the liver. Thus women consuming comparable amounts of alcohol to men have a higher circulating BAC, where their organs are exposed to the toxic effects of alcohol for a longer period of time. Sex differences in gastric ADH may be age related, as activity appears higher in young men compared to young women and reverses to higher in middle-aged women compared to middle-aged men (Parlesak et al., 2002).

Sex differences in neuroinflammation and the corresponding stress response to chronic alcohol exposure may provide another mechanism of increased vulnerability to alcohol neurotoxicity in females. Females and males respond to stress differently; although males are at an increased risk for adverse consequences of stress in association with maternal adversity during pregnancy, females are at greater risk to early life and peripubertal adversity to negative outcomes throughout life (see Bale & Epperson, 2015 for review). The same corticolimbic regions that are vulnerable to chronic stress-induced glucocorticoid activity are also vulnerable to alcohol induced damage (Crews et al., 2004a; Duman, 2009). Additionally, inflammatory and immune responsivity to stress is greater in females than males (Gallucci et al., 1993; Spitzer, 1999), while alcohol represents a physiological stressor (Ogilvie & Rivier, 1997) that preferentially activates the HPA axis in females in rodent models as well as in humans (Jenkins & Connolly, 1968; Rivier, 1993, 1996).

Females are more vulnerable to the neuroinflammatory effects of alcohol and have a sexually dimorphic neuroadaptive response. Hashimoto and Wiren (2008) investigated the consequences of withdrawal from chronic alcohol intoxication using selectively bred lines of mice that provide a model of neuroadaptation and represent distinct alcoholic phenotypes in sensitivity to alcohol and divergent withdrawal severity. Transcriptional profiling demonstrated that genes involved in apoptosis and cell death were a predominant functional category in females compared with males identified through bioinformatics (Hashimoto & Wiren, 2008). Confirmation analysis showed a highly significant increase in dead cells in females, but instead, potential neuroprotection in males, supporting sex as important influence on gene expression (not genotype or selected phenotype) and increased vulnerability in females (Hashimoto & Wiren, 2008).

A follow up to this study using the same mouse lines found distinct sexual dimorphic signaling in the medial prefrontal cortex (mPFC); a proinflammatory inflammotoxic phenotype was found in females, in contrast to immune suppression and relative neuroprotection observed in males (Wilhelm et al., 2014), consistent with previous work (Hashimoto & Wiren, 2008). This study also found altered NF-κB signaling in the mPFC of alcohol treated males and females, however they were found in completely distinct gene networks indicative of a sexually dimorphic response (Wilhelm et al., 2014). NF-κB is signaling mediates many of the detrimental effects of alcohol on the hippocampus (McClintick et al., 2013), however it also has a role in survival and neuroplasticity (Li & Verma, 2002). Therefore it is possible that NF-κB signaling may contribute to neuroinflammation and damage in females, and neuroprotection in males following chronic alcohol consumption.

In another series of studies, Walls and colleagues evaluated the consequences of repeated chronic alcohol exposure and withdrawal on cell death and associated cellular signaling pathways in hippocampal slices. First, they found that chronic alcohol exposure and withdrawal resulted in increased sensitivity to toxicity and damage in the cornu ammonis 1 (CA1) and dentate gyrus hippocampal subregions from females but not males (Walls et al., 2013). Increased hippocampal sensitivity to alcohol neurotoxicity has also been found in cultures from female but not male postnatal week two pups (Barron et al., 2008).

To evaluate the impact of distinct signaling pathways on hippocampal toxicity, Walls investigated the toxic effects of glucocorticoids and the potential interaction with alcohol-mediated damage. As previously noted, chronic alcohol increases glucocorticoids in males and females (Gianoulakis et al., 2003), and high levels of glucocorticoids can lead to hippocampal neurodegeneration and cell death (Sato et al., 2010) where there is an abundance of glucocorticoid receptors (Sorrells & Sapolsky, 2007). Using corticosterone (the predominant glucocorticoid in rodents), Walls et al. (2013) found glucocorticoid administration after repeated chronic alcohol exposure resulted in a surprising, highly sexually dimorphic response. Glucocorticoid treatment alone resulted in increased toxicity and damage in CA1 in both male and female control cultures, consistent with previous results (You et al., 2009). In the immediate withdrawal period, glucocorticoid signaling in female chronic alcohol-exposed cultures increased sensitivity to ethanol induced damage in the CA1, however glucocorticoids blocked all ethanol-induced damage in male cultures, consistent with neuroprotection (Walls et al., 2013). These results provide further evidence of a distinct neuroadaptive response between males and females following chronic alcohol exposure similar to previous findings (Hashimoto & Wiren, 2008).

Other models of chronic alcohol exposure have demonstrated a sexually dimorphic response to neuroinflammation. Following chronic alcohol treatment, female mice have significantly higher cortical levels of the inflammatory mediators (e.g. COX-2) and proinflammatory cytokines (e.g. TNF-α) than male alcohol treated mice (Alfonso-Loeches et al., 2013). Increased levels of these inflammatory mediators was associated with significantly higher alcohol-induced gliosis and up-regulation of GFAP (astrocyte marker), caspace-3 activation (an apoptotic-associated enzyme), and reduced levels of the neuronal markers NeuN and MAP-2 in alcohol treated females than alcohol treated males (Alfonso-Loeches et al., 2013). Reduced NeuN and MAP-2 is correlated with more significant up-regulation of caspace-3 activation, and suggests the same alcohol treatment induced greater neuronal injury and brain damage in females than males, suggesting enhanced vulnerability in females (Alfonso-Loeches et al., 2010; Alfonso-Loeches et al., 2013).

Wilhelm and colleagues provide further support for the hypothesis of a sexually dimorphic stress response to chronic alcohol exposure and withdrawal, where females exhibit a proinflammatory response and males an immunosuppressed response. In both males and females, at peak withdrawal from chronic alcohol exposure via vapor inhalation, serum corticosterone concentrations was significantly increased compared to control animals (Wilhelm et al., 2015b). However, females exhibited changes in gene expression of down-stream signaling consistent with activation of inflammation and cell death associated pathways;  astrocyte-enriched genes associated with inflammatory signaling and tissue damage were particularly increased in females (Wilhelm et al., 2015b). Additionally, an increase in the number of dead or dying cells within the anterior cingulate cortex was observed in females, while a decrease was seen in males (Wilhelm et al., 2015b), consistent

with the finding in hippocampal cultures of enhanced CORT and toxicity in females in contrast to neuroprotection in males (Walls et al., 2013).

Further study into the effects of alcohol exposure targeting astrocytes provides more evidence that females and males have a distinct biological response during withdrawal following chronic intoxication. Alcohol increased astrocytic markers in the ACC and various subregions of the hippocampus of female mice, including the DG and hilus, but no significant effect on labeled cells in any subregion for males was found (Wilhelm et al., 2015a). Furthermore, female but not male astrocytes exhibited increased expression of the pro-inflammatory cytokines and reduced expression of neuroprotective cytokines, indicating that female astrocytes increased inflammatory signaling in the presence of a reduced neuroprotective response (Wilhelm et al., 2015a). The downstream effects of such cellular dysfunction are inflammation, oxidative stress, and compromised BBB integrity, which can contribute to neurotoxicity and neurodegeneration in females (Claycomb et al., 2013; Wilhelm et al., 2015b). Additionally, glial cells shifted to proinflammatory activation no longer provide trophic factors and other neuroprotective signaling, providing another mechanism of potential cell loss in females due to increased neuroinflammation.

Taken together, these studies indicate sexually dimorphic neuroadaptation following chronic alcohol exposure characterized by a proinflammatory response mediated by glial cells and increased sensitivity to glucocorticoids and neurotoxicity in females, in contrast to immune suppression and neuroprotection seen in males (Alfonso-Loeches et al., 2013; Hashimoto & Wiren, 2008; Walls et al., 2013; Wilhelm et al., 2014). Both male and female chronic alcoholics show significant shrinkage of grey and white matter in the brain (Kubota et al., 2001; Mann et al., 2001; Pfefferbaum et al., 1992; Sullivan & Pfefferbaum, 2005), and

other animal models have found alcohol-induced damage in males (Zou & Crews, 2012 for example). However, it may be the case that in other models that employ repeated dosing and/or larger volumes of alcohol, neuroadaptation may shift what is initially an acute neuroprotective stress response in males, to a chronic proinflammatory response similar to that seen in females after less alcohol exposure. Thus consistent with the hypothesized telescoping effect, neuroinflammation and resulting medical consequences occur in females sooner and after a shorter history of alcohol abuse, and only after more prolonged, chronic use do similar problems occur in males. This idea is consistent with the notion that inflammatory and immune responses are more marked in females than in males (Spitzer, 1999), and the fact that females have higher prevalence rates of neuroinflammatory diseases, including multiple sclerosis (Johansson et al., 2012), Alzheimer's (Streit, 2010), and Parkinson's disease (Amor et al., 2010).

The potential role of gonadal hormones in increased female vulnerability to chronic alcohol-induced damage has not been thoroughly investigated and remains unclear. Although evidence indicates that ovarian hormones provide neuroprotection for females against various brain insults (Hoffman et al., 2006; Petrone et al., 2014) and decline with aging (Daniel et al., 2015), women are more vulnerable to neurotoxicity from alcohol. Both human and animal studies indicate that the female reproductive cycle and associated hormones are altered due to alcohol ingestion (Emanuele et al., 2001; Gavaler et al., 1993; Rettori et al., 1987); alteration in estrogen levels is associated with changes in glucocorticoid concentrations (Burgess & Handa, 1992) and inflammatory signaling (Straub, 2007). Circulating levels of estradiol in particular results in greater activation of the HPA axis in both females and castrated males in response to alcohol indicated by increases in adrenocorticotropic hormone (ACTH; Ogilvie

& Rivier, 1997; Rivier, 1996) and corticosterone (Rivier, 1993); the latter effect is reversed by administration of testicular androgens (Ogilvie & Rivier, 1997). Interestingly, the influence of estradiol on glial proinflammatory responses may underlie the sexual dimorphic response and greater incidence of neuroinflammation in females. Loram and colleagues found that estradiol incubation produced anti-inflammatory effects in microglia from adult male rats but a proinflammatory effect in microglia from adult female rats (Loram et al., 2012). These findings support the idea that the neuroadaptive response to alcohol in males is immunosuppressive and relatively neuroprotective in males and proinflammatory in females. Gonadal hormones may either drive, or contribute to this difference in HPA axis activation that results in an increased vulnerability to chronic alcohol-induced damage in females.

**Abstinence and Brain Regeneration**

Despite significant reductions in brain volume as a result of chronic alcohol consumption, human studies provide substantial evidence that abstinence from alcohol allows for natural healing to occur in the brain (Carlen et al., 1978; Sullivan et al., 2000b). Longitudinal imaging studies of brain structures reveal increases in cortical gray matter volume within a month of abstinence, followed by increases in white matter and reversal of ventricular enlargement after longer periods of abstinence (Mann et al., 2005; O'Neill et al., 2001; Pfefferbaum et al., 1995). Neuroimaging approaches demonstrate that abstinence from alcohol could partially reverse alcohol-related macro-structural, micro-structural, and metabolic abnormalities in white matter (Gazdzinski et al., 2010). These improvements in overall brain volume within the first months of abstinence are associated with improvements in cognition, such as nonverbal memory and attention (Sullivan et al., 2000a; Sullivan et al., 2000b), while very long periods of abstinence have been reported to resolve most

neurocognitive deficits associated with alcoholism (Fein et al., 2006). In general, studies suggest that recovery during abstinence is associated with structural changes in the brain that include growth of both white and gray matter as well as improved overall cognitive functioning. It is important to note however, that even after longer periods of abstinence (6-8 months) from chronic alcohol consumption, significant shrinkage persists compared to controls within the frontal lobe, anterior parietal lobe, temporal lobe, cingulate gyrus, insula, thalamus, and cerebellum (Cardenas et al., 2007; Chanraud et al., 2007). Additionally, despite the apparent reversal of some cognitive impairment by abstinence, detoxification only reverses alcoholic related deficits in learning, memory, and executive function in less half of all cases (de la Monte & Kril, 2014). The persistent shrinkage of brain volume and associated cognitive deficits highlights the importance of long periods of abstinence for brain recovery, and the need for better understanding of the mechanisms involved in endogenous self-repair from alcohol-induced damage.

### *Endogenous Recovery*

Animal models provide evidence of some of the endogenous processes going on during abstinence that may facilitate recovery from alcohol-induced damage. In particular, the regenerative sequelae that follow alcohol intoxication are well established in the binge model of alcohol dependence used in the current series of experiments. The combined effects of cell death and decreased cell birth are associated with neurodegeneration in multiple alcohol-vulnerable regions of the brain in this model follow binge alcohol exposure (Crews et al., 2004a; Nixon & Crews, 2002; Obernier et al., 2002a). However, abstinence results in increased cell proliferation in multiple brain regions after just one day (Nixon & Crews, 2004; Nixon et al., 2008). Cells genesis continues to increase into cortical gray and white

matter areas as well as many non-cortical regions after two days of abstinence (Crews & Nixon, 2009). In particular, proliferation in the hippocampal dentate gyrus increases from binge alcohol-inhibited levels to control levels after one day of abstinence followed by increases several times that of controls that persists for more than a week (Nixon & Crews, 2004). Following binge exposure, two burst of proliferation occur in the first week of abstinence: the first occurs two days after the last dose of alcohol that primarily differentiates into microglia. These microglia persist for months and migrate to many brain regions (Nixon et al., 2008). These new microglia are believed to aid various brain regions in recovery by taking on a partially activated state and secreting trophic factors and anti-inflammatory cytokines (Marshall et al., 2013; McClain et al., 2011). The second proliferative burst is region specific and occurs seven days after the last dose of alcohol. This burst of proliferation occurs in the dentate gyrus subgranular zone and produces cells that will primarily differentiate into new neurons that, given enough time, will mature into new dentate gyrus granule neurons (Nixon & Crews, 2004). The mechanisms underlying increased proliferation and its contribution to recovery following binge alcohol exposure need further study.

One mechanism that may contribute to recovery is reactive gliosis and an associated increase in trophic support after both were suppressed during binge-alcohol intoxication. The temporal pattern of these proliferative bursts in the binge model suggests that the initial increase in microglia is necessary to aid in the “clean-up” process following alcohol induced damage and then create a more pro-neurogenic environment in which new neurons can be born, mature, and integrate into the existing infrastructure (Zhao et al., 2013). Consistent with this idea is the fact that glia change in number in response to various insults to the CNS (Block et al., 2007; Ridet et al., 1997; Tagliaferro et al., 1997; Zhou et al., 1995), including

alcohol. Alcohol kills and inhibits cell genesis during intoxication, however several studies have found that abstinence following intoxication produces reactive gliosis across multiple brain regions leading to an increase in astrocyte (Rosengren et al., 1985; Satriotomo et al., 1999; Vongvatcharanon et al., 2010), microglia (Marshall et al., 2013; Nixon et al., 2008; Riikonen et al., 2002), and oligodendrocytes (Tateno et al., 2005). In the 4-day binge model, the glial response appears to have two primary phases: an initial proinflammatory phase intended to isolate damage and prevent it from spreading, while also cleaning up dead and dying cells, followed by a pro-neurogenic phase during which glia release neurotrophins and other factors that help stimulate recovery and production of new neurons.

Along with an increase in glial cells and trophic factors, an increase in pCREB transcription that rebounds from alcohol-suppressed levels during abstinence may also contribute to regeneration. Many studies have suggested that pCREB transcription increases plasticity and survival of neurons following insult (Mabuchi et al., 2001; Walton & Dragunow, 2000). Evidence has linked pCREB transcription with synaptic, pro-survival NMDA receptors (Hardingham & Bading, 2002), which may result in an increase of trophic support. During withdrawal from chronic alcohol intoxication, the brain enters a hyper-excitability state characterized by rebound of high extracellular glutamate levels. Increased synaptic glutamate release might activate NMDA receptors resulting in an increase in pCREB formation and drive BDNF synthesis and secretion (Papadia & Hardingham, 2007).

### *Exercise Neurorestoration*

Despite some recovery happening during abstinence from alcohol, binge drinking by definition happens multiple times, potentially limiting the brain's ability to recover. Evidence indicates that longer periods of abstinence result in better recovery, however repeated binge

episodes may disrupt these endogenous attempts. Therefore, there is a need to develop interventions that could potentially bolster the self-repair processes. Prior research indicates that post-injury neural events can be influenced by behavior (Kozlowski et al., 2013; Will et al., 2004); exercise is one such behavior, which provides numerous psychological and physiological benefits that promote healthy living (see Voss et al., 2013 for review). Several studies provide evidence for prescribing exercise as an effective medicine in the treatment of several chronic diseases including depression (Josefsson et al., 2014), Parkinson's disease (Konerth & Childers, 2013), Multiple Sclerosis (Latimer-Cheung et al., 2013), and various cancers (George et al., 2011; Kenfield et al., 2011; see Pedersen & Saltin, 2015 for full review). As such, exercise may prove efficacious for augmenting endogenous repair in the brain of alcohol abstinent patients, and especially those who drank in a binge pattern.

Physical exercise provides many benefits that enhance overall health (Cotman et al., 2007), as well as cognition across the lifespan (Bass et al., 2013; Erickson & Kramer, 2009; Pajonk et al., 2010). Exercise has vast benefits to the cardiovascular system including increased blood flow capacity, nutrient delivery, cardioprotective proteins, and angiogenesis, and decreased baseline blood pressure (Black et al., 1990; Noble et al., 1999; Pescatello et al., 2004; Swain et al., 2003). Studies have linked physical activity to improved immune system function and anti-inflammatory processes in the CNS (Kohut et al., 2006; Skalicky & Viidik, 1999). Exercise helps develop and maintain cardiorespiratory, musculoskeletal, and neuromotor fitness in health adults (Garber et al., 2011). Additionally, exercise increases muscle mitochondrial proteins (Moraska et al., 2000) and help bolster antioxidant activity (Alessio et al., 2005).

Exercise is a powerful promoter of neuroplasticity, which is believed to underlie its significant benefits for cognition and brain health, and potential to restore the binge damaged brain. In contrast to binge alcohol intoxication, exercise increases proliferation of glia in various regions (de Senna et al., 2011; Ehninger & Kempermann, 2003; Li et al., 2005), and influences all aspects of new neuron maturation, including cell proliferation, survival, and neuronal differentiation in the hippocampal dentate gyrus (Brown et al., 2003; Kobilo et al., 2011; van Praag et al., 1999a; van Praag et al., 1999b; Wu et al., 2008). The ability to create new cells and help them survive is important for the brain's ability to respond to damage and is a key mechanism behind the restorative potential of exercise. Exercise also supports the quality of new and existing neurons and signal transmission by increasing the density of dendritic spines and enhancing long-term potentiation (LTP) in the DG (Farmer et al., 2004; Redila & Christie, 2006; van Praag et al., 1999a). These cellular and signaling changes are associated with improvements in cognition, including learning and memory formation (Gomez-Pinilla et al., 2008; van Praag et al., 1999a; van Praag et al., 1999b)

Improved trophic factor signaling has been considered as the most popular hypothesis to explain the positive effects of physical activity on the brain and cognition (see Phillips et al., 2014 for review). Many studies have demonstrated that physical exercise of varying durations upregulates both BDNF (Ding et al., 2011; Neeper et al., 1995; Neeper et al., 1996) and IGF-1 (Carro et al., 2001; Ding et al., 2006; Trejo et al., 2001) in several brain regions, both of which appear to be key mediators of the effects of exercise on the brain, neurogenesis in particular (Farmer et al., 2004; Trejo et al., 2001; Yu et al., 2014). Increases in the high affinity receptors TrkB and IGF-1R, for BDNF and IGF-1 respectively, have also been found following exercise (Ding et al., 2011; Vaynman et al., 2003). Concurrently, physical exercise

elevates the expression of other genes involved with signal transduction pathways (MAPK/ERK) and transcription (CREB), which influence expression and activation of BDNF and IGF-1 (Arthur et al., 2004; Choi et al., 2008; Vaynman et al., 2003). Considering all of the effects of BDNF and IGF-1 on a variety of functions including growth, proliferation, survival and neuroprotection, it is likely that increases in both responsible for many of the benefits of exercise. Indeed, studies blocking the effects of BDNF and IGF-1 have shown to either reduce or eliminate the positive effects of physical activity, including neurogenesis (Ding et al., 2006; Trejo et al., 2001; Vaynman et al., 2004). There is also evidence that exercise enhances neurogenesis by reducing the number of proinflammatory microglia (Vukovic et al., 2012) and increasing the number of proneurogenic microglia that provide trophic support (Kohman et al., 2012; Ziv et al., 2006).

Physical exercise provides many benefits to cell survival and protection that directly contrasts many of the negative consequences of chronic alcohol intoxication, such as opposing effects on trophic support (BDNF and IGF-1) for example. Therefore it is possible that exercise may provide an effective treatment for healing the alcohol-damaged brain. Using the 4-day binge model of alcohol dependence, we previously found that two weeks of voluntary exercise prior to alcohol administration was neuroprotective against alcohol-induced loss of DG granule neurons in females (Leasure & Nixon, 2010). More recently, we found that binge consumption resulted in a persistent loss of DG granule neurons in females 35 days after the last dose of alcohol, an effect that was reversed by four weeks of voluntary exercise (Maynard & Leasure, 2013). Moreover, exercise has been shown to help the brain recover from developmental alcohol exposure (Helfer et al., 2009; Redila et al., 2006; Thomas et al., 2008) and can counteract the deleterious effect of alcohol on neural stem cell

proliferation when access is given coincident with alcohol self-administration (Crews et al., 2004b). The mechanism(s) via which exercise provides neuroprotection and neurorestoration to the binge-damaged brain has not been thoroughly investigated. In chapter five, we investigate the duration of time necessary for exercise to restore the loss of granule neurons in the DG of the female brain and the mechanisms that underlie that restoration.

**Project Overview**

Despite evidence in humans indicating that females are more sensitive to the neurotoxic effects of alcohol and resulting medical consequences, little research has been done to investigate this effect, while even fewer studies have investigated the effects of alcohol on both males and females in the same experiments. Therefore, there is a need to assess the deleterious effects of alcohol on both males and females at the same time, and to develop a rodent model that mirrors the enhanced vulnerability in females to alcohol neurotoxicity that is seen in humans. Developing such a model, and understanding the mechanisms that underlie enhanced female vulnerability, may shed light on the progression of AUD development in women and inform strategies. Additionally, support for physical exercise as an effective medical treatment for the many negative consequences of alcohol intoxication has been shown, however the mechanisms that underlie these benefits are not well understood. The current dissertation used a rat model of an AUD known to cause neurodegeneration to examine potential sex differences in alcohol-induced neurodegeneration in the hippocampus, corresponding cognitive deficits, and altered trophic support as a potential mechanism of increased vulnerability in females. Additionally, the current dissertation examines increased trophic support as a potential mechanism of exercise-driven recovery from binge alcohol-induced neurodegeneration. The overarching hypothesis for this project is that **females are more vulnerable than males to the structural and functional consequences of binge alcohol exposure, and that exercise repairs damage due to binge alcohol.**

**Aim 1**: Determine whether the binge alcohol exposure results in significantly greater cell death and reduced cell birth in the hippocampus of females than males. (Chapt2)

We hypothesize that binge alcohol exposure will result in a significantly greater reduction of hippocampal dentate gyrus granule neurons and proliferating cells, and a significantly greater increase in cell death in female but not male rats. Remaining DG granule neurons (labeled with a Nissl stain), proliferating cells (Ki67+ cells) and cell death (FJB+ cells) will be quantified following alcohol exposure using immunostaining, brightfield microscopy, and stereology.

**Aim 2**: Determine whether cognitive deficits corresponding to binge alcohol-induced neurodegeneration are more severe in females than males (Chapter 3).

We hypothesize that binge-induced neurodegeneration in the hippocampus and associated cortical regions will correspond with significantly greater spatial and recognition memory deficits in female but not male rats. Spatial memory deficits will be determined using the Morris water maze (MWM) and recognition memory deficits using the novel object recognition (NOR) task following alcohol exposure.

**Aim 3**: Determine whether decreased trophic support underlies increased vulnerability to binge alcohol-induced neurodegeneration in females (Chapter 4).

We hypothesize that binge-induced neurodegeneration will correspond with significantly greater decreases in trophic support and signaling molecules in the hippocampus of female but not male rats. Alcohol's effects on trophic support and signaling molecules will be determined using the Western blotting analytical technique for BDNF, TrkB, IGF-1R, CREB, pCREB.

**Aim 4**: Determine the length of time needed for exercise-driven hippocampal recovery and altered protein expression associated with the restoration of the female rat dentate gyrus granule cell layer following binge-alcohol induced damage. (Chapter 5)

We hypothesize that voluntary access to running wheels will reverse the binge-induced reduction of dentate gyrus granule neurons in female rats. We further hypothesize that exercise restoration of the granule cell layer will be associated with an increase in trophic support and signaling molecules compared to sedentary animals with persistent damage. Remaining DG granule neurons (labeled with a Nissl stain) will be determined using immunohistochemistry and stereology. Exercise effects on trophic support and signaling molecules will be determined using the Western blotting analytical technique for BDNF, TrkB, IGF-1R, CREB, pCREB, and the microglia marker Iba1.

## Chapter 2: Evidence for enhanced vulnerability in females in a model of binge alcohol-induced neurodegeneration

Although not traditionally classified as a neurodegenerative disease due to its preventable nature, alcohol use disorders (AUDs) and specifically the characteristic excessive consumption of alcohol, result in significant volume loss in cortical and subcortical brain structures that underlies a variety of cognitive deficits in alcoholics (Crews & Nixon, 2009; de la Monte & Kril, 2014; Pfefferbaum et al., 1992; Sullivan et al., 2000b). Chronic alcohol intoxication causes neurodegeneration in various areas of the brain, while corticolimbic areas, and more specifically the hippocampus, are particularly sensitive to insult (Agartz et al., 1999; Beresford et al., 2006; Sullivan et al., 1995). In the hippocampus, the effects of alcohol on neurogenesis, including disruption of neural stem cell's ability to form colonies, proliferate, differentiate, and survive, contribute to the pathology of physiological and cognitive consequences of alcohol (Crews & Braun, 2003; Nixon & Crews, 2002; Tateno et al., 2005). The mechanisms of alcohol-induced cell death and inhibition of cell birth and survival are complex but appear to involve proinflammatory cytokines, oxidative stress, and loss of trophic factors, mechanisms that overlap with many neurodegenerative diseases (Crews, 2008; Crews & Nixon, 2009).

Like many diseases, alcohol affects women and men differently, yet compared with alcoholic men, alcoholic women have received little research attention. Men have an increased risk of developing alcohol dependence and higher rates of alcoholism than women (Kessler et al., 1994; York & Welte, 1994), yet evidence suggests women progress faster to alcohol dependence (Hernandez-Avila et al., 2004; Schuckit et al., 1995; Schuckit et al., 1998). Despite fewer years of heavy drinking, women are more vulnerable than men to the

adverse medical consequences of heavy alcohol consumption, including cardiomyopathy (Fernandez-Sola & Nicolas-Arfelis, 2002), peripheral neuropathy (Ammendola et al., 2000), and cirrhosis of the liver (Loft et al., 1987). Evidence also indicates that women are more sensitive to the neurotoxic effects of alcohol than men (Hommer et al., 2001; Hommer, 2003), including larger reductions in hippocampi volume (Agartz et al., 2003), additionally, cognitive and structural impairments in the brain emerge faster in the course of the disease for women than for men (Mann et al., 1992; Nixon et al., 1995). The accelerated course of AUDs in females, wherein medical consequences emerge faster over the course of the disease despite a shorter history of alcohol abuse compared to males, is labeled the "telescoping effect" (Piazza et al., 1989).

Despite evidence indicating that females are more vulnerable to alcohol-induced brain damage than men, few experimental studies have directly compared neurodegeneration in males and females. Therefore the goal of this set of experiments was to examine sex differences in alcohol-induced neurodegeneration using a well-established AUD model (Collins et al., 1996; Majchrowicz, 1975). This model mimics the level of intoxication seen in binge drinking humans and causes neurodegeneration in brain regions consistent with human chronic binge drinkers: the olfactory bulbs, frontal lobes, and multiple corticolimbic structures including the agranular insular cortex, anterior piriform cortex, perirhinal cortex, and entorhinal cortex, as well as the hippocampal dentate gyrus (Collins et al., 1996; Corso et al., 1998; Crews et al., 2004a; Hamelink et al., 2005; Obernier et al., 2002b). However, remarkably little is known about the consequences of binge alcohol consumption in females within this model because the vast majority of the work has focused on males. Using females with this model, we have previously shown binge exposure results in alcohol-induced cell

death in brain regions consistent with males, a decrease in cell proliferation, and a significant decrease of granule neurons in the hippocampal dentate gyrus (Leasure & Nixon, 2010). More recently, we have shown that the loss of DG granule neurons in females persist 35 days after the last dose of alcohol. Currently, no studies have directly compared neurodegeneration between males and females using this model.

In the current set of experiments, neurodegeneration is defined and measured as a decrease in Nissl-stained dentate gyrus granule neurons, an increase in cell death (FJB+ cells) in the dentate gyrus, entorhinal/perirhinal cortex, and piriform cortex, and a decrease in cell proliferation (Ki67+ cells) in the dentate gyrus. We hypothesize that females will have a greater relative reduction of DG granule neurons and Ki67+ cells, and increase in FJB+ cells compared to males, indicating greater vulnerability to binge alcohol.

## METHODS

### *Animals*

A total of 55 (32 females and 23 males) Long-Evans rats aged nine weeks were used across all experiments. Females were 175-200g and males 275-300g upon arrival, were group housed by sex in clear Plexiglas cages and given 1 week to acclimate to vivarium conditions, which includes *ad libitum* rat chow and water and a reversed light/dark cycle (lights off at 9:00/ on at 21:00). Prior to beginning the experiments, all rats were tamed by gentle handling to acclimate them to the experimenters and make them amenable to gavage. All experimental procedures were conducted in accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health and approved by the University of Houston Institutional Animal Care and Use Committee (protocol number 14-013).

Twenty-eight rats were divided into four groups of comparable weights in a 2x2 design to compare the effects of Sex (Female, Male) and Diet (Binge, Control) on remaining dentate gyrus granule neurons, Ki67+ cells, and FJB+ cells. The remaining twenty-seven rats were included in three control experiments described in detail later.

***Binge-Alcohol Administration Model***

Alcohol was administered via intragastric gavage according to a previously used paradigm modified from Majchrowicz (1975). This model was chosen because it mimics the high blood alcohol levels of binge-pattern drinkers (Hunt, 1993; Tomsovic, 1974) and for its well documented neurodegeneration profile (Crews & Nixon, 2009; Kelso et al., 2011). During binge exposure, food was removed from all animals but water was always available. Rats were gavaged with alcohol diet (25% alcohol w/v in vanilla Ensure™; Abbot Laboratories, Columbus, OH) or isocaloric control diet (Dextrose w/ vanilla Ensure™) every 8 hours for 4 days, starting on the first day of the experiment (12 doses total). The initial dose for each animal was 5g/kg and caused significant intoxication; further doses were then determined based on a 6-point behavioral intoxication scale (Table 2.1). Each point on the scale corresponds to an accompanying dose of alcohol, such that the greater the observed behavioral intoxication, the smaller the subsequent dose. Control animals received isocaloric diet at the average volume given to the alcohol group of the same sex to control for neuroplastic changes associated with caloric intake (Gillette-Guyonnet & Vellas, 2008; Loncarevic-Vasiljkovic et al., 2012).

**Table 2.1 Behavioral Intoxication Scale**

| Intoxication Score | Behavioral Attributes | Dose (g/kg) |
|---|---|---|
| 0 | Normal animal | 5 |
| 1 | Hypoactive, mildly ataxic | 4 |
| 2 | Ataxic, elevated abdomen | 3 |
| 3 | Ataxic, absence of abdominal elevation, delayed righting reflex | 2 |
| 4 | Loss of righting reflex, retain eye blink reflex | 1 |
| 5 | Loss of righting reflex, loss of eye blink reflex | 0 |

Table 2.1 Animal's CNS depression (intoxication) was scored based on behavioral score to determine alcohol dose

***Blood Alcohol Concentration***

For all alcohol treated animals, blood alcohol concentration (BAC) was determined from tail blood samples taken 90 minutes after the morning dose on day 3 (dose 7), during the period of peak BAC (Morris et al., 2010b). For an additional 12 alcohol treated animals, blood samples were collected via saphenous vein 90 minutes after the third, fifth, and seventh doses of alcohol to track BAC progression during the binge and determine potential differences between doses and sexes. Although tail bleeds are the most common technique for blood collection, this method was chosen for the latter groups of animals because of the ability to take multiple blood samples over a short period of time with minimal duress to the animal. Both methods of collection provided BACs consistent with those typically found in this model. Previous work has also shown no differences in stress-associated changes between the two methods of collection or an increase in corticosterone over time after multiple collections (Abatan et al., 2008). Samples were centrifuged, and then stored at -20°C until analysis. Serum was extracted and BEC determined using an AMI Analyzer based on external standards (Analox, Waltham, MA).

### *Estrous Cycle Monitoring*

Previous research has shown that levels of progesterone and estrogen improve outcome in experimental models of brain injury for female rats (Roof et al., 1993). The estrous cycle can also acutely influence cell genesis, with the highest number of new cells born during proestrous (Tanapat et al., 1999). We have previously determined that stage of estrous has no effect on remaining granule neurons or cell survival 35 days after the last dose of alcohol in this model (Maynard & Leasure, 2013). However, given the shortened time frame of the present experiment, stages of estrous were tracked for eight days in a separate group of five female rats prior to starting the alcohol paradigm. Each rat was then given the first dose of alcohol diestrous. To determine stage of estrous, vaginal smears were taken once each day between 8:00 and 9:00 A.M. Each sample was placed onto a slide, stained with cresyl violet and coverslipped. Samples were viewed under a light microscope at 10x magnification and the determination of the stage of estrous (proestrous, estrous, metestrous, diestrous) was made consistent with prior methods (Marcondes et al., 2002).

### *Tissue Collection for Immunohistochemistry*

Eight hours after the last dose of alcohol or control diet, rats were given an overdose of anesthetic (cocktail of ketamine, xylazine and acepromazine) and intracardially perfused with cold saline, followed by 4% paraformaldehyde until the upper body was stiff. Brains were removed, post-fixed overnight, and then refrigerated in 30% sucrose. Brains were cut in 50 µm coronal sections on a freezing microtome. Sections were stored in cryprotectant in 96-well microtiter plates at -20°C until further processing.

***Immunohistochemistry***

To assess cell proliferation, every sixth serial section was processed for Ki67 using our standard immunohistochemistry (IHC) protocol (Maynard & Leasure, 2013). Free floating sections were rinsed with 0.1 M tris-buffered saline (TBS) three times at room temperature for 10 minutes each. Sections were then quenched for 30 minutes at room temperature in 0.6% hydrogen peroxide (to exhaust the activity of endogenous peroxidases) followed by three 10-minute washes in TBS. Next, sections were treated with a 30 minute citrate buffer antigen retrieval step at 80° C, and then allowed to return to room temperature for 5 minutes. Following three additional TBS washes, sections were blocked for 60 minutes in 3% normal donkey serum (Sigma-Aldrich, St. Louis, MO, USA), followed by incubation at 4° C for 72 hours in primary antibody (rabbit anti-Ki67, Vector Laboratories, Burlingame, CA; 1:1800). After two TBS rinses for 15 minutes each and 15 minutes blocking in 3% normal donkey serum, sections were incubated overnight at room temperature in secondary antibody (biotinylated donkey anti-rabbit, Jackson ImmunoResearch Laboratories, West Grove, PA, USA; 1:250). Next, sections were rinsed three times in TBS for 10 minutes each, treated for 60 minutes in avidin-biotin complex (ABC, Vector Labs, Burlingame, CA, USA) and then rinsed three times in TBS for 10 minutes each. Sections reacted and were visualized with diaminobenzidine (DAB) and then rinsed four times in TBS for 10 minutes each, before being mounted onto gelatinized slides and allowed to dry overnight. Once dried, all brain sections were counterstained with Methyl Green, cleared in xylene, and coverslipped using Permount. Slides were coded such that the investigators performing cell counts were blind to experimental condition. The same slides were also used for quantification of dentate gyrus granule neurons using the methyl green Nissl counterstain.

### *Quantification of Proliferating Cells (Ki67+ cells)*

The number of Ki67-positive (Ki67+) cells in the dentate gyrus was quantified using a Nikon Eclipse 80i upright microscope. The granule cell layer (GCL) and subgranular zone (SGZ) of the DG were traced using the 10x objective and cells counted using a 40x oil objective. Each labeled soma in the granule cell layer and subgranular zone (defined as zero to two cell bodies from the inner molecular layer) was counted in every sixth section from Bregma -1.88 µm and ending at Bregma -6.04 (Paxinos & Watson, 2009), using a 40 x oil objective. This resulted in 10-12 sections per brain.

### *Quantification of Remaining Dentate Gyrus Granule Neurons*

The number of methyl green Nissl stained granule neurons in the hippocampal dentate gyrus was determined by unbiased stereological methods using the optical fractionator method applied via an automated stereology system (StereoInvestigator, MicroBrightField, VT, USA). Using a Nikon Eclipse 80i upright microscope, the granule cell layer of the DG was traced using the 10x objective and cells were counted with two-dimensional counting frames using a 100x oil objective. The average mounted section thickness was approximately 37µm, thus top and bottom guard zones were set at 5µm each, for an optical dissector height of 27µm. Granule neurons were counted in every twelfth section in a single hemisphere beginning at the earliest emergence of the DG at Bregma -1.88 µm and ending at Bregma -6.04 (Paxinos & Watson, 2009). This resulted in 5–6 sections per brain. The counting frame size was 40 x 40 µm and the grid size was 200 x 200 µm. The number of granule cell profiles was totaled and used to estimate the total number of granule neurons per dentate gyrus consistent with previously published methods (Maynard &

Leasure, 2013). Coefficients of error (CE) were calculated using the method of Gunderson and were less than 0.05 for all groups (Gundersen et al., 1999)

### *FluoroJade B Labeling and Quantification*

Fluorojade B staining of degenerating cells was conducted following published methods (Leasure & Nixon, 2010; Schmued & Hopkins, 2000). Every twelfth section from Bregma -1.80 µm and ending at Bregma -6.04 (Paxinos & Watson, 2009) was mounted to Superfrost Plus ® slides (Fisher Scientific, Waltham, MA) and left to dry overnight. Slides were processed through graded alcohols, rinsed in distilled water, and then incubated in 0.06% potassium permanganate for 10 minutes on a shaker. Slides were then washed in distilled water for one minute before being incubated in FJB and DAPI (41-6-diamidino-2-phenylindole counterstaining to visualize nucleic material) in the dark for 20 minutes on a shaker. After three additional washes in distilled water, sections were air dried and then coverslipped in Cytoseal 40 ® (Thermo Fisher Scientific, Waltham, Massachusetts). Quantification of FJB-positive (FJB+) cells was conducted on a Nikon Eclipse 80i upright microscope fitted with epifluorescence including a 488λ cube for blue light excitation. Regions of interest were traced using a 10x objective and every FJB+ cell within those regions was counted using a 40x oil objective. FJB+ cells were counted in one hemisphere of the hippocampal dentate gyrus (combined GCL and SGZ) from Bregma -1.88 µm and ending at Bregma -6.04 µm and entorhinal/perirhinal cortex and piriform cortex from Bregma -3.12 µm and ending at Bregma -4.52 µm (see Figure 2.1 adapted from Paxinos & Watson, 2009), following previously published methods with this model (Leasure & Nixon, 2010). FJB+ cells are reported as the mean number of cells per section for each brain region.

**Figure 2.1 Regions included in quantification of cell death and birth**

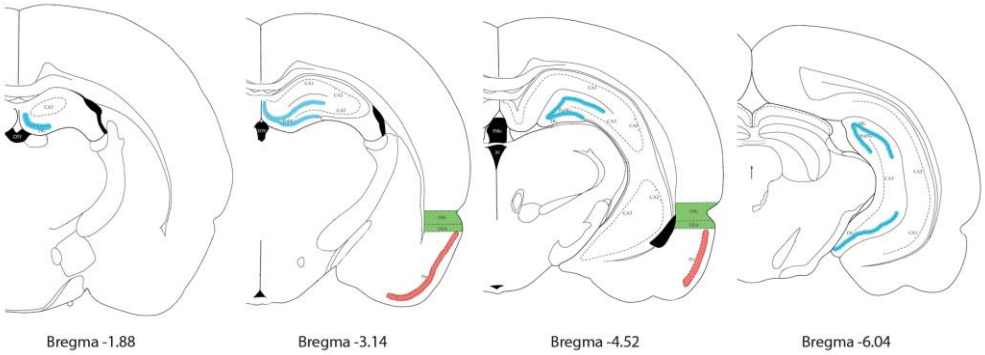


Figure 2.1 Coronal sections illustrating areas for quantification of quantification of markers of cell birth and death. Granule neurons were quantified in the dentate gyrus and Ki67+ and FJB+ cells were quantified in the DG and SGL highlighted in blue. FJB+ cells were quantified in the entorhinal and perirhinal cortex highlighted in green, and piriform cortex highlighted in red. Images adapted from Paxinos and Watson (2009).

### *Statistical Analyses*

Data were analyzed and graphed using Excel 2014 (Microsoft Office Excel, WA, USA) and SPSS Statistics 17.0 (IBM SPSS Statistics, IL, USA). BACs were analyzed with t-tests or one-way ANOVA. Comparisons of granule neurons of more than two groups within sex were analyzed by one-way analysis of variance (ANOVA) and cell counts for DG granule neurons, FJB+ and Ki67+ cell were analyzed by two-way ANOVA with post hoc tests as appropriate. Behavioral scores were analyzed with appropriate non-parametric tests.

## RESULTS

### *Behavioral Response to Binge Alcohol Administration*

Measures of intoxication across all experiments are shown in Table 2.2, and are similar to those reported in past studies with this model (Maynard & Leasure, 2013; Morris et al., 2010b). To determine if there was a difference in BACs between males and females and the three time points that samples were collected, a two-way ANOVA for Sex, Dose, and the Sex x Dose interaction for BAC was analyzed. This revealed no significant main effect of Sex or Sex x Dose interaction, indicating no sex differences in BAC during binge exposure. However, a significant main effect of Dose [$F_{(2,27)} = 13.862$, $p < .0001$], followed by planned

post-hoc *t*-tests indicated a significant decrease in BAC for both sexes by Dose 7 ($p < .0001$), an indication of the development of tolerance. Despite no differences in BAC, Mann-Whitney non-parametric tests indicated males appeared significantly more behaviorally intoxicated on average than females ($p < .05$) and received a significantly smaller average dose (g/kg) of alcohol per day ($p < .05$). This observation prompted a yoke-dose control experiment described below.

***Behavioral Response to Binge Alcohol Administration (Yoked Control Experiment)***

Dose of alcohol in this binge model is determined based on behavioral intoxication, thus since males appeared more behaviorally intoxicated, they ultimately ended up receiving less alcohol (g/kg) than females (Table 2.2). To control for the possibility that a sex difference in remaining granule neurons was due to female rats receiving higher doses of alcohol, a separate group of ten rats (6 females and 4 males) were included, wherein females were yoked to the average male alcohol dose and males were yoked to the average female alcohol dose. Thus, instead of doses being determined by behavioral intoxication, they were determined by the average dose of opposite sex counterparts. For example, if the average female dose was 2.5 g/kg for the 8$^{th}$ dose but the yoked male had an intoxication score of 1, it would receive the 2.5 g/kg dose of alcohol instead of a 4 g/kg dose in accordance with the intoxication scale. A yoked animal with an intoxication score of 5 however, received no dose of alcohol instead of the average dose, in order to avoid mortality.

A two-way ANOVA comparing BACs of typically binged females and binge yoked females revealed a significant main effect of Group [$F_{(1,29)} = 12.303$, $p < .001$] however no significant Group x Dose interaction [$F_{(2,29)} = .202$, $p = .818$] indicating that the typically binged females had significantly higher BACs during binge exposure than binge yoked

females. In line with this, an independent samples *t*-test revealed that binge yoked females behaved significantly less intoxicated on average than typically binged females [$t_{(12)} = 2.851$, $p > .05$]. For males, a two-way ANOVA comparing BACs of typically binged males and binge yoked males revealed no significant main effect of group [$F_{(1,27)} = 3.583$, $p = .069$], nor a significant Group x Dose interaction [$F_{(2,27)} = 1.458$, $p = .251$]. Non-parametric tests revealed binge yoked males did not behave more intoxicated than the typically binged males.

**Table 2.2 Intoxication measures for chapter two**

| Sex | Group | Intoxication behavior | Dose (g/kg/day) | BAC (mg/dl) Dose 3 | BAC (mg/dl) Dose 5 | BAC (mg/dl) Dose 7 |
|---|---|---|---|---|---|---|
| **Female** | Binge | 1.9 ± 0.1 | 9.2 ± 0.3 | 377.8 ± 16.8 | 401.8 ± 12 | 275.3 ± 21.6 |
| | Diestrous | 1.9 ± 0.1 | 9.4 ± 0.2 | # | # | 298.1 ± 14.6 |
| | Yoked | 1.0 ± 0.3* | 7.4 ± 0.3 | 309.1 ± 9.8 | 336.6 ± 24.4 | 180.7 ± 42.2 |
| **Male** | Binge | 2.5 ± 0.1* | 7.4 ± 0.3* | 347.2 ± 13.1 | 402 ± 31.9 | 259 ± 35.5 |
| | Yoked | 2.1 ± 0.4 | 9.2 ± 0.3 | 361 ± 32.6 | 429.4 ± 33.1 | 372.5 ± 35.2 |

Table 2.2 Measures of various intoxication parameters of the Majchrowicz model. # Blood samples were not taken from animals after dose 3 and 5 in the estrous cycle experiment. No significant differences in BACs were observed despite differences in intoxication behavior and dose per day in males compared to females *p< 0.05.

***Binge exposure decreased the number of dentate gyrus granule neurons in females***

Binge alcohol exposure was associated with a significant 13% decrease in remaining DG granule neurons in females, but not males (Figure 2.2B). A two-way ANOVA revealed a significant main effect of Sex [$F_{(1,24)} = 19.977$, $p < .0001$, $\eta p^2 = .454$], Diet [$F_{(1,24)} = 5.015$, $p < .05$, $\eta p^2 = .173$] and a significant Sex x Diet interaction [$F_{(1,24)} = 4.511$, $p < .05$, $\eta p^2 = .158$]. This indicated that females but not males had significant neurodegeneration in this hippocampal region.

To control for the possibility that neuron loss in the DG was linked to stage of estrous, remaining granule neurons were quantified in a separate group of females that all began binge exposure during diestrous. Additionally, to determine whether the larger daily dose of alcohol in females compared to males was responsible for the reduction of granule

neurons in females, remaining granule neurons were quantified in the yoked binge groups. A one-way ANOVA comparing remaining granule neurons in female control, typically binged, yoked binge, and diestrous binge animals revealed a significant main effect of group [$F_{(3,22)}$ = 5.561, $p$ < .005, $\eta p^2$ = .431]. Bonferroni corrected post-hoc comparisons revealed that the all of the female binged groups had significantly fewer remaining granule neurons than female controls (Figure 2.2C; $p$<.05 for all comparisons). However, there were no significant differences between the different female binge-exposed groups. For males, a one-way ANOVA comparing remaining granule neurons in male control, typically binged, and yoked binge animals revealed no significant main effect of group [$F_{(2,14)}$ = .045, p = .956, $\eta p^2$ = .006]. These results indicated that binge-exposed females had a significant reduction of DG granule neurons and similar BACs despite controlling for the estrous cycle, and receiving the same dose of alcohol as the males, yet binge-exposed males had no corresponding reduction when give the same dose of alcohol as the females (Figure 2.2).

**Figure 2.2 Binge exposure decreased dentate gyrus granule neurons in females**

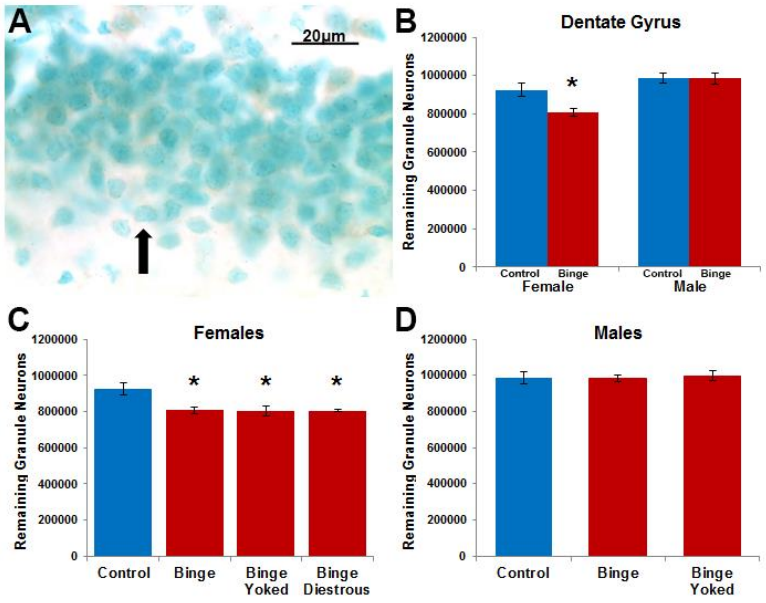


Figure 2.2 Remaining dentate gyrus granule neurons were quantified following binge exposure using Nissl-stained cells (A). Results indicate a binge-induced decrease of granule neurons in female but not male animals compared to controls (B), even after controlling for estrous cycle and dose of alcohol (C, D). *$p$< 0.05.

***Binge exposure resulted in reduced proliferation of progenitor cells in the dentate gyrus***

Ki67-positive (Ki67+; a marker of cell proliferation) cells were observed in clusters along the SGZ (Figure 2.3B-E). A two-way ANOVA for Sex, Diet and the Sex x Diet interaction revealed a significant main effect of Sex [$F_{(1,23)} = 11.801$, $p < .05$, $\eta p^2 = .339$] and Diet [$F_{(1,23)} = 22.263$, $p < .05$, $\eta p^2 = .492$] but no significant Sex x Diet interaction [$F_{(1,23)} = .090$, $p = .767$, $\eta p^2 = .004$]. A single binge exposure resulted in a significant decrease in the number of proliferating cells Ki67+ cells) in both sexes compared to control animals, however females had a larger (non-significant compared to binge males) percentage loss of Ki67+ cells (47%) compared to female controls than the loss in binge-exposed males (38%; Figure 2.3A). Additionally, a significant main effect of sex indicated that males on average had more Ki67+ cells than females regardless of binge exposure. It has previously been reported that there are no sex differences in basal levels of proliferation, however males have greater immature neuron production than females (Hillerer et al., 2013).

**Figure 2.3 Binge exposure decreased cell proliferation in males and females**

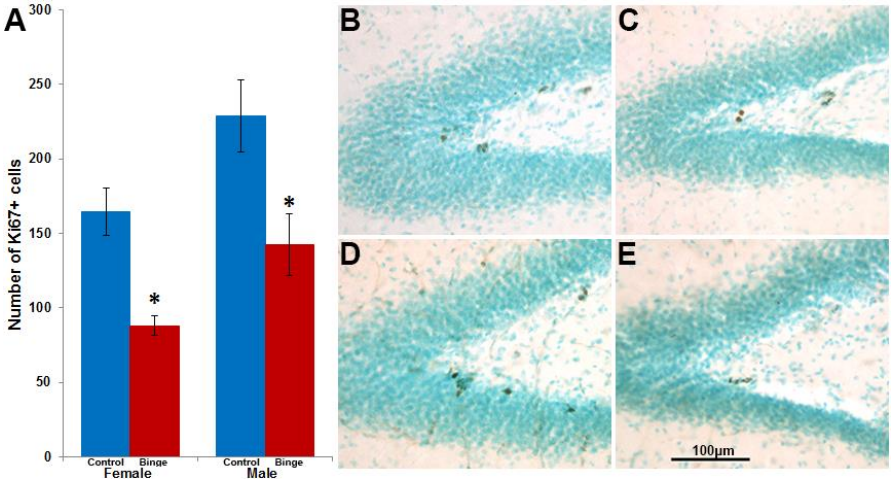


Figure 2.3 Binge exposure decreased the number of Ki67+ cells in the dentate gyrus of alcohol exposed rats (A). Representative photomicrographs show Ki67+ cells along the SGZ of the DG in female control (B), female binge (C), male control (D), and male binge (E) rats. *p< 0.05 alcohol-exposed versus controls.

***Binge exposure resulted in more cell death in the dentate gyrus for females than males***

Cell death has been consistently observed in this model in male and female rats, therefore FJB labeling was examined in the hippocampus and associated cortical regions (Entorhinal/perirhinal and Piriform cortices). Similar to past reports, binge alcohol exposure resulted in FJB+ cells in expected locations with little to no FJB+ cells observed in any regions of control female and male animals (Kelso et al., 2011; Leasure & Nixon, 2010). Binge exposure resulted in an increase in FJB+ cells in each region for both males and females compared to control animals (Figure 2.4), which is supported by main effects of Diet in all regions examined (detailed below). However, two-way ANOVA revealed that binge exposure affected the hippocampus of females differently than males. In the dentate gyrus, binge exposure resulted in a significantly greater increase in FJB+ cells in females than males as revealed by significant main effects of Diet [$F_{(1,23)} = 68.721$, $p < .0001$, $\eta p^2 = .749$] and Sex [$F_{(1,23)} = 4.331$, $p < .05$, $\eta p^2 = .158$] as well as a significant Sex x Diet interaction [$F_{(1,23)} = 5.104$, $p < .05$, $\eta p^2 = .182$].

In the entorhinal/perirhinal cortex, binge exposure increased the number of FJB+ cells in both males and females over controls reflected by the main effect of Diet [$F_{(1,23)} = 63.469$, $p < .001$, $\eta p^2 = .734$]. However, there was no sex differences in the number of FJB+ cells revealed by no main effect of Sex or a significant Sex x Diet interaction [$F_{(1,23)} = .741$, $p = .398$] (Figure 2.4B). A similar pattern was observed for the piriform cortex, ANOVA revealed a significant main effect of Diet [$F_{(1,23)} = 102.783$, $p < .001$, $\eta p^2 = .817$], but no main effect of Sex or a significant Sex x Diet interaction (Figure 2.4C). Thus, binge exposure had a sex-specific effect on alcohol induced FJB labeling of degenerating cells in the hippocampus (Figure 2.4A).

**Figure 2.4 Cell death in the hippocampus and associated cortical regions**

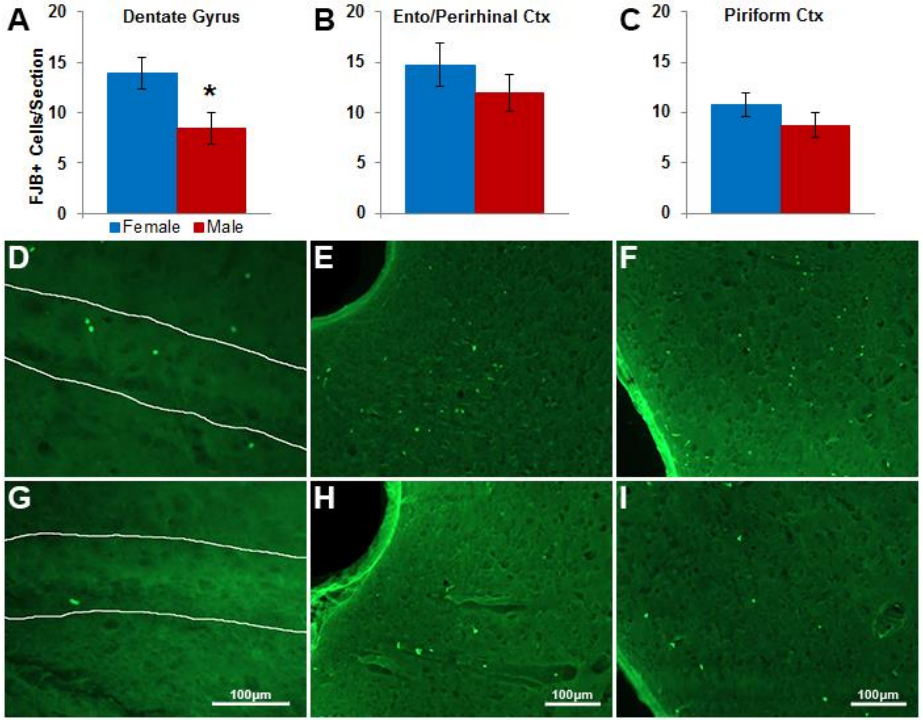


Figure 2.4 Binge exposure induced cell death in all regions as evidenced by FJB labeling (A,B,C). As all control groups averaged 0.4 cells per section or less, only binge groups are graphed. Binge exposure increased the number of degenerating cells (FJB+ cells) in the dentate gyrus of females compared to males (A), but not in the entorhinal/perirhinal cortex and piriform cortex (B, C). Representative pictographs are shown for female binge rats for the dentate gyrus (D), entorhinal/perirhinal cortex (E), and piriform cortex (F), and for male binge rats for the dentate gyrus (G), entorhinal/perirhinal cortex (H), and piriform cortex (I). *p< 0.05 female versus male.

## DISCUSSION

Alcohol affects men and women differently, with women seemingly more sensitive to the neurotoxic effects and resulting negative consequences of alcohol abuse. Despite this, few studies have investigated sex differences in neurodegeneration following binge alcohol, and even fewer have done so using animal models of AUDs. To our knowledge, this is the first time the 4 day binge model has been used to directly compare the effects of binge-alcohol consumption on neurodegeneration in males and females. The major finding of this work is that the female hippocampus is more sensitive to binge-induced neurodegeneration than the male hippocampus.

First, we found a significant 13% binge-induced reduction of remaining DG granule neurons in female rats compared to controls. These data support our previous finding that 4 days of binge alcohol exposure causes a detectable loss of granule neurons in the female dentate gyrus (Leasure & Nixon, 2010; Maynard & Leasure, 2013). While neurodegeneration was assumed to occur from alcohol-induced cell death in the male hippocampus in this model of binge consumption (Kelso et al., 2011; Obernier et al., 2002a), we are the first to report that males had no significant loss of fully differentiated dentate gyrus granule cells compared to controls. This effect persists even after controlling for the daily dose of alcohol consumed, and controlling for the stage of estrous in females, consistent with our previous findings that binge-induced brain damage is not influenced by the estrous cycle (Maynard & Leasure, 2013). In addition, no differences were seen in blood alcohol concentration between males and females, therefore BAC was likely not responsible for the sexually dimorphic sensitivity to neurodegeneration in the hippocampus. It is interesting that despite similar BAC's, males acted more behaviorally intoxicated, yet received a smaller average daily dose of alcohol.

Second, although a binge-induced increase in FJB labeling of necrotic cell death has been reported in the DG of males (Kelso et al., 2011; Morris et al., 2010a) and females (Leasure & Nixon, 2010), we are the first to report a significantly larger increase of FJB+ cells in the DG of binged-exposed females than males. These data provide further support of the conclusion that the female hippocampus is more sensitive than male's to binge-induced neurodegeneration. Consistent with previous work in this model, binge exposure increased the number of FJB+ cells in the entorhinal/perirhinal cortex and the piriform cortex in both sexes (Kelso et al., 2011; Leasure & Nixon, 2010), however we found no difference in the number of FJB+ cells between binge-exposed males and females for either region. It is

possible that of the regions we investigated, increased female vulnerability in this model is specific to the hippocampus. Despite signs of cell death in the DG of males, the lack of a corresponding loss of granule neurons highlights the importance of looking at *what remains*, and not just *what is lost*.

Third, binge alcohol exposure inhibited the number of proliferating progenitor cells in the dentate gyrus (Ki67+ cells) in both males and females compared to controls. Although not significant, there was a larger (47%) decrease in proliferating cells in binged females compared to control females versus a smaller (38%) decrease in binged males compared to control males. These effects are consistent with previous studies indicating binge alcohol inhibition of cell proliferation and neurogenesis in both males and females (Leasure & Nixon, 2010; Nixon & Crews, 2002). Recent work suggests alcohol affects cell birth as a mechanism of producing detrimental effects on hippocampal integrity (Crews & Nixon, 2009; Morris et al., 2010a). The finding of binge-induced inhibition of proliferating progenitor cells in our current study may have contributed to the loss of replacement or protection of existing cells in females, however it did not appear to have an impact on remaining granule neurons in males.

Our conclusion that the female hippocampus is more vulnerable to alcohol-induced damage than males mirrors previous findings from human alcoholics. Using MRI scans, alcoholic women were found to have smaller hippocampi than alcoholic men, despite having a later onset of heavy drinking (Agartz et al., 2003). Similar support has been found in other animal models of alcohol-induced neurodegeneration. Walls and colleagues evaluated the consequences of repeated chronic alcohol exposure and withdrawal on hippocampal cell death in male and female rats. Using hippocampal slice cultures, they found increased

sensitivity to toxicity and alcohol-induced damage in the CA1 and DG hippocampal subregions in females but not males (Walls et al., 2013). Hippocampal slices from post-natal pups also indicate increased hippocampal sensitivity to alcohol and neurodegeneration in females but not males (Barron et al., 2008). The hippocampus may represent a unique area of the brain that is more sensitive to alcohol in females due to its dynamic nature, or could instead be more representative of alcohol-induced brain damage and sensitivity in females more broadly. Human and animal studies have demonstrated other areas that appear to have a sexually dimorphic vulnerability to alcohol, including the frontal lobes (Schweinsburg et al., 2003), more specifically the prefrontal cortex (Hashimoto & Wiren, 2008; Medina et al., 2008) and anterior cingulate cortex (Wilhelm et al., 2015a; Wilhelm et al., 2015b), in addition to the corpus callosum (Hommer et al., 1996).

While the current series of experiments indicated no reduction of DG granule neurons in males, evidence from imaging studies and post mortem analysis of the brains from alcoholic men indicate significant alcohol-induced reductions in hippocampal volume (Beresford et al., 2006; De Bellis et al., 2000; Sullivan et al., 1995). It is possible that a longer period of binge exposure or repeated binge exposures would produce similar deleterious effects in males as females. However, we have previously found that a subsequent binged timed seven days after the last dose of alcohol did not result in a further cumulative loss of DG granule neurons in females (Maynard & Leasure, 2013). Considering we are the first to report on remaining granule neurons in males after a single binge exposure in this model, the effect of a second binge exposure in males needs further investigation. Our evidence of fewer remaining granule neurons in the female DG after a single binge exposure supports the “telescoping” effect; that is, negative consequences of alcohol consumption

emerge sooner in females after a shorter history of alcohol abuse than males. Despite receiving the same amount of alcohol over the same period of time, female animals exhibited a binge-induced reduction of granule neurons not seen in males.

One intriguing finding is that males had on average more Ki67+ cells than females, regardless of diet, indicating a potential mechanism of neuroprotection. In fact, a recent report indicated that males have a higher basal level of immature neuron production than females (Hillerer et al., 2013). However, because animals were sacrificed eight hours after the last dose of alcohol, this would not be enough time for immature neurons to proliferate, mature, and integrate into the GCL to replace those potentially lost during binge exposure. Additionally, despite a greater on average number of Ki67+ cells in our current study, there was still a robust effect of binge on the number of proliferating cells in males (-38%) and therefore not likely to be a source of neuroprotection. Instead, our finding of a binge-induced loss of granule neurons in females but not males is likely due to differences in the cause of and resiliency to cell death.

Although not directly assessed in this series of experiments, prior work investigating the sexually dimorphic sensitivity to alcohol-induced neurodegeneration has revealed potential mechanisms. For example, men and women respond to stress differently such that inflammatory and immune responsivity to stress is greater in females than males (Gallucci et al., 1993; Spitzer, 1999). Alcohol represents a physiological stressor (Ogilvie & Rivier, 1997) that preferentially activates the HPA axis in females in rodent models as well as in humans (Jenkins & Connolly, 1968; Rivier, 1993, 1996). In line with this, recent evidence indicates females are more vulnerable to the neuroinflammatory effects of alcohol and have a sexually dimorphic neuroadaptive response. First, Walls and colleagues found increased

alcohol and glucocorticoid sensitivity in female hippocampal slices that resulted in cell death, however glucocorticoids blocked all alcohol-induced damage in male cultures (Walls et al., 2013). Other groups have demonstrated that, following chronic alcohol intoxication, females exhibited changes in gene expression of signaling events involved in apoptosis, activation of inflammation, and cell death, compared with neuroprotection in males (Hashimoto & Wiren, 2008; Wilhelm et al., 2015b; Wilhelm et al., 2014). Consistent with this, female alcohol-treated mice express higher cortical levels of inflammatory mediators (e.g. COX-2) and proinflammatory cytokines (e.g. TNF-α) than males (Alfonso-Loeches et al., 2013). The shift from neuroprotection to neuroinflammation in females may be mediated by reactive glia, astrocytes in particular. Following alcohol exposure, female but not male astrocytes exhibit increased expression of pro-inflammatory cytokines and reduced expression of neuroprotective cytokines, indicating that female astrocytes increased inflammatory signaling in the presence of a reduced neuroprotective response (Wilhelm et al., 2015a). The downstream effects of such cellular dysfunction include further inflammation and oxidative stress contributing to neurodegeneration (Claycomb et al., 2013; Wilhelm et al., 2015b). Additionally, glial cells shifted to proinflammatory activation no longer provide trophic factors and other neuroprotective signaling, providing another mechanism of potential cell loss in females due to increased neuroinflammation.

These studies indicate a sexually dimorphic neuroadaptation to binge alcohol exposure characterized by a proinflammatory response in females in contrast to neuroprotective response in males. A possible consequence or even cause of this response to alcohol in females is a void in trophic support, leaving the brain and hippocampus vulnerable to binge-induced damage. Intact trophic support however may provide a source of

neuroprotection from alcohol-induced damage in males. The role of trophic support in the increased sensitivity of the female hippocampus to binge-induced damage and loss of granule neurons is investigated in chapter 4.

## Chapter 3: Evidence for sex-specific cognitive deficits following binge alcohol-induced neurodegeneration

In humans, alcohol dependence is associated with significant structural and functional injury to the brain characterized by significant volume loss in cortical and subcortical brain structures due to shrinkage of both gray and white matter (Kubota et al., 2001; Mann et al., 2001; Pfefferbaum et al., 1992; Sullivan & Pfefferbaum, 2005). Alcohol dependence is also associated with repercussions in several cognitive domains: verbal fluency/language, speed of processing, working memory, attention, problem solving, learning and memory, visuospatial abilities (see Stavro et al., 2013 for review). In fact, alcohol-induced cognitive impairments are the second leading cause of dementia, behind only Alzheimer's disease (Eckardt & Martin, 1986; Saxton et al., 2000). However, it was not until the advent of more sophisticated techniques that studies began to associate cognitive deficits with neurodegeneration within specific brain regions and the integrity of their circuits (Alfonso-Loeches & Guerri, 2011; Zahr et al., 2011). For example, multiple neuropsychological studies have revealed that severe deficits in executive functioning and complex cognitive skills are associated with alcohol-induced damage to the frontal lobes and its connections (Medina et al., 2008; Sullivan & Pfefferbaum, 2005). Alcohol-induced damage to this region is thought to be the cause of impaired judgements, blunted affect, poor insight, social withdrawal, reduced motivation, distractibility, and the attention and impulse-control deficits often seen in alcoholics (Crews & Boettiger, 2009; Oscar-Berman & Hutner, 1993; Parsons, 1993; Sullivan & Pfefferbaum, 2005; Sullivan et al., 2000a; Sullivan et al., 2000b).

The hippocampus is another structure that is sensitive to alcohol-induced neurodegeneration and is associated with a range of cognitive deficits. The compromised

hippocampal integrity and connections to cortical regions (e.g. entorhinal cortex) in alcoholics may underlie some of the behavioral deficits in executive functioning (Beresford et al., 2006; Chanraud et al., 2010) , as well as impairments in learning, memory, and visuospatial abilities (Beatty et al., 1996; Crews et al., 2004a; Pitel et al., 2009; Townshend & Duka, 2005). Through its widespread corticolimbic connections, disruption of the hippocampus can affect many other areas and vice versa. For example, due to its connections with frontal cortical regions, hippocampal damage can affect behaviors associated with the frontal lobes (Godsil et al., 2013) and may have a role in drug addiction (Hyman et al., 2006; Nixon et al., 2011). The hippocampus is highly interconnected with the entorhinal cortex, therefore its damage affects hippocampal integrity and contributes to cognitive deficits (Bott et al., 2013; Harich et al., 2008).

Despite extensive investigations of the cognitive deficits associated with binge drinking and alcohol dependence, the majority of studies have only sampled males. In fact, an extensive meta-analysis of 63 studies on cognitive deficits associated with alcoholism found that only 19% assessed both males and females at a comparable rate, and only 5% studied strictly female samples (Stavro et al., 2013). This is concerning, given that evidence suggests women are more sensitive to the neurotoxic effects of alcohol than men (Hommer et al., 2001; Hommer, 2003), including larger reductions in hippocampi volume (Agartz et al., 2003) and frontal lobe gray and white matter volume and integrity (Medina et al., 2008; Schweinsburg et al., 2003). Additionally, cognitive and structural impairments in the brain emerge faster in the course of the disease for women despite a shorter history and lower total lifetime consumption than men (Mann et al., 1992; Nixon et al., 1995) evidence of a "telescoping" effect (Piazza et al., 1989).

Of the few characterizations of neuropsychological deficits in alcoholic women, older ones seem to indicate women have more mild cognitive deficits than men, although deficits tend to be in the same functional domains (Silberstein & Parsons, 1981). In contrast, more recent characterizations indicate similar standard deviations from controls in alcoholic women as alcoholic men for deficits including executive functioning and visuospatial composites (Sullivan et al., 2002; Sullivan et al., 2000b). However, alcoholic women were at a greater performance disadvantage compared to controls as alcoholic men on short-term memory tests, nonverbal tests in particular (Sullivan et al., 2002; Sullivan et al., 2000b). Alcoholic men on the other hand had greater deficits in gait and balance function than women. An important point to consider for these findings is that alcoholic men in this sample had be abstinent on average for only a month compared to an average of 3.6 months in women. Therefore, it is possible that given similar period of abstinences, impairment may have been even greater in women and in more faculties. Other investigations of sex differences have found that heavy drinking is associated with more impaired response inhibition in women (Nederkoorn et al., 2009). Adolescent binge drinking is also associated with greater impairment on tests of attention, working memory, and visuospatial abilities for females than males (Medina et al., 2008; Squeglia et al., 2011; Squeglia et al., 2012).

Animal models provide an opportunity to link alcohol-induced neurodegeneration with associated cognitive deficits; however findings are dependent on many things, including dose, timing, and administration method of alcohol, as well as the difficulty and various parameters of the behavior test itself. Using the Morris water maze (MWM), a task highly sensitive to detect hippocampal cognitive impairment, previous work revealed spatial reference memory and working memory impairments in males following six months of

chronic alcohol intake associated with hippocampal damage (Arendt et al., 1988), while others report reference memory but not working memory deficits (Lukoyanov et al., 1999). Other studies investigating repeated alcohol treatment just prior to testing have reported disruptions hippocampal dependent learning including context conditioning (Melia et al., 1996) and spatial memory in the MWM in males (Shimizu et al., 1998; White et al., 1998), although impaired spatial learning and memory are not consistently found in this model (Acheson et al., 2001; Markwiese et al., 1998).

The 4-day binge model used in the current series of experiments has also been used to investigate similar cognitive deficits. Following a 4-day binge exposure, male rats showed normal acquisition of spatial reference memory in finding the platform in the MWM, yet were impaired in the reversal learning task (Cippitelli et al., 2010a; Obernier et al., 2002b). In addition, neither of these studies found effects on binge exposure on nonspecific motor function and exploration behavior indicated by the open-field test and elevated plus-maze (EPM). In another study, spatial and non-spatial impairments following binge exposure were found using the object exploration task (OET) in male animals (Cippitelli et al., 2010b). Interestingly, non-spatial object recognition ability was reversed after 10 weeks, but spatial memory remained impaired. Finally, using the same behavior testing methods as the previous studies, impairments in spatial reference memory, reversal learning, and object recognition memory were found in male animals by repeating the 4-day binge exposure four times (Zhao et al., 2013). Although the preceding studies provide evidence of the associations between alcohol-induced neurodegeneration and cognitive deficits, they focused on males and did not include females. Few studies have investigated potential cognitive deficits following binge

alcohol exposure in females, and none have directly compared performance between males and females using the 4-day binge model.

To address this, the current experiments investigated potential sex differences in cognitive deficits using the 4-day binge model of alcohol dependence. Excessive alcohol is known to cause neurodegeneration of the hippocampus and entorhinal cortex (EC) in humans and animals. In addition, impairments in spatial learning and memory, and abnormal response perseveration are commonly seen in alcoholics. The hippocampus and EC are closely interconnected regions, and known to be critically involved in spatial learning and memory formation (Aggleton et al., 2000; Jarrard, 1993). The reciprocal connections between the EC and hippocampus suggest a close functional link. Along with this, evidence indicates that lesions of the hippocampus result in robust and reliable spatial reference memory deficits in the Morris water maze (MWM; Galani et al., 1998; Morris et al., 1982), and that lesions of the EC produce similar outcomes in the same task (Nagahara et al., 1995; Schenk & Morris, 1985). Additionally, the novel object recognition (NOR) task is a useful tool for evaluation of object recognition memory based on the integrity of the perirhinal/entorhinal-hippocampal region (Parron & Save, 2004; Save et al., 1992; Winters et al., 2008). Degeneration of cells in the entorhinal cortex and loss of their projections into the hippocampus are sufficient to induce a recognition memory deficit (Galani et al., 1998; Mumby & Pinel, 1994).

Therefore we investigated sex differences in binge-induced impairment of spatial reference memory, reversal learning, and object recognition memory using the Morris water maze and novel object recognition tasks. Specifically, spatial reference memory impairment was defined as an increased path length and escape latency to find the platform compared to

controls. Reversal learning impairment was defined as an increased path length and latency to find the new platform location, and greater time spent in and number of entries into the previous platform location quadrant. Finally, object recognition memory impairment is defined as more time spent with and contacts of familiar objects than novel objects. Based on our data indicating hippocampal neurodegeneration in females but not males, we hypothesize that females will have greater impairments in spatial reference, reversal learning, and object recognition memory, indicating greater vulnerability to the consequences of alcohol in females.

## METHODS

### *Animals*

A total of 44 (22 females and 22 males) Long-Evans rats aged nine weeks were used across all experiments. All rats were housed and handled as previously described in chapter two. All experimental procedures were conducted in accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The relevant animal protocol was approved by the University of Houston Institutional Animal Care and Use Committee (protocol number 14-013). Rats were divided into four groups (12 binged females, 12 binged males, 10 control females, and 10 control males) in a 2x2 design to compare the effects of Sex (Female, Male) and Diet (Binge, Control) on cognitive deficits in spatial reference memory, reversal learning, and object recognition memory using the MWM and NOR tasks.

### *Binge-Alcohol Administration Model and Blood Alcohol Concentration*

Animals received alcohol or isocaloric control diet as previously described in chapter two. For all binge-exposed animals, blood samples were collected via tail bleed taken 90

minutes after the morning dose on day 3 and BAC assessed as previously described in chapter two.

### *Monitoring of Withdrawal Symptoms*

Eight hours after the last dose of alcohol, food was replaced in the animals' home cages. Spontaneous withdrawal behavior in all alcohol treated rats was monitored for hours 10–26 after the last dose. This range corresponds to the peak period of withdrawal in this model (Faingold, 2008; Majchrowicz, 1975; Penland et al., 2001). Rats were observed in their home cages and scored for spontaneous withdrawal behaviors in 30-minute intervals. Red lights were used during the dark cycle so as to not disturb Circadian rhythms. Behaviors were scored based on the withdrawal scale of Penland et al. (2001) but identical to that reported previously (Tables 3.1; Morris et al., 2010b). Spontaneous withdrawal symptoms of each animal were observed and the most severe symptom recorded at every time point. Mean withdrawal severity and peak withdrawal severity scores were calculated. The mean withdrawal score refers to the average of the scores observed, and peak withdrawal score refers to the average of the most severe withdrawal symptom observed in each animal during the withdrawal period.

**Table 3.1 Withdrawal Scale**

| Withdrawal Score | Behavioral Attribute | Withdrawal Score | Behavioral Attribute |
|---|---|---|---|
| 1 | Hyperactivity | 3.0 | Head Tremor |
| 1.4 | Tail Tremor | 3.2 | Induced Running |
| 1.6 | Tail Spasm | 3.4 | Wet Dog Shakes |
| 2.0 | Caudal Tremor | 3.6 | Chattering Teeth |
| 2.2 | Tip Toe Arch | 3.8 | Spontaneous Convulsions |
| 2.4 | Splayed Limbs | | |
| 2.6 | General Tremor | 4.0 | Death |

Table 3.1 Animal's behavior was scored based on a modified scale of withdrawal symptoms (Penland et al., 2001).

***Behavioral Testing***

Animals in alcohol groups remained in their home cages after the last dose of alcohol and remained abstinent for roughly 4.5 days before behavioral testing. After this time, all binged animals were free of any signs of withdrawal and were indistinguishable from control diet animals based on weight and appearance. After 4.5 days, animals began 8 days of behavioral testing for cognitive deficits including open field , Morris water maze, and novel object recognition tasks (Figure 3.1).

**Figure 3.1 Timeline of binge treatment and behavioral testing**

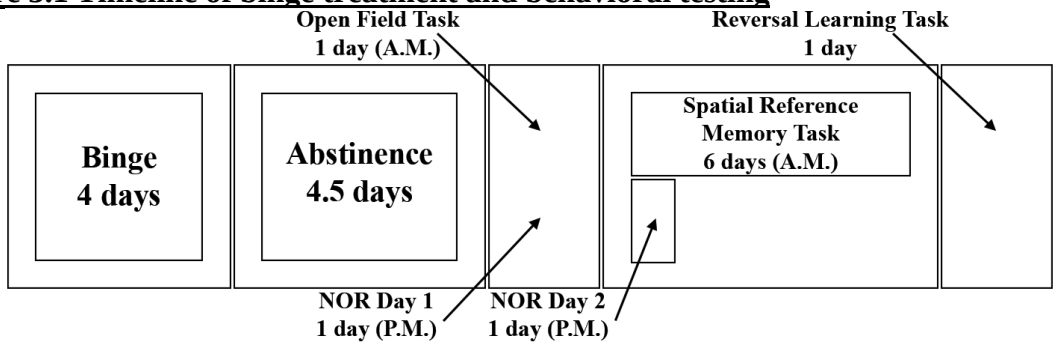


Table 3.1 Four and a half days after the last dose of alcohol, animals began a 8 day battery of behavioral testing.

***Analysis of Exploratory Behavior in Open Field***

The open field test is commonly used to assess general locomotor activity levels and anxiety-like behavior in rodents (Prut & Belzung, 2003). Four and a half days after the last dose of alcohol each animal was tested in an open field, which consisted of a plywood box with a white plastic bottom divided into 25 equally-sized squares (each 15 x 15 cm) as previously reported (Leasure & Jones, 2008). Each animal was placed in the center of the open field and video recorded for 5 minutes. Locomotor activity was assessed analyzed using an automated tracking system (Noldus, Amsterdam, Netherlands) for the following outcome measures: distance traveled and percentage of time spent in the center of the arena. Most

rodents stay close to the walls of the apparatus, and are considered less anxious if they venture into the middle of the open field.

### *Morris Water Maze (MWM)*

The Morris water maze (MWM) is widely used to study spatial learning and memory and is very accurate in assessing damage to cortical regions and the hippocampus (D'Hooge & De Deyn, 2001). The apparatus used consisted of a circular, galvanized steel pool, 167 cm in diameter and 76 cm deep as previously reported (Leasure & Jones, 2008). The pool was placed in a room with different extramaze cues on the walls surrounding the pool. The pool and a 12-cm circular platform were painted and sealed with white non-toxic paint. The pool was filled with water until it reached 2cm above the platform surface and then made opaque with white non-toxic paint. Water was allowed to equalize to room temperature before testing began.

### *Analysis of Spatial Reference Learning and Memory*

Twenty-four hours after the open field task (5.5 days post alcohol) rats were trained in the MWM to escape from the water onto the submerged platform using a difficult two-trial per day paradigm that has been shown to detect subtle differences in performance (van Praag et al., 1999b; Vaynman & Gomez-Pinilla, 2005). The platform was placed in the northwest quadrant and remained constant throughout spatial learning, but the release point changed each day. Each rat was given two trials per day for six consecutive days. Each trial had a 60s ceiling and a 60s inter-trial interval. Rats were released facing the wall, from one of the four randomly chosen points, and allowed 60s to find the hidden platform. Once the animal reached the platform, it was allowed to remain on the platform for approximately 10s. If the animal failed to reach the platform within the trial ceiling, the animal was gently guided to

the platform by the experimenter and placed on the platform for 10s. The animal was then removed from the water, dried off, and placed in its home cage to await its next trial. Pool water was agitated between animals and trials to prevent non visual cues. Escape latency, swim speed, and path length were recorded and analyzed by an automated tracking system (Noldus, Amsterdam, Netherlands).

### *Analysis of Reversal Learning*

Following six days of spatial reference memory testing, the animals were tested in a reversal learning task for one day in the MWM. The submerged platform was moved to the southeast corner (opposite quadrant from learning) and the animals were given four trials, each consisting of a 60s ceiling and a 60s inter-trial interval to locate the platform. During reversal learning, the release point for an animal changed for each trial. Rats were released facing the wall from each of the four quadrant edges and given 60s to find the platform. Once the animal reached the platform, it was allowed to remain on the platform for approximately 10s. If the animal failed to reach the platform with the trial ceiling, the animal was gently guided to the platform by the experimenter and placed on the platform for 10s. The animal was then removed from the water, dried off, and placed in its home cage to await its next trial. Length of time and number of entries into the former platform quadrant were recorded and analyzed.

### *Novel Object Recognition (NOR)*

The novel object recognition task is a common test that uses rat's natural tendency to interact with novel objects as a means to assess recognition memory. This task is preferable to other methods of assessing learning because it does not cause undue stress on animals, does not require prolonged training, and requires no punishment or reinforcement to motivate

behavior (Bevins & Besheer, 2006). In the afternoon, four and a half days after the last dose of alcohol, animals began the novel object recognition (NOR) protocol. The NOR test was broken into two days of testing: Day 1, familiarization to "familiar objects", Day 2, recognition to the novel object (Figure 3.3)

***Familiarization (Day 1)***

During the familiarization period, 2.5 cm spherical wooden beads (www.craftparts.com) designated as "familiar" objects were placed in each rat's home cage (3 per animal) 24 hours prior to Day 2 testing (Figure 3.2A). These objects were introduced to the home cage so that they could acquire the odor of the animals, and also so that the animals would become familiar with beads of this size and shape. During this time, objects designated as novel one (NO1), either a 2 7/8" star or a 1 ¾" peg (www.craftparts.com) were placed into separated sealed plastic bags with bedding from respective animal cages (Figure 3.2B). Although we did not want the animals to become familiar with the shape of these objects yet, we wanted these objects to acquire the scent of the animals' home cage, so that the rat would not identify these objects as novel based on scent. Novel objects were counter balanced for all of the animals to prevent a preference for one object or another beyond habituation (half of the animals star was NO1 and for other half peg was NO1). Experimenters were careful to keep track of which rats belonged to which cage, and made sure that objects that each rat would see only came into contact with that rat's bedding.

**Figure 3.2 Shapes and sizes of objects used in NOR task**

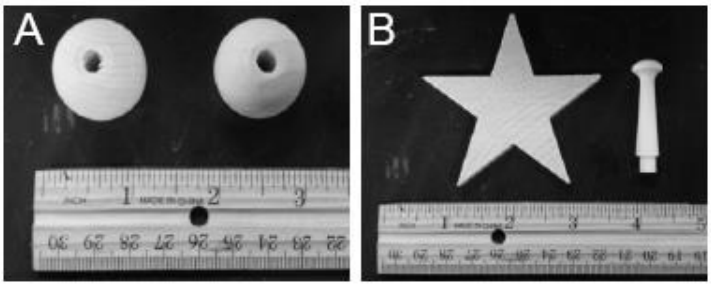


Figure 3.2 The size and shape of the novel and familiar objects are depicted.

***Recognition of NO1 (Day 2)***

During the recognition phase of the task (24 hours after familiarization to the spherical beads in the home cage), the now familiar spherical beads were removed from the home cages for one hour and placed in a sealed plastic bag with the soiled bedding and other objects for each animal. Rats were acclimated to the darkened testing room with red lights for 15 min before testing began. Testing occurred in the rats' home cage. Right before the trials began, the cage mates were removed and placed into a separate clean cage. The experimenter placed three familiar objects (spherical beads) in the cage along with one of the NO1 objects. Rats were exposed to these four bead shapes for three 1 minute trials with 1 minute inter-trial intervals during which the beads were removed from the cage. This procedure produces habituation to NO1 while eliminating odor as a cue, since every bead the animal sees smells like his home cage. To dismiss scent-marking as a confound, the NO1 bead was discarded after each trial and replaced by another taken from the same plastic bag. The experimenter changed gloves whenever switching between cages, to avoid cross-contaminating the beads with the smell of another cage.

For each 1 minute trial, the three familiar beads and the NO1 bead were placed along the front wall of the testing cage, and the rats were allowed 1 min to explore the beads. The spatial arrangement of the beads was randomly altered between trials. The latency to

approach the first bead was recorded using a stopwatch, and this first approach to a bead initiated the 1-min trial. Exploration time for each of the four beads was recorded using OD Log (Macropod Software) and an external keypad that had 4 keys coded for each object. To maximize the sensitivity of the test, one novel (NO1) and three familiar beads were used during habituation trials rather than only one familiar and one novel object. Presenting the rats with four choices when assessing relative object preference greatly increases power (sensitivity and reliability) compared to two-choice procedures typically used in recognition memory tests. To avoid both confusion and fatigue in the rats, only three one-minute trials were administered to each rat.

**Figure 3.3 Flowchart of the NOR task.**

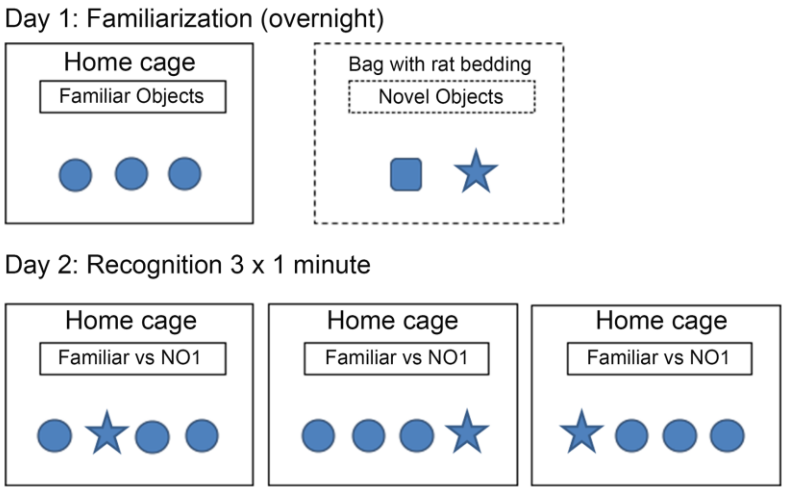


Figure 3.3 In the Familiarization phase, rats become accustomed to the presence of wooden objects (Familiar) in the homecage. In the Recognition phase, they discriminate between 3 familiar objects and a novel object (NO1).

***Statistical Analyses***

Data were analyzed and graphed using Excel 2014 (Microsoft Office Excel, WA, USA) and SPSS Statistics 17.0 (IBM SPSS Statistics, IL, USA). All two group comparisons for intoxication behavior scores were analyzed with Mann-Whitney non-parametric tests and BACs were analyzed by student's *t*-test. Open field, Morris water maze, and novel object

recognition data were analyzed using repeated measures ANOVA and two-way factorial ANOVAs. All data are reported as the mean ± standard error of the mean and analyses considered significantly different if $p<0.05$.

## RESULTS

### *Behavioral Response to Binge Alcohol Administration*

Intoxication parameters across all experiments are shown in Table 3.2, and were similar to those previously reported with this model (Maynard & Leasure, 2013; Morris et al., 2010b) and chapter 2. Mann-Whitney non-parametric tests revealed no significant differences between males and females for intoxication ($p$ = 1.00), dose ($p$ = .913), average withdrawal ($p$ = .274), and peak withdrawal ($p$ = .491). Independent $t$-test revealed no difference in BAC [$t_{(22)}$ = .624, p = .539] between males and females.

**Table 3.2 Intoxication measures for chapter three**

| Sex | Group | Intoxication behavior (0-5 scale) | Dose (g/kg/day) | BAC (mg/dl) | Average Withdrawal Score | Peak Withdrawal Score |
|---|---|---|---|---|---|---|
| **Female** | Binge | 2.3 ± 0.11 | 8.3 ± 0.3 | 251 ± 18.8 | 1.5 ± 0.1 | 2.8 ± 0.1 |
| **Male** | Binge | 2.3 ± 0.06 | 8.2 ± 0.2 | 232.4 ± 23 | 1.6 ± 0.1 | 2.9 ± 0.1 |

Table 3.2 Measures of various intoxication parameters were similar between males and females.

### *Binge exposure had no effect on open field behaviors*

In order to assess the effects of binge alcohol exposure on nonspecific motor function and exploration, we assessed behavior in the open field test. Relative to control animals: an increase in locomotion would be considered a stimulant effect while the opposite would be related to sedation; an increased percent of time spent in the central area would be interpreted as anxiolytic-like behavior, and a decrease anxiogenic-like behavior (Prut & Belzung, 2003). A two-way ANOVA revealed no significant effects of Sex [$F_{(1,40)}$ = 2.359, $p$ = .132], Diet [$F_{(1,40)}$ = .233, $p$ = .632] or a significant Sex x Diet interaction [$F_{(1,40)}$ = .046, $p$ = .831] for

percent time spent in the center. A similar pattern was observed for distance traveled, ANOVA revealed no significant effects of Sex [$F_{(1,40)} = .115$, $p = .737$], Diet [$F_{(1,40)} = 1.82$, $p = .185$] or a significant Sex x Diet interaction [$F_{(1,40)} = .217$, $p = .644$]. These results indicate there were no differences between groups on nonspecific motor functions and exploration, thus binged animals were indistinguishable from control diet animals prior to behavioral testing.

***Binge exposure resulted in spatial reference memory impairments in females***

Acquisition of the spatial reference memory task was measured by escape latency and path length to the platform, and swim speed. The average of each animal's two daily trials was used for analysis. A three-way mixed model ANOVA, revealed that for escape latency significant main effects of Training Day [$F_{(5,200)} = 60.75$, $p < 0.05$, $\eta p^2 = .930$] and Diet [$F_{(1,40)} = 4.76$, $p < 0.05$, $\eta p^2 = .106$] and significant Sex x Diet [$F_{(1,40)} = 4.96$, $p < 0.05$, $\eta p^2 = .110$] and Diet x Training Day [$F_{(5,200)} = 2.67$, $p < 0.05$, $\eta p^2 = .253$] interactions. Thus, all groups improved performance over time, and Diet, when averaged across time, had a significant, sex-dependent effect. Bonferroni-corrected *post hoc* comparisons showed that binged females had significantly longer escape latencies compared to control females ($p = 0.003$) and binged males ($p = 0.07$) (Figure 3.4A, B). For path length, there was a significant main effect of Training Day [$F_{(5,200)} = 90.36$, $p < 0.05$, $\eta p^2 = .904$] and significant Sex x Diet [$F_{(1,40)} = 4.97$, $p < 0.05$, $\eta p^2 = .111$] and Diet x Training Day [$F_{(5,200)} = 2.56$, $p < 0.05$, $\eta p^2 = .211$] interactions (Figure 3.4C). Thus, all groups decreased path length over time, and Diet, when averaged across time, had a significant, sex-dependent effect. Bonferroni-corrected *post hoc* comparisons showed that binged females had significantly longer path lengths compared to control females ($p = 0.006$) and binged males ($p = 0.014$) (Figure 3.4D).

**Figure 3.4 Spatial reference memory impairment in females**

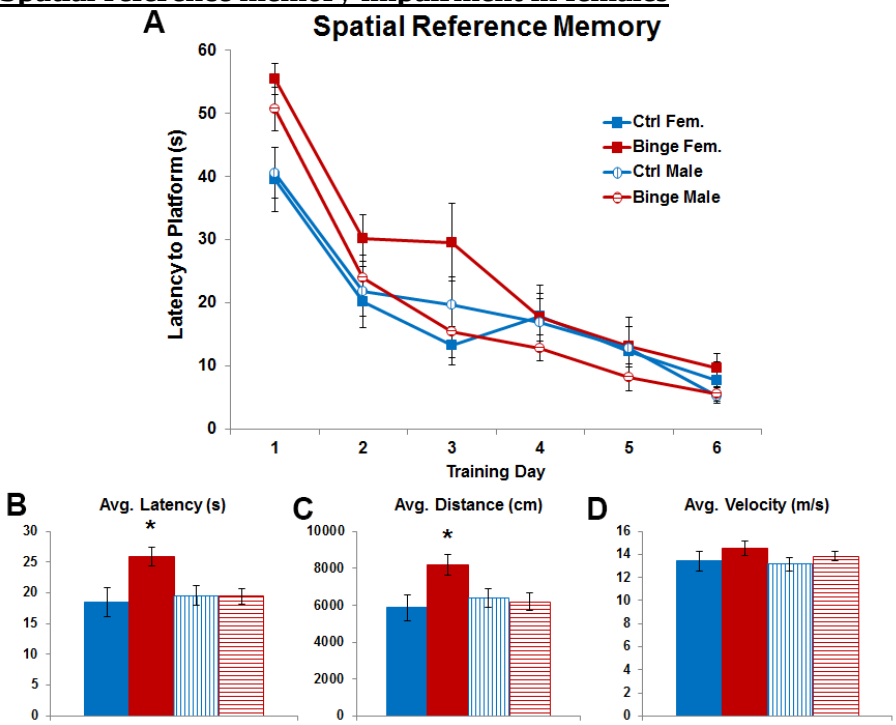


Figure 3.4 Binge exposer caused females to take longer to learn the location of the platform but not males. Females had significantly longer average escape latency (D), path length (E), despite no difference in swim speed (F). *$p$< 0.05 compared to female control and male binge.

***Binge exposure had no effect on performance in the reversal learning task***

Following the completion of the 6-day reference memory task, the submerged platform was move to the opposite quadrant used in the reference memory task. Animals were tested the next day in a reversal learning task for four trials to determine ability to learn the new location of the platform. Number of entries into and time spent in the original platform quadrant during the first trial were measured as a sign of perseverative behavior. ANOVA revealed a significant effect of Trial [$F_{(3,120)} = 41.165$, $p < .05$, $\eta p^2 = .507$] indicating that all animals decreased escape latency over the course of the trials. There were no significant effects of Sex [$F_{(1,40)} = .490$, $p = .488$] and Diet [$F_{(1,40)} = .048$, $p = .829$] or significant interactions [$F_{(1,40)} = .033$, $p = .856$] indicating that binge exposure had no effect on learning the new location of the platform (Figure 3.5A). This was supported by no significant effects for average latency, path distance, or average velocity. Binge exposure had

no effect on perseverative behavior during the first trial of the reversal learning task. ANOVA revealed no significant effects of Sex [$F_{(1,40)}$ = .063, p = .803], [$F_{(1,40)}$ = .542, $p$ = .466] or Diet [$F_{(1,40)}$ = .767, $p$ = .386], [$F_{(1,40)}$ = .088, $p$ = .769] for percent of time spent in the original platform quadrant or number of entries into the original quadrant (Figure 3.5B,C).

**Figure 3.5 Binge exposure had no effect on reversal learning**

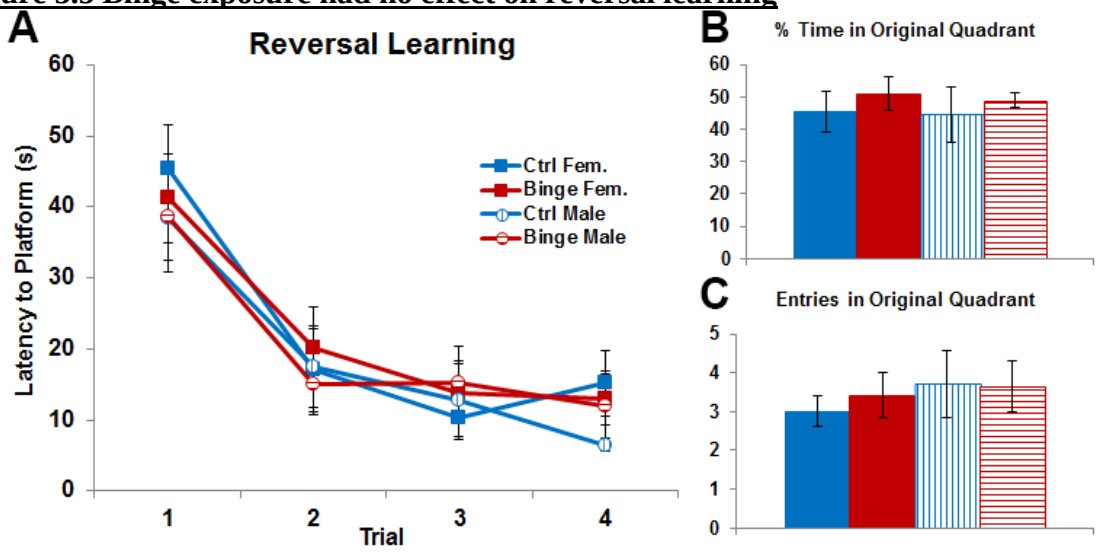


Figure 3.5 All animals learned the new location of the platform (A) and there were no signs of perseverative behaviors during the first trial identified by similar percent time (B) and entries into the original quadrant (C).

***Binge exposure had no effect on object recognition memory or learning***

Object recognition memory was tested during three trials. For each 1 minute trial, three familiar beads and the NO1 bead were placed along the front wall of the testing cage, and the rats were allowed 1 min to explore the beads. More exploration of NO1 than familiar objects (duration and contacts) on the first trial was regarded as evidence for novel object recognition. Also, a significant reduction in NO1 exploration time between the first and last trials was taken as evidence of habituation to NO1 (i.e., learning). As shown in Table 3.3, one-way ANOVA for each group indicated that all animals spent significantly more time with NO1 than the familiar objects in the first trial, indicating no effect of binge exposure on novel object recognition. Furthermore, repeated measures ANOVA for each group revealed

that control females [$F_{(2,18)} = 4.998$, $p < .05$], binge females [$F_{(2,20)} = 5.462$, $p < .05$], control males [$F_{(2,18)} = 5.905$, $p < .05$], and binge males [$F_{(1,20)} = 10.067$, $p < .05$] showed a significant reduction in the amount of time spent exploring NO1 between trial 1 and trial 3, indicating robust habituation (Figure 3.6).

**Table 3.3 Time spent with novel and familiar objects**

| Group | NO1 | Familiar 1 | Familiar 2 | Familiar 3 | *F* value | *P* value |
|---|---|---|---|---|---|---|
| Female Ctrl. | 74 ± 4.6 | 8.7 ± 2.5 | 8.5 ± 3 | 8.7 ± 1.4 | 111.37 | <.0001 |
| Female Binge | 68.1 ± 6 | 10.2 ± 2.1 | 11.3 ± 4 | 10.4 ± 2 | 54.90 | <.0001 |
| Male Ctrl | 75.9 ± 4.1 | 10.6 ± 2 | 7.3 ± 1.8 | 6.2 ± 2.2 | 160.47 | <.0001 |
| Male Binge | 78.1 ± 3.3 | 5 ± 1.4 | 5.2 ± 1.1 | 11.7 ± 2.5 | 249.97 | <.0001 |

Table 3.3 All groups spent significantly more time with NO1 than the familiar s.

**Figure 3.6 Binge exposure had no effect on object recognition**

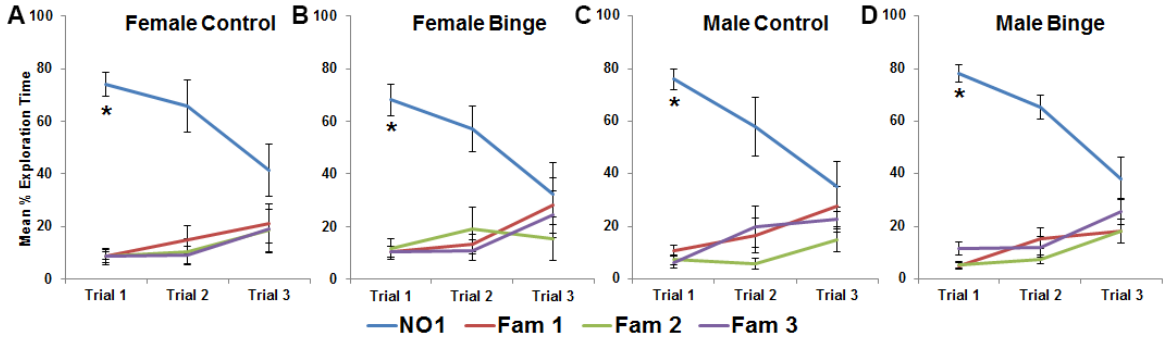


Figure 3.6 All groups demonstrated habituation, spending more time with NO1 on trial 1 than trial 3. *p<.05.

## DISCUSSION

Chronic alcohol consumption in humans is known to cause neurodegeneration and cognitive dysfunction. Alcoholics show decreased neuropsychological performance on tests of learning, memory, problem solving, visual spatial and perceptual motor functioning, and information processing (Parsons, 1993). Despite extensive investigations of the cognitive deficits associated with binge drinking and alcohol dependence, the majority of studies have only sampled males. Evidence suggests women are more sensitive to the neurotoxic effects of alcohol than men (Hommer et al., 2001; Hommer, 2003), as a result, structural and cognitive impairments emerge sooner in women despite shorter drinking histories and lower

total lifetime consumption than men (Mann et al., 1992; Nixon et al., 1995). To our knowledge, the present study represents the first time the 4-day binge model has been used to directly compare the effects of binge-alcohol consumption on potential cognitive deficits in males and females. The major finding of this work is that females are more sensitive to binge-induced spatial navigation impairment than males.

We found that binge-exposed females had a significant spatial reference memory impairment indicated by a significantly longer escape latency and path distance in learning the location of the submerged platform in the MWM. Males on the other hand showed no such impairment and learned the location of the platform as well as control animals. This data is supported by findings from our previous experiments indicating that the female hippocampus is more sensitive binge exposure and the 4-day binge causes a significant neurodegeneration in the DG of females but not males. The hippocampus is known to be critically involved in spatial learning and memory formation (Aggleton et al., 2000; Jarrard, 1993) and evidence indicates that lesions result in robust and reliable spatial reference memory deficits in the MWM (Galani et al., 1998; Morris et al., 1982). Compromised hippocampal integrity and connections to cortical regions in alcoholics are thought to underlie some of the behavioral deficits in executive functioning (Beresford et al., 2006; Chanraud et al., 2010), as well as impairments in learning, memory, and visuospatial abilities (Beatty et al., 1996; Crews et al., 2004a; Pitel et al., 2009; Townshend & Duka, 2005). It is likely then that neurodegeneration of the female hippocampus within this model resulted in the spatial reference memory impairment as neither were found in males. Even though we found cell death in the entorhinal cortex of males and females, some evidence suggests that spatial learning can proceed normally without the EC (Bannerman et al., 2001; Galani et al.,

1998), further implicating neurodegeneration of the female hippocampus specifically in the present spatial memory impairment. Importantly, the spatial reference memory deficit in our study was not a function of nonspecific locomotor impairment or anxiety, because measures of both in the Open field test were unaffected by prior binge alcohol exposure.

Few studies have investigated spatial learning and memory in female animals following binge alcohol exposure, however spatial memory impairments were found in female adolescent and adult animals when testing was concurrent with alcohol administration (Sircar et al., 2009), and following chronic prenatal alcohol exposure (An & Zhang, 2013). The lack of a MWM spatial reference memory impairment in males is consistent with other work using the 4-day binge model (Cippitelli et al., 2010a; Obernier et al., 2002b), however one group found an impairment but only after testing males that were exposed to a total of four 4-day binge exposures (Zhao et al., 2013). Other models of alcohol exposure have found similar spatial deficits in males but only with associated with hippocampal damage after 6 months of chronic alcohol intake (Arendt et al., 1988; Lukoyanov et al., 1999) or thiamine deficiency (Carvalho et al., 2006), when testing occurred concurrent with alcohol administration (Matthews et al., 1995). In our previous experiment (described in Chapter 2), we found no loss of DG granule neurons in males, and spatial learning and memory was tested well after alcohol had been cleared.

Second, we found no effect of binge alcohol exposure on reversal learning for females or males. Although not a classical test of perseveration, behavior in this test could indicate perseverative responses, similar to the abnormal response inhibition and perseveration often manifested in chronic alcoholics (Oscar-Berman & Hutner, 1993). In the current study there was no evidence of this however, as binge-exposed animals did not have a

greater number of entries or increased amount of time spent in the original quadrant compared to control animals. This is in contrast to the findings of two other studies that found reversal learning impairments in males using the same task (Cippitelli et al., 2010a; Obernier et al., 2002b). One possible explanation is that in the current experiment animals were only trained for two trials a day to learn the location of the platform prior to reversal learning compared to four trials a day in the other two studies. It is possible that the platform location was "overlearned" by binge animals after four trials per day, thus they spent more time searching in the original goal quadrant during the reversal learning task. Although it is difficult to compare results between protocols, binge animals in the current study spent an average of 10 seconds less and made 6 fewer entries into the original quadrant than binge animals in a prior study (Obernier et al., 2002b). MWM performance can also be influenced by other factors such as apparatus or methodology (D'Hooge & De Deyn, 2001). Another explanation is that the binge-induced neurodegeneration in our current study was localized to the hippocampus. Lesions of the entorhinal-perirhinal cortex cause modest spatial learning deficits (Nagahara et al., 1995) but deficits in reversal learning of a spatial task (Hagan et al., 1992; Pouzet et al., 1999), while hippocampal lesions cause more global spatial reference and working memory deficits (Jarrard, 1993). This research suggests that significant neurodegeneration of the female hippocampus we saw in the previous chapter was associated with a spatial reference memory impairment, however the apparent lack of significant entorhinal cortex neurodegeneration preserved reversal learning.

Finally, we found no effect of binge alcohol exposure on object recognition memory or habituation. The protocol used was chosen because it controls for odor, and presents the rats with four choices when assessing relative object preference which greatly increases

power (sensitivity and reliability) compared to two-choice procedures. Additionally, because animals were tested over three trials with a random arrangement of the objects, this was considered non-spatial task, different than learning in the MWM. Evidence indicates that encoding of geometric arrangement for place navigation in a spatial NOR task is dependent on the hippocampal-entorhinal connections (Save et al., 1992), however the perirhinal/entorhinal connections appear to be more important for the identification of a novel geometric arrange of objects in a non-spatial NOR task (Parron & Save, 2004). This research suggests that novel object recognition and learning was preserved in our current study possibly because limited neurodegeneration was found in the perirhinal and entorhinal cortex following binge exposure. Similar to what we found in the MWM, it appears as though the female hippocampal dentate gyrus is particularly sensitive to neurodegeneration and resulting deficits in hippocampal dependent tasks, however damage in other areas might not be as severe or pervasive. Even though FJB+ cells are commonly found in the perirhinal and entorhinal cortex within this model, by the time testing occurs a week later, it may be that recovery has already taken place or that there was not a significant amount of neurodegeneration in the first place.

Prior to the current experiment, only two studies have investigated the effect of binge exposure on novel object recognition memory using the 4-day binge model. The first found impairments in males for a non-spatial NOR task seven days after the last dose of alcohol, however they used a methodology that lacked the sensitivity of the current protocol and failed to report the number of contacts and percent of time exploring (Cippitelli et al., 2010b). The second used the same methodology as the first, but only found a significant novel object recognition impairment after repeating the 4-day binge exposure four times in

males (Zhao et al., 2013). Using an intermittent alcohol exposure paradigm in adolescent male rats, one group found impairments in NOR that were associated with significant cell-death and inflammation in the hippocampus and cortex after exposure to 3.0 g/kg once per day for 14 days (Pascual et al., 2007). In a different study, impairments in NOR were found using a chronic feeding paradigm where female mice received alcohol for 5 weeks starting in adolescence (Golub et al., 2015). Interestingly, the latter study found that disruption of novel object recognition memory was linked to alcohol-induced suppression of neurogenesis.

Taken together, the results indicate that females are more sensitive to binge alcohol-induced deficits in hippocampal dependent behavior, such as spatial reference memory tested in the MWM. This is likely associated with the increased sensitivity and neurodegeneration seen in the female hippocampus, as males had neither impaired spatial reference memory nor a reduction of dentate gyrus granule neurons. Binge exposure had no effect on novel objection recognition memory or reversal learning, a possible indication that resulting sex specific cognitive deficits are reserved to hippocampal-dependent tasks.

***Chapter 4: Sex differences in trophic support and signaling underlie female vulnerability to binge alcohol-induced neurodegeneration***

The extent of alcohol-induced neurodegeneration is dependent on many factors including pattern of intake, genetics, and age. More recently, evidence suggests that sex is an important factor that affects the extent of alcohol-induced neurodegeneration. Females appear more sensitive to the neurotoxic effects of alcohol and cognitive and structural impairments in the brain emerge faster in the course of the disease for women than for men (Mann et al., 1992; Nixon et al., 1995), despite a shorter history of alcohol abuse. Despite this, few studies have directly compared alcohol-induced damage between males and female, and the majority have only included males. Animal models and studies of human alcoholics have increased the understanding of the following mechanisms that may underlie alcohol-induced neurodegeneration: oxidative stress, neuroinflammation, reduced cell genesis and altered glial functioning, and loss of trophic support. These four mechanisms appear to be both causes and consequences of one another, and thus work in conjunction to contribute to alcohol-induced neurodegeneration. It is not well understood however, if these mechanisms affect male and female brains differently and how they contribute to enhanced vulnerability to alcohol-induced neurodegeneration in women.

Alcohol may have different effects on trophic support that are dependent on sex. Binge exposure is thought to result in a loss of trophic factors that promote overall health of the brain including cell proliferation, growth, and survival. The transcription factor cAMP-responsive element-binding protein (CREB), the neurotrophic factor brain-derived neurotrophic factor (BDNF), and the growth factor insulin-like growth factor 1 (IGF-1), have been implicated as negatively affected by alcohol. CREB, BDNF, and IGF-1 have been

shown to have multifaceted roles in diverse processes such as neurodevelopment, synaptic plasticity, neurogenesis, and neuroprotection (Aberg et al., 2003; Alonso et al., 2002; Lonze & Ginty, 2002; Masters et al., 1991; Reichardt, 2006; Sakamoto et al., 2011).

The disruption of CREB, BDNF, and IGF-1 leaves the brain more vulnerable to damage and is implicated in alcohol-induced neurodegeneration. The reduction of CREB transcription has been shown to contribute to alcohol neurotoxicity (Zou & Crews, Wand, 2005; 2006). The activation of oxidative stress by alcohol coincides with the loss of CREB transcription (Zou & Crews, 2006), while binge alcohol treatment results in a decrease in pCREB (activated CREB) immunoreactivity that is most pronounced in regions of the brain showing degeneration (Bison & Crews, 2003). Alcohol has profound effects on BDNF expression that appear to be dependent on the alcohol dose and brain region, with a pattern towards reduced BDNF with higher chronic doses (Moonat et al., 2010; Stevenson et al., 2009) that corresponds with alcohol-induced neurodegeneration (Davis, 2008) and reduced survival of new DG granule neurons (Herrera et al., 2003; Nixon & Crews, 2002). A number of studies indicate that alcohol-induced toxicity is mediated in part by interference with the production and functioning of IGF-1 (Cohen et al., 2007; de la Monte et al., 2008; Hallak et al., 2001; Sonntag & Boyd, 1988). Alcohol mediates its effects on IGF-1 signaling by inhibiting gene expression and impairing receptor binding (de la Monte et al., 2005; Soscia et al., 2006), which results in increased apoptosis (de la Monte et al., 2001; Ikonomidou et al., 2000; Zhang et al., 1998), mitochondrial dysfunction (de la Monte et al., 2001), and increased oxidative stress (Cohen et al., 2007; de la Monte et al., 2008). Chronic alcohol may also contribute to cell death by increasing proinflammatory cytokines that inhibit IGF-1 and its pro-survival signals (Venters et al., 1999).

Trophic support appears to be very important in maintaining a healthy brain, including neuronal survival and cognitive functioning. The loss of that support has been implicated in alcohol-induced neurodegeneration and may contribute to impairments in behavior; however it is not known if alcohol's effects on trophic support are sex dependent and a source of enhanced female vulnerability. Therefore, we investigated whether trophic support is differently affected by binge alcohol exposure. We found significant neurodegeneration in the female hippocampus associated with spatial memory impairments; therefore we investigated the effect of binge exposure on trophic support in the dorsal hippocampus because spatial learning and memory has been shown to be dependent on this area (Pothuizen et al., 2004; Vann & Albasser, 2011). Specifically, we measured the effect of a 4-day binge exposure on the protein expression of BDNF, its high affinity receptor TrkB, the IGF-1 receptor, CREB, and its activated state phosphorylated CREB (pCREB) using Western blotting methods. Due to potential sex differences in basal levels of protein expression and the limitations in the Western blotting quantification, it is necessary to compare protein expression in binge-exposed males and females to sex-matched control animals. We hypothesize that binge exposure will result in a significantly greater reduction of BDNF, TrkB, IGF-1R, CREB, and pCREB protein expression in binged females compared to female controls, but not binged males compared to binge controls. This finding would reveal a mechanism associated with our earlier results indicating the female hippocampus is more vulnerable to binge induced neurodegeneration and associated deficits.

# METHODS

## *Animals*

A total of 22 (11 females and 11 males) Long-Evans rats aged nine weeks were used across all experiments. All rats were housed and handled as previously described in chapter two. All experimental procedures were conducted in accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The relevant animal protocol was approved by the University of Houston Institutional Animal Care and Use Committee (protocol number 14-013). Rats were divided into four groups of comparable weights (~6 per group) in a 2x2 design to compare the effects of Sex (Female, Male) and Diet (Binge, Control) on protein expression of trophic support and transcription factors.

## *Binge-Alcohol Administration Model and Blood Alcohol Concentration*

Animals received alcohol or isocaloric control diet as previously described in chapter two. For all binge-exposed animals, blood samples were collected via tail bleed taken 90 minutes after the morning dose on day 3 and BAC assessed as previously described in chapter two.

## *Tissue Collection for Western Blotting*

Eight hours following the last dose of alcohol rats were overdosed with anesthetic (cocktail of ketamine, xylazine and acepromazine). and observed for loss of toe pinch reflex as an indicator of anesthetic depth. Each animal was then decapitated, and brains were quickly removed. The hippocampi were dissected from whole brains and separated into dorsal and ventral halves. The hippocampi of each rat were flash frozen using dry ice and subsequently stored at -80°C until further processing. The dorsal hippocampus was homogenized in lysis buffer and centrifuged in order to isolate protein from the lysate. The

dorsal hippocampus was chosen because our prior results implicated this area as particularly damaged (Chapter 2 and 3). . The supernatant was taken and stored for further use. A sample of this was used for quantification of protein concentration via BCA protein assay (Thermo Scientific). Protein concentration was then adjusted with a portion of sample in loading buffer to 1 µg/µL for all samples and heated at 70°C for 10 min to denature proteins.

***Western Blotting***

For each Western blot, 20 µL of each sample (standardized to 20 µg total protein content) was separated by molecular weight via gel electrophoresis and transferred to polyvinylidene (PVDF) membranes. Following transfer, membranes were washed six times for 10 min each in Tris-buffered saline with Tween-20 (TBST). Membranes were then incubated for 1h at room temperature in a blocking solution consisting of 5% powdered nonfat milk in TBST. Primary antibodies were then added to the blocking solution and transferred to 4°C, where they were allowed to incubate overnight (Table 4.1). Primary antibodies for either COX IV or GAPDH were included on each blot depending on molecular weight of other target proteins, as a positive loading control for basal protein expression. Membranes were then washed six times for 10 min each in TBST. The membranes were then incubated in a solution of 5% skim milk in TBST containing secondary antibody (goat anti-rabbit, 1:10,000) conjugated against horseradish peroxidase (HRP) that was optimized for use with the C-Digit blot scanner. Incubation lasted 1h at room temperature, after which membranes were washed a final six times in TBST for 10 min each. Visualization of proteins was achieved by chemiluminescence using the Li-Cor C-Digit blot scanner (NE, USA) and luminol substrate from the same manufacturer.

**Table 4.1 Primary antibody information for chapter four**

| Primary | Molecular Wt. | Dilution | Vendor |
|---|---|---|---|
| BDNF | 14 kDa | 1:100 | Santa Cruz |
| TrkB | 140 kDa | 1:1,000 | Cell Signaling Tech. |
| IGF-1R | 95 kDa | 1:1,000 | Cell Signaling Tech. |
| CREB | 43 kDa | 1:1,000 | Cell Signaling Tech. |
| pCREB | 43 kDa | 1:1,000 | Cell Signaling Tech. |
| COX IV | 17 kDa | 1:1,000 | Cell Signaling Tech |
| GAPDH | 36 kDa | 1:10,000 | GeneTex |

Table 4.1 Primary antibodies used for investigation of binge exposures effect on protein expression of trophic support (BDNF, TrkB, IGF1R) and transcription factors (CREB, pCREB).

Band intensity was quantified using the accompanying software from Li-Cor for the C-Digit blot scanner, Image Studio Digits Ver 4.0. Background, signal, and midtone levels were adjusted for each blot for fidelity in reporting, and a region of interest containing each band was drawn with minimal background included. The intensity of the band was expressed in arbitrary units as the median intensity within the area of interest. The expression of a protein was then weighted by the corresponding intensity of the loading control in the same lane. This weight was obtained by dividing each loading control by the mean intensity of all loading controls within the same blot in order to account for any possible variability between protein concentrations in individual lanes. The mean of the weighted intensity values was obtained for non-binged controls on the blot, and all weighted intensity values from all treatment groups were normalized against this average (percent of control). This was done out of necessity due to the large number of animals, which prevented simultaneously running every animal on a single blot, and the inability to compare data from separate blots directly.

### *Statistical Analyses*

Data were analyzed and graphed using Excel 2014 (Microsoft Office Excel, WA, USA) and SPSS Statistics 17.0 (IBM SPSS Statistics, IL, USA). Normalized data for protein expression from binged animals was compared against sex-matched controls for statistical

significance determined via independent *t*-tests. All data are reported as the mean percent of control ± standard error and analyses considered significantly different if $p<0.05$.

## RESULTS

### *Behavioral Response to Binge Alcohol Administration*

Intoxication parameters across all experiments are shown in Table 3.2, and were similar to those previously reported with this model (Maynard & Leasure, 2013; Morris et al., 2010b) and chapter 2. Mann-Whitney non-parametric tests revealed no significant differences between males and females for intoxication ($p$ = .364], dose ($p$ = .295), nor independent *t*-test for BAC [$t_{(8)}$ = .452, p = .663].

**Table 4.2 Intoxication measures for chapter four**

| Sex | Group | Intoxication behavior (0-5 scale) | Dose (g/kg/day) | BAC (mg/dl) |
|---|---|---|---|---|
| **Female** | Binge | 2.3 ± 0.1 | 8.3 ± 0.3 | 244.6 ± 16.9 |
| **Male** | Binge | 2.4 ± 0.1 | 8.0 ± 0.3 | 229.5 ± 29 |

Table 4.2 Measures of various intoxication parameters were similar between males and females.

### *Binge exposure resulted in a greater disruption of trophic support in females than males*

Relative expression of trophic factor proteins in the dorsal hippocampus, as determined by Western blotting, is summarized in Figure 4.1. Compared to control females, binge-exposed females showed a significant decrease in BDNF expression (61.18 ± 8.35%) [$t_{(9)}$ = 2.899, $p < .05$] and expression of its high affinity receptor TrkB (41.91 ± 3.83%) [$t_{(9)}$ = 3.397, $p < .05$]. Compared to control males, binge-exposed males showed a significant decrease in BDNF expression (68.9 ± 8.92%) [$t_{(9)}$ = 2.489, p < .05] but no significant decrease in the expression of its receptor TrkB (82.81 ± 8.89%) [$t_{(9)}$ = .993, $p$ = .47]. Binge exposure had no significant effect on IGF-1R expression in females (109.31 ± 14.67%) [$t_{(9)}$ = -.478, $p$ = .644] or males (115.38 ± 15.16%) [$t_{(9)}$ = -.675, $p$ = .516]. Although there was a

significant decrease in the expression of BDNF for both binged exposed males and females, only females had a significant decreased in the expression of TrkB, indicating a greater disruption of trophic support in the dorsal hippocampus of females.

**Figure 4.1 Binge exposure decreased BDNF and TrkB in females**

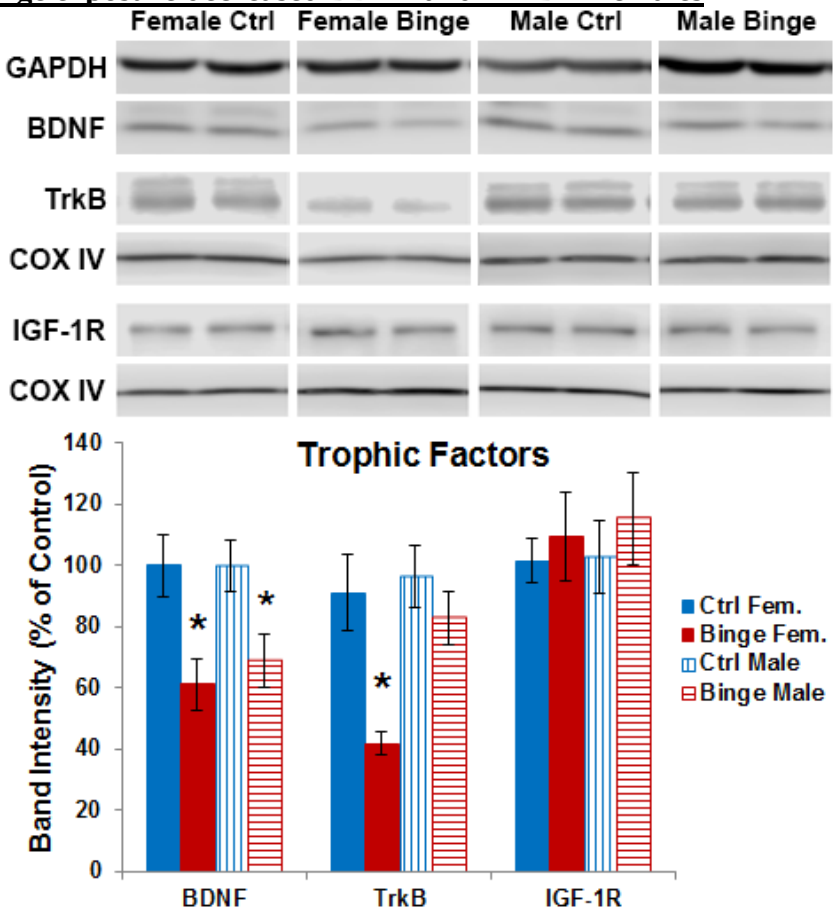


Figure 4.1 Relative expression of trophic factor proteins. Binge exposure resulted in a significance decrease in BDNF and TrkB for binged females compared to controls, but only BDNF for binged males. *$p$< 0.05.

***Binge exposure resulted in a decrease in transcription factors in females***

Relative expression of transcription factor proteins in the dorsal hippocampus, as determined by Western blotting, is summarized in Figure 4.2. Compared to control females, binge-exposed females showed a significant decrease in CREB expression (69.35 ± 12.27%) [$t_{(9)}$ = 2.669, $p$ < .05] and pCREB expression (52.07 ± 11.89%) [$t_{(9)}$ = 2.385, $p$ < .05] However, compared to control males, binge-exposed males shown no significant decrease in

CREB expression (83.57 ± 6.25%) [$t_{(9)}$ = 1.688, $p$ = .123] or pCREB expression (87.35 ± 12.77%) [$t_{(9)}$ = .781, $p$ = .460]. These results indicate that binge exposure resulted in disruption of transcription via a decrease in the expression of CREB and its phosphorylated state pCREB in females but not males.

**Figure 4.2 Binge exposure decreased CREB and pCREB in females**

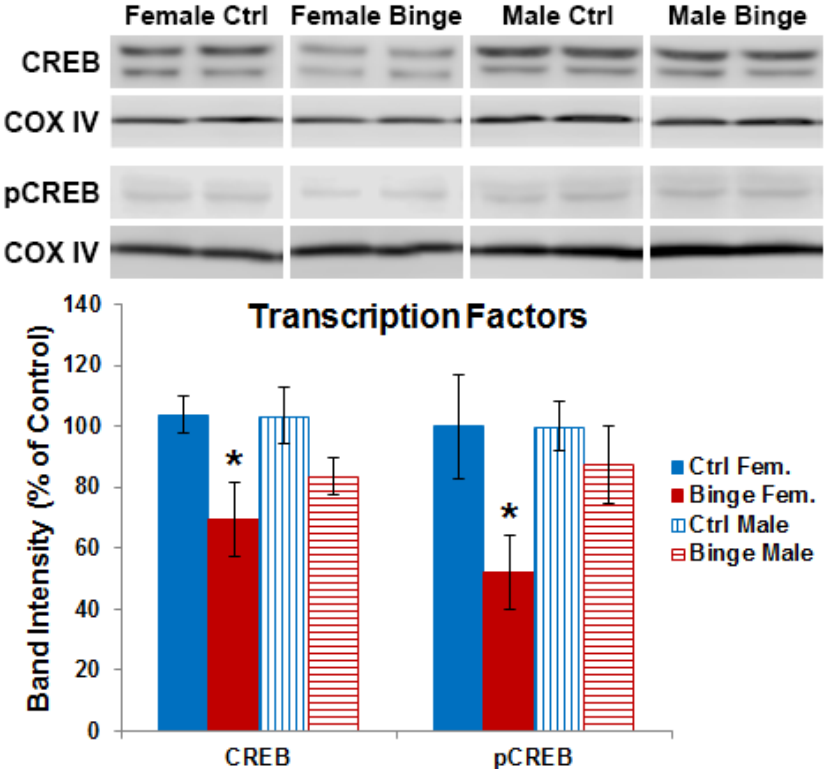


Figure 4.2 Relative expression of transcription factor proteins. Binge exposure resulted in a significance decrease in CREB and pCREB in females but not males. *$p$< 0.05.

## DISCUSSION

Animal models and post mortem studies of the brains of human alcoholics have revealed some of the mechanisms thought to underlie alcohol-induced neurodegeneration, including oxidative stress, neuroinflammation, reduced cell genesis, and the loss of trophic support. It is possible that sex differences in these mechanisms may contribute to the increased sensitivity to binge alcohol-induced neurodegeneration in females relative to males. Trophic factors in particular are a possible target because they are vital for the health

and functioning of neurons and their disruption is implicated in many neurodegenerative diseases. However, whether binge alcohol affects trophic support differently in males and females has not been investigated and to our knowledge, this is the first time the 4-day binge model has been used to directly compare the effects of binge alcohol consumption on trophic support and transcription factors in males and females. The major finding of this experiment is that the female brain is more sensitive to binge-induced disruptions of BDNF-TrkB signaling and CREB signaling in the dorsal hippocampus.

We found that binge-exposed females had a significant decrease in the protein expression of BDNF and its high-affinity receptor TrkB, in addition to the transcription factor CREB and its activated form pCREB in the dorsal hippocampus. By comparison, binge-exposed males only showed a significant decrease in BDNF protein expression, while its receptor and CREB-related proteins were preserved. It is likely then that this widespread disruption of trophic support was associated with the binge-induced reduction of dentate gyrus granule neurons in females within this model as neither of these were found in males. Alcohol has been shown to have profound effects on BDNF expression with a pattern towards reduced BDNF with higher chronic doses of alcohol (Moonat et al., 2010; Stevenson et al., 2009) that leaves the brain more sensitive to and corresponds with alcohol-induced neurodegeneration (Davis, 2008; Zou & Crews, 2006) and reduced survival of new DG granule neurons (Herrera et al., 2003; Nixon & Crews, 2002). This corresponds with our data as we found a decrease in BDNF protein expression in both males and females, as well as reduced proliferation of cells labeled with Ki67 in the dentate gyrus in both sexes (Chapter 2). Evidence also indicates that BDNF in the hippocampus specifically may be more susceptible to alcohol-induced effects than in the entorhinal cortex (Miller, 2004; Miller &

Mooney, 2004). Even though we didn't asses BDNF levels in the entorhinal cortex, we saw no further damage there in females compared to males, nor associated cognitive impairments.

A binge-induced reduction in BDNF during intoxication may disrupt the brain's ability to respond to damage through the genesis of new cells. However, we saw no loss of dentate gyrus granule neurons in males. The reduction in proliferation and new cell survival associated with a decrease in BDNF may not have been as impactful in males as it seemed to be in females. This may be in part because we saw no decrease in protein expression of the high-affinity BDNF receptor TrkB in males. Females did however show a significant reduction of TrkB protein expression in the dorsal hippocampus. BDNF controls neuronal survival and plasticity through binding to TrkB (Givalois et al., 2001). The BDNF-TrkB interaction promotes survival and differentiation of neurons, and is also involved in learning ability and memory function (Koponen et al., 2004; Pietropaolo et al., 2007). The combined loss of BDNF and TrkB signaling may have left the female brain more vulnerable to alcohol-induced neurodegeneration by negatively affecting cell birth as well as leaving a void in trophic support needed to protect against cell death. In fact, low levels of both BDNF and TrkB are associated with the loss of dentate gyrus granule neurons in other models of neurodegeneration (Mesquita et al., 2002).

It is believed that optimal available amounts of both BDNF and TrkB are critical for the promotion of their neurotrophic effects (Connor & Dragunow, 1998; Murer et al., 2001). There is substantial evidence that BDNF signaling through TrkB can prevent the death of cultured hippocampal neurons injured by numerous deleterious factors such as energy deprivation (Cheng & Mattson, 1994), excitotoxins (Mattson, 2000), and elevated concentrations of free radicals (Mattson et al., 1995). BDNF-TrkB signaling may protect

these neurons by inducing an increase in levels of proteins that suppress oxidative stress (Mattson, 2000), one of the proposed key mechanisms in alcohol-induced neurodegeneration. It is possible then, that despite reduced BDNF protein expression, intact TrkB expression enabled the male hippocampus to be more resilient and protected against a loss of dentate gyrus granule neurons. Because there is evidence that BDNF and TrkB are critical for long-term potentiation (Kovalchuk et al., 2002) and complex cognitive abilities (Yamada & Nabeshima, 2003), chronic disturbance of the BDNF-TrkB signal transduction system may also be associated with behavioral impairments. Recovery from spatial reference memory deficits are also mediated by BDNF-TrkB signaling (Ortiz et al., 2014). Although BDNF levels were not assessed in the week following binge exposure, binge-induced spatial memory deficits in females may be associated with hippocampal neurodegeneration and persistent BDNF-TrkB disruption.

Finally, our results indicated that females, but not males, had a significant decrease in protein expression of the transcription factor CREB and its activated form pCREB. CREB promotes neuroprotection by regulating the transcription of other pro-survival factors to protect neurons from excitotoxicity and apoptosis (Lonze & Ginty, 2002; Mantamadiotis et al., 2002) and is critical for proliferation, differentiation, survival, and maturation of all types of cells, including new neurons in the rodent hippocampus (Fujioka et al., 2004; Nakagawa et al., 2002). CREB helps protect against oxidative stress driven neurodegeneration by regulating ROS detoxification and increasing expression of antioxidants (Bedogni et al., 2003; Kronke et al., 2003), while disruption of CREB-mediated transcription results in increased vulnerability to ROS-induced cell toxicity (Lee et al., 2009).Therefore the loss of

CREB may have contributed to the binge-induced reduction of granule neurons in females, while preserved CREB signaling may have provided a source of neuroprotection in males.

Reduced CREB transcription has been shown to contribute to alcohol neurotoxicity (Zou & Crews, Wand, 2005; 2006) and coincides with the activation of oxidative stress by alcohol (Zou & Crews, 2006). Additionally, binge exposure results in a decrease in pCREB immunoreactivity that is most pronounced in regions of the brain showing neurodegeneration (Bison & Crews, 2003). This corresponds with our current findings of reduced pCREB expression in the dorsal hippocampus in line with a binge-induced increase in cell death and reduction of granule neurons in females; binge exposure had no effect on pCREB and granule neurons in males. Increasing the transcription of CREB though the use of antioxidants blocks neuroinflammation and alcohol-induced neuronal death (Pandey et al., 2005; Pluzarev et al., 2008; Zou & Crews, 2004). Although we found no increase in CREB or pCREB expression in males, it is possible that their maintenance at control levels provided a source of neuroprotection.

BDNF and TrkB are intimately linked with CREB and together they contribute to each other's roles in plasticity. When BDNF binds to TrkB, it initiates a signaling cascade that increases the transcription and phosphorylation of CREB which results in the expression of genes important for plasticity and survival, including CREB-mediated anti-apoptotic genes (Arthur et al., 2004; Watson et al., 2001; Xing et al., 1998). In turn, pCREB can also increase the transcription of BDNF (Conti et al., 2002; Katoh-Semba et al., 2008; Tabuchi et al., 2002). Increasing alcohol administration results in decreased expression of BDNF, TrkB, and pERK (part of the signaling cascade that ends with CREB) in the hippocampus (Kim et al., 2012). In line with this, studies of hippocampal slice cultures have found reductions in

CREB-regulated BDNF that coincided with sensitivity to neurodegeneration (Zou & Crews, 2006). It is not surprising then that binge exposure resulted in a decrease in CREB and pCREB expression in females, given that they also had a decrease in BDNF and TrkB expression. Interestingly, we found no differences in the IGF-1 receptor protein expression. Previous work seems to indicate that alcohol has more robust effects on IGF-1 than its receptor (Lynch et al., 2001).

Taken together, these results indicate that females are more sensitive than males to binge alcohol-induced disruption of trophic and transcription factors in the dorsal hippocampus. The decrease in BDNF-TrkB and CREB/pCREB protein expression may underlie female sensitivity to binge-induced neurodegeneration of the dentate gyrus and its associated cognitive deficits.

## *Chapter 5: Exercise-driven repair of damage due to binge alcohol is associated with increased trophic support*

Chronic alcohol consumption, particularly in a binge pattern, results in significant volume loss in various cortical and subcortical structures, including shrinkage of both gray and white matter (Kubota et al., 2001; Mann et al., 2001; Pfefferbaum et al., 1992; Sullivan & Pfefferbaum, 2005). This overwhelming alcohol-induced loss of brain mass is likely due to loss of neurons, glia, and their white matter fiber tracts, but may also include a reduction in the brain parenchyma, that is, the actual volume of surviving cells and their processes (Crews, 1999). Despite all this damage, human studies provide substantial evidence that abstinence from alcohol allows for natural hearing to occur in the brain (Carlen et al., 1978; Sullivan et al., 2000b). Longitudinal imaging studies of brain structures reveal increases in cortical gray matter volume within a month of abstinence, followed by increases in white matter and reversal of ventricular enlargement after longer periods of abstinence (Mann et al., 2005; O'Neill et al., 2001; Pfefferbaum et al., 1995). Improvements in overall brain volume occur within the first months of abstinence and are associated with improvements in cognition (Sullivan et al., 2000a; Sullivan et al., 2000b). Thus, it appears that endogenous recovery during abstinence is time dependent, with greater recovery in structural and cognitive deficits happening with longer periods of abstinence (Fein et al., 2006).

Although reversal of both structural and functional loss occurs during abstinence, there appears to be a limit to recovery. Even after 6-8 months of abstinence from alcohol consumption, significant shrinkage persists compared to controls within the frontal lobe, anterior parietal lobe, temporal lobe, cingulate gyrus, insula, thalamus, and cerebellum (Cardenas et al., 2007; Chanraud et al., 2007). In addition, evidence indicates that abstinence

only reverses alcoholic related deficits in learning, memory, and executive function in less than half of all cases (de la Monte & Kril, 2014). Additionally, binge pattern drinking, the most common pattern of consumption among those who abuse alcohol, happens multiple times. Some even report binge drinking anywhere from four to eight times a month, consuming about eight drinks per binge (CDC, 2012). With this pattern of drinking there are not many opportunities for long periods of abstinence to facilitate recovery. Therefore, there is a need to develop interventions that could potentially bolster the self-repair processes.

Physical exercise is a promising potential intervention because it provides many beneficial effects that may facilitate recovery and directly contrast with the more deleterious effects of binge alcohol consumption. For example, physical exercise has been linked to improved immune system function and anti-inflammatory processes in the CNS (Kohut et al., 2006; Skalicky & Viidik, 1999) and helps bolster antioxidant activity (Alessio et al., 2005). Exercise is a powerful promoter of neuroplasticity, which is believed to underlie its significant benefits for cognition and brain health, and potential to restore the binge damaged brain. In contrast to binge alcohol , exercise increases proliferation of glia in various regions (de Senna et al., 2011; Ehninger & Kempermann, 2003; Li et al., 2005), and influences neurogenesis, survival, and differentiation in the DG (Brown et al., 2003; Kobilo et al., 2011; van Praag et al., 1999a; Wu et al., 2008). The ability to create new cells and help them survive is important for the brain's ability to respond to damage and is a key mechanism behind the restorative potential of exercise. In line with this, evidence indicates physical exercise is effective in the treatment of several chronic diseases (see Pedersen & Saltin, 2015 for full review). It is possible that physical exercise may be an effective intervention to treat the neurodegenerative and cognitive consequences associated with alcohol abuse.

Previous work has indicated that exercise is associated with the reversal of some of the consequences of alcohol exposure. For example, exercise has been shown to help the brain recover from developmental alcohol exposure (Helfer et al., 2009; Redila et al., 2006; Thomas et al., 2008) and can counteract the deleterious effect of alcohol on neural stem cell proliferation when access is given coincident with alcohol self-administration (Crews et al., 2004b). We have previously shown that two weeks of voluntary exercise protects the dentate gyrus from a binge-induced loss of granule neurons in females (Leasure & Nixon, 2010). More recently, we found that the binge-induced reduction of DG granule neurons in females persists for 35 days after the last dose of alcohol, however four weeks of voluntary exercise reversed this loss (Maynard & Leasure, 2013). It remains unclear however, how exercise drives the restoration of the granule cell layer, or the period of time needed for exercise-driven hippocampal recovery.

Improved trophic factor signaling is thought to underlie the positive effects of physical activity on the brain and cognition (see Phillips et al., 2014 for review). Many studies have demonstrated that physical exercise of varying durations upregulates both BDNF (Ding et al., 2011; Neeper et al., 1995; Neeper et al., 1996) and IGF-1 (Carro et al., 2001; Ding et al., 2006; Trejo et al., 2001) in several brain regions, both of which appear to be key mediators of the effects of exercise on the brain, neurogenesis in particular (Farmer et al., 2004; Trejo et al., 2001; Yu et al., 2014). There is some evidence that exercise helps the brain recover from oxidative damage through its effects on BDNF expression. Exercise-induced upregulation of BDNF stimulates DNA repair by activating CREB, which, in turn, induces the expression of key enzymes in repair pathways (Yang et al., 2014). Additionally, exercise increases pCREB expression, however, this effect and the benefits of exercise on

synaptic plasticity and cognition are negated by blocking the binding of BDNF to TrkB (Vaynman et al., 2004). It is also possible that the increase in BDNF and cell survival signaling resulting from physical exercise is actually dependent on CREB activation and phosphorylation itself (Chen & Russo-Neustadt, 2009).

It is possible that exercise-driven increases in trophic support contribute to the recovery from binge-induced damage. In the current set of experiments, we determined the period of time necessary for exercise-driven hippocampal recovery. Because we previously found restoration of the binged-induced loss of granule neurons in females after four weeks of exercise, the current experiment investigated the effects of access to voluntary exercise wheels for one, two and three weeks of exercise. New granule cells can express mature neuronal markers as early as two to four weeks after proliferation (Shors et al., 2001; Snyder et al., 2009a). At this time new granule neurons are integrated into the granule cell layer and contribute electrophysiologically and behaviorally to hippocampal function (Schmidt-Hieber et al., 2004; Snyder et al., 2005; Winocur et al., 2006). Evidence indicates that exercise can actually accelerate the maturation of young neurons as well as increase their survival (Snyder et al., 2009b). Therefore we hypothesized that access to voluntary exercise wheels for three weeks will be associated with the repopulation of the binge-induced loss of DG granule neurons compared to sedentary animals.

We also investigated whether repair of the binge-damaged DG was associated with increased expression of neurotrophins and associated signaling molecules, including BDNF, TrkB, IGF-1R, CREB, and pCREB. We hypothesized that exercise-driven repair of binge induced hippocampal damage will be associated with an increase in trophic factor protein expression (BDNF, TrkB, IGF-1R, CREB, and pCREB) compared to sedentary animals.

Finally, we also determined the effect of exercise on microglia following binge-induced neurodegeneration. The normal response of microglia following neuronal injury is to upregulate production of BDNF (Miller & Mooney, 2004; Nakajima et al., 2001), and enhanced BDNF expression by microglia can afford neuroprotection (Liao et al., 2012). Additionally there is evidence that exercise increases the number of microglia that provide trophic support (Kohman et al., 2012; Ziv et al., 2006). . We hypothesized that exercise-driven repair of binge induced hippocampal damage will be associated with an increase in protein expression of Iba1, a marker of microglia.

## METHODS

### *Animals*

A total of 30 female Long-Evans rats aged nine weeks were used across all experiments. All rats were housed and handled as previously described in chapter two. All experimental procedures were conducted in accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The relevant animal protocol was approved by the University of Houston Institutional Animal Care and Use Committee (protocol number 14-013). Animals were divided into four groups: animals that remained sedentary, or animals that exercised for one week (T14), two (T21), or three weeks, (T28), starting 7 days after the last dose of alcohol (Table 5.1).

**Table 5.1 Animals exercised or remained sedentary for 1, 2, or 3 weeks**

| Group | T14 | D21 | D28 |
|---|---|---|---|
| Binge Exercise | Granule (n=6); Westerns (n=5) | Granule (n=6) | Granule (n=4) |
| Binge Sedentary | Westerns (n=5) | N/A | Granule (n=4) |

Table 5.1 Animals were sedentary or started exercise 7 days after the last dose of alcohol. For T14 separate animals were used to assess the effect of exercise on remaining granule neurons and trophic factor expression.

### *Binge-Alcohol Administration Model and Blood Alcohol Concentration*

Animals received diets as previously described in chapter two. For all binge-exposed animals, blood samples were collected via tail bleed taken 90 minutes after the morning dose on day 3 and BAC assessed as previously described in chapter two.

### *Monitoring of Withdrawal Symptoms*

All animals were monitored for withdrawal as previously described in chapter four.

### *Voluntary Exercise Paradigm*

On the seventh day following the last dose of alcohol, rats in exercise groups were given access to voluntary exercise wheels for a maximum of four hours daily for one week, two weeks, or three weeks, beginning at the onset of their dark cycle (9:00 AM). Exercise began on the seventh post-binge day because binge alcohol exposure damages the brain, and the initial seven days following brain injury is a vulnerable period during which increased activity can exacerbate damage and limit recovery (Griesbach et al., 2004; Humm et al., 1998). In order to precisely monitor distance travelled, rats were removed from home cages and placed into individual running wheels equipped with counters. During the exercise period, animals had access to food and water ad libitum. After exercise, animals were returned to their home cages in the vivarium. Sedentary animals remained in their home cages in the vivarium during this period.

### *Tissue Collection and Staining for Immunohistochemistry*

Following the last day of exercise, animals were sacrificed and brains extracted as previously described in chapter two. Sections used to quantify the number of remaining DG granule neurons were processed and stained as previously described in chapter two.

### *Granule Cell Quantification*

The number of methyl green Nissl stained granule neurons in the hippocampal dentate gyrus was determined by unbiased stereological methods as previously described in chapter two. To determine if exercise reversed the binge-induced reduction of DG granule neurons and if the reduction persisted in animals that remained sedentary, estimates of the total number of granule neurons per dentate gyrus was compared with those of female binge-exposed and female control animals sacrificed after the last dose of alcohol in chapter 2.

### *Tissue Collection and Western Blotting*

After it was determined that two weeks of exercise was sufficient to restore binge-induced damage to the hippocampus, we investigated the effect of one week of exercise post-binge on trophic support in two separate groups of animals. This time was chosen to try and capture the dynamic effect of exercise on trophic support before recovery had completed. Following the last day of exercise animals were sacrificed and brains extracted as previously described in chapter three. Additionally, samples were analyzed for protein expression using the same methods and antibodies as described in chapter three. Additionally, we used a primary antibody for Iba1 (17 kDa, 1:250, Wako Laboratory Chemicals) to assess the effect of exercise on microglia. Because we wanted to determine the effect of exercise on trophic support in a binged-exposed animal and the limitations of the number of animals that can be included on western blots, we used binge-exposed animals that remained sedentary as our control group.

### *Statistical Analyses*

Data were analyzed and graphed using Excel 2014 (Microsoft Office Excel, WA, USA) and SPSS Statistics 17.0 (IBM SPSS Statistics, IL, USA). Cell counts for remaining

DG granule neurons were analyzed by one-way ANOVA with post hoc tests as appropriate. Normalized data for protein expression from binged exercise animals was compared against binged sedentary animals for statistical significance determined via independent *t*-tests. Pearson correlations were performed for variables where appropriate and significance was determined using the critical value table for Pearson's Correlation Coefficient. All data are reported as the mean ± standard error of the mean and analyses considered significantly different if $p<0.05$.All data are reported as the mean percent of control ± standard error and considered significantly different if $p<0.05$.

## RESULTS

### *Behavioral Response to Binge Alcohol Administration*

Measures of intoxication across all experiments are shown in Table 5.2, and are similar to those reported in past studies with this model (Maynard & Leasure, 2013; Morris et al., 2010b) and chapters 3 and 4. Kuskal Wallis test for activity and Mann-Whitney non-parametric tests revealed no significant differences between any groups for intoxication, dose, average withdrawal, and peak withdrawal. This is to be expected because all animals had received the same treatment at the time of behavioral intoxication and withdrawal.

**Table 5.2 Intoxication measures for chapter five**

| Time | Activity | Intoxication behavior | Dose (g/kg/day) | BAC (mg/dl) | Average Withdrawal Score | Peak Withdrawal Score |
|---|---|---|---|---|---|---|
| **T14** | Sedentary | 2.0 ± 0.1 | 9.1 ± 0.2 | # | 1.8 ± 0.2 | 2.8 ± 0.2 |
| **T14** | Exercise | 2.1 ± 0.1 | 8.9 ± 0.2 | # | 1.9 ± 0.1 | 2.8 ± 0.1 |
| **T21** | Exercise | 2.0 ± 0.2 | 9.3 ± 0.5 | # | 1.9 ± 0.2 | 3.0 ± 0.2 |
| **T28** | Sedentary | 2.2 ± 0.1 | 8.7 ± 0.4 | 262.9 ± 20.6 | 1.9 ± 0.2 | 2.8 ± 0.1 |
| **T28** | Exercise | 2.1 ± 0.1 | 9.0 ± 0.3 | 252.1 ± 14.7 | 2.0 ± 0.1 | 3.0 ± 0.1 |

Table 5.2. Measures of various intoxication parameters were statistically similar between all groups. # BACs from these groups were omitted due to sample degradation but commonalities between behavioral intoxication measurement and dose suggest that the BACs should be comparable.

***Distance Run***

There were no significant differences in average distance run during the first week of exercise [$F_{(2,20)}$ = .310, $p$ = .737] or differences in average distance run during the second week of exercise [$t_{(8)}$ = .615, $p$ = .615] (Figure 5.1A). However, a one-way ANOVA for average distance run across the entire experiment revealed that animals that exercised for three weeks (T28) ran significantly more per day than animals that only exercised for one week (T14) [$F_{(2,20)}$ = 4.198, $p$ < .05] likely because animals had two extra weeks of access to exercise wheels. No differences were found however in average distance run between animals that had access for one week (T14) and two weeks (T21; $p$ = 0.10). In line with this, all animals increased distance run over the course of the experiment [$F_{(6,120)}$ = 8.357, $p$ < .05]. As expected, more weeks of access to exercise wheels resulted in greater total distances run [$F_{(2,20)}$ = 10.805, $p$ < .05] (Figure 5.1B).

**Figure 5.1 Animals increased distance run over time**

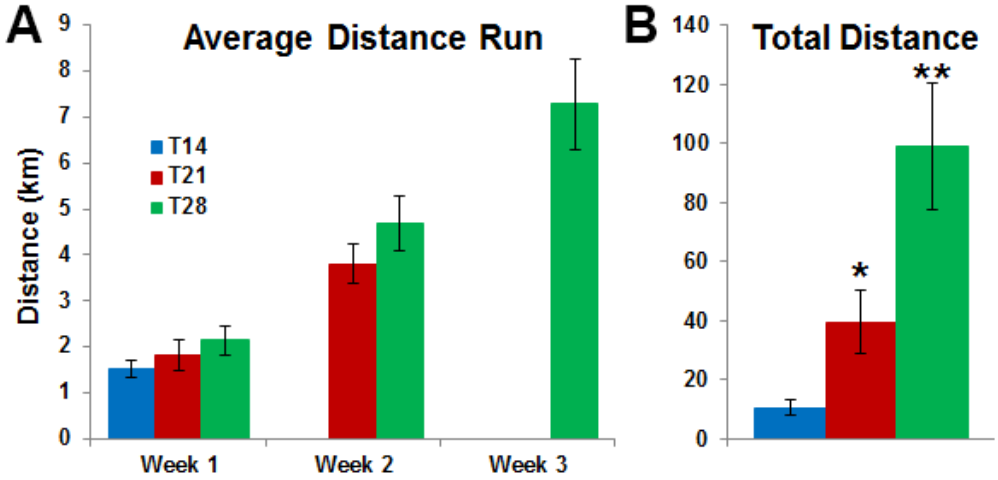


Figure 5.1 All animals increased distance run over time. For each week, animals ran similar average distances (A). Animals that exercise for three weeks ran further than all other groups (B). *, ** p<.05.

***Two of weeks exercise reverses the binge-induced loss of DG granule neurons***

Binge exposure resulted in a significant loss of DG granule neurons that persisted for four weeks after the last dose of alcohol [$F_{(2,18)}$ = 6.598, $p$ < .05] compared to control animals

from experiment two, consistent with our previous findings (Figure 5.2A; Maynard & Leasure, 2013). One-way ANOVA for the effect of exercise on remaining granule neurons after binge exposure revealed a significant effect of Time [$F_{(3,21)} = 9.69$, $p < .05$]. Planned post hoc tests compared the number of remaining granule neurons after exercise compared to control animals revealed that two (p = 1.00) and three (1.00) weeks of exercise were associated with recovery from binge-induced granule cell loss but not one week of exercise ($p < .05$) when compared to control animals (Figure 5.2B). We found no significant correlation between average distance run and remaining granule neurons ($r = .38$), however there was a significant correlation between total distance run and remaining granule neurons ($r = .51$). These results indicate that between seven and fourteen days of exercise are necessary to repopulate the DG following binge exposure.

**Figure 5.2 Exercise reverses binge-induced loss of granule neurons**

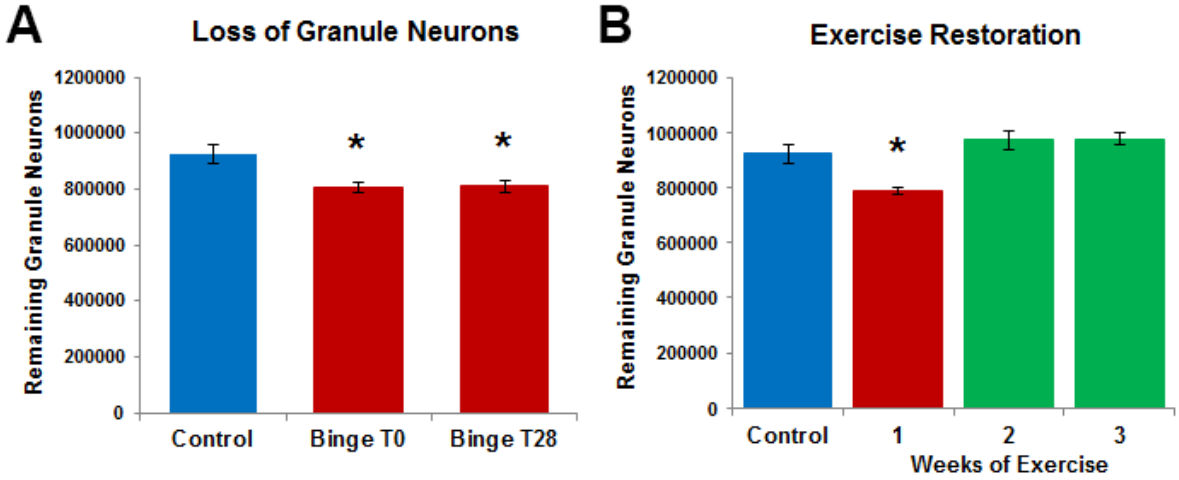


Figure 5.2 Binge exposure resulted in a persistent loss of granule neurons four weeks later (A).Two but not one week of exercise restored the loss of DG granule neurons. *p<.05.

***One week of exercise resulted in an increase of BDNF following binge exposure***

Because our results indicate that between seven and fourteen days of exercise is needed for hippocampal recovery, we assessed the effect of one week of exercise on the expression of trophic support. Relative expression of trophic factor proteins in the

hippocampus, as determined by Western blotting, is summarized in Figure 5.3. Compared to binge-exposed sedentary animals, one week of exercise was associated with a significant increase in BDNF expression (197.58 ± 19.53%) [$t_{(8)}$ = -3.299, $p$ < .05], but no difference in TrkB expression (110.82 ± 11.61%) [$t_{(8)}$ = -1.558, p = .163], or IGF-1R expression (95.22 ± 8.14%) [$t_{(8)}$ = .297, $p$ = .775]. These results indicate that exercise may have facilitated restoration of the DG via an increase in BDNF expression.

**Figure 5.3 Exercise increased the expression of BDNF post-binge**

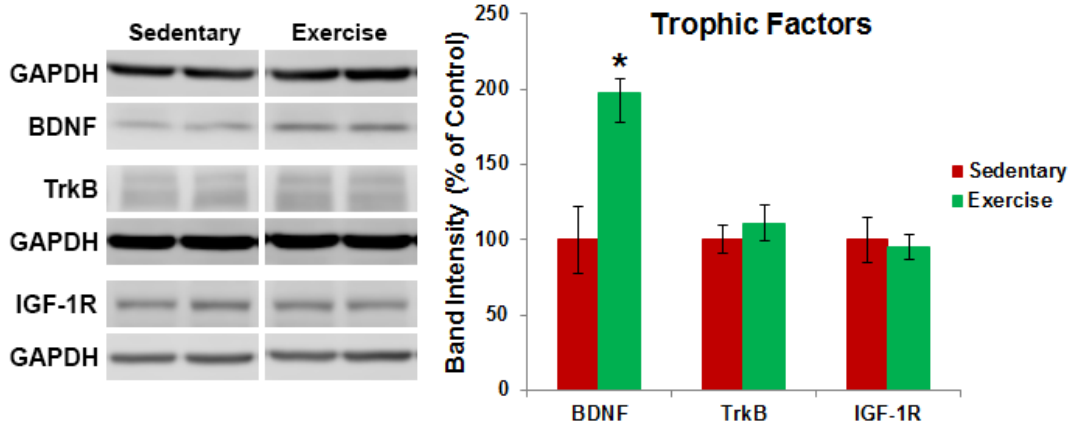


Figure 5.3 Relative expression of trophic factor proteins. Exercise resulted in a significance increase of BDNF compared to sedentary binged animals. *p< 0.05.

***One week of exercise resulted in an increase of pCREB and Iba1 following binge exposure***

Relative expression of transcription factor proteins in the hippocampus, as determined by Western blotting, is summarized in Figure 5.4. Compared to binge-exposed sedentary animals, one week of exercise was associated with a significant increase in pCREB expression (128.77 ± 3.6%) [$t_{(8)}$ = -2.638, $p$ < .05] and Iba1 expression (181 ± 16.6%) [$t_{(8)}$ = -3.747, $p$ < .05], but no difference in CREB expression (106.99 ± 14.16%) [$t_{(8)}$ = -.428, $p$ = .682]. These results indicate that exercise may have facilitated repair of the DG via an increase in pCREB and microglia.

**Figure 5.4 Exercise increased the expression of pCREB and Iba1 post-binge**

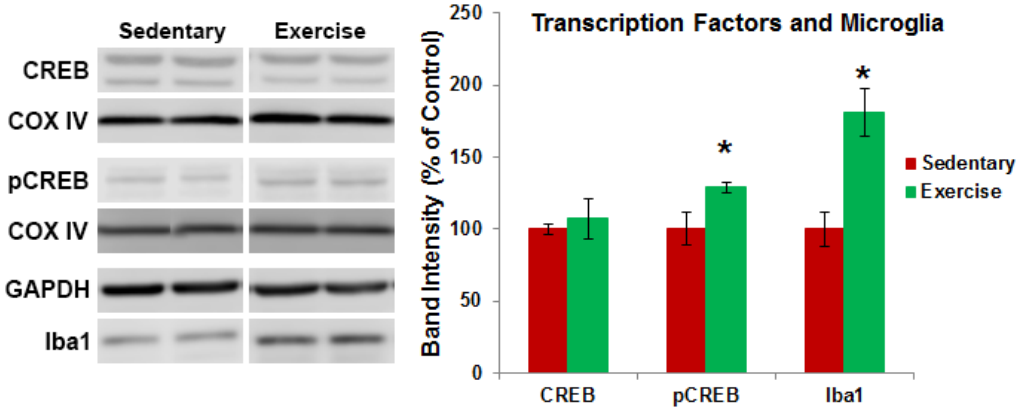


Figure 5.4 Relative expression of trophic factor proteins. Exercise resulted in a significance increase of pCREB and Iba1 compared to sedentary binged animals. *p< 0.05.

## DISCUSSION

Abstinence from alcohol allows for natural brain healing to occur. Imaging studies reveal that longer periods of abstinence are associated with increases in cortical gray and white matter that in turn are associated with improvements in cognition (Sullivan et al., 2000a; Sullivan et al., 2000b). However, recovery tends to be incomplete. Therefore, there is need to develop interventions that could potentially bolster the self-repair process. Physical exercise is one such potential intervention. We have previously found that voluntary exercise is both neuroprotective and neurorestorative of the binge-induced loss of DG granule neurons in females (Leasure & Nixon, 2010; Maynard & Leasure, 2013). Improved trophic factor signaling is thought to underlie the positive effects of physical activity on the brain, however, the effect of exercise on trophic support in the binged-damaged brain has not been investigated. To our knowledge, this is the first time the 4-day binge model has been used to investigate the duration of exercise necessary for repair, as well as the effect of exercise on trophic factors associated with repair of the binge-damaged dentate gyrus. The major finding of this experiment is that binged-induced damage to the female DG is repaired after two

weeks of voluntary exercise. Additionally, exercise-driven recovery of the DG was associated with increases of BDNF, pCREB, and Iba1 protein expression.

First, we found that between seven and fourteen days of access to voluntary exercise wheels was necessary to repopulate the binge-induced loss of DG granule neurons. Thus, it appears exercise repairs the female DG in a shorter amount of time than the 28 days of exercise we had previously reported (Maynard & Leasure, 2013). Evidence indicates that newly proliferated progenitor cells in the SGZ need anywhere from two to four weeks to mature into neurons and integrate into the granule cell layer (GCL; Shors et al., 2001; Snyder et al., 2009a). Additionally, exercise can accelerate the maturation process of young neurons as well as increase their survival (Snyder et al., 2009b). Our current findings are consistent with this, as two weeks of exercise was sufficient to replace granule neurons lost from binge exposure. Previous work with the 4-day binge model indicates that on the seventh day following binge exposure there is a large burst in proliferation in the DG (Crews et al., 2004b). This burst produces cells that primarily differentiate into new neurons that, given enough time, will mature into DG granule neurons (Nixon & Crews, 2004). It is possible that exercise was able to increase the survival and number of these newly generated cells and aid their maturation and integration into the GCL. We have previously shown that exercise-driven recovery of the DG 35 days post-binge was associated with an increase in number and survival of new cells generated during abstinence (Maynard & Leasure, 2013).

One week of exercise may not have repaired the female DG because it was simply not enough exercise itself to be restorative. Although there was no difference in the average distance run between animals that had access to exercise wheels for one week and two weeks, animals that had had access for two weeks ran almost four times further total.

Additionally, remaining granule neurons was positively correlated with total distance but not average distance run. It is possible that a greater distance run during the one week of exercise wheel access may have restored the DG in T14 animals. However this is still unlikely as newly generated cells would have had to mature and integrate faster than previously reported and may indicate that exercise driven recovery is time and distance dependent.

Second, we found increased protein expression of BDNF, pCREB, and microglia in binge-exposed animals that exercised for one week compared to binge-exposed animals that remained sedentary. Although one week of exercise was not enough to reverse the binge-induced loss of granule neurons, it does appear however, to initiate a cascade of trophic factors and signaling molecules that lead to eventual recovery with more time. Evidence indicates that exercise's effects on cell survival and neurogenesis are dependent on BDNF signaling (Farmer et al., 2004; Trejo et al., 2001; Yu et al., 2014). Exercise may also help the brain recover from oxidative damage through increased BDNF expression. Exercise-induced upregulation of BDNF stimulates DNA repair by increasing the activation of CREB into pCREB which, in turn, induces the expression of key enzymes in repair pathways (Yang et al., 2014). Along with BDNF, exercise increases pCREB expression, however, this effect and the benefits of exercise on synaptic plasticity and cognition are negated by blocking the binding of BDNF to TrkB (Vaynman et al., 2004). It is also possible that the increase in BDNF and cell survival signaling resulting from physical exercise is actually dependent on phosphorylation of CREB itself (Chen & Russo-Neustadt, 2009). This is consistent with our current findings as we show an increase in pCREB and BDNF after one week of exercise.

Along with an increase in BDNF, an increase in pCREB transcription that rebounds from alcohol-suppressed levels during abstinence may also contribute to regeneration. Many

studies have suggested that pCREB transcription increases plasticity and survival of neurons following insult (Mabuchi et al., 2001; Walton & Dragunow, 2000). Evidence has linked pCREB transcription with synaptic, pro-survival NMDA receptors (Hardingham & Bading, 2002), which may result in an increase of trophic support. One week of exercise may have been able enhance the endogenous recovery process from binge-induced damage by increasing the phosphorylation of CREB. Therefore, exercise-induced upregulation of BDNF and pCREB likely resulted in an increase in new neurons generated during abstinence, as well as an increase in anti-apoptotic and other signals important for survival and maturation of these newly generated cells. By aiding in the creation of new cells and survival, one week of exercise may have created an environment that facilitated the recovery from binge-induced DG damage in the following week.

. One week of exercise also resulted in an increase in the expression of the microglia marker Iba1, indicating an increase in the number of microglia. Previous evidence indicates that exercise can increase the number of microglia that provide trophic support (Kohman et al., 2012; Ziv et al., 2006). The normal response of microglia following neuronal injury is to upregulate production of BDNF (Miller & Mooney, 2004; Nakajima et al., 2001) and enhanced BDNF expression by microglia can afford neuroprotection (Liao et al., 2012). Microglia have been shown to proliferate in large numbers during the first couple of days of abstinence after alcohol in this model (Nixon et al., 2008) and are believed to aid various brain regions in recovery by taking on a partially activated state and secreting trophic factors and anti-inflammatory cytokines (Marshall et al., 2013; McClain et al., 2011). Therefore it is possible that one week of exercise was able to enhance the endogenous recovery process from binge-induced damage by increasing the number of microglia. An important thing to

note however, is that this study cannot directly tie the increase in BDNF solely to microglial function. BDNF is also secreted by astrocytes, oligodendrocytes, and neurons, particularly dentate gyrus neurons (Mannion et al., 1999; Wilkins et al., 2003; Ziv et al., 2006)

It was surprising that one week of exercise did not result in an increase in the TrkB and IGF-1R receptors. Increases in the high affinity receptors TrkB and IGF-1R have previously been found after just 7 days of exercise (Ding et al., 2011; Vaynman et al., 2003). It is possible that binge exposure has a lasting effect on the protein expression for these two receptors, preventing an exercise-induced upregulation after one week. A lack of an increase in these two receptors may also underlie why one week of exercise was insufficient to restore granule cell loss because they are important binding sites for BDNF and IGF-1. However, animals in the preceding studies had access to exercise wheels in their home cages for the entirety of the seven days (Vaynman et al., 2003). Therefore, it is possible that animals in the current study that only ran for one week simply did not run far enough to effect protein expression for TrkB and IGF-1R. Animals that exercised for two weeks however, ran nearly four times further than animals that only exercised for one week. Although protein expression was not assessed in animals that exercised for two weeks, it is possible their increased total distance run was associated with increases in both TrkB and IGF-1R.

Taken together, these results indicate that exercise-driven repair of binge-induced damage to the DG is associated with increased trophic support and signaling molecules. An exercise-induced increase in microglia may contribute to this recovery process by increasing trophic factor signaling and cleaning up dead and dying cells. Finally, we conclude that between 7 and 14 days of exercise is necessary for the reversal of binge-induced damage to the female hippocampal dentate gyrus.

***Chapter 6: Overall Conclusions***

## DISCUSSION

Alcoholism is a chronic disease that negatively affects many aspects of society. Nearly 88,000 people die from alcohol related causes annually, making it the third leading preventable cause of death in the United States (CDC, 2015). Binge drinking, the most common pattern of consumption among those who abuse alcohol, is particularly problematic. In fact, binge pattern drinking is more predictive of neurodegeneration than total lifetime alcohol intake (Bobak et al., 2004; Hunt, 1993). Alcohol abuse causes neurodegeneration characterized by significant volume loss of gray and white matter that is associated with cognitive deficits that compromise executive functioning, learning, and memory (Pfefferbaum et al., 1992; Sullivan & Pfefferbaum, 2005).

Alcohol affects women differently than men. Evidence suggests that women are more sensitive to the neurotoxic effects of alcohol and are more vulnerable to the adverse medical consequences of heavy alcohol consumption than men (Hommer, 2003; Nixon et al., 1995), despite the fact that alcoholic women tend to start drinking later in life and consume less alcohol in their lifetimes (Agartz et al., 2003; Hommer et al., 2001). The course of alcohol use disorders is thus considered by some to be accelerated or compressed in women compared to men, and has been labeled the “telescoping” effect (Piazza et al., 1989). Few studies have directly compared alcohol-induced brain damage and cognitive dysfunction between males and females, and the mechanisms that underlie increased female vulnerability remain poorly understood. Therefore, these studies investigated sex differences in alcohol-induced neurodegeneration, and associated cognitive deficits and disruption of trophic support, using a rodent model of an AUD. In addition, we investigated the effect of post-

binge exercise on the reversal of binge-induced neurodegeneration and changes in trophic support associated with neurorestoration.

**Aim 1**: Determine whether the binge alcohol exposure results in significantly greater cell death and reduced cell birth in the hippocampus of females than males. (Chapt2)

The hypothesis that binge alcohol exposure would result in significantly greater neurodegeneration in the hippocampal dentate gyrus of females compared to males was supported by experiments herein. Reduced DG granule neurons and significantly more degenerating and dead cells labeled with FJB suggests the female DG is more sensitive to binge-induced neurodegeneration than the male DG. However, the hypothesis that females would have a greater reduction of proliferating cells in the DG and more cell death in associated cortical regions was not supported. Binge exposure reduced the number of proliferating cells labeled with Ki67 at similar rates between females (47%) and males (38%). In addition, there was no difference in the number of FJB+ cells in the entorhinal/perirhinal cortex or piriform cortex between binge-exposed males and females.

**Aim 2**: Determine whether cognitive deficits corresponding to binge alcohol-induced neurodegeneration are more severe in females than males (Chapter 3).

The hypothesis that binge-induced neurodegeneration in the hippocampus would be associated with spatial reference memory deficits in females, but not males, was supported. Binge-exposed females had a significantly longer escape latency and path distance to find the location of the platform than males, despite no difference in swim speed. However, the hypothesis that binge exposure would result in greater recognition memory deficits in females compared to males was not supported. More time spent with NO1 than familiar

objects during trial one, and decreased time spent with NO1 between trial one and three, suggests that neither males nor females had deficits in object recognition learning or memory.

**Aim 3**: Determine whether decreased trophic support underlies increased vulnerability to binge alcohol-induced neurodegeneration in females (Chapter 4).

The hypothesis that alcohol-induced neurodegeneration in the hippocampus is associated with decreased trophic support and signaling molecules in females, but not males, was partially supported. Decreased expression of BDNF, TrkB, CREB, and pCREB in females suggests that the female hippocampus was vulnerable to cell death and loss of proliferation due to binge exposure. Males also had decreased expression of BDNF, likely associated with the binge-induced reductions in cell proliferation, yet no changes in the expression of TrkB, CREB, and pCREB suggests a mechanism of neuroprotection against binge-induced loss of DG granule neurons. Neither binge exposed males nor females showed decreased expression of IGF-1R.

**Aim 4**: Determine the length of time needed for exercise-driven hippocampal recovery and altered protein expression associated with the restoration of the female rat dentate gyrus granule cell layer following binge alcohol-induced damage. (Chapter 5)

The hypothesis that voluntary exercise would reverse the binge-induced reduction of DG granule neurons in females was partially supported. Two and three weeks of exercise restored the binge-induced loss of DG granule neurons, however one week was insufficient for repair. The hypothesis that exercise-driven hippocampal recovery was associated with increased trophic support was partially supported. One week of exercise increased the protein

expression of BDNF, pCREB, and Iba1, suggesting an ongoing recovery process had started associated with an increase in microglia. However, one week of exercise had no effect on TrkB and IGF-1R expression, possibly explaining why more than one week of exercise was necessary to restore the binge-induced loss of granule neurons.

### *Conclusions*

The purpose of this dissertation was to determine whether the female brain is selectively vulnerable to binge alcohol exposure. More specifically, we investigated sex differences in the effect of binge exposure on neurodegeneration, associated cognitive deficits and alterations in trophic support. An additional purpose was to investigate whether post-binge exercise could reverse binge-induced damage. The data reported here suggests the females are more vulnerable to the negative consequences of binge exposure, however exercise may help alleviate or even reverse those consequences.

The data showing a loss of granule neurons and more FJB+ cells indicate that the female dentate gyrus is more sensitive to binge-induce neurodegeneration than the male dentate gyrus. This is consistent with volumetric MRI scans of gray and white matter that indicate there is greater hippocampal shrinkage found among alcoholic women than among alcoholic men, despite a later onset of heavy drinking (Agartz et al., 2003). Even when controlling for the dose of alcohol consumed, neurodegeneration still occurred in the female DG and not the male DG. This increased vulnerability is likely due in part to a loss of trophic support that would protect granule neurons from cell death. In chapter four, we found that binge-exposure resulted in a loss of BDNF, TrkB, CREB, and pCREB protein expression. Signaling between BDNF and TrkB can protect neurons against cell death, in particularly degeneration brought on by oxidative stress (Mattson, 2000). Consistent with this, chronic

alcohol has been shown to downregulate BDNF, which leaves the brain more sensitive to alcohol-induced neurodegeneration (Davis, 2008; Zou & Crews, 2006). Decreased BDNF signaling corresponds with alcohol-induced neurodegeneration (Davis, 2008). The loss of BDNF is also associated with reduced proliferation and survival of new DG granule neurons (Herrera et al., 2003; Nixon & Crews, 2002). Both males and females demonstrated reduced BDNF protein expression, which likely contributed to the binge-induced decrease in proliferating cells seen in both sexes. Despite a loss of BDNF and associated survival of newly generated cells, preserved TrkB, CREB, and pCREB was likely protective in binge-exposed males, resulting in fewer degenerating and dead cells in the DG, and no loss of DG granule neurons. These results provide evidence that the combination of cell death and loss of cell birth contribute to neurodegeneration in dentate gyrus, however reduced cell birth alone may not be sufficient to reduce the number of granule neurons in males.

In contrast, binge-exposed females showed decreased CREB and pCREB expression, and therefore did not have the same resiliency against cell death as the males. Transcription of pCREB promotes neuroprotection by regulating the transcription of other pro-survival factors to protect neurons from excitotoxicity and apoptosis (Lonze & Ginty, 2002; Mantamadiotis et al., 2002), while binge exposure results in a decrease of pCREB immunoreactivity that is most pronounced in regions of the brain showing neurodegeneration (Bison & Crews, 2003). Therefore, the loss of pCREB expression in the hippocampus corresponds with the neurodegeneration seen in the female dentate gyrus; neither of which were found in males. In addition, CREB helps protect against oxidative stress-driven neurodegeneration by regulating ROS detoxification and increasing expression of antioxidants (Bedogni et al., 2003; Kronke et al., 2003), while disruption of CREB-mediated

transcription results in increased vulnerability to ROS-induced cell toxicity (Lee et al., 2009). Therefore, the loss of CREB may have contributed the binge-induced cell death in females.

Interestingly, we found no increase in degenerating and dying cells in the entorhinal/perirhinal cortex and piriform cortex of binged-exposed females compared to binge-exposed males. It is possible that increased female sensitivity to binge-induced neurodegeneration is limited to the hippocampus, the dentate gyrus more specifically. Another possibility is that, in binge-exposed females, there actually was a reduction in the number of neurons in these regions compared to males, however it was not detected with FJB labeling. Additionally, we did not assess the effect of binge exposure on trophic support in these regions, and thus are unable to determine if signs of neurodegeneration or vulnerability were present. The fact that we found FJB+ cells in the male DG but no corresponding reduction in DG granule neurons highlighted the importance of looking at *what remains*, and not just *what is lost*. It is also possible that a second binge exposure, or a prolonged single exposure, would reveal sex differences in neurodegeneration within these regions. The hallmark of the telescoping effect is that neurodegeneration and consequences emerge sooner in females despite a shorter history of alcohol abuse than males. These areas may still be more sensitive to binge-induced damage in females compared to males, it is just that more binge exposures are necessary to reveal that.

Alcohol is known to affect various cortical regions, and imaging studies reveal that alcoholic females have larger decreases in cortical thickness and total cerebral volume than alcoholic males (Agartz et al., 2003; Momenan et al., 2012). Regions such as the corpus callosum (Hommer et al., 1996) and frontal cortex (Medina et al., 2008; Schweinsburg et al., 2003) have also been implicated as more vulnerable to alcohol exposure in females than

males. Therefore, it would be surprising that the hippocampus would be the only area of the female brain showing increased sensitivity to binge-induced neurodegeneration.

The spatial reference memory impairment in females, but not males, was associated with neurodegeneration and loss of trophic support data in chapters two and four. The hippocampus is known to be critically involved in spatial learning and memory formation (Aggleton et al., 2000; Jarrard, 1993) and evidence indicates that lesions result in robust and reliable spatial reference memory deficits in the MWM (Galani et al., 1998; Morris et al., 1982). Compromised hippocampal integrity in alcoholics is thought to underlie impairments in learning, memory, and visuospatial abilities (Beatty et al., 1996; Crews et al., 2004a; Pitel et al., 2009; Townshend & Duka, 2005). Spatial reference memory has been shown to be dependent on the hippocampus, dorsal hippocampus more specifically (Pothuizen et al., 2004; Vann & Albasser, 2011), therefore it is not surprising that females, but not males, showed a binge-induced spatial memory impairment. Previous work with the 4-day binge model is consistent with our finding of no impairment in males (Cippitelli et al., 2010a; Obernier et al., 2002b). This is also consistent with neuropsychological studies in humans that indicate female alcoholics have more severe performance impairments in short-term memory, nonverbal tests in particular, compared to controls than alcoholic men (Sullivan et al., 2002; Sullivan et al., 2000b). Greater impairment on tests of attention, working memory, and visuospatial abilities for females than males is also seen in adolescents who binge drink (Medina et al., 2008; Squeglia et al., 2011; Squeglia et al., 2012).

We, however, found no impairments in reversal learning or object recognition memory following binge exposure. Object recognition memory has been shown to be heavily reliant on entorhinal/perirhinal cortex (Parron & Save, 2004), while reversal learning

involves frontal lobe functioning (Oscar-Berman & Hutner, 1993). Although we did not assess neurodegeneration in the frontal cortex, as described in chapter two we found no difference in degenerating cell or cell death between binge-exposed males and females in the entorhinal/perirhinal cortex. Even though we found cell death in these regions, it is possible that it was not associated with a significant loss of neurons sufficient to produce a deficit in object recognition learning and memory. Like neurodegeneration in the entorhinal/perirhinal cortex, it is possible that a second binge exposure, or a prolonged single exposure, would reveal sex differences in cognitive deficits within these regions. In fact, following four repeated four-day binge exposures, reversal learning and object recognition memory deficits are seen in males (Zhao et al., 2013). Impairments in these two tasks may present themselves after repeated binge exposure sooner in females than males.

Finally, as described in chapter five, data showing that one week of exercise increases protein expression of BDNF, pCREB and Iba1 upholds the idea that exercise can enhance endogenous repair from binge-induced damage. Evidence indicates that the effects of exercise on cell survival and neurogenesis are dependent on BDNF signaling (Farmer et al., 2004; Trejo et al., 2001; Yu et al., 2014). Increased levels of pCREB also help increase plasticity and survival of neurons following injury (Mabuchi et al., 2001; Walton & Dragunow, 2000). The increase in BDNF and cell survival signaling resulting from physical exercise may actually be dependent on phosphorylation of CREB itself (Chen & Russo-Neustadt, 2009). Therefore, the increases in BDNF and pCREB following a single week of exercise likely started a signaling cascade that resulted in an increase in the number of newly generated neurons, and increased survival of those generated during abstinence. With another week of exercise, these cells would have ample support to fully mature and integrate into the

granule cell layer to replace those lost during binge exposure. An increase in microglia due to exercise would also provide another source of support, specifically BDNF, to drive recovery. These data indicate that 7 to 14 days of exercise is necessary for reversal of binge-induced damage to the female hippocampal dentate gyrus, likely due to an increase in trophic support that facilitates the birth, survival, and maturation of new neurons. This finding has important implications in the treatment of alcoholism and other consequences of alcohol abuse, as exercise could potentially provide a cheap and easy intervention.

## LIMITATIONS

First, a limitation of these studies is that all animals were alcohol naïve at the beginning of the experiment, and their first exposure to alcohol in adulthood was in a very large quantity over a prolonged period. This type of exposure does not necessarily reflect the human condition as people generally experiment with lower concentrations of alcohol during adolescence before consuming the neurotoxic levels used within these experiments (Guilamo-Ramos et al., 2004).

Another potential limitation is that the changes in protein expression due to binge exposure (Chapter 4) or exercise (Chapter 5) were investigated in the dorsal hippocampus, and not specifically the dentate gyrus. It is possible that protein expression in other dorsal hippocampal subregions influenced our results, thus our findings were not dentate gyrus specific. However, qualitative analysis of tissue immuno-stained for BDNF, TrkB, and IGF-1R revealed staining of positive cells in the dentate gyrus in a pattern consistent with the changes in protein expression indicated by Western blotting.

## FUTURE STUDIES

An important future direction would be to investigate whether sex differences in neurodegeneration was linked to a sexually dimorphic inflammatory response to binge exposure. Inflammatory and immune responsivity to stress is greater in females than males (Gallucci et al., 1993; Spitzer, 1999), while alcohol represents a physiological stressor (Ogilvie & Rivier, 1997) that preferentially activates the HPA axis in females in rodent models as well as in humans (Jenkins & Connolly, 1968; Rivier, 1993, 1996). Recent evidence indicates females are more vulnerable to neuroinflammatory effects of alcohol, resulting in significant greatly neurotoxicity in the frontal lobes and hippocampi of females compared to males (Walls et al., 2013), greater transcription of genes associated with apoptosis and cell death (Hashimoto & Wiren, 2008; Wilhelm et al., 2014), and increased production of proinflammatory cytokines and inflammatory mediators (Alfonso-Loeches et al., 2010; Alfonso-Loeches et al., 2013). A greater inflammatory response in binge exposed females compared to males may underlie their increased sensitivity to binge-induced neurodegeneration.

Increased inflammation may also cause neurodegeneration by disrupting trophic support seen in chapter four. Inflammation may cause glial cells to degenerate and die, or even enter an activated state that exacerbates inflammation (Block & Hong, 2005; Block et al., 2007; Reynolds et al., 2007). Inflammation has direct effects on trophic factors as well; proinflammatory cytokines, like those released with chronic alcohol consumption, causes a significant reduction in BDNF in multiple brain regions (Calabrese et al., 2014; Guan & Fang, 2006; Lapchak et al., 1993) and contribute to cell death by inhibiting IGF-1 and its pro-survival signals (Venters et al., 1999). Prior evidence with the 4-day binge model has

shown little indication of an increase in inflammation following binge exposure (Marshall et al., 2013; McClain et al., 2011), however these studies only investigated male animals. It is important for future studies to characterized inflammation in females using this model, as it may be a possible source of increased vulnerability to binge-induce neurodegeneration.

Further investigation of the effect of binge exposure on sex differences in cell loss in other areas of the brain, including other hippocampal subregions, is needed. Although the dentate gyrus is a particularly vulnerable area, evidence indicates that the CA1 and CA3 hippocampal subregions may also be sensitive to binge-induced neurodegeneration (Bengoechea & Gonzalo, 1991; Risher et al., 2015). There is indication that these areas may also be more sensitive to binge-induced neurodegeneration in females than males (Walls et al., 2013; You et al., 2009). Evidence from human alcoholics and rodent models reveal that the frontal lobes may also be more vulnerable to alcohol-induced neurodegeneration in females than males (Medina et al., 2008; Schweinsburg et al., 2003; Wilhelm et al., 2014). Future studies using this model should investigate potential sex differences in in vulnerability to binge-induced neurodegeneration in other regions, including the frontal lobes and other hippocampal subregions.

Finally, BDNF appears to have an important role in both the vulnerability and recovery from binge-induced damage. The current work found strong associations between decreased BDNF and binge-induced loss of dentate gyrus granule neurons in females, as well as between increased BDNF and exercise-driven recovery. Future studies should investigate BDNF mechanistically to better understand its role in these diverse processes. Signaling between BDNF and TrkB can protect neurons against cell death, in particular degeneration brought on by oxidative stress (Mattson, 2000). It is possible that supplementing females

with BDNF and/or pharmacologically increasing expression of the TrkB receptor would block binge-induced damage. On the other hand, studies blocking the effects of BDNF either reduce or eliminate the positive effects of physical activity, including neurogenesis (Ding et al., 2006; Trejo et al., 2001; Vaynman et al., 2004). Therefore it is possible that blocking BDNF binding to the TrkB receptor during exercise would prevent the restoration of the granule cell layer. These future directions would improve the understanding of the dynamic role of BDNF during and after binge exposure.

## FINAL COMMENTS

The current dissertation work investigates sex differences in the consequences of binge alcohol in the hippocampus using a 4-day binge model. The results indicate the female dentate gyrus is more sensitive to binge-induced neurodegeneration and associated cognitive deficits than the male dentate gyrus. This is likely caused by sex differences in the effect of binge exposure on trophic support and signaling molecules. These data support the idea that the female brain is more sensitive to alcohol than the male brain, and that damage and behavioral impairments emerge earlier despite a shorter history of alcohol abuse. However, voluntary exercise can enhance the endogenous repair process and reverse binge-induced damage by increasing trophic support available to support the process.

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